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RESEARCH ARTICLE

Profiling of intracellular and extracellular antibiotic resistance genes in municipal wastewater treatment plant and their effluent-receiving river

Peiyuan Deng[1] · Xiaojia Hu[2] · Wentao Cai[3] · Zuoxu Zhang[4] · Yuli Zhang[5] · Yihe Huang[1] · Yingying Yang[1] · Changkan Li[1] · Shu Ai[2]

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Abstract

The presence of antibiotic resistance genes (ARGs) and heavy metal resistance genes (MRGs) in extracellular and intracellular DNA (eDNA and iDNA) has received considerable attention in recent years owing to the potential threat to human health and the ecosystem. As a result, we investigated six ARGs, three MRGs, and two mobile genetic elements (MGEs) in the municipal wastewater treatment plant (MWWTP) and its adjacent environments. Results revealed that the absolute abundances of eARGs and eMRGs were lower than iARGs and iMRGs in MWWTP. By contrast, eARGs and eMRGs were higher in river sediments. Among ARGs, aminoglycoside resistance genes (aadA) was the most abundant gene (3.13×10^2 to 2.31×10^6 copies/mL in iDNA; 1.27×10^3 to 7.23×10^5 copies/mL in eDNA) in MWWTP, while *zntA* gene (9.4×10^2 to 3.97×10^6 copies/mL in iDNA; 3.2×10^3 to 6×10^5 copies/mL in eDNA) was amongst the MRGs. Notably, *intI1* was enriched and positively correlated with iDNA (*tetA*, *sul1*, *blaCTX-M*, *ermB*, and *merA*) and eDNA (*blaCTX-M*, *ermB*, and *merA*), demonstrating its function in the proliferation of resistance genes. This widespread distribution of ARGs, MRGs, and MGEs in MWWTP and its adjacent river sediments will help clarify the transmission routes within these environments and provide a theoretical basis for better monitoring and mitigation of such dissemination.

Keywords Antibiotic resistance genes · Heavy metal resistance genes, Extracellular DNA · Municipal wastewater treatment plants · River sediment

Introduction

In recent years, antimicrobial agents have been widely used in human medicine and veterinary practices, resulting in the evolution of antibiotic-resistant bacteria (ARB) and corresponding ARGs (Kumar et al. 2019). As a consequence, xenogenic pollution has evolved into a worldwide ecological and public health issue. Examining the risks associated with the transmission and growth of pathogens, ARGs, and MGEs across waterways and ecosystems under is ongoing. According to reports, infection-related deaths are more than 700,000 each year worldwide, and the predicted antimicrobial-resistant infection numbers could reach 10 million by 2050 (O'Neill 2016). Therefore, the increasing prevalence of antibiotic resistance has been considered as a major global challenge and threat to human health (Matthiessen et al. 2016). The World Health Organization (WHO) Global Action Plan has emphasized the urgency to monitor

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✉ Shu Ai
yuanajhj@163.com

[1] Henan Engineering Research Center of Bird-Related Outage, Zhengzhou Normal University, Zhengzhou 450044, Henan, China

[2] School of Environmental and Municipal Engineering, North China University of Water Resources and Electric Power, Zhengzhou 450046, Henan, China

[3] Ural Institute, North China University of Water Resources and Electric Power, Zhengzhou 450045, Henan, China

[4] School of Environment, Beijing Jiaotong University, Beijing 100044, China

[5] Zhengzhou Veterinary Drug Feed Quality and Safety Inspection Center, Zhengzhou 450052, Henan, China

the prevalence and spread of antibiotic resistance in various settings (Yin et al. 2021).

