ASCE

An Extraction Method to Quantify the Fraction of Extracellular and Intracellular Antibiotic Resistance Genes in Aquatic Environments

Kassidy O'Malley[1]; Walter McDonald, A.M.ASCE[2]; and Patrick McNamara[3]

Abstract: As the abundance and diversity of antibiotic resistance genes increases in the environment, there is a concurrent increase in the threat to public and ecosystem health. Extracellular antibiotic resistance genes (eARGs) are cell-free DNA that can promote the development of antibiotic resistance via transformation by competent bacterial cells. Despite this role, eARGs have not been well characterized in different environmental waters. Their small size and low concentrations in some aquatic environments render them difficult to extract. The aim of this research was to modify an eARG extraction method to determine the abundance of both eARGs and intracellular ARGs (iARGs) in the same water sample. The modified method, consisting of sequential filtration to separate iARGs from eARGs, adsorption-elution with aluminum hydroxide–coated silica gel, and precipitation, extracted eARGs and iARGs with a recovery rate between 79.5% and 99.0%. The novel method was then utilized for the extraction of the extracellular and intracellular fractions of four ARGs, one mobile genetic element, and the 16S rRNA in tap water, river surface water, lake surface water, stormwater, and wastewater effluent. This is the first instance in which the extracellular and intracellular fractions of the 16S rRNA, *intI*1, bla_{TEM}, *erm*F, *sul*1, and *tet*C genes in stormwater and lake surface water are reported. In addition, this modified method enabled the quantification of the extracellular concentration of the erythromycin resistance gene *erm*F in environmental waters for the first time; the gene's abundance ranged from 1.26×10^5 to 8.82×10^6 gene copies/L across the aquatic waters sampled. The extracellular abundance of the mobile genetic element *intI*1, moreover, was quantified in tap water (7.00×10^4 gene copies/L) for the first time. The validation and application of this method to diverse environmental matrices should allow for further research to be conducted to better understand the role of eARGs in the spread of antibiotic resistance. **DOI: 10.1061/(ASCE)EE.1943-7870.0001993.**

Author keywords: Environmental antibiotic resistance genes (ARGs); Aquatic environment; Extracellular genetic material; Adsorption-elution; Droplet digital polymerase chain reaction (PCR).

Introduction

Both the natural and engineered environment are reservoirs of antibiotic resistance elements, including antibiotic-resistant bacteria (ARB), antibiotic resistance genes (ARGs), and other genetic determinants (e.g., integrons, transposons, and plasmids) (He et al. 2021; Pruden et al. 2018). The occurrence of antibiotic resistance in the environment is a natural phenomenon (D'Costa et al. 2011), yet human activity, specifically the mishandling of antibiotics and the discharge of effluents carrying resistance elements, is drastically intensifying the spread of antibiotic resistance (Finley et al. 2013; Kumar and Pal 2018). Environmental disturbances originating from anthropogenic influence have impacted microbial ecology in several ways, including the amplification and diversification of the environmental resistome, an increase in the abundance and distribution of ARGs, and the emergence of novel resistance elements (Finley et al. 2013; Surette and Wright 2017). The proliferation of ARGs is facilitated primarily through horizontal gene transfer, of which three mechanisms have been identified: (1) conjugation, (2) transduction, and (3) transformation (Levy 1989; Von Wintersdorff et al. 2016). Transformation, specifically, is the uptake, integration, and functional expression of extracellular DNA (eDNA) by competent bacterial cells (Thomas and Nielsen 2005). eDNA can originate in the environment indirectly from input sources containing eDNA, or directly from the extrusion of intracellular DNA (iDNA) from microbial cells or passive release as a result of cell lysis (Nagler et al. 2018; Nielsen et al. 2007). In the environment, eDNA can act as a nutrient and energy source (Johnsborg et al. 2007), serve in the formation of biofilms (Jakubovics et al. 2013; Nagler et al. 2018), and is a source of genetic material for microbes to acquire by gene transformation (Nielsen et al. 2007). The dynamic pool of extracellular genetic elements found in the environment ranges from integrons, transposons, and gene cassettes to eARGs present as chromosomal or plasmid eDNA fragments (Barnes et al. 2014). Such genetic material has the potential to host various resistance determinants and be integrated via transformation into competent bacteria, thereby enriching the cell with resistance mechanisms (Dong et al. 2019; Lu et al. 2010; Mao et al. 2014).

eDNA can represent a relevant fraction of the total DNA in a given environment, and it differs comparatively from iDNA in its fate, stability, and transport (Mao et al. 2014; Pietramellara et al. 2009; Zarei-Baygi and Smith 2021). Numerous studies have

[1]Dept. of Civil, Construction, and Environmental Engineering, Marquette Univ., Milwaukee, WI 53233.

[2]Assistant Professor, Dept. of Civil, Construction, and Environmental Engineering, Marquette Univ., Milwaukee, WI 53233. ORCID: https://orcid.org/0000-0002-9217-7908

[3]Assistant Professor, Dept. of Civil, Construction, and Environmental Engineering, Marquette Univ., 1637 W Wisconsin Ave., Milwaukee, WI 53233 (corresponding author). Email: patrick.mcnamara@marquette.edu

Note. This manuscript was submitted on September 8, 2021; approved on December 21, 2021; published online on March 14, 2022. Discussion period open until August 14, 2022; separate discussions must be submitted for individual papers. This paper is part of the ***Journal of Environmental Engineering***, © ASCE, ISSN 0733-9372.

evaluated the persistence of eDNA and found that it can range from a few days to years, while demonstrating that the fate of eDNA is specific to the conditions of its environment (Barnes et al. 2014; Levy-booth et al. 2007; Mao et al. 2014; Nielsen et al. 2007; Zhu 2006). Notably, the adhesion with clay minerals, sand, humic substances, and other organic molecules has been shown to protect and stabilize eDNA (Corinaldesi et al. 2005; Mao et al. 2014). Adsorption protects eDNA from nuclease-mediated enzymatic hydrolyzation, specifically by DNase enzymes (Levy-booth et al. 2007). As a result, soil environments have been reported to harbor the largest fractions of eDNA (Dong et al. 2019; Mao et al. 2014; Pietramellara et al. 2009; Zhao et al. 2020).

The eDNA adsorbed to soil particles, as well as eDNA floating in the water column, remains bioavailable for transformation, the rate of which can be comparable to that of conjugation in certain environments (Zarei-Baygi and Smith 2021). Moreover, transformation has a broad capacity for the exchange of resistance determinants efficiently between phylogenetically distant species (Domingues et al. 2012; Lu et al. 2020; Mantilla-Calderon et al. 2019; Von Wintersdorff et al. 2016). Consequently, eARGs are at risk of being assimilated by pathogens of clinical relevance (Chancey et al. 2015; Von Wintersdorff et al. 2016) and thus could play a critical role in the proliferation of antibiotic resistance.

Despite the potentially significant role of eARGs in the propagation of antibiotic resistance, their abundance in aquatic environments has not been well characterized. This gap exists in part due to the small size and low concentrations of eARGs in aquatic environments (Liu et al. 2020; Zarei-Baygi and Smith 2021), leading to variable extraction methods targeting different environmental waters. Chemical precipitation methods have been widely and successfully applied for the extraction of eARGs from sediment and sludge (Corinaldesi et al. 2005; Dong et al. 2019; Mao et al. 2014; Sui et al. 2019; Zhang et al. 2013; Zhao et al. 2020; Zhou et al. 2019) but scarcely utilized for the extraction of eARGs from aquatic environments (Mao et al. 2014; Zhang et al. 2018). In the instances in which precipitation was used, aquatic environments of higher eARG concentrations were the focus, thus keeping sample volumes and chemical requirements low. Precipitation methods, however, are difficult to apply across aquatic environments of variable eARG concentrations.

Additional methods that have recently emerged have utilized hydroxyl magnetic beads (Yuan et al. 2019) and consecutive ultrafiltration with silica adsorption (Liu et al. 2020) for the extraction of eARGs from environmental waters. The use of magnetic beads for the extraction of eARGs has only been applied to small-volume (2–5 mL) wastewater samples and was developed and optimized based on the recovery of eDNA (16S rDNA gene) only, from which the recovery efficiency was 85.3%. Liu et al. (2020), however, showed that recovery of extracellular genes is highly dependent on gene length. The consecutive ultrafiltration with a silica adsorption method was only able to achieve 38.8% and 44.5% recovery of the bla_{TEM} (1,043 bp) and *tet*A (472 bp) eARGs, respectively, compared to the 62.2% recovery of the 16S rRNA gene (approximately 10,000 bp) (Liu et al. 2020). An adsorption-elution method developed by Wang et al. (2016) was optimized based on the recovery of eARGs from synthetic and environmental waters and was able to achieve greater than 90% recovery. This method has been further applied for the extraction of eARGs from wastewater effluent, tap water, and the effluent of a bench-scale anaerobic membrane bioreactor cotreating domestic wastewater and a manure slurry in Liu et al. (2018), Hao et al. (2019), and Lou et al. (2020), respectively. The method established by Wang et al. (2016), however, was not developed to extract both eARGs and iARGs from an environmental water sample. In addition, subsequent studies utilizing this method did not use consistent techniques for either the separation of iARGs from eARGs or the extraction of eARGs following concentration. Moreover, no study has evaluated this method's ability to simultaneously extract eARGs and iARGs from multiple aquatic environments of varying ARG concentrations. To fill the research gap concerning the role of eARGs relative to iARGs in aquatic environments, a reliable method must be applicable to diverse water samples.

