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# Cell-free DNA: A Neglected Source for Antibiotic Resistance Genes Spreading from WWTPs

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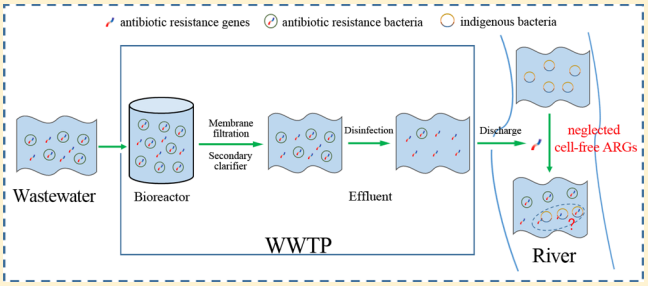
Supporting Information

**ABSTRACT:** Cell-associated ARGs in wastewater treatment plants (WWTPs) has been concerned, however, cell-free ARGs in WWTPs was rarely studied. In this study, the abundances of four representative ARGs, *sulII*, *tetC*, $bla_{PSE-1}$, and *ermB*, in a large municipal WWTP were investigated in both cell-associated and cell-free fractions. Cell-associated ARGs was the dominant ARGs fraction in the raw wastewater. After biological treatment, sludge settling, membrane filtration, and disinfection, cell-associated ARGs were substantially reduced, though the ratios of ARG/16S rRNA gene were increased with disinfection. Cell-free ARGs persisted in the WWTP with a removal of 0.36 log to 2.68 logs, which was much lower than the removal of cell-associated ARGs (3.21 logs to 4.14 logs). Therefore, the abundance ratio of cell-free ARGs to cell-associated ARGs increased from 0.04−1.59% to 2.00−1895.08% along the treatment processes. After 25-day-storage, cell-free ARGs in both biological effluent and disinfection effluent increased by 0.14 log to 1.99 logs and 0.12 log to 1.77 logs respectively, reflecting the persistence and low decay rate of cell-free ARGs in the discharge water. Therefore, cell-free ARGs might be a kind of important but previously neglected pollutant from WWTPs, which added potential risks to the effluent receiving environments.




## INTRODUCTION

The prevalence and persistence of antibiotic resistance in bacterial pathogens have become an emerging threat to public health.[1−3] Antibiotic resistance causes the failure of antibiotics on pathogens, so that ordinary infectious diseases currently treatable with antibiotics might soon become untreatable and life threatening.[4−6] Therefore, the generation and dissemination of antibiotic resistance have raised deep concern, and need to be controlled.

Selective pressure from antibiotics primarily takes place in human and animal guts,[7] which may enhance the antibiotic resistance of intestinal bacteria. A large part of the antibiotic resistance bacteria (ARB) is excreted into environments, and further spread antibiotic resistance by horizontal transfer of antibiotic resistance genes (ARGs).[8] In addition, antibiotics are only partially absorbed and metabolized by human and animals after medication, up to 75% of antibiotics and antibiotic metabolites are excreted.[9] In the receiving environments, antibiotic residues sustained a selective pressure, thus contributing to the proliferation of ARGs and ARBs in microbial communities.[10]

In cities, sewers and wastewater treatment plants (WWTPs) collect domestic wastewater as well as excreta, remove the wastes, and discharge the effluents to natural environments. The excreted antibiotics and intestinal ARBs in domestic wastes create a favorable condition in wastewater collection and treatment systems for antibiotic resistance selection and spread.[7] In order to attenuate the dissemination of ARGs from WWTPs to natural environments, it is essential to reduce ARGs in WWTPs' effluents. However, numerous studies[11−15] indicated WWTPs as ARGs reservoirs based on the investigation of ARGs' abundance at different WWTPs. Although conventional biological treatment could reduce certain ARGs significantly by about 3 orders of magnitude,[13,16,17] the remained ARGs in the effluents still ranged in $10^5$−$10^9$ gene abundance/L.[18] Such effluents may favor the persistence and spread of antibiotic resistance in the microbial

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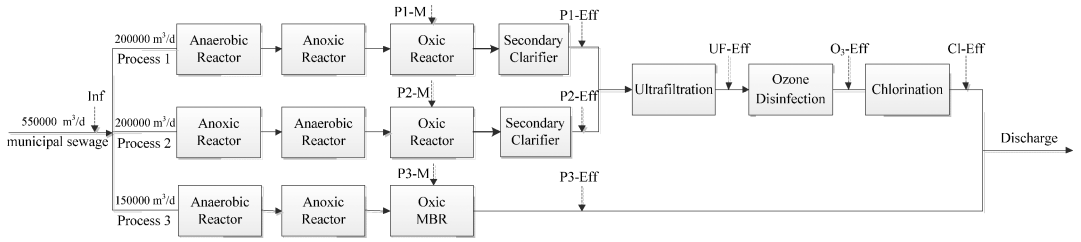


**Figure 1.** Treatment processes of the WWTP and the sampling sites. The solid-lined arrows show the wastewater flows; and the dashed arrows indicate the sampling points.

communities of the receiving environments.[19] Therefore, studies on ARB inactivation by effluent disinfection using UV,[20,21] chlorination,[22] ozone,[23] photocatalytic ozonation,[24] $UV/H_2O_2$,[25,26] $UV/TiO_2$,[27] and Fenton-$UV/H_2O_2$[28] are emerging. Here comes a matter of concern that almost all previous studies focused on cell-associated ARGs, which can be intercepted by 0.22 $\mu$m filter; while ARGs in cell-free DNA, which can pass through 0.22 $\mu$m filters, were neglected.

The proliferation of ARGs among bacteria occurs frequently through one of the following three mechanisms of horizontal gene transfer (HGT): (1) conjugation by cell-to-cell contact; (2) transduction by bacteriophage infection; and (3) transformation by direct uptake of extracellular DNA (eDNA).[1,29] eDNA generates from the secretion of live cells as well as the lysis of dead cells.[30] The persistence of eDNA in soil and sediment has been reported, which suggests an important role of eDNA-based transformation in environments. The estimated transformation frequency in marine sediments was $10^{-6}$–$10^{-7}$ transformants per recipient cell,[31] which was higher than the frequencies of conjugation ($10^{-7}$ transconjugants per donor cell) and transduction ($10^{-7}$–$10^{-9}$ transductants per plaque forming unit) in seawater.[32,33] A big risk is that eDNA-based transformation may be an important pathway of HGT in ARGs dissemination, as Zhang et al.[34] reported a high extracellular ARG abundance of $1.7 \times 10^3$ to $4.2 \times 10^8$ copies $g^{-1}$ dry sludge in livestock wastes. The originated eDNA can be partially adsorbed to cells or particles, and partially dissolved in waters. The 0.22 $\mu$m filter intercepts intercellular DNA (iDNA) and cell/particle-adsorbed eDNA (here we call them as cell-associated DNA), while the filtrates contains the eDNA (here we call it as cell-free DNA) passed through 0.22 $\mu$m filter. Up to now, the abundance of ARGs in the cell-free DNA in WWTPs has not been reported, and the risk of antibiotic resistance spreading from WWTPs' effluents is probably underestimated.

In this study, cell-associated DNA and cell-free DNA were simultaneously extracted from wastewater samples from a municipal WWTP. The abundances of four ARGs, that is, *tetC*, *sulII*, *ermB*, and $bla_{PSE-1}$, in both cell-associated and cell-free DNA were determined by quantitative PCR (qPCR). Among them, *tetC*, *sulII*, and *ermB* are the representative resistance genes of tetracyclines, sulfonamides, and macrolides, respectively, and were frequently detected in WWTPs with relatively high abundances.[35] $Bla_{PSE-1}$ was a rarely reported $\beta$-lactams resistance gene in WWTPs, which may have relatively low abundance. The removal of cell-associated ARGs and cell-free ARGs by three different biological treatment processes and follow-up disinfection processes were evaluated. The abundance variations of cell-associated and cell-free ARGs in biological effluents and disinfected effluents were also investigated in a 25-day-period after sample collection, in order to evaluate the decay of ARGs in the effluents after discharge. The purpose of this study was to investigate the abundance of cell-associated and cell-free ARGs in the WWTP and to assess whether cell-free ARGs is an important type of ARGs spreading from WWTPs.

