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Antibiotics Removal in Biological Sewage Treatment Plants

Ghosh, G.C.[1,2*], Hanamoto, S[1], Yamashita, N.[1], Huang, X.[3] and Tanaka, H.[1]

1. Research Center for Environmental Quality Management,
Graduate School of Engineering, Kyoto University, Kyoto 615–8540, Japan
2. Departments of Environmental Science and Technology, Jessore University of Science and Technology, Jessore 7408, Bangladesh
3. Department of Environmental Science and Engineering, Tsinghua University, Beijing 100084, China

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ABSTRACT: This study investigated the occurrence and removal of 12 antibiotics (ciprofloxacin, enrofloxacin, levofloxacin, norfloxacin, nalidixic acid, azithromycin, clarithromycin, roxithromycin, lincomycin, novobiocin, sulfamethoxazole, trimethoprim) at four sewage treatment plants (STPs): two STPs in Kyoto, Japan and two STPs in Beijing, China. The STPs differed in design and operation conditions, utilized a variety of secondary treatment processes. The antibiotics were frequently detected in influents and effluents, and ranged from ng/L up to lower µg/L. In influent, clarithromycin (1.1–1.6 µg/L) and levofloxacin (3.6–6.8 µg/L) were detected in the highest concentration in Japanese and Chinese STPs, respectively. The overall elimination of the antibiotics were differed between STPs and ranged from negative to >90%. These data demonstrate that there are detectable levels of antibiotics are discharging from STPs, and only some of these antibiotics are being removed in a significant proportion by STPs. It was also observed that biological nutrient removal based sewage treatment processes (anaerobic–anoxic–oxic: A^2O; and anoxic–oxic: AO) have relatively higher antibiotics removal efficiencies than oxidation ditch (OD) processes.

Keywords: antibiotics, effluent, influent, sewage treatment.

INTRODUCTION

The release of active pharmaceuticals and their metabolites into the environment has become an increasing concern over recent years. Due to advancements in analytical capabilities, residues of a wide range of active pharmaceutical ingredients have been detected in various compartments of the environment. This has lead to an increasing interest in the assessment of fate, environmental risk, and potential regulations of these emerging contaminants, mirrored by the large number of publications and reviews available in recent years, (Daughton and Ternes, 1999; Halling-Sørensen *et al*., 1998; Heberer, 2002; Kümmerer, 2004). Among pharmaceuticals, antibiotics are of particular concern, as they are designed to be highly bioactive and can induce bacterial resistance, even at low concentrations through continuous exposure (Costanzo *et al*., 2005). After administration, significant parts of the original antibiotics with possible metabolites are excreted with urine and feces, and finally ending into STPs. Antibiotics have been detected in STPs, surface water, ground water, sewage sludge, soil or even in drinking water at concentration between ng /L up to µg /L (Golet *et al*., 2002; Petrovic *et al*., 2003; Batt *et al*., 2006a; Batt *et al*.,

* Corresponding Author Email: gopales8@hotmail.com

2006b; Daughton and Ternes, 1999; Ghosh *et al.*, 2009). Nowadays, antibiotics resistance is an emerging issue considering that the discovery of new antibiotics is not keeping pace with the growing antibacterial resistance.

Occurrence of sulfonamides, macrolides and quinolones groups antibiotics were more documented than others, with variable removal efficiencies (Golet *et al.*, 2002; Lindberg *et al.*, 2005; Göbel *et al.*, 2007). Quinolone antibiotics are eliminated in conventional waste water treatment by 88–91%, with sorption to sewage sludge being the main process responsible (Golet *et al.*, 2002). A high sorption to sludge for fluoroquinolones was also found in STPs (Lindberg *et al.*, 2005). In Spanish STPs, removal efficiency of 60% for sulfamethoxazole was reported by Clara *et al.* (2004).