The fate of eARGs relative to iARGs is important to understand for characterizing the threat and extent of antibiotic resistance originating from different environments. Therefore, the objectives of this study were to (1) adapt a previously developed method (Wang et al. 2016) for the simultaneous extraction of eARGs and iARGs from the same water sample; and (2) verify that the method can quantify the fraction of eARGs and iARGs in different aquatic environments of varying ARG concentrations, including tap water, wastewater effluent, river surface water, lake surface water, and stormwater. The abundances of four ARGs, the Class 1 integron, *inti*1, and the 16S rRNA gene in both extracellular and intracellular DNA were quantified via droplet digital polymerase chain reaction (ddPCR) from environmental samples.

Materials and Methods

Extracellular DNA Extraction

The eDNA extraction method utilized in this study was adapted from a previously described method (Wang et al. 2016). Nucleic acid adsorption particles (NAAPs), aluminum hydroxide–coated silica gel, were first produced according to Wang et al. (2016). The modified laboratory extraction procedure is depicted in Fig. 1. Step 1, i.e., separation of iDNA from eDNA, was specifically added to modify the Wang et al. (2016) method for iDNA and eDNA fractionation. Step 2, concentration of eDNA, and Step 3, extraction via precipitation, were thus modified to only concentrate extracellular material with NAAPs and limit the concentration of material to recover.

In detail, the environmental samples were first filtered through a vacuum filtration apparatus using a 0.22-μm Millipore Express Plus hydrophilic polyethersulfone (PES) membrane filter (Fisher Scientific, Hampton, New Hampshire) (Fig. 1, Step 1). A 0.22-μm sized filter is applicable to the capture of intracellular DNA (Kaboosi et al. 2010) and has been consistently applied to the extraction of cellular material from environmental waters (Chen et al. 2020; Corinaldesi et al. 2005; Dong et al. 2019; Reynolds et al. 2020). Moreover, 0.22-μm PES filters have been reported to recover minimal eDNA, between 4.96% and 5.54%, when spiked at environmental concentrations between 5×10^4 and 5×10^6 copies/mL (Liang and Keeley 2013). Total suspended solids (TSS) as well as organic particles and solution pH were found to be the primary factors influencing eDNA retention. Consequently, a 0.22-μm filter was selected to fractionate iDNA from eDNA in the environmental samples. The filter, thus, represents the fraction of DNA contained within a cell as well as the minimal eDNA adsorbed to a cell or solid and captured on a filter. The filtrate represents the cell-free fraction of DNA and the small number of intact cells that pass through a 0.22-μm filter. The filter was utilized for iDNA extraction (see the section "Intracellular DNA Extraction"), and the filtrate was collected for eDNA extraction.

To concentrate eDNA present within the filtrate, a glass column (1.5×50 cm, Bio-Rad Laboratories, Hercules, California) was first sealed with 18 g of NAAPs, and then the filtrate was pumped (30 mL/min) through the column using a peristaltic pump (Fig. 1, Step 2). Following the passage of the sample filtrate, 100 mL of an

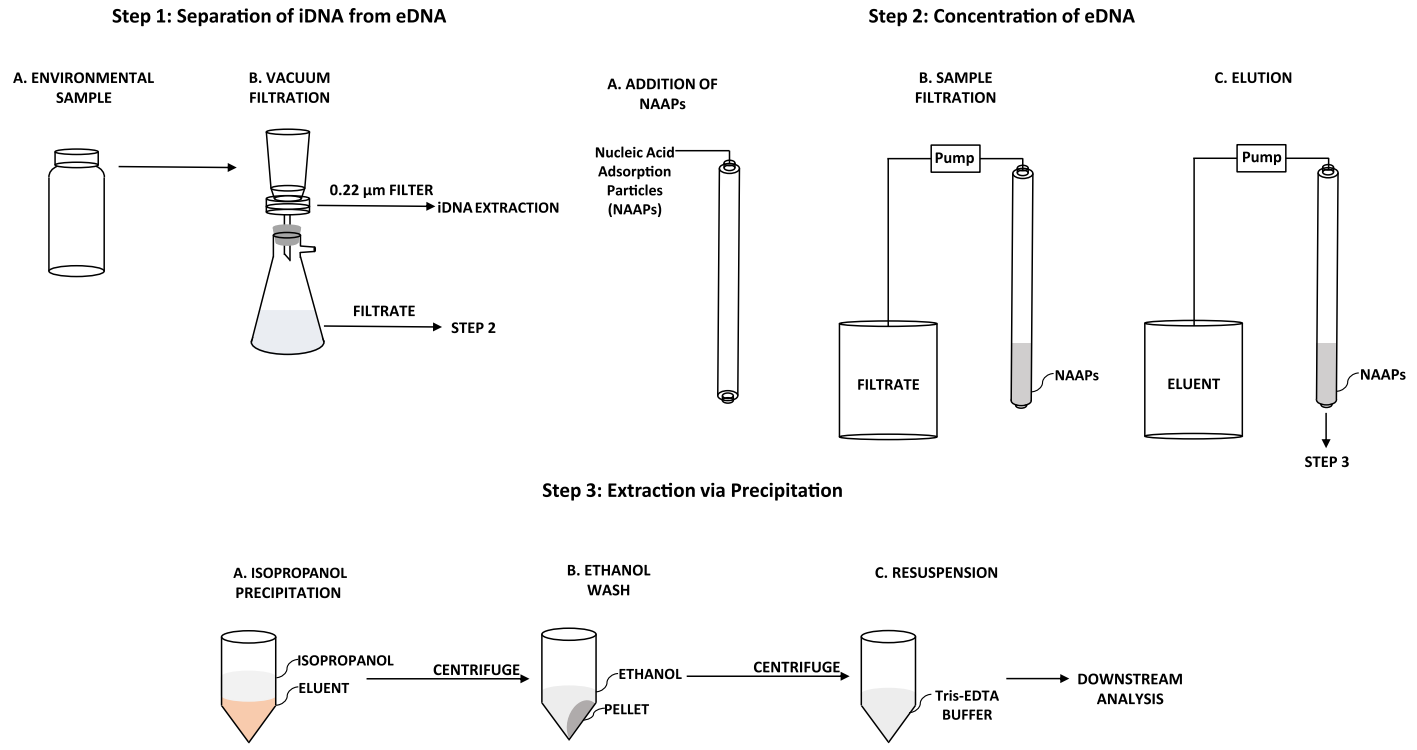

Fig. 1. eDNA extraction laboratory workflow.

eluent [15 g/L NaCl, 30 g/L tryptone, 15 g/L beef extract, 3.75 g/L glycine, 0.28 g/L Na(OH), pH $= 9.3 \pm 0.2$; autoclaved at 120°C for 20 min] was pumped through the column and collected in a centrifuge tube. After the eluent was transferred to the centrifuge tube, an equal volume of isopropanol was added, and the solution was maintained at room temperature for 16 h (Fig. 1, Step 3). After precipitation, the mixture was centrifuged ($10{,}000 \times g$ for 10 min at room temperature), and the supernatant was decanted. The pellet was washed with 10 mL of 70% ethanol and centrifuged once more ($10{,}000 \times g$ for 5 min at room temperature) and the supernatant decanted. The pellet was air dried and resuspended with a Tris–ethylenediamine tetraacetic acid (EDTA) buffer. The final eDNA extracts were stored at −20°C for subsequent analyses.

Intracellular DNA Extraction

After vacuum filtration (see the section "Extracellular DNA Extraction"), the filters were cut into fragments, and iDNA was extracted from the filters according to the FastDNA Spin Kit manufacturer's protocol (MP Biomedicals, Santa Ana, California). Briefly, 1.0 mL of cell lysing solution for tissues and cells (CLS-TC) was used, and in modification to the specified protocol, cells were lysed using liquid nitrogen freeze thaw cycling rather than homogenization (Kappell et al. 2019). The following steps included binding, washing, and eluting the DNA in a DNase/pyrogen-free water. DNA extracts were stored at −20°C until further analysis.

eDNA Extraction Method Validation: Negative Control, Spike and Recovery, and Reproducibility

The eDNA extraction method utilized for this study was validated first by processing triplicate sterilized Milli-Q water samples as a negative control through the method to quantify residual contamination. Second, Milli-Q water was spiked with lysed DNA extracts to quantify the simultaneous recovery of ARGs via the eDNA extraction method. The spike for this experiment was obtained from an environmental river water sample selected because it was assumed to contain similar targets and concentrations of eDNA. The river water was processed through the iDNA extraction method, and the lysed DNA extract was utilized as the spike. The initial concentration of the extract was determined via ddPCR. The spiked DNA was then added to 1 L Milli-Q water, and the spiked water underwent the eDNA extraction procedure. Then the abundances of five genes (*erm*B, *sul*1, bla_{TEM}, *intI*1, and the 16S rRNA gene) were quantified by ddPCR, and the recovery was calculated as follows using Eq. (1):

$$\text{eARG recovery rate}\,(\%) = \frac{B - C}{A - C} \times 100 \tag{1}$$

where A is the absolute concentration (gene copies $(\mathrm{GC})/\mu\mathrm{L}$) of eARGs added to the water samples (spike determined by ddPCR); B is the absolute concentration $(\mathrm{GCs}/\mu\mathrm{L})$ of ARGs quantified by ddPCR following eDNA extraction; and C is the residual absolute concentration $(\mathrm{GCs}/\mu\mathrm{L})$ of eARGs in the control group (negative control) (Wang et al. 2016).