Several research have looked into ARGs in MWWTP that confer resistance to a broad range of antibiotics, including β-lactam, fluoroquinolones, macrolides, sulfonamides, and tetracyclines, but few have looked into their persistence and distribution in iDNA and eDNA. Municipal wastewater treatment plants (MWWTP) are considered as the dominant reservoirs of ARB and ARGs and play an important role in altering the magnitude and distribution of ARGs in the receiving environments (Di Cesare et al. 2016; Mokracka et al. 2012; Qiao et al. 2018). Previous studies have also demonstrated that the suitable biotic (enzymatic degradation, microbial community structure, nutrients, etc.) and abiotic (temperature, pH, water content, adsorbent, etc.) conditions in MWWTP contribute to the prevalence and persistence of iARGs and eARGs (Barnes et al. 2014; Hao et al. 2019; Zhang et al. 2013). Municipal wastewater treatment plants have the ability to eliminate ARBs; nonetheless, the residual intact DNA may persist even after ARBs are entirely deactivated by the process of disinfection. Previously, *intI*1 gene concentrations in eDNA tend to range from 1.74×10^9 to 2.59×10^9 copies/g dw in sludge samples and 6.34×10^6 copies/g dw in lake sediment samples, respectively, whereas in iDNA, concentration levels varied from 3.0×10^8 to 1.22×10^{11} copies/g dw in sludge samples and 7.37×10^7 copies/g dw in lake sediment samples, respectively (Dong et al. 2019). Furthermore, it was observed that the abundance of *ermB* in sediment samples was consistent in iDNA and eDNA (Dong et al. 2019). Similarly, the occurrence of sulfonamides and tetracyclines resistance genes were reported in sludge samples as reported by Li et al. (2021). The prevalence of eARGs and integrons, combined with concentrations of antibiotics and bacteria in environmental settings, may encourage the spread of resistance, posing health concerns to people through numerous routes.

Antibiotic resistance genes (ARGs) may be present in the natural environment as intracellular (iARGs) or extracellular (eARGs) that participate in the horizontal gene transfer (HGT) process (Mao et al. 2014). The horizontal gene transfer of ARGs facilitates the spread of ARBs through mobile genetic elements (MGEs) such as transposons, integrons, plasmids, and bacteriophages (Mao et al. 2014). Intracellular ARGs (iARGs) may accelerate the prevalence of antibiotic resistance via conjugation (cell–cell contact) or transduction (phage infection). On the other hand, eDNA originating from cell auto-secretion, death, lysis, predation, phage infection, etc. represents a significant proportion of the total DNA (Hao et al. 2019; Vlassov et al. 2007) and are assimilated by bacteria through transformation (Guo et al. 2018; Liu et al. 2018; Zarei-Baygi & Smith 2021).

Previous studies have demonstrated that eDNAs in sediments are generally more persistent and easier to propagate than in water, which might be attributed to the adsorption of clay particles, sand, and organic matter by eDNA (Mao et al. 2014; Zhao et al. 2020). The adsorption concentration of eDNA was up to 10^3 μg/g in the soil colloids and particles, and eDNA could persist in the sediment for months and even years (Dong et al. 2019; Poly et al. 2000), thereby accelerating the dissemination and proliferation of ARGs in these environments. Therefore, differential separation and thorough analysis of iDNA and eDNA are required to explain the processes behind the transmission of these replicating mobile DNA pollutants and their distribution in different environments.

However, little information is available on the distribution of iARGs and eARGs in MWWTP and its receiving environments. A recent study has also shown that chlorine disinfection could increase both iARGs and eARGs in MWWTP (Liu et al. 2018). Generally, the processes of disinfection in MWWTP could kill ARB, and iARGs associated with iDNA could be released into water and adsorbed by the competent non-resistant bacteria, resulting in the dissemination of antibiotic resistance (Liu et al. 2018). Therefore, it is crucial to investigate the prevalence of ARGs in MWWTP and the fate of ARGs in the receiving environments directly or indirectly associated with the dissemination and proliferation of ARGs.

Recently, the extraction of DNA (iDNA, eDNA) and the occurrence of ARGs (iARGs, eARGs) have become the research hotspots (Mao et al. 2014; Zarei-Baygi and Smith 2021; Zhang et al. 2017), because the knowledge of the abundance and distribution of heavy metal resistance genes (iMRGs, eMRGs) in MWWTP and its adjacent river is still lacking. Therefore, the objective of this study is as follows: (1) to verify the occurrence of ARGs and MRGs in MWWTP and their receiving environments (river sediments); (2) to quantify the concentration of eDNA and iDNA and their corresponding ARGs and MRGs; (3) to investigate the correlation between ARGs, MRGs, and MGEs. The study results will provide new insights into the diversity and abundance of ARGs and MRGs in MWWTP and its receiving environments.

