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Monitoring and evaluation of antibiotic-resistant bacteria at a municipal wastewater treatment plant in China

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ABSTRACT

The prevalence of antibiotic-resistant bacteria in municipal wastewater treatment plants (WWTPs) is becoming a concern of public health. In order to acquire information on the emission of antibiotic-resistant bacteria from WWTP effluents into natural waters, both average antibiotic tolerance and concentrations of antibiotic-resistant bacteria in the effluent of a WWTP in Beijing, China were investigated. A new index of IC_{50}/MIC ratio (the antibiotic concentration required to inhibit 50% of total heterotrophic bacteria compared to the highest minimum inhibitory concentration value of a group of pathogens according to a specific antibiotic, as defined by CLSI) was used to reflect the average antibiotic tolerance of total heterotrophic bacteria in the secondary effluent. The results showed that the IC_{50}/MIC ratios of heterotrophic bacteria in the secondary effluent to penicillin, ampicillin, cephalothin, chloramphenicol and rifampicin were >2, >1, >1, and 1.08, respectively, which reflected a significantly high general level of heterotrophic bacteria found in the secondary effluent resistant to these five antibiotics. The concentrations of penicillin-, ampicillin-, cephalothin-, and chloramphenicol-resistant bacteria were as high as 1.5×10^4–1.9×10^5, 1.2×10^4–1.5×10^5, 8.9×10^3–1.9×10^5 and 2.6×10^4–2.0×10^5 CFU/mL, and the average percentages in relation to total heterotrophic bacteria were 63%, 47%, 55%, and 69%, respectively. The concentrations of tetracycline- and rifampicin-resistant bacteria were 840–6.1×10^3 and 310–6.1×10^4 CFU/mL with average percentages of 2.6% and 11%, respectively. Furthermore, our study found that five- and six-antibiotic-resistant bacteria were widely distributed in four types of enterobacteria from the secondary effluent. The presence of multiple-antibiotic-resistant bacteria from effluents of WWTPs into natural waters could pose a serious problem as a secondary pollutant of drinking water.

1. Introduction

Antibiotic resistance is common in pathogens, opportunistic pathogens, and even non-pathogenic bacteria in various environmental conditions, such as hospitals, communities, etc. (Séveno et al., 2002; Baquero et al., 2008). Recent studies on antibiotic resistance of bacteria showed serious results: mortality rates doubled in cases where resistant infections were present; furthermore, length of treatments increased often requiring the use of more expensive antibiotics or antibiotic cocktails (WHO, 2007). Hence, the prevalence of antibiotic-resistant pathogens is becoming a major public health issue all over the world. Antibiotics and antibiotic resistance genes play fundamental ecological roles in shaping the structures of microbial communities (Martines et al., 2009). Horizontal gene transfer among bacteria and the overuse of antibiotics intensify the antibiotic resistance in pathogens and environmental bacteria (Baquero, 2004). The prevalence of antibiotic-resistant bacteria in the environment threatens to be conducive to the emergence of antibiotic resistance in bacterial pathogens (Baquero, 2004).

A microorganism is usually categorized to be clinically antibiotic-resistant by applying the appropriate breakpoint in a defined phenotypic test system (CSLI, 2006b). Agar diffusion and broth susceptibility tests are two common phenotypic test systems used to determine the breakpoint; these serial assays investigate the antibiotic concentration at which bacterial growth is inhibited (CSLI, 2006b). However, microorganisms in environmental samples are always mixed with multiple species present, rather than a single strain. Most studies on antibiotic-resistant bacteria worked on single strains and tested the antibiotic resistance of these single strains to describe the status of antibiotic-resistant bacteria in environmental samples (Reinthaler et al., 2003; Hu et al., 2008; Goñi-Urriza et al., 2000). There is limited research on general antibiotic resistance level of environmental samples, such as effluents from wastewater treatment plants (WWTPs). Nevertheless, in both agar diffusion and broth susceptibility test methods, exposing of bacteria to antibiotics is the most practical way to confirm antibiotic tolerance. This method can be used to analyze the general level of antibiotic tolerance of environmental bacteria in a mixed system.