The final step taken to validate the extraction method was to evaluate its reproducibility. On August 22, 2020, three 1-L water grab samples were taken from the Kinnickinnic River in Milwaukee, Wisconsin. The environmental river water samples were then processed through the iDNA and eDNA extraction method. Three genes, 16S rRNA, *erm*B, and *sul*1, were quantified via ddPCR from the DNA extracts, and reproducibility was assessed by calculating the relative standard deviation of the mean absolute abundance across the triplicate samples.

Environmental Water Sampling

Five different aquatic environments were targeted for the quantification of iARGs and eARGs: tap water, river surface water, lake surface water, stormwater from an outfall, and wastewater effluent.

For each environment, triplicate samples were taken on one occasion. The selected environments were chosen to represent a variety of water types and environmental conditions, and the objective of sampling was to validate and test the capacity of this method to extract iARGs and eARGs from environments of varying water quality. Future work will aim to fully characterize eARGs in the environment with further sampling over time to determine how environmental processes impact the fate of eARGs.

For tap water, 5 L tap water were collected in triplicate from the laboratory cold-water faucet on February 3, 2021, after being thoroughly flushed for 5 min. The drinking water treatment processes preceding distribution are detailed in the Supporting Information in the Supplemental Materials (Fig. S1). The wastewater effluent sample was collected on the same day at a full-scale conventional wastewater treatment plant that services residential, industrial, and commercial sources and had an average daily flow of approximately 281 million liters per day in 2021. Five liters were collected from the surface of the chlorination tank prior to dechlorination. The wastewater treatment processes preceding chlorination are detailed in Fig. S2. On November 5, 2020, 1-L grab samples were collected in triplicate from the Menomonee River and Lake Michigan in Wauwatosa and Milwaukee, Wisconsin, respectively. River water samples were collected in the center of the stream cross section at a depth of 0.3 m (1 ft), while the lake water samples were collected off a dock at the surface of the water; both samples were collected during baseflow conditions. During a storm event on November 10, 2020, stormwater grab samples were collected from a stormwater outfall structure in triplicate in Wauwatosa, Wisconsin. The depth of rainfall that fell during the storm on November 10, 2020 was 11.43 mm (0.45 in.), according to Milwaukee Metropolitan Sewerage District rain gauge data (MMSD 2020). Following collection, all samples were transported to the lab, stored at 4°C, and underwent iDNA and eDNA extraction within 24 h.

ARG Quantification

From the five environmental sampling locations, four ARGs, the integrase gene of the Class 1 integron, *intI*1, and the 16S rRNA gene, were quantified from the iDNA and eDNA extracts. The ARGs selected, *tet*C, *sul*1, bla_{TEM}, and *erm*F, are of the tetracycline, sulfonamide, beta-lactam, and macrolide antibiotic classes, respectively, and were quantified due to the frequency of their intracellular fraction being detected in the environment (Zhang et al. 2018). The 16S rRNA was quantified because it is a representative measurement of the total biomass in the sample, and *intI*1 was selected because it is a mobile genetic element, frequently associated with ARG horizontal gene transfer. For the validation of the eDNA extraction method, five genes, bla_{TEM}, *erm*B, *intI*1, *sul*1, and 16S rRNA, were quantified via ddPCR from the spike and recovery extracts for evaluating simultaneous recovery (i.e., determine whether the presence of one gene would impact the recovery of another gene). The genes selected represent a range of sequence length, 245–1,500 bp. The same five genes were also quantified from negative control experiments to measure background contamination and calculate recovery. Three genes, 16S rRNA gene, *erm*B, and *sul*1, were selected to be quantified from the Kinnickinnic River water sample to assess reproducibility.

ddPCR was conducted as previously described (Kimbell et al. 2021). Briefly, the ddPCR assays used consisted of 11 μL QX200 ddPCR EvaGreen Supermix (Bio-Rad Laboratories, Hercules, California), 0.55 μL forward and reverse primers (250 nM each) (Table S7), 4 μL diluted DNA extracts, and 5.9 μL nuclease-free water, for a total reaction mixture volume of 22 μL. The assays were pipetted into a 96-well plate, sealed, vortexed, and centrifuged to ensure homogenization. Droplets were generated in the QX200 Droplet Generator (Bio-Rad) with 20 μL of each reaction mixture and 70 μL of QX200 Droplet Generation Oil for EvaGreen (Bio-Rad) being dispersed into the separate wells of the eight-channel cartridges. The generated droplets (approximately 40 μL) were pipetted into a new 96-well PCR plate and sealed with pierceable foil heat seals at 180°C using a PX1 PCR Plate Sealer (Bio-Rad). The plate was subsequently transferred to the C1000 Touch Thermal Cycler (Bio-Rad) for thermal cycling under the following conditions: 5 min at 95°C for activation of DNA polymerase, 39 cycles at 95°C for 30 s, and 60°C for 60 s, followed by signal stabilization at 4°C for 5 min and 90°C for 5 min. The targeted genes were then quantified using the QX200 Droplet Reader (Bio-Rad) using QuantaSoft software (version 1.7.4.0917).

QA/QC for ddPCR

The quantitative digital PCR experiments (dMIQE) checklist (Table S8) was completed to document quality assurance and quality control steps taken for ddPCR analysis (Huggett et al. 2013). In addition, the limit of blanks, detection, and quantification were quantified for each gene in accordance with the MIQE guidelines (Bustin et al. 2009; Deprez et al. 2016; Taylor et al. 2017).

Water Quality Analysis

Temperature readings were gathered for all samples in situ, except for the wastewater effluent sample, which was measured after being transported to the lab. pH and conductivity were measured via Thermo Scientific Orion probes (Thermo Fisher Scientific, Waltham, Massachusetts) immediately after being transported to the lab. Total nitrogen (TN) was analyzed using the Hach Total Nitrogen Reagent Set (Hach Company, Ames, Iowa). The Hach set applies a persulfate digestion test method for the determination of TN at a range of 0.5–25 mg N/L. Hach Phosphorus TNTplus Vial Tests (Hach Company) were utilized for total phosphorus (TP) quantification by the ascorbic acid test method, equivalent to EPA Method 365.1 (USEPA 1993). The range of TP measurement is 0.05–1.5 mg P/L. TSS from a 1,000-mL sample was processed according to Standard Method 2540D. Dissolved organic carbon (DOC) and ultraviolet–visible (UV-Vis) spectrophotometry were determined according to the USEPA Method 415.3 (Potter and Wimsatt 2009) using a TOC-V_{CSN} analyzer (Shimadzu, Kyoto, Japan) and GENESYS 50 UV-Vis spectrophotometer (Thermo Fisher Scientific), respectively. All water quality analysis results can be found in Table S1.

Statistical Analysis

ddPCR results were analyzed using QuantaSoft Analysis Pro Software (version 1.0.596). If a low number of droplets were measured (<10,000 per 20 μL PCR), the reaction was rejected (Košir et al. 2017); the average number of droplets accepted across all samples was 14,487. The Shapiro-Wilk test ($\alpha = 0.05$) was used to confirm that the absolute abundance of the gene data from each sampling location was not normally distributed. Consequently, the data were log-transformed, Shapiro-Wilk was performed once more to confirm normal distributions, and all subsequent statistical analyses were performed on the transformed data. A one-way ANOVA with the post hoc Tukey's multiple comparisons test was performed to evaluate the significant relationships (p-value ≤ 0.05) between data sets. All statistical analyses were performed, and figures were produced using GraphPad Prism 7 (GraphPad Software, La Jolla, California).

Results and Discussion

Validation of Adapted Adsorption-Elution Extracellular DNA Extraction Method

The steps taken to validate this adapted eDNA extraction method included (1) running negative controls to quantify background contamination, (2) conducting spike and recovery experiments to evaluate recovery of multiple ARGs, and (3) employing the method on environmental samples to determine the method's reproducibility in real-world water matrices. The results of the negative control experiments indicated that there was minimal contamination throughout the extraction process. Only bla_{TEM} and *intI*1 were detectable above their limit of quantification in the negative control eDNA extracts (Table S2), and the detect values were more than two orders of magnitude lower than environmental concentrations.

The recovery efficiency of the extracellular genes extracted via adsorption-elution using aluminum hydroxide–coated silica gel can be found in Fig. 2. The average percentage recoveries were calculated to be $99.0\% \pm 0.01\%$ for the 16S rRNA gene, while the mobile genetic element *intI*1 was $84.9\% \pm 0.02\%$, and the ARGs bla_{TEM}, *erm*B, and *sul*1 were $94.4\% \pm 0.02\%$, $97.0\% \pm 0.06\%$, and $79.5\% \pm 0.02\%$, respectively (Table S3). These results were found to be similar to those obtained in Wang et al. (2016), in which the pUC19 plasmid was recovered at a rate exceeding 90%.

