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

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While antibiotic pollution has attracted considerable attention due to its potential in promoting the dissemination of antibiotic resistance genes in the environment, the antibiotic activity of their related substances has been neglected, which may underestimate the environmental impacts of antibiotic wastewater discharge. In this study, a real-time quantitative approach was established to evaluate the residual antibacterial potency of antibiotics and related substances in antibiotic production wastewater (APW) by comparing the growth of a standard bacterial strain (*Staphylococcus aureus*) in tested water samples with a standard reference substance (e.g. oxytetracycline). Antibiotic equivalent quantity (EQ) was used to express antibacterial potency, which made it possible to assess the contribution of each compound to the antibiotic activity in APW. The real-time quantitative method showed better repeatability (Relative Standard Deviation, RSD 1.08%) compared with the conventional fixed growth time method (RSD 5.62–11.29%). And its quantification limits ranged from 0.20 to 24.00 $\mu\text{g L}^{-1}$, depending on the antibiotic. We applied the developed method to analyze the residual potency of water samples from four APW treatment systems, and confirmed a significant contribution from antibiotic transformation products to potent antibacterial activity. Specifically, neospiramycin, a major transformation product of spiramycin, was found to contribute 13.15–22.89% of residual potency in spiramycin production wastewater. In addition, some unknown related substances with antimicrobial activity were indicated in the effluent. This developed approach will be effective for the management of antibacterial potency discharge from antibiotic wastewater and other waste streams.

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Environmental impact

Antibiotic pollution from antibiotic production wastewater has caused increasing concern worldwide. Previous studies have focused on the discharge of antibiotics by determining their concentrations. However, some antibiotic related substances in effluents still possess antibacterial activity, which might induce emergence and proliferation of antibiotic resistant bacteria and genes in the environment. Therefore, it is important to investigate residual antibacterial potency in antibiotic production wastewater with high concentrations of antibiotics and related substances. In this study, a real-time quantitative method was developed and applied for the analysis of residual antibacterial potency of antibiotic production wastewater. The results showed that spiramycin production wastewater contained transformation products with high potency. This method made it possible to assess the contribution of each compound and should be a useful tool for environmental risk management for providing the basis for identification of transformation products with antibacterial activity.

Introduction

The presence of residual antibiotics can induce antibiotic resistance in environmental bacteria,^{1–6} which is of considerable concern. Antibiotic production wastewater (APW) contains

high concentrations of residual antibiotics,⁷ and treated wastewater has been identified as an important point source of antibiotic pollution in the environment.^{8,9}

Precursors, intermediates and byproducts can also accompany the production of objective antibiotics.^{10–12} Importantly, these substances can possess antibiotic activity, yet have received little attention with regard to their occurrence in APW. At the same time, some antibiotics in wastewater can be converted into transformation compounds through hydrolysis or other mechanisms during APW treatment.^{1,13–17} Such biotransformation or hydrolysis products can maintain a structure similar to their mother compounds, and can possess antibiotic activity,¹⁴ though at lower levels than their parent compounds.¹⁸

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These transformation products may also exert selective pressure on bacteria in the environment, favoring the emergence and spread of antibiotic resistance.¹⁹ While the discharge of antibiotics during APW treatment has raised great concern,^{3,4,20} the potential impacts of related substances remain unclear. It is important, therefore, that effective methods be developed for the assessment of antibacterial potency in wastewater.

In pharmacopoeias,^{21,22} antibacterial potency is determined by comparing the inhibition effect of a target antibiotic on a sensitive standard microorganism (e.g. *Staphylococcus aureus* and *Escherichia coli*) with that of a standard reference substance. Both cylinder-plate and turbidimetric methods have been used to assess the change in the potency of antibiotic solutions in environmental processes.^{14,15,23–28} Turbidimetry is simple, non-time consuming, and suitable for environmental sample analysis and management. Previous studies have established different turbidimetric methods to assess the potency change of artificial antibiotic solutions during environmental processes using *E. coli*^{23–27} and *Bacillus subtilis*.^{26,28} In these studies, changes in antibiotic activities were expressed by using EC₅₀, MIC₅₀ or similar indicators. However, these approaches are not effective in assessing the contribution of each compound to antibacterial potency in environmental samples.