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In recent years, more and more studies have shown that antibiotic-resistant bacteria are widely spread in different environments, from pastures and rivers to bays and raw sewage (Sapkota et al., 2007; Ash et al., 2002; McKeon et al., 1995; Walter and Vennes, 1985; Reinthaler et al., 2003). Antibiotic-resistant bacteria in effluents of municipal WWTPs have also been continuously investigated by researchers all over the world (Baquero et al., 2008; Reinthaler et al., 2003; Walter and Vennes, 1985; da Costa et al., 2006, 2008; Schwartz et al., 2003). Several surveys inferred that effluents from WWTPs could be a source of antibiotic-resistant bacteria and antibiotic resistance genes, which may enhance the spread of antibiotic-resistant bacteria into the natural environment, and also the transfer of antibiotic resistance to more pathogenic bacteria (Pruden et al., 2006; Goñi-Urriza et al., 2000; Baquero et al., 2008; Li et al., 2009; Zhang et al., 2009a,b). However, most of the investigations about antibiotic-resistant bacteria in WWTPs were based on an antibiotic resistance determination of isolates by antibiotic susceptibility testing such as the agar diffusion method (Reinthaler et al., 2003; da Costa et al., 2006; Goñi-Urriza et al., 2000; Boczek et al., 2007; Pignato et al., 2009). The role of antibiotic-resistant bacteria as a contributor of antibiotic resistance to bacteria found in secondary effluents of WWTPs is still not clear, because there are few studies on the concentrations of antibiotic-resistant bacteria in secondary effluents.

In China, overuse of antibiotics and antibiotic resistance is severe (Heddini et al., 2009). The average growth of antibiotic resistance in hospitals and communities reached about 22% from 1994 to 2000, which could possibly be the most rapid growth rate of resistance all over the world (Zhang et al., 2006). Wastewaters from hospitals (likely to contain antibiotic-resistant bacteria) are also discharged into WWTPs, increasing the prevalence of antibiotic-resistant bacteria in WWTPs. However, very few studies on the pollution of antibiotic-resistant bacteria in the effluents of WWTPs have been carried out in China. Thus, the purpose of this study was to obtain a general level of antibiotic tolerance of total heterotrophic bacteria and investigate the concentration distribution of bacterial resistance to six different antibiotics in the secondary effluent of the WWTP to provide useful information on antibiotic-resistant bacteria and suspected risk of antibiotic resistance during reclaimed water reuse in China. Multiple-antibiotic-resistant bacteria were isolated and their resistances to six antibiotics were tested. The six antibiotics chosen in this study included penicillin G, ampicillin, cephalothin, chloramphenicol, tetracycline and rifampicin. Penicillin G, ampicillin and cephalothin are widely applied to cure respiratory tract infection as basic antimicrobial drugs in China (Ministry of Health of PRC, 2009). Chloramphenicol is a general antimicrobial drug in ophthalmology and rifampicin is an antituberculous drug (Ministry of Health of PRC, 2009). Previously, tetracyline was also widely used in China (Hu, 1999).

2. Materials and methods

2.1. Water samples

Water samples used in this study were collected from the effluents of secondary sedimentation in a WWTP. The WWTP has a treatment capacity of 200,000 m^3/d, serving a community in the northwest of Beijing. The basic treatment process of the WWTP is primary sedimentation, followed by an anaerobic–anoxic–oxic process as its secondary treatment. Eight groups of samples were collected from November 2009 to October 2010. The water samples were transported to laboratory on ice, stored at 4 °C and analyzed on the day of collection.

2.2. Antibiotics and stock solutions

Six antibiotics were used to detect antibiotic-resistant bacteria in the secondary effluents. The basic properties of the six target antibiotics are listed in Table S1 (in supporting information). All stock solutions of the antibiotics were prepared at a concentration of 800 mg/L by dissolving in suitable solvents, including distilled deionized water (ddH_2O), methanol and 70% (v/v) ethanol solution. The stock solutions were stored at −20 °C for ≤30 days.

2.3. Bacteria enumeration and antibiotics exposure

2.3.1. Enumeration of total heterotrophic bacteria

The total heterotrophic plate counts (HPC) (ISO 6222, 1999) were enumerated with 1 mL diluted water sample in 10 mL nutrient agar (peptone: 10.0 g/L, beef extract: 3.0 g/L, NaCl: 5.0 g/L, agar: 15.0 g/L, pH = 7.2). Water samples were diluted using phosphate-buffered saline (PBS, pH = 7.4) to obtain colony counts between 30 and 300 per plate. The plates were incubated at 37 °C for 24 h.

2.3.2. Antibiotic exposure of total heterotrophic bacteria

Antibiotic exposures of total heterotrophic bacteria were carried out in 10 mL nutrient agar mixed with 1 mL diluted water sample with a range of antibiotic concentrations (0, 4, 8, 16 and 32 mg/L). The plates were incubated at 37 °C for 24 h. Water samples were diluted using phosphate-buffered saline (PBS, pH = 7.4) to obtain colonies between 30 and 300 per plate. The colony counts were obtained after 24 h of incubation and characterized as survival after antibiotic exposure (Walter and Vennes, 1985; Watkinson et al., 2007).

2.3.3. Enumeration of antibiotic-resistant bacteria

Antibiotic-resistant bacteria were enumerated with 1 mL diluted water sample in 10 mL nutrient agar with an individual antibiotic at a given concentration. Water samples were diluted using phosphate-buffered saline (PBS, pH = 7.4) to obtain 30–300 colonies of antibiotic-resistant plates per plate. The plates were incubated at 37 °C for 24 h.

2.4. Isolation of multiple-antibiotic-resistant bacteria and antibiotics susceptibility testing

2.4.1. Isolation of multiple-antibiotic-resistant bacteria

Multiple-antibiotic-resistant bacteria were screened on nutrient agar plates with 16 mg/L tetracycline from one secondary effluent sample. The secondary effluent sample was collected from secondary sedimentation in the WWTP on April 17th, 2010. Representative isolates were purified on nutrient agar plate with 16 mg/L tetracycline twice. The isolates were identified by 16S ribosomal RNA sequencing (by Takara Bio Inc.) and by performing a BLAST search (NCBI, National Center of Biotechnology Information).

2.4.2. Growth curve of the isolates

The end of logarithmic phase of the isolate was determined to prepare the inoculants for antibiotic susceptibility testing. The single colony was inoculated into nutrient broth (peptone: 10.0 g/L, beef extract: 3.0 g/L, NaCl: 5.0 g/L, pH = 7.2) with 16 mg/L tetracycline and incubated at 37 °C in an airbath with 110–120 rpm. OD_{600} was measured every 2 h to determine the growth curves of the isolates.

2.4.3. Antibiotic susceptibility testing

Antibiotic susceptibility was determined by the microtiter broth dilution method (CLSI, 2006a). Antibiotic susceptibility testing was carried out in a 96-well microtiter plate. The bacteria at the end of the logarithmic phase were inoculated into Mueller Hinton broth (M-H broth, beef infusion solids: 2.0 g/L, starch: 1.5 g/L, casein hydrolysate: 17.5, pH = 7.2–7.6, from Beijing Aoboxing Biotech Company Ltd.) with a series of tetracycline concentrations (2, 4, 8, 16, 32, 64, 128, 256, 512, and 1024 mg/L). Each inoculation concentration analysis was performed in triplicate via parallel wells. The 96-well microtiter plates were incubated at 37 °C over 18 h, and scanned with a microplate reader with absorbency values at 600 nm. The minimum inhibitory concentration (MIC) of a tested single strain to an antibiotic was determined as the concentration just when the survival of the tested strain to an antibiotic was ≤1%.

2.5. Normalization of survival of bacteria exposed to antibiotics

The survival of bacteria in the effluent spiked with antibiotics was normalized to determine the exposure to antibiotics a shown in Eq. (1).

$$\begin{aligned} survival\,(\%) &= \frac{plate\ count\ exposed\ to\ an\ antibiotic}{total\ heterotrophic\ plate\ count} \times 100 \text{ or} \\ survival\,(\%) &= \frac{OD_{600}\ exposed\ to\ an\ antibiotic}{OD_{600}\ without\ antibiotic} \times 100 \end{aligned} \quad (1)$$

2.6. Logistic dose response to antibiotics

The dose response of bacteria to an antibiotic is always mathematically fitted by the general form of the four-parameter logistic function as Eq. (2) (DeLean et al., 1978).

$$survival\,(\%) = \frac{A_1 - A_2}{1 + (x/x_0)^p} + A_2 \quad (2)$$

Here, survival is the calculated percentage of antibiotic-resistant bacteria in the total heterotrophic bacteria population (in Eq. (1)).

A_1, the response when there is no antibiotic during antibiotic exposure, initial value is 100.

A_2, the response for "infinite" dose, initial value is 0.

x_0, is the 50% inhibitory concentration (IC_{50}), i.e. the dose resulting in a response halfway between A_1 and A_2.

x, the arithmetic dose.

p, is a "slope factor" that determines the steepness of the curve.

The antibiotic exposure of total heterotrophic bacteria in the secondary effluent was fitted using the logistic function (Eq. (2)) to get an IC_{50} of total heterotrophic bacteria to an antibiotic as an average antibiotic tolerance of total heterotrophic bacteria in the effluent of the WWTP. Logistic dose responses to antibiotics were fitted by the Origin 8.0 software using sigmoidal fitting with logistic dose response in Pharmacology or Chemistry.

3. Results and discussion

3.1. Survival of total heterotrophic bacteria in the presence of six antibiotics

The survival of total heterotrophic bacteria from secondary effluent in the presence of antibiotics could represent the average antibiotic tolerance and the ratio of antibiotic-resistant bacteria in the microbial community. Fig. 1 illustrates the survival of total heterotrophic bacteria in the secondary effluent sample when exposed to various concentrations of penicillin, ampicillin, cephalothin, chloramphenicol, tetracycline or rifampicin. The results showed that the survival of total heterotrophic bacteria in the presence of the six antibiotics can be divided into two groups. Group I included exposure to penicillin, ampicillin, cephalothin or chloramphenicol, while group II included tetracycline or rifampicin exposures. Total heterotrophic bacteria in group I decreased less than 50% when exposed to antibiotic concentrations of 32 mg/L. The curves remained stable when the concentrations of these four antibiotics were between 16 mg/L and 32 mg/L. However, there was an approximate 99% decrease in group II when exposed to antibiotics at a concentration of 32 mg/L. Hence, total heterotrophic bacteria in the municipal wastewater effluent had a higher tolerance to penicillin G, ampicillin, cephalothin and chloramphenicol than to tetracycline and rifampicin.

Survival fitting by the logistic function (Eq. (2)) was analyzed to determine the IC_{50} value in order to compare the average level of antibiotic tolerance of total heterotrophic bacteria with maximum value of MICs of pathogens defined in the directory of CLSI (Clinical and Laboratory Standards Institute, 2006b). Both IC_{50} values of total heterotrophic bacteria to antibiotics and the goodness of fit were gained from the fitting the curves of total heterotrophic bacteria exposed to the six antibiotics (as shown in Table 1). The maximum value of clinical MICs of pathogens for each antibiotic was determined in the directory of CLSI (2006b). The IC_{50}/MIC ratios were calculated to illustrate the average level of antibiotic tolerance of total heterotrophic bacteria in the secondary effluent (as shown in Table 1).

The R-squared values in Table 1 suggested that most dose response curves of total heterotrophic bacteria to antibiotics in the effluents fit well in the four-parameter logistic equation (Eq. (1)). The goodness of fit tests of group II were better than those of group I.

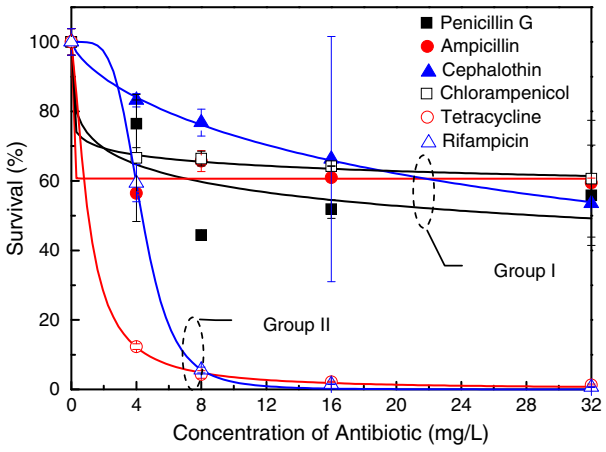

Fig. 1. Four-parameter logistic fitting curves for survival of total heterotrophic bacteria to the antibiotics in the secondary effluent. Error bars indicate standard deviation for replicates from a single sample.

IC_{50} values of total heterotrophic bacteria to the six antibiotics in the secondary effluent were different, even for penicillin and ampicillin, both of which belong to the penicillin group. Generally, IC_{50} values of total heterotrophic bacteria exposed to antibiotics in group I were higher than those in group II. IC_{50} values of total heterotrophic bacteria to ampicillin, cephalothin and chloramphenicol were greater than 32 mg/L. However, IC_{50} values of total heterotrophic bacteria exposed to tetracycline and rifampicin were 1.0 mg/L and 4.3 mg/L, respectively. However, the IC_{50}/MIC ratio showed that penicillin, ampicillin, cephalothin, chloramphenicol and rifampicin tolerance of total heterotrophic bacteria in the secondary effluent were all greater than 1, which were significantly higher than tetracycline tolerance (0.06). The results showed that total heterotrophic bacteria had a significant tolerance to penicillin, ampicillin, cephalothin, chloramphenicol and rifampicin. It could be inferred that there could be a prevalence of penicillin-, ampicillin-, cephalothin-, chloramphenicol- and rifampicin-resistant bacteria in the wastewater. This inference is also supported by the rate of antibiotic consumption in Beijing, which showed that beta-lactam antibiotics were used in 46% of cases to treat infectious diseases in humans, making them the most common antibacterial agent (Li et al., 2008).

3.2. Concentration distribution of antibiotic-resistant bacteria in the effluents

It is difficult to estimate the concentration of antibiotic-resistant bacteria in the effluent of a WWTP. This is because the MIC of each single strain to an antibiotic is unique, and there are multiple species of microorganisms in a secondary effluent. In order to evaluate antibiotic-resistance bacteria in the effluents, MIC of total heterotrophic bacteria to an antibiotic was confirmed as the maximum clinical MIC of typical pathogens to the antibiotic according to the performance standards from CLSI (2006b). MIC values of the six antibiotics used in this study to estimate antibiotic-resistant bacteria were shown in Table 1.

The antibiotic-resistant bacteria in the secondary effluents were monitored from November 2009 to October 2010. Concentrations of penicillin-, ampicillin-, cephalothin-, chloramphenicol-, tetracycline- and rifampicin-resistant bacteria in the secondary effluent samples are shown in Fig. 2.

The average concentration of total heterotrophic bacteria in the secondary effluents was 1.5×10^5 colony-forming units (CFU)/mL, which fell in a typical range observed in the secondary effluents of municipal wastewaters (Xie et al., 2007; Qin et al., 2004; Jackson et al., 2000). The concentration of antibiotic-resistant bacteria can be divided into three groups. The first group includes penicillin-, ampicillin-, cephalothin-, and chlorampenicol-resistant bacteria, in which the concentrations were as high as 1.5×10^4–1.9×10^5, 1.2×10^4–1.5×10^5, 8.9×10^3–1.9×10^5, and 2.6×10^4–2.0×10^5 CFU/mL, respectively. Rifampicin-resistant bacteria ranked in the second group, with a concentration of 310–6.1×10^4 CFU/mL. The concentration of tetracycline-resistant bacteria was the lowest at 840–6.1×10^3 CFU/mL. The results showed that penicillin-, ampicillin-, cephalothin-, and chloramphenicol-resistant bacteria were much more prevalent than tetracycline-resistant bacteria. Furthermore, the sum of one of the average concentrations among penicillin-, ampicillin- and cephalothin-resistant bacteria and the average concentration of chloramphenicol-resistant heterotrophic bacteria was larger than the average concentration of total heterotrophic bacteria. For example, over 50% of heterotrophic bacteria had both penicillin and chloramphenicol resistance in the secondary effluents. Thus, multiple-antibiotic-resistant heterotrophic bacteria could be widespread in the secondary effluent of this WWTP.