## MATERIALS AND METHODS

**WWTP and Sampling.** The treatment processes of the WWTP and the sampling sites are shown in Figure 1. The WWTP has three parallel biological treatment processes: process 1 (P1), Anaerobic-Anoxic-Oxic; process 2 (P2), Anoxic-Anaerobic-Oxic; process 3 (P3), Anaerobic-Anoxic-Oxic membrane bioreactor (MBR). Main operation parameters of the biological treatment processes are shown in Supporting Information (SI) Table S1. The effluents of P1 and P2 were further treated by ultrafiltration (UF), ozonation, and chlorination, successively. The pore size of UF as well as membranes in the MBR was 0.02 $\mu$m. The ozone dose was 3.5 mg $L^{-1}$ and the contact time of ozonation were 25 min. NaClO was used in the chlorination with a dose of 4.2 mg $Cl_2$ $L^{-1}$ and contact time of 5 h. The effluent of this WWTP was discharged to a river nearby.

Two sets of samples were collected on August 10, 2016 and March 17, 2017, respectively. On August 10, 2016, the influent of the WWTP (Inf), the mixtures of oxic units in P1, P2, and P3 (P1-M, P2-M, P3-M), the effluents of P1, P2, and P3 (P1-Eff, P2-Eff, P3-Eff), and the effluents of UF and ozonation (UF-Eff, $O_3$-Eff) were collected, while the effluent of chlorination (Cl-Eff) was missed. On March 17, 2017, all the 10 samples were collected. For shortening sample storage and thus avoiding potential variation of gene abundance caused by DNA degradation of cell-free DNA and DNA release of dead cell, instantaneous sampling method was applied. Five L sample was collected from each sampling point, stored in a polyethylene barrel at 4 °C, and brought back to our lab in about 1 h. Physical and chemical analysis and DNA extraction of the samples were conducted immediately in the lab. All the containers were washed with bleach, and then rinsed with tap water, deionized water, and Millipore water successively before use. The properties of the samples are shown in SI Table S2.

After water property analysis and DNA extraction, the remained P1-Eff and $O_3$-Eff from March 17, 2017 were stored under 20 °C in dark. The samples after 3, 6, 10, 15, and 25-day-storage were also collected for DNA extraction and ARGs determination.

**DNA Extraction.** For the nine samples collected on August 10, 2016, cell-associated DNA and cell-free DNA were separately extracted from Inf, P1-M, P2-M, P3-M, P1-Eff, and P2-Eff. However, considering few cells might remain in P3-Eff, UF-Eff, and $O_3$-Eff after 0.02 $\mu$m membrane filtration in the MBR and UF, the cell-associated DNA of these three samples


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were not collected separately. For a more accurate analysis, DNA in all the samples collected on March 17, 2017 were extracted in cell-associated and cell-free fractions.

For cell-associated DNA extraction, the samples were filtrated through 0.22 $\mu$m membranes (Millipore, MA) for cell interception. Volume of the samples for cell-associated DNA extraction, shown in SI Table S3, was determined based on SS concentration of the samples to ensure enough DNA could be obtained from the intercepted cells. For cell-free DNA extraction, the samples were placed in 4 °C refrigerator statically to settle suspended solids. The supernatants were filtered through 0.22 $\mu$m membranes to remove residual cells. The cell-free DNA in the filtrates was collected with precipitation method.[36,37] 726 mL of absolute ethanol and 33 mL of 3 M sodium acetate were added to 330 mL of filtrate sample, and the mixed solution was stored at −20 °C overnight. The precipitates were obtained by centrifuging at 10000 × g for 10 min and discarding supernatants. The air-dried pellets were used for cell-free DNA extraction. For P3-Eff, UF-Eff, and $O_3$-Eff from August 10, 2016, each 330 mL of unfiltered sample was directly added with 726 mL of absolute ethanol and 33 mL of 3 M sodium acetate, stored at −20 °C overnight, and precipitated to obtain the total DNA. The schematic diagram of cell separation and DNA extraction procedures are shown in SI Figure S1.

PowerSoil DNA Isolation Kit (MoBio, CA) was used to extract DNA from cell intercepted membranes and the precipitates for its consistent yield and high quality of DNA from water samples.[38] All DNA extractions were conducted with two biological replicates. The DNA concentration was detected by a NanoDrop 2000 UV−vis spectrophotometer (Thermo Fisher, Wilmington, MA).

**Quantification of ARGs Copy Numbers.** The qPCR was performed on a quantitative thermocycler (CFX-96, Bio-Rad, CA) using SYBR Green I method. The primers and thermal programs for the ARGs and 16S rRNA gene quantification are listed in SI Table S4. The qPCR mixture was 20 $\mu$L, that is, 10 $\mu$L of SYBRs Premix Ex Taq (Takara, Dalian, China), 2.0 $\mu$L of template DNA, 0.4 $\mu$L of each primer, and 7.2 $\mu$L of $ddH_2O$. Standard curves were constructed as previously described.[39] Briefly, the target gene fragments were amplified from the sludge samples, separated by 1.5% agarose gel electrophoresis, purified and recovered with a QIA-quick gel extraction (Qiagen, Hilden, Germany), and then cloned into pGEM-T Easy vectors (Promega, Madison, WI). The recombinant plasmids were transformed into competent *E. coli* DH5$\alpha$ (TIANGEN, Beijing, China). Positive clones were randomly selected and checked with PCR. The plasmids extracted from the positive clones were used as standards for qPCR. 10-fold serial dilutions of the plasmid DNA were adopted to create a standard curve. The correlation coefficients of the standard curves in this study were all >0.985, with amplification efficiencies of 90.1% to 101.9%. All detections in the qPCR assay were conducted with three technical replicates.

After qPCR assay, gene quantity was evaluated in relative abundance and absolute abundance. Absolute abundance was obtained by normalizing gene copies with the volume of extracted sample to evaluate the ARGs amount in unit volume of water samples. Relative abundance was obtained by normalizing gene copies with extracted DNA amount to evaluate the relative proportion of ARGs in DNA of the samples. More specifically, the relative abundances of cell-associated genes were obtained by normalizing cell-associated gene copies with cell-associated DNA amount; the relative abundances of cell-free genes were obtained by normalizing cell-free gene copies with cell-free DNA amount; and the relative abundances of total (cell-associated plus cell-free) genes were obtained by normalizing total gene copies with total DNA amount. Moreover, the ratio of ARG abundance to 16S rRNA gene abundance was obtained by normalizing average ARG abundance of the duplicate biological samples to average 16S rRNA gene abundance of the same duplicate biological samples, which could evaluate the relative proportion of ARB in microbial community of the samples.

## RESULTS AND DISCUSSION

**DNA Content Variation along Treatment Processes in the WWTP.** The DNA content of the samples in this study are shown in Table 1. The cell-associated DNA content in the Inf was 450.6 ± 40.4 ng $mL^{-1}$ and 806.7 ± 134.2 ng $mL^{-1}$, respectively on August 10, 2016 and on March 17, 2017, whereas the cell-free DNA content in the Inf was 2.5 ± 0.4 ng $mL^{-1}$ and 5.7 ± 1.5 ng $mL^{-1}$, respectively, indicating that cell-associated DNA was the dominant DNA in the Inf with a cell-free DNA/cell-associated DNA ratio of 0.63%. In the mixtures from oxic units (P1-M, P2-M, and P3-M), the cell-associated DNA became more predominant (5657.5 ± 555.1−20843.3 ± 4077.6 ng $mL^{-1}$) compared to the cell-free DNA (3.3 ± 3.4−15.9 ± 0.4 ng $mL^{-1}$) due to the high concentration of MLSS (SI Table S2). Cell-free DNA content in P1-M, P2-M, and P3-M did not increase as much as that of cell-associated DNA, probably owing to the degradation of cell-free DNA which affected by different water properties.[38]