In this study, a real-time quantitative method was established to evaluate the residual antibacterial potency of antibiotic and related compounds in APW by comparing the growth of a standard bacterial strain (*Staphylococcus aureus*) in tested water samples to a standard reference substance. Equivalent quantity (EQ, mg L⁻¹) was used to express antibacterial potency to allow for comparison with instrumental analytical results, and assess the contribution of each compound in environmental samples. Samples from four APW treatment systems treating spiramycin or oxytetracycline containing wastewater were used to evaluate the effectiveness of the developed method, and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was used to detect the concentrations of target antibiotics and known transformation products in wastewater. The results showed that neo-spiramycin, a transformation product of spiramycin, contributed to antibacterial potency (13.15–22.89%) in spiramycin production wastewater. This study can provide a useful tool for the evaluation and management of APW.

Materials and methods

Chemicals, reagents and model bacterial strain

Antibiotic assay medium no. 3 (Table S1,† used in turbidimetric assay²²) and nutrient agar were purchased from Beijing AoBoXing Bio-Tech Co. Ltd (China). For potency tests, erythromycin (938 u mg⁻¹), spiramycin (1343 u mg⁻¹), kanamycin (694 u mg⁻¹), tetracycline (971 u mg⁻¹), paromomycin (718 u mg⁻¹), ribostamycin (695 u mg⁻¹) and streptomycin (711 u mg⁻¹) were purchased from the National Institutes for Food and Drug Control (NIFDC, Beijing, China). Oxytetracycline (≥95% (HPLC)), tigecycline (≥98% (HPLC)), neospiramycin and formic acid were purchased from Sigma-Aldrich (St. Louis, MO,

USA). 4-Epi-oxytetracycline, α-apo-oxytetracycline, and β-apo-oxytetracycline were acquired from Acros Organics (Geel, Belgium). Methanol, acetonitrile and acetone were supplied by Fisher Scientific (Fair Lawn, NJ, USA). Ultra-pure water (18.2 MΩ cm) was prepared using water purification equipment (Milli-Q, Millipore, Bedford, MA, USA).

Experiments were performed with sterilized phosphate buffer solution (PBS) at different pHs (Table S2†), which supplied suitable growing conditions for bacteria. Solutions of all antibiotic standards for potency assay were prepared by dissolving in water/ethanol/HCl (Table 1) and diluting antibiotic standards in PBS at different concentrations on the day of assay. The successive solutions in increasing stepwise concentrations were usually in the ratio of 1 : 1.25 or smaller for quantitative assay.

Nutrient agar medium was used for the preparation of slants. Antibiotic assay medium no. 3 was used to culture *Staphylococcus aureus* and prepare the bioassay solution. The medium was dissolved in distilled water, and sterilized in an autoclave at 115 °C for 30 min.

Staphylococcus aureus (CMCC (B) 26003) (NIFDC, Beijing, China), a representative Gram-positive bacterium, was used as the model bacterial strain for the antibacterial potency test according to pharmacopoeias.²² A 200 μL frozen preserved *Staphylococcus aureus* test strain was rapidly thawed and grown in antibiotic assay medium no. 3 at 37 °C for 6 h with vigorous shaking. The strains were inoculated on slants of nutrient agar media, and incubated at 37 °C for 16–18 h. The slants were stored at 4 °C and used for bioassay within one week. On the day of assay, the organisms were washed from the agar slants with 10 mL sterilized normal saline solution (0.9%) and diluted by antibiotic assay medium no. 3 to prepare the test strain suspension, whose absorbance at 580 nm reached 0.30.

Sampling sites

Four APW treatment systems, including two spiramycin production wastewater treatment systems (WWTS) (a pilot and a full-scale system) at Wuxi Fortune Pharmaceutical Co. Ltd. (Jiangsu Province, China), one lab-scale spiramycin WWTS, and a full-scale oxytetracycline production WWTS at North China Pharmaceutical Group Co. (Hebei Province, China), were selected for this study. The full-scale spiramycin WWTS, consisting of an anoxic reactor and oxidation ditch (OD), has been in use since 2012. Water samples were collected at an equal proportion three times during a 24 h period in November 2013 and December 2014, and included full-scale anoxic effluent samples (SPM (F)-W1_{1–2}) and full-scale final effluent samples (SPM (F)-W2_{1–2}) (Fig. S1(b)†). The pilot WWTS consisted of a sequential up-flow anaerobic sludge bed (UASB), an anoxic reactor and an oxic reactor, and was operated for nine months, as described in our previous study.²⁹ One UASB effluent sample (SPM (P)-W1₁), three anoxic samples (SPM (P)-W2_{1–3}) and three aerobic samples (SPM (P)-W3_{1–3}) were collected from the pilot system over the experimental period (Fig. S1(a)†). The simulated lab-scale WWTS, consisting of a biological contact oxidation process, was operated with a hydraulic retention time of 24 h

