

Fluoroquinolones in Hospital Wastewater: Analytical Method, Occurrence, Treatment with Ozone and Residual Antimicrobial Activity Evaluation

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Fluoroquinolones antimicrobials are partially excreted *in natura* after administration. Their occurrence was investigated in hospital raw sewage (HRS) and treated wastewater (TW) using an online solid phase extraction-ultra high-performance liquid chromatography-tandem mass spectrometry method (SPE-UHPLC-MS/MS). The analytical curves ranged from 0.25 to 46 ng mL⁻¹ and the matrix effect, assessed for marbofloxacin and lomefloxacin, ranged from -63 to +1%. All HRS samples contained ciprofloxacin (1-34 ng mL⁻¹) and ofloxacin (0.9-27 ng mL⁻¹), while norfloxacin was detected in 17% of the samples (0.8-4.4 ng mL⁻¹). Only ciprofloxacin (0.5-5.6 ng mL⁻¹) was detected in TW samples. The antimicrobial activity of the HRS samples for *E. coli* was higher than a ciprofloxacin solution of 1 mg L⁻¹, due to the presence of other antimicrobial agents. Ozonation (10 mg O₃ L⁻¹ min⁻¹; 10 min) degraded up to 84% of the fluoroquinolones and removed the antimicrobial activity of HRS samples.

Keywords: fluoroquinolones, hospital wastewater, SPE-UHPLC-MS/MS, antimicrobial activity, advanced oxidation

Introduction

Fluoroquinolones are one of the most important and successful class of antimicrobials in use nowadays.¹ In 2016, the WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR)² classified ciprofloxacin, an antimicrobial from the fluoroquinolones class, as a drug of critical importance for human medicine. However, when these drugs are excreted from the human body, they can reach the many environmental compartments through sewage and irregular disposal, contributing to the development of bacteria resistant to fluoroquinolones and other antimicrobial agents, due to cross-resistance risks.³ The development of antimicrobial resistant genes concerns the scientific community since there is no forecast of new antimicrobial drugs to be available in the market in the near future.³

Data collected in 71 countries, from 2000 to 2010, revealed a growth of more than 30% of total global antibiotic consumption. Among them, cephalosporins,

penicillins, and fluoroquinolones had the largest absolute increase in consumption over the same period.⁴ It is important to highlight that Brazil, India, China, Russia, and South Africa (BRICS countries) were responsible for 76% of the total increase.⁴ A study conducted in a private hospital in the state of Rio Grande do Sul (Brazil) concluded that antimicrobials correspond to 52% of the total amount of drugs prescribed to patients. Of those drugs, 13% were fluoroquinolones.⁵ In general, about 26% of antimicrobials used for human purposes are administered in hospitals and, therefore, these drugs and their metabolites end up in the hospital wastewater and, ultimately, in the environment.⁶

Reports focusing on the occurrence of fluoroquinolones traces in Brazilian hospital wastewaters are scarce. Worldwide, some authors⁷⁻¹³ reported that the predominant fluoroquinolones present in hospital raw wastewater effluents were ciprofloxacin (0.85-101 ng mL⁻¹), ofloxacin (25-35 ng mL⁻¹) and norfloxacin (0.2-17 ng mL⁻¹). Unexpectedly, enrofloxacin, a veterinary drug, was also identified in hospital wastewater (0.1 ng mL⁻¹).¹¹ In another study,¹⁴ it was reported that the ciprofloxacin concentration in hospital wastewater (8.3-13.8 ng mL⁻¹)

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was approximately 10 times higher than in the effluents of a wastewater treatment plant (WWTP) (0.6-1.3 ng mL⁻¹). In addition, it was concluded that the WWTP was not able to fully remove the fluoroquinolones (maximal removal of 90%), contributing to their persistence and spread in river waters.¹⁵ It is noteworthy that none of these works evaluate or correlate the presence of the antimicrobials and the effluents antimicrobial activity. Halling-Sørensen *et al.*,¹⁶ assessing the risk of antimicrobials in the aquatic environment, concluded that effluents with an antimicrobial activity higher or equal to a 0.31 mg L⁻¹ ciprofloxacin solution could impact the efficacy of WWTP based on active sludge. Thus, investigating the biological activity of the effluents is as important as determining the presence of antimicrobials in the environmental compartments, since the residual antimicrobial activity is critical for evaluating the risk of bacterial resistance development. Hospital wastewaters are a complex mixture of compounds, and researches on the quantitative evaluation of their antimicrobial activity are scarce. Caianelo *et al.*¹⁷ described a method to monitor the residual antimicrobial activity in solutions after treatment of UV/H₂O₂. This method is also suitable to monitor the antimicrobial activity of other effluents.

Since the presence of antimicrobial resistant bacteria in hospital wastewater is a reality, it is of paramount importance to monitor and control the load of antimicrobials, such as fluoroquinolones, in wastewater, which should now be added to the list of pollutants of emergent concern.¹⁸

It is also essential to investigate processes that can remove these compounds from wastewater samples. Ozonation (E⁰ = 2.07 V) was proven efficient on removing fluoroquinolones from aqueous media.¹⁹⁻²¹ Hydroxyl radicals (E⁰ = 2.8 V) (formed during ozonation processes at pH around 7 or higher) are highly reactive and non-selective, thus very useful to degrade and/or mineralize recalcitrant compounds such as fluoroquinolones.²²

While the concentration of antimicrobials in wastewater vary from ng L⁻¹ to µg L⁻¹ levels, the analytes need to be concentrated before quantification by liquid chromatography coupled to a tandem mass spectrometer (LC-MS/MS). For this purpose, traditional solid phase extraction (SPE) procedures have been successfully used.^{8,18} However, to allow the determination of these very low concentrations, large sample volumes filtered over membrane filters need to be percolated through SPE cartridges,^{9,23} which is considerably time-consuming. To overcome these limitations, online solid-phase extraction and ultra high-performance liquid chromatography coupled to tandem mass spectrometry (SPE-UHPLC-MS/MS) is a promissory technique, since the system is automatized and small sample volumes (about 100 to 1000 µL) are

required. Recently, several authors^{13,24-28} reported the use of this technique for the determination of antimicrobial and antiparasitic drugs in environmental matrices.