The reproducibility of the method was determined by quantifying the relative standard deviation of three genes extracted in triplicate from a river surface water sample. The relative standard deviation of the mean environmental concentrations of the 16S rRNA gene, *erm*B, and *sul*1 were 3.12%, 0.37%, and 4.24%, respectively (Tables S4–S6). The recoveries and consistency in gene extraction demonstrate this method's ability for simultaneous and reproducible extracellular DNA extraction to quantify eARGs. Moving forward, this method was applied to more complex environmental waters. The environmental waters sampled—tap water, river and lake surface water, stormwater, and wastewater effluent—varied in ARG contamination, as well as pH, specific conductance, TSS, TN, TP, DOC, and UV-Vis (Table S1), signifying that this adsorption-elution method was successful in handling a range of environmental waters.

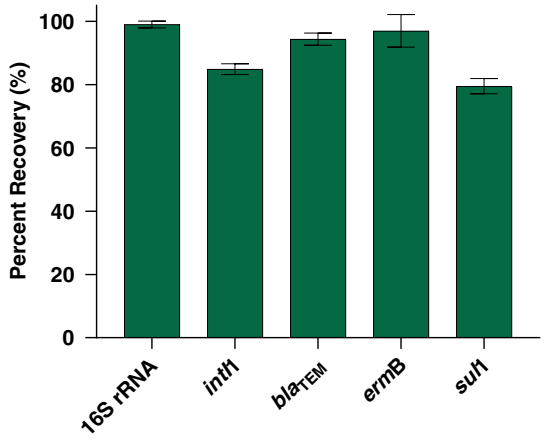

Fig. 2. Percent recovery of extracellular DNA (16S rRNA gene), the class 1 integron (*intI*1), and eARGs (bla_{TEM}, *erm*B, and *sul*1) by an adsorption-elution method with nucleic acid adsorption particles, analyzed via ddPCR ($n = 9$). Error bars represent the standard deviation of the mean recovery. Experiments performed in Milli-Q water.

Detection of Extracellular DNA and ARGs in Environmental Waters

Tap Water

The mean extracellular gene abundances detected from the tap water sample were statistically lower ($p < 0.05$) than all other environmental samples, indicating the sensitivity of this method for the extraction of eARGs at low concentrations (Fig. 3). For the first time, the extracellular concentration of the *erm*F resistance gene and the *intI*1 mobile genetic element were quantified in tap water; the mean concentrations were 1.26×10^5 and 7.00×10^4 gene copies/L, respectively. Compared to reports of eARGs extracted from tap water through different methods, the concentrations of the ARGs *tet*C, *sul*1, and bla_{TEM} in this study were slightly higher than those reported in Hao et al. (2019), and the fraction of the 16S rRNA gene was much lower than that of Sakcham et al. (2019), who reported the fraction of eDNA to be between 29% and 48% of the total DNA from a distribution system utilizing monochloramine as a secondary disinfectant. The residual in the distribution systems from this study is also a chloramine, while free chlorine was used in Hao et al. (2019). Chloramines have been reported to provide better penetration of biofilms in distribution systems compared to free chlorine (Lee et al. 2011). Moreover, disinfectants and disinfection byproducts have been shown to provide a selective pressure on microbial communities, promoting horizontal gene transfer (Mantilla-Calderon et al. 2019). These results suggest that the residuals applied by drinking water utilities could be playing a crucial role in the distribution systems and ultimately influencing the abundance of eDNA and eARGs in tap water. Because only one sample was evaluated in this study, more research will be needed to elucidate the specific function residuals play on eARG abundance.

Wastewater

In full-scale wastewater treatment plants, disinfection and biological treatment have a demonstrated ability to significantly alter the abundance of DNA and ARGs, leading to wastewater treatment plants being labeled as hotspots for antibiotic resistance (LaPara et al. 2011; Liu et al. 2018; Yuan et al. 2019; Zhang et al. 2018). The disinfection process that preceded the effluent sample for this study was chlorine disinfection. Liu et al. (2018) documented that the concentrations of both iARGs ($n = 22$) and eARGs ($n = 19$) were significantly increased following chlorine disinfection, including *tet*C, *sul*1, and bla_{TEM}. This enhancement of ARGs within chlorine processes was observed in previous research due to the coselection of disinfection and antibiotic resistance (Jin et al. 2020; Li et al. 2016). Moreover, it has been demonstrated that chlorination can cause the release of viable eARGs from cell lysis while simultaneously promoting competent bacterial cells, resulting in a rise of gene transfer via transformation (Jin et al. 2020). The wastewater effluent sample taken for this study consistently displayed the greatest mean absolute abundance for all eARGs quantified, bla_{TEM}, *erm*F, *sul*1, and *tet*C (Fig. 3). The absolute abundance concentrations of the eARGs ranged from 8.82×10^6 to 2.49×10^7 gene copies/L, consistent with the effluent samples from Liu et al. (2018), Yuan et al. (2019), and Zhang et al. (2018). In addition, the concentration of the extracellular mobile genetic element, *intI*1, which has been reported in wastewater effluent, was quantified at 2.10×10^7 gene copies/L (Calderón-Franco et al. 2021). The extracellular concentration of *erm*F (8.82×10^6 gene copies/L) was reported for the first time in the effluent wastewater of a full-scale wastewater treatment plant.

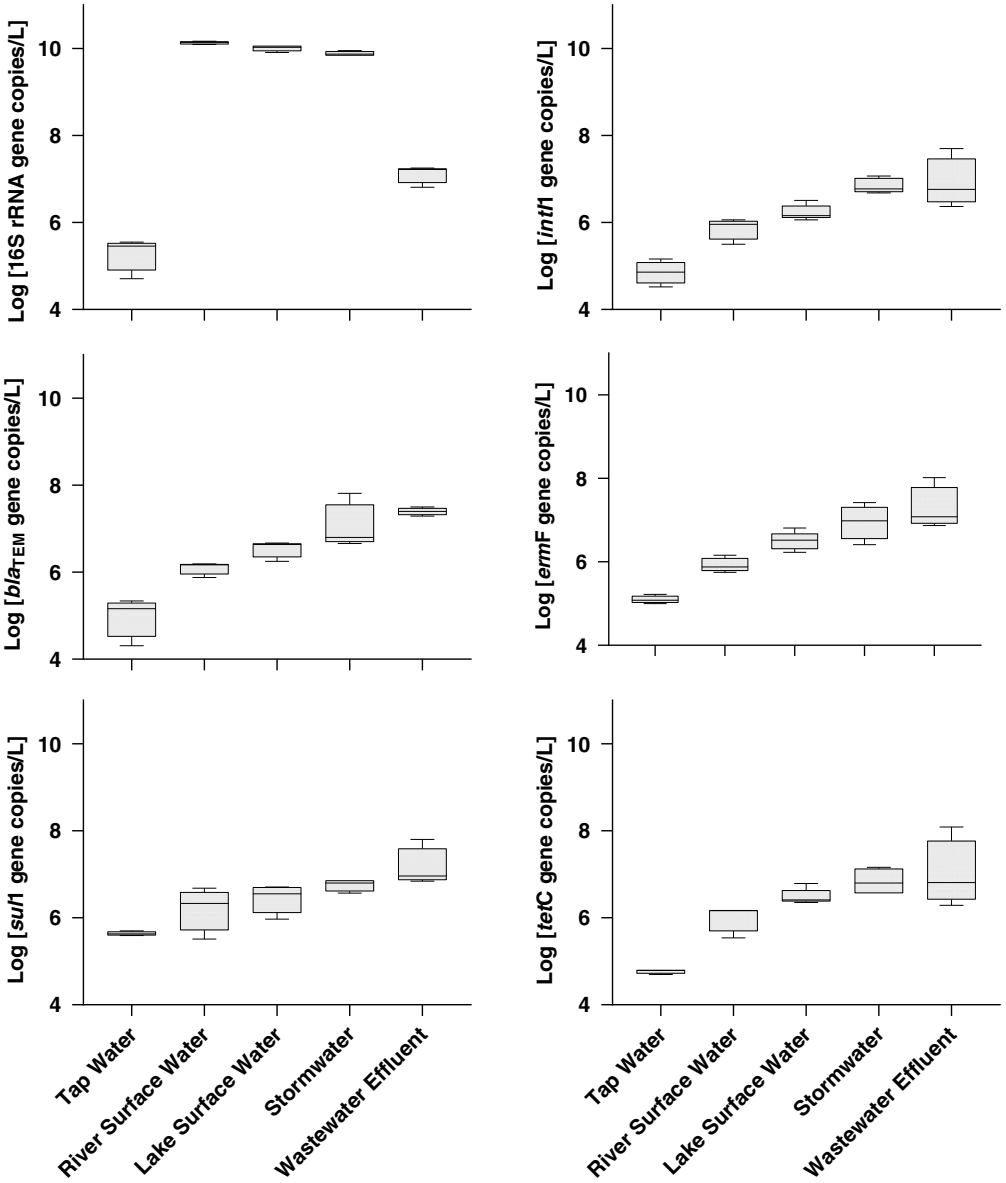

Fig. 3. Box plots displaying median distribution of absolute gene abundance (gene copies/L) of extracellular 16S rRNA gene, mobile genetic element, *intI*1, and eARGs bla_{TEM}, *erm*F, *sul*1, and *tet*C sampled from various environments ($n = 3$). Within each box plot, the upper and lower cross pieces represent the maximum and minimum values, respectively. The top of the box itself denotes the upper quartile (75th percentile) and the bottom of the box is the lower quartile (25th percentile). Statistically significant relationships between the abundance of the individual genes and locations were evaluated by Tukey's posthoc test (p-value < 0.05).

