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Antibiotic resistance, plasmid-mediated quinolone resistance (PMQR) genes and *ampC* gene in two typical municipal wastewater treatment plants

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Antibiotic resistant bacteria and plasmid-mediated quinolone resistance genes and *ampC* gene were investigated for *Escherichia coli* isolates from two typical municipal wastewater treatment plants in both dry and wet seasons by using the antibiotic susceptibility test and PCR assay, respectively. The results showed that 98.4% of the isolates (1056) were found resistant to antibiotic(s) tested and 90.6% showed multiple resistances to at least three antibiotics. Tetracycline was found to have the highest resistance frequency (70.8%), followed by ampicillin (65.1%), whereas ceftazidime had the lowest resistance frequency of 9.0%. Moreover, 39.2% of the *E. coli* isolates were carrying plasmids. *intI1* had the highest detection rate in the plasmids (38.1%), followed by *qnrS*, *ampC*, *qnrB*, *intI2* and *aac(6′)-Ib-cr*. The disinfection process (UV and chlorination) could significantly reduce the number of bacteria, but percentage of the resistant bacteria, resistance frequency for each antibiotic, MAR index and detection rate of the plasmid-mediated resistance genes were all found increasing in the effluents of biological units. The results of this study showed that a more frequent horizontal gene transfer occurred in the biological units. Wastewater treatment plants were an important medium for the recombination and dissemination of antibiotic resistance genes in the environment.

Environmental impact

The wide application of antibiotics can lead to environmental contamination with antibiotic residues and development of bacterial resistance to antibiotics. This has become a global public concern. A variety of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) were widely detected in various environmental media. Effluents from wastewater treatment plants (WWTPs) are suspected to be one of the main anthropogenic sources for ARGs and ARB. Therefore, it is important to understand the removal and dissemination of ARGs and ARB in WWTPs. Plasmids are the major vectors of horizontal gene transfer (HGT). Quinolone resistance genes and ampC gene are two kinds of ARGs contained in the plasmids normally found in many *Enterobactericeae* isolated from clinical samples. Unfortunately, little is known about their occurrence and characteristics in WWTPs. This study aimed to investigate the antibiotic resistance profiles in *Escherichia coli* isolated from two typical municipal wastewater treatment plants (activated sludge and oxidation ditch processes), to characterize plasmid-mediated quinolone resistance genes, ampC gene and integrons by the polymerase chain reaction (PCR), and to assess the effects of treatment processes on the removal of ARB and ARGs in the WWTPs. The results showed that the treatment processes especially disinfection processes (UV and chlorination) could significantly reduce the number of bacteria, but increase bacterial resistance in the biological treatment units. The findings reflect the importance of WWTPs as a reservoir of ARB and ARGs, and as a medium for their recombination and dissemination in the environment.

1. Introduction

Antibiotics are extensively used to prevent and/or treat diseases in humans and animals.[1,2] In China, approximately 210 000 tons of antibiotics are produced each year, with 46% being used for animal husbandry.[3] The wide application of antibiotics can lead to environmental contamination with antibiotic residues and development of bacterial resistance to antibiotics.[4] This has become a global public health concern.

A variety of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) were widely detected in various environmental media, for instance, wastewaters from wastewater treatment plants (WWTPs),[5–7] surface water and sediments.[8–11] Effluents from WWTPs are suspected to be the main anthropogenic sources for ARGs and ARB.[12] ARB and ARGs in the environment could threaten human health, as suggested by increasing medical events. According to the US CDC (2013), at least two million Americans are infected with resistant pathogens each year, and at least 23 000 people die as a direct result of these infections.[13] Therefore, it is important to understand the removal and dissemination of ARGs and ARB in WWTPs.

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Plasmids are the major vectors of horizontal gene transfer (HGT). They are considered to play a key role in the transfer of ARGs because of their autonomous physical movement (*via* conjugation) and autonomous replication.[14–16] Quinolone resistance genes and *ampC* gene are two kinds of ARGs contained in the plasmids normally found in many *Enterobacteriaceae* isolated from clinical samples. Quinolone resistance was considered to be acquired only by chromosomal mutations until plasmid-mediated quinolone resistance (PMQR) was first reported in a *Klebsiella pneumoniae* clinical strain isolated in 1994 in Birmingham, Alabama,[17] and so far PMQR strains have been reported worldwide from unrelated species.[18–20] Quinolone resistance genes consist of *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, encoding DNA gyrase and/or topoisomerase IV; *qepA*, regulating the expression of efflux pumps and *aac(6′)-Ib-cr*, encoding a variant aminoglycoside acetyltransferase that modifies ciprofloxacin.[17,18,21] Besides PMQR genes, plasmid-mediated *ampC* is under the spotlights because of its ability of hydrolyzation of third-generation cephalosporins. AmpC enzymes are classified as miscellaneous extended-spectrum β-lactamase (ESBL) enzymes. High level expression of *ampC* confers bacterial resistance to penicillins, monobactams, oxyimino-cephalosporins and cephamycins. In combination with porin deficiency ampC β-lactamases can also confer resistance to carbapenems. Normally tightly regulated chromosomal genes encoding AmpC enzymes are found in several species of *Enterobacteriaceae*.[22] However, mobilization of plasmid-mediated *ampC* from the chromosome onto plasmids has also led to the occurrence and high level expression of *ampC* in species lacking *ampC*.[23] Although plasmid-mediated quinolone resistance genes and *ampC* gene were often detected in the clinical isolates, unfortunately little is known about their occurrence and characteristics in WWTPs.

