Proliferation of Multidrug-Resistant New Delhi Metallo-β-lactamase Genes in Municipal Wastewater Treatment Plants in Northern China

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S Supporting Information

ABSTRACT: The New Delhi metallo-β-lactamase (NDM-1) increases bacterial resistance to a broad range of antibiotics, and bacteria that produce it can cause infections that are very difficult to treat, thus posing great risks to human health. This paper addresses the occurrence of NDM-1 genes through different processes in wastewater treatment plants (WWTPs). NDM-1 genes prevailed through several treatment units (including disinfection by chlorination) in two WWTPs in northern China. Significant NDM-1 gene levels were present in the effluent discharged from both WWTPs (from 1316 ± 232 to 1431 ± 247 copies/mL, representing from 4.4 to 93.2%, respectively, of influent levels). NDM-1 genes were present at much higher concentrations in dewatered waste sludge that is applied to soils [$(4.06 \pm 0.98) \times 10^7$ to $(6.21 \pm 2.23) \times 10^7$ copies/g of dry weight], raising the possibility of propagation to indigenous bacteria. This concern was validated by a conjugation experiment with Haihe River sediment not harboring NDM-1 genes at detectable levels, where an NDM-1-positive *Achromobacter* sp. isolated from a WWTP transferred the NDM-1 gene to an indigenous *Comamonas* sp. The discharge of NDM-1 genes in the effluent and dewatered waste sludge from WWTPs (even at rates higher than influent values) underscores the need to better understand and mitigate their proliferation and propagation from WWTPs.
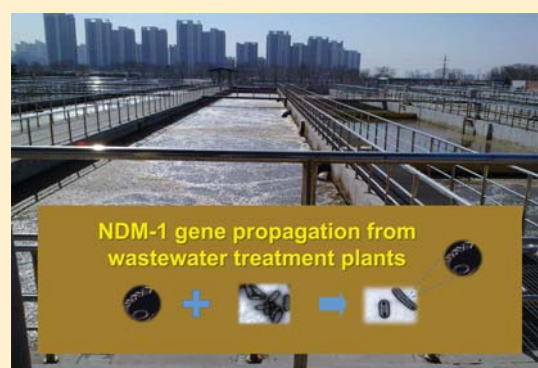
INTRODUCTION

Multidrug-resistant bacteria (MRB) pose an imminent threat to global health.[1,2] The emergence of MRB is commonly acknowledged to be due to the widespread, indiscriminate, and increasing use of antibiotics, of which β-lactam antibiotics are among the most commonly prescribed. Bacterial genes encoding β-lactamases, which hydrolyze β-lactam rings and disrupt the antibiotic properties of the molecules, are continuously being identified as major determinants of multidrug resistance. In particular, the metallo-β-lactamases (MBL) are the most troubling because of their zinc-catalyzed mechanism of hydrolysis, and bacteria that produce them can cause infections that are very difficult to treat.[3]

In 2009, a broad-spectrum antibiotic-resistant strain of *Klebsiella pneumonia* was isolated from a Swedish patient previously hospitalized in India.[4] The antibiotic resistance determinant was identified as a novel MBL and designated the New Delhi metallo-β-lactamase (NDM-1). To date, cases of multidrug-resistant bacteria (mostly enterobacteria) harboring the encoding gene, bla_{NDM-1}, have been found on every continent except Antarctica. The widespread proliferation of bla_{NDM-1} is particularly concerning because it endows its host with resistance to all β-lactam antibiotics except aztreonam.

Furthermore, bla_{NDM-1} is plasmid-borne and can propagate rapidly. In some cases, bla_{NDM-1}-positive isolates have been found to be resistant to all antibiotics, including tigecycline and colistin to which these isolates are normally susceptible.[5,6]

NDM-1 genes are appearing more frequently in nonclinical samples and were recently detected in *Acinetobacter lwoffii* isolated from chicken in eastern China[7] and in seepage and tap water samples from New Delhi.[8] Most recently, the NDM-1 gene was detected in hospital sewage;[9] however, there are few or no data regarding propagation of the NDM-1 gene and its fate in the environment.

