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Rapid analysis of multiclass antibiotic residues and some of their metabolites in hospital, urban wastewater and river water by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry

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Abstract

The present work describes the development of a fast and robust analytical method for the determination of 53 antibiotic residues, covering various chemical groups and some of their metabolites, in environmental matrices that are considered important sources of antibiotic pollution, namely hospital and urban wastewaters, as well as in river waters. The method is based on automated off-line solid phase extraction (SPE) followed by ultra-high-performance liquid chromatography coupled to quadrupole linear ion trap tandem mass spectrometry (UHPLC-QqLIT). For unequivocal identification and confirmation, and in order to fulfil EU guidelines, two selected reaction monitoring (SRM) transitions per compound are monitored (the most intense one is used for quantification and the second one for confirmation). Quantification of target antibiotics is performed by the internal standard approach, using one isotopically labelled compound for each chemical group, in order to correct matrix effects. The main advantages of the method are automation and speed-up of sample preparation, by the reduction of extraction volumes for all matrices, the fast separation of a wide spectrum of antibiotics by using ultra-high-performance liquid chromatography, its sensitivity (limits of detection in the low ng/L range) and selectivity (due to the use of tandem mass spectrometry) The inclusion of ß-lactam antibiotics (penicillins and cephalosporins), which are compounds difficult to analyze in multi-residue methods due to their instability in water matrices, and some antibiotics metabolites are other important benefits of the method developed.

As part of the validation procedure, the method developed was applied to the analysis of antibiotics residues in hospital, urban influent and effluent wastewaters as well as in river water samples.

Keywords: *antibiotics, ultra-high-performance liquid chromatography, quadrupole-linear ion trap, multi-residue analytical method, analysis of hospital and urban wastewater, analysis of river water.*

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Introduction

Various types of pharmaceutical residues are being constantly detected in environmental waters (waste, surface and drinking water) at relatively low concentrations. Recent research investigations pointed out that some pharmaceuticals (and within this group antibiotics are included) can exert adverse ecological and human health effects even at the low concentrations found in the environment [1-3]. Furthermore, some PhACs such as antidepressants and antibiotics can be prone to bioconcentration/bioaccumulation in aquatic organisms, particularly in fish [4-7]. Antibiotics are one of the pharmaceutical classes with higher usage and consumption worldwide. They are widely used in both human and veterinary medicine mainly for treating bacterial infections. However, besides their therapeutic usage, they are also used as growth promoters in livestock animal production, as feed additives in fish farming and as coccidiostatic drugs in the poultry industry [8].
The most notorious and significant negative effects attributed to the occurrence of antibiotics is the development of antibiotic resistance [9-12]. While antibiotic-resistant bacteria are found in the natural environment, significantly higher numbers of these bacteria are present in wastewater or even in treated wastewater [8]. Some studies indicated that WWTP can serve as potential reservoirs of antibiotic resistance genes which can be transferred to human-associated bacteria through water and food webs, and thus contribute to antibiotic resistance proliferation [9,13]. Indeed, some studies revealed that WWTP discharges can be an important vehicle of antibiotic-resistance in natural waters [14] and in soils irrigated with wastewater effluents [13]. Antibiotic resistant genes have even been found in drinking waters [10,15]. Furthermore, it was observed that differences in treatment plant designs and their operation may influence the fate of resistant bacteria and resistance genes in wastewater [8,16-18].
In this context, it is important to set up fast, sensitive and reliable analytical methods that enable the determination of a wide range of antibiotic residues in environmental waters, such as hospital, urban wastewater and river waters, at the low concentration levels that they are found. These methods are particularly needed to support the studies dealing with the proliferation of antibiotic resistance in environmental waters polluted by antibiotic residues, in order to draw correlations between their presence and the occurrence of antibiotic resistance genes.
Nowadays, a large number of multi-residue analytical methods are already available for the determination of a wide spectrum of antibiotics in foodstuffs of animal origin [19-21], such as milk [22-24], eggs [25], honey [26] animal muscle [27] and meat [28], among others. Regarding water matrices, the vast majority of existing multi-residue methods generally include sulfonamides, trimethoprim, nitroimidazole antibiotics [29] whereas the methods that also take into account several ß-lactam antibiotics (penicillins and cephalosporins) are more scarce [30,31]. Zhou et al. [32] recently developed a multi-residue method to determine a wide range of antibiotics in several aqueous and solid environmental matrices. However, in this methodology, only one penicillin and cephalosporin antibiotic was taken into account. Furthermore, the vast majority of analytical methods published in the literature, focus their attention on parent antibiotics and rarely include metabolites. The addition of metabolites is of special interest since they can be still bioactive, they can be found at higher concentrations than the original substance and they may have high stability and mobility in the environment.
In all these methodologies, the instrumental technique per excellence is liquid chromatography coupled to tandem mass spectrometry. The cost-effectiveness of analytical procedures is becoming a priority issue in all current experimental designs.

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The goal is to maximize the number of compounds that can be determined in a single simple procedure, by developing multi-residue methods, to increase sample throughput (by reducing chromatographic analysis time), to minimize sample manipulation (by automating sample preparation devices and decreasing sample volumes used) and to increase method efficiency, in terms of selectivity and sensitivity.

With respect to liquid chromatography, the current trend involves the use of ultra high performance liquid chromatography (UHPLC). One of the drivers for the growth of this technique has been the evolution of packing materials used in the columns for the chromatographic separation. The underlying principles of UHPLC are governed by the van Deemter equation. According to this equation, by decreasing the particle sizes of the stationary phase in the analytical column to sub-2-µm, there is a significant gain not only in efficiency but also this efficiency does not diminish at increased flow rates or linear velocities. In this way, a much faster chromatographic separation of a large number of compounds can be achieved, in comparison with conventional HPLC, together with narrower peaks, improved sensitivity and higher resolution. On the other hand, since chromatographic efficiency is proportional to the column length and inversely proportional to the particle size, columns can be shortened by the same factor as the particle size without loss of resolution. Then, by using a flow rate three times higher than in HPLC, due to smaller particles, and shortening the column by one third (again due to smaller particle sizes), the separation can be completed in 1/9 the time invested in HPLC while maintaining resolution.

Concerning tandem mass spectrometry, current trends are focused towards the use of hybrid tandem mass spectrometers, such as quadrupole-time-of-flight (QqTOF) and quadrupole-linear ion trap (QqLIT), due to the advantages offered in comparison with triple quadrupole tandem mass spectrometers. While QqTOF instruments provide high confidence in compound identification due to exact mass measurements, evidence on isotopic patterns and their capability to distinguish isobaric mass interferences, QqLIT mass spectrometers can operate in a wide variety of scan modes, which can be combined in one single experiment through the Information Dependent Acquisition (IDA) function [33]. Furthermore, these instruments offer high sensitivity equal or even higher than triple quadrupole instruments [34] and when operating under SRM mode, large number of transitions can be monitored within one single retention time window [35].

The present work describes the development of an analytical method based on automated off-line solid phase extraction (SPE) followed by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap (QqLIT) tandem mass spectrometry, for the fast and simultaneous determination of 53 multiple-class antibiotics as well as some of their metabolites in hospital and urban wastewater and in river water. Antibiotic classes cover various chemical groups such as fluoroquinolones, quinolones, penicillins, cephalosporines, macrolides, tetracyclines, lincosamides, sulfonamides, dihydrofolate reductase inhibitors and nitroimidazoles. Target antibiotics were selected because of their high human and veterinary usage worldwide as well as their high occurrence and ubiquity in the aquatic environment, according to the information found in the scientific literature.