Materials and methods

Sample collection

Samples (i.e., the raw influent, final effluent, and activated sludge in the aeration tank) were collected from one tertiary MWWTP located in Zhengzhou City, Henan Province, China, which has a total treatment capacity of 6×10^5 m^3/day. Treatment processes in MWWTP consists of grit chamber, primary settlement, anaerobic-anoxic-aerobic (A/A/O) process, secondary settlement, coagulation, settlement, sand filtration, and disinfection by NaClO. The average flow rate

of treated wastewater is 350,000 m^3/day. In the receiving river, the top ~ 20 cm of the sediment samples were obtained from approximately 600 m upstream (UM) and downstream (DM) of the MWWTP discharge points.

The wastewater and solid samples were collected independently, and the sampling campaigns were conducted on three different days (between March and July 2020). Composite samples of influent wastewater were collected. Approximately 1 L of wastewater was collected every 3 h for a period of 15 h, and the hydraulic retention time (HRT) of the MWWTP was taken into account to eliminate the impact of hydraulic loading changes throughout sample periods. Similarly, a total of 5 L flow-proportionate effluent samples were collected as wastewater samples every 3 h to avoid the effect of hydraulic loading fluctuations during the sampling periods. Bacteria have a high rate of survival in natural environments, and they cannot withstand various treatment process. In this study, bacteria were more prevalent in the influent; however, it is likely that some bacteria will survive after passing through various processes of tertiary municipal wastewater treatment plant (MWWTP), and they may be discharged into the effluents. In order to extract sufficient quantity and quality of DNA for the analysis, we took a larger amount of effluents sample, i.e., a total of 5 L for the extraction of DNA compared to influents. Approximately 500 g of solid subsamples were collected from the river sediment samples. All the samples were collected in sterile containers, chilled in the icebox, and transported to the laboratory for immediate processing.

Extraction of iDNA and eDNA

iDNA and eDNA were extracted from the wastewater and sludge samples according to the previously reported method (Dong et al. 2019; Mao et al. 2014) with minor modifications (data provided in supplementary information). In general, 20 mL of influent (Inf), 200 mL of effluent (Eff), 10 mL of the activated sludge in the aeration tank (Aet), and 2.5 g (dry weight, calculated by water content) of the river sediment were used for DNA extraction. The sample was added to 4–15 mL of NaH_2PO_4 (0.12 M, pH 8.0) and 0.2–1 g of polyvinylpolypyrrolidone (PVPP), and then the mixture was shaken at 250 rpm for 10 min and centrifuged at 10,000 g, 4 °C for 10 min. The resulting solution was filtered through a sterile filtration membrane of 0.22-μm pore size (PVDF, Millipore, USA). The membrane and the above residues on filter were combined and used for the extraction of iDNA, while the filtrate was used for the extraction of eDNA. The iDNA was extracted using the FastDNA® SPIN Kit Soil (MP Biomedicals, USA), and the eDNA was extracted using the TIANamp Bacteria DNA Kit (TIANGEN, China) according to the manufacturer's instructions. The extraction of eDNA and iDNA from the same sample was replicated multiple times, and the extracted DNA was combined for further analysis. The quality and quantity of the extracted DNA were evaluated using 1% agarose gel electrophoresis and NanoDrop One^C (Thermo Scientific, USA). All the DNA samples were stored at − 20 °C until analysis.