In order to compare this investigation with those carried out by other researchers, the percentages of antibiotic-resistant heterotrophic bacteria in the secondary effluents investigated in this study were calculated and are shown in Fig. 3. The average percentages

Table 1
IC_{50} of total heterotrophic bacteria exposed to the six antibiotics in the secondary effluent.

	PEN	AMP	CEP	TET	CHL	RIF
Adj. R^2	0.71	0.95	0.61	>0.99	0.73	>0.99
IC_{50} (mg/L)[a]	>32 [35.5(±17.0)]*	>32	>32 [39.5(±20.2)]*	1.0 (±0.3)	>32	4.3 (±0.1)
MIC (mg/L)[b]	16	32	32	16	32	4
IC_{50}/MIC ratio	>2	>1	>1	0.06	>1	1.08

Data express as mean ± SE. The significant difference was calculated by Tukey's test ($p<0.05$).
[a] IC_{50}: 50% inhibitory concentration of heterotrophic bacteria to an antibiotic.
[b] MIC: Minimum inhibitory concentration, which was determined as the maximum MIC of typical pathogens in the directory of Clinical and Laboratory Standards Institute (CLSI, 2006b).
* The data in brackets were from the fitting curve of dose response.

of penicillin-, ampicillin-, cephalothin-, and chloramphenicol-resistant heterotrophic bacteria were 63%, 47%, 55%, and 69%, respectively, while tetracycline- and rifampicin-resistant heterotrophic bacteria were 2.6% and 11%, respectively. Meanwhile, the median value percentages of penicillin-, ampicillin-, cephalothin-, chloramphenicol-, tetracycline-, and rifampicin-resistant bacteria in the effluents were 75%, 47%, 58%, 71%, 2.0%, and 4%, respectively. Generally, the ratios of tetracycline- and rifampicin-resistant bacteria in the secondary effluents were found to be much lower than the other four.

The percentages of antibiotic-resistant bacteria reported by researchers in different countries or regions are summarized in Table 2. Compared with the percentages of antibiotic-resistant bacteria including *E. coli*, lactose-fermenting *Enterobacteriaceae*, *Enterococcus*, *Acinetobacter* spp. etc., the percentages of penicillin-, ampicillin-, cephalothin- and chloramphenicol-resistant bacteria in the effluent in this study occurred at higher rates, while tetracycline- and rifampicin-resistant bacteria were in a lower range. Penicillin-, ampicillin- and cephalothin-resistant bacteria however, were widespread in WWTPs in the six countries, while the percentages of chloramphenicol-, tetracycline- and rifampicin-resistant bacteria were quite different between these countries (Meckes, 1982; Murray et al., 1984; Schwartz et al., 2003; da Costa et al., 2006, 2008; Pignato et al., 2009; Zhang et al., 2009a,b). This implies that the percentage of antibiotic-resistant bacteria in a WWTP may be related to medication habits in one region or country.

Basing levels of antibiotic-resistant bacteria on either total heterotrophic bacteria counts or specific pathogenic bacteria cultured in the laboratory however, represents only a fraction of bacteria present in the environment. Molecular methods should be developed to detect those antibiotic-resistant viable but non-culturable bacteria in the environment.