For unequivocal identification and confirmation two Selected Reaction Monitoring (SRM) transitions were monitored per compound. Quantification was performed by the internal standard approach, by using isotopically labeled antibiotics, which is indispensable to correct matrix effects.

The work presented in this manuscript offer several advantages such as: (i) the minimization and speed-up of sample manipulation by automating the sample

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preparation step and by using low sample volumes (i.e. 25 mL for influent wastewaters, 50mL for hospital and urban effluent wastewater and 100 mL for river waters), (ii) the inclusion of antibiotic metabolites (methods already available focus their attention on parent compounds and rarely include metabolites) and (iii) its high sensitivity when working in the Selected Reaction Monitoring (SRM) mode (limits of detection are in the low ng/L range, even though less sample volumes for sample pre-concentration are used) and (iv) the inclusion, with good analytical performances of penicillins and cephalosporins in the multi-residue method (these compounds are quite unstable in water medium and therefore they present several difficulties in their analysis in water matrices, especially for their analysis in multi-residue methodologies).
Finally, the developed method was successfully applied to the analysis of antibiotics residues in hospital, waste and river waters from one hospital, several WWTPs and river waters in the area of Catalonia (North East of Spain). Results indicate that antibiotics are widespread pollutants in these types of matrices.

2. Materials and methods

2.1. Chemicals and reagents

All antibiotic standards were of high purity grade (>90%). All compounds were purchased from Sigma-Aldrich. According to **table 1,** substances with number 33, 34, 36 and 38 were purchased as hydrochloride salts, compounds with number 19, 21, 22 and 25 were acquired as sodium salts, substances with number 16 and 17 as potassium salt, antibiotics with number 14 and 15 were purchased as trihydrate salts and compounds with number 30 and 35 were acquired as tartrate and hyclate salts, respectively. Isotopically labeled compounds, used as internal standards, were, ofloxacin-d_3, ciprofloxacin-d_8 (as hydrochloride hydrate salt), erythromycin-N,N-dimethyl-^{13}C, ampicillin-^{15}N and ronidazole-d_3, purchased from Sigma-Aldrich, and azithromycin-d_3, sulfamethoxazole-d_4 and lincomycin-d_3, which were purchased from Toronto Research Chemicals (Ontario, Canada). On the other hand, sulfadimethoxine-d_6 and sulfadoxine-d_3, which were used as surrogate standards, were purchased from Sigma-Aldrich.
Individual stock standard, isotopically labeled internal standard and surrogate standard solutions were prepared at a concentration of 1000 mg/L, by dissolving 10 mg of solid reference standard in 10 mL of an appropriate solvent. Thus, the cephalosporins and penicillins cefalexin, cefazolin sodium salt, cefatoxime sodium salt, cefapirin and amoxicillin trihydrate were dissolved in HPLC water whereas cefuroxime sodium salt, ceftiofur, ampicillin trihydrate, penicillin G and penicillin V potassium salts were dissolved in AcN/H_2O (50:50 v/v) whereas oxacillin sodium hydrate was prepared using HPLC/MeOH (50:50 v/v), as described in Kantiani et al. [36]. The rest of compounds were dissolved in methanol. However, the addition of 100 µL NaOH 1M was necessary for the proper dissolution of fluoroquinolone and quinolone antibiotics as described by Ibáñez et al. [30].
After preparation, standards were stored at -20ºC. Special precautions have to be taken into account for tetracyclines, which have to be stored in the dark in order to avoid their exposure to light, since it has been demonstrated that tetracycline antibiotics are liable to photodegradation [37]. In addition, to ensure stability of penicillins and cephalosporins different aliquots were used for each freeze-thaw cycle (each aliquot is used only once when preparing working standard solutions), as recommended in Kantiani et al. [36]. Fresh stock antibiotic solutions were prepared every six months

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while fluoroquinoles and quinolones were prepared every two-three months and penicillins and cephalosporins monthly, due to their limited stability.
Working standard solutions, containing all antibiotics were prepared in methanol/ water (50:50, v/v) and were renewed before each analytical run by mixing appropriate amounts of intermediate standard solutions. Separate mixtures of isotopically labeled internal standards, used for internal standard calibration, and surrogates were prepared in methanol, with the exception of ampicillin-^{15}N, which was diluted in HPLC water/AcN (50:50 v/v). Further dilutions were also prepared in a methanol/water (50:50, v/v) mixture.
The cartridges used for solid phase extraction were Oasis HLB (60 mg, 3 mL) and Oasis MCX (60 mg, 6 mL), both from Waters Corporation (Milford, MA, U.S.A.). Glass fiber filters (1 μm) and nylon membrane filters (0.45 μm) were purchased from Whatman (U.K.). HPLC grade methanol, acetonitrile, water (Lichrosolv) were supplied by Merck (Darmstadt, Germany). Ammonium hydroxide, hydrochloric acid 37% and ethylenediaminetetraacetic acid disodium salt solution (Na_2EDTA) at 0.1 mol/L were from Panreac. Formic acid 98% was from Merck (Darmstadt, Germany). Nitrogen for drying was from Abelló Linde S.A (Spain) and it was of 99.9990% purity. A Milli-Q-Advantage system from Millipore Ibérica S.A. (Spain) was used to obtain HPLC-grade water.

2.2. Water samples collection, sample pre-treatment and analysis

The method was optimized using hospital wastewater, urban influent and effluent wastewater and river water. Specifically, hospital effluent wastewater was taken from Josep Trueta hospital, one of the main hospitals in the area of Girona (Catalonia, Spain), with 400 beds and that gives service to approximately 795363 people. Wastewater samples were collected from Girona's wastewater treatment plant facility, which receives the wastewater coming from Josep Trueta hospital and treats water from an area of 143975 inhabitants, with a design of 206250 population equivalents. Besides hospital wastewater, this treatment plant also receives urban and domestic wastewater. It has a primary and secondary treatment operating with conventional activated sludge. On the other hand, river water for method optimization was taken from river Onyar, which crosses the city of Girona before flowing into the river Ter.
For method validation, grab hospital and urban wastewater as well as river water were used. Amber glass bottles pre-rinsed with ultrapure water were used for sample collection. Hospital wastewaters were filtered through 2.7 µm followed by 1 µm glass fiber filters and after that, they were further filtered through 0.45 µm nylon membrane filters (Whatman, U.K.), whereas waste and river water samples were only filtered through 1 µm glass fiber filters and 0.45 µm nylon membrane filters.
A suitable volume of a Na_2EDTA solution, having a concentration of 0.1 M, was added to the different types of water to achieve a final concentration of 0.1% (g solute/g solution) and sample pH was adjusted to 2.5 with hydrochloric acid. Moreover, water samples were spiked, with an appropriate volume of a standard mixture containing surrogate standards, in order to have a concentration of 200 ng/L in urban influent wastewaters, 100 ng/L in urban and hospital effluent wastewaters and 50 ng/L in river water, respectively.
Water samples were automatically extracted by a GX-271 ASPEC™ system (Gilson, Villiers le Bel, France) using Oasis HLB cartridges (60 mg, 3 mL) for all types of matrices. SPE cartridges were conditioned with 5 mL of methanol followed by 5 mL of HPLC-grade water, acidified at pH 2.5 with hydrochloric acid, at a flow rate of 2 mL/min. 25 mL of urban influent wastewater, 50 mL of urban and hospital effluent