In the present study, the occurrence and fate of the antibiotics (Fig. 1) at four full–scale STPs located in Japan (STP–A and STP–B, Table 1) and China (STP–C and STP–D, Table 1) were investigated. The selected STPs represent a wide variety of biological sewage treatment technologies. The target antibiotics comprised a wide range of antibiotic groups (e.g. Macrolide antibiotics: azithromycin, clarithromycin, roxithromycin; fluoroquinolone antibiotics: ciprofloxacin, enrofloxacin, levofloxacin, lincomycin, nalidixic acid, norfloxacin; and others: novobiocin, sulfamethoxazole and trimethoprim). The choices of these antibiotics were based upon their annual consumption, reported detection in surface water/ waste water, and analytical capabilities.

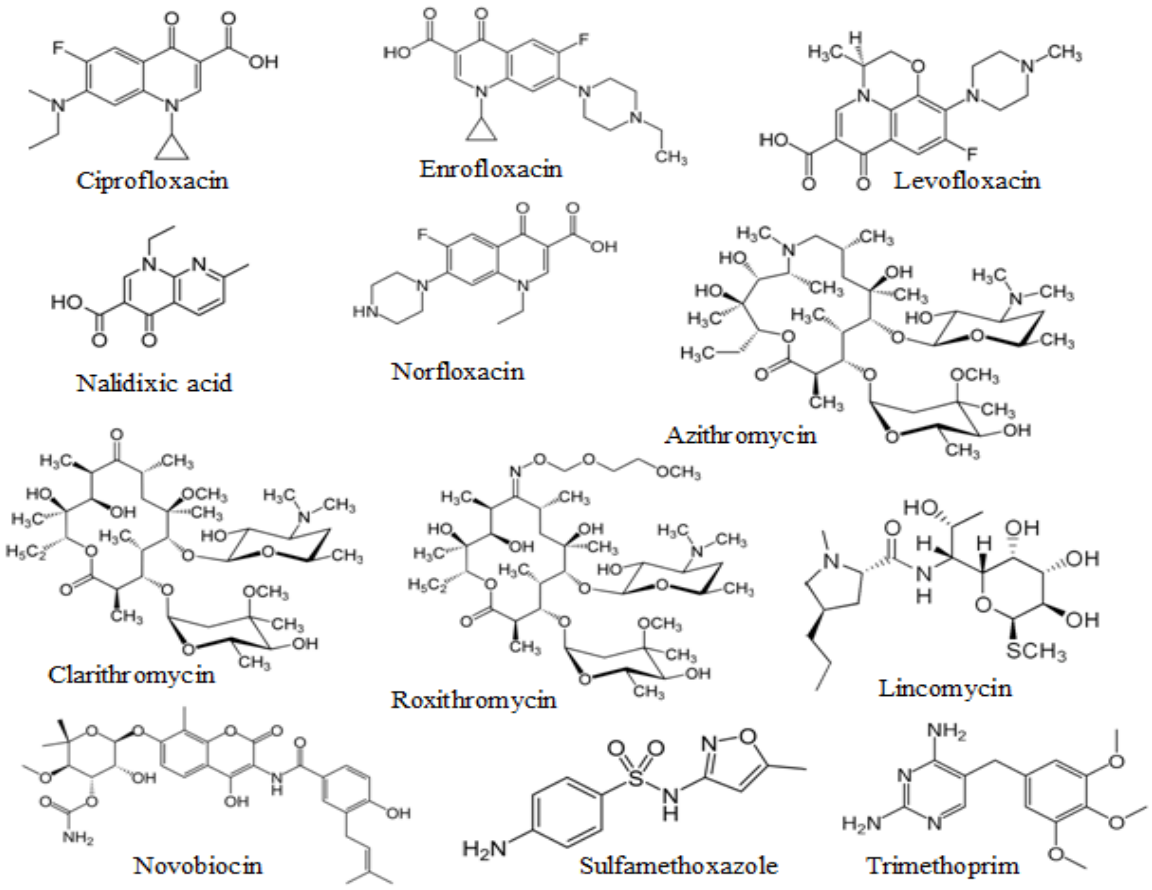

Fig. 1. Molecular structure of the selected antibiotics

Table 1. A summary of the sewage treatment plants characteristics and operating conditions