**Table 1. DNA Contents in the Samples**

| | August 10, 2016 | | March 17, 2017 | |
|---|---|---|---|---|
| sample | cell-associate DNA content (ng/mL) | cell-free DNA content (ng/mL) | cell-associate DNA content (ng/mL) | cell-free DNA content (ng/mL) |
| inf | 450.6±40.4 | 2.5 ± 0.4 | 806.7 ± 134.2 | 5.7 ± 1.5 |
| P1-M | 5657.5 ± 555.1 | 9.5 ± 1.6 | 13 350.0 ± 1352.9 | 7.7 ± 1.7 |
| P2-M | 8512.5 ± 1757.2 | 8.7 ± 2.1 | 12 058.3 ± 2265.1 | 3.3 ± 3.4 |
| P3-M | 10 255.0 ± 162.6 | 15.9 ± 0.4 | 20 843.3 ± 4077.6 | 7.8 ± 2.8 |
| P1-Eff | 5.4 ± 0.8 | 9.8 ± 0.0 | 12.5 ± 1.3 | 1.7 ± 0.2 |
| P2-Eff | 7.1 ± 0.6 | 9.0 ± 0.2 | 11.7 ± 1.4 | 3.2 ± 2.4 |
| P3-Eff | 1.1 ± 0.2 | | 7.5 ± 1.5 | 4.2 ± 1.0 |
| UF-Eff | 1.7 ± 0.5 | | 6.6 ± 1.1 | 1.5 ± 0.3 |
| $O_3$-Eff | 1.4 ± 0.2 | | 1.1 ± 0.3 | 1.1 ± 0.0 |
| Cl-Eff | | | 0.1 ± 0.0 | 1.6 ± 1.7 |

Owing to the good performance of secondary clarifiers, little sludge was remained in P1-Eff and P2-Eff. The cell-associated DNA content on August 10, 2016 and March 17, 2017 were 5.4 ± 0.8 ng $mL^{-1}$ and 12.5 ± 1.3 ng $mL^{-1}$ in P1-Eff, and 7.1 ± 0.6 ng $mL^{-1}$ and 11.7 ± 1.4 ng $mL^{-1}$ in P2-Eff. The cell-free DNA content in P1-Eff and P2-Eff were 9.8 ± 0.0 ng $mL^{-1}$ and 9.0 ± 0.2 ng $mL^{-1}$, respectively on August 10, 2016, which were higher than the cell-associated DNA fraction. This indicated that cell-free DNA could be the dominant DNA in the effluent from a well-operated process. The cell-free DNA content in P1-Eff and P2-Eff on March 17, 2017 was lower than that on


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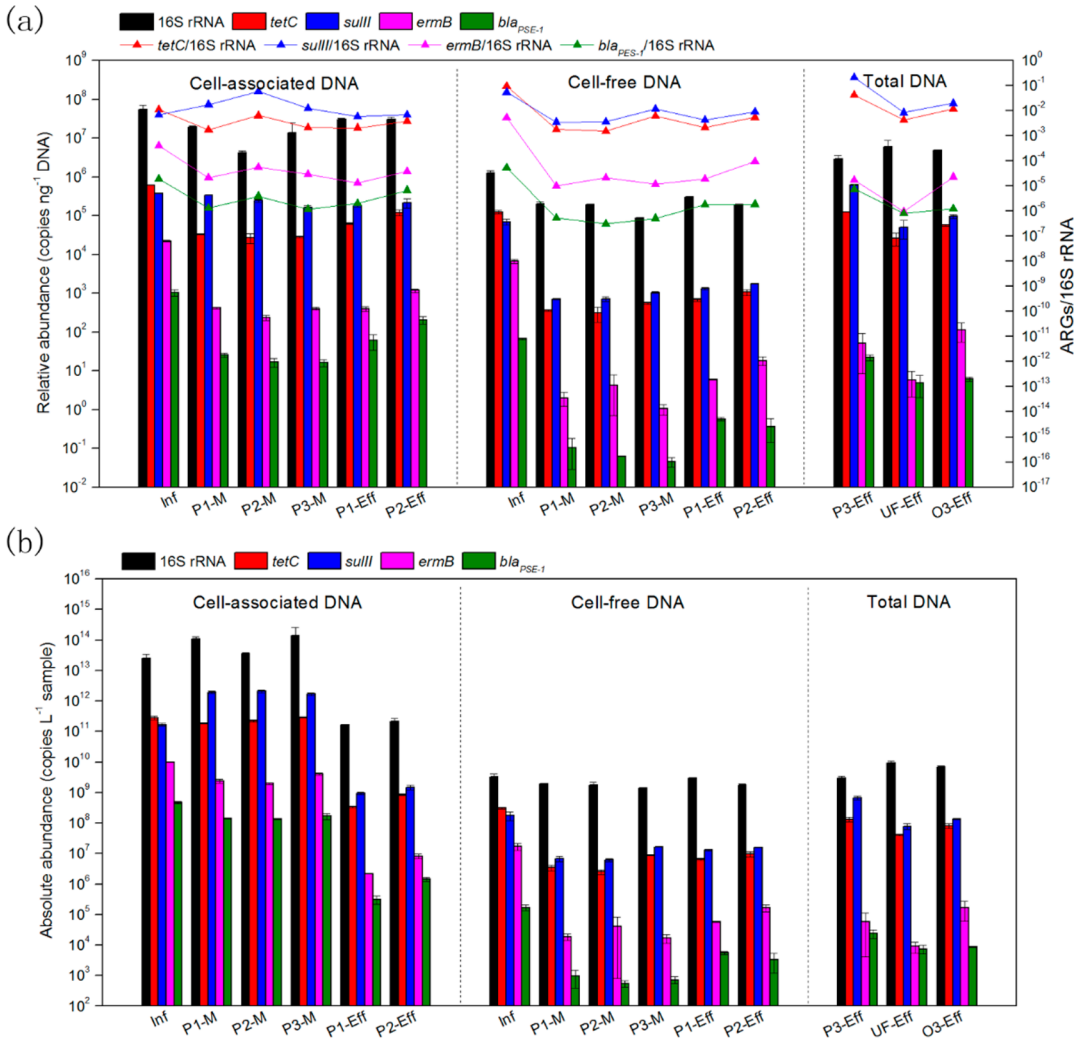


**Figure 2.** Gene abundances in the samples collected on August 10, 2016. (a) Relative abundances and the ratios of ARG abundance to 16S rRNA gene abundance; and (b) absolute abundances. *Error bars* represent standard errors of the duplicate biological samples.

August 10, 2016, but still accounted for 11.97% and 21.48% of the total DNA content in the samples.

The concentration of total DNA in P3-Eff and UF-Eff on August 10, 2016, was only 1.1 ± 0.2 ng $mL^{-1}$ and 1.7 ± 0.5 ng $mL^{-1}$, much lower than the concentration of cell-free DNA in P3-M (15.9 ± 0.4 ng $mL^{-1}$), and in P1-M (9.5 ± 1.6 ng $mL^{-1}$) and P2-M (8.7 ± 2.1 ng $mL^{-1}$). The reduced DNA in MBR effluent compared to that in the mixture of the MBR was owing to the separation and high retention capacity of membrane,[40] the same with the function of UF. On March 17, 2017, cell-associated DNA and cell-free DNA were reduced by 99.96% and 46.15% respectively by interception of the membrane in MBR. The decreased cell-associated DNA and cell-free DNA in P3-Eff and UF-Eff indicated that 0.02 $\mu$m membrane in MBR and UF intercepted not only cells but also part cell-free DNA.

However, the extracted cell-associated DNA from P3-Eff and UF-Eff on March 17, 2017 indicated that a small amount of microorganisms remained in the P3-Eff and UF-Eff, the same with previous report.[41] Ozonation can cause the direct destruction of cell wall and multiple oxidation reactions with intracellular substances, including the purines and pyrimidines of DNA, via $O_3$ and hydroxyl radicals,[42] therefore reduced 83.3% of cell-associated DNA and 26.7% of cell-free DNA in UF-Eff on March 17, 2017. Chlorination also conducts oxidation reactions, via HOCl and $OCl^-$ with lower redox potential,[43] and reduced cell-associated DNA to 0.1 ± 0.0 ng $mL^{-1}$, however, increased cell-free DNA to 1.6 ± 1.7 ng $mL^{-1}$, in Cl-Eff on March 17, 2017. The data indicated that cell-free DNA persisted in the wastewater with relatively stable content during the whole treatment processes (Table 1). The high proportion of cell-free DNA in the final effluent might be a potential risk of ARGs spreading, if high abundance ARGs existed in the cell-free DNA.