Quantification of ARGs and MGEs

The resistance genes considered in this study were frequently detected as antibiotic resistance genes (*tet*A, *sul*1, *aad*A, bla_{CTX-M}, *erm*B, *qnr*A), heavy metal resistance genes (*zntA*, *merA*, *czcA*), the integrase genes (*int*I1 and *int*I2), and the 16S rRNA genes. The primers and amplification reactions of the determined genes are listed in Table S1. The reactions were conducted by an Accurate96 qPCR System (Drawell, China) using the AceQ® qPCR SYBR Green Master Mix (Vazyme, Nanjing, China). Plasmids (*E. coli* DH5a) containing the target gene fragments were constructed with the pMD™19-T vector (Takara, Japan) and used as controls for qPCR standard curves. The detailed procedure used for DNA extraction, qPCR conditions, and ARGs quantification was adopted from previous study (Wang et al. 2019). Briefly, the qPCR was performed in 20-μL reaction mixtures, which contained 10 μL of AceQ SYBR® Green Master Mix, 0.2 mM each primer, and 2 μL of DNA template. The amplification reaction was as follows: initial denaturation at 95 °C for 5 min and 40 cycles consisting of 95 °C for 10 s, the annealing temperature for 15 s, and 72 °C for 15 s. The standard curves for the qPCR primer sets covered at least five orders of magnitude in concentration ($R^2 \geq 0.99$) (Wang et al. 2019).

Data analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS), the IBM version 22 software, and the GraphPad Prism software 6.01 (La Jolla, CA, USA). Network analysis based on Spearman analysis between ARGs and MRGs as well as MGEs was determined using Gephi (version 0.9.2). The paired sample *t*-test was used to assess the significance of the differences between different samples based on the *p*-values.

Results and discussion

Concentrations of eDNA and iDNA

The average concentrations of the detected iDNA and eDNA in the samples are depicted in Fig. 1 and Table S2. The concentration of iDNA in the influents, aeration tank, and effluents of MWWTP was 274.11 ± 18.38, 1471.93 ± 193.28, and 4.34 ± 0.31 ng/mL, respectively, whereas the eDNA

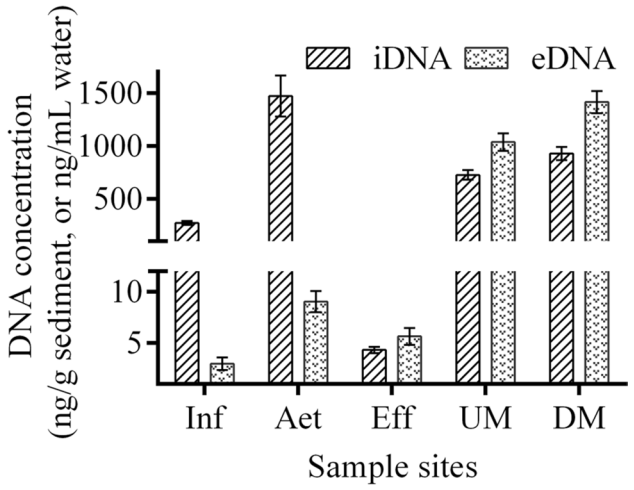

Fig. 1 The concentration of iDNA (intracellular DNA) and eDNA (extracellular DNA) in municipal wastewater treatment plants (the mean ± SD of three independent replicates of experiments). *Inf = Influent; Aet = Aeration Tank; Eff = Effluent; UM = Upstream; DM = Downstream

content was 2.98 ± 0.62, 9.05 ± 1.03, and 5.56 ± 0.83 ng/mL. Although the ratios of eDNA to the total DNA in the effluents of MWWTP (56.57%) was higher than the influents (1.08%) and aeration tank (0.61%), the concentration of the total DNA in the effluents was reduced by 96.4% and 99.32%. The sharp decreased of iDNA and eDNA in the effluents indicated that MWWTP had a good cell and DNA removal efficiency. However, the extracted iDNA from the effluent indicated that a small number of microorganisms remained in the effluent, which was consistent with the previous study results (Yang et al. 2014). The eDNA yields from the effluents were higher than the influent and aeration tank of MWWTP, which might be due to microbial death by MWWTP disinfection, resulting in the release of DNA molecules and resisting the effluents. Therefore, MWWTP was considered as a dominant reservoir of eDNA, and the effluents were directly discharged into the receiving rivers, affecting the river sediments.