Plant ID	Location (City/Country)	Average flow (m^3 d^{-1})	Treatment processes		HRT (h)	SRT (d)
			Primary	Secondary		
STP–A	Kyoto/ Japan	32000	Yes	A^2O	13.0	18
STP–B	Shiga/ Japan	27500	Yes	AO+ suspended carrier	10.9	17
STP–C	Beijing/China	480000	Yes	OD	17.4	16
STP–D	Beijing/China	810000	Yes	A^2O	11.45	7

HRT: Hydraulic retention time; SRT: Sludge retention time; A^2O: Anaerobic/Anoxic/Oxic; AO: Anoxic/Oxic

METHODOLOGY

Chemical and reagents

Macrolide antibiotics: azithromycin, clarithromycin, roxithromycin; fluoroquinolone antibiotics: ciprofloxacin, enrofloxacin, levofloxacin, lincomycin, nalidixic acid, norfloxacin; and others novobiocin, sulfamethoxazole and trimethoprim were purchased from Wako pure chemical industries (Osaka, Japan) and Sigma–Aldrich (Taufkirchen, Germany). All antibiotics were of analytical grade (purity >95%). Formic acid (LC/MS grade), ascorbic acid, Na_2EDTA and methanol (LC/MS grade) were also purchased from Wako pure chemical industries. Individual standard solutions at 1mg m/L of each antibiotic were prepared by weighing and dissolving in methanol. All working mixed standards were prepared before analysis.

Sample collection and solid–phase extraction

Brief descriptions of STPs are listed in Table 1. Each 1L of sample was collected in glass bottle and immediately acidified (pH= 3) by ascorbic acid at sampling location to reduce microbial activity, further degradation with chlorine and enhance trapping of the antibiotics on the solid–phase extraction (SPE) cartridge. In the laboratory, samples were filtered (GF/B) immediately prior to solid–phase extraction SPE.

Each sample was divided into two representative sub–samples of 200ml and Na_2EDTA at 1 g/L was added. One was spiked with mixed standards of antibiotics (50 ng of each antibiotics), and the other was considered as blank sample. Recoveries were calculated by comparing spiked and blank sample. Oasis hydrophilic–lipophilic balance (HLB) cartridges (200 mg, 6 mL, Waters, Corp., and Milford, MA) were used for solid phase extraction. Cartridges were pre–conditioned with 3ml methanol, followed by 3 mL of Milli–Q water. Samples (each 200 ml) were passed through the cartridge at a flow rate of 10mL min^{-1} using concentrator and then vacuum dried for 120 min. For the Chinese samples, cartridges were kept in 4˚C after enrichment and transported to Japan. There were no effect of cartridge storage and transport in this study (data not shown). Elution was carried out with 6 mL of methanol in 10ml glass vial. Methanol was evaporated under a gentle nitrogen stream at 37˚C to dryness and reconstituted with acidified Milli–Q water (0.01% formic acid): methanol solution of 90: 10, to final volume of 1mL (i.e. enrichment factor of 200).

Liquid chromatography–tandem mass spectrometry

Chromatographic separation of the antibiotics were achieved with a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) separation module with a binary pump system equipped with UPLC BEH C18 column (100× 2.1 mm , 1.7 µm particle size). Optimum separation was achieved with a binary gradient consisting of 0.01% formic acid (v/v) in water (solvent A) and methanol (solvent B) at a flow rate of 0.35 ml min^{-1}. The gradient elution setting was: 0–2min: 10% B; 2–8 min: 10–25% B; 8–14 min: 25–55% B; 14–16 min: 55% A; 16–19 min: 55–95% B; 19–21min: 95–10% B (return to initial conditions); 21–23min:

equilibration of the column. The column temperature was kept at 60°C and injected sample volume was 10 µL. The UPLC system was coupled to a Quattro Micro API mass spectrometer with the electrospray ionization (ESI) source Z–spray (Waters company Ltd.).