**ARGs Abundance Variation along Treatment Processes in the WWTP.** All the four target ARGs were detected in both cell-associated and cell-free fractions in the WWTP. The gene abundances in samples collected on August 10, 2016 and on March 17, 2017 are respectively shown in Figure 2 and Figure 3.

*Cell-Associated Gene Abundances.* Shown in Figure 2a and Figure 3a, the relative abundances of the four ARGs in the Inf on August 10, 2016 ($1.05 \times 10^3$-$6.17 \times 10^5$ copies $ng^{-1}$ DNA) were about 1 order of magnitude higher than that in the Inf on March 17, 2017 ($3.34 \times 10^2$-$4.79 \times 10^4$ copies $ng^{-1}$ DNA). The $bla_{PSE-1}$ showed the lowest abundance, whereas the *tetC* and *sulII* were much higher (Figure 2 and Figure 3). Tetracyclines was the first broad-spectrum antibiotic family, which has been widely used for more than 70 years,[44] therefore resulted in the



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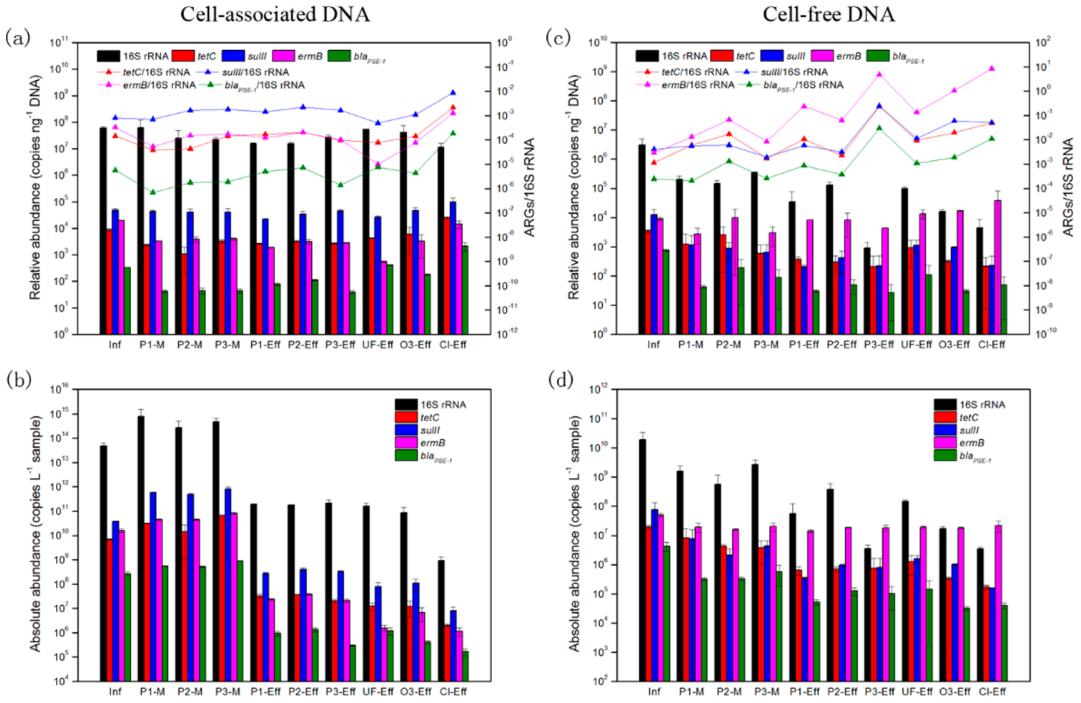


**Figure 3.** Gene abundances in the samples collected on March 17, 2017. (a) Relative gene abundances and the ratios of ARG abundance to 16S rRNA gene abundance in cell-associated DNA; (b) absolute gene abundances in cell-associated DNA; (c) relative gene abundances and the ratios of ARG abundance to 16S rRNA gene abundance in cell-free DNA; and (d) absolute gene abundances in cell-free DNA. *Error bars* represent standard errors of the duplicate biological samples.

worldwide distribution and high abundance of tetracycline resistance genes in a variety of natural and artificial environments.[45−47] The *sulII* also was an abundant ARG in surface water[48] and wastewater treatment systems.[35,49] The data showed consistent abundance variation of the cell-associated ARGs between the two set of samples. The relative abundance of *sulII* kept relatively stable with *sulII*/16S rRNA gene increased in the oxic mixtures and the biological effluent, revealed the enrichment of *sulII*-carried ARBs in the community after biological treatment. While both the relative abundances of *tetC*, $bla_{PSE-1}$, and *ermB*, and the abundance ratio of these three ARG/16S rRNA gene were reduced, indicated the competitive disadvantage of *tetC*-, *ermB*-, and $bla_{PSE-1}$-carried ARBs during biological treatment. However, the absolute abundances of *tetC*, $bla_{PSE-1}$, and *ermB* in P1-M, P2-M, P3-M were still similar to (Figure 2b) or even slightly higher than (Figure 3b) that in Inf, owing to the high MLSS in the oxic reactors. While the absolute abundance of *sulII* was about 1 order of magnitude increased in the oxic mixture (Figure 23b). As cell-associated ARGs exists in wastewater in the form of SS, a WWTP could achieve a good cell-associated ARGs removal if the SS in the treated wastewater was removed well. In this WWTP, high removal of cell-associated ARGs by biological treatment processes (1.98−3.67 logs, SI Table S5) was achieved with good performance of the secondary clarifiers in P1 and P2, and the SS interception by membrane in P3. In previous studies, two $A^2/O$ WWTPs in northern China (ARGs removal of 89.0%−99.8%),[50] an $A^2/O$ WWTP (1.16−2.44 logs) and a triple oxidation WWTP (1.10−2.25 logs)[49] also showed efficient removal of ARGs. However, negative removal rates were also reported,[41,50]and we conjecture that it might be due to high concentration of suspended sludge in the effluents.

The data of samples from March 17, 2017 showed that the relative abundance of cell-associated ARGs and ratios of ARG/16S rRNA gene in UF-Eff was $4.11 \times 10^{2}$- $2.67 \times 10^{4}$ copies $ng^{-1}$ DNA and $7.56 \times 10^{-6}$ to $4.91 \times 10^{-4}$, respectively, similar to those in P1-Eff and P2-Eff. Tertiary UF and two disinfection processes, i.e. ozonation and chlorination, were applied in this WWTP. Disinfection processes could kill or inactive microorganisms, and reduce cell-associated ARGs remained in biological effluents. In a previous study,[28] both Fenton oxidation and UV/$H_2O_2$ reduced cell-associated ARGs in the biological effluent by up to 3.5 logs. Chlorine disinfection, UV irradiation, and ozone disinfection achieved acceptable cell-associated ARGs removals by 2.98−3.24 logs, 2.48−2.74 logs, and 1.68−2.55 logs, respectively.[51] In this study, the absolute abundance of ARGs in cell-associated fraction reduced by ozonation was very limited with removal of −0.63 log -0.47 log, and chlorination removed cell-associated ARGs in the water by 0.38−1.13 logs (SI Table S5). The total removals of cell-associated ARGs by P1/P2-UF-$O_3$−Cl were 3.21 logs −4.14 logs (SI Table S5), which was satisfactory.