Table 1 IDL, LOQ and linear range of antibiotics inhibiting *Staphylococcus aureus*

Antibiotics	R^2	Linear range (mg L ⁻¹)	Slope ^a	PBS ^b	IDL ^c (mg L ⁻¹)	LOQ ^d (µg L ⁻¹)
Spiramycin ^e	0.997 ± 0.001	4.50–17.00	0.592 ± 0.103	7.8	2.92	18.00
Erythromycin ^e	0.995 ± 0.003	0.10–1.00	0.556 ± 0.138	7.8	0.10	0.40
Oxytetracycline ^f	0.997 ± 0.002	0.10–0.40	0.480 ± 0.196	6.0	0.10	0.40
Tetracycline ^f	0.998 ± 0.001	0.05–0.30	0.095 ± 0.008	6.0	0.05	0.20
Paromomycin	0.995 ± 0.002	0.80–3.00	0.628 ± 0.182	7.8	0.62	3.20
Ribostamycin	0.998 ± 0.001	6.00–18.00	0.646 ± 0.201	7.8	3.44	24.00
Streptomycin	0.998 ± 0.001	5.00–30.00	0.531 ± 0.140	7.8	4.41	20.00
Kanamycin	0.994 ± 0.003	4.00–18.00	0.184 ± 0.023	7.8	2.11	16.00

^a Values are the means of multiple determinations ($n \geq 3$). ^b pH of PBS used in assay. ^c Instrumental detection limits (the antibiotic EQ mg L⁻¹ resulting in a 90% OD_{580 nm} value of positive control). ^d Quantification limits of the method. ^e Standard solution was dissolved in 4 mL ethanol/10 mg antibiotic. ^f Standard solution was dissolved in 1 mL HCl/10 mg antibiotic.

continuously for more than 600 days with six stages (spiramycin concentrations: 0, 0.1, 1, 5, 25, and 50 mg L⁻¹). The effluent from the reactor (SPM (L)-W) was collected at the sixth stage, in which the influent spiramycin concentration was 50 mg L⁻¹ over three successive days (Fig. S1(c)†). The full-scale oxytetracycline production WWTS has been in use since 2000, as described in our previous study.⁴ Water was collected as 24 h composite samples in January 2010 and January 2012, and included influent (OTC-W1₁₋₂), aerobic (OTC-W2₁) and final effluent samples (OTC-W3₁₋₂) (Fig. S1(d)†).

Sample pretreatment

Wastewater samples were first filtered through GF/F glass fiber filters (0.7 µm, Whatman, Maidstone, Kent, UK) and stored at 4 °C until solid phase extraction (SPE) on 6 mL Oasis HLB sorbent cartridges (500 mg; Waters, Milford, MA, USA)³⁰⁻³² within two days. The SPE technique was used to remove the background interfering compounds in wastewater with detailed procedures described in the ESI.† After re-dissolution, the extract solution (called the test sample in potency assay) was diluted with ultra-pure water and PBS (according to Table 1) to obtain readings in the middle of the standard curve for UPLC-MS/MS and potency assessment, respectively.

UPLC-MS/MS analyses

Concentrations of oxytetracycline and its transformation products (three hydrolysates, 4-epi-oxytetracycline, α -apo-oxytetracycline, and β -apo-oxytetracycline), spiramycin and neospiramycin 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) as described in the ESI.† according to previous studies.^{20,30-33} The operational parameters of the tandem MS are listed in Tables S3 and S4.† All samples were analyzed in triplicate.