During quantification optimization each antibiotic was individually infused as a standard solution into the initial mobile phase (50% solvent A, 50% solvent B) directly into the mass spectrometer at a concentration of 5 mg /L. During the infusion, the parameters (cone voltage, collision energy, ionization mode) were optimized for each compound in order to obtain the maximum sensitivity with the highest amount of product ions available and the most sensitive Multiple Reaction Monitoring (MRM) transitions were determined for each antibiotic. Table 2 shows the MS/MS parameter optimized for transitions selected in the multiresidue quantitative method.

Table 2. Target antibiotics list and optimized ESI–MS/MS conditions for the analysis of antibiotics

Antibiotics	ESI	Precursor ion (m/z)	Product ion (m/z)	Cone Voltage (V)	Collision Energy (eV)	Retention Time	Instrument LOQ (ng /L)
Sulfamethoxazole	+	254	155.9	25	15	5.8	1.02
Azithromycin	+	749.5	591.4	40	25	10.9	0.16
Clarithromycin	+	748.9	157.9	30	20	14.8	1.31
Roxithromycin	+	837.7	679.4	25	20	15.0	0.30
Ciprofloxacin	+	332.2	231	25	35	5.4	0.51
Enrofloxacin	+	360.2	245.2	30	26	6.1	0.91
Levofloxacin	+	362.1	318	30	20	4.8	0.61
Nalidixic acid	+	233.3	215.1	35	14	3.6	0.24
Norfloxacin	+	320.2	276.2	25	18	4.9	0.47
Lincomycin	+	407.5	126.1	30	22	4.4	0.73
Novobiocin	-	611.3	205.2	50	50	3.2	0.55
Trimethoprim	+	291.4	230.2	35	20	4.2	0.35

The parameters of the mass spectrometer were as follows: electrospray source block and desolvatation temperature: 120 and 400°C respectively; capillary voltages: 2.5 kV; cone and desolvatation gas flow 50 and 900 L h^{-1} respectively. The instrument control, data acquisition and quantification were performed by Mass Lynx 4.1 software.

Validation of the analytical procedures

Every antibiotic was analyzed with MRM, using the highest precursor ion/ product ion transitions. Calibration curves were obtained by analyzing mixture standard solutions at six levels of concentration ranging between 1 µg/L and 300 µg/L. Precision and accuracy of the overall analytical procedure were evaluated with wastewater samples, spiked at the two following levels of concentration: 0, 200 and 500 ng/L, and compared with a direct injection of a standard mixture, and reproducibility was assessed. The method was considered accurate if recoveries were in the 50–150% range, and precision was satisfactory if the RSD was lower than 15%. Blank samples were previously analysed to confirm the presence/ absence of any significant peak at the selected transitions. For the sewage water limits of quantification (LOQs) were difficult to determine because the samples already contained some of the selected analytes and thus, the matrix interference was serious. Therefore, LOQs in the sewage water and sludge samples were defined as signal to noise (S/N) ratios of 10 or higher. LOQ for each compound in sewage were from 1 to 10 ng /L.

RESULTS AND DISCUSSION

Occurrence of antibiotics in the STPs influent

The antibiotics concentrations in influent at four STPs are shown in Figure 2A. All 12 selected antibiotics were detected in all influent samples, and concentration varied among STPs. At STP–A, antibiotics concentrations in influent are ranging from 37 ng/L (lincomycin) to 1129 ng/L (clarithromycin), however, in STP–B, it is ranging from 26 ng/L (lincomycin) to 3077 ng/L (clarithromycin). Among selected antibiotics, clarithromycin occurred at the highest concentration in influent of the Japanese STPs, followed by azithromycin (160–1866 ng/L), levofloxacin (532–425 ng/L), norfloxacin (155–514 ng/L), Ciprofloxacin (231–37 ng/L) and sulfamethoxazole (159–174 ng/L). Clarithromycin, levofloxacin and azithromycin in influent at 883, 981 and 371 ng/L, respectively, was reported previously from Japan (Yasojima *et al*., 2006). In STP–D influent, located in China, levofloxacin (6800 ng/L), norfloxacin (2775 ng/L), trimethoprim (1578 ng/L) and sulfamethoxazole (1280 ng/L) were detected at the highest concentration among all the STPs.

