Supplementary Information:

Evaluation of residual antibacterial potency in antibiotic production wastewater using a real-time quantitative method

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Materials and methods

Sample pretreatment

The solid phase extraction (SPE) procedures for oxytetracycline (OTC) production wastewater were carried out as follows[1]: (1) adjust the pH of wastewater sample (500 mL each) to 2.5–3.0 with 40% H_2SO_4, and add 0.2 g of Na_2EDTA to the sample to complex potential interfering metals; (2) precondition an Oasis HLB cartridge with 5 mL of methanol, 5 mL of 0.5 M HCl and 5 mL of ultra-pure water sequentially; (3) extract the sample with the HLB cartridge at a flow rate of approximately 0.5 mL/min, and wash the cartridge with 5 mL of 5% methanol aqueous solution and 5 mL ultra-pure water; (4) elute the antibiotics with 10 mL of a dichloromethane/acetone mixture (3:2, v/v); (5) dry the extract under a gentle stream of N_2; (6) dissolve the dried residue with 0.8 mL of methanol and dilute with 1.2 mL of ultra-pure water.

The SPE procedures for spiramycin (SPM) production wastewater were carried out as follows[2]: (1) Oasis HLB cartridges were preconditioned sequentially with 10 mL of methanol, 10 mL of water, 10 mL of 2% NaCl, and 2 mL of 0.1 M phosphate buffer (pH 8.0); (2) extract 500 mL wastewater sample (after filtered) with the HLB cartridge at a flow rate of 0.5 mL/min, and wash the cartridge with 5 mL of ultra-pure water; (3) the cartridge was then dried under a gentle stream of N_2; (4) antibiotics were eluted from the cartridge with 10 mL of 95% methanol; (5) dry the extract under a gentle stream of N_2; (6) dissolve the dried residue with 2 mL of solvent buffer (prepared by mixing ACN and 0.1 M ammonium acetate in a ratio of 15:85).

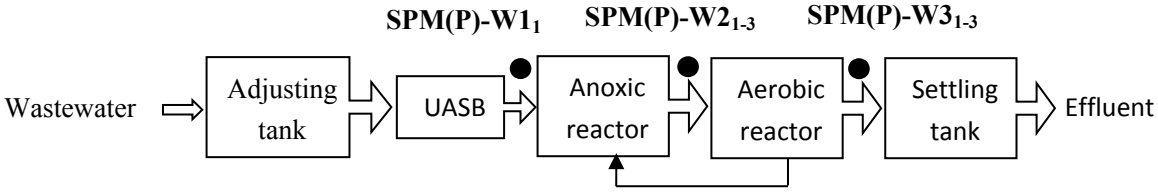
UPLC-MS/MS analyses

Concentrations of OTC and its transformation products (three hydrolysates, 4-epi-oxytetracycline (EOTC), α-apo-oxytetracycline (α-apo-OTC), β-apo-oxytetracycline (β-apo-OTC)) and SPM and neospiramycin (NeoSPM) were detected by UPLC-MS/MS (Acquity UPLC system, Waters, USA) equipped with an Acquity UPLC BEH C18 column (2.1 mm×100 mm, 1.7 μm particles, Waters, USA) according to procedures reported previously[1-5].

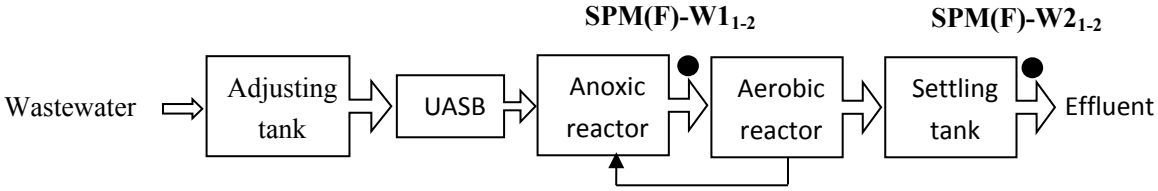
OTCs: Stock solutions of OTCs (1 mg/mL) were prepared in methanol and diluted to the range of 0.05–1 mg/L to obtain the standard curves. Ultrapure water added with 0.1% formic acid was used as solution A, while methanol was used as solution B. Separation conditions are as following: solution A decreased from 90% to 70% in the first 4 min, then decreased from 70% to 10% in 4.5 min, and then maintained at 10% for 1 min, and increased back to 90% in 0.5 min, finally balancing at 90% for 3 min. The flow rate was 0.25 mL/min. The electrospray ionization was operated in the positive ion mode. The capillary was 3.5 kV, source temperature was 110 °C, and desolvation temperature was 600 °C. Nitrogen gas was used as the desolvation gas with a flow rate of 600 L/h and the cone gas of 60 L/h. The operational parameters of the tandem MS were listed in Table S3.