Assay of antibacterial potency by the real-time quantitative method and potency calculation

We added 1 mL of antibiotic standard solution or the test sample (diluted with PBS from extracts after re-dissolution) and 9 mL of the strain suspension to test tubes. We used 1 mL of PBS

and 9 mL of antibiotic medium no. 3 without strain suspension for the blank control, and 1 mL of PBS and 9 mL of strain suspension for the positive control. The test tubes were incubated on a microbiological turbidimetric apparatus (WBS-100, Beijing Xianqu Weifeng Technology Development Co., China) in the dark at 37 °C for 4 h, and were shaken at regular intervals. After 45 min, the optical density of each test tube at 580 nm (OD_{580 nm}), which reflects bacterial growth, was automatically recorded every 10 min until 20 sets of data were acquired. One set of data for potency assessment was chosen from the 20 sets on the basis of the correlation coefficient of linearity. All samples were analyzed for potency in triplicate and average values were reported.

The linear response of different concentrations of antibiotics and the calibration curves were generated using linear regression analysis. The residual potency was transformed into antibiotic equivalent quantities (EQ mg L⁻¹), which were calculated by linear regression. Recovery rates were determined by adding known amounts of oxytetracycline/spiramycin to the samples at the beginning of the process. Potency was determined after SPE pretreatment.

To compare the real-time quantitative method and the conventional fixed growth time method²³ (optical density of the suspension determined after stopping bacterial growth at a fixed culture time in the logarithmic phase), the inhibition ratio of the simulated oxytetracycline wastewater (0.30 mg L⁻¹) was evaluated five times. The inhibition ratio (I) (%) was calculated as follows:¹⁴

$$I = ([D_t - E_t]/D_t) \times 100 \quad (1)$$

where t is the selected culture time (min); E_t = the OD_{580 nm} value of the tested samples at the time of t min; D_t = OD_{580 nm} of the positive control without the antibiotic or sample at t min. The inhibition ratio values ranged from 0 to 1.

Data analysis

Statistical analyses were performed using SPSS version 17.0. Paired sample t -tests were used to assess significant differences between the different systems and samples based on p values. Pearson's bivariate correlation analysis was used to assess the

relationship between the antibiotic concentration using UPLC-MS/MS and the antibacterial potency (antibiotic EQ mg L^{-1}) using real-time quantitative assay. Differences were considered significant only when p values were less than 0.05.

Results and discussion

Method validation

In previous studies,^{23–28} antibacterial potency was assayed after killing bacteria to stop their growth at a fixed time (usually 6 h or 8 h) (Table S5†). The bacterial growth rates, however, are affected by many different factors^{23–28} including initial cell number, temperature and mixing speed, and it can be difficult to acquire a good repeatability over a fixed growth time period. Therefore, the repeatability of the real-time quantitative assay was compared with that of the conventional fixed growth time method in Fig. 1 (data shown in Table S6†). The RSD of the real-time quantitative method was 1.08%, much lower than that of the conventional fixed growth time method (5.62–11.29%), indicating that the former had higher repeatability.

Antibacterial potency has been expressed by growth inhibition (%), EC_{50} (mg L^{-1}) or PEQ (ranging from 0 to 1) in previous studies (Table S5†); however, such approaches only determine changes during different treatment processes. In our research, an equivalent quantity (antibiotic EQ, mg L^{-1}) was used to express antibacterial potency in wastewater. This approach is more suitable for risk evaluation because the results can be compared with the concentration determined using UPLC-MS/MS to assess the contribution of each compound in environmental samples.

In the present study, antibacterial potency EQ (mg L^{-1}) was calculated on the basis of the standard curves. Different antibiotics exhibit different antibacterial potencies due to their unique antibacterial properties. Although our method was developed specifically for APW assessment, which usually contains only one type of antibiotic at a relatively high concentration, it may also be applied to other environmental

samples such as animal breeding wastewater, which usually contains one or two types of known antibiotics at relatively high concentrations. For other environmental samples, such as sewage and surface water, further studies are required to evaluate the applicability of the current method. The major challenge is that, in such environmental samples, there normally exist multiple types of antibiotics, making it difficult to choose an antibiotic standard.

The standard curves ($R^2 > 0.99$) of eight antibiotics, including two macrolides, three tetracyclines and four aminoglycosides, are shown in Table 1. The linear ranges and slopes of the various antibiotics were quite different. Among them, the linear ranges of spiramycin and oxytetracycline were 4.5–17.0 mg L^{-1} with a slope of 0.592 ± 0.103 and 0.10–0.40 mg L^{-1} with a slope of 0.480 ± 0.196 , respectively. The instrumental detection limits (IDLs) ranged from 0.05 to 4.41 mg L^{-1} , which was the antibiotic EQ resulting from the 90% $\text{OD}_{580 \text{ nm}}$ value of the positive control (antibiotic-free) calculated by the linear equation³⁴ (Table 1). The quantification limits of the real-time quantitative method (LOQ) were determined by the linear range of different antibiotics (Table 1) and ranged from 0.20 to 24.00 $\mu\text{g L}^{-1}$.