In this work, an SPE-UHPLC-MS/MS method for the determination of fluoroquinolone antimicrobials was developed and validated for wastewaters samples. The method was applied for monitoring these antimicrobials over a period in hospital wastewater and effluents of an urban WWTP. In addition, the correlation between the concentration of antimicrobials in wastewater and its residual antimicrobial activity were evaluated and discussed. The samples containing fluoroquinolones were subjected to an ozonation process to degrade these target compounds and to investigate the capability of the said process to remove the residual antimicrobial activity.

Experimental

Chemicals and reagents

All solvents used were HPLC grade. The other chemicals were of analytical grade or purer. Fluoroquinolones analytical standards: ciprofloxacin (99% purity), norfloxacin (99% purity), ofloxacin (99% purity), gatifloxacin (98% purity), lomefloxacin (98% purity), marbofloxacin (98% purity) and the surrogate ciprofloxacin-*d*₈ (99% purity), were purchased from Sigma-Aldrich (Saint Louis, USA). Standard fluoroquinolones stock solutions were prepared in the concentration of 1000 µg mL⁻¹ in methanol:2% acetic acid, v/v and stored under refrigeration at 4 °C. Ciprofloxacin-*d*₈ was prepared at a concentration of 100 µg mL⁻¹ in ultra-pure water (Milli-Q purification system; Millipore, USA). Working standard solutions were prepared daily by dilution of the stock solutions in ultra-pure water. Potassium iodide and sodium thiosulfate were purchased from Ecibra (São Paulo, Brazil). Mueller-Hinton broth and Mueller-Hinton agar were supplied by Himidia (Mumbai, India).

Hospital wastewater and wastewater treatment plant samples

The Hospital of the University of Campinas (Hospital de Clínicas) serves an estimated population of 10,000 people *per* day (including 409 beds with 85% of occupancy), and generate, in average, an effluent volume of 1.8 L s⁻¹. At this location, no treatment of the hospital wastewater is performed, and the raw effluent is directly discharged in the wastewater sewage system of the Campinas City. The WWTP treats approximately 80 L s⁻¹ using an upflow anaerobic sludge blanket (UASB) followed

by a biological trickling filter and clarifier tank.

The effluent sampling campaign was carried out from September 2016 to March 2017 at two different sampling sites selected to evaluate the occurrence and variations in antimicrobial concentrations: (A) hospital raw sewage (HRS) from a drain connected to the hospital sewer network of the University of Campinas, and (B) treated wastewater (TW) from a wastewater treatment plant located in Campinas, Brazil. A pump collected the effluent samples into a 200 L tank. After homogenization, an aliquot of 1 L of wastewater was collected. This procedure took place in different days, always at 7 a.m. After each sampling, the wastewater was characterized as follows: pH, conductivity, ammoniacal nitrogen, total Kjeldahl nitrogen (TKN), total phosphorus (P), total suspended solids (TSS), turbidity, chemical oxygen demand (COD), and dissolved organic carbon (DOC), according to the guidelines established in the Standard Methods for the Examination of Water and Wastewater.²⁹ The samples were filtered through a 0.45 µm membrane for the analysis of dissolved organic carbon. The parameters for the physicochemical analysis are listed in Table 1. All samples were collected and kept under refrigeration until analysis. The samples were processed to be analyzed on the same day of the collection.

Online SPE-UHPLC-MS/MS system

The analytes of interest were detected, identified and quantified using an online SPE system coupled to an Acquity UPLC IClass Waters and a Xevo TQD Zspray mass spectrometer (Waters), fitted with an electrospray ionization interface operating in positive mode (ESI⁺). The online SPE system was thoroughly described in a previous work.²⁷ Oasis HLB and XBridge C8 SPE columns were assessed for the online solid phase extraction carried out at 30 °C. The filtered samples were loaded using the quaternary solvent manager (QSM) pump with 95:5 v/v of 0.1% formic acid:methanol at a flow rate of 1 mL min⁻¹. At 0.50 min the valve position was inverted and the retained analytes on the SPE column were eluted, in backflush mode, directly to the analytical column (Acquity UPLC BEH C18 analytical column, 2.1 × 50 mm, 1.7 µm, Waters) using a mixture of 85:15 v/v aqueous 0.1% formic acid:acetonitrile, at a flow rate of 0.500 mL min⁻¹, propelled by the binary solvent manager (BSM) pump (Table 2). Finally, the SPE sorbent was washed and conditioned using a mixture of water:methanol with 0.1% formic acid under the gradient described in Table 2, between 2.16 and 4.00 min, using the QSM. The auto-sampler temperature was maintained at 15 °C.