Lake Surface Water

The levels of eARGs discovered in the wastewater effluent samples indicate that the discharge of this effluent water into the environment could be disseminating a high concentration of eARGs into surface waters, suggesting that the receiving environment could also be important reservoirs for extracellular resistance elements. This theory is confirmed by the results of this study, in which the extracellular abundance of the *intI*1, bla_{TEM}, *erm*F, *sul*1, and *tet*C genes in the lake surface water sample did not differ significantly ($p > 0.05$) from that of the wastewater effluent (Fig. 3). A previous study, Liu et al. (2020), assessed the impact discharges from a wastewater treatment plant had on a receiving river surface water. A higher concentration of all eARGs ($n = 6$) quantified was observed in the downstream samples when compared with the upstream samples (Liu et al. 2020). Lake Michigan, however, is a much larger body of water, where dilution is expected to play a role, and many sources containing resistance elements could impact the waters. In addition to wastewater effluent discharge, runoff from surrounding soils, underlying sediments, and discharge from local rivers could also be contributing to the abundance of eARGs in these environmental waters (Torti et al. 2015; Vuillemin et al. 2017). This study is the first to quantify the level of eARG contamination in lake surface water.

River Surface Water

Similarly, in the river surface water samples, the eARGs *intI*1, *sul*1, and *tet*C, again, did not present a significant difference ($p > 0.05$) from the eARGs quantified in the wastewater effluent (Fig. 3). Moreover, the absolute abundances of all eARGs were not statistically different ($p > 0.05$) between the river and lake

samples, implying a possible similarity in the impact and persistence of eDNA pollution in different surface water environments. The occurrence of the extracellular 16S rRNA, *intI*1, *sul*1, and bla_{TEM} genes was previously reported in aquatic river water by Liu et al. (2020) and Mao et al. (2014). The eARG abundances were comparable to those quantified in this study; the 16S rRNA gene abundance was much higher than previously reported. *erm*F and *tet*C had yet to be quantified, and the mean concentrations of these eARGs were 8.50×10^5 and 9.10×10^5 gene copies/L, respectively. Interestingly, previous research had evaluated the role of adsorption in river systems by either extracting eDNA directly from the sediment (Mao et al. 2014) or by extracting the fraction of eDNA adsorbed to particles in the water (Liu et al. 2020). In both instances, adsorption was revealed to play an important role in the protection and subsequent persistence of eDNA, resulting in sediment communities being predominantly eDNA and eARG (Mao et al. 2014). It can be concluded that, while eDNA is an artifact of iDNA conversion, the protection imparted by adsorption to sediments allows for eDNA stability, persistence, and cycling in soil environments, conceivably fostering the genetic transformation of eARGs to environmental bacteria (Levy-booth et al. 2007; Mao et al. 2014).

Stormwater

The fraction of eARGs to iARGs has yet to be evaluated in stormwater. The mean concentrations of 16S rRNA, *intI*1, bla_{TEM}, *erm*F, *sul*1, and *tet*C discovered were 7.81×10^9, 7.46×10^6, 1.23×10^7, 8.62×10^6, 5.29×10^6, and 8.23×10^6 gene copies/L, respectively. The indicated absolute abundances for the genes present in the stormwater samples were not statistically different than that in wastewater for *intI*1, bla_{TEM}, *erm*F, *sul*1, and *tet*C. Wastewater has been characterized as an important reservoir and proliferator of antibiotic resistance (Dong et al. 2019; Zhou et al. 2019). Consequently, this research indicates that stormwater can be a comparable reservoir for both iARGs and eARGs, and further research is warranted to characterize the distribution across multiple storms and the impact of stormwater runoff on receiving water bodies.

Abundance of Intracellular and Extracellular DNA and ARGs in Environmental Samples

Across the five sampling locations, all intracellular and extracellular genes quantified via ddPCR were detectable. The quantification of the absolute abundance of these genes revealed, for the first time, the fraction of iDNA to eDNA as well as iARGs to eARGs when extracted consistently and simultaneously from environmental waters of varying ARG concentrations (Fig. 4). Statistically, the intracellular mean abundance of every gene in all samples was significantly greater than the extracellular mean abundance, except in one instance where no statistical difference was observed between the intracellular and extracellular fractions of the *erm*F gene in wastewater.

The extracellular genes *intI*1, bla_{TEM}, *erm*F, *sul*1, and *tet*C were not significantly different ($p < 0.05$) from each other in the river, lake, stormwater, and wastewater effluent samples. In tap water, only the extracellular *sul*1 gene was found to be significantly elevated above the extracellular *intI*1, bla_{TEM}, and *tet*C genes. This result contrasts with that of the intracellular genes, where many significant relationships were found between the genes, particularly in the river, lake, and stormwater samples. There was no significant difference between the concentrations of the intracellular *intI*1, bla_{TEM}, *erm*F, *sul*1, and *tet*C genes in tap water. A similar result was found in wastewater effluent for the intracellular *intI*1, bla_{TEM}, *erm*F, and *tet*C genes. Previous research had suggested that DNA characteristics, such as length and conformation, as well as abiotic (e.g., light, oxygen, and sediments) and biotic (microbial community and extracellular enzymes) environmental conditions, were the primary factors influencing eDNA occurrence and persistence in the environment (Barnes et al. 2014). The results of this study indicate homogeny between the different eARGs in the sampled environments, suggesting that eARG occurrence is driven primarily by the environment. More research, however, is needed to confirm this conclusion and elucidate the relative importance of DNA and environmental characteristics on eDNA persistence.

Comparative analysis between the ARGs and the 16S rRNA genes also yielded some significant results. In the river, lake, and stormwater samples, the intracellular and extracellular 16S rRNA genes were found to be statistically greater ($p < 0.0001$) than each iARG and eARG, respectively. In various environments, ARGs have been found at lower concentrations than the 16S rRNA gene, but ARGs could also be subject to targeted degradation via DNase enzymes, UV inactivation from sunlight exposure, and natural decay (Liu et al. 2020; Mao et al. 2014). More research is needed, however, to elucidate the fate of ARGs in the environment compared to the 16S rRNA gene. In the tap water and wastewater effluent samples, no statistical difference was found between the extracellular 16S rRNA gene and the eARGs, while both statistically significant and nonsignificant relationships were observed between the intracellular 16S rRNA genes and iARGs. This result suggests that through drinking water and wastewater treatment plants, the extracellular 16S rRNA gene and eARGs might be behaving similarly through treatment processes, whereas the intracellular genes are not. Previous research in treatment plants showed mixed results. Yuan et al. (2019), for instance, found that UV disinfection led to no distinction between iARG and eARGs and the 16S rRNA gene, whereas the 16S rRNA gene reported by Calderón-franco et al. (2021) was significantly greater than iARGs and eARGs following tertiary treatment.

The relative abundances of the ARGs quantified (normalized to the 16S rRNA gene) are presented in Fig. 5. These data revealed many instances in which the extracellular fraction was not significantly different than that of the intracellular fraction, as well as two cases where the eARG abundance was statistically greater than that of the iARG: *sul*1 for tap water ($p < 0.001$) and *erm*F for wastewater effluent ($p < 0.05$). The targeted degradation of the 16S rRNA gene relative to ARGs has been observed by previous researchers (He et al. 2021; Liu et al. 2020; Mao et al. 2014; Zhang et al. 2013). The difference was attributed to the different locations of the genes, with the 16S rRNA gene being located on the chromosome, while ARGs are frequently associated with mobile genetic elements, particularly plasmids (Liu et al. 2020). Plasmids display a higher resistance to degradation factors compared to chromosomal DNA, attributable to their size and structure (He et al. 2021). Moreover, chlorination has been demonstrated to reduce the presence of chromosomal-associated DNA and ARGs more readily than plasmid-borne genes (Zhang et al. 2019). This could explain the notable finding that the relative abundance of every eARG for the tap water and wastewater samples was either not statistically different or greater than the iARG abundance.