In Alexander's study,[23] the ozone dose was 0.9 ± 0.1 g ozone per 1g dissolved organic carbon (DOC, average 10.0 ± 2.3 mg $L^{-1}$) presented in the wastewater, the *ermB* was reduced by 2 orders of magnitude, while the removal of other three ARGs (*vanA*, *blaVIM*, and *ampC*) were −764% to 9%, which were similar to the low ARGs removal by ozonation in this study. The reaction time of chlorination in this WWTP were 5 h, which was much longer than that in Yuan's study (30 min),[22] resulting in a better ARGs removal under low dose of 4.2 mg $Cl_2$ $L^{-1}$. While only more than 60% of erythromycin resistance gene and more than 20% of tetracycline resistance gene were removed under 10.0 mg $Cl_2$ $L^{-1}$ in Yuan's study.[22] In addition, with disinfection treatment, the relative abundance of the ARGs as well as the ratios of ARG/16S rRNA gene were gradually increased from $4.11 \times 10^{2}$- $2.67 \times 10^{4}$ copies $ng^{-1}$ DNA and $7.56 \times 10^{-6}$ to $7.76 \times 10^{-4}$ in UF-Eff to $2.13 \times 10^{3}$ to $9.95 \times$


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$10^4$ copies $ng^{-1}$ DNA and $1.86 \times 10^{-4}$ to $8.69 \times 10^{-3}$ respectively in Cl-Eff. The increased ARBs in the community indicated an ARBs selection effect of disinfection processes, which has been reported under UV[20] and ozonation[23] treatment. Ferro et al.[25] also revealed increased ratio of antibiotic resistant *E. coli* to total coliforms after UV/$H_2O_2$ treatment (UV ~ 17.36 $\mu W\ cm^{-2}$; $H_2O_2$ 20 mg $L^{-1}$) within treatment time less than 60 min, however, the ratio was significantly decreased when the treatment time extended to 90 min or longer. Besides, after 40 min treatment of $O_3$ (23 mg $L^{-1}$), $O_3$+sonolysis ($O_3$ 23 mg $L^{-1}$; sonolysis 70 W $L^{-1}$), and $O_3$+sonolysis ($O_3$ 23 mg $L^{-1}$; sonolysis 100 W $L^{-1}$), no viable cells in the disinfected hospital wastewater were observed by Petri dishes inoculation, in which $O_3$+sonolysis ($O_3$ 23 mg $L^{-1}$; sonolysis 100 W $L^{-1}$) reduced cell-associated DNA by 81.4%. A highly rigorous disinfection is needed to effectively remove cell-associated ARGs.

*Cell-Free Gene Abundances.* The persistence of cell-free ARGs in the wastewater during treatment was revealed in this study (Figure 2 and Figure 3). On August 10, 2016, the relative abundances of cell-free ARGs in P1-M, P2-M, P3-M, P1-Eff, and P2-Eff, were generally 1−3 orders of magnitude lower than that in cell-associated DNA. The reduced relative abundances of *tetC*, *sulII*, and $bla_{PSE\text{-}1}$ in the samples from March 17, 2017 were similar to that on August 10, 2016, except that the relative abundance of *ermB* kept relative stable. Mainly originated from dead cell, the gene composition in new-generated eDNA should be similar to that in iDNA. However, open duplexes DNA is more adsorptive than supercoil DNA owing to the higher availability of surface charges and molecule flexibility,[52−54] and the adsorbed DNA was more resistant against degradation by DNase I.[52] Adsorption selectivity and unequal DNase I resistance could induce different genetic composition between cell-free DNA and cell-associated DNA. In addition, due to fracture and/or incomplete degradation of gene, some DNA fragments could not be detected by gene targeted PCR but were measurable by UV spectrophotometer, which reduced the relative abundance of cell-free ARGs. From the absolute abundance of the ARGs in cell-free DNA (middle column of Figure 2b, Figure 3d), it could be seen that the ARGs in cell-free DNA in the wastewater was substantially reduced by biological treatment.

As cell-free DNA of P3-Eff, UF-Eff, and $O_3$-Eff from August 10, 2016 was not separately extracted, the gene abundances in total DNA in these samples are presented in Figure 2 (right column). The ozonation did not show a ARGs removal, as the absolute abundance of ARGs in $O_3$-Eff was higher than that in UF-Eff. Whereas the data from March 17, 2017 showed that the relative abundances of *tetC*, *sulII*, and $bla_{PSE\text{-}1}$ in cell-free DNA were reduced to a certain extent with treatment of ozonation and chlorination, however, that of *ermB* increased slightly (Figure 3b). The reason for the stable relative abundance of *ermB* in cell-free DNA along the whole treatment processes was unknown. The cell-free ARGs removals of ozonation, and chlorination on March 17, 2017 were 0.03 log −0.65 log, and −0.09 log −0.81 log (shown in SI Table S5), respectively, which were much lower than that of biological treatment processes (0.43 log -2.34 logs). The removals of cell-free ARGs by the whole combined process of P1/P2-UF-$O_3$−Cl were 0.36 log -2.68 logs, much lower than cell-associated ARGs removals. The lower removal of cell-free ARGs than cell-associated ARGs resulted in an increased absolute abundance ratio of cell-free ARGs to cell-associated ARGs, which increased from 0.04%−0.17% in Inf to 1.35%−2.76% in P1-Eff and 0.23%−1.99% in P2-Eff on August 10, 2016, and increased from 0.20%−1.59% in Inf to 0.13%−85.93% in P1-Eff, P2-Eff, and P3-Eff, and further to 2.00%−1895.08% in Cl-Eff on March 17, 2017 (shown in Table 2). The difficulty of cell-free ARGs reduction

**Table 2. Absolute Abundance Ratios of Cell-Free ARGs to Cell-Associated ARGs in the Samples from the WWTP**

| | sample | *tetC* | *sulII* | *ermB* | $bla_{PSE\text{-}1}$ |
|---|---|---|---|---|---|
| August 10, 2016 | Inf | 0.11% | 0.11% | 0.17% | 0.04% |
| | P1-Eff | 1.97% | 1.35% | 2.76% | 1.77% |
| | P2-Eff | 1.13% | 1.06% | 1.99% | 0.23% |
| March 17, 2017 | Inf | 0.28% | 0.20% | 0.32% | 1.59% |
| | P1-Eff | 1.94% | 0.13% | 60.36% | 5.41% |
| | P2-Eff | 1.92% | 0.24% | 50.55% | 9.43% |
| | P3-Eff | 3.64% | 0.24% | 85.93% | 34.59% |
| | UF-Eff | 10.15% | 2.00% | 1244.70% | 11.91% |
| | $O_3$-Eff | 2.75% | 0.95% | 269.79% | 7.96% |
| | Cl-Eff | 8.57% | 2.00% | 1895.08% | 23.50% |

may be attributed to two possible reasons: first, as dissolved in wastewaters, cell-free ARGs are hard to be reduced by settling or membrane filtration; second, cell-free ARGs was mainly released by lysed microorganisms. As long as there are ARBs in wastewaters, cell-free ARGs will inevitable generate after cell lysis of dead ARBs. From this perspective, cell-associated ARGs removal is the priority step prior to cell-free ARGs control. In addition, ARBs can spread ARGs via conjugation, transduction, and transformation. While cell-free ARGs can disseminate ARGs via transformation only, which means cell-free ARGs may less threatening than cell-associated ARGs. Nevertheless, a watchful eye should be kept on cell-free ARGs, especially after ARBs removal or inactivation by WWTP.

Advanced oxidation processes (AOPs) has been reported to effectively disinfect microorganisms and reduce ARBs.[20,23−28,51,55] However, most of the above-mentioned studies neglected the variation of cell-free ARGs during disinfection. Antibiotic resistant *E. coli* was reduced by more than 4 logs with UV/$H_2O_2$ treatment, however, the $bla_{TEM}$ abundance in total DNA only decreased by less than 1 log.[26] Ferro Giovanna et al.[25] found stable or increased abundance of $bla_{TEM}$ and *qurS* in total DNA of UV/$H_2O$ treated samples, though the abundances of the two ARGs in intracellular DNA were decreased after disinfection. These reports indicated that though ARBs could be effectively reduced by AOPs, ARGs in cell-free DNA might increase during disinfection and resulted in faint changes of ARGs abundance in total DNA. In this study, the improved cell-free ARGs proportion might increase the spread risk of ARGs via cell-free sources. Although there was an opinion that screening of ARG pools overestimated the risk of antibiotic resistance transmission from environments to humans, ARB based assessment should be applied to evaluate the antibiotic resistance transmission to humans.[56] However, the threat posed by ARBs will be aggravated by the capacity to disseminate ARGs via cell-free DNA by HGT was recognized.[1,29,56] The increased ratio of cell-free ARGs/cell-associated ARGs in disinfected water, as well as the high ratio (0.23−85.93%) in biological effluents, indicated that the ARGs spreading risk via cell-free DNA transformation might play an important role in ARGs proliferation after treated wastewater discharge, especially for the disinfected one. Though the