Table 1. Physicochemical characterization of the collected samples

Parameter	Unit	Hospital raw sewage (n = 12)			Treated wastewater (n = 7)		
		Min	Max	Median	Min	Max	Median
pH	-	6.3	8.6	7.8	6.16	7.7	7.0
Chemical oxygen demand (COD)	mg O ₂ L ⁻¹	133	897	642	11	77	17
Total Kjeldahl nitrogen (TKN-N)	mg N L ⁻¹	87.6	125.8	98.3	25.6	34.6	29.0
Ammoniacal nitrogen (NH ₃ -N)	mg NH ₃ L ⁻¹	31.2	68.5	40.0	3.5	14.0	4.0
Nitrate (NO ₃ -N)	mg NO ₃ L ⁻¹	0.62	3.2	1.0	0.5	0.98	0.6
Dissolved organic carbon (DOC)	mg L ⁻¹	89.3	214.9	124.0	3.45	11.0	4.0
Phosphorus (PO ₄ ³⁻)	mg P L ⁻¹	2.7	10.4	7.0	4.7	11.0	4.0
Conductivity	µS cm ⁻¹	975	1800	1083	389	874	487
Turbidity	NTU	75	304	123	3.5	15.6	6.0
Total suspended solids	mg L ⁻¹	15.4	485.0	218.5	2.5	48	3.5

Table 2. Conditions for the quaternary and binary solvent manager pumps

time / min	Quaternary solvent manager		time / min	Binary solvent manager	
	Water:methanol (v/v) with 0.1% formic acid	Flow rate / (mL min ⁻¹)		Water:methanol (v/v) with 0.1% formic acid	Flow rate / (mL min ⁻¹)
0-2.15	95:5				
2.16-3.15	95:5-25:75				
3.16-3.99	25:75-95:5	1.0	0-4	85:15	0.5
4.00	95:5				

The mass spectrometer conditions were as follows: 3.5 kV capillary voltage, desolvation gas temperature of 400 °C, source temperature of 150 °C, desolvation gas flow rate of 900 L h⁻¹ and cone gas flow of 100 L h⁻¹. The analytes were identified and determined using individual transitions from both quantification and confirmation ions in the selected reaction monitoring (SRM) mode. The optimum conditions of the collision energy, cone voltage and selected ions for each analyte are shown in Table 3. The chemical structures and physicochemical properties of the assessed fluoroquinolones are shown in Table S1 (Supplementary Information (SI) section).

Table 3. Quantification and confirmation transitions monitored in the SRM method for all the analytes and surrogate, with the respective cone voltages and collision energies

Antimicrobial	SRM transition	Cone voltage / V	Collision energy / eV
Norfloxacin	320.2 > 233.1 ^q	20	22
	320.2 > 276.1 ^c	25	22
Ciprofloxacin	332.1 > 314.1 ^q	20	22
	332.1 > 288.1 ^c	20	18
Ciprofloxacin- <i>d</i> ₈	340.1 > 322.1 ^q	20	22
	340.1 > 296.1 ^c	20	18
Lomefloxacin	352.1 > 265.1 ^q	39	16
	352.1 > 308.1 ^c	39	26
Ofloxacin	362.1 > 261.1 ^q	20	30
	362.1 > 318.1 ^c	20	20
Gatifloxacin	376 > 261 ^q	48	30
	376.1 > 332.1 ^c	42	19
Marbofloxacin	363.1 > 72 ^q	35	20
	363.1 > 345.8 ^c	40	19

^q: Quantification; ^c: confirmation.

Method validation

The method was in-house validated considering the following parameters: linear range, linearity, matrix effect, intra-day precision and limit of quantification (LOQ). The linear range was established in ultra-pure water fortified with each fluoroquinolone in nine concentration levels: 0.25; 0.50; 1.15; 5.9; 11.5; 17.5; 23.1; 35.0; 46.0 ng mL⁻¹. Ciprofloxacin-*d*₈ was added at a concentration of 11.5 ng mL⁻¹ as an internal standard (surrogate). Triplicate samples were analyzed for each point on the calibration curve. The calibration curves were obtained by ordinary least squares after the homogeneity of variances was confirmed by the Levene's test. Residues were randomly distributed, with no obvious patterns and no outliers (standardized residual test). The matrix effect was evaluated comparing the angular coefficients of the

curves obtained in the wastewater samples fortified with the veterinary fluoroquinolone lomefloxacin (not previously identified in the fortified sample) and the calibration curves in ultrapure water. The intra-day precision was determined using a wastewater sample fortified with ciprofloxacin-*d*₈ at concentration levels of 5.9 and 17.4 ng mL⁻¹ and it was expressed as the relative standard deviation (RSD) of the area response for each injection cycle. The analyses were made in quintuplicate (n = 5) using the same method and equipment by the same analyst. The limits of quantification were determined by analyzing the filtered (0.22 μm membrane filters) wastewater samples fortified with ciprofloxacin-*d*₈ in decreasing concentrations, until a signal-to-noise ratio of 10 was achieved.

The accuracy of the method was established through a recovery test. For this purpose, HRS samples were added of ciprofloxacin, ofloxacin, and norfloxacin at two concentration levels (5.9 and 17.4 ng mL⁻¹) and analyzed by the developed method.

Sample analysis: occurrence assay

A volume of 9.6 mL of the wastewater samples (i.e., HRS and TW) was transferred to a 50 mL Falcon tube containing 400 μL of the surrogate (i.e., ciprofloxacin-*d*₈) at a concentration of 300 ng mL⁻¹. The mixture was homogenized, and the final solution was filtered (0.22 μm) and injected directly into the online SPE-UHPLC-MS/MS system. Each sample was analyzed in triplicate.