Conclusions

An extraction method, an eDNA extraction method was successfully adapted and validated for the simultaneous extraction of eARGs and iARGs. The extraction method was consequently

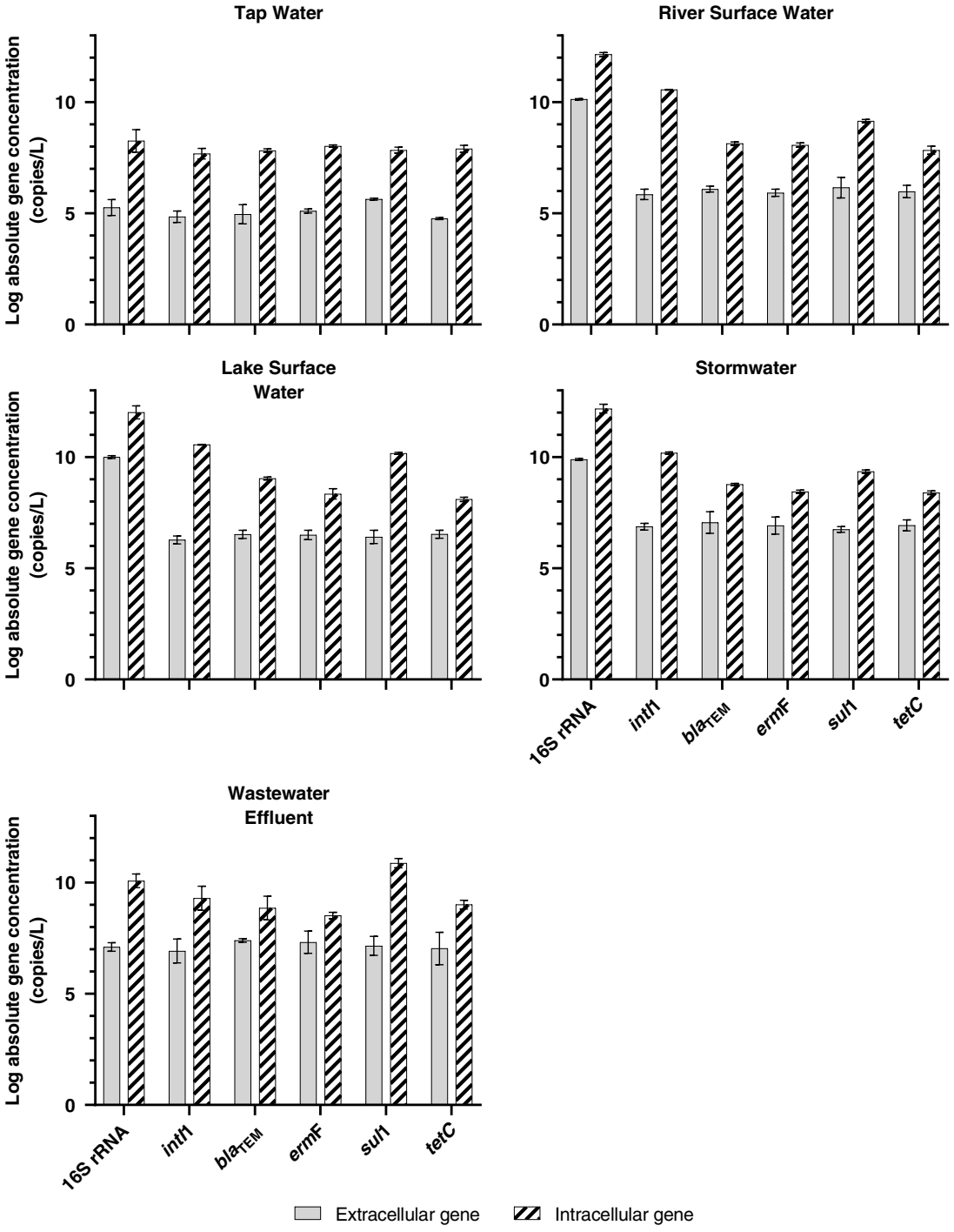

Fig. 4. Absolute abundance of ARGs, 16S rRNA gene, and mobile genetic element *intI*1 in eDNA and iDNA from environmental water samples. The abundance represents the mean ($n = 3$), and error bars represent the standard deviation of the mean. Statistical significance relationships between the abundance of the individual genes and locations were evaluated by Tukey's posthoc test (p-value < 0.05). A statistically significant difference in the intracellular and extracellular mean abundance ($p < 0.05$) was found for every gene at each location, except between the intracellular and extracellular abundance of the *erm*F gene in the wastewater effluent sample.

applied to environmental waters, thereby providing, for the first time, the abundance of several eARGs and iARGs from various environments via one extraction method. In each environment—tap water, wastewater effluent, river surface water, lake surface water, and stormwater—eARGs were detected at quantifiable levels. Though eDNA and eARGs did not dominate the overall resistance profile of the different environments, as a portion of ecological metagenomes, they still hold relevance for the proliferation of antibiotic resistance and the interactions that lead to gene persistence. Furthermore, the persistence of such elements as mobile genetic elements indicates that a large source of extracellular genetic material could be disseminating in the environmental metagenome and be available for horizontal gene transformation. This work is still limited, however, in that the DNA extracted was not

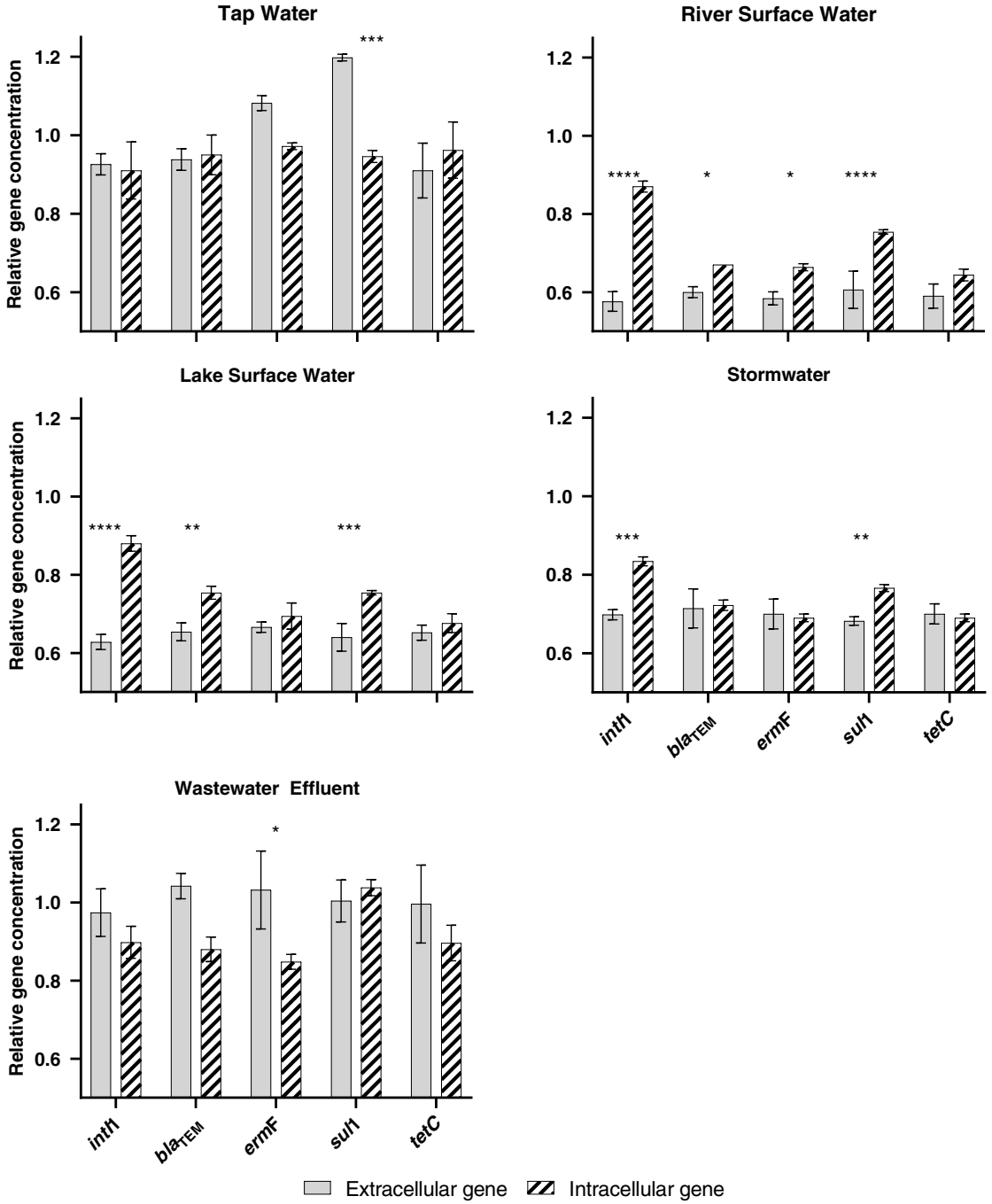

Fig. 5. Relative abundance of ARGs and *intI*1 (relative to the 16S rRNA gene) in eDNA and iDNA from environmental water samples. The abundance represents the mean ($n = 3$), and error bars represent the standard deviation of the mean. Statistically significant relationships between the relative abundance of the extracellular and intracellular ARG fractions were evaluated by Tukey's posthoc test (one asterisk: $p < 0.05$; two asterisks: $p < 0.01$; three asterisks: $p < 0.001$; and four asterisks: $p < 0.0001$).

evaluated for its viability for natural transformation. Thus, future research should focus on the risks posed by eARGs by considering the competency of the extracellular material for horizontal transformation. The work done for this study and the method that was validated will enable further eARG analysis from environments of varying levels of contamination, including metagenomic analyses that will be needed to better understand the diversity, as well as the fate and transport, of eARGs compared to iARGs under different environmental conditions.

Data Availability Statement

All data generated or used during the study appear in the published article.

Acknowledgments

This material is based upon work supported by the National Science Foundation Graduate Research Fellowship under

Grant No. 2041851. Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors(s) and do not necessarily reflect the views of the National Science Foundation. This research was also supported by an Opus College of Engineering Seed Grant funded by the GHR Foundation.

Supplemental Materials

Figs. S1 and S2 and Tables S1–S8 are available online in the ASCE Library (www.ascelibrary.org).