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wastewater and 100 mL of river water were loaded onto the cartridge at a flow rate of 1 mL/min. After sample pre-concentration, cartridges were rinsed with 6 mL of HPLC grade water, at a flow rate of 2 mL/min, and were dried with air for 5 min, to remove excess of water. Finally, analytes were eluted with 6 mL of pure methanol at a flow rate of 1 mL/min. Extracts were evaporated to dryness under a gentle nitrogen stream and reconstituted with 1 mL of methanol/ water (50:50, v/v). Finally, 10 μL of a 1 ng/μL standard mixture containing all isotopically labeled standards were added in the extract before instrumental analysis for internal standard calibration. Labeled standards included in the internal standard mixture were ofloxacin-d_3, ciprofloxacin-d_8, ampicillin-^{15}N, Erythromycin-N,N-dimethyl-^{13}C, azithromycin-d_3, lincomycin-d_3, sulfamethoxazole-d_4 and ronidazole-d_3.

2.3. Solid phase extraction optimization

To optimize the extraction method, the lipophilic/hydrophilic balanced Oasis HLB (60 mg, 3 mL) and the mixed reversed phase/cationic exchange sorbent Oasis MCX (60 mg, 3 mL), both from Waters Corporation (Milford, MA, USA), were compared, operating under different conditions. To evaluate which of these experiments yielded higher recoveries of target antibiotics, preliminary experiments were performed with MilliQ water. In all cases, water samples were spiked with appropriate concentrations of a standard mixture containing all target antibiotics and surrogate standards. After that, an appropriate volume of a Na_2EDTA solution to achieve a final concentration of 0.1% (g solute/g solution) was added to Milli-Q-water. For Oasis MCX cartridges, samples were acidified, prior to the extraction, with hydrochloric acid until pH=2.5, whereas for Oasis HLB two experiments were performed: (i) one with no sample pH adjustment and the other one by adjusting the sample pH at 2.5 also using hydrochloric acid. In the experiments where water samples were acidified, cartridges were conditioned with 5 mL methanol followed by 5 mL HPLC grade water acidified with hydrochloric acid at pH 2.5, while in the experiments carried out without pH adjustment, SPE cartridges were conditioned with 5 mL methanol and 5 mL HPLC grade water. In all cases, 50 mL of Milli-Q-water were loaded onto the cartridges at a flow rate of 1 mL/min, cartridges were washed with 5 mL of HPLC grade water and analytes were afterwards eluted at a flow rate of 1 mL/min, using 6 mL of pure methanol for Oasis HLB, whereas for Oasis MCX, 3 mL of pure methanol followed by 3 mL of 5% of NH_4OH in methanol were used (these two solvents were pooled in one single collection vial).
In all situations, cartridges were dried with air for 5 min, to remove excess of water, then extracts were evaporated to dryness under a gentle nitrogen stream and reconstituted with 1 mL of methanol/ water (50:50, v/v), adding an appropriate concentration of internal standard mixture, as described in the previous section.
Comparing these experiments, Oasis HLB cartridges with sample acidification prior to extraction were the conditions providing higher recoveries for almost all antibiotics classes under study, and therefore, these conditions were the ones selected for further recovery experiments and analysis of water samples.

2.4. Ultra-high-performance-ESI-(QqLIT) MS/MS analysis

Chromatographic separations were carried out with a Waters Acquity Ultra-Performance™ liquid chromatograph system, equipped with two binary pumps system (Milford, MA, USA) using an Acquity HSS T_3 colum (50 mm x 2.1 mm i.d., 1.8 µm particle size) for also from Waters Corporation. The optimized separation conditions were as follows: solvent (A) acetonitrile, solvent (B) HPLC grade water acidified at 0.1% with formic acid at a flow rate of 0.5 mL/min. The gradient elution was: initial

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conditions 5% A; 0–3.0 min, 5–70% A; 3.0-3.5 min, 100% A; 3.5–5.0 min, 100% A; from 5.0 to 5.1 return to initial conditions; 5.1 to 6.0, equilibration of the column. The sample volume injected was 5 µL. The UPLC instrument was coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a turbo Ion Spray source. Compound dependent MS parameters (declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP)) were optimized by direct infusion of individual standard solutions of each compound at concentrations ranging from 20 to 50 µg/L. For quantitative purposes, two MRM transitions were monitored for each antibiotic and a summary of the optimum SRM transitions and conditions is available in **table 2**. All transitions were recorded by using the Scheduled MRM™ algorithm with the purpose to increase sensitivity and to achieve reproducible chromatographic peaks. Target scan time (TST) was set at 0.25 seconds, with a MRM detection window of 20 seconds. Resolution at the first quadrupole (Q1) was set at unit, and at the third quadrupole (Q3), it was set at low and the pause between mass ranges was 5 ms. Settings for source-dependent parameters were determined by Flow Injection Analysis and are as follows: curtain gas (CUR), 30V; nitrogen collision gas (CAD) medium; source temperature (TEM) was 650ºC; ion spray voltage was 5500 V; ion source gases GS1 and GS2 were set 60 and 50V, respectively and entrance potential (EP) was set at 10. All data were acquired and processed using Analyst 1.5.1 software.

Results and discussion

3.1. Solid phase extraction optimization

Figure 1 shows the recoveries of representative antibiotics of each chemical group in Milli-Q water under the different conditions and polymeric phases tested: (i) adding Na_2EDTA prior to sample pre-concentration using Oasis HLB cartridges without sample pH adjustment (sample pH is around 4.5-5), (ii) adding Na_2EDTA and adjusting sample pH at 2.5 before extraction, using also Oasis HLB cartridges, and (iii) adding Na_2EDTA followed by sample acidification at pH 2.5 and extraction with Oasis MCX.

Na_2EDTA was added in all protocols since the addition of a chelating agent, such as EDTA, oxalic or citric acid is recommended in the analysis of antibiotic residues in environmental samples [29]. The addition of the strong chelating agent, such as EDTA in water samples prior to extraction is mostly to complex metals or multivalent cations (residual metal ions) that are soluble in water, on SPE cartridges and glassware [37]. The antibiotics from the groups of tetracyclines, fluoroquinolones and macrolides have a high tendency to complex with those ions, resulting in lower extraction recoveries. The addition of the chelating agent is thus necessary to achieve good extraction efficiencies.

Regarding polymeric cartridges, only Oasis MCX and HLB sorbents were tested since, based on the author's previous experience, they are the ones yielding higher recoveries for a big number of pharmaceuticals [34], including antibiotics, and they are the ones mostly recommended in literature reviews for the analysis of different classes of antibiotics [29,37]. In fact, due to the chemical composition of Oasis HLB, which contain lipophilic divinylbenzene units and the hydrophilic N-vinylpyrrolidone units, allow the efficient extraction of organic contaminants in a wide range of pH (from pH 1 to 14).

On the other hand, the pH of the sample solution plays a significant role in the extraction efficiency of antibiotics. In the majority of multi-residue methods published so far, sample pH is generally adjusted within the range from 2.5 to 4 [29,38] to achieve

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good extraction recoveries for the majority of chemical groups included. The acidification of at least 2 units under pKa values of target analytes in water samples is recommended, in order to obtain their neutral or acidic forms, which may significantly improve their retention onto the SPE polymeric sorbent [38].