Residual antimicrobial activity

Antimicrobial activity assays were performed in consonance with the method described by Caianelo *et al.*,¹⁷ a detailed method scheme is provided in the Supplementary Information (Figure S1, SI section). Gram-positive bacteria *Bacillus subtilis* (ATCC 168) and the Gram-negative bacteria *Escherichia coli* (K12, ATCC 23716) were used as microorganism test. SpectraMax microplate reader (Molecular Devices), with 620 nm lenses filter, was used to measure the absorbance of each sample well. Dose-response curves for ciprofloxacin solution (1 mg L⁻¹) were used as a control parameter. The samples were evaluated in duplicate.

Ozonation assays

The experimental setup was described in a previous work²¹ and consisted of an ozone gas generator (O3R-model generator, Philozon) and two cylindrical glass reactors (V = 2.5 L, h = 50 cm and d_{inner} = 8 cm), one of them

containing a potassium iodide solution to consume the residual ozone (Figure S2, SI section). The ozone feed rate was kept at $10 \text{ mg O}_3 \text{ L}^{-1} \text{ min}^{-1}$ using atmospheric air as the feed gas at a flow rate of 4 mL min^{-1} . The temperature was fixed at $25 \pm 1 \text{ }^\circ\text{C}$ during all experiments. A fine-pore diffuser (porosity $16\text{--}40 \mu\text{m}$) was used for better distribution of the inflow gas inside the reactors. The unconsumed ozone flew out of the reactor and was bubbled into the second reactor containing 500 mL of potassium iodide solution (2% , m/v). The potassium iodide titration method was used to determine the inflow and outflow ozone dosages.²⁹ Ozonated samples collected between 0 and 10 min were promptly flushed with nitrogen for 5 min to remove any residual ozone.

Results and Discussion

Based on reported studies^{7,8,11} and previous analyses, ciprofloxacin, ofloxacin, and norfloxacin are the most frequently detected fluoroquinolones for human use in wastewater. Moreover, ciprofloxacin is the fourth most consumed antimicrobial drug in the Hospital of the University of Campinas. Therefore, fluoroquinolones were prioritized to be assessed in the HRS and TW samples.

Optimization of the online SPE

To enhance detectability and minimize the matrix effect in the determination of residues of fluoroquinolones in wastewater, the following parameters were evaluated: the sorbent phase, the solvent for sample loading and elution, the volume of solvent for the washing step, and the injection volume.

The target compounds were strongly retained by both tested sorbent phases (Figure S3, SI section). Therefore, the Oasis HLB column ($2.1 \times 30 \text{ mm} \times 10 \mu\text{m}$; at $30 \text{ }^\circ\text{C}$) was selected based on its common use in offline SPE,^{13,26,30,31} due to the strong interactions between fluoroquinolones and the lipophilic-hydrophilic balanced polymer Oasis HLB sorbent.

The chromatographic strength of the sample loading solvent should be weak enough to allow preconcentration of the analytes on the sorbent without losses and, at the same time, should be strong enough to be able to remove some concomitants from the sample, which might otherwise enhance the matrix effect. Different proportions of water:methanol, with 0.1% formic acid were assessed: $100:0$; $95:5$; $90:10$ and $80:20$ v/v. The results were depicted in Figure S4 (SI section). A loading solvent composed of $95:5$ (v/v) water:methanol, with 0.1% formic acid was selected for the subsequent studies, because greater

proportions of methanol led to fluoroquinolones losses, diminishing the analyte response.

The concentration factor in the online SPE increased with the increase of the injection volume, enhancing detectability. The following volumes were evaluated: 50 , 100 , 150 , 200 and $250 \mu\text{L}$ (Figure S5, SI section). No significant recovery losses were observed, indicating that when the maximum sample injection volume in a single injection was used (i.e., $250 \mu\text{L}$), the compounds were adequately retained by the SPE sorbents. Considering the maximum injection volume of $1 \mu\text{L}$ in the chromatographic system without the online SPE, the volume injection of $250 \mu\text{L}$ represented a 250-fold increase in the pre-concentration factor.

The evaluation of the sample loading time is essential to allow analytes pre-concentration on the SPE sorbent and to avoid analyte losses during this stage. First, the SPE column was directly connected to the detector. Then, $250 \mu\text{L}$ of the sample was injected with different loading times. Analyte losses were observed with loading times higher than 0.5 min , especially for the more polar compounds (Figure S6, SI section).

Briefly, the optimized SPE conditions to the UHPLC-MS/MS system were as follows: HLB Oasis SPE sorbent column; loading solvent water:methanol $95:5$ (v/v), with 0.1% of formic acid at a flow rate of 1.00 mL min^{-1} ; sample volume of $250 \mu\text{L}$; elution using backflush mode for 1.65 min with $85:15$ (v/v) water:acetonitrile, with 0.1% of formic acid at a flow rate of 0.50 mL min^{-1} . The total run time for a single analysis was 5.0 min (0.50 min to retain the analytes from the sample onto the SPE sorbent, 1.65 min to elute them from the SPE column, separate them on the analytical column and quantify them by MS/MS, and 2.85 min to wash, condition and equilibrate the online SPE system for the next injection cycle). A characteristic chromatogram is shown in Figure S7 (SI section). Using this method, more than 500 analyses cycles were performed without signal loss or SPE column clogging.

Due to the presence of organic matter, suspended solids and colloids, wastewater samples must be filtered prior to analysis by SPE-UHPLC-MS/MS. However, it is possible that some fluoroquinolone molecules are sorbed by the many particles present on these samples since their sorption on charged soil particles is already reported.³² Moreover, the colloids commonly present in wastewater are known to bind to emerging organic contaminants, including antimicrobials, affecting their fate in the environment and contributing to the underestimation of their concentration in environmental samples.^{33,34} Hence, an internal standard must be used to minimize the matrix effect and correct the losses during the sample preparation steps and the analysis.

The isotope-labeled internal standard needs to be added to the samples before any sample preparation step, including the filtration.

Method validation

The method was in-house validated and showed linearity (r) higher than 0.98 for norfloxacin, marbofloxacin, and lomefloxacin, while for ciprofloxacin, ofloxacin, and gatifloxacin the coefficients were 0.99, within the concentration range of 0.25–46.0 ng mL⁻¹. The analytical curves were obtained using water samples fortified with the analytes of interest and the SPE-UHPLC-MS/MS system for the analysis. Ciprofloxacin-*d*₈ was used as the surrogate.

The parameters presented in Table 1 show that each wastewater sample collected has a different matrix composition. Thus, the matrix effect was investigated in each sample to avoid inaccurate quantification. As lomefloxacin and marbofloxacin are veterinary drugs and were not present in the hospital wastewater, these compounds were also used to estimate the matrix effect. For this purpose, ciprofloxacin-*d*₈ was used as the surrogate. The marbofloxacin and lomefloxacin signals were suppressed in the hospital wastewater samples (Figure S8, SI section) in comparison to the signals obtained for aqueous solutions of the same concentration prepared in ultrapure water. The matrix effect for marbofloxacin ranged from –30 to –63%, while the matrix effect for lomefloxacin varied between –19 and +1%. Nevertheless, these values are an estimate for the matrix effect of the analytes of interest and were used only to adjust the sample preparation step. The LOQ of the method was determined by filtered wastewater samples fortified with ciprofloxacin-*d*₈ in decreasing concentrations until signal-to-noise ratios of 10 were achieved. The LOQ values for the fluoroquinolones were stated as 0.50 ng mL⁻¹ for the HRS and 0.25 ng mL⁻¹ for the TW samples. These values are appropriate to quantify fluoroquinolones in the concentration range in which they commonly occur in wastewater effluents.^{7,8,11} The intra-

day precision ($n = 5$) was evaluated in both wastewater matrices. The relative standard deviations (in percentage) of the response areas for filtered wastewater samples fortified with the ciprofloxacin-*d*₈ in two different concentrations levels, 5.9 and 17.4 ng mL⁻¹, were, respectively, 3.5 and 10.0% for HRS and 3.2 and 3.3% for TW. The accuracy was evaluated in three concentration levels (5.9, 11.5 and 23.1 µg L⁻¹) for marbofloxacin and lomefloxacin and the values varied between 90 and 114%.

Occurrence of fluoroquinolone in hospital wastewater

Fluoroquinolones were monitored in both HRS and TW samples for six months, between September 2016 and March 2017. Figure 1 shows the average occurrence data for the fluoroquinolones identified and quantified in the analyzed samples. As expected, the HRS samples presented higher concentration of fluoroquinolones when compared to the effluents collected from the WWTP. Using the online SPE-UHPLC-MS/MS method previously described, three fluoroquinolones were detected in the HRS samples (norfloxacin, ciprofloxacin, and ofloxacin). Ciprofloxacin (1.3–33.9 ng mL⁻¹) and ofloxacin (0.9–27.1 ng mL⁻¹) were identified in all analyzed samples, while norfloxacin was found in lower concentrations (0.8–4.4 ng mL⁻¹) and frequency (17%). In addition, the twelve days stability test did not show a degradation of the fluoroquinolones higher than 6.3% in the HRS samples. This confirms the high stability of fluoroquinolones even in complex matrices.

Ciprofloxacin (0.5–5.6 ng mL⁻¹) was the only fluoroquinolone identified in TW samples, with a lower concentration and frequency (53%) when compared to the HRS samples. This finding is in accordance with the occurrence data reported in the literature:³⁵ ciprofloxacin is one of the most common fluoroquinolone found in the aqueous environment. Since the conventional biological treatment system was not able to completely remove the fluoroquinolones from wastewater, traces of these

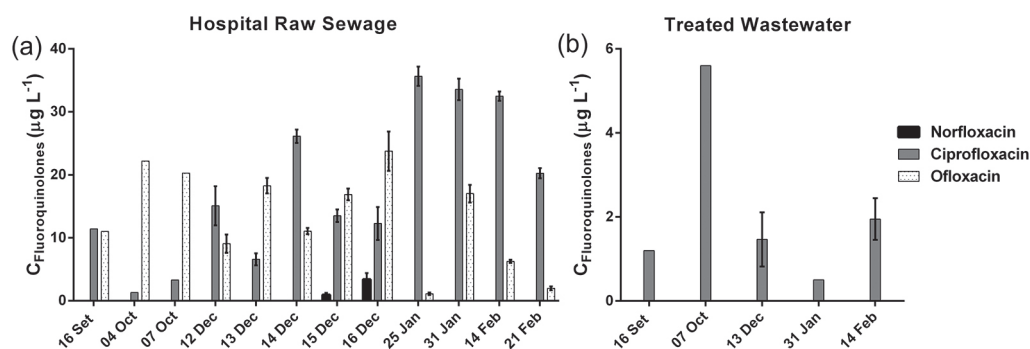


Figure 1. Occurrence of the fluoroquinolones: (a) hospital raw sewage samples and (b) treated wastewater samples.

antimicrobials were still present in the TW samples, indicating that these substances are recalcitrant to conventional processes in the WWTP. Polishing treatments, such as ozonation, should be considered as an alternative solution to remove these compounds or to transform these recalcitrant pollutants into more biodegradable ones. In addition, no correlation was observed between the characterization parameters reported in Table 1 and the concentration of the fluoroquinolone antimicrobials in the samples.