Some previous studies haveinvestigated the antibiotic resistance and occurrence of ARB and ARGs in WWTPs.[6,24–26] Multiple antibiotic resistance was observed for fecal coliforms and *enterococci* in municipal WWTPs.[24] Auerbach *et al.* observed diverse tetracycline resistance genes in activated sludge treatment plants.[25] Munir *et al.* found that disinfection (chlorination and UV) processes did not contribute to the significant reduction of ARGs and ARB in conventional utilities.[6] Rizzo *et al.* also found that conventional disinfection processes may not be effective in the activation of ARB based on the laboratory experiments.[26] However, further research is still required to investigate the effects of treatment processes on the removal of resistant bacteria. Temperature is also a crucial factor for *E. coli* bacterial growth, and its effect on the bacterial resistance pattern is also unknown.

The objective of this study was to investigate the antibiotic resistance profiles in *E. coli* isolated from two typical municipal WWTPs (activated sludge and oxidation ditch processes) in both dry and wet seasons, to detect plasmid-mediated quinolone resistance genes, *ampC* gene and integrons by polymerase chain reaction (PCR), and to assess the effects of treatment processes on the removal of ARB and ARGs in WWTPs. The results from this study could help better understand the dissemination of plasmid-mediated resistance genes from WWTPs to the aquatic environment, and their potential environmental impacts.

2. Materials and methods

2.1 Study sites and sample collection

Two municipal WWTPs (Plant A and Plant B) in Guangdong Province, South China were chosen for this study. The treatment technologies (activated sludge, and oxidation ditch) used in the two plants are typical in the region. Plant A serves a population of 425 000 equivalent inhabitants and treats up to 70 000 m^3 of municipal wastewater per day. The wastewater treatment process in Plant A consists of pre-treatment (screens), a grit chamber and a cyclic activated sludge system (CASS), which includes an anoxic tank, an anaerobic tank and an aerobic tank (AAO process), and followed by a secondary clarifier. Part of activated sludge is returned to the anoxic tank from the aerobic tank. The secondary effluent is further treated with chlorination before discharge as the final effluent. Plant B serves a population equivalent of around 380 000 inhabitants and treats around 100 000 m^3 of municipal wastewater per day. The treatment processes in Plant B includes pre-treatment (screens), a grit chamber, followed by an oxidation ditch and a secondary clarifier. The tertiary treatment in Plant B employs the Newland NLQ series UV C open channel water disinfection system (Newland Entech, Fujian).

Water samples were collected from Plant A and Plant B in May 2010 (wet season) and November 2010 (dry season). Basic information, process flow charts and sampling locations of the two WWTPs are shown in Table 1 and Fig. 1. The 24 h composite water samples were aseptically collected with sterile polyethylene bottles (500 ml). A composite sample split in three bottles was taken from each sampling point, immediately placed on ice, and transported back to the laboratory for further processing.

2.2 Isolation and identification of *Escherichia coli*

Ten-fold serial dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) of each water sample were made in sterile saline solution (0.85% NaCl). Then 0.1 ml volume from each dilution was spread on nutrient agar (Oxoid, UK) in triplicates to determine the total culturable bacteria. The inoculations were incubated at 35 °C for 24 h. Colony forming units (CFUs) on the nutrient agar plates were recorded to calculate the number of total culturable bacteria (CFUs per ml).

In order to achieve the recommended target range of *E. coli* (20–80 CFUs per filter), ten-fold serial dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) of each water sample were made in a sterile saline solution (0.85% NaCl). 0.1 ml of each dilution was filtered through a sterile membrane filter (0.45 μm pore diameter) with a vacuum filtration apparatus. Then the membrane filter was aseptically removed by using a sterile forcep and rolled on the modified membrane-thermotolerant *E. coli* agar (modified mTEC agar, BD, USA).[27] The plates were incubated at 35 ± 0.5 °C for 2 h and then sealed with parafilm and incubated at 44.5 ± 0.2 °C for 22–24 h.

Table 1 Parameters of Plant A and Plant B[a]

Process	Water flow m^3 per day	TSS mg L^{-1}	COD mg L^{-1}	BOD_5 mg L^{-1}	TP mg L^{-1}	NH4–N mg L^{-1}	pH	Conductivity ms cm^{-1}
Plant A								
Influent	74 400	150	170–180	70–80		16	8.1	
Grit chamber	74 400							
Anoxic	74 400	3000						
Anaerobic	74 400	3000						
Aerobic	74 400	3000						
Final effluent	19 680	10	20	15		3	6.7	60
Return sludge	67 200	10000						
Plant B								
Influent	96 000	175	185	110	2.7	23		
Grit chamber	96 000	3000						
Oxidation ditch	96 000	3000						
Secondary clarifier	96 000							
Final effluent	170 800	16	22	10	0.8	35		
Return sludge		4000						

[a] TSS, total suspended solids; COD, chemical oxygen demand; BOD_5, 5d biochemical oxygen demand; TP, total phosphorus.

According to the protocols of EPA,[27] red or magenta colonies on the modified mTEC agar were counted and inoculated onto the eosin methylene blue (EMB) agar and incubated at 35 ± 0.5 °C for 24 h. Then the purple-black colonies with distinctive metallic green sheen were identified as *E. coli*, inoculated onto nutrient agar and enriched at 35 ± 0.5 °C for 24 h.

2.3 Antibiotic susceptibility test

Antibiotic susceptibility was tested with the Kirby–Bauer disk diffusion method on Mueller-Hinton agar plates (Oxoid, UK), according to the guidelines of the Clinical and Laboratory Standards Institute.[28] A panel of twelve antibiotic discs (Oxoid, UK) was tested: ampicillin (AMP, 10 μg), piperacillin (PRL, 100 μg), cefazolin (KZ, 30 μg), ceftazidime (CAZ, 30 μg), gentamicin (CN, 10 μg), streptomycin (S, 10 μg), ciprofloxacin (CIP, 5 μg), levofloxacin (LEV, 5 μg), sulfamethoxazole/trimethoprim (SXT, 25 μg), trimethoprim (W, 5 μg), tetracycline (TE, 30 μg) and chloramphenicol (C, 30 μg).