This is the first report of the occurrence, persistence, and fate of NDM-1 genes through different processes in wastewater treatment plants (WWTPs). Conjugation experiments with an NDM-1-positive *Achromobacter* sp. isolated from a WWTP and indigenous bacteria in Haihe River sediment were also conducted to address the potential propagation of NDM-1 genes in environments receiving WWTP discharges. Knowl-
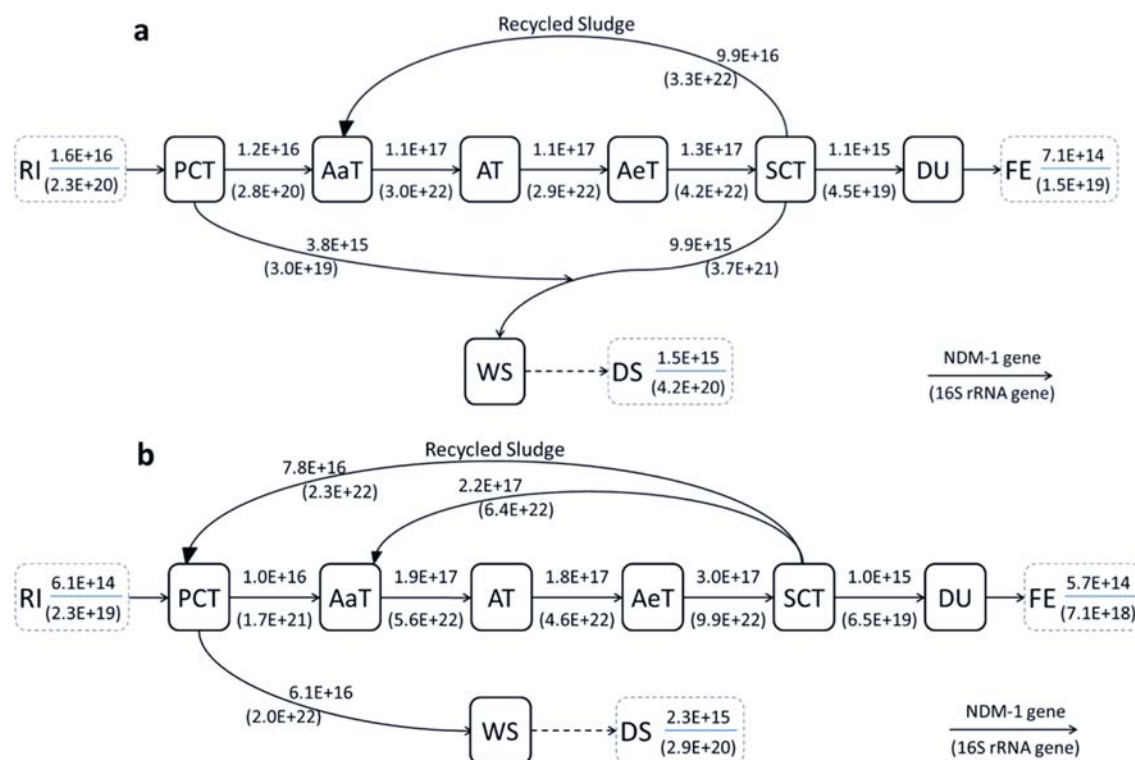
Figure 1. Process configuration and gene flow (copy numbers per day) through two wastewater treatment plants (WWTPs) in northern China: (a) WWTP1 and (b) WWTP2. Flows of NDM-1 genes are given above the arrow, and flows of 16S rRNA genes (in parentheses) are given below the arrow. Influent-normalized flows and associated balances are provided in Table S3 of the Supporting Information for WWTP1 and Table S4 of the Supporting Information for WWTP2. Abbreviations: RI, raw influent; PCT, primary clarifier tank; AaT, anaerobic tank; AT, anoxic tank; AeT, aerated tank; SCT, second clarifier tank; DU, disinfection unit; FE, final effluent; WS, waste sludge; DS, dewatered sludge (also lime-treated for WWTP2).

edge of the distribution of NDM-1 genes in WWTPs and the associated discharge patterns helps inform strategies for mitigating the propagation of multidrug resistance determinants and the associated risks to public health.