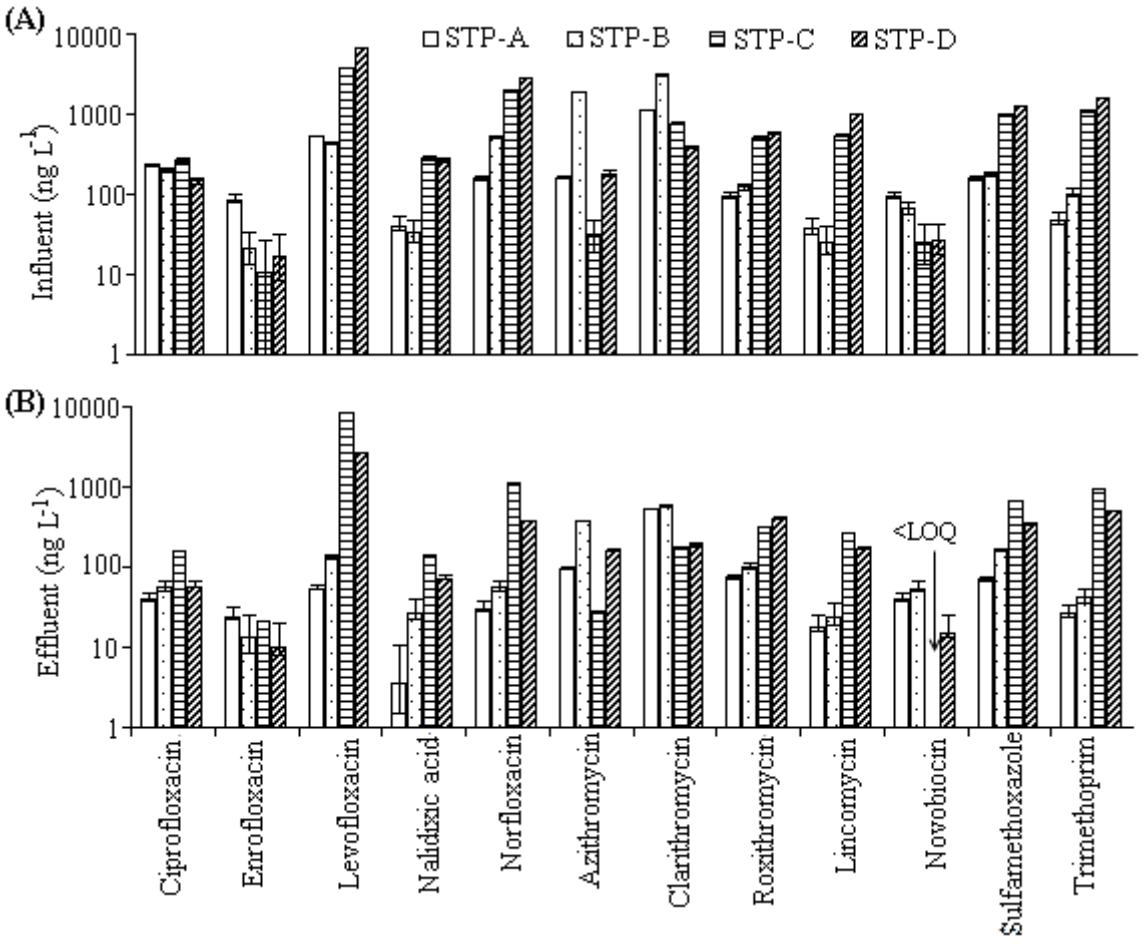

Fig. 2. Comparison of the antibiotic concentrations in influent (A) and effluent (B) samples in the STPs in Japan (STP–A and STP–B) and China (STP–C and STP–D)