The amount of iDNA in the sediments collected from the downstream of MWWTP (929.33 ± 62.41 ng/g dried sediment) was approximately 1.3-folds higher than that in the sediments from the downstream of MWWTP (725.91 ± 45.46 ng/g). Additionally, the eDNA content in the sediments from upstream (1035.79 ± 83.84 ng/g) and downstream (1413.27 ± 104.19 ng/g) of MWWTP was higher than iDNA, confirming that the sediments could serve as a dominant reservoir for eDNA. This result was consistent with the previous study results (Mao et al. 2014). This might be attributed to the fact that the majority of the eDNA in aquatic sediments is bound and absorbed by complex organic molecules, large particles, and clay, decreasing the susceptibility to nuclease attack (Nielsen et al. 2007). Apparently, the persistence of large amounts of eDNA in the sediments is a source of nutrients and gene pools for bacteria, which might provide a source of nitrogen and phosphorous and/or exogenous genetic material and contribute to DNA repair and the horizontal gene transfer through natural transformation, protecting against nuclease degradation (Tani and Nasu 2010; Vlassov et al. 2007).

Occurrence of ARGs in eDNA and iDNA

The absolute abundances of six ARGs, including one tetracycline resistance gene (*tet*A), one sulfonamide resistance gene (*sul*1), one aminoglycoside resistance gene (*aad*A), one β-lactam resistance gene ($bla_{\text{CTX-M}}$), two integrase genes (*int*I1, *int*I2), and 16S rRNA gene, were quantified by qPCR. All the six target ARGs were detected in eDNA and iDNA in the MWWTP, while *int*I2 and $bla_{\text{CTX-M}}$ were absent in the effluents of MWWTP (Fig. 2). The aminoglycoside resistance genes (*aad*A) were the most abundant ARGs in MWWTP, with the concentrations ranging from 3.13×10^2 to 2.31×10^6 copies/mL in iDNA and 1.27×10^3 to 7.23×10^5 copies/mL in eDNA, which were closely associated with integrons, promoting horizontal ARG gene transfers (Laroche et al. 2009). The above results were consistent with the previous study results (Zhao et al. 2020).

The absolute abundances of eARGs were usually 1–2 orders of magnitude lower than that of iARGs in the influent and aeration tank samples, while eARGs in the effluent of MWWTP were higher than iARGs. The abundance of most of the eARGs was significantly increased in the effluent of MWWTP, which might be attributed to the disinfection process by NaClO that killed many bacteria in MWWTP and allowed the release of free DNA into the environment from the dead and lysed bacteria (Liu et al. 2018; Zhang et al. 2017). It is worth noting that MWWTP had better removal efficiency for $bla_{\text{CTX-M}}$ (4.54-log), *tet*A (4.47-log), *sul*1 (4.07-log), *aad*A (4.02-log), and *erm*B (3.64-log). The results suggested that the incomplete removal efficiency from MWWTP could increase the abundance of ARGs in MWWTP effluent (Jelic et al. 2011; Mao et al. 2015) and further affect the receiving river. Notably, most ARGs, including iARGs and eARGs, showed higher abundance in the sediment samples collected from the downstream of MWWTP than in the upstream sediments. The results further indicated that MWWTP could significantly increase the prevalence and abundance of ARGs and the potential risks to the effluent receiving environments (Zhang et al. 2017). Furthermore, the absolute abundance of eARGs in the sediments was higher than that of iARGs, indicating slower degradations rates and higher persistence of eARGs in the plasmid than in the chromosomal (Dong et al. 2019; Zhao et al. 2020).