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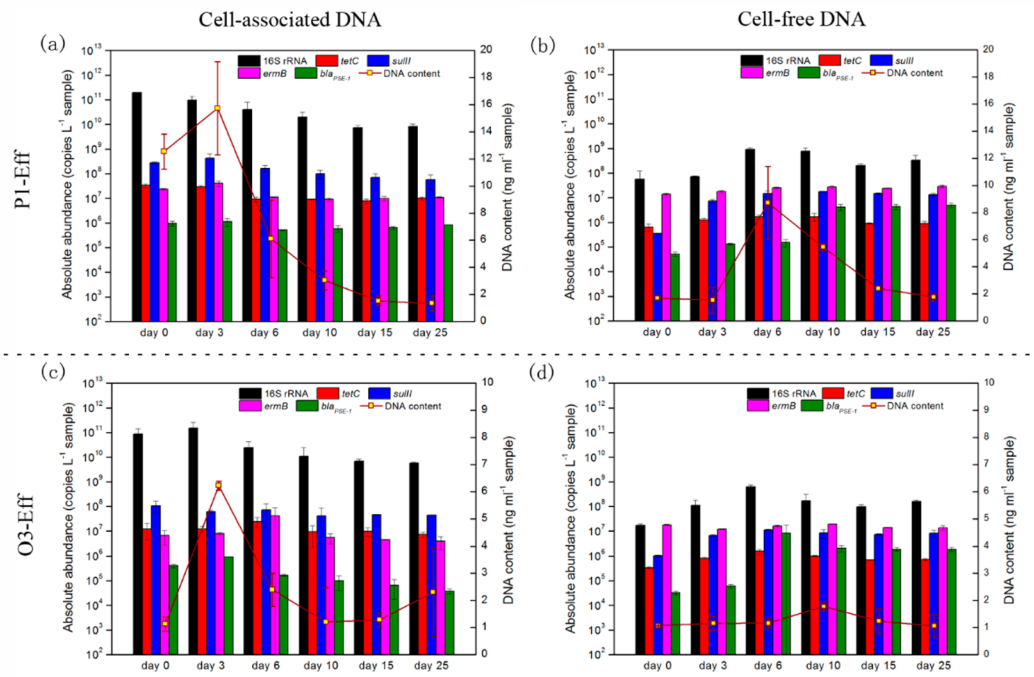


**Figure 4.** Variations of absolute gene abundance in (a) cell-associated DNA in P1-Eff; (b) cell-free DNA in P1-Eff; (c) cell-associated DNA in $O_3$-Eff; and (d) cell-free DNA in $O_3$-Eff during storage. Error bars represent standard errors of the duplicate biological samples.

removal of cell-free ARGs by AOPs has not been reported, considering the strong oxidizing ability, AOPs could probably be potential methods for cell-free ARGs control. In addition, adsorption of cell-free DNA on montmorillonite, kaolinite, Illite, and biochar have been reported, with capacity of 0.04−38 mg/g, 0.2−4.9 mg/g, 0.4−1.2 mg/g, and 1.89−5.12 mg/g, respectively.[57−59] However, further investigation is needed on whether adsorption could be an efficient process for cell-free ARGs removal from disinfected effluent laden with low cell-free DNA concentration. Cell-free ARGs also could be degraded by DNA-degrading enzymes, for example DNase, however, application of DNA-degrading enzymes in WWTP would be expensive.

**ARGs Abundance Variation during Storage.** Considering the decay ratio of cell-free ARGs is an essential factor for evaluating the proliferation risk of ARGs, abundance variations of cell-associated and cell-free ARGs in the biological effluent, P1-Eff, and the disinfected effluent, $O_3$-Eff, during sample storage (in dark under 20 °C) were investigated in this study. Though the sample storage environment was quite different from effluent receiving environment, the abundance variations could, to some extent, reflect the ARGs decay tendency after effluent discharge.

During storage, cell-associated DNA in both P1-Eff and $O_3$-Eff increased in first 3 days, and gradually decreased in the following days. In P1-Eff, cell-free DNA kept relatively stable in first 3 days, then increased by 7.17 ng $mL^{-1}$ on day 6, and gradually decreased in the following days. In $O_3$-Eff, cell-free DNA also showed such an increase and decrease variation, though the maximum DNA content, appeared on day 10, was only 0.7 ng $mL^{-1}$ higher than that on day 0.

The relative abundance of the four ARGs and ratios of ARG/16S rRNA gene in cell-associated DNA in the two samples were gradually increased during storage (SI Figure S2), indicated the increase of the ARBs in the microbial community of the samples. The absolute abundances of all the four ARGs in cell-associated DNA in the two samples showed an increase followed with a decrease or relative stabilization trend during storage (Figure 4a,c). The maximum absolute abundances of the cell-associated ARGs appeared on day 3 or day 6. For cell-free fraction in P1-Eff (Figure 4b), all ARGs increased in first 6 days during storage. Subsequently, *sulII* and *ermB* kept stable during following storage days, $bla_{PSE-1}$ further increased on day 10, then kept stable in the following days, whereas *tetC* decreased after 15 days storage. In cell-free DNA of $O_3$-Eff (Figure 4d), *ermB* kept stable during 25-day-storage, whereas *tetC*, *sulII*, and $bla_{PSE-1}$ increased in the first 6 days storage and then kept relatively steady too. After 25-day-storage, cell-associated ARGs in P1-Eff and $O_3$-Eff reduced by 0.06 log -0.69 log and 0.22 log -1.03 logs, respectively, while cell-free ARGs in P1-Eff and $O_3$-Eff increased by 0.14 log -1.99 logs and −0.12 log -1.77 logs, respectively (SI Table S6). The absolute abundance ratios of cell-free ARGs to cell-associated ARGs in 25-day-stored P1-Eff and $O_3$-Eff were 8.84%−608.12% and 9.70%−5004.90% respectively (Table 3). Low decay ratio and long-term persistence of cell-free ARGs in discharged effluents were indicated. Once cell-free ARGs were discharge to the effluent receiving environment, the risk of ARGs proliferation via cell-free DNA would be in long-term. Therefore, cell-free ARGs should be reduced as much as possible before effluent discharge, while cell-associated ARGs removal is the precursor

**Table 3. Absolute Abundance Ratios of Cell-Free ARGs to Cell-Associated ARGs in the 25 Days Stored Samples**

| sample | *tetC* | *sulII* | *ermB* | $bla_{PSE-1}$ |
|---|---|---|---|---|
| P1-Eff | 8.84% | 23.58% | 268.35% | 608.12% |
| $O_3$-Eff | 9.70% | 18.87% | 350.58% | 5003.90% |



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step as cell-associated ARGs will transform to cell-free ARGs after cell lysis.

Currently, ARGs is a hot topic, and has been considered as a group of emerging contaminants,[60] which exhibits severe potential threats to human health. The export of ARGs from ARGs pools, such as WWTPs, must be controlled. Previous studies have investigated the fates of cell-associated ARGs in WWTPs[41,50] and explored the inactivation of ARBs by disinfection.[20,23,61] This study revealed the significantly increased proportion of cell-free ARGs in effluents after SS removal, especially after disinfection. The persistence of cell-free ARGs in the discharged effluent also was indicated. Therefore, this study raises a previously neglected fact that cell-free ARGs in WWTPs' effluents might be an important pollution type of ARGs. However, the transformation potential of cell-free ARGs in the receiving environment is still unknown. The evaluation of practical risk of cell-free ARGs dissemination through HGT in different effluent receiving environments is strongly suggested, and effective technologies of cell-free ARGs control, for example optimized AOPs, are also demanded.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b04283.

Table S1Main operation parameters of the biological treatment processes. Table S2: Properties of the samples. Table S3: Volume of samples used for cell-associated DNA extration. Table S4: Primers and thermal programs for gene quantification. Table S5: ARGs removal by different processes in the WWTP. Table S6: ARGs removals during water sample storage. Figure S1: Schematic dragram of cell separation and DNA extration procedure, and the cell separation filter. Figure S2: Variations of relative gene abundance and ratios of ARG/16S rRNA gene in cell-associated and cell-free DNA in P1-Eff, and cell-associated and cell-free DNA in $O_3$-Eff during storage (PDF)

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### Notes

The authors declare no competing financial interest.

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## ■ REFERENCES


(1) Andersson, D. I.; Hughes, D. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat. Rev. Microbiol.* **2010**, *8* (4), 260−271.

(2) Czekalski, N.; Sigdel, R.; Birtel, J.; Matthews, B.; Burgmann, H. Does human activity impact the natural antibiotic resistance background? Abundance of antibiotic resistance genes in 21 Swiss lakes. *Environ. Int.* **2015**, *81*, 45−55.