Staphylococcus aureus, which was selected as the standard bacterial strain in our study according to pharmacopoeias,²² is Gram-positive and sensitive to most broad spectrum antibiotics. However, it should be noted that there are some narrow spectrum antibiotics acting only on Gram-negative bacteria. For such APW, we used *E. coli* as the standard bacterial strain (data not shown).

The recovery rates of the potency assay are shown in Table S7.† The average recovery rates for oxytetracycline (spiked concentrations: 2, 10, 50 mg L^{-1}) and spiramycin (spiked concentrations: 2, 10, 100 mg L^{-1}) were 100.04% and 99.18% in deionized water, respectively, with a similar recovery rate obtained for oxytetracycline in oxytetracycline wastewater (spiked concentration: 5 mg L^{-1}).

Detection of antibacterial potencies in APW

The residual potency values of treated oxytetracycline wastewater and spiramycin wastewater are shown in Tables S8 and S9,† respectively, together with the antibiotic concentrations. As indicated in Tables S8 and S9,† we could assess the antibiotic potencies in different APWs by using the same standard bacterial strain (*Staphylococcus aureus*) with the standard reference antibiotic. Table S8† shows that the oxytetracycline EQ values acquired with the potency assay for oxytetracycline wastewater samples were quite close to the oxytetracycline concentrations acquired using UPLC-MS/MS. The oxytetracycline concentrations in the influent wastewater ranged from 6.26–7.80 mg L^{-1} , which decreased to 0.62–2.03 mg L^{-1} in the effluent after biological treatment, while the potency in the influent wastewater ranged from 6.58–8.43 oxytetracycline EQ mg L^{-1} , which decreased to 0.66–1.52 (82–90% removal) in the effluent after wastewater treatment. As shown in Table S8,† large amounts of oxytetracycline and 4-epioxytetracycline remained in the treated wastewater. Pearson's correlation coefficient suggested a strong and positive correlation between

Fig. 1 Comparison of repeatability of the real-time quantitative method ($n = 5$) with the conventional fixed growth time one. t_x was determined on the basis of the correlation coefficient of linearity in the real-time quantitative method.

Fig. 2 Correlation between the antibiotic concentration by UPLC-MS/MS and the antibacterial potency equivalent quantity (antibiotic EQ mg L⁻¹) by real-time quantitative assay of APW.

potency (oxytetracycline EQ mg L⁻¹) and oxytetracycline concentration determined by UPLC-MS/MS ($R^2 = 0.999$; $p < 0.01$), showing that oxytetracycline was mainly responsible for the antibacterial potency in the wastewater.

Table S9† shows that all spiramycin EQ values acquired with the potency assay for spiramycin wastewater samples were higher than the spiramycin concentrations acquired using UPLC-MS/MS. For the pilot system, the spiramycin concentrations in the influent ranged from 1.88–2.26 mg L⁻¹, which decreased to 0.77–0.84 mg L⁻¹ in the effluent after biological treatment, while potency in the influent was 11.01 spiramycin EQ mg L⁻¹, which decreased to 1.54–1.82 spiramycin EQ mg L⁻¹ in the effluent after biological treatment. In the full-scale system, the spiramycin concentrations decreased from 0.38–1.41 to 0.31–0.39 mg L⁻¹, while the potency of the wastewater decreased from 1.65–2.05 to 0.71–1.50 spiramycin EQ mg L⁻¹ after wastewater treatment. A relatively strong and positive correlation was still observed between potency (spiramycin EQ mg L⁻¹) and spiramycin concentrations ($R^2 = 0.896$; $p < 0.01$). However, it was clear that spiramycin might not be the sole compound contributing to the observed potency.