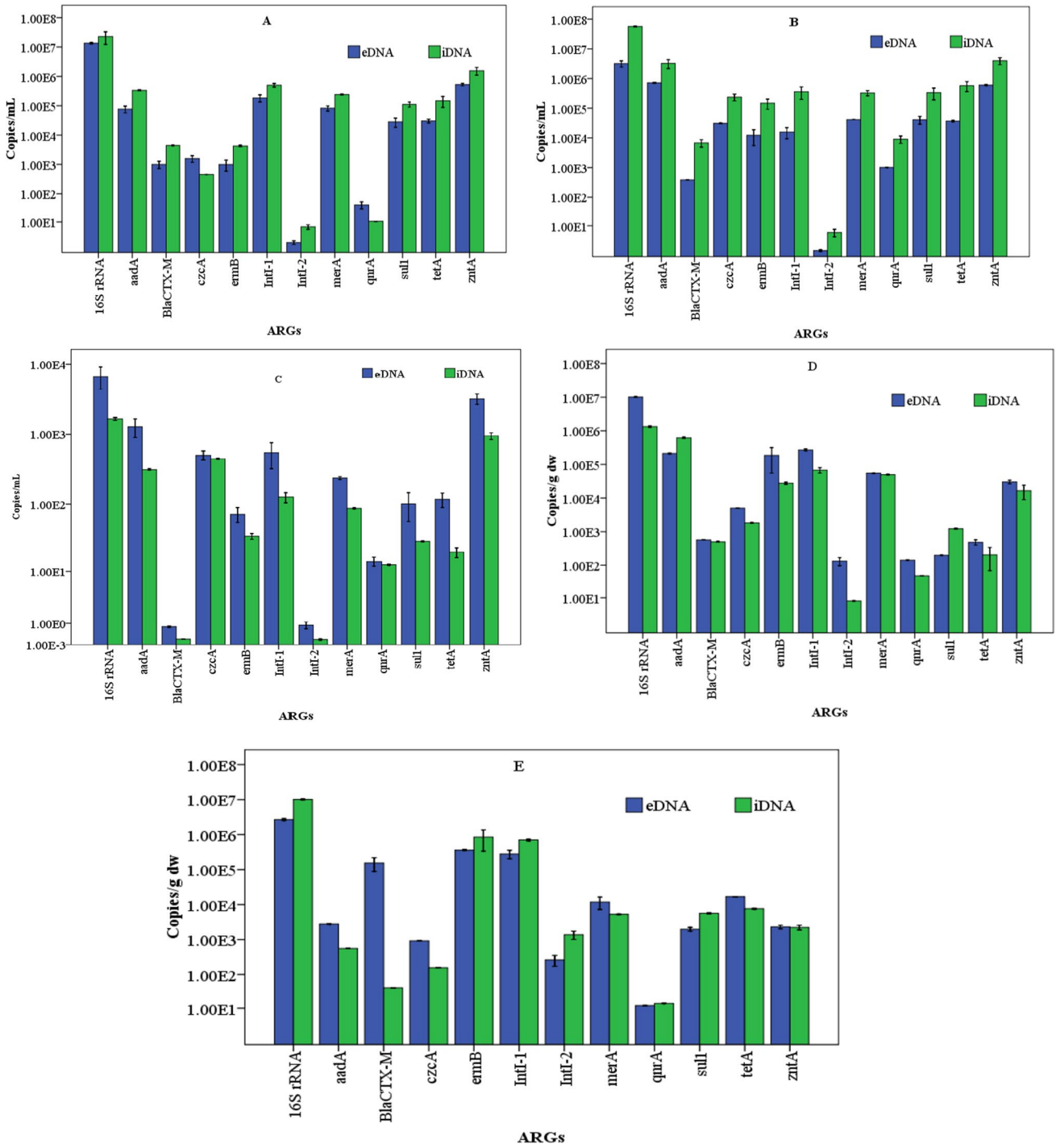

Fig. 2 Absolute abundance of ARGs, MRGs and MGEs (copies/mL and copies/g) in iDNA and eDNA in **a**) influent of MWWTP; **b**) aeration tank of MWWTP; **c**) effluent of MWWTP; **d**) upstream of MWWTP; and **e**) downstream of MWWTP samples

Previous studies have indicated that eARGs could exist in the sediment for a long time, proliferate and disseminate among different bacteria through lateral gene transfer, and could be a potential threat to human health (Mao et al. 2014; Zhao et al. 2020).