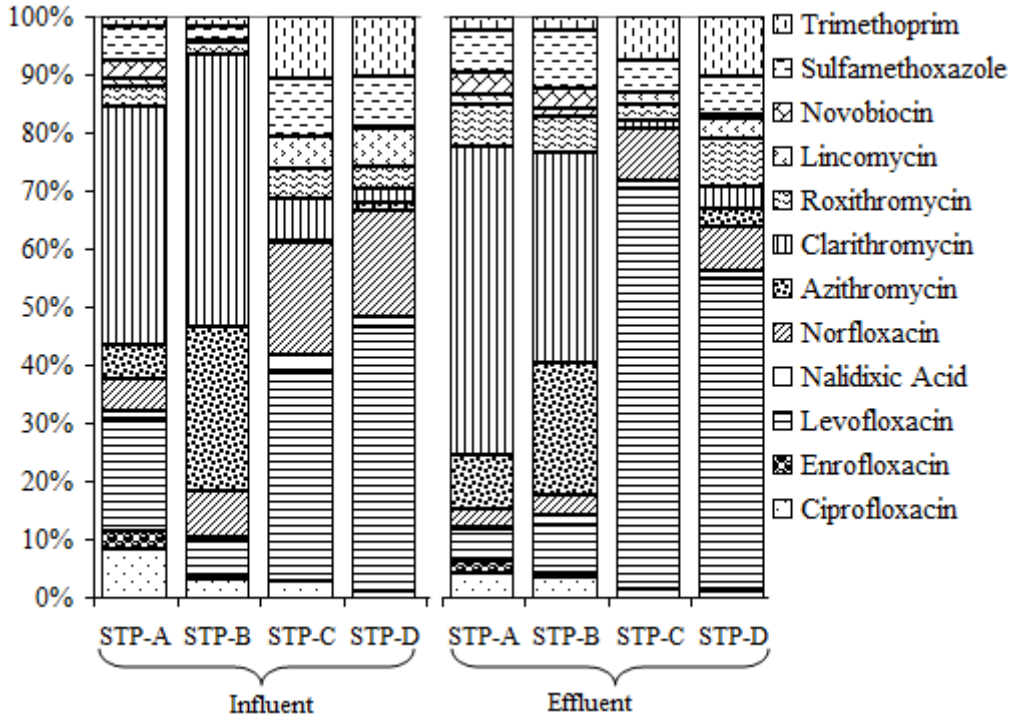

Fig. 3. Comparison of composition profile of the selected antibiotics in influent and effluent in the STPs in Japan (STP–A and STP–B) and China (STP–C and STP–D)

Antibiotics in STP–C and STP–D followed a similar pattern with little difference in concentration (Fig. 2A). Clarithromycin concentration (377–762 ng /L) in the STPs influent in China (STP–C and STP–D) was similar to Europe (300–600 ng /L; Göbel *et al*., 2004) but three to five orders lower than the concentration found in STPs in Japan (STP–A and STP–B). Sulfamethoxazole and trimethoprim are often use together and were found in higher concentration in STP–C and STP–D (> 1578ng /L). In Europe, sulfamethoxazole 230–570 ng /L, trimethoprim 220–440 ng /L and roxithromycin 10–40 ng /L was detected in influent (Göbel *et al*., 2007), which is similar to the STP–A and STP–B. Like STP–C and STP–D, a relatively similar concentration of trimethoprim was also detected in Sweden (1300 ng/L; Lindberg *et al*., 2005) and New Mexico, USA (1400 ng/L; Brown *et al*., 2006)

In contrast, a completely different pattern of antibiotics occurrences in influent were observed in Japan (STP–A and STP–B) and China (STP–C and STP–D). Only macrolide antibiotics: azithromycin and clarithromycin contribute 45–75% load within the selected antibiotics in influent in STP–A and STP–B (Fig. 3). On the other hand, it was fluoroquinolone antibiotics–levofloxacin and norfloxacin in STP–C and STP–D (Fig. 3). In this study levofloxacin, norfloxacin, trimethoprim, and sulfamethoxazole were detected up to µg/L in influent in China, whereas, only clarithromycin were found in µg/L in influent in Japan.