(3) Zhai, W. C.; Yang, F. X.; Mao, D. Q.; Luo, Y. Fate and removal of various antibiotic resistance genes in typical pharmaceutical wastewater treatment systems. *Environ. Sci. Pollut. Res.* **2016**, *23* (12), 12030−12038.

(4) Karkman, A.; Johnson, T. A.; Lyra, C.; Stedtfeld, R. D.; Tamminen, M.; Tiedje, J. M.; Virta, M. High-throughput quantification of antibiotic resistance genes from an urban wastewater treatment plant. *FEMS Microbiol. Ecol.* **2016**, *92*, (3): fiw014, DOI: 10.1093/femsec/fiw014.

(5) De Leener, E.; Martel, A.; Decostere, A.; Haesebrouck, F. Distribution of the erm(B) gene, tetracycline resistance genes, and Tn1545-like Transposons in macrolide- and lincosamide-resistant enterococci from pigs and humans. *Microb. Drug Resist.* **2004**, *10* (4), 341−345.

(6) Kim, S. R.; Nonaka, L.; Suzuki, S. Occurrence of tetracycline resistance genes tet(M) and tet(S) in bacteria from marine aquaculture sites. *FEMS Microbiol. Lett.* **2004**, *237* (1), 147−156.

(7) Caucci, S.; Karkman, A.; Cacace, D.; Rybicki, M.; Timpel, P.; Voolaid, V.; Gurke, R.; Virta, M.; Berendonk, T. U. Seasonality of antibiotic prescriptions for outpatients and resistance genes in sewers and wastewater treatment plant outflow. *FEMS Microbiol. Ecol.* **2016**, *92*, (5): fiw060, DOI: 10.1093/femsec/fiw060.

(8) Fall, S.; Mercier, A.; Bertolla, F.; Calteau, A.; Gueguen, L.; Perriere, G.; Vogel, T. M.; Simonet, P. Horizontal gene transfer regulation in bacteria as a "Spandrel" of DNA repair mechanisms. *PLoS One* **2007**, *2* (10), e1055.

(9) Luo, Y.; Xu, L.; Rysz, M.; Wang, Y. Q.; Zhang, H.; Alvarez, P. J. J. Occurrence and transport of tetracycline, sulfonamide, quinolone, and macrolide antibiotics in the Haihe river basin, China. *Environ. Sci. Technol.* **2011**, *45* (5), 1827−1833.

(10) Li, C.; Lu, J. J.; Liu, J.; Zhang, G. L.; Tong, Y. B.; Ma, N. Exploring the correlations between antibiotics and antibiotic resistance genes in the wastewater treatment plants of hospitals in Xinjiang, China. *Environ. Sci. Pollut. Res.* **2016**, *23* (15), 15111−15121.

(11) Reinthaler, F. F.; Posch, J.; Feierl, G.; Wust, G.; Haas, D.; Ruckenbauer, G.; Mascher, F.; Marth, E. Antibiotic resistance of E-coli in sewage and sludge. *Water Res.* **2003**, *37* (8), 1685−1690.

(12) da Costa, P. M.; Vaz-Pires, P.; Bernardo, F. Antimicrobial resistance in Enterococcus spp. isolated in inflow, effluent and sludge from municipal sewage water treatment plants. *Water Res.* **2006**, *40* (8), 1735−1740.

(13) Munir, M.; Wong, K.; Xagoraraki, I. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Res.* **2011**, *45* (2), 681−693.

(14) Novo, A.; Manaia, C. M. Factors influencing antibiotic resistance burden in municipal wastewater treatment plants. *Appl. Microbiol. Biotechnol.* **2010**, *87* (3), 1157−1166.

(15) Yuan, Q. B.; Guo, M. T.; Yang, J. Monitoring and assessing the impact of wastewater treatment on release of both antibiotic-resistant bacteria and their typical genes in a Chinese municipal wastewater treatment plant. *Environ. Sci. Proc. Impacts* **2014**, *16* (8), 1930−1937.

(16) Auerbach, E. A.; Seyfried, E. E.; McMahon, K. D. Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Res.* **2007**, *41* (5), 1143−1151.

(17) Zhang, T.; Zhang, M.; Zhang, X. X.; Fang, H. H. Tetracycline resistance genes and tetracycline resistant lactose-fermenting enterobacteriaceae in activated sludge of sewage treatment plants. *Environ. Sci. Technol.* **2009**, *43* (10), 3455−3460.

(18) Gao, P.; Munir, M.; Xagoraraki, I. Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. *Sci. Total Environ.* **2012**, *421*, 173−183.

(19) Proia, L.; von Schiller, D.; Sanchez-Melsio, A.; Sabater, S.; Borrego, C. M.; Rodriguez-Mozaz, S.; Balcazar, J. L. Occurrence and