Monitoring antimicrobial activity

Effluent samples are complex mixtures of substances that may concur to increase the antimicrobial activity of the solution. Because of that, it is difficult to assign their antimicrobial activity to a specific compound of the effluent. Nevertheless, it is possible to relate the dose-response shift curves of the effluent samples to a ciprofloxacin solution of known concentration. The correlation can be determined as the potency equivalent (PEQ value = $EC_{50, \text{ciprofloxacin}} / EC_{50, \text{sample}}$). The EC_{50} represents the dilution factor applied in the ciprofloxacin solution ($C_{\text{ciprofloxacin}} = 1 \text{ mg L}^{-1}$) or effluent sample at which 50% growth inhibition was observed from the dose-response curves of each sample.^{17,36}

The dose-response curve for ciprofloxacin was obtained for the concentration range between 1 mg L^{-1} and 0.13 ng mL^{-1} , and the EC_{50} for ciprofloxacin was approximately 8 ng mL^{-1} for *E. coli* (Gram-negative) and 4 ng mL^{-1} for *B. subtilis* (Gram-positive). The Gram-positive bacteria have a thick layer of peptidoglycan and are less

resistant to antimicrobials than the Gram-negative bacteria, which have a more impenetrable cell wall.

The primary goal in evaluating the antimicrobial activity of the wastewater samples was to show that the antimicrobial activity of the sampling sites varies in a short period. It also provides an overview of how this effluent could impact the environment if discharged without proper treatment. The monitoring of the antimicrobial activity over five days demonstrated that the HRS samples presented higher antimicrobial activity than a corresponding 1 mg L^{-1} ciprofloxacin solution, as shown in the dose-response curves depicted in Figure 2. The PEQ demonstrated that these samples possessed an antimicrobial activity between 2.2 and 8.5 times higher than the ciprofloxacin solution for Gram-negative bacteria, while for Gram-positive bacteria the PEQ varied between 0.3 and 1.6. This means that complex wastewater matrices present a high concentration of antimicrobial agents, which include other pharmaceutical drugs besides the fluoroquinolones. Furthermore, the presence of antimicrobial activity higher than a 1 mg L^{-1} ciprofloxacin solution is critical for the WWTP systems: Halling-Sørensen *et al.*¹⁶ concluded, in their studies about risk assessments of antimicrobials in the aquatic environment, that effluents with antimicrobial activities higher or equal to a 0.31 mg L^{-1} ciprofloxacin solution can impact the WWTP system. In addition, it is important to report that the TW samples presented a PEQ lower than 0.04 for both tested microorganisms.

The use of serial dilution assays allows the estimation of EC_{50} with high accuracy, even if a variance in the cell growth occurs due to the complexity of the sample matrices. The results presented as dose-shift curves provide

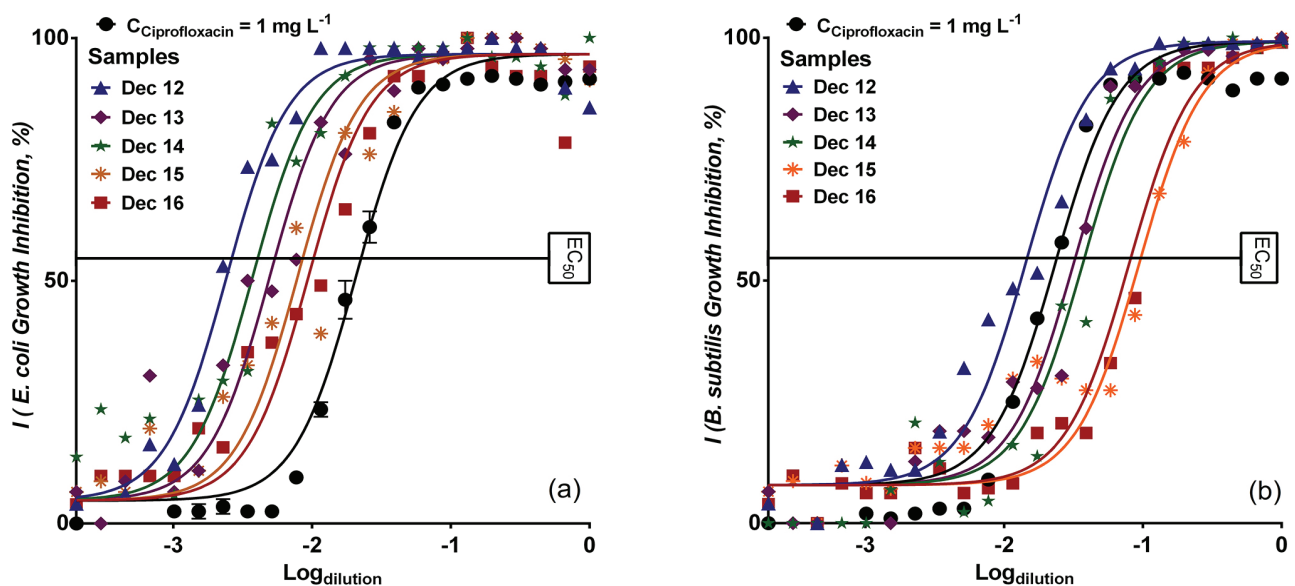


Figure 2. Antimicrobial activity monitoring of the hospital raw sewage (HRS) samples for (a) *E. coli* and (b) *B. subtilis*.

information about the minimum inhibitory concentration.

The antimicrobial residues and resistance genes may be more abundant in hospital wastewaters than in municipal wastewaters because the hospital wastewater is diluted once it is discharged in the municipal sewage system, which minimizes its impact in the WWTP system.^{37,38}

The antimicrobial usage varies with the patient population, the different healthcare treatments available and the season of the year. Nevertheless, cephalosporins, fluoroquinolones, and penicillin derivatives are the most prescribed antimicrobial drugs in hospitals.⁵ Among the antimicrobials excreted into hospital waste streams, sulfonamides and fluoroquinolones are the most persistent, while beta-lactam derivatives are rapidly hydrolyzed and do not appear to persist in the environment.³⁹ Typical concentrations of pharmaceuticals in wastewater samples (hospital, livestock, and pharmaceutical manufacturer facilities) range from 1.5 to 44 ng mL⁻¹.⁴⁰ It is important to highlight that, once excreted, the antimicrobial residues and their metabolites can both still be active, increasing the antimicrobial activity of the effluent. This mixture of compounds may cause an additive or synergic effect in the antimicrobial activity of the sample. Due to that, the presented antimicrobial activity assay is an accurate method to monitor the antimicrobial activity of effluents.

Degradation of the antimicrobials in the wastewater samples

The operational conditions were mostly based on those applied in a previous work.²⁷ For this reason, no attempt was made to achieve a strict optimization of the experimental conditions required for the process. The HRS and TW samples (respectively collected on January 25th, 2017 and February 14th, 2017), in which fluoroquinolones were quantified, were subjected to ozonation as described in the Experimental section. The effluent characterization showed that the pH of the HRS and TW samples was around 7, indicating that the degradation by ozonation occurred by both molecular ozone and hydroxyl radicals. A previous work⁴¹ showed that reactions at pH 7 or higher are more efficient than reactions at a pH lower than 7, due to ozone decomposition in hydroxyl radicals. At this pH value, the fluoroquinolone molecules are mainly in their zwitterionic form.

Ozone doses of 10 mg L⁻¹ min⁻¹ applied for 5 min resulted in the degradation of 36 and 41% of the initial concentration determined in the HRS sample for ciprofloxacin and ofloxacin, respectively. When increasing the reaction time to 10 min, the concentration of ciprofloxacin and ofloxacin dropped 66 and 84%, respectively. Because of the lower concentration of pharmaceutical drugs in

the TW sample, lower ozone doses were required: after 5 min of ozonation, no fluoroquinolone was detected in the effluent sample. Vasconcelos *et al.*²⁰ also observed the degradation of ciprofloxacin in hospital sewage samples and concluded that ozone-based processes are good prospects for degrading fluoroquinolones, even in a complex effluent. Moreover, De Witte *et al.*¹⁹ observed that, due to the sorption of ciprofloxacin on suspended solids at pH 7, the reaction time applied in deionized water needed to be doubled to achieve the same 95% degradation of ciprofloxacin in wastewater.

The HRS is eventually discharged in the affluent of the WWTP monitored in this work. The high concentrations of fluoroquinolones in the HRS may impact the bacterial colonies of the activated sludge used for the wastewater treatment, reducing its efficiency. In cases like this, previous ozonation may increase the capability of the WWTP system to degrade these recalcitrant compounds. However, ozonation processes have been preferably applied in WWTPs after the biological treatments, due to the high organic load of the effluents.⁴²

Traces of antimicrobials were still present in the samples subjected to ozonation, meaning that these molecules may still reach the aqueous environment, promoting the development of antimicrobial resistant bacteria.^{38,43} Therefore, the antimicrobial activity of the samples subjected to ozonation was monitored. It was verified that the antimicrobial activity of the samples for Gram-positive and Gram-negative microorganisms decreased during the reaction period, indicating that the antimicrobial agents were degraded. The dose-response curves obtained for the untreated and ozonized samples are illustrated in Figure 3. When compared with the untreated samples, the antimicrobial activity of the HRS samples for Gram-positive and Gram-negative bacteria decreased by 81 and 92%, respectively. No antimicrobial activity was observed in the TW samples subjected to ozonation for 5 min. De Witte *et al.*¹⁹ evaluated the residual antimicrobial activity of ozonated ciprofloxacin solutions using the agar diffusion test. The authors observed that ciprofloxacin ozonation at pH 7 did not generate degradation products with antimicrobial activity, unlike the reactions performed at pH 3 and 10. Dodd *et al.*³⁶ observed a stoichiometric relationship between ciprofloxacin degradation and the reduction of the antimicrobial activity against *E. coli*. They have shown that ozonation at pH 7 did not lead to the formation of degradation products with higher antimicrobial activity than the original compound, which is an advantage compared to the rapid degradation rate promoted at ozonation at pH 3 and 10.

The antimicrobial activity was reduced in the ozonated solutions, which indicates changes in the active sites of the

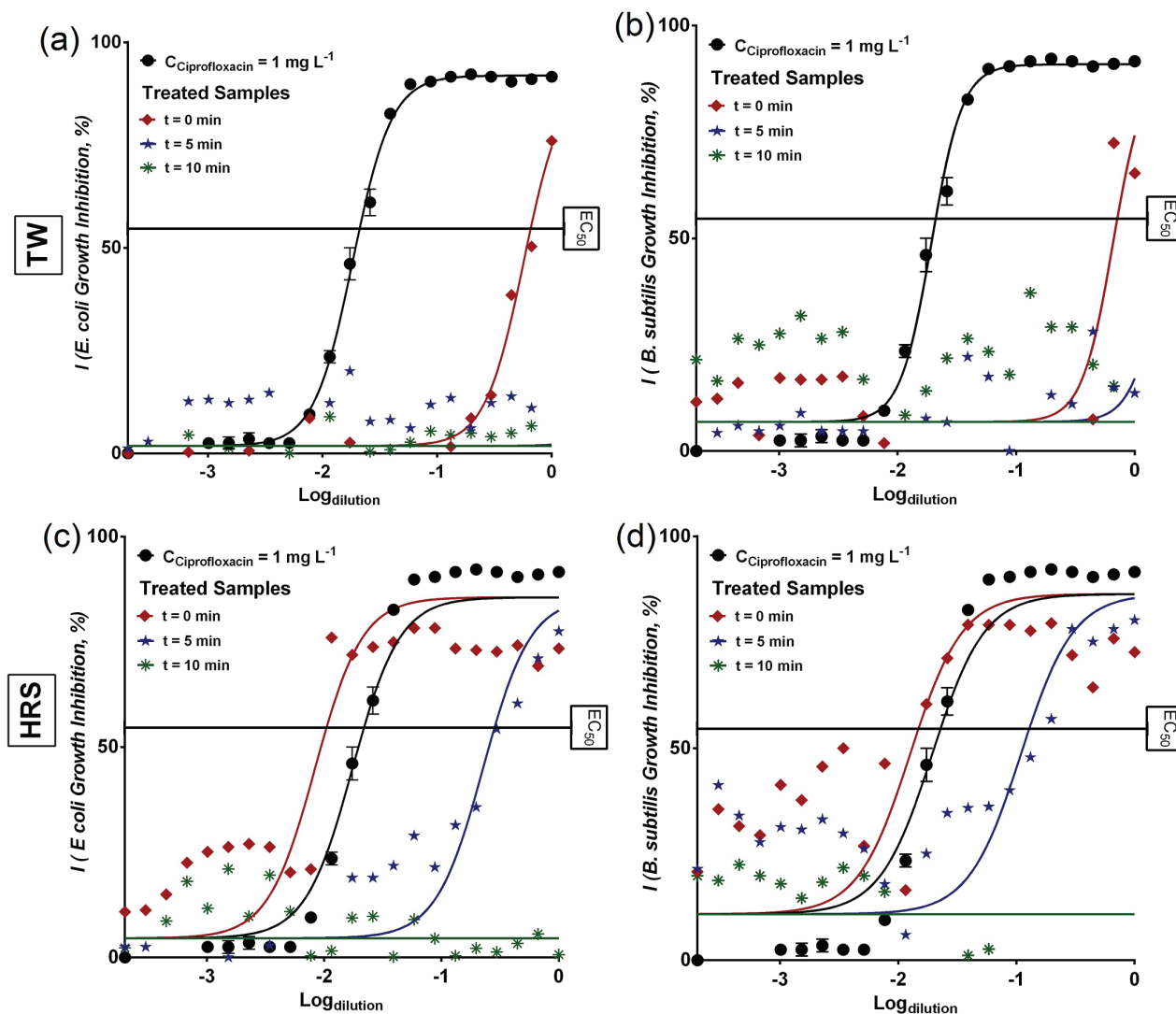


Figure 3. Antimicrobial activity monitoring during effluent ozonation: for *E. coli* (a) treated wastewater (TW) and (c) hospital raw sewage wastewater (HRS); for *B. subtilis* (b) treated wastewater (TW) and (d) hospital raw sewage wastewater (HRS).

antimicrobials. For the fluoroquinolones, the antimicrobial activity is related to the formation of hydrogen bonds between the C=O, COOH, F, and piperazinyl ring acceptor groups and the DNA gyrase of the microorganism.¹⁷ Furthermore, any changes in this acceptor groups result in an intermediate molecule with less antimicrobial activity than the original compound.¹⁷

Conclusions

The online SPE-UHPLC-MS/MS method is appropriate for the monitoring of fluoroquinolones in wastewater samples, with adequate detectability and selectivity. Compared to the traditional SPE, this method is less time-consuming and requires less solvent use and sample handling. This study proves the importance of the isotope-labeled internal standard (surrogate) addition before sample

preparation steps to correct losses during filtration and to eliminate matrix effect.

The occurrence of fluoroquinolone in the HRS was higher than in TW samples. Ciprofloxacin, norfloxacin, and ofloxacin were majorly detected in the HRS samples, while only ciprofloxacin was detectable in the TW samples.

The HRS samples presented an antimicrobial activity for *E. coli* higher than a 1 mg L⁻¹ ciprofloxacin solution, which could impact the efficacy of the upflow anaerobic sludge blanket at the WWTP. The ozonation of the effluents was capable to degrade the fluoroquinolones and remove the antimicrobial activity of the samples.

Supplementary Information

Supplementary information is available free of charge at <http://jbcs.sbq.org.br> as PDF file.

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