Each inoculum of *E. coli* was suspended in sterile saline solution (0.85% NaCl) with a sterile swab to adjust turbidity to 0.5 McFarland standard, and streaked evenly on

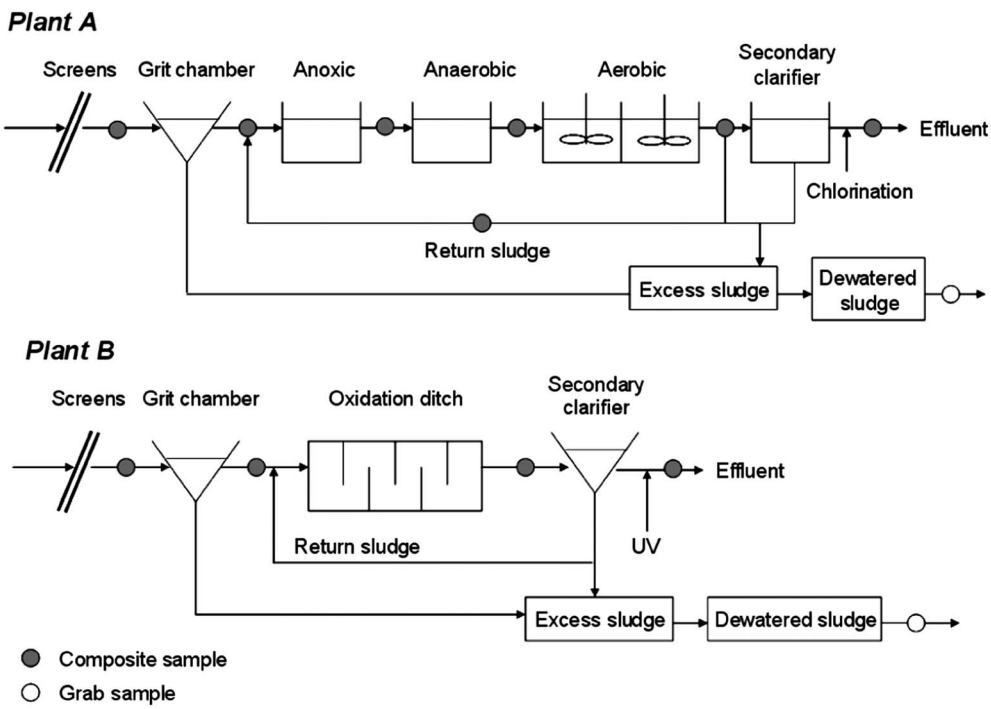

Fig. 1 Treatment process flow charts and sampling locations of the two wastewater treatment plants.

Mueller-Hinton agar plates. The antibiotic discs were placed on the agar using a disc dispenser (Oxoid, UK). Plates were incubated inverted at 35 °C for 16–18 h. Then the inhibition zone diameters were measured to the nearest millimeter, and the strains were characterized as susceptible (*S*), intermediate (*I*) or resistant (*R*) to the antibiotics based on the guidelines of the Clinical and Laboratory Standards Institute.[28] *Escherichia coli* ATCC 25922 was used as the control strain.

2.4 Extraction of plasmids and PCR assays for detection of plasmid-mediated resistance genes

E. coli isolates resistant to quinolones or beta lactamase were chosen to extract plasmids using the TIANprep Mini Plasmid Kit (TIANGEN, China) according to the manufacturer's recommendations, and the plasmid extracts were stored at −20 °C for PCR assays.

Universal PCR assays were performed to detect the plasmid-mediated quinolone resistance genes (*qnrA*, *qnrB*, *qnrS*, and *aac(6′)-Ib-cr*), *ampC* gene and integrons (*intI1*, *intI2*, and *intI3*). The primers used for amplifications of these genes are listed in Table 2. PCR assays were carried out in 25 μl volumes containing 2 μl of template DNA (*ca.* 40 ng), 0.2 mM of each dNTP (TaKaRa, Japan), 1× PCR buffer (TaKaRa, Japan), 2.0 U Taq polymerase (TaKaRa, Japan), 1.6 mM $MgCl_2$ (TaKaRa, Japan), and 0.4 μM of each primer. The temperature profile for amplification was given as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, annealing temperature of each gene (Table 2) for 30 s, 72 °C for 2 min, and a final extension step at 72 °C for 10 min. The PCR products were subjected to gel electrophoresis. Amplified DNA (5 μl) was mixed with 1 μl 6× loading buffer dye and loaded on 2% (w/v) agarose gel containing 1× Gelred nucleic acid stain (Biotium, USA), followed by running in 1× TAE buffer at 5V cm^{-1} for 40 min, and visualizing by UV transillumination.

Amplifications were performed with a Bio-Rad S1000 thermal cycler (Bio-Rad, USA). Each PCR run contained positive controls, a negative control (DNA extraction of *E. coli* ATCC 25922) and a blank control (distilled water instead of DNA extraction). The cloned DH5α *E. coli* strains of *qnrA*, *qnrB*, *qnrS*, *aac(6′)-Ib-cr*, *ampC*, *intI1*, *intI2*, *intI3* were used as a positive control for the corresponding resistance gene, respectively. Each PCR assay was run in two replicates.

2.5 Statistical analysis

The antibiotic resistance frequency (%) was calculated by the equation: $m/n \times 100$, where m is the number of *E. coli* resistant to antibiotics, and n is the number of *E. coli* isolated from the samples. To understand the potential resistance of *E. coli* to multiple antibiotics, the multiple antibiotic resistance (MAR) index was calculated for each sample by the equation: $a/(b \times c)$, where a is aggregate number of antibiotics to which test isolates displayed resistance, b is the number of antibiotics tested, and c is the number of *E. coli* strains isolated from the sample.[29,30]

One-way ANOVA was used to evaluate the statistical significance of difference with p-value < 0.05 using SPSS version 13.0 (IBM, New York). Averages and standard deviations were calculated with Microsoft Excel, 2003.

3. Results

3.1 Removal of bacteria

The numbers of the total culturable bacteria and *E. coli* are given in Table 3. Overall, the trends observed in concentration ranges at different sampling points were: raw influent > pre-disinfection effluent > post-disinfection effluent (One-way ANOVA, $p < 0.05$). The total culturable bacteria ranged from $(1.7 \pm 0.2) \times 10^3$ to $(5.4 \pm 0.7) \times 10^6$ CFUs per ml in Plant A and from $(2.5 \pm 0.3) \times 10^3$ to $(4.0 \pm 0.5) \times 10^6$ CFUs per ml in

Table 2 Primers used for detection of quinolone resistance (*qnr*) genes, *ampC* and integrons in this study[a]

Target gene	Primer pair	Sequences (5′ to 3′)	Amplicon size (bp)	Annealing temperature (°C)	Ref.
qnrA	FW	AGA GGA TTT CTC ACG CCA GG	580		
	RV	TGC CAG GCA CAG ATC TTG AC			
qnrB	FW	GGM ATH GAA ATT CGC CAC TG	264	58	44
	RV	TTT GCY GYY CGC CAG TCG AA			
qnrS	FW	GCA AGT TCA TTG AAC AGG GT	428		
	RV	TCT AAA CCG TCG AGT TCG GCG			
aac(6′)-Ib-cr	FW	TTG CGA TGC TCT ATG AGT GGC TA	482	55	45
	RV	CTC GAA TGC CTG GCG TGT TT			
ampC	FW	GGGAATGCTGGATGCACAA	643	58	22
	RV	CATGACCCAGTTCGCCATATC			
intI1	FW	ACG AGC GCA AGG TTT CGG T	565	52	46
	RV	GAA AGG TCT GGT CAT ACA TG			
intI2	FW	GTG CAA CGC ATT TTG CAG G	403	52	46
	RV	CAA CGG AGT CAT GCA GAT G			
intI3	FW	CAT TTG TGT TGT GGA CGG C	717	52	46
	RV	GAC AGA TAC GTG TTT GGC AA			
	RV	GATGCCATCGCAAGTACGAG			

[a] FW, forward; RV, reverse.

Table 3 The number of total culturable bacteria and *E. coli* of two wastewater treatment plants in dry and wet seasons

Samples			No. of total culturable bacteria[a] (CFU per ml)	No. of *E. coli* (CFU per ml)	Percentage of resistant *E. coli* (%)
Plant A	Dry season	Influent	$(3.4 \pm 0.4) \times 10^6$	$(2.7 \pm 0.4) \times 10^4$	59.4 ± 1.5
		Grit chamber	$(2.3 \pm 0.3) \times 10^6$	$(2.2 \pm 0.3) \times 10^4$	58.3 ± 2.9
		Anoxic	$(5.0 \pm 0.7) \times 10^5$	$(3.3 \pm 0.4) \times 10^4$	81.3 ± 2.7
		Anaerobic	$(3.2 \pm 0.4) \times 10^4$	$(1.3 \pm 0.2) \times 10^4$	90.6 ± 7.4
		Aerobic	$(2.0 \pm 0.3) \times 10^4$	$(1.5 \pm 0.2) \times 10^3$	93.8 ± 5.9
		Effluent	$(1.7 \pm 0.2) \times 10^3$	$(1.6 \pm 0.2) \times 10^2$	61.5 ± 4.4
	Wet season	Influent	$(5.4 \pm 0.7) \times 10^6$	$(4.3 \pm 0.6) \times 10^4$	55.2 ± 1.5
		Grit chamber	$(3.6 \pm 0.5) \times 10^6$	$(5.6 \pm 0.7) \times 10^4$	60.4 ± 2.5
		Anoxic	$(8.0 \pm 1.1) \times 10^5$	$(2.6 \pm 0.3) \times 10^4$	88.5 ± 4.4
		Anaerobic	$(5.1 \pm 0.7) \times 10^4$	$(8.7 \pm 1.1) \times 10^3$	93.8 ± 2.9
		Aerobic	$(3.2 \pm 0.4) \times 10^4$	$(2.0 \pm 0.3) \times 10^3$	91.7 ± 5.9
		Effluent	$(2.7 \pm 0.4) \times 10^4$	$(1.9 \pm 0.3) \times 10^2$	79.2 ± 2.3
Plant B	Dry season	Influent	$(2.5 \pm 0.3) \times 10^6$	$(5.0 \pm 0.7) \times 10^4$	56.3 ± 2.8
		Grit chamber	$(1.9 \pm 0.2) \times 10^6$	$(3.2 \pm 0.4) \times 10^4$	55.2 ± 1.5
		Oxidation ditch	$(2.5 \pm 0.3) \times 10^6$	$(2.4 \pm 0.3) \times 10^4$	94.8 ± 4.4
		Secondary clarifier	$(2.4 \pm 0.3) \times 10^4$	$(3.3 \pm 0.4) \times 10^3$	89.6 ± 2.5
		Effluent	$(2.5 \pm 0.3) \times 10^3$	$(2.5 \pm 0.3) \times 10^2$	66.7 ± 2.9
	Wet season	Influent	$(4.0 \pm 0.5) \times 10^6$	$(7.9 \pm 1.1) \times 10^4$	55.7 ± 1.9
		Grit chamber	$(3.0 \pm 0.4) \times 10^6$	$(5.1 \pm 0.7) \times 10^4$	55.2 ± 4.4
		Oxidation ditch	$(3.9 \pm 0.5) \times 10^6$	$(3.9 \pm 0.5) \times 10^4$	91.7 ± 2.7
		Secondary clarifier	$(3.8 \pm 0.5) \times 10^4$	$(5.2 \pm 0.7) \times 10^3$	81.3 ± 3.2
		Effluent	$(4.0 \pm 0.5) \times 10^3$	$(3.9 \pm 0.5) \times 10^2$	61.5 ± 4.5

[a] Total culturable bacteria refer to all bacteria that can grow on the nutrient agar plate at 35 °C.

Plant B. The highest number was found in the influent samples of Plant A and Plant B in both seasons. The number of *E. coli* was observed to be much lower, ranging between $(1.6 \pm 0.2) \times 10^2$ and $(4.3 \pm 0.6) \times 10^4$ CFUs per ml in Plant A, and between $(2.5 \pm 0.3) \times 10^2$ and $(7.9 \pm 1.1) \times 10^4$ CFUs per ml in Plant B. The percentage of resistant *E. coli* in the isolates ranged from 55.2% to 93.8% in Plant A, and from 55.2% to 94.8% in Plant B. The highest percentage of resistant *E. coli* was observed in biological units (*i.e.*, anaerobic process for Plant A and oxidation ditch for Plant B). The lowest percentage of resistant *E. coli* was found in the influent for Plant A and in the grit chamber process for Plant B. The number of total culturable bacteria and E. *coli* in the wet season was significantly higher than that in the dry season (One-way ANOVA, $p < 0.05$), because the higher temperature in the wet season facilitated faster bacterial growth. The removal of total culturable bacteria and *E. coli* was 2.3–3.3 log reduction in Plant A and 2.3–3.0 log reduction in Plant B. No significant difference in abundance of total culturable bacteria and *E. coli* was observed between Plant A and Plant B (One-way ANOVA, $p > 0.05$).

3.2 Antibiotic resistance

High prevalence of antibiotic resistance was found in the two municipal WWTPs. A total of 1056 *E. coli* isolates were collected from the water samples in May 2010 (wet season) and December 2010 (dry season) in Plant A and Plant B. Among 576 *E. coli* isolates in Plant A, 567 isolates (98.4%) were resistant to at least one of the 12 antibiotics tested. In addition, the frequency of multiple resistances (resistance to at least three antibiotics) was high up to 90.6%. High resistance frequencies were also found in Plant B, with 476 out of 480 *E. coli* isolates (99.2%) being resistant. The multiple resistance rate was 95.4%, and 49 isolates (10.2%) were observed resistant to all 12 antibiotics tested.

For determination of the resistance rate of each antibiotic, the frequencies of antibiotic resistance for *E. coli* isolates are calculated and shown in Table 4. Among the 12 antibiotics tested, the most frequently detected resistance was found for tetracycline, with an average frequency of 70.8% and 68.8% in Plant A and Plant B, respectively, followed by ampicillin. Ceftazidime was found with the lowest resistance frequencies, *i.e.* 9.0% for Plant A, and 12.9% for Plant B. The bacterial resistance to the rest antibiotics in the two plants was listed as follows: for Plant A, ampicillin (69.8%), streptomycin (61.5%), trimethoprim (53.1%), piperacillin (52.4%), sulfamethoxazole/trimethoprim (52.1%), chloramphenicol (36.1%), cephazolin (30.9%), ciprofloxacin (26.4%), gentamicin (25.3%) and levofloxacin (19.1%); and for Plant B, ampicillin (60.4%), trimethoprim (59.6%), sulfamethoxazole/trimethoprim (58.8%), piperacillin (51.3%), streptomycin (48.8%), chloramphenicol (29.6%), ciprofloxacin (25.8%), levofloxacin (25.4%), cephazolin (24.6%), and gentamicin (23.8%). The MAR indices for multiple antibiotic resistances ranged from 0.28 to 0.70 in Plant A and from 0.49 to 0.78 in Plant B (Table 4). The highest MAR values were all found in the effluents of biological units in both Plant A and Plant B. It should be noted that the MAR indices for the effluents were in most cases higher than those for the influents (One-way ANOVA, $p < 0.05$) except for Plant A in the dry season. No significant difference for the antibiotic resistance frequencies and MAR indices was observed between dry and wet seasons (One-way ANOVA, $p > 0.05$).

Table 4 Antibiotic resistance frequencies and MAR indices of two different types of municipal wastewater treatment plants in dry and wet seasons

Samples (no. of isolates)			Resistance frequencies of resistant *E. coli* (%)[a]												MAR index[b]
			AMP	PRL	KZ	CAZ	CN	S	CIP	LEV	SXT	W	TE	C	
Plant A	Dry season	Influent (48)	54.2	54.2	37.5	8.3	20.8	54.2	12.5	12.5	45.8	45.8	54.2	16.7	0.45 ± 0.05b
		Grit chamber (48)	50.0	20.8	12.5	4.2	4.2	41.7	16.7	16.7	20.8	25.0	37.5	12.5	0.35 ± 0.04c
		Anoxic (48)	54.2	62.5	20.8	4.2	20.8	70.8	20.8	12.5	45.8	45.8	83.3	29.2	0.48 ± 0.06b
		Anaerobic (48)	95.8	83.3	50.0	12.5	54.2	91.7	37.5	33.3	62.5	62.5	95.8	25.0	0.70 ± 0.08a
		Aerobic (48)	91.7	41.7	25.0	12.5	20.8	95.8	16.7	12.5	87.5	87.5	95.8	87.5	0.67 ± 0.07a
		Effluent (48)	91.7	87.5	37.5	12.5	29.2	83.3	37.5	12.5	33.3	33.3	62.5	16.7	0.45 ± 0.05b
	Wet season	Influent (48)	54.2	45.8	45.8	12.5	33.3	54.2	45.8	29.2	54.2	54.2	50.0	12.5	0.36 ± 0.04c
		Grit chamber (48)	58.3	45.8	16.7	4.2	20.8	41.7	20.8	20.8	37.5	45.8	62.5	37.5	0.28 ± 0.03c
		Anoxic (48)	75.0	62.5	41.7	12.5	37.5	41.7	25.0	25.0	33.3	33.3	70.8	37.5	0.40 ± 0.05b
		Anaerobic (48)	62.5	29.2	29.2	4.2	20.8	37.5	37.5	16.7	62.5	62.5	62.5	45.8	0.48 ± 0.06b
		Aerobic (48)	79.2	41.7	20.8	8.3	20.8	58.3	12.5	12.5	87.5	87.5	95.8	83.3	0.60 ± 0.07a
		Effluent (48)	70.8	54.2	33.3	12.5	20.8	66.7	33.3	25.0	54.2	54.2	79.2	29.2	0.43 ± 0.05b
		Average (576)	69.8	52.4	30.9	9.0	25.3	61.5	26.4	19.1	52.1	53.1	70.8	36.1	0.47 ± 0.13
Plant B	Dry season	Influent (48)	75.0	58.3	16.7	8.3	20.8	58.3	41.7	29.2	45.8	45.8	58.3	33.3	0.62 ± 0.06b
		Grit chamber (48)	54.2	37.5	33.3	16.7	33.3	37.5	20.8	20.8	50.0	50.0	54.2	29.2	0.55 ± 0.07b
		Oxidation ditch (48)	70.8	66.7	29.2	16.7	33.3	58.3	37.5	33.3	70.8	75.0	79.2	45.8	0.78 ± 0.08a
		Secondary clarifier (48)	58.3	54.2	29.2	16.7	25.0	66.7	37.5	33.3	70.8	70.8	83.3	25.0	0.72 ± 0.08a
		Effluent (48)	66.7	62.5	37.5	12.5	33.3	16.7	16.7	41.7	79.2	79.2	91.7	33.3	0.72 ± 0.08a
	Wet season	Influent (48)	66.7	50.0	8.3	12.5	12.5	50.0	33.3	20.8	37.5	37.5	50.0	25.0	0.59 ± 0.07b
		Grit chamber (48)	45.8	29.2	25.0	8.3	25.0	29.2	12.5	12.5	41.7	41.7	45.8	20.8	0.49 ± 0.05c
		Oxidation ditch (48)	62.5	58.3	20.8	16.7	25.0	50.0	29.2	25.0	62.5	66.7	70.8	37.5	0.77 ± 0.08a
		Secondary clarifier (48)	50.0	45.8	20.8	12.5	16.7	58.3	16.7	25.0	62.5	62.5	75.0	16.7	0.67 ± 0.07a
		Effluent (48)	54.2	50.0	25.0	8.3	12.5	62.5	12.5	12.5	66.7	66.7	79.2	29.2	0.70 ± 0.08a
		Average (480)	60.4	51.3	24.6	12.9	23.8	48.8	25.8	25.4	58.8	59.6	68.8	29.6	0.66 ± 0.09

[a] AMP: ampicillin; PRL: piperacillin; KZ: cephazolin; CAZ: ceftazidime; CN: gentamicin; S: streptomycin; CIP: ciprofloxacin; LEV: levofloxacin; SXT: sulfamethoxazole/trimethoprim; W: trimethoprim; TE: tetracycline; C: chloramphenicol. [b] MAR index: multiple antibiotic resistance index (mean ± SD); abc: significant difference indicated by different letters, one-way ANOVA, $p < 0.05$.

3.3 Identification and characterization of plasmid-mediated quinolone resistance (PMQR) genes and *ampC* gene

A total of 452 out of 1056 *E. coli* isolates (42.8%) resistant to quinolones or beta lactamase antibiotics were chosen to identify plasmid-mediated quinolone resistance (PMQR) genes and *ampC* gene. The plasmid-carrying rates were 39.6% and 38.8% in Plant A and Plant B, respectively (Table 5). Except for *intI3* and *qnrA* genes, *intI1*, *intI2*, *ampC*, *aac(6′)-Ib-cr*, *qnrB* and *qnrS* were all detected in each treatment unit of Plant A and Plant B in both dry and wet seasons. Amongst the eight plasmid-mediated resistance genes above, *intI1* was the highest, with the detection frequency of 38.1%, followed by *qnrS*, 37.1%. The lowest was *aac(6′)-Ib-cr*, a quinolone resistance determinant, with the detection frequency of 5.1%. The detection frequency of *intI2* was the second lowest, 9.3%, much lower than *intI1*. *ampC*, expressing resistance to cephalosporin, was 32.6%. Increasing plasmid carriage rates and resistance genes detection frequencies were observed in post-biological units in both Plant A and Plant B in both dry and wet seasons.

4. Discussion

The results in the present study showed high prevalence of antibiotic resistance in the *E. coli* isolates from the two typical WWTPs (Plant A and Plant B). This is consistent with a previous report for WWTP effluents.[31] But Pignato *et al.*[32] reported much lower resistance frequencies against ampicillin (22.7%), tetracycline (19.4%), sulfamethoxazole (16.8%) and streptomycin (14.3%) in Italian raw and treated wastewaters than those in the present study. The multiple resistance frequency of *E. coli* was very high up to 90.6% in the present study, which is much higher than 24.2% for the Italian study.[32] It suggested that more serious contamination with ARB occurred in Plant A and Plant B. Tetracycline had the highest resistance frequency of 70.8%, which was much higher than that of Łuczkiewicz *et al.* (23%),[24] followed by ampicillin (65.1%) (Table 4). As a third generation cephalosporin, ceftazidime was observed to have the lowest frequency of 9.0% in the present study. Therefore, further measures are needed to eliminate ARB from the effluents of WWTPs.

Significant reduction in the number of total culturable bacteria and *E. coli* in the final effluents was observed in comparison with the influents. This is consistent with the results from previous studies.[31,32] The present study and the two previous studies all found that the disinfection process (chlorination and UV) could eliminate the cultured bacteria effectively. But no significant change in the bacterial number was found between pre- and post-disinfected effluents by Munir *et al.*[6] Nevertheless, no significant difference in bacteria removal was observed between Plant A and Plant B with different treatment technologies (One-way ANOVA, $p > 0.05$). No significant

Table 5 Detection of resistance plasmids and resistance genes in *E. coli* isolated from two different types of municipal wastewater treatment plants in dry and wet seasons

Samples (no. of isolates)			No. of *E. coli* resistant to quinolones or beta lactamase	No. of *E. coli* carrying plasmids	*intI1*	*intI2*	*intI3*	*ampC*	*aac(6′)-Ib-cr*	*qnrA*	*qnrB*	*qnrS*
Plant A	Dry season (288)	Influent (48)	12 (25.0%)	9 (18.8%)	9 (18.8%)	4 (8.3%)	ND[a]	7 (14.6%)	1 (2.1%)	ND	6 (12.5%)	7 (14.6%)
		Grit chamber (48)	10 (20.8%)	7 (14.6%)	7 (14.6%)	3 (6.3%)	ND	5 (10.4%)	1 (2.1%)	ND	5 (10.4%)	5 (10.4%)
		Anoxic (48)	17 (35.4%)	13 (27.1%)	13 (27.1%)	4 (8.3%)	ND	12 (25.0%)	3 (6.3%)	ND	8 (16.7%)	16 (33.3%)
		Anaerobic (48)	15 (31.3%)	11 (22.9%)	12 (25.0%)	4 (8.3%)	ND	9 (18.8%)	2 (4.2%)	ND	8 (16.7%)	14 (29.2%)
		Aerobic (48)	23 (47.9%)	30 (62.5%)	29 (60.4%)	7 (14.6%)	ND	28 (58.3%)	5 (10.4%)	ND	15 (31.3%)	28 (58.3%)
		Effluent (48)	45 (93.8%)	44 (91.7%)	42 (87.5%)	8 (16.7%)	ND	39 (81.3%)	4 (8.3%)	ND	12 (25.0%)	42 (87.5%)
	Wet season (288)	Influent (48)	13 (27.1%)	10 (20.8%)	11 (22.9%)	3 (6.3%)	ND	6 (12.5%)	1 (2.1%)	ND	5 (10.4%)	8 (16.7%)
		Grit chamber (48)	10 (20.8%)	8 (16.7%)	9 (18.8%)	2 (4.2%)	ND	4 (8.3%)	0 (0.0%)	ND	4 (8.3%)	5 (10.4%)
		Anoxic (48)	18 (37.5%)	15 (31.3%)	15 (31.3%)	3 (6.3%)	ND	11 (22.9%)	1 (2.1%)	ND	7 (14.6%)	15 (31.3%)
		Anaerobic (48)	15 (31.3%)	12 (25.0%)	12 (25.0%)	3 (6.3%)	ND	10 (20.8%)	1 (2.1%)	ND	6 (12.5%)	13 (27.1%)
		Aerobic (48)	36 (75.0%)	33 (68.8%)	33 (68.8%)	6 (12.5%)	ND	28 (58.3%)	4 (8.3%)	ND	14 (29.2%)	26 (54.2%)
		Effluent (48)	38 (79.2%)	36 (75.0%)	34 (70.8%)	7 (14.6%)	ND	37 (77.1%)	3 (6.3%)	ND	10 (20.8%)	39 (81.3%)
		Average (576)	252 (43.8%)	228 (39.6%)	226 (39.2%)	54 (9.4%)	—	196 (34.0%)	26 (4.5%)	—	100 (17.4%)	218 (37.8%)
Plant B	Dry season (240)	Influent (48)	12 (25.0%)	11 (22.9%)	10 (20.8%)	4 (8.3%)	ND	6 (12.5%)	2 (4.2%)	ND	5 (10.4%)	11 (22.9%)
		Grit chamber (48)	10 (20.8%)	9 (18.8%)	8 (16.7%)	3 (6.3%)	ND	4 (8.3%)	2 (4.2%)	ND	4 (8.3%)	9 (18.8%)
		Oxidation ditch (48)	29 (60.4%)	30 (62.5%)	30 (62.5%)	8 (16.7%)	ND	23 (47.9%)	7 (14.6%)	ND	16 (33.3%)	29 (60.4%)
		Secondary clarifier (48)	20 (41.7%)	20 (41.7%)	18 (37.5%)	5 (10.4%)	ND	14 (29.2%)	4 (8.3%)	ND	8 (16.7%)	19 (39.6%)
		Effluent (48)	35 (72.9%)	34 (70.8%)	30 (62.5%)	8 (16.7%)	ND	37 (77.1%)	5 (10.4%)	ND	9 (18.8%)	30 (62.5%)
	Wet season (240)	Influent (48)	10 (20.8%)	9 (18.8%)	9 (18.8%)	2 (4.2%)	ND	5 (10.4%)	1 (2.1%)	ND	3 (6.2%)	8 (16.7%)
		Grit chamber (48)	7 (14.6%)	5 (10.4%)	5 (10.4%)	2 (4.2%)	ND	3 (6.3%)	1 (2.1%)	ND	1 (2.1%)	5 (10.4%)
		Oxidation ditch (48)	27 (56.3%)	22 (45.8%)	22 (45.8%)	5 (10.4%)	ND	21 (43.8%)	3 (6.3%)	ND	8 (16.7%)	26 (54.2%)
		Secondary clarifier (48)	20 (41.7%)	17 (35.4%)	16 (33.3%)	2 (4.2%)	ND	13 (27.1%)	1 (2.1%)	ND	3 (6.3%)	14 (29.2%)
		Effluent (48)	30 (62.5%)	29 (60.4%)	28 (58.3%)	5 (10.4%)	ND	22 (45.8%)	2 (4.2%)	ND	5 (10.4%)	23 (47.9%)
		Average (480)	200 (41.7%)	186 (38.8%)	176 (36.7%)	44 (9.2%)	—	148 (30.8%)	28 (5.8%)	—	62 (12.9%)	174 (36.3%)
		Total isolates (1056)	452 (42.8%)	414 (39.2%)	402 (38.1%)	98 (9.3%)	—	344 (32.6%)	54 (5.1%)	—	162 (15.3%)	392 (37.1%)

[a] ND, not detected.

difference for the antibiotic resistance frequencies and MAR indices was observed between dry and wet seasons either (One-way ANOVA, $p > 0.05$), indicating that temperature has little effect on bacterial resistance.