3.3. Characteristic of multiple-antibiotic-resistant bacteria

The results of the concentration of antibiotic-resistant bacteria showed that multiple-antibiotic-resistant bacteria could be widespread in the secondary effluent of this WWTP. Thus, five tetracycline-resistant bacteria species were randomly isolated and screened from the secondary effluent, identified by 16S rRNA, and tested against antibiotic susceptibility. MICs of each isolate to the six antibiotics are shown in Table 3.

The five isolates were identified as *Aeromonas*, *Enterobacter*, *Escherichia* (or *Shigella*), and *Klebsiella*, all of which belong to enterobacteria. The results in Table 3 showed that the MICs of the five isolates to penicillin, ampicillin, chloramphenicol and tetracycline were significantly higher than standard MICs for enterobacteria (including Enterobacteriaceae and *Enterococcus*) from CLSI. Three of the five isolates were resistant to all six antibiotics. The isolate of *Aeromonas* (No. 2) had no rifampicin resistance, and the isolate of *Klebsiella* (No. 5) had no cephalothin resistance. The result proved not only the existence of multiple-antibiotic-resistant bacteria but also the wide distribution of five- and six-antibiotic-resistant bacteria in four types of enterobacteria in the secondary effluent of the WWTP. Hence, the prevalence of multiple-antibiotic-resistant bacteria could be a serious problem in the municipal WWTP.

4. Conclusions

The survival of total heterotrophic bacteria when exposed to the six antibiotics, the concentration of antibiotic-resistant bacteria and analysis of multiple-antibiotic-resistant bacteria showed that penicillin-, ampicillin-, cephalothin-, and chloramphenicol-resistant bacteria were widespread in the secondary effluent of the WWTP in Beijing, China. The IC_{50}/MIC ratios of total heterotrophic bacteria in the secondary effluent to penicillin, ampicillin, cephalothin, chloramphenicol and rifampicin were >2, >1, >1, and 1.075, respectively. The concentrations of penicillin-, ampicillin-, cephalothin-, chloramphenicol-, tetracycline-, and rifampicin-resistant bacteria in the secondary effluents were 1.5×10^4–1.9×10^5, 1.2×10^4–1.5×10^5, 8.9×10^3–1.9×10^5, 2.6×10^4–2.0×10^5, 840–6.1×10^3, and 310–6.1×10^4 CFU/mL, respectively. The average percentages of penicillin-, ampicillin-, cephalothin-, chloramphenicol-, tetracycline-, and rifampicin-resistant heterotrophic bacteria in the effluents were 63%, 47%, 55%, 69%, 2.6%, and 11%, respectively. Five- and six-antibiotic-resistant in four types of

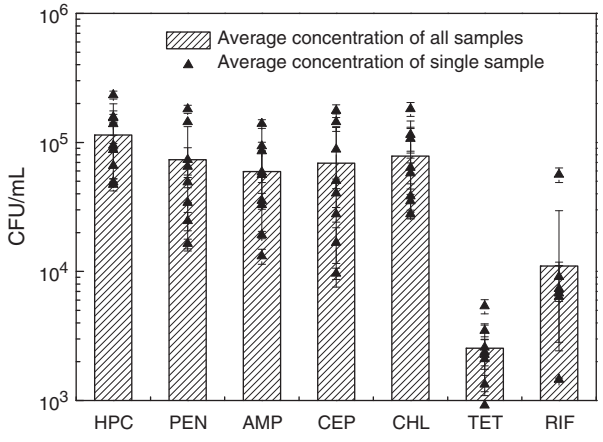

Fig. 2. Concentration distribution of antibiotic-resistant bacteria in the secondary effluents. Error bars indicate standard deviation for replicates from single sampling events. (HPC: total heterotrophic bacteria; PEN: penicillin-resistant bacteria; AMP: ampicillin-resistant bacteria; CEP: cephalothin-resistant bacteria; CHL: chloramphenicol-resistant bacteria; TET: tetracycline-resistant bacteria; RIF: rifampicin-resistant bacteria).