Occurrence of antibiotics in the STPs secondary effluent

Similar to influent samples, concentration of the antibiotics varied among secondary effluent in the STPs (Fig. 2). Clarithromycin was detected in higher concentration in secondary effluent at STP–A (536 ng/L) and STP–B (583 ng/L). In STP–A, except clarithromycin, all antibiotics were detected <100 ng/L. Levofloxacin, azithromycin, roxithromycin, and sulfamethoxazole were detected between 50–100 ng/L, and others

were <50 ng/L in STP–A effluent. Like STP–A effluent, clarithromycin (583 ng/L) was detected in the highest concentration in effluent of STP–B. Comparatively, a higher level of levofloxacin (2623–8628 ng/L), norfloxacin (370–1116 ng/L), azithromycin (65–847 ng/L), sulfamethoxazole (335–683 ng/L), and trimethoprim (503–960 ng/L) were detected in effluent of STP–C and STP–D. Same as STP–C, high concentration of sulfamethoxazole (700 ng/L) was also detected form New York, USA (Batt *et al*., 2007). Trimethoprim was measured in higher concentration in effluents from Sweden (1300 ng/L; Lindberg *et al*., 2005), New York, USA (2500 ng/L; Batt *et al*., 2007) which are similar to STP–D effluent. Norfloxacin was detected 350–370 ng/L at Shenzhen Nan Shan, China (Gulkowska *et al*., 2008) which is similar to STP–D (370 ng/L) but three times lower than STP–C (1116 ng/L). Only enrofloxacin and levofloxacin were detected around two to three order higher concentration in effluent than influent in STP–C. This can be explained by the presence of substances, e.g. human metabolites/conjugates which can subsequently be transformed to parents compound in biological treatment (Göbel *et al*., 2005, 2007) and/or adsorption and desorption mechanism of compounds which may led to first sorption of the compounds in biological reactor and later desorption during return sludge mixing.

Elimination of antibiotics in the STPs

The removal efficiencies of the selected antibiotics in STPs in Japan (STP–A and STP–B) and China (STP–C and STP–D) are presented in Figure 4. Antibiotics removal efficiencies in the STPs were calculated from the concentration difference in dissolved phase between influent and effluent samples, and varied among compounds (Fig. 4). Removal efficiency of each antibiotic was varied among STPs due to specific treatment technology employed by individual STP, the hydraulic and solid residence time at different STPs, and moreover physical and chemical properties of the antibiotics.

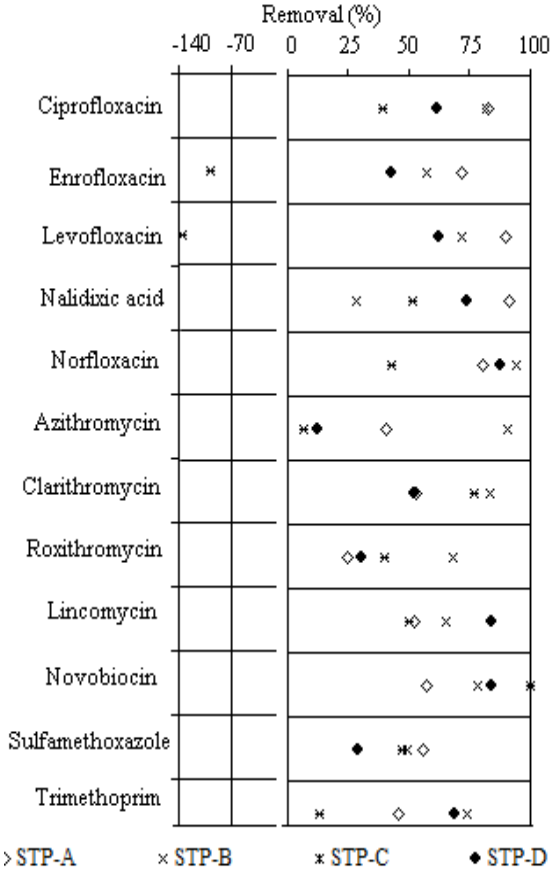

Fig.4. Comparison of removal efficiencies of the selected antibiotics in STPs in Japan (STP–A and STP–B) and China (STP–C and STP–D