References

Barnes, M. A., C. R. Turner, C. L. Jerde, M. A. Renshaw, W. L. Chadderton, and D. M. Lodge. 2014. "Environmental conditions influence eDNA persistence in aquatic systems." *Environ. Sci. Technol.* 48 (3): 1819–1827. https://doi.org/10.1021/es404734p.

Bustin, S. A., et al. 2009. "The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments." *Clin. Chem.* 55 (4): 611–622. https://doi.org/10.1373/clinchem.2008.112797.

Calderón-Franco, D., M. C. M. Van Loosdrecht, T. Abeel, and D. G. Weissbrodt. 2021. "Free-floating extracellular DNA: Systematic profiling of mobile genetic elements and antibiotic resistance from wastewater." *Water Res.* 189 (Feb): 116592. https://doi.org/10.1016/j.watres.2020.116592.

Chancey, S. T., S. Agrawal, M. R. Schroeder, M. M. Farley, H. Tettelin, and D. S. Stephens. 2015. "Composite mobile genetic elements disseminating macrolide resistance in *Streptococcus pneumoniae*." *Front. Microbiol.* 6 (Feb): 1–14. https://doi.org/10.3389/fmicb.2015.00026.

Chen, L., Y. Xu, X. Dong, and C. Shen. 2020. "Removal of intracellular and extracellular antibiotic resistance genes in municipal wastewater effluent by electrocoagulation." *Environ. Eng. Sci.* 37 (12): 783–789. https://doi.org/10.1089/ees.2020.0189.

Corinaldesi, C., R. Danovaro, and A. Dell'Anno. 2005. "Simultaneous recovery of extracellular and intracellular DNA suitable for molecular studies from marine sediments." *Appl. Environ. Microbiol.* 71 (1): 46–50. https://doi.org/10.1128/AEM.71.1.46-50.2005.

D'Costa, V. M., et al. 2011. "Antibiotic resistance is ancient." *Nature* 477 (7365): 457–461. https://doi.org/10.1038/nature10388.

Deprez, L., P. Corbisier, A. M. Kortekaas, S. Mazoua, R. Beaz Hidalgo, S. Trapmann, and H. Emons. 2016. "Validation of a digital PCR method for quantification of DNA copy number concentrations by using a certified reference material." *Biomol. Detect. Quantif.* 9: 29–39. https://doi.org/10.1016/j.bdq.2016.08.002.

Domingues, S., K. Harms, W. F. Fricke, P. J. Johnsen, G. J. da Silva, and K. M. Nielsen. 2012. "Natural transformation facilitates transfer of transposons, integrons and gene cassettes between bacterial species." *PLoS Pathog.* 8 (8): e1002837. https://doi.org/10.1371/journal.ppat.1002837.

Dong, P., H. Wang, T. Fang, Y. Wang, and Q. Ye. 2019. "Assessment of extracellular antibiotic resistance genes (eARGs) in typical environmental samples and the transforming ability of eARG." *Environ. Int.* 125 (Apr): 90–96. https://doi.org/10.1016/j.envint.2019.01.050.

Finley, R. L., P. Collignon, D. G. J. Larsson, S. A. Mcewen, X. Li, W. H. Gaze, R. Reid-smith, M. Timinouni, D. W. Graham, and E. Topp. 2013. "The scourge of antibiotic resistance: The important role of the environment." *Clin. Infect. Dis.* 57 (5): 704–710. https://doi.org/10.1093/cid/cit355.

Hao, H., et al. 2019. "Profiling of intracellular and extracellular antibiotic resistance genes in tap water." *J. Hazard. Mater.* 365 (Mar): 340–345. https://doi.org/10.1016/j.jhazmat.2018.11.004.

He, T., L. Jin, J. Xie, S. Yue, P. Fu, and X. Li. 2021. "Intracellular and extracellular antibiotic resistance genes in airborne PM2.5 for respiratory exposure in urban areas." *Environ. Sci. Technol. Lett.* 8 (2): 128–134. https://doi.org/10.1021/acs.estlett.0c00974.

Huggett, J. F., et al. 2013. "The digital MIQE guidelines: Minimum information for publication of quantitative digital PCR experiments." *Clin. Chem.* 59 (6): 892–902. https://doi.org/10.1373/clinchem.2013.206375.

Jakubovics, N. S., R. C. Shields, N. Rajarajan, and J. G. Burgess. 2013. "Life after death: The critical role of extracellular DNA in microbial biofilms." *Lett. Appl. Microbiol.* 57 (6): 467–475. https://doi.org/10.1111/lam.12134.

Jin, M., L. Liu, D. W. Dong, Y. W. Liu, J. Yin, Z. Y. H. Wang, Z. Q. Z. Shen, D. S. H. Li, and J. G. J. Li. 2020. "Chlorine disinfection promotes the exchange of antibiotic resistance genes across bacterial genera by natural transformation." *ISME J* 14 (7): 1847–1856. https://doi.org/10.1038/s41396-020-0656-9.

Johnsborg, O., V. Eldholm, and L. S. Håvarstein. 2007. "Natural genetic transformation: Prevalence, mechanisms and function." *Res. Microbiol.* 158 (10): 767–778. https://doi.org/10.1016/j.resmic.2007.09.004.

Kaboosi, H., M. R. Razavi, and S. Noohi. 2010. "Efficiency of filtration technique for isolation of leptospires from surface waters: Role of different membranes with different pore size and materials." *Afr. J. Microbiol. Res.* 4 (9): 671–676.

Kappell, A. D., K. R. Harrison, and P. J. McNamara. 2019. "Effects of zinc orthophosphate on the antibiotic resistant bacterial community of a source water used for drinking water treatment." *Environ. Sci.: Water Res. Technol.* 5 (9): 1523–1534. https://doi.org/10.1039/C9EW00374F.

Kimbell, L. K., J. Huo, E. L. Lamartina, A. D. Kappell, Y. Wang, J. Newton, and P. J. McNamara. 2021. "Cast iron drinking water pipe biofilms support diverse microbial communities containing antibiotic resistance genes, metal resistance genes, and class 1 integrons." *Environ. Sci. Water Res. Technol.* 7 (3): 584–598. https://doi.org/10.1039/D0EW01059F.

Košir, A. B., C. Divieto, J. Pavšič, S. Pavarelli, D. Dobnik, T. Dreo, R. Bellotti, M. P. Sassi, and J. Žel. 2017. "Droplet volume variability as a critical factor for accuracy of absolute quantification using droplet digital PCR." *Anal. Bioanal. Chem.* 409 (28): 6689–6697. https://doi.org/10.1007/s00216-017-0625-y.

Kumar, A., and D. Pal. 2018. "Antibiotic resistance and wastewater: Correlation, impact and critical human health challenges." *J. Environ. Chem. Eng.* 6 (1): 52–58. https://doi.org/10.1016/j.jece.2017.11.059.

LaPara, T. M., T. R. Burch, P. J. McNamara, D. T. Tan, M. Yan, and J. J. Eichmiller. 2011. "Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-Superior Harbor." *Environ. Sci. Technol.* 45 (22): 9543–9549. https://doi.org/10.1021/es202775r.

Lee, W. H., D. G. Wahman, P. L. Bishop, and J. G. Pressman. 2011. "Free chlorine and monochloramine application to nitrifying biofilm: Comparison of biofilm penetration, activity, and viability." *Environ. Sci. Technol.* 45 (4): 1412–1419. https://doi.org/10.1021/es1035305.

Levy, S. B. 1989. *Gene transfer in the environment*. New York: McGraw-Hill.

Levy-booth, D. J., R. G. Campbell, R. H. Gulden, M. M. Hart, R. Powell, J. N. Klironomos, K. P. Pauls, C. J. Swanton, J. T. Trevors, and K. E. Dunfield. 2007. "Cycling of extracellular DNA in the soil environment." *Soil Biol. Biochem.* 39 (12): 2977–2991. https://doi.org/10.1016/j.soilbio.2007.06.020.

Li, D., S. Zeng, M. He, and A. Z. Gu. 2016. "Water disinfection byproducts induce antibiotic resistance-role of environmental pollutants in resistance phenomena." *Environ. Sci. Technol.* 50 (6): 3193–3201. https://doi.org/10.1021/acs.est.5b05113.

Liang, Z., and A. Keeley. 2013. "Filtration recovery of extracellular DNA from environmental water samples." *Environ. Sci. Technol.* 47 (16): 9324–9331. https://doi.org/10.1021/es401342b.

Liu, M., A. Hata, H. Katayama, and I. Kasuga. 2020. "Consecutive ultrafiltration and silica adsorption for recovery of extracellular antibiotic resistance genes from an urban river." *Environ. Pollut.* 260 (May): 114062. https://doi.org/10.1016/j.envpol.2020.114062.

Liu, S., et al. 2018. "Chlorine disinfection increases both intracellular and extracellular antibiotic resistance genes in a full-scale wastewater treatment plant." *Water Res.* 136 (Jun): 131–136. https://doi.org/10.1016/j.watres.2018.02.036.

Lou, E. G., M. Harb, A. L. Smith, and L. B. Stadler. 2020. "Livestock manure improved antibiotic resistance gene removal during co-treatment of domestic wastewater in an anaerobic membrane bioreactor." *Environ.*

Sci. Water Res. Technol. 6 (10): 2832–2842. https://doi.org/10.1039/d0ew00387e.