persistence of antibiotic resistance genes in river biofilms after wastewater inputs in small rivers. *Environ. Pollut.* **2016**, *210*, 121−128.
(20) Huang, J. J.; Xi, J. Y.; Hu, H. Y.; Li, Y.; Lu, S. Q.; Tang, F.; Pang, Y. C. UV light tolerance and reactivation potential of tetracycline-resistant bacteria from secondary effluents of a wastewater treatment plant. *J. Environ. Sci.* **2016**, *41*, 146−153.
(21) Guo, M. T.; Yuan, Q. B.; Yang, J. Ultraviolet reduction of erythromycin and tetracycline resistant heterotrophic bacteria and their resistance genes in municipal wastewater. *Chemosphere* **2013**, *93* (11), 2864−2868.
(22) Yuan, Q.; Guo, M.; Yang, J. Genes during wastewater chlorination: Implication for antibiotic resistance control. *PLoS One* **2015**, *10* (3), e0119403.
(23) Alexander, J.; Knopp, G.; Dotsch, A.; Wieland, A.; Schwartz, T. Ozone treatment of conditioned wastewater selects antibiotic resistance genes, opportunistic bacteria, and induce strong population shifts. *Sci. Total Environ.* **2016**, *559*, 103−112.
(24) Moreira, N. F. F.; Sousa, J. M.; Macedo, G.; Ribeiro, A. R.; Barreiros, L.; Pedrosa, M.; Faria, J. L.; Pereira, M. F. R.; Castro-Silva, S.; Segundo, M. A.; Manaia, C. M.; Nunes, O. C.; Silva, A. M. T. Photocatalytic ozonation of urban wastewater and surface water using immobilized TiO2 with LEDs: Micropollutants, antibiotic resistance genes and estrogenic activity. *Water Res.* **2016**, *94*, 10−22.
(25) Ferro, G.; Guarino, F.; Castiglione, S.; Rizzo, L. Antibiotic resistance spread potential in urban wastewater effluents disinfected by UV/H2O2 process. *Sci. Total Environ.* **2016**, *560*, 29−35.
(26) Ferro, G.; Guarino, F.; Cicatelli, A.; Rizzo, L. Beta-lactams resistance gene quantification in an antibiotic resistant Escherichia coli water suspension treated by advanced oxidation with UV/H2O2. *J. Hazard. Mater.* **2017**, *323*, 426−433.
(27) Dunlop, P. S. M.; Ciavola, M.; Rizzo, L.; McDowell, D. A.; Byrne, J. A. Effect of photocatalysis on the transfer of antibiotic resistance genes in urban wastewater. *Catal. Today* **2015**, *240*, 55−60.
(28) Zhang, Y. Y.; Zhuang, Y.; Geng, J. J.; Ren, H. Q.; Xu, K.; Ding, L. L. Reduction of antibiotic resistance genes in municipal wastewater effluent by advanced oxidation processes. *Sci. Total Environ.* **2016**, *550*, 184−191.
(29) Jain, R.; Rivera, M. C.; Lake, J. A. Horizontal gene transfer among genomes: The complexity hypothesis. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96* (7), 3801−3806.
(30) Vlassov, V. V.; Laktionov, P. P.; Rykova, E. Y. Extracellular nucleic acids. *BioEssays* **2007**, *29* (7), 654−667.
(31) Stewart, G. J.; Sinigalliano, C. D. Detection of horizontal gene-transfer by natural transformation in native and introduced species of bacteria in marine and synthetic sediments. *Appl. Environ. Microb.* **1990**, *56* (6), 1818−1824.
(32) Chandrasekaran, S.; Venkatesh, B.; Lalithakumari, D. Transfer and expression of a multiple antibiotic resistance plasmid in marine bacteria. *Curr. Microbiol.* **1998**, *37* (5), 347−351.
(33) Jiang, S. C.; Paul, J. H. Gene transfer by transduction in the marine environment. *Appl. Environ. Microb.* **1998**, *64* (8), 2780−2787.
(34) Zhang, Y. P.; Snow, D. D.; Parker, D.; Zhou, Z.; Li, X. Intracellular and extracellular antimicrobial resistance genes in the sludge of livestock waste management structures. *Environ. Sci. Technol.* **2013**, *47* (18), 10206−10213.
(35) Tao, W. D.; Zhang, X. X.; Zhao, F. Z.; Huang, K. L.; Ma, H. J.; Wang, Z.; Ye, L.; Ren, H. Q. High levels of antibiotic resistance genes and their correlations with bacterial community and mobile genetic elements in pharmaceutical wastewater treatment bioreactors. *PLoS One* **2016**, *11* (6), e0156854.
(36) Ficetola, G. F.; Miaud, C.; Pompanon, F.; Taberlet, P. Species detection using environmental DNA from water samples. *Biol. Lett.* **2008**, *4* (4), 423−425.
(37) Eichmiller, J. J.; Miller, L. M.; Sorensen, P. W. Optimizing techniques to capture and extract environmental DNA for detection and quantification of fish. *Mol. Ecol. Resour.* **2016**, *16* (1), 56−68.
(38) Eichmiller, J. J.; Best, S. E.; Sorensen, P. W. Effects of temperature and trophic state on degradation of environmental DNA in lake water. *Environ. Sci. Technol.* **2016**, *50* (4), 1859−1867.
(39) Bai, Y.; Sun, Q.; Wen, D.; Tang, X. Abundance of ammonia-oxidizing bacteria and archaea in industrial and domestic wastewater treatment systems. *FEMS Microbiol. Ecol.* **2012**, *80* (2), 323−330.
(40) Zielinska, M.; Cydzik-Kwiatkowska, A.; Bulkowska, K.; Bernat, K.; Wojnowska-Baryla, I. Treatment of Bisphenol A-Containing Effluents from Aerobic Granular Sludge Reactors with the Use of Microfiltration and Ultrafiltration Ceramic Membranes. *Water, Air, Soil Pollut.* **2017**, *282*, 228.
(41) Wang, J. L.; Mao, D. Q.; Mu, Q. H.; Luo, Y. Fate and proliferation of typical antibiotic resistance genes in five full-scale pharmaceutical wastewater treatment plants. *Sci. Total Environ.* **2015**, *526*, 366−373.
(42) Sousa, J. M.; Macedo, G.; Pedrosa, M.; Becerra-Castro, C.; Silva, S. C.; Pereira, M. F. R.; Silva, A. M. T.; Nunes, O. C.; Manaia, C. M. Ozonation and UV254 nm radiation for the removal of micro-organisms and antibiotic resistance genes from urban wastewater. *J. Hazard. Mater.* **2017**, *323*, 434−441.
(43) Sharma, V. K.; Johnson, N.; Cizmas, L.; McDonald, T. J.; Kim, H. A review of the influence of treatment strategies on antibiotic resistant bacteria and antibiotic resistance genes. *Chemosphere* **2016**, *150*, 702−714.
(44) Roberts, M. C. Update on acquired tetracycline resistance genes. *FEMS Microbiol. Lett.* **2005**, *245* (2), 195−203.
(45) Donato, J. J.; Moe, L. A.; Converse, B. J.; Smart, K. D.; Berklein, F. C.; McManus, P. S.; Handelsman, J. Metagenomic analysis of apple orchard soil reveals antibiotic resistance genes encoding predicted bifunctional proteins. *Appl. Environ. Microb.* **2010**, *76* (13), 4396−4401.
(46) Glad, T.; Kristiansen, V. F.; Nielsen, K. M.; Brusetti, L.; Wright, A.-D.; Sundset, G. M. A. Ecological characterisation of the colonic microbiota in Arctic and sub-Arctic seals. *Microb. Ecol.* **2010**, *60* (2), 320−330.
(47) Rahman, M. H.; Sakamoto, K. Q.; Nonaka, L.; Suzuki, S. Occurrence and diversity of the tetracycline resistance gene tet(M) in enteric bacteria of Antarctic adelie penguins. *J. Antimicrob. Chemother.* **2008**, *62* (3), 627−628.
(48) Pei, R.; Kim, S.-C.; Carlson, K. H.; Pruden, A. Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res.* **2006**, *40* (12), 2427−2435.
(49) Li, J. N.; Cheng, W. X.; Xu, L. K.; Jiao, Y. N.; Baig, S. A.; Chen, H. Occurrence and removal of antibiotics and the corresponding resistance genes in wastewater treatment plants: effluents' influence to downstream water environment. *Environ. Sci. Pollut. Res.* **2016**, *23* (7), 6826−6835.
(50) Mao, D. Q.; Yu, S.; Rysz, M.; Luo, Y.; Yang, F. X.; Li, F. X.; Hou, J.; Mu, Q. H.; Alvarez, P. J. J. Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants. *Water Res.* **2015**, *85*, 458−466.
(51) Zhuang, Y.; Ren, H. Q.; Geng, J. J.; Zhang, Y. Y.; Zhang, Y.; Ding, L. L.; Xu, K. Inactivation of antibiotic resistance genes in municipal wastewater by chlorination, ultraviolet, and ozonation disinfection. *Environ. Sci. Pollut. Res.* **2015**, *22* (9), 7037−7044.
(52) Romanowski, G.; Lorenz, M. G.; Wackernagel, W. Adsorption of plasmid dna to mineral surfaces and protection against DNase-I. *Appl. Environ. Microb.* **1991**, *57* (4), 1057−1061.
(53) Poly, F.; Chenu, C.; Simonet, P.; Rouiller, J.; Monrozier, L. J. Differences between linear chromosomal and supercoiled plasmid DNA in their mechanisms and extent of adsorption on clay minerals. *Langmuir* **2000**, *16* (3), 1233−1238.
(54) Melzak, K. A.; Sherwood, C. S.; Turner, R. F. B.; Haynes, C. A. Driving forces for DNA adsorption to silica in perchlorate solutions. *J. Colloid Interface Sci.* **1996**, *181* (2), 635−644.
(55) Calero-Caceres, W.; Muniesa, M. Persistence of naturally occurring antibiotic resistance genes in the bacteria and bacteriophage fractions of wastewater. *Water Res.* **2016**, *95*, 11−18.
(56) Manaia, C. M. Assessing the risk of antioiotic resistance transmission from the environment to humans: Non-direct


DOI: 10.1021/acs.est.7b04283
*Environ. Sci. Technol.* 2018, 52, 248−257

proportionality between abundance and risk. *Trends Microbiol.* **2017**, *25* (3), 173−181.
(57) Yu, W. H.; Li, N.; Tong, D. S.; Zhou, C. H.; Lin, C. X.; Xu, C. Y. Adsorption of proteins and nucleic acids on clay minerals and their interactions: A review. *Appl. Clay Sci.* **2013**, *80*−*81*, 443−452.
(58) Wang, C.; Wang, T.; Li, W.; Yan, J.; Li, Z.; Ahmad, R.; Herath, S. K.; Zhu, N. Adsorption of deoxyribonucleic acid (DNA) by willow wood biochars produced at different pyrolysis temperatures. *Biol. Fertil. Soils* **2014**, *50* (1), 87−94.
(59) Gardner, C. M.; Gunsch, C. K. Adsorption capacity of multiple DNA sources to clay minerals and environmental soil matrices less than previously estimated. *Chemosphere* **2017**, *175*, 45−51.
(60) Pruden, A.; Pei, R. T.; Storteboom, H.; Carlson, K. H. Antibiotic resistance genes as emerging contaminants: Studies in northern Colorado. *Environ. Sci. Technol.* **2006**, *40* (23), 7445−7450.
(61) Yuan, Q. B.; Guo, M. T.; Yang, J. Fate of antibiotic resistant bacteria and genes during wastewater chlorination: implication for antibiotic resistance control. *PLoS One* **2015**, *10* (3), e0119403.



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