The potency assessment and UPLC-MS/MS analysis results are compared in Fig. 2. The oxytetracycline potency values of the wastewater samples were in close accordance with the analyzed oxytetracycline concentrations (slope = 0.8915), while the spiramycin potency values were significantly higher than the analyzed concentrations (slope = 0.1319). Therefore, some other compounds likely contributed to spiramycin potency, suggesting the necessity to regulate transformation compounds and their mother compounds in wastewater and better control dissemination of antibiotic resistant genes. For this purpose, the real-time quantitative approach to determine antibacterial potency established in this study should be a better option considering that many related substances remain unknown.

Table 2 Relative potency of transformation products to parent antibiotics

Parent antibiotics	Transformation products	Relative potency
Oxytetracycline (OTC)	4-Epi oxytetracycline (EOTC)	1
	α -Apo-oxytetracycline (α -OTC)	0.007 ^a
	β -Apo-oxytetracycline (β -OTC)	0.003 ^a
Spiramycin (SPM)	Neospiramycin (NeoSPM)	1
		0.874 ^b

^a Relative potency of spiramycin degradation products = EC₅₀ of degradation products/EC₅₀ of parent antibiotics. ^b Relative potency of neospiramycin = potency of 10 mg L⁻¹ neospiramycin (spiramycin EQ mg L⁻¹)/potency of 10 mg L⁻¹ spiramycin (spiramycin EQ mg L⁻¹).

Assessment of transformation product contributions to wastewater potencies

Spiramycin, a 16-membered macrolide antibacterial agent, can be transformed into neospiramycin by losing a mycarose group, which can be further transformed into forocidin by losing a forosamine group through hydrolysis.^{35,36} Some minor spiramycin-related substances such as methylenespiramycin and deoxydihydrospiramycin have also been reported.³⁷ Among these substances, neospiramycin was identified as the major transformation product of spiramycin^{32,38} reaching up to 5.3 ± 0.4 mg L⁻¹ in spiramycin production wastewater.³⁹

As shown in Table 2, the relative potency of neospiramycin to spiramycin was 0.874, close to previously reported values (88% compared with spiramycin) (European Agency for the Evaluation of Medicinal Products, Committee for Veterinary Medical Products, 1999). This result indicated that the contribution of neospiramycin to the potency of wastewater was not to be neglected. So the concentration of neospiramycin in wastewater samples (SPM (F)-W and SPM (L)-W) was also analyzed (Table 3). Neospiramycin potency ranged from 0.14–3.79 spiramycin EQ mg L⁻¹, which was a contribution of 13.15–22.89% to the residual wastewater potency of the five wastewater samples.

It should be noted that residual potency (spiramycin EQ mg L⁻¹) was still higher than the total potency of spiramycin and neospiramycin, leaving 9.09–60.26% of wastewater potency unexplained. Thus, some other unknown substances with antibacterial activity were present in these wastewater samples. Both antibiotics and their related substances with antibiotic activity in WWTs can disturb the function of an ecosystem by spreading antibiotic resistance genes and affecting the microbial community structure. Hence, antibacterial potency assays, which can express the presence of all potent substances, are an important tool for environmental management. As discussed above, however, some unknown transformation products or intermediates with antimicrobial activity might be transformed from spiramycin and await future investigation.

As described above, oxytetracycline explained most of the wastewater potency for oxytetracycline production wastewater. However, oxytetracycline can be easily transformed into 4-epi-oxytetracycline through an equilibrium reaction and to

Table 3 Contribution of neospiramycin to wastewater residual potency in spiramycin production wastewater

Sample ^a	Spiramycin concentration ^b (mg L ⁻¹)	Neospiramycin concentration ^b (mg L ⁻¹)	NeoSPM potency ^c (spiramycin EQ mg L ⁻¹)	Wastewater residual potency ^d (spiramycin EQ mg L ⁻¹)	Contribution of NeoSPM to wastewater potency ^e	Contribution of unknown substances to wastewater potency ^f
SPM (F)-W1 ₁	0.38 ± 0.01	0.32 ± 0.02	0.28 ± 0.01	1.65 ± 0.22	16.71%	60.26%
SPM (F)-W2 ₁	0.31 ± 0.01	0.16 ± 0.01	0.14 ± 0.01	0.71 ± 0.09	19.72%	36.88%
SPM (F)-W1 ₂	1.41 ± 0.05	0.52 ± 0.02	0.46 ± 0.01	2.05 ± 0.04	22.29%	9.09%
SPM (F)-W2 ₂	0.39 ± 0.01	0.39 ± 0.01	0.34 ± 0.01	1.50 ± 0.05	22.89%	51.21%
SPM (L)-W	17.03 ± 0.52	4.33 ± 0.13	3.79 ± 0.11	28.79 ± 2.73	13.15%	27.72%