Occurrence of MRGs in eDNA and iDNA

The occurrence of three common MRGs in the form of eDNA and iDNA was detected at a higher abundance (Fig. 2). The absolute abundance of iMRGs ranged from

8.57×10^1 to 3.97×10^6 copies/mL, and those of eMRGs ranged from 2.36×10^2 to 6.00×10^5 copies/mL in MWWTP. Notably, the *znt*A genes were the most abundant MRGs in MWWTP, with the concentrations ranging from 9.4×10^2 to 3.97×10^6 copies/mL in iDNA and 3.2×10^3 to 6×10^5 copies/mL in eDNA, which were 1–4 orders of magnitude higher than merA and czcA in iDNA and 1–2 orders of magnitude in eDNA. In the present study, the absolute abundance of most eMRGs were higher than iMRGs in the sediment samples collected from the downstream of MWWTP, while MRGs showed a higher abundance in the sediment samples collected from the downstream of MWWTP than in the upstream sediments. The results were consistent with ARGs, suggesting that MRGs and ARGs persisted in the effluent of MWWTP with a relative content after the whole treatment processes. A higher proportion of MRGs and ARGs in the final effluent posed a potential risk to the prevalence of antibiotic resistance in their receiving environments.

Network analysis between MGEs and ARGs

The integrase gene *int*I1 was detected in all eDNA and iDNA, which played an important role in the dissemination of antibiotic resistance through horizontal gene transfer of ARGs (Gaze et al. 2011). The enrichment of *int*I1 gene in eDNA was up to 1.85×10^5 copies/mL in the influent, 1.58×10^4 copies/mL in the aeration tank, and 5.4×10^2 copies/mL in the effluent samples, respectively. On the other hand, the concentrations of *int*I1 in iDNA were up to 5.02×10^5 copies/mL, 3.6×10^5 copies/mL, and 1.25×10^2 copies/mL, respectively, which were lower than the previous document (Sui et al. 2019).

There was a strong ($r > 0.7$) and positive correlation between ARGs and MGEs in the iDNA and eDNA, which was consistent with the previous study results (Lu et al. 2015; Zhang et al. 2018) (Fig. 3). Notably, *int*I1 was more correlated with ARGs in iDNA (*tet*A, *sul*1, $bla_{\text{CTX-M}}$, *erm*B, and merA) than in eDNA ($bla_{\text{CTX-M}}$, *erm*B, and *mer*A), suggesting that the ARGs located in MGEs might be degraded by nucleases when they release from bacteria. Furthermore, MGEs (*int*I1and *int*I2) carrying the ARGs could reduce the immediate degradation by nucleases by protecting the sediment particles (Demaneche et al. 2001). Additionally, *int*I2 was only correlated with *erm*B in iDNA and eDNA. Notably, *znt*A and *mer*A were correlated with *tet*A, *sul*1, *aad*A, and $bla_{\text{CTX-M}}$ in iDNA, and *czc*A was correlated with *aad*A, *qnr*A, and *znt*A in eDNA, suggesting the co-occurrence and co-transfer of ARGs and MRGs in iDNA and eDNA (Di Cesare et al. 2016). So far, little emphasis has been put on the occurrence of ARGs, MRGs, and MGEs in iDNA and eDNA collected from MWWTP and its receiving river sediments.

To the best of our knowledge, this is the first study to quantify the *znt*A, *mer*, and *czc*A genes and evaluate the abundance of eMRGs in MWWTP and its adjacent sediments through qPCR, providing the valuable data for assessing the contribution of ARGs and MRGs in MWWTP and its adjacent environments.