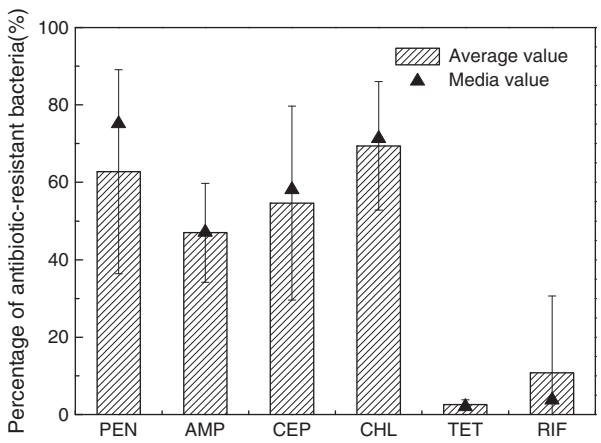

Fig. 3. The percentage of antibiotic-resistant bacteria in the secondary effluents. Error bars indicate standard deviation for replicates from single sampling events. (Abbreviations for antibiotic-resistant bacteria are the same as those in Fig. 3).

Table 2
Percentages of antibiotic-resistant bacteria in municipal wastewater treatment plants in different countries/regions.

Country	Sample[a]	Bacteria	Percentages of antibiotic-resistant bacteria (%)[b]						
			PEN	AMP	CEP	TET	CHL	RIF	Reference
Canada	TE	Heterotrophic bacteria		73	53	4	0		Murray et al. (1984)
China	SE	Heterotrophic bacteria	28–98	27–60	19–92	1.5–4.8	40–96	0.8–59	This study
China, Hong Kong	S	*Enterobacteriaceae*				32			Zhang et al. (2009b)
Germany	S	Heterotrophic bacteria	22–38		27–61				Schwartz et al. (2003)
Germany	SE	Heterotrophic bacteria	13–27		19–59				Schwartz et al. (2003)
Italy	SE	*E. coli*		16–20	20–28	13–17	0–10		Pignato et al. (2009)
Portugal	M	*Enterococcus*		0–17		23–53	0–13		da Costa et al. (2006)
Portugal	M	*E. coli*		56.5		80.7			da Costa et al. (2008)
USA	SE	*Acinetobacter* spp.					35	77.5	Zhang et al. (2009a)
USA	SE	Coliforms				2.6–5.1	0.1–0.3		Meckes (1982)

[a] E: secondary effluents; TE: tertiary effluents; S: sludge; M: mixed samples of raw influent, sludge and effluent.
[b] PEN: penicillin-resistant bacteria; AMP: ampicillin-resistant bacteria; CEP: cephalothin-resistant bacteria; CHL: chloramphenicol-resistant bacteria; TET: tetracycline-resistant bacteria; RIF: rifampicin-resistant bacteria.

Table 3
MICs of multiple-antibiotic-resistant bacteria to the six antibiotics isolated from the secondary effluent.

No.	Genus	MICs of multiple-antibiotic-resistant bacteria (mg/L)					
		PEN	AMP	CEP	CHL	TET	RIF
1	*Aeromonas*	>1024	256	512	1024	128	4
2	*Aeromonas*	>1024	>1024	1024	>1024	128	2
3	*Enterobacter*	256	32	128	>1024	128	4
4	*Escherichia* (or *Shigella*)[a]	>1024	>1024	>1024	>1024	256	8
5	*Klebsiella*	64	64	2	>1024	256	512
6	Enterobacteriaceae[b]	–	≥32	≥32	≥32	≥16	–
7	*Enterococcus*[b]	≥16	≥16		≥32	≥16	≥4

[a] The 16S rRNA sequencing and gene blasting can't make a distinction between Escherichia and Shigella.
[b] Values of MICs are for determining antibiotic resistance in Performance Standards for Antimicrobial Susceptibility Testing of CLSI (2006b).

enterobacteria were widely distributing in the secondary effluent. Multiple-antibiotic-resistant bacteria could pose a serious threat to the widespread of antibiotic resistance from the effluent of the WWTP to natural waters as a secondary pollutant of drinking water.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.envint.2011.03.001.

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