SPMs: Stock solutions of SPM and NeoSPM (1 mg/mL) were prepared in methanol and diluted to the range of 0.05–1 mg/L to obtain the standard curves. Ultrapure water added with 0.1% formic acid was used as solution A, while methanol was used as solution B. Separation conditions are as following: solution A decreased from 90% to

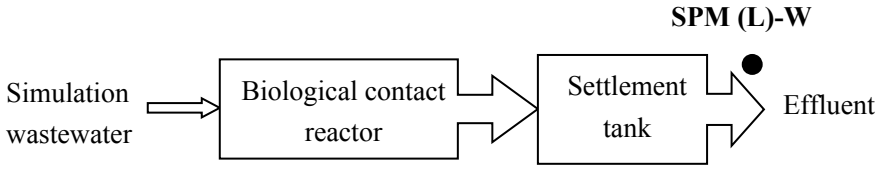
60% in the first 3 min, then maintained at 60% for 3 min, and increased back to 90% in 0.1 min, finally balancing at 90% for 3.9 min. The flow rate was 0.25 mL/min. Electro-sprayed ionization mode (positive) was used for the MS/MS system. Ion source and dissolvent gas temperatures were 120 °C and 600 °C, respectively. Capillary was 3.5 kV. The dissolvent gas flow rate was 600 L/h. The operational parameters of the tandem MS were listed in Table S4.



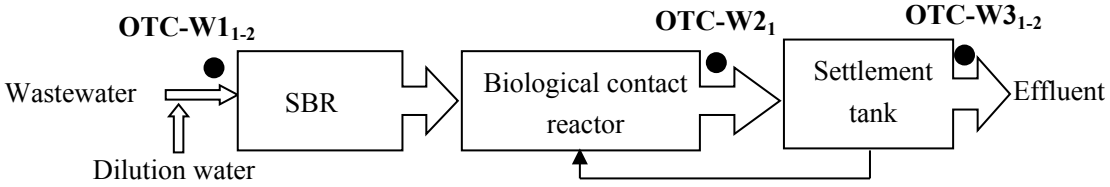
(a) Pilot-scale SPM production wastewater treatment system



(b) Full-scale SPM production wastewater treatment system



(c) Lab-scale SPM simulation wastewater treatment system



(d) Biological OTC production wastewater treatment system

Fig.S1 The flow chart and sampling sites of four wastewater treatment systems treating Spiramycin (SPM) in Pilot-scale (a), Full-scale (b), Lab-scale (c), and Oxytetracycline (OTC) (d).

" • ": Sampling sites. **UASB**: Up-flow Anaerobic Sludge Bed; **SBR**: Sequential Batch Reactor.

SPM (P)-W1, W2, W3: UASB effluent, Anoxic reactor effluent, Aerobic reactor effluent from the pilot-scale system for treating SPM wastewater; **SPM (F)-W1, W2**: Anoxic reactor effluent, Final effluent from full-scale system for treating SPM wastewater; **SPM (L)-W**: Final effluent from lab-scale system for treating SPM simulation wastewater; **OTC-W1, W2, W3**: influent,

Table S1 Composition of antibiotic assay medium No.3

Subsance	g/L	Substance	g/L
Peptone	5	Sodium chloride	3.5
Beef extract powder	1.5	Dipotassium hydrogen phosphate	3.68
Yeast extract powder	3	Potassium dihydrogen phosphate	1.32
Glucose	1	pH	7.0-7.2