Macrolides antibiotics (*e.g.* azithromycin, clarithromycin and roxithromycin) removal efficiencies were lower than fluoroquinolones antibiotics (*e.g.* ciprofloxacin, enrofloxacin, levofloxacin, nalidixic acid and norfloxacin) in the STP–A (Fig. 4). Except levofloxacin (-135%) and enrofloxacin (-97%) in STP–C, all quinolones antibiotics were removed moderately (40–90%) in STP–A, STP–B and STP–D. Only clarithromycin (77%) and nalidixic acid (51%) were removed >50% in STP–C. Macrolide antibiotics have been shown to be more persistent than some of the other antibiotics (Huang *et al*., 2001). Varying results, including negative elimination was

also reported in literature (Göbel *et al*., 2007). A moderate removal efficiency for levofloxacin (30–55%), clarithromycin (30–50%) and azithromycin (40–55%) were also reported from Japan (Yasojima *et al*., 2006). A higher removal of norfloxacin (78%) was observed from STPs in southern china (Gulkowska *et al*., 2008) which is similar to STP–D (86%) to this study. Norfloxacin was removed around 40% in STP–C and there was no detection of novobiocin in effluent form STP–C. Sulfamethoxazole was removed 56% in STP–A, 9% in STP–B, 31% in STP–C and 73% in STP–D. Sulfamethoxazole was removed in higher proportion in STP–A and STP–D than STP–B and STP–C. A similar removal of sulfamethoxazole (29–60%) was observed in CAS and Fixed bed reactor in Switzerland in winter but negative removal in summer (Göbel *et al*., 2007). Lower removal of antibiotics in STP–C could be related with its operation technology which is Oxidation Ditch (OD) based secondary treatment. In general, OD process operated with relatively lower dissolved oxygen level than CAS, AO and A^2O process, and oxygen level is a limiting factor for biological activity in aerobic process. Trimethoprim was not removed effectively in OD based process in STP–C, however, removal efficiency was 40–70% in A^2O (STP–A and STP–D) and AO (STP–B) process. Longer HRT and SRT generally results in the higher removal of antibiotics in STPs (Batt *et al*., 2006; Clara *et al*., 2005). In this study, except STP–C (HRT 17.4h) all STPs were operated relatively in similar HRT (9–12 h). On the other hand STP–D had shorter SRT (7 d) than other three STPs (16–19days). Based on the antibiotics removal performance in STPs in Japan (STP–A and STP–B), except macrolides antibiotics (azithromycin and clarithromycin), most of the antibiotics were removed higher in STP–A (A^2O process) than STP–B (suspended carrier based AO process). In Chinese STPs (STP–C and STP–D), except clarithromycin in STP–C (OD process), all antibiotics were removed more than two order magnitude in STP–D (A^2O process).

CONCLUSIONS

The occurrence of antibiotics varied among STPs location in different geographical location, in Japan and China. In influent samples, llevofloxacin (6800 ng/L), norfloxacin (2775 ng/L), trimethoprim (1578 ng/L) sulfamethoxazole (1280 ng/L) were detected in higher concentration in the Chinese STPs (STP–C and STP–D), however, azithromycin (1866 ng/L) and clarithromycin (1134 ng/L) were detected in higher concentration in Japanese STPs. Except Novobiocin in effluent of STP–C, all antibiotics were detected in all sample analyzed. In general, removal efficiency of the antibiotics varied with individual treatment technologies applied, from negative to >90% removal, and in some extend A^2O (STP–A and D) and AO (STP–B) based biological nutrient removal processes were superior to OD based biological treatment.

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