^a SPM (L)-W was collected from the effluent of the simulation reactor by aerobic biological treatment; SPM (F)-W1₁, W2₁ and SPM (F)-W1₂, W2₂ were collected from the spiramycin production wastewater treatment plant in November 2013 and December 2014, respectively. ^b Concentration was determined by UPLC-MS/MS. ^c Neospiramycin potency was calculated by multiplying the neospiramycin concentration by coefficient 0.874 (relative potency shown in Table 2). ^d Wastewater residual potency was determined by the real-time quantitative method and expressed by spiramycin equivalent quantity. ^e Contribution of neospiramycin to wastewater potency = neospiramycin potency/potency × 100 (%). ^f Contribution of unknown to wastewater potency = (1 - (spiramycin potency + neospiramycin potency)/potency) × 100 (%), in which spiramycin potency = spiramycin concentration × 1 (relative potency shown in Table 2).

anhydroxytetracycline by irreversible dehydration, which is unstable and can be further transformed into α -apo-oxytetracycline and β -apo-oxytetracycline.^{40,41}

4-Epioxytetracycline, α -apo-oxytetracycline and β -apo-oxytetracycline were previously reported as the main transformation products of oxytetracycline in wastewater.^{15,20} As shown in Table S8,† some oxytetracycline transformation compounds (4-epioxytetracycline) were detected in oxytetracycline production wastewater. This was in accordance with previous research, in which the concentrations of the three oxytetracycline transformation products were 0.36–29.61 mg L⁻¹ and 35.13–39.34 μ g L⁻¹ in wastewater and surface water receiving oxytetracycline wastewater, respectively.²⁰

In the present study, the relative potencies of 4-epioxytetracycline, α -apo-oxytetracycline and β -apo-oxytetracycline to oxytetracycline were 0.007, 0.003 and 0.007, respectively (Table 2). Thus, the transformation products were less potent than their parent compounds, in agreement with previous research.¹⁵ Halling-Sorensen *et al.* reported that 4-epioxytetracycline, α -apo-oxytetracycline and β -apo-oxytetracycline exhibited lower inhibition (concentration for 50% of the maximal effect (EC₅₀), 50% minimum inhibitory concentration (MIC₅₀)) to soil and sludge bacteria than the parent compound.¹⁵ Unlike spiramycin, oxytetracycline lost most of its potency once transformed. However, further studies are needed to clarify whether the low potencies of the transformed compounds mean low ability at inducing antibiotic resistance genes.

Antibiotics and their related substances may potentially influence the structure and function of organism communities in ecosystems receiving wastewater effluents. Moreover, these potent substances could potentially promote or maintain bacterial resistance and antibiotic resistant genes. Therefore, determining antibiotic concentrations alone might not fully reflect the harmful effects of wastewater effluents on the environment. Wastewater residual potency might be more effective at evaluating the risks to the surrounding environment.

It should be noted that different standard bacterial strains (*e.g.* *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*) have been used for potency assays of different

antibiotics in pharmacopoeias.²² In this study, only *Staphylococcus aureus*, the most commonly used strain, was employed to assess residual potency of antibiotic wastewater. Despite this limitation, the real-time quantitative method was convenient and should be easy to implement for environmental management.

Conclusions

A real-time quantitative approach with high repeatability was developed to evaluate the residual antibacterial potency of antibiotic wastewater. Based on the experimental results, the following conclusions can be drawn:

1. The real-time quantitative method showed higher repeatability (RSD 1.08%) than the conventional fixed growth time method (RSD 5.62–11.29%).
2. Residual antibacterial potency was expressed by an antibacterial potency equivalent quantity (antibiotic EQ, mg L⁻¹), which could be compared with antibiotic concentration and made it possible to assess the contribution of each compound. Neospiramycin retained most of the spiramycin antibacterial activity, and was found to contribute to antibacterial potency (13.15–22.89%) in spiramycin production wastewater. The real-time quantitative method developed here will be a useful tool for environmental risk management.
3. This method was developed specific to APW, which contained high concentrations of antibiotics and their related substances in order to evaluate the risk of antibiotic wastewater discharge. Further study should be conducted to extend its application to other environmental waters.

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