Table S2 Preparation of phosphate buffer solution (PBS)

PBS	Dipotassium hydrogen phosphate	Potassium dihydrogen phosphate
pH=7.8	5.59 g/L	0.41 g/L
pH=6.0	2.00 g/L	8.00 g/L

Table S3 ESI-MS/MS parameters for OTCs

Analyte	MRM transition (m/z)	Cone voltage (V)	Collision energy (kV)
OTC	461.5 > 443, 461.5 > 426*	25	15
EOTC	461.5 > 443, 461.5 > 426*	25	15
α-apo-OTC	443 > 426*, 443 > 408	23	18
β-apo-OTC	443 > 426*, 443 > 408	23	18

*Predominant ion defined as a base peak for quantification.

Table S4 ESI-MS/MS parameters for spiramycin and neospiramycin

Analyte	MRM transition (m/z)	Cone voltage (V)	Collision energy (kV)
NeoSPM	699.0 > 174.0*; 699.0 > 159.9	18	15
SPM	843.6 > 174.0*; 843.6 > 100.9	25	25

*Predominant ion defined as a base peak for quantification.

Table S5 Brief review about potency assay in this study and previous studies

Target sample		Environmental process	Tested method		Tested strain	Potency expression	Antibiotic analyses	Reference
Environmental samples	Antibiotic production wastewater	Wastewater treatment systems	Real-time quantitative assay method	37 °C, agitation, real-time (≤ 4 h)	*Staphylococcus aureus*	Antibiotic equivalent quantity (EQ, mg/L)	UPLC-MS/MS	This study
Artificial antibiotic solutions	Sulfa drug solution	Photolysis	Fixed growth time method	37 °C, 200 rpm agitation, 8 h	*Escherichia coli* DH5a	EC_{50}	HPLC	6
	Tetracycline solution	Photolysis		37 °C, 190 rpm agitation, 6 h	*Escherichia coli* DH5a	EC_{50}	-	7
	Lincomycin, ciprofloxacin, trimethoprim solutions	Oxidization by potassium permanganate		37 °C, gently shaken, 6 h	*Escherichia coli DH5R*	Growth inhibition (I, %); EC_{50}	LC-MS/MS; HPLC-PDA	8
	Antibacterial molecules (13)[a]; biocide triclosan solutions	O_3, •OH treatment		37 °C or 30 °C, 200 rpm agitation, 8 h	*Escherichia coli* K12 wildtype; *Bacillus subtilis* Marburg	Growth inhibition (I, %); PEQ = $EC_{50,0}/EC_{50,x}$	-	9
	Ciprofloxacin solution	Aqueous photolytic and photocatalytic batch reactions		37 °C, 200 rpm agitation, 8 h	*Escherichia coli*	Growth inhibition (I, %); PEQ = $EC_{50,0}/EC_{50,x}$	HPLC	10

	β-lactam, β-lactam-(R)-sulfoxide solutions	O_3, •OH treatment	30 °C, 200 rpm agitation, 8 h	*Bacillus subtilis* Marburg	Growth inhibition (I, %); PEQ = $EC_{50,0}/EC_{50,x}$	HPLC-MS/MS; HPLC-UV	11

a. Roxithromycin, azithromycin, tylosin, ciprofloxacin, enrofloxacin, penicillinG, cephalexin, sulfamethoxazole, trimethoprim, lincomycin, tetracycline, vancomycin, amikacin.

Table S6 Comparison of repeatability of the real-time quantitative method (n=5) with the conventional fixed growth time one

Inhibition ratio [a] (%)	Conventional fixed growth time method					Real-time quantitative method (t_x [b])
	100 min	120 min	140 min	160 min	180 min	
Oxytetracycline solution (n=5)	61.35	61.64	60.51	53.96	46.68	50.32 (170 min [b])
	52.77	55.15	54.34	50.69	42.88	50.69 (160 min [b])
	58.76	56.68	54.45	48.62	38.54	51.46 (150 min [b])
	57.59	55.96	52.54	46.65	36.23	50.00 (150 min [b])
	53.57	55.53	55.52	53.08	46.82	50.51 (170 min [b])
Average	56.81	56.99	55.47	50.60	42.23	50.60
RSD (%)	6.33	4.66	5.43	6.01	11.29	1.08

[a] Results were expressed by inhibition ratio (%) of bacterial growth.

[b] t_x was determined on the basis of the correlation coefficient of linearity in the real-time quantitative method.

Table S7 Recovery rate of the real-time quantitative potency assay (n=6)

Sample	Spiked concentration (mg/L)	Average potency (EQ mg/L)	Recovery rate (%)	Average recovery rate (%)
Oxytetracycline in deionized water	50	49.68	99.36	100.04
	10	9.97	99.75	
	2	2.02	101.01	
Oxytetracycline in oxytetracyciline wastewater[+]	5	4.97	99.40	99.40
Spiramycin in deionized water	100	98.05	98.05	99.18
	10	10.01	100.11	
	2	1.99	99.37	

[+] Addition of 5 mg/L oxytetracycline standards to oxytetracycline wastewater.

Table S8 Potencies and oxytetracycline and its transformation products concentrations of oxytetracycline production wastewater ($R^2 = 0.999$. $P < 0.01$)

Sample	Potency [a] (oxytetracycline EQ mg/L)	OTC Conc. [b] (mg/L)	EOTC Conc. [b] (mg/L)	α-OTC Conc. [b] (mg/L)	β-OTC Conc. [b] (mg/L)
OTC-$W1_1$	8.43 ± 0.49	7.80 ± 0.67	0.61 ± 0.04	-	-
OTC-$W2_1$	2.27 ± 0.14	2.24 ± 0.01	-	-	-
OTC-$W3_1$	1.52 ± 0.16	2.03 ± 0.01	-	-	-
OTC-$W1_1^+$	13.41 ± 0.76	12.23 ± 0.04	0.75 ± 0.01	-	-
OTC-$W1_2$	6.57 ± 0.24	6.26 ± 0.16	0.41 ± 0.03	-	-
OTC-$W3_2$	0.66 ± 0.04	0.62 ± 0.09	-	-	-

[a] Antibacterial potency determined by real-time quantitative method expressed by oxytetracycline equivalent quantity.

[b] Concentrations of oxytetracycline (OTC) and its transformation products (4-epioxytetracycline (EOTC), α-apo-oxytetracycline (α-OTC), β-apo-oxytetracycline (β-OTC)) determined by UPLC-MS/MS.

[+] Addition of 5 mg/L oxytetracycline standards.

Table S9 Potencies and spiramycin concentrations of spiramycin production wastewater ($R^2 = 0.896$. $P < 0.01$)

Sample	Potency [a] (spiramycin EQ mg/L)	SPM Conc. [b] (mg/L)
SPM (P)-$W1_1$	11.01 ± 0.08	1.88 ± 0.03
SPM (P)-$W2_1$	8.56 ± 0.000	1.61 ± 0.01
SPM (P)-$W3_1$	1.81 ± 0.11	0.84 ± 0.01
SPM (P)-$W2_2$	8.91 ± 0.04	2.26 ± 0.04
SPM (P)-$W3_2$	1.54 ± 0.01	0.77 ± 0.01
SPM (P)-$W2_3$	10.91 ± 0.83	1.50 ± 0.07
SPM (P)-$W3_3$	1.81 ± 0.12	0.81 ± 0.03
SPM (F)-$W1_1$	1.65 ± 0.22	0.38 ± 0.01
SPM (F)-$W2_1$	0.71 ± 0.09	0.31 ± 0.01
SPM (F)-$W1_2$	2.05 ± 0.04	1.41 ± 0.05
SPM (F)-$W2_2$	1.50 ± 0.05	0.39 ± 0.01

[a] Antibacterial potency determined by real-time quantitative method expressed by spiramycin equivalent quality.

[b] Spiramycin (SPM) concentration determined by UPLC-MS/MS.

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