For this reason, in this work, the efficiency of Oasis HLB cartridges was tested at pH 2.5 and without sample pH adjustment, while for Oasis Mixed Mode Cation Exchange (MCX) SPE extraction performance was only tested at low sample pH. This is, in fact, the recommended protocol for these cartridges, because at low pH values, basic, acidic and neutral substances can be retained in the mixed mode polymer (basic drugs are positively charged and therefore, they can be strongly bound to the polymer by positive cation exchanger, while neutral and acidic compounds are retained by reversed phase).

As it is depicted in **figure 1**, in general terms, the methodology using Oasis HLB cartridges at low sample pH was the protocol that yielded higher recoveries for the majority of antibiotic classes whereas Oasis MCX was the one that showed worst performances. However, in some cases, differences between protocols were not significant (like for sulfonamides and fluoroquinolones), while in some other situations, Oasis HLB without sample pH adjustment showed better recoveries than the treatment with the same polymeric phase but with sample pH adjustment (especially for lincosamides and macrolides). Macrolide antibiotics contain a basic dimethylamine [-$N(CH_3)_2$] group. Thus, according to their chemical structure, they are basic compounds with pKa values around 8, and probably, better extraction efficiencies would be expected at pH values higher than 2.5 [38].

For quinolones, recoveries achieved with Oasis HLB with and without sample pH adjustment were very similar, but performances decreased when using Oasis MCX cartridges. Quinolones contain a carboxylic group which makes all these compounds acidic, and they have only one pKa in the range between 6.0 and 6.9 [38]. Therefore, in acidic conditions they are in neutral form, having a good retention in Oasis HLB cartridges.

Regarding fluoroquinolones, they have an amino group in the heterocyclic ring (namely piperazinyl), and they have two dissociation constants. The reported values of pKa_1 and pKa_2 are in the 5.5-6.3 and 7.6-8.5 range, respectively and thus, the intermediate form is a zwitterion. At acidic conditions they are in cationic form and it has been observed that cationic, zwitterionic and neutral species of these antibiotics are well retained on the polymeric Oasis HLB column [38]. Furthermore, due to their presence as cationic forms at low pH values would explain the good extraction when using mixed mode polymeric phases (Oasis MCX).

Concerning sulfonamides, they contain one basic amine group ($-NH_2$) and one acidic sulfonamide group ($-SO_2NH-$). They are ampholytes with weakly basic and acidic characteristics, having two pKa values, pKa_1 (2-2.5) and pKa_2 (5-8), respectively [38]. Thus, sulfonamides are positively charged at pH 2 and 5, and negatively charged at alkaline conditions above pH 5, explaining their good retention under all conditions tested.

Major differences were observed for penicillins, chephalosporines and tetracyclines, showing higher recoveries when using Oasis HLB at low sample pH. In fact, penicillins and cephalosporines showed extremely low extraction recoveries when using Oasis MCX cartridges. One explanation could be that they degrade during the elution step, since methanol with ammonia is used, and it has been reported that these substances are prone to degradation under acidic and basic conditions [29]. Indeed, it is recommended to acidify the samples right before extraction, to avoid any analyte losses due to degradation. Even though neutral sample pH is the recommended protocol for the

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analysis of penicillins and cephalosporines, in this case they showed better recoveries when acidifying the sample to pH 2.5 before extraction. Recoveries were even better than without sample pH adjustment, where sample pH is around 5. Good recoveries at sample pH around 2 and 3 are supported by other authors, who also found satisfactory recoveries for these substances under these conditions [30,39,40]. For tetracyclines, extraction at sample pH below their pKa (3.3-9) increases retention on the SPE cartridges [41].
The main objective of this work was to develop an extraction procedure that enables the simultaneous analysis of a wide range of multiple-class antibiotics in one single extraction step. Having that in mind and based on the results obtained, the extraction method based on Oasis HLB cartridges with sample pH adjustment and Na_2EDTA as a cation complexing agent, was selected as the optimum protocol to apply to the multi-residue extraction of antibiotic residues in several water matrices. Recoveries for hospital effluent wastewater, urban influent and effluent wastewaters and river water (mean of three replicates ±RSD) under the optimum conditions (Oasis HLB at low pH values) are given in **table 3** and discussed in the method performance section.

3.2. Ultra-high-performance liquid chromatography separation optimization and QqLIT MS/MS Conditions for quantification and identification of antibiotics

In order to optimize chromatographic separation, different mobile phases and additives were tested. For the aqueous phase, buffered mobile phases consisting of formiate/formic acid at different concentration levels (5 mM and 10 mM at pH=3.2) and HPLC water with 0.1% formic acid were (FA) evaluated, whilst methanol and acetonitrile were tested as organic solvents. Only these mobile phases were tested because they are the ones mainly used in the analysis of multiple-class antibiotics [29,37]. The use of acidic aqueous mobile phases is very common for the analysis of antibiotics, improving their ionization efficiency.
To test which combinations of aqueous and organic mobile phases performed the best, a linear gradient from 5% to 95% of organic solvent in 6min and a flow rate of 0.4 mL/min were used as starting conditions. All these mobile phase combinations were tested using two different UHPLC columns: i) an Acquity HSS T_3 column (50 mm x 2.1 mm i.d., 1.8 μm particle size and ii) an Acquity BEH C_{18} column (50 mm x 2.1 mm i.d., 1.7 μm particle size). These columns were tested because the Acquity HSS T_3 column is recommended and can be a good choice when developing separations for highly polar and medium polar compounds, such as pharmaceuticals, while C_{18} stationary phases are the most common ones in the chromatographic analysis of antibiotics. In fact Acquity HSS T_3 bonding utilizes a trifunctional C_{18} alkyl phase bonded at a ligand density that promotes polar compound retention and aqueous mobile phase compatibility. Furthermore, the proprietary T_3 endcapping process is much more effective than traditional trimethylsilane (TMS) endcapping. This unique combination of bonding and endcapping provides superior polar compound retention and aqueous compatibility while also enhances column performance, lifetime, peak shape and stability. Columns with 50 mm length (50 mm x 2.1 mm) were used because the principal objective was to achieve fast separation, keeping a good resolution.
Between all the combinations mentioned, the use of an Acquity HSS T_3 column, with acetonitrile as organic phase and HPLC grade water containing 0.1% of formic acid as the aqueous phase were the conditions providing better resolution, peak shapes and responses.
Once the best mobile phases and UHPLC column were established, the elution gradient and flow rate were adjusted and further optimized with the aim to improve

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chromatographic resolution and peak shapes (by obtaining narrower chromatographic peaks) and to reduce total analysis time. In UHPLC, with the use of stationary phases containing small particles (typically <2 μm in size), chromatographic separation is performed with higher resolutions, sensitivities and reduced analysis time (chromatographic runs are approximately 3 times shorter than in HPLC). Furthermore, the use of UHPLC may help in reducing matrix effects produced by isobaric co-eluting sample compounds, thanks to the enhanced chromatographic resolving power provided by UHPLC in comparison with HPLC. The efficiency gained by using columns with particle sizes lower than 2 μm does not diminish at increased flow rates or linear velocities. Therefore, chromatographic separations are carried out at higher flow rates than in conventional HPLC, for increased separation speed.

Different flow rates were tested (from 0.4 to 0.8 mL/min) and the optimum one was set to 0.5 mL/min. Finally, different temperatures were tested (30ºC, 40ºC and 50ºC). For the vast majority of pharmaceuticals, peak shapes and chromatographic response improved when 30ºC was used.

It should be remarked that 100% organic content is kept during one minute in the elution gradient to clean the column and to avoid carry over contamination. In **figure 2**, the total ion current (TIC) chromatogram from a standard mixture and real samples containing some of the compounds analyzed are displayed.

Regarding tandem mass spectrometry analysis, $[M+H]^+$ ions were selected as precursor ion. Two SRM transitions between the precursor ion and the two most abundant fragment ions were monitored for each compound, except for the isotopically labeled internal standards, which are not likely to be found in environmental matrices, and therefore, only one transition was monitored. The first transition is used for quantification purposes, whereas the second one is to confirm the identity of the target compounds. Besides the monitoring of the SRM transitions, other identification criteria were used for quantification: (i) the matching of the UHPLC retention time of the compound in the standard with those in the samples, (the retention time in the sample must be within ±2% the retention time of the compound in the standards), and (ii) the comparison between the relative abundances of the two selected analyte SRM transitions in the sample with those in the standards. These relative abundances in the samples must be within ±20% of the two SRM ratios in the analytical standards (see **table 2**).

As mentioned before in section 2.4, all SRM transitions were monitored by using the Scheduled MRM™ algorithm. With this option, all SRM transitions of a certain analyte are monitored only around its expected elution retention time. Thus, automated SRM scheduling decreases the number of concurrent SRM transitions, allowing both the cycle time and the dwell time to be automatically optimized for the highest sensitivity, accuracy and reproducibility. In addition, this algorithm allows the monitoring of many more SRM transitions in a single acquisition run, which is especially important when using fast liquid chromatography, such as UHPLC, without compromising reproducibility and accuracy. Chromatographic peaks in UHPLC are much narrower, and thus, it is difficult to achieve enough points per peak when monitoring a large number of transitions by just using a fixed value for the dwell time for each SRM transition monitored.

3.3. Method performance and matrix effects

The performance of the method was evaluated through the estimation of the linearity, extraction recoveries, sensitivity (by calculating instrumental detection limits, method

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detection and quantification limits), repeatability and reproducibility as well as matrix effects.
Quantification was based on linear regression calibration curves, by the internal standard approach. Regarding method performance in terms of dynamic range, linear response generally covered three orders of magnitude. Calibration curves gave good fits ($r2>0.99$) over the established concentration points ranging from 0.5 or 1 µg/L, to 50 or 100 µg/L, depending on the compounds.
Calibration standards were measured at the beginning and at the end of each sequence, and one calibration standard was measured repeatedly throughout the sequence, after every 20-25 samples, to check for signal stability.
Instrumental limits of detection (IDLs) were estimated from signal to noise ratios (S/N=3) of low concentration calibration standards. IDLs ranged from 0.1 to 5 pg injected. These values indicate the high sensitivity of the mass spectrometer used and its capabilities to detect target antibiotics at the low concentrations found in complex environmental samples.
Recoveries were determined by spiking hospital effluent wastewater, urban influent and effluent wastewater and river water, in triplicate, with two standard mixtures: (i) one containing penicillins and cephalosporins in HPLC grade water and (ii) another one containing sulfonamides, dihydrofolate reductase inhibitors, nitroimidazole antibiotics, quinolones, fluoroquinolones, macrolides, tetracyclines and lincosamides in methanol. The final spiking concentration in hospital effluent wastewater, urban influent and effluent wastewater was 400 ng/L while for river water it was 50 ng/L. These concentrations were selected as representative values since some antibiotics, like ofloxacin and ciprofloxacin can be found at high concentrations (high ng/L-low µg/L) in these matrices [39,42,43]. For river water, the spiking level selected was one order of magnitude lower than the one selected for wastewaters, since an important dilution factor occurs when pharmaceuticals enter surface waters. Moreover, for some compounds high method limits of quantification are achieved (around 50-100 ng/L) in wastewaters and therefore, these spiking levels were considered the most appropriate ones for method validation purposes.
Futhermore, it should be worth mentioning that although some of the target antibiotics (especially some tetracyclines and sulfonamides) are more frequently used in veterinary medicine, they have been also included as part of the method validation for hospital wastewaters.
The spiking mixture containing penicillins and cephalosporines was prepared freshly to ensure their stability. Moreover, this mixture was prepared in HPLC grade water to avoid the degradation that these substances can suffer in methanol [30]. In fact, extracts were eluted, evaporated and reconstituted just before analysis, to avoid the possible degradation of ß-lactam antibiotics in methanol as well as to ensure integrity of the sample extracts. Different proportions of methanol/water were selected to reconstitute sample extracts and the proportion consisting of methanol/water (50/50, v/v) was selected because it provided better chromatographic peak shape and sensitivity for macrolide and tetracycline antibiotics. However for the analysis of only penicillins and cephalosporines it would be more recommendable to reconstitute the extracts with pure HPLC grade water or mobile phase initial conditions.
Relative recoveries were determined by comparing the concentrations obtained after the whole SPE procedure, calculated by internal standard calibration, with the initial spiking levels. Since water samples can contain target antibiotics, blanks (non-spiked samples) were also analyzed and the levels found subtracted from those obtained from spiked samples. Results for each matrix are presented in **table 3**. Generally, in all type

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of waters analyzed, recoveries achieved for all target antibiotics ranged from 50 to over 100% in some cases. However, some substances such as amoxicillin, penicillin G and hydroxyl-metronidazole in all water samples, oxacillin and orbifloxacin in urban wastewater influent and orbifloxacin, cinoxacin, oxolinic acid, penicillin G and V and ceftiofur in hospital effluent wastewaters showed low recovery rates (between 20 and 30%, see **table3**). Low recoveries obtained for some penicillins can be explained by their unstability in water media, attributed to their chemical structure [38]. This is, in fact, one limitation of multi-residue methodologies, where not the best conditions for all target analytes are achieved and therefore, a compromise on the final analytical conditions has to be reached. The overall method precision, calculated as the relative standard deviation (%RSD) was satisfactory (see **table 3**), ranging from 1 to 15% in a general extent, with some compounds showing %RSD until 20% (**see table 3**).

Method detection (MDL) and quantification limits (MQL) were estimated from the signal to noise ratio (S/N=3 for detection limits and S/N=10 for quantification limits) of real samples and recovery replicates (MDLs and MQLs were calculated as the average of those estimated in real samples and in the spiked samples). When antibiotics were not detected in real samples, they were estimated only from the spiked replicates. MDLs calculated for hospital and urban effluent wastewaters ranged from 1 to approximately 30 ng/L, from 3 to 30 ng/L for influent wastewaters and from 0.5 to 15 ng/L for river waters, respectively. Higher MDLs were achieved for some substances, such as norfloxacin (55 ng/L in urban effluent wastewater and 78 ng/L in influent effluent wastewater), oxacillin (40 and 48 ng/L in hospital, urban effluent and influent wastewater, respectively), cefazolin (around 50 ng/L in hospital and urban effluent wastewater) and doxycycline (approximately 70 ng/L in urban influent and effluent wastewaters). Regarding MQLs, they ranged from approximately from 5 to 50 ng/L in both hospital and urban effluent wastewater, and from 10 to 60 ng/L for urban influent wastewater, with some exceptions such as danofloxacin, norfloxacin, oxacillin, cefazolin, doxycycline and tylosin, whose limits of quantification were around (and in some cases exceeded) 100 ng/L (for more details, see **table 4**). For river waters, MQLs were much lower than in wastewaters, roughly ranging from 1 to 50 ng/L.

It is worth mentioning that with this method, low MDLs and MQLs were achieved for the vast majority of antibiotics, even though low sample volumes are used for a sample pre-concentration. By reducing the sample volume of complex samples, such as hospital and urban wastewaters, matrix effects may be decreased. In fact, the MDLs and MQLs calculated in this study are comparable to those obtained by other analytical methods where more volume has to be loaded for the SPE [30,31,41,44] and even very close to those obtained by using an on-line SPE instrumental system [45].

Run-to-run variations (repeatability) were assessed from 5 consecutive injections of a 10 μg/L calibration curve standard, while day-to-day (reproducibility) variations were evaluated by measuring a standard over 3 consecutive days. The RSD values achieved for intra-day analysis were below 7%, with the exception for cefuroxime and doxycycline, showing RSD of 16 and 12%, respectively. Concerning inter-day analysis, RSD ranged approximately from 10 to 25%, with some exceptions (see **table 3**).

Regarding matrix effects, they were only evaluated for complex matrices, namely urban (influent and effluent) and hospital wastewater To evaluate to what extent target compounds and isotopically labeled substances were sensitive to signal suppression or enhancement, matrix effects were evaluated using equation (1). According to this equation, the peak areas of urban and hospital wastewater extracts, all spiked with target antibiotics (area matrix), are first subtracted by the peak areas corresponding to the native analytes present in the sample (area blank). The values obtained are then

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compared with the peak areas in the solvent (methanol-water 50:50, v/v) spiked with target antibiotics at the same concentration (area solvent). The spiked concentration was 10 μg/L for all the matrices considered.

(1) $$Signal\,suppression(\%) = 100 - \left(\frac{(areamatrix - areablank)x100}{areasolvent}\right)$$

Almost all compounds were subjected to ion suppression, with the exception of fluoroquinolone and quinolone antibiotics, which showed signal enhancement in all matrices. Furthermore, tetracycline antibiotics chlortetracycline, doxycycline and oxytetracycline also showed signal enhancement in urban influent and effluent wastewaters, tetracycline only in urban effluents and chlortetracycline in hospital effluent wastewaters. While antibiotics were subjected to significant ion suppression (even severe, up to 80 and 90% for some substances) in hospital and influent wastewaters, in effluent wastewaters the degree of ion suppression was significantly reduced (suppression was between 20 and 50%, being 50% the maximum value). For this reason, figures showing the percentage of ion suppression only in hospital wastewater and urban influent wastewaters are included. Therefore, in **figures 3A and 3B**, representative antibiotics of each chemical group subjected to ion suppression are depicted, indicating the percentage of signal reduction in (A) hospital effluent wastewater and (B) urban influent wastewaters. Values presented in the figures correspond just to the wastewater samples used for method validation and this parameter should be evaluated with each set of samples analyzed. These results show that it is of high significance to use a plausible approach to correct these effects, in order to avoid inaccurate quantification (by overestimation or underestimation) when analyzing real samples. In this study, internal standard calibration including a wide range of isotopically labeled antibiotics was used as the strategy to correct matrix effects. Since no isotopically labeled standards were available for each antibiotic, one internal standard was selected for each chemical group. The criterion to select internal standards was based on their similarity with the compounds under study in terms of the mass spectrometric response, the chemical structure, the chromatographic retention time and the degree and type of matrix effects (it was checked that internal standards and analytes were affected by a similar degree of ion suppression or enhancement).

3.4. Application of the method to the analysis of hospital and urban wastewaters and river waters

To demonstrate the applicability of the analytical method developed, hospital wastewater, urban influent and effluent wastewater from three WWTPs, and five river waters, which receive the discharge of WWTPs, all located in the area of Girona, were analyzed. Hospital wastewater samples were collected from Josep Trueta hospital, which is one of the main hospitals in the area of Girona, with around 400 beds and that gives service to approximately 795.363 people. Grab samples were collected at two different days, specifically in November 29th and December 12th 2011.

Regarding wastewaters, three WWTP were monitored. The first one (WWTP1) corresponds to the WWTP located at the outskirts of Girona city, which treats water from an area of 143975 inhabitants, with a design of 206250 population equivalents, and it mainly receives urban, domestic and hospital wastewaters from Josep Trueta hospital. The second WWTP (WWTP2) corresponds to Celrà's treatment plant, which serves a population of 4638 inhabitants, with a design of 18900 population equivalents, and besides municipal sewage, it also receives wastewater from an industrial area, where two pharmaceutical industries are located. It should be mentioned that some of

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these industries have their own treatment systems and therefore, wastewaters that reach WWTP2 may have suffered some previous treatment. Finally, the third WWTP (WWTP3) corresponds to Castell-Platja d'Aro WWTP, which has a design of 175000 population equivalents and it mainly treats municipal wastewater from several coastal towns. This plant is located in an important touristic area in Costa Brava (one of the most important touristic areas along the Spanish Mediterranean coast).

All three WWTP have a primary and secondary treatment operating with conventional activated sludge (CAS). Furthermore, WWTP2 and WWTP3 have also a tertiary treatment with UV radiation, but in WWTP2 it only operates for internal recirculation. It should be worth mentioning that in WWTP3 an MBR-RO pilot plant is installed, which treats the secondary effluent of CAS treatment.

Furthermore, WWTP1 operates with a hydraulic retention time of 27 hours, whereas WWTP2 and WWTP3 uses 48 hours and 14 hours respectively. Influent and effluent wastewaters were collected as 24h composite samples, except for WWTP3, where it was not possible to achieve composite samples and therefore, grab samples were analyzed. Furthermore, in WWTP2 samples were collected flow proportionally. Samples from WWTP1 and WWTP3 were collected in December 2011, whereas samples from WWTP2 were taken in January 2012. Moreover, for WWTP2 samples after tertiary treatment with UV were analyzed.

Hospital, waste and river waters selected are significant regarding the occurrence of antibiotics. On one hand, hospital wastewaters analyzed correspond to an important hospital, where it is expected that a wide range of antibiotics are used on a daily basis. Moreover, WWTP selected besides urban wastewater (WWTP3), they receive hospital (WWT1) and industrial wastewater (WWTP2), which may be important sources of contamination by antibiotics of human consumption mainly.

Concerning river waters, five samples have been determined, all of them corresponding to the river Ter (TR1_A, TR1_B, TR2_A, TR2_B and TR2_C). The river Ter starts in Ulldeter, at 2400 meters height, and it travels across important rural areas in Catalonia (North East of Spain), such as Ripollès, Osona, Selva, Gironès and Baix Empordà. Moreover, this river is one of the main rivers that crosses Girona city before flowing into the Mediterranean Sea. Due to its trajectory, it is expected to detect antibiotics of both human and veterinary use. Then, TR1_A and TR2_A correspond to sampling points located before WWTP1 and WWTP2, respectively, whereas TR1_B and TR2_B are referred to the sampling points located at the point of discharge of WWTP1 and WWTP2, respectively. Finally TR2_C is located 1000 meters downstream WWTP2.

Levels of antibiotics detected in hospital and urban wastewaters are summarized in **table 5**, whereas levels found in river waters are depicted in **figure 4**. Concerning hospital wastewaters, approximately 14 compounds out of the 53 antibiotics investigated were detected. Antibiotics found at highest concentrations corresponded to the fluoroquinolone antibiotics ofloxacin (from around 3 to 10 μg/L) and ciprofloxacin (from approximately 5 to 8 μg/L). The lincosamide clindamycin was also detected at high concentrations during the first sampling (1.5 μg/L), followed by hydroxyl-metranidazole and metranidazole (0.9 and 0.6 μg/L, respectively). It is worth mentioning that hydroxyl-metronidazole was detected at significant concentrations being even more prominent than the parent antibiotic and that, to the author's knowledge, this is the first study reporting the occurrence of hydroxymetronidazole in wastewater samples. Other antibiotics found at significant concentrations were the macrolides azithromycin and clarithromycin (from approximately 100 ng/L up to 1 μg/L for clarithromycin in the second sampling campaign). Sulfamethoxazole and trimethoprim were also found but at lower concentrations (from approximately 50 to

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200 ng/L). Significant differences in antibiotic concentrations between sampling campaigns were observed, which is not surprising since different usage of antibiotics is expected along the days. In fact, in a study conducted by Lindberg et al. [39] significant differences in antibiotic concentrations were observed within the same day. However, a similar antibiotic residue profile was detected in the two sampling campaigns, which can give information about antibiotic usage and consumption in this hospital.

Concentration levels detected are in good agreement with those found in the scientific literature. Verlicchi et al. in a review paper [42] reported high levels for ciprofloxacin and ofloxacin as well, between 10 ng/L up to 100 μg/L and from approximately 0.5 μg/L to more than 10 μg/L, respectively. Moreover, in this review paper, the authors gave a list of the pharmaceutical classes most frequently found in hospital wastewaters, being fluoroquinolones, penicillins, lincosamides, sulfamethoxazole, tetracyclines and trimetoprim the most ubiquitous antibiotics. On the other hand, Won-Jin et al. [46] analyzed a wide range of pharmaceuticals, including antibiotics, in household, hospital, livestock and municipal wastewater, and they also found high concentrations for ciprofloxacin (median concentration of 3 μg/L). The other antibiotics that they found were sulfamethoxazole (with a concentration range from 0.1 to 4 μg/L), trimethoprim (from 0.02 to 95 μg/L) and lincomycin (from 0.2 to 48 μg/L). Another representative study is the aforementioned manuscript of Lindberg et al. [39], who detected concentrations of ofloxacin, ciprofloxacin, sulfamethoxazole, trimethoprim, metronidazole and doxycycline in the high μg/L range (from 2 up to 100 μg/L) in hospital wastewater samples.

Regarding urban wastewaters, between 11 and 14 out of the 53 antibiotic compounds investigated were detected in influent wastewaters whereas nearly 11 out of the 53 were found in effluent wastewaters. Regarding the effluent wastewater after tertiary treatment, a similar number of antibiotics than in effluent wastewaters after biological treatment were detected and at similar concentrations. Levels found in these matrices were in the high ng/L range, and as expected, lower than in hospital wastewaters. Even though concentrations found in influent wastewaters were much higher than the ones found in effluents, still significant levels (in the low and high ng/L range) were detected in these treated waters (see **table 5**). Higher levels in influent and effluent wastewaters in all WWTP were found for the fluoroquinolones ciprofloxacin and ofloxacin (and norfloxacin only in WWTP2), macrolides azithromycin and clarithromycin, the sulfonamide sulfamethoxazole, the hydrofolase reductase inhibitor trimethoprim and the nitroimidazole antibiotics metronidazole and its metabolite hydroxylmetronidazole. It is worth mentioning that hydroxymetronidazole was found in both influent and effluent wastewaters from WWTP1 and WWTP3 at significant concentrations, equal or even more prominent than the parent compound.

On the other hand, tertiary treatment applied in WWTP2, which consists of UV disinfection, did not have a significant effect in further mitigating the antibiotics concentrations found in effluent wastewaters after biological treatment, since similar concentrations have been found in both matrices. These findings are also in good agreement with a study conducted by Radjenovic et al [47], where it was found that pharmaceuticals, and among this group some antibiotics were studied, were not removed from drinking water after UV radiation. This was attributed to the fact that the UV dose applied for disinfection was insufficient for the breakdown of low molecular weight compounds [48]. In another study conducted by Prados-Joya et al. [49], where they investigated the efficiency of UV radiation in the direct photodegradation of nitroimidazole antibiotics, it was also concluded that the dose habitually used for water disinfection is not sufficient to remove this type of compounds.

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Regarding river waters, and as expected, concentrations found were much lower than the ones detected in wastewaters, indicating that an important dilution factor occurs once antibiotics enter river waters (see **figure 4**). In a great extent, concentrations found ranged from 1-3 ng/L to nearly 200 ng/L. Like in wastewaters, antibiotics most frequently detected and at highest concentrations were the fluoroquinolones ofloxacin and ciprofloxacin, the macrolides azithromycin and clarithromycin, the lincosamides clindamycin, the sulfonamide sulfamethoxazole, the hydrofolase reductase trimethoprim and the nitroimidazole metronidazole. Furthermore, like in wastewaters, quinolone antibiotics were also detected but at much lower concentrations (from 2 to 10 ng/L approximately).

Concentration levels found in river water samples before and after wastewater treatment plants are very similar for the vast majority of antibiotics, except for ofloxacin, azithromycin, clarithromycin and sulfamethoxazole in TR1 samples (see **figure 4**A) and for azythromycin in TR2 samples (see **figure 4B**). For these particular substances, WWTP effluents might have an important contribution to their occurrence in river waters. However, no firm conclusions on the effect of WWTP as main sources of antibiotic contamination in river waters can be drawn for the other antibiotics. Antibiotics present in the samples located before the wastewater treatment plants may come from both urban and veterinary sources, since as previously mentioned, river Ter crosses rural areas along its trajectory.

Finally, similar concentrations than the ones presented here have been reported by several monitoring programs conducted in different countries where they analyzed urban wastewater and river waters [43,50,51].

Conclusions

The multi-residue analytical method developed, based on automated off-line SPE-UHPLC-MS/MS (QqLIT) allowed the simultaneous extraction of a wide spectrum of multiple-class antibiotics and some representative metabolites in hospital and urban wastewater as well as in river water. The inclusion of relevant antibiotic metabolites in analytical methods is important in order to have a complete picture about their environmental occurrence, fate and risks. The method developed present several advantages such as high throughput, due to the minimization of sample manipulation and the use of ultra-high-performance liquid chromatography, which speeds up chromatographic analysis and separation of target antibiotics is performed with high resolution; the inclusion of relevant metabolites; high sensitivity and selectivity, due to the use of last generation, cutting-edge hybrid tandem mass spectrometers, such as the quadrupole-linear ion trap tandem mass spectrometry; and finally, good analytical performances within the multi-residue method for ß-lactam antibiotics (penicillins and cephalosporines). The method developed yielded limits of detection in the low ng/L range for complex environmental matrices, such as hospital and urban wastewaters, and also for river waters, thus providing a reliable and robust tool that can be used for routine analysis of multiple-class antibiotics in aqueous samples. Indeed, this method can be valuable to support studies dealing with the occurrence of antibiotic resistance in environmental waters polluted by antibiotic residues. Finally, the application of the method to the analysis of hospital, municipal wastewater and river water showed that antibiotics are quite ubiquitous pollutants in such matrices. The high concentrations found in hospital wastewaters and the low removal rates achieved during conventional wastewater treatment indicate that both hospital and urban wastewater are an important source of antibiotic pollution in the aquatic environment. Even though levels found in

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wastewaters were in the low µg/L and high ng/L range, an important dilution occurs in river waters, leading to low ng/L levels detected.

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Figure captions

Figure1. Recoveries obtained for selected antibiotics in Milli-Q-water using Oasis HLB cartridges, with and without sample pH adjustment, and Oasis MCX cartridges.

Figure2. Representative total ion current (TIC) chromatograms of (A) a 50 ng/mL standard mixture of antibiotics and chromatograms showing some of the most ubiquitous compounds detected in (B) an urban influent wastewater and (C) a river water sample.

Figure3. Matrix effects evaluation. Bars show the percentage of signal reduction (ionization suppression) for some representative antibiotics in (A) hospital wastewater and (B) urban influent wastewater.

Figure4. Concentration levels of the antibiotic residues detected above the method limits of quantification in the river Ter (A) at the sampling locations before and after WWTP1 and (B) at the sampling points before and after WWTP2 and 1km downstream.

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Table1. Antibiotics analyzed, classified by their chemical group, and the isotopically labelled internal standards assigned for their quantification.

Chemical group	Compounds	Number	Chemical formula	Corresponding internal standard
Fluoroquinolones	Ofloxacin	1	$C_{18}H_{20}FN_3O_4$	Ofloxacin-d_3
	Ciprofloxacin	2	$C_{17}H_{18}N_3FO_3$	Ciprofloxacin-d_8
	Enrofloxacin	3	$C_{19}H_{22}FN_3O_3$	Ofloxacin-d_3
	Danofloxacin	4	$C_{19}H_{20}FN_3O_3$	Ofloxacin-d_3
	Norfloxacin	5	$C_{16}H_{18}N_3FO_3$	Ofloxacin-d_3
	Orbifloxacin	6	$C_{19}H_{20}F_3N_3O_3$	Ofloxacin-d_3
	Marbofloxacin	7	$C_{17}H_{19}FN_4O_4$	Ofloxacin-d_3
	Cinoxacin	9	$C_{12}H_{10}N_2O_5$	Ofloxacin-d_3
Quinolones	Flumequine	10	$C_{14}H_{12}FNO_3$	Ofloxacin-d_3
	Oxolinic acid	11	$C_{13}H_{11}NO_5$	Ofloxacin-d_3
	Nalidixic acid	12	$C_{12}H_{12}N_2O_3$	Ofloxacin-d_3
	Pipemidic acid	13	$C_{14}H_{17}N_5O_3$	Ofloxacin-d_3
Penicillins	Amoxicillin	14	$C_{16}H_{19}N_3O_5S$	Ampicillin-^{15}N
	Ampicillin	15	$C_{16}H_{19}N_3O_4S$	Ampicillin-^{15}N
	Penicillin G	16	$C_{16}H_{17}N_2O_4S$	Ampicillin-^{15}N
	Penicillin V	17	$C_{16}H_{17}N_2O_5S$	Ampicillin-^{15}N
	Oxacillin	19	$C_{19}H_{18}N_3O_5S$	Ampicillin-^{15}N
Cephalosporins	Cefalexin	20	$C_{16}H_{17}N_3O_4S$	Ampicillin-^{15}N
	Cefazolin	21	$C_{14}H_{13}N_8O_4S_3$	Ampicillin-^{15}N
	Cefotaxime	22	$C_{16}H_{16}N_5O_7S_2$	Ampicillin-^{15}N
	Cefuroxime	23	$C_{16}H_{16}N_4O_8S$	Ampicillin-^{15}N
	Ceftiofur	24	$C_{19}H_{17}N_5O_7S_3$	Ampicillin-^{15}N
	Cefapirin	25	$C_{17}H_{16}N_3O_6S_2$	Ampicillin-^{15}N
Macrolides	Azithromycin	27	$C_{38}H_{72}N_2O_{12}$	Azithromycin-d_3
	Clarithromycin	28	$C_{38}H_{69}NO_{13}$	Azithromycin-d_3
	Roxithromycin	29	$C_{41}H_{76}N_2O_{15}$	Azithromycin-d_3
	Tylosin	30	$C_{46}H_{77}NO_{17}$	Azithromycin-d_3 or Lincomycin-d_3
	Tilmicosin	31	$C_{46}H_{80}N_2O_{13}$	Azithromycin-d_3 or Lincomycin-d_3
	Spiramycin	32	$C_{43}H_{74}N_2O_{14}$	Azithromycin-d_3 or Lincomycin-d_3
Tetracyclines	Tetracycline	33	$C_{22}H_{24}N_2O_8$	Sulfamethoxazole-d_4
	Chlortetracyline	34	$C_{22}H_{23}ClN_2O_8$	Sulfamethoxazole-d_4
	Doxycycline	35	$C_{22}H_{24}N_2O_8$	Sulfamethoxazole-d_4
	Oxytetracycline	36	$C_{22}H_{24}N_2O_9$	Sulfamethoxazole-d_4
Lincosamides	Clindamycin	37	$C_{18}H_{33}ClN_2O_5S$	Lincomycin-d_3
	Lincomycin	38	$C_{18}H_{34}N_2O_6S$	Lincomycin-d_3
Sulfonamides	Sulfamethoxazole	39	$C_{10}H_{11}N_3O_3S$	Sulfamethoxazole-d_4
	Sulfadiazine	40	$C_{10}H_{10}N_4O_2S$	Sulfamethoxazole-d_4
	Sulfisomidin	41	$C_{12}H_{14}N_4O_2S$	Sulfamethoxazole-d_4
	Sulfathiazole	42	$C_9H_9N_3O_2S_2$	Sulfamethoxazole-d_4
	Sulfadimethoxine	43	$C_{12}H_{14}N_4O_4S$	Sulfamethoxazole-d_4
	Sulfapyridine	44	$C_{11}H_{11}N_3O_2S$	Sulfamethoxazole-d_4
	Sulfamerazine	45	$C_{11}H_{12}N_4O_2S$	Sulfamethoxazole-d_4
	Sulfamethizole	46	$C_9H_{10}N_4O_2S_2$	Sulfamethoxazole-d_4
	Sulfamethoxypiridazine	47	$C_{11}H_{12}N_4O_3S$	Sulfamethoxazole-d_4
	Sulfisoxazole	48	$C_{11}H_{13}N_3O_3S$	Sulfamethoxazole-d_4
	Sulfanitran	49	$C_{14}H_{13}N_3O_5S$	Sulfamethoxazole-d_4
	Sulfabenzamide	50	$C_{13}H_{12}N_2O_3S$	Sulfamethoxazole-d_4
	N-acetylsulfadiazine*	51	$C_{12}H_{12}N_4O_3S$	Sulfamethoxazole-d_4
	N-acetylsulfamethazine*	52	$C_{14}H_{16}N_4O_3S$	Sulfamethoxazole-d_4

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	N-acetylsulfamerazine*	54	$C_{13}H_{14}N_4O_3S$	Sulfamethoxazole-d_4
Dihydrofolate reductase inhibitors	Trimethoprim	55	$C_{14}H_{18}N_4O_3$	Sulfamethoxazole-d_4
Nