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Persistence of Extracellular DNA in River Sediment Facilitates Antibiotic Resistance Gene Propagation

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Supporting Information

ABSTRACT: The propagation of antibiotic resistance genes (ARGs) represents a global threat to both human health and food security. Assessment of ARG reservoirs and persistence is therefore critical for devising and evaluating strategies to mitigate ARG propagation. This study developed a novel, internal standard method to extract extracellular DNA (eDNA) and intracellular DNA (iDNA) from water and sediments, and applied it to determine the partitioning of ARGs in the Haihe River basin in China, which drains an area of intensive antibiotic use. The concentration of eDNA was higher than iDNA in sediment samples, likely due to the enhanced persistence of eDNA when associated with clay particles and organic matter. Concentrations of *sul1*, *sul2*, *tetW*, and *tetT* antibiotic resistance genes were significantly higher in sediment than in water, and were present at higher concentrations as eDNA than as iDNA in sediment. Whereas ARGs (frequently located on plasmid DNA) were detected for over 20 weeks, chromosomally encoded 16S rRNA genes were undetectable after 8 weeks, suggesting higher persistence of plasmid-borne ARGs in river sediment. Transformation of indigenous bacteria with added extracellular ARG (i.e., kanamycin resistance genes) was also observed. Therefore, this study shows that extracellular DNA in sediment is a major ARG reservoir that could facilitate antibiotic resistance propagation.

INTRODUCTION

The widespread use and misuse of antibiotics in medical and agricultural settings has been a major driving force behind the increasing occurrence of antibiotic resistance genes (ARGs), and the evolution of multidrug resistant bacteria. It is commonly believed that hospitals are major focal points for resistance evolution because of their high per capita antibiotic use and host density. However, continual antibiotic discharge into the environment from animal agriculture facilities also creates selective pressure for antibiotic resistance.[1,2] Approximately 50% of all antibiotics given to humans and animals are excreted in their original active form in urine,[3] producing local antibiotic concentration gradients that, while orders of magnitude lower than that obtained in vivo during therapeutic dosing, are nevertheless high enough to drive ARG enrichment.[4] Importantly, sublethal concentrations of antibiotics promote ARG diversity by providing a fitness advantage to those bacteria able to grow more quickly at these concentrations, and by increasing mutation rates.[2] Although antibiotics are known to persist in both soil and aquatic environments,[4,5] ARGs may persist even after antibiotics are removed or the host bacteria have died, which is known as their "easy-to-get, hard-to-lose" trait.[6] Understanding the spatial and temporal dynamics of ARG propagation, and locating their main environmental reservoirs, is therefore critical for developing methods to mitigate widespread antibiotic resistance.

Many ARGs are located on mobile genetic elements, such as plasmids, integrons, or transposons, that promote their broad dissemination via horizontal gene transfer (HGT) mechanisms. ARGs present as intracellular DNA (iDNA) may be spread through either conjugation or transduction. Conversely, extracellular DNA (eDNA) is assimilated by naturally competent bacteria via transformation. It is important to recognize that the fate and transport of these two forms of DNA are fundamentally different, and thus may contribute to ARG propagation in different manners. Previous studies with soil,[7−9] marine sediments,[10] freshwater,[11] biofilms[12,13] and river epilithon[14] showed that natural transformation can occur broadly in environmental media, and may play a key role in HGT for some species. Notably, the transformation frequency for certain species (*A. calcoaceticus*) was as high as 4.7×10^{-3}.[8] Of the approximately 90 bacterial species that have been determined to be naturally transformable,[15,16] many are human pathogens, including representatives of the genera *Campylobacter*, *Haemophilus*, *Helicobacter*, *Neisseria*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus*.[17] Since ARGs present as either iDNA or eDNA may possess significant differences in their mobility and availability to indigenous bacteria,[18] it is important

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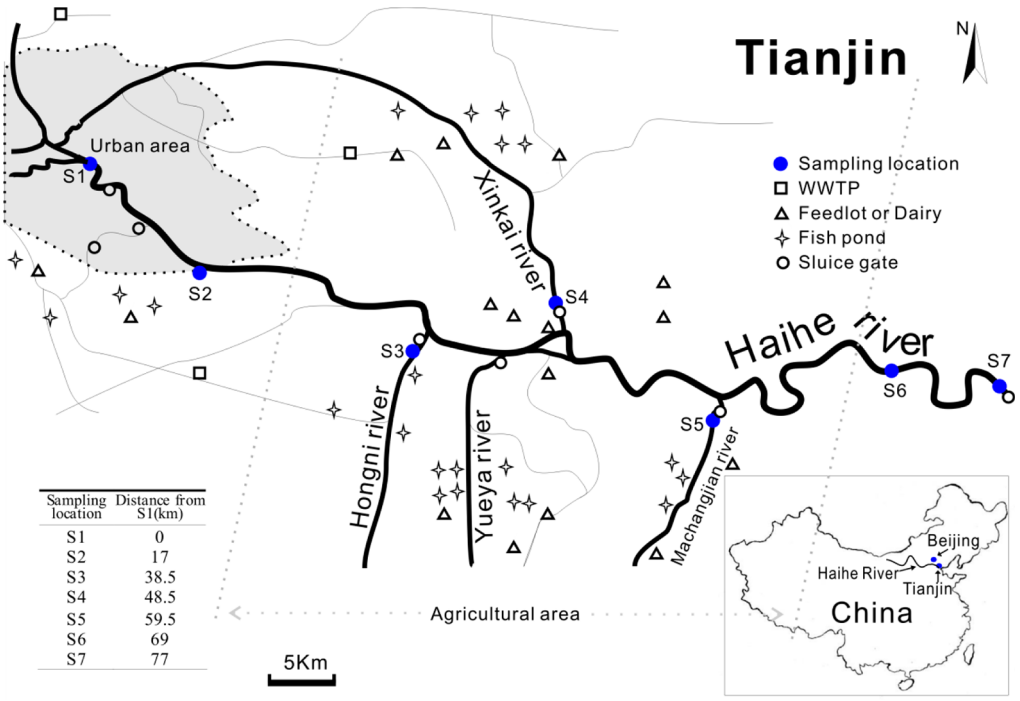

Sampling location	Distance from S1(km)
S1	0
S2	17
S3	38.5
S4	48.5
S5	59.5
S6	69
S7	77

Figure 1. Map of sampling sites along the Haihe River.

to understand the partitioning and fate of ARGs in the environment.

eDNA is relatively stable in soil or sediment,[19−24] often persisting for months,[25] and is typically found at higher concentrations in sediment than in water.[26] Specifically, eDNA has been detected at 3 to 4 orders of magnitude higher concentrations in sediment than in water, and to be present at 10- to 70-fold higher concentrations than iDNA in the same sediment.[27] Other studies have demonstrated similar results using various extraction methods and environmental media.[28−30] Several studies have indicated this is due to the absorption of eDNA to clay minerals, sand, and humic substances, which protect it from nuclease attack by both partial nuclease inactivation as well as physical separation.[31−34] This may have implications regarding the persistence and propagation of ARGs if they were found to be differentially enriched in eDNA. However, no studies to date have explored this possibility.

Several attempts have been made to extract and separate eDNA and iDNA fractions from sediments or soil.[35−38] Dell'Anno et al.[39] reported the recovery of eDNA in aquatic sediments; however, they were not able to avoid contamination by iDNA. Another study by Dell'Anno[27] utilized samples amended with plasmid DNA as internal standard to monitor whether coextraction of iDNA occurred. Ogram et al.[26] reported a method in which they added radio labeled DNA to sediment samples for the isolation of both eDNA and iDNA from a range of sediments. However, quantitative quality control [including limit of quantification (LOQ) and extraction recovery] was absent from the above procedures. In this study, we describe a procedure for isolating and tracking eDNA and iDNA which utilizes two internal standards for improved quality control, as well as to enhance accurate quantification of DNA concentrations.

To better understand ARG fate, and locate ARG reservoirs in the environment, we chose to quantify their distribution in the Haihe River basin in China (Figure 1), which is the largest water system in Northern China and is known to harbor high levels of both antibiotics and ARGs.[4,40] The objectives of this study were as follows: (1) develop a reliable method to quantify eDNA and iDNA in sediment and water; (2) quantify the concentrations of eDNA and iDNA, as well as their corresponding ARG abundance, in the Haihe River basin; and (3) study the persistence of eDNA and the propagation of ARGs from eDNA to indigenous bacteria (i.e., transformation). To our knowledge, this is the first study to associate high levels of ARGs with eDNA, and to establish sediment-associated eDNA as a major reservoir of ARGs.

MATERIALS AND METHODS

Sampling Sites and Sample Collection. Seven geographically distinct regions of the Haihe River in China were chosen as sampling sites (Figure 1). The sampling sites were located in both the main stream and the tributaries of the Haihe River, and are representative of the urban and agricultural areas in the region. Specifically, the sites were as follows: (1) Dagu Bridge; (2) Fumin Bridge; (3) Hongni River; (4) Xinkai River; (5) Machangjian River; (6) Haimen Bridge; and (7) Haihe Estuary, at the end of the Haihe River. Sampling sites 1, 2, 6, and 7 were on the main stream of the Haihe River, and sampling sites 3, 4, and 5 were on tributaries of the Haihe River.

Surface water and superficial sediment were collected in December 2010. Samples were immediately transferred into sterile plastic bags and stored on ice in the dark during sampling and stored at 4 °C after transporting to the lab.

DNA Extraction and Quality Control. Sediment samples were subdivided into two equivalent subsamples; one subsample was lyophilized to calculate the percentage of moisture for further DNA quantification. Another subsample (equivalent to 1 g/dry weight) was added to 4 mL of NaH_2PO_4 (0.12 M, pH 8.0) and 0.2 g of polyvinyl polypyrrolidone (PVPP). The mixture was shaken at 250 rpm and 25 °C for 10

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min, followed by centrifugation (10 000*g*, 4 °C, 10 min). The supernatant was filtered through a sterile membrane (0.22 μm pore size, PVDF, Osmonics, U.S.) and stored on ice. Another 4 mL of NaH_2PO_4 was added to the pellets, which was subjected to the same extraction cycle three times. Supernatants were combined and centrifuged for 20 min at 10 000*g* (at 4 °C). All supernatant fractions were collected, combined, and stored on ice until subsequent eDNA extraction using the cetyltrimethylammonium bromide (CTAB) method.[27] The collected cell pellets were combined to extract intracellular DNA. Four mL of DNA extraction buffer (100 mM Tris-HCl, 100 mM Na_2-EDTA, 100 mM Na_3PO_4, 1.5 M NaCl, 1% cetyltrimethylammoniun bromide (CTAB), 50 mg/mL proteinase K) and 0.5 g of 1 mm glass beads (Acid-washed) were added to the cell pellets, then vortexed at 2500 rpm for 10 min. One ml of SDS (10%) was then added, and the mixture was frozen in liquid nitrogen for 1 min, followed by immediate incubation at 60 °C for 20 min. This procedure was repeated three times. The mixture was separated by centrifugation at 13 000*g* at 4 °C for 20 min, and the supernatant was stored on ice. The precipitate was resuspended with 2 mL of DNA extraction buffer and vortexed at 2500 rpm for 5 min, then incubated at 37 °C for 2 h. One mL of SDS was added, and the mixture was incubated at 60 °C for 20 min, followed by centrifugation at 13 000*g*, 4 °C for 20 min. The supernatants for each sample were pooled and kept on ice until purification.

Water samples (0.2 L) were filtered through a 0.22-μm membrane (PVDF, Osmonics, U.S.) and stored on ice. The filtrates were then centrifuged (10 000*g*, 4 °C, 10 min), and the supernatants were concentrated and stored on ice for subsequent eDNA extraction as described above. The remaining cell pellets were collected from each filtration and centrifugation step and were combined to extract intracellular DNA as described above. The DNA extractions (eDNA and iDNA extracts) were purified separately using a commercially available kit (3S Spin DNA Purification Kit, Shenergy Biocolor, China). All extractions were stored at −20 °C.

The extraction yield and quality of the DNA were verified by spectrophotometry (Nanodrop 1000) and gel electrophoresis. The concentrations of DNA in sediments (μg/g) and water (μg/mL) were calculated according to the value of OD_{260}.[41] The purity indexes of the DNA were calculated by adsorption ratio of $OD_{260/280}$ and $OD_{260/230}$. The $OD_{260/280}$ of the DNA extractions were required to be above 1.8, and $OD_{260/230}$ above 2.0 to ensure no PCR reaction inhibitors were present. The DNA yields were revised according to their recoveries.

CESA9, which codes for cellulose synthase A9 in *Arabidopsis thaliana* ecotype Columbia[42] and was not detected in any samples, was used as the eDNA internal standard. *CESA9* was amplified using standard PCR and inserted into the pEASY-T1 TA cloning vector (pEASY-T1 Simple Cloning Kit, Transgene, China). The primers are described in Table S1 of theSupporting Information, SI. Chemically competent *E. coli* DH5a was then transformed with the construct using the heat-shock method. The strain was cultured in Luria−Bertani (LB) broth, and the vector was extracted using an EasyPure Plasmid MiniPrep Kit (Transgen, Beijing, China). The purified vector containing *CESA9* was added to 0.2 L water samples or 1 g sediments at varying concentrations to estimate recovery (see below).

Xanthomonas campestris pv *campestris* 8004 (*Xcc*8004), a pathogen of cruciferous plants,[43] was used as the iDNA internal standard. The gene *XC2068* is present in the genome as a single copy and is specific to strain *Xcc*8004, so it was used as an indicator for the presence of the strain, which was not detected by PCR assays in samples. The strain was cultured in Luria−Bertani (LB) broth, and 1 mL (OD600 = 0.803) cultures were added to 0.2 L water samples or 1 g sediments to estimate recovery (see below).

To ensure that there was no coextraction of intracellular and extracellular DNA, control tests were performed in which the internal standards of iDNA (*Xcc*8004-*XC2068*) and eDNA (pEASY-T1-*CESA9*) were both added to an sample (0.2 L water sample or 1 g sediment). The *CESA9* and *XC2068* genes were measured by PCR and qPCR to determine the purity quotient of both eDNA and iDNA. DNA recovery was calculated by normalizing to the extraction efficiency. We found no cross-contamination during extraction of eDNA and iDNA, as detailed in the SI (Section 3. Internal standards).

Polymerase Chain Reaction. Polymerase chain reaction (PCR) assays were used to test the methods of extraction and broad-scale screening of the presence and absence of antibiotic resistance genes. PCR amplifications were performed using a Biometra thermocycler (Biometra T Gradient, Germany). The PCR reaction mixture contained 12.5 μL of 2 × Taq PCR Master Mix [(0.1 U Taq polymerase/μL, 0.5 mM dNTP (dATP, dCTP, dGTP, dTTP each), 3 mM $MgCl_2$, 100 mM KCl, 20 mM Tris-HCl, pH 8.3) and other stabilizing agents] (Tiangen Biotech Ltd., Beijing, China), 0.5 μL of each primer (1 μM), 1 μg of template DNA, and ddH_2O to a final volume of 25 μL. Negative controls contained all of the components of the PCR mixture except DNA template. Positive controls were extracellular DNA extracted from sediment used for antibiotic resistance gene amplication, total DNA extracted from *E.coli* BH5α for 16S rRNA genes, and plasmid DNA (Plasmid Miniprep Kit, Axyprep) to test for separation of plasmid and chromosomal DNA. Both positive and negative controls were included in every run.

16S rRNA and antibiotic resistance genes were amplified in each DNA extracted sample. PCR amplification conditions were as follows: an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 15s, annealing at (°C) for 30 s, and elongation at 72 °C for 1 min. A final extension cycle was performed at 72 °C for 7 min. Primers and annealing temperatures are as previously reported.[4]

Real-Time Quantitative PCR (qPCR). All qPCR assays were performed using a Bio-Rad IQ5 instrument (Bio-Rad Company, U.S.). Calibration standard curves for positive controls were generated as described previously.[5] The establishment of negative and positive controls and qPCR reactions were described previously.[4] Amplification details are given in SI-1.

Assessment of Extracellular DNA Persistence of in Sediments. DNA endurance tests were based on a simplified OECD 308 test.[44] For the treatments, sediment (300g dry weight with 5 cm thickness of sediment layer) and river water sampled from the Haihe River (1.5 L) was stored in 2L beakers with a water/sediment volume ratio of 3:1 and equilibrated under test conditions, All microcosms were incubated in triplicate at 20 °C with alternation of 10 h light and 14 h of darkness using artificial climate boxes (SPX-250/300IC, Shanghai Boxun Industry & Commerce GO., Ltd. Medical Equipment Factory, Shanghai, China). The endurance tests lasted for 20 weeks, and eDNA and iDNA were extracted from periodical samples taken from both sediment and water at 0, 1, 2, 4, 6, 8, 12, 16, and 20 weeks as described above.

Bacterial Assimilation of ARGs from Sedimental Extracellular DNA. A 15-day microcosm experiment based on the OECD 308 test[44] was used to determine the potential of extracellular ARGs to transform indigenous bacteria. A kanamycin resistance (Kr) gene, located on the pEASY plasmid (Transgene, Beijing, China), was used as the extracellular ARG. The recipients were indigenous bacteria lacking a Kr gene. Two treatments (0 or 20 mg/L kanamycin) were utilized for microcosms amended with the pEASY plasmid. Kanamycin (20 mg/L) was included in control microcosms without the pEASY plasmid. For all microcosms, 2 L beakers were filled with sediment (300 g dry weight with 5 cm thickness of sediment layer) and river water (1.5 L) with a water/sediment volume ratio of 3:1. The microcosms were incubated at 20 °C with alternating light cycles (10 h light/14 h dark). The plasmid-borne Kr genes (10^9 copies/g sediment) were then added into all but the control microcosms. Bacterial iDNA was extracted following periodic sampling and conventional PCR was used to detect the presence of Kr genes. The isolated Kr gene positive bacteria were then identified by 16S rRNA gene sequencing. Total DNA extract from the periodic microcosm samples was used for qPCR analysis of the Kr gene copy number.

RESULTS AND DISCUSSION

Concentrations of Extracellular DNA (eDNA) and Intracellular DNA (iDNA) in Sediment and Water. Sediment-associated eDNA was the dominant form of DNA in samples from the Haihe River basin. To quantify the differences in DNA concentration and ARG abundance between iDNA and eDNA, we first developed and assessed an internal standard method to extract each simultaneously from sediment and water samples. The concentration of eDNA from Haihe River sediment (96.8 ± 19.8 μg/g) was significantly higher ($p \leq 0.05$) than that of iDNA (76.7 ± 13.0 μg/g) (Figure 2), suggesting sediment may serve as an important

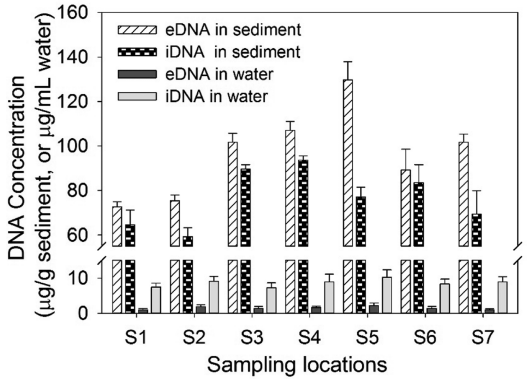

Figure 2. The concentration of eDNA and iDNA in different samples from the Haihe River. Error bars represent standard deviation from the mean of triplicate samples.

reservoir for eDNA; a result in line with previous observations at other sites.[27,45] This is likely due to the adsorption of eDNA to clay and organic matter in sediment, which decreases susceptibility to nuclease attack.[30] Conversely, considering water samples, the concentration of iDNA (9.7 ± 1.5 μg/mL) was higher ($p < 0.05$) than that of the eDNA (2.2 ± 0.8 μg/mL).

Notably, the sampling location (S5) with the highest concentration of sediment-associated eDNA (Figure 2) also had the highest percentage of clay (Table 1). In general, we observed a significant correlation ($r^2 = 0.850$, $p < 0.05$) between

Table 1. Composition of the Sediments from Different Sampling Locations[a]

	sampling locations						
sediment	S1	S2	S3	S4	S5	S6	S7
sand (%)	27.4	18.4	26.4	16.4	17.6	28.6	18.8
silt (%)	65.4	72.8	72.8	62.4	60.1	60.2	63.6
clay (%)	7.2	8.8	20.8	21.2	22.3	11.2	17.6
pH	6.8	6.3	6.5	7.1	6.0	6.3	6.5
TOC (mg/g)	17.1	23.2	29.6	30.3	34.2	32.0	26.7

[a]S1: Dagu bridge, S2: Fumin bridge, S3: Hongni River, S4: Xinkai River, S5: Machangjian River, S6: Haimen bridge S7: Haihe estuary.

eDNA concentrations and clay content (SI Figure S1). However, larger particles, like sand and silt, showed no correlation with concentrations of eDNA, implying that clay sediment particles provide more protection from eDNA biodegradation than larger particles.[22,29,34,46] In addition, a significant correlation ($r^2 = 0.668$, $p < 0.05$) was also found between eDNA concentrations and total organic carbon (TOC) in sediment, which suggests that sediment-associated organic matter may enhance the persistence of eDNA.

Detection of ARGs and 16S rRNA Genes in eDNA and iDNA from Sediment and Water. The presence of twelve common ARGs, in the form of iDNA or eDNA, was determined for each sampled site (Table 2). Of these, *sul1*, *sul2*, *tetW*, and *tetT* were present in each sample, and only three were not detected at any site (*sul3*, *sulA*, *tetL*). Significantly higher concentrations of the detected ARGs were found in sediment than in water (paired *t* test on the data in SI, $p < 0.05$), and ARG concentrations were higher in eDNA than in iDNA in sediment ($p < 0.05$) (Figure 3a). This further suggests that sediment-associated eDNA is an important reservoir for ARGs, and could increase the possibility of ARG propagation in the environment through horizontal gene transfer. On the contrary, all assessed ARG concentrations from water samples were higher for iDNA than for eDNA. Moreover, the presence of *tetO* and *tetS* in eDNA and its absence in iDNA from sediment samples at the same site (Table 2) corroborates that sediment-associated eDNA is an important ARG reservoir.

Importantly, the ratio of ARGs/16S rRNA genes was also significantly higher for eDNA than that for iDNA ($p < 0.05$ in sediment and water), implying slower degradation rates and higher persistence of (usually plasmid-borne) ARGs compared to (chromosomal) 16S rRNA genes in eDNA. Previous studies have indicated that DNA fragment size is inversely correlated to soil adsorption, which may provide a potential explanation for the persistence of plasmid-borne ARGs in this study.[47]

Persistence of Extracellular DNA in Sediments. DNA stability and decay rates were characterized to assess the persistence of both eDNA and iDNA in sediment and water samples. First-order degradation rate constants (k) for iDNA and eDNA were not statistically different ($p > 0.05$) over the first six weeks (Table 3), resulting in half-lives ($t_{1/2}$) of 4.7 and 5.2 days, respectively. A significant difference in k values was observed after six weeks ($p < 0.05$), when DNA concentrations were lower than 0.1 μg/g, and half-lives of 77 days for eDNA and 6.5 days for iDNA (11.8-fold lower) were estimated. Note that conversion of iDNA to eDNA occurs during cell death and, accompanied by bacterial proliferation and degradation of

Table 2. PCR Detection of Antibiotic Resistance Genes Harbored in iDNA or eDNA Extracted from Sediments Collected from Different Locations[a]

	sampling locations													
	intracellular DNA							extracellular DNA						
ARGs	1	2	3	4	5	6	7	1	2	3	4	5	6	7
sul1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sul2	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sul3	−	−	−	−	−	−	−	−	−	−	−	−	−	−
sulA	−	−	−	−	−	−	−	−	−	−	−	−	−	−
tetB	+	+	−	−	+	+	−	+	+	+	−	+	+	−
tetL	−	−	−	−	−	−	−	−	−	−	−	−	−	−
tetM	−	−	+	+	+	−	+	−	−	+	+	+	−	+
tetO	−	−	−	−	−	−	−	−	−	+	+	−	+	+
tetQ	−	+	−	+	+	−	+	−	+	−	+	+	−	+
tetS	−	−	−	+	−	−	+	−	−	−	+	−	+	+
tetT	+	+	+	+	+	+	+	+	+	+	+	+	+	+
tetW	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a]"+" means ARG detected, "−" means no detection. Sampling Locations (Figure 1): Sample Locations: 1: Dagu Bridge 2: Hongni River 3: Xinkai River 4: Machangjian River 5: Haihe Estuary.

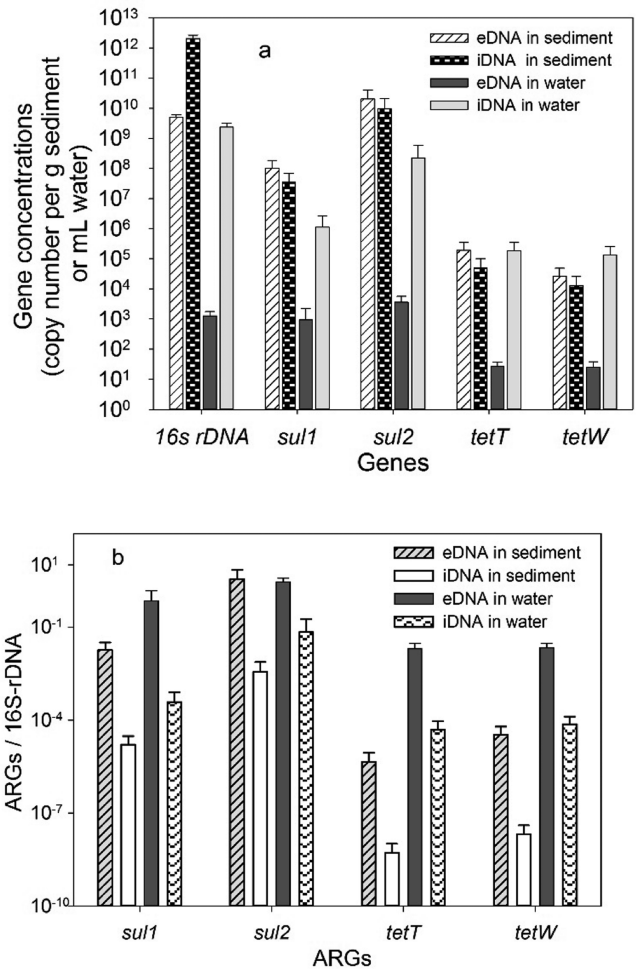

Figure 3. Abundance of ARGs and 16S-rDNA genes in eDNA and iDNA in sediment and water (a) Copies of ARGs/g sediment or ARGs/ml water; (b) Copies of ARGs/16S-rDNA. The abundance of ARGs and 16S-rDNA genes represent the average detected from all sampling sites (S1−S7).

iDNA, it confounds the calculation of eDNA degradation rate constants. The decay of eDNA after the eighth week was also evaluated (k = 0.009 day^{-1} and $t_{1/2}$ = 77 days). Since the iDNA concentration (0.021 ± 0.007 μg/g) was fairly low by the eighth week, and it was lower than the limit of detection after the ninth week, the conversion of iDNA to eDNA could be discounted, and a significant difference ($p < 0.05$) in k values between iDNA and eDNA degradation was still observed. This provides strong evidence that the persistence of eDNA in sediment is not just an artifact of iDNA conversion, but is due to innate factors which impart greater stability in this environment. Indeed, previous work has shown that the degradation kinetics of eDNA can vary significantly depending on environmental conditions.[22,48,49]

Both forms of DNA were much more persistent in sediment than in water, and eDNA was more persistent in sediment samples than iDNA (Figure 4). Although concentrations of both eDNA and iDNA rapidly decreased in water, and were undetectable by the second week, sediment-associated eDNA was still detected after 12 weeks at 0.06 μg eDNA/g. Furthermore, ARGs were still detectable after 12 weeks; *sul2* and *tetW* were at concentrations between limit of detection and the LOQ (SI Tables S2 and S3), *sul1* and *tetT* were at concentrations close to the LOQ (SI Tables S2 and S3). In contrast, 16S rRNA genes were not detected (lower than 8 copies per 25 μL of reaction mixture, SI) Table S2, which corroborates that chromosomal DNA was more rapidly degraded than the plasmid DNA harboring the ARGs. The prevalence of *sul1* and *sul2* genes in the Haihe River has been demonstrated in our previous study,[4] and attenuation of 16S rRNA genes was consistent with the attenuation of iDNA (Figure 4). This strongly supports the hypothesis that sediment-associated eDNA is a reservoir for antibiotic resistance genes, which could increase the potential for ARG proliferation and propagation through indigenous microbial communities.

Bacteria Assimilate Extracellular ARGs in Sediments. The transformation of extracellular kanamycin resistance (Kr) genes to indigenous bacteria was observed (Table 4), and a single kanamycin resistant isolate was identified by 16S rRNA sequencing. The isolate was phylogenetically closest to *Proteus* sp. NC (expect = 0.0, Identities 774/791 = 98%) as determined by BLAST analysis.

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Table 3. First-Order Decay Constants for eDNA and iDNA in Sediment (Raw Data Is Given in SI Table S4)

concentration	DNA in sediments	initial concentration (μg/g)	first-order rate constant, k (day^{-1})	*P*-value	R^2	$t_{1/2}$ (d)	interval
higher concentration (>100 μg/g)	eDNA	202.956 ± 1.116	0.133 ± 0.002	<0.05	0.9998	5.212	0–6 weeks
	iDNA	120.234 ± 0.762	0.147 ± 0.002	<0.05	0.9998	4.715	
lower concentration (<0.1 μg/g)	eDNA	0.086 ± 0.008	0.009 ± 0.003	<0.05	0.7641	77.016	6–20 weeks
	iDNA	0.087 ± 0.005	0.106 ± 0.012	<0.05	0.9807	6.539	
	eDNA	0.076 ± 0.011	0.009 ± 0.005	<0.05	0.6498	77.016	8–20 weeks

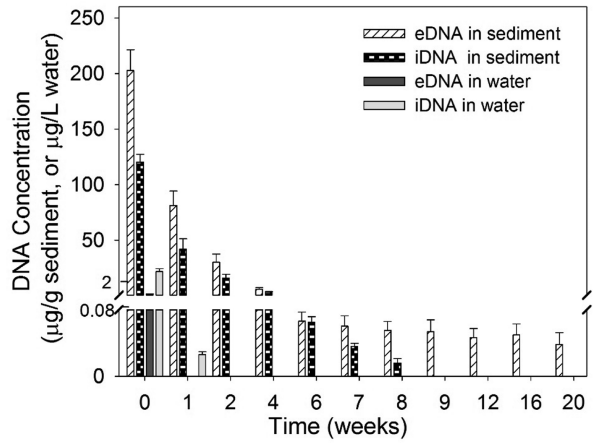

Figure 4. Persistence of eDNA and iDNA in sediment and water samples from the Haihe River.

Table 4. Assimilation of eDNA (Kr genes) into iDNA[a]

	Kr genes in iDNA (copies per L water)	
	10^{th} day	15^{th} day
control[b]	0	0
treatment 1[c]	388.6 ± 63.1	481.0 ± 77.9
treatment 2[d]	346.7 ± 55.2	801.0 ± 94.7

[a]Donor: Kr genes in pEASY plasmid, Recipient: Sediment lacking a kanamycin resistance gene. [b]Control: Recipient + 20 mg/L Kanamycin. [c]Treatment 1: Donor + Recipient. [d]Treatment 2: Donor + Recipient +20 mg/L Kanamycin.

During bacterial assimilation of extracellular ARGs, the presence of antibiotics exerts selective pressure, which facilitates their transformation (Kr genes presented as eDNA in this experiment). The abundance of Kr genes was significantly higher (*t* test, $p < 0.05$) in kanamycin treatment than the nonkanamycin group 1 by the 15th day (Table 4), demonstrating that antibiotics are capable of exerting sufficient selective pressure to facilitate the transformation of Kr genes from eDNA.

In summary, this work presents a reliable internal standard method to extract eDNA and iDNA from water and sediment and quantitatively discern their concentrations. By applying this method, higher concentrations of eDNA (compared to iDNA) were obtained in sediment from the Haihe River basin, likely reflecting enhanced persistence of eDNA in sediments. ARGs including *sul1*, *sul2*, *tetW*, and *tetT* were present at higher concentrations in eDNA than in iDNA, and predominated in sediments. This suggests that sediment-associated eDNA can serve as an important reservoir for ARG maintenance and propagation. Given the importance of assessing the risk posed by ARGs in the environment, further studies should focus on characterizing mechanisms and rates of ARG propagation through natural transformation from eDNA to indigenous bacteria.

■ ASSOCIATED CONTENT

Supporting Information

PCR conditions and primers, soil texture, correlation of clay content and eDNA concentration in sediments, Nonlinear regression and half-life calculation for eDNA and iDNA, and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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