Activated sludge and oxidation ditch are two kinds of treatment processes useful in eliminating organic and inorganic pollutants applied extensively around the world. However, the highest values of percentage of resistant *E. coli*, resistance frequency of each antibiotic, MAR index and detection frequency of resistance genes were all found in the effluents of biological units (*i.e.*, anoxic, anaerobic and aerobic processes in Plant A and oxidation ditch process in Plant B) in the present study (shown in Tables 3–5). It suggested that a more frequent horizontal gene transfer occurred in the biological units compared to that in the natural environments. Although the treatment processes in the present study were efficient in reducing the bacterial number, an increasing percentage of resistant *E. coli* was found from influent to effluent. This can be explained by the increasing proportion of resistant bacteria in the total culturable bacteria in the biological units and effluents. In fact, an increase in resistant bacteria proportion and antibiotic resistance in *Escherichia* spp. isolates was also observed in the treated effluent in comparison with the raw wastewater.[33,34] Rizzo *et al.* also found that *E. coli* population of mutation could survive the disinfection process.[26]

Multiple antibiotic resistant bacteria can disseminate antibiotic resistance determinants to susceptible strains of the same species or to other species or genera by different mechanisms, mainly by plasmids.[35] In the present study, out of 1056 *E. coli* strains isolated from the wastewater samples, 39.2% contained plasmids and 14.0% were obtained from final effluent samples. A previous study showed that the frequency of integrons was high in Gram-negative clinical isolates, with class 1 integrons being up to 57% in *E. coli* isolates.[36] In the present study, the detection rates of class 1 and class 2 integrons were 38.1% and 9.3% of the *E. coli* isolates, respectively (Table 5), suggesting that class 1 integrons were an important mechanism in dissemination of resistance genes. Three of four plasmid-mediated quinolone resistance genes were detected in the isolates. The detection frequencies were as follows: *qnrS* > *qnrB* > *aac(6′)-Ib-cr* > *qnrA*, suggesting that the mechanism of quinolone resistance was mainly topoisomerase protection peptides, which bind to and protect topoisomerases from inhibition by quinolones.[19] Intrinsically resistant to clavulanic acid, *ampC* producing strains are a cause of great concern as carbapenems are the only antibiotics effective against such strains.[37] Compared to the previous study of Sobia *et al.*,[38] *ampC* carriage rate in the present study was at a relatively high level, with 32.6% of the *E. coli* isolates containing this gene. McKinney and Pruden found that *ampC* was the most resistant to UV compared with *tetA*, *mecA* and *vanA* genes.[39] In addition, *ampC* is also a plasmid-mediated resistance gene. Thus, the *ampC* gene may spread in an easy way amongst *E. coli* strains and even different species by conjugation.[15,40,41] And resistant bacteria carrying the *ampC* gene were more resistant to the UV disinfection process in comparison with bacteria containing other resistance genes.

Integrons and plasmids are the two most important mechanisms that facilitate multiple resistance acquisition.[15,16,42] Integrons are a recombination system which can effectively capture and express individual mobile resistance gene cassettes in the environment. The key role of plasmids in contributing to horizontal gene transfer is undisputed.[16] The results of the present study showed a trend of increasing percentage of resistant *E. coli* and increasing detection frequencies of plamid-mediated resistance genes in the WWTPs, although the disinfection processes (UV and chlorination) could significantly reduce the number of total culturable bacteria. A proportion of multiple antibiotic resistant bacteria, which carry transferable plasmids containing resistance genes, could survive the disinfection processes and enter the environment eventually. In addition, a more frequent horizontal gene transfer was observed in the biological units in the present study. Therefore, wastewater treatment plants are an important reservoir of diverse mobile antibiotic resistance elements and play a key role in recombination and dissemination of antibiotic resistance genes in the environment.[43] Thus, more effective treatment processes are required to deal with the surviving ARB and ARGs in the final effluents.

5. Conclusion

This study provided the monitoring data on prevalence of antibiotic resistance and characteristics of plasmid-mediated quinolone resistance genes and *ampC* gene in *E. coli* isolated from two typical municipal WWTPs. High prevalence of antibiotic resistance and multiple resistances was observed in the wastewaters from the two WWTPs. Among the plasmid-mediated genes detected in *E. coli* strains, *intI1* had the highest detection rate in the plasmids, followed by *qnrS*, *ampC*, *qnrB*, *intI2* and *aac(6′)-Ib-cr.* The disinfection processes (UV and chlorination) could significantly reduce the number of bacteria, but the percentage of resistant *E. coli*, resistance frequency for each antibiotic, MAR index and detection rate of plasmid-mediated resistance genes were all found increasing in the effluents of biological units, suggesting that a more frequent horizontal gene transfer occurred in the biological units. A proportion of multiple antibiotic resistant bacteria, which carry transferable plasmids containing resistance genes, could survive the disinfection process and enter the receiving environment. The findings of the present study reflect the importance of WWTPs as a reservoir of ARB and ARGs, and a medium for their recombination and dissemination in the environment.

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