Lu, J., Y. Wang, S. Zhang, P. Bond, Z. Yuan, and J. Guo. 2020. "Triclosan at environmental concentrations can enhance the spread of extracellular antibiotic resistance genes through transformation." *Sci. Total Environ.* 713 (Apr): 136621. https://doi.org/10.1016/j.scitotenv.2020.136621.

Lu, N., J. L. Zilles, and T. H. Nguyen. 2010. "Adsorption of extracellular chromosomal DNA and its effects on natural transformation of *Azotobacter Vinelandii*." *Appl. Environ. Microbiol.* 76 (13): 4179–4184. https://doi.org/10.1128/AEM.00193-10.

Mantilla-Calderon, D., M. J. Plewa, G. Michoud, S. Fodelianakis, D. Daffonchio, and P. Y. Hong. 2019. "Water disinfection byproducts increase natural transformation rates of environmental DNA in *Acinetobacter baylyi* ADP1." *Environ. Sci. Technol.* 53 (11): 6520–6528. https://doi.org/10.1021/acs.est.9b00692.

Mao, D., Y. Luo, J. Mathieu, Q. Wang, L. Feng, Q. Mu, C. Feng, and P. J. J. Alvarez. 2014. "Persistence of extracellular DNA in river sediment facilitates antibiotic resistance gene propagation." *Environ. Sci. Technol.* 48 (1): 71–78. https://doi.org/10.1021/es404280v.

MMSD (Milwaukee Metropolitan Sewerage District). 2020. *Milwaukee weather center: MMSD rain gauge data*. Milwaukee, WI: MMSD.

Nagler, M., H. Insam, G. Pietramellara, and J. Ascher-Jenull. 2018. "Extracellular DNA in natural environments: Features, relevance and applications." *Appl. Microbiol. Biotechnol.* 102 (15): 6343–6356. https://doi.org/10.1007/s00253-018-9120-4.

Nielsen, K. M., P. J. Johnsen, D. Bensasson, and D. Daffonchio. 2007. "Release and persistence of extracellular DNA in the environment." *Environ. Biosaf. Res.* 6 (1–2): 37–53. https://doi.org/10.1051/ebr:2007031.

Pietramellara, G., J. Ascher, F. Borgogni, M. T. Ceccherini, G. Guerri, and P. Nannipieri. 2009. "Extracellular DNA in soil and sediment: Fate and ecological relevance." *Biol. Fertil. Soils* 45 (3): 219–235. https://doi.org/10.1007/s00374-008-0345-8.

Potter, B. B., and J. Wimsastt. 2009. "Method 415.3, Rev. 1.2: Determination of total organic carbon and specific UV absorbance at 254 nm in source water and drinking water." Accessed February 23, 2022. https://cfpub.epa.gov/si/si_public_record_report.cfm?Lab=NERL&dirEntryId=214406.

Pruden, A., et al. 2018. "An environmental science and engineering framework for combating antimicrobial resistance." *Environ. Eng. Sci.* 35 (10): 1005–1011. https://doi.org/10.1089/ees.2017.0520.

Reynolds, L. J., L. Sala-Comorera, N. A. Martin, T. M. Nolan, J. H. Stephens, A. Gitto, G. M. P. O'Hare, J. J. O'Sullivan, and W. G. Meijer. 2020. "Correlation between antimicrobial resistance and faecal contamination in small urban streams and bathing waters." *Sci. Total Environ.* 739 (Oct): 140242. https://doi.org/10.1016/j.scitotenv.2020.140242.

Sakcham, B., A. Kumar, and B. Cao. 2019. "Extracellular DNA in monochloraminated drinking water and its influence on DNA-based profiling of a microbial community." *Environ. Sci. Technol. Lett.* 6 (5): 306–312. https://doi.org/10.1021/acs.estlett.9b00185.

Sui, Q., Y. Chen, D. Yu, T. Wang, Y. Hai, J. Zhang, M. Chen, and Y. Wei. 2019. "Fates of intracellular and extracellular antibiotic resistance genes and microbial community structures in typical swine wastewater treatment processes." *Environ. Int.* 133 (Dec): 105183. https://doi.org/10.1016/j.envint.2019.105183.

Surette, M. D., and G. D. Wright. 2017. "Lessons from the environmental antibiotic resistome." *Annu. Rev. Microbiol.* 71 (Sep): 309–329. https://doi.org/10.1146/annurev-micro-090816-093420.

Taylor, S. C., G. Laperriere, and H. Germain. 2017. "Droplet Digital PCR versus qPCR for gene expression analysis with low abundant targets: From variable nonsense to publication quality data." *Sci. Rep.* 7 (1): 1–8. https://doi.org/10.1038/s41598-017-02217-x.

Thomas, C. M., and K. M. Nielsen. 2005. "Mechanisms of, and barriers to, horizontal gene transfer between bacteria." *Nat. Rev. Microbiol.* 3 (9): 711–721. https://doi.org/10.1038/nrmicro1234.

Torti, A., M. A. Lever, and B. B. Jørgensen. 2015. "Origin, dynamics, and implications of extracellular DNA pools in marine sediments." *Mar. Geonomics* 24 (Dec): 185–196. https://doi.org/10.1016/j.margen.2015.08.007.

USEPA. 1993. "Method 365.1, Revision 2.0: Determination of phosphorus by semi-automated colorimetry." Accessed February 23, 2022. https://www.epa.gov/sites/production/files/2015-08/documents/method_365-1_1993.pdf.

Von Wintersdorff, C. J. H., J. Penders, J. M. Van Niekerk, N. D. Mills, S. Majumder, L. B. Van Alphen, P. H. M. Savelkoul, and P. F. G. Wolffs. 2016. "Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer." *Front. Microbiol.* 7 (Feb): 1–10. https://doi.org/10.3389/fmicb.2016.00173.

Vuillemin, A., F. Horn, M. Alawi, C. Henny, D. Wagner, S. A. Crowe, and J. Kallmeyer. 2017. "Preservation and significance of extracellular DNA in ferruginous sediments from Lake Towuti, Indonesia." *Front. Microbiol.* 8 (Jul): 1–15. https://doi.org/10.3389/fmicb.2017.01440.

Wang, D. N., L. Liu, Z. G. Qiu, Z. Q. Shen, X. Guo, D. Yang, J. Li, W. L. Liu, M. Jin, and J. W. Li. 2016. "A new adsorption-elution technique for the concentration of aquatic extracellular antibiotic resistance genes from large volumes of water." *Water Res.* 92 (Apr): 188–198. https://doi.org/10.1016/j.watres.2016.01.035.

Yuan, Q. B., Y. M. Huang, W. B. Wu, P. Zuo, N. Hu, Y. Z. Zhou, and P. J. J. Alvarez. 2019. "Redistribution of intracellular and extracellular free & adsorbed antibiotic resistance genes through a wastewater treatment plant by an enhanced extracellular DNA extraction method with magnetic beads." *Environ. Int.* 131 (Oct): 104986. https://doi.org/10.1016/j.envint.2019.104986.

Zarei-Baygi, A., and A. L. Smith. 2021. "Intracellular versus extracellular antibiotic resistance genes in the environment: Prevalence, horizontal transfer, and mitigation strategies." *Bioresour. Technol.* 319 (Jan): 124181. https://doi.org/10.1016/j.biortech.2020.124181.

Zhang, M., S. Chen, X. Yu, P. Vikesland, and A. Pruden. 2019. "Degradation of extracellular genomic, plasmid DNA and specific antibiotic resistance genes by chlorination." *Front. Environ. Sci. Eng.* 13 (3): 1–12. https://doi.org/10.1007/s11783-019-1124-5.

Zhang, Y., A. Li, T. Dai, F. Li, H. Xie, L. Chen, and D. Wen. 2018. "Cell-free DNA: A neglected source for antibiotic resistance genes spreading from WWTPs." *Environ. Sci. Technol.* 52 (1): 248–257. https://doi.org/10.1021/acs.est.7b04283.

Zhang, Y., D. D. Snow, D. Parker, Z. Zhou, and X. Li. 2013. "Intracellular and extracellular antimicrobial resistance genes in the sludge of livestock waste management structures." *Environ. Sci. Technol.* 47 (18): 10206–10213. https://doi.org/10.1021/es401964s.

Zhao, Z., K. Zhang, N. Wu, W. Li, W. Xu, Y. Zhang, and Z. Niu. 2020. "Estuarine sediments are key hotspots of intracellular and extracellular antibiotic resistance genes: A high-throughput analysis in Haihe Estuary in China." *Environ. Int.* 135 (Feb): 105385. https://doi.org/10.1016/j.envint.2019.105385.

Zhou, S., Y. Zhu, Y. Yan, W. Wang, and Y. Wang. 2019. "Deciphering extracellular antibiotic resistance genes (eARGs) in activated sludge by metagenome." *Water Res.* 161 (Sep): 610–620. https://doi.org/10.1016/j.watres.2019.06.048.

Zhu, B. 2006. "Degradation of plasmid and plant DNA in water microcosms monitored by natural transformation and real-time polymerase chain reaction (PCR)." *Water Res.* 40 (17): 3231–3238. https://doi.org/10.1016/j.watres.2006.06.040.