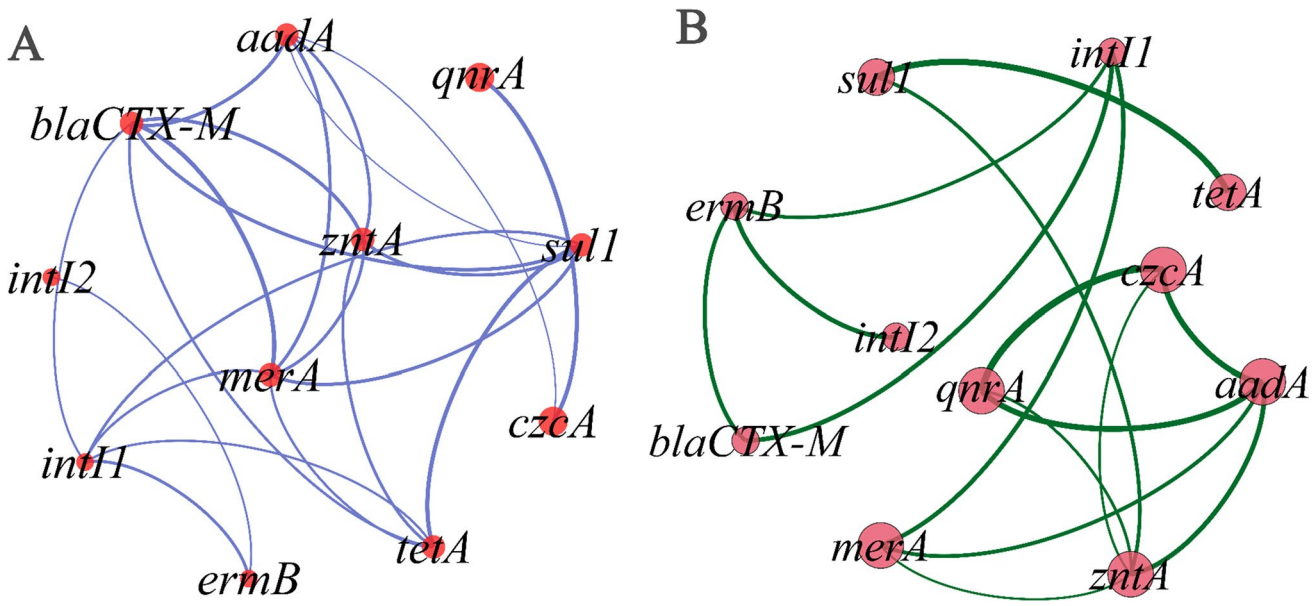

Fig. 3 Network analysis revealing the co-occurrence pattern of ARGs, MRGs and MGEs in iDNA (A) and eDNA (B). A node represents ARGs, MRGs or MGEs (the bigger size of each node, the more numbers of connections). The blue (iDNA) and green (eDNA) edges represents a strong ($r > 0.75$) and significantly positive correlation ($p < 0.05$) based on Spearman correlation analysis

Conclusions

Municipal wastewater treatment plant can play an important role in altering the magnitude and distribution of ARGs in the receiving environments. In this study, the incomplete removal of ARGs, MRGs, and MGEs in MWWTP resulted in the persistence and their accumulation in the receiving river. Although disinfection processes in MWWTP killed bacteria, they released substantial quantities of free DNA into the environment that promoted the propagation of resistance genes. The enrichment of i*ntI1* gene in iDNA and eDNA might have also increased the dissemination of ARGs and MRGs in the environment through the lateral gene transfer process. This widespread distribution of contaminants in MWWTP and its adjacent river sediments will help clarify the transmission routes of antibiotics and heavy metal resistance within these environments. Although in the present study only one MWWTP and its receiving river was selected for the profiling of intracellular and extracellular antibiotic resistance genes, but overall, the study will provide a theoretical basis for better monitoring and prevention of antibiotic resistance dissemination. Secondly, we suggest to detect the transfer ability of ARG and MRGs as well as to carry out metagenomics and metatranscriptomics to acquire a greater understanding of the resistance mechanism.

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Author contribution Peiyuan Deng, Shu Ai, and Xiaojia Hu designed the experiment. Wentao Cai, Zuoxu Zhang, Yuli Zhang, and Yihe Huang executed the study. Xiaojia Hu and Yingying Yang finished data statistic and analysis. Peiyuan Deng and Shu Ai drafted the manuscript, and Changkan Li revised the manuscript.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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