■ MATERIALS AND METHODS

Sampling. Water and sludge samples were collected from the outlet of each unit of two WWTPs in northern China on December 12, 2011. These WWTPs use anaerobic and anoxic lagoons followed by a conventional activated sludge process (Figure 1). WWTP1 receives mainly domestic sewage, whereas WWTP2 treats both domestic and industrial wastewater (Table S1 of the Supporting Information). To avoid confounding effects associated with hydraulic loading fluctuations, samples from the effluent of various treatment units were collected in triplicate every 2 h for a 24 h period and stored on ice in the dark before being processed further.

DNA Extraction and Polymerase Chain Reaction (PCR) of NDM-1 Genes. Water samples (0.5 L) were vacuum-filtered (0.22 μm filters), and the filters were placed in extraction tubes provided in the Ultraclean Water DNA Kit (MoBio Laboratories, Inc.). For sludge, DNA was extracted with the Soil DNA Isolation Kit (MoBio Laboratories, Inc.). The extracted DNA was further purified using the DNA pure-spin kit (Vigorousbio, Beijing, China) to minimize PCR inhibition. An internal standard (*Escherichia coli* DH5α cloned with the CESA9 gene) was used to determine DNA extraction efficiency as detailed previously.[10] The abundances of NDM-1, 16S rRNA genes, and DNA extraction recoveries in the effluent

of each stage of WWTPs are listed in Table S2 of the Supporting Information.

PCR of NDM-1 Genes. PCR primers for the NDM-1 gene were designed according to reported NDM-1 sequences (GenBank entry FN396876). Primer sequences were CTCG-CACCGAATGTCTGGC (NDM-1−533 forward), GCGGC-GTAGTGCTCAGTGTC (NDM-1−533 reverse), GGAATT-GCCCAATATTATGC (NDM-1−807 forward), and CGCA-GCTTGTCGGCCATG (NDM-1−807 reverse). The NDM-1−533 primer pair was used for environmental samples, and the NDM-1−807 pair was used for pure cultures of isolates, using previously described procedures.[10] For quantitative PCR, the NDM-1 gene primers used were CGCCATCCCTGACG-ATCAAA (NDM-1−214a) and CTGAGCACCGCATTAGC-CG (NDM-1−214s). The target genes (NDM-1 and 16S rRNA genes) were cloned into *E. coli* DH5α and were sequenced to verify the absence of nonspecific amplification. For details about molecular methods, please refer to the Supporting Information (section SI-1 and Table S3).

Isolation of NDM-1-Positive Bacteria. Multiresistance plates containing various antibiotics, sulfamethoxazole (50 mg/L), ampicillin (100 mg/L), erythromycin (50 mg/L), chloromycetin (50 mg/L), streptomycin (50 mg/L), kanamycin (25 mg/L), neomycin (50 mg/L), and tetracycline (50 mg/L), were used to isolate NDM-1-positive strains from WWTP samples. NDM-1 genes were verified by PCR and DNA sequencing of the isolates as detailed in section SI-2 of the Supporting Information. The susceptibility of the isolated bacteria to antibiotics was assessed by the broth microdilution method as set by the Clinical and Laboratory Standards

Institute.[11] Minimal inhibitory concentrations (MICs) were determined using standard methods,[11] as detailed in section SI-3 of the Supporting Information.

NDM-1 Gene Transfer. A 9 day microcosm experiment based on the OECD 308 test[12] was used to determine the potential of the NDM-1 gene to transform indigenous bacteria. A NDM-1-positive strain isolated from the aeration tank (AeT) of WWTP2 was used as the NDM-1 gene donor. The recipients were indigenous bacteria from Haihe River sediment, which tested negative for the NDM-1 gene prior to the addition of the donor strain. Microcosms without the NDM-1 gene donor were used as controls. Beakers (2 L) were filled with sediment (300 g dry weight from the top 5 cm of the sediment layer) and river water (1.5 L). The microcosms were incubated at 20 °C with alternating light cycles (light for 10 h and dark for 14 h) using artificial climate boxes (SPX-250/300IC, Medical Equipment Factory, Shanghai Boxun Industry & Commerce GO., Ltd., Shanghai, China). The isolate carrying the NDM-1 gene (10^9 copies/mL, 5 mL) was added to all but the control microcosms. Colonies of multidrug-resistant bacteria were screened on multiresistance plates containing sulfamethoxazole (50 mg/L), ampicillin (100 mg/L), erythromycin (50 mg/L), chloromycetin (50 mg/L), streptomycin (50 mg/L), kanamycin (25 mg/L), neomycin (50 mg/L), and tetracycline (50 mg/L). PCR and DNA sequencing were used to confirm that the NDM-1 gene had been transferred.

■ RESULTS AND DISCUSSION

Prevalence of NDM-1 Genes in WWTPs. NDM-1 genes were present in the influent and prevailed through each stage of the WWTPs considered. These NDM-1 genes had a DNA sequence that was 99.8% identical (532 bp match) to the reported NDM-1 genes carried by *Acinetobacter baumannii*. NDM-1 genes were also detected in the final effluent (FE) and waste dewatered sludge (DS) of both WWTPs, which raises the possibility of propagation to indigenous bacteria following DS land application and effluent discharge. NDM-1 gene levels in the disinfected effluent were 1316 ± 232 copies/mL for WWTP1 and 1431 ± 247 copies/mL for WWTP2. Although these concentrations account for a relatively small fraction of the corresponding 16S rRNA gene concentration in the effluent (i.e., 4.9×10^{-5} and 8.1×10^{-5}, respectively), they represent 4 and 93%, respectively, of influent NDM-1 gene flow levels (Figure 1). NDM-1 gene concentrations in DS were much higher, reaching $(4.06 \pm 0.98) \times 10^7$ copies/g for WWTP1 and $(6.21 \pm 2.23) \times 10^7$ copies/g for WWTP2 (Figure 2). These values also correspond to a small fraction of the respective 16S rRNA gene concentration (3.6×10^{-6} and 8.0×10^{-6}, respectively) but represent 9 and 370%, respectively, of influent NDM-1 gene flow levels.

Flow of NDM-1 Genes through Various Treatment Units. The total load of NDM-1 genes (as well as 16S rRNA genes, as a surrogate for total bacteria) entering the WWTPs and flowing through each unit was determined by multiplying volumetric flow rates by the corresponding gene concentrations (Figure 1). Both WWTPs discharge NDM-1 genes to the environment, predominantly through land application of dewatered sludge but also through the disinfected effluent. Whereas a reduction in the total NDM-1 load (i.e., 86% of influent) occurred through WWTP1 (Figure 1a), the overall discharge of NDM-1 for WWTP2 was 4.6-fold greater than influent values (0.9-fold in the FE and 3.7-fold in the DS) (Figure 1b), underscoring the potential for NDM-1 prolifer-
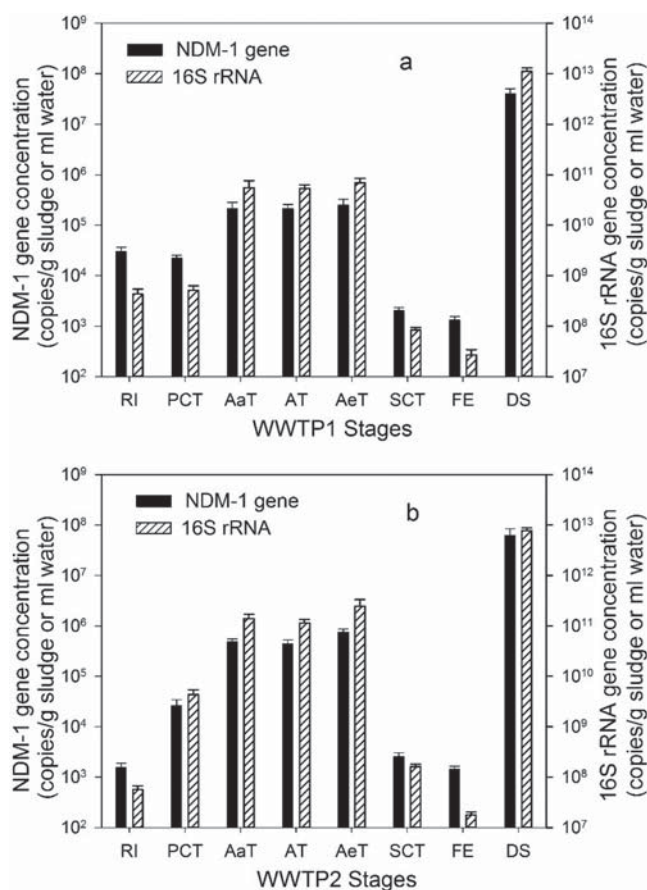

Figure 2. NDM-1 and 16S rRNA gene concentrations in the effluent of different stages of (a) WWTP1 and (b) WWTP2. Please refer to Figure 1 for plant configuration and stage abbreviations.

ation in some WWTPs. The significant differences in NDM-1 discharges are surprising because both WWTPs disinfect (chlorinate) their effluents, although WWTP2 exhibits an unusual system configuration by recycling sludge from the SCT to the PCT to enhance the settling of suspended solids. This increases total microbial concentrations (i.e., 16S rRNA) by 2 orders of magnitude from the RI to the PCT (Figure 2b), resulting in NDM-1 gene concentrations in water exiting the PCT that are 17-fold higher than influent values (Figure 1b). Mass balances of NDM-1 and 16S genes flowing in and out of each unit of WWTP1 (Table S4 of the Supporting Information) and WWTP2 (Table S5 of the Supporting Information) reflect proliferation of NDM-1 genes in units experiencing microbial growth during sewage biodegradation.

Variation in NDM-1 Gene Concentrations through Various Treatment Units. Changes in NDM-1 gene abundance through each treatment unit (excluding the DU) followed a trend similar to that seen for 16S rRNA genes (Figure 2). A positive correlation ($p < 0.01$) was found between the abundance of NDM-1 and 16S rRNA genes in both plants (Figure S1 of the Supporting Information), which implies that fluctuations in NDM-1 concentrations through various treatment units were primarily attributable to changes in total microbial abundance (e.g., proliferation in biological reactors and densification due to settling and dewatering) rather than to differential enrichment or removal. One exception was the removal efficiencies through the disinfection units, which were significantly lower ($p < 0.05$) for NDM-1 than for 16S rRNA

Table 1. Minimal Inhibitory Concentrations (MICs) of Antibiotic Resistance of NDM-1-Positive Bacteria from This Study and the Literature

	antibiotics	MIC^a (mg/L)	ref
β-lactam antibiotics	imipenem IMP	0.5–64	4, 7, 15–20
	meropenem MEM	0.5–256	4, 7, 15, 17–20
	ertapenem ERT	2–512	4, 7, 16, 18, 19
	doripenem DOR	1 to >32	18, 19
	cefotaxime COT	16 to >512	4, 7, 15–17
	ceftazidime CAZ	32 to >512	4, 7, 16–19
	cefepime CEP	1 to >256	4, 7, 16–19
	cefradine CRA	16 to >512	7
	2:1 cefoperazone/ sulbactam CPS2/1	2 to >256	17
	ceftriaxone CEF	>256	18
	cefoxitin CEF	2–256	4
	cefuroxime CEX	8–256	4
	cephalothin CEP	4–256	4
	aztreonam AZT	0.064 to >256	4, 7, 17–19
	ampicillin AMP	32 to >256, **>128**	4, 7, 17, this study
	piperacillin/tazobactam TZP	4 to >256	4, 16–18
	amoxicillin-clavulanate AMX/CLAc	>16	18
aminoglycoside	amikacin AK	0.5–256	15–19
	kanamycin KAN	0.5–64, **>64**	7, this study
	gentamicin GEN	0.25 to >256	7, 17, 18
	tobramycin TOB	>16	18
	streptomycin	**>512**	this study
	neomycin	**>64**	this study
quinolone	levofloxacin LEV	0.25 to >32	15, 19
	ciprofloxacin CIP	0.004 to >32	4, 7, 16–18
polypeptide	polymyxin B POL	0.5–5	7, 18
	colistin COL	0.12 to >8	4, 15–17, 19
tetracycline antibiotics	minocyclin MN	0.125 to <32	15, 17
	tetracycline TET	2–32, **>64**	7, 18, this study
	tigecycline TGC	0.12–16	16–19
chloramphenicols	chloramphenicol CHL	2–32, **>128**	7, this study
sulfonamide	sulfamethoxazole	**>64**	this study
macrolides	erythromycin	**>64**	this study

^{a}Bold numbers are the MIC values obtained in this study.

genes. Specifically, removal efficiencies by chlorination in WWTP1 were 68.3 ± 4.0% for 16S rRNA versus 34.5 ± 3.7% for NDM-1 genes, whereas the corresponding efficiencies for WWTP2 were 88.8 ± 2.3 for 16S rRNA and 43.1 ± 3.8% NDM-1 genes. The lower removal efficiencies for NDM-1 versus those for 16S rRNA genes through the disinfection (by chlorination) units of both WWTPs suggest that some bacteria harboring NDM-1 genes may be coincidentally coresistant to chlorination, as suggested by previous research.[13,14]

Isolation of an NDM-1-Positive Bacterium. A multidrug-resistant strain was isolated from multiple treatment units of WWTP2 using multiresistance plates, and colonies from AeT were identified by 16S rRNA gene sequencing (99% identical) as *Achromobacter* sp. The NDM-1 gene in this isolate was 99.9% (806 bp match) identical to that in the NCBI database associated with *A. baumannii*. Our isolate was resistant to the

eight tested antibiotics, with a minimal inhibitory concentration (MIC) of >64 mg/L (Table 1). For comparison, the NDM-1-positive *Acinetobacter lwoffii* isolated from chicken in eastern China was less resistant, with corresponding MIC values of <32 mg/L.[7] Such multidrug-resistant bacteria in WWTPs could pose risks to public health if they propagate through the environment.

NDM-1 Gene Transfer. A novel NDM-1 gene-positive bacterium that was resistant to multiple antibiotics (Table 1) was isolated after 9 days from NDM-1-negative Haihe River sediment that had been inoculated with the NDM-1-positive *Achromobacter* sp. The NDM-1 gene in this isolate was 100% identical (806 bp match) to that in the *Achromobacter* sp. donor, yet colony morphology was different for this isolate (it was circular, possessed a smooth edge and surface, and was translucent with a light yellow color). Using 16S rRNA sequencing and BLAST alignment using the GenBank database, this strain was determined to be phylogenetically close (99% identical) to *Comamonas* sp. Y14B, an indigenous bacterium commonly found in the environment. This illustrates the potential for propagation of NDM-1 genes discharged from WWTPs, which increases risks to public health and underscores the need to develop more effective disinfection or barrier approaches.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed PCR and MIC protocols, operation parameters for the two WWTPs, and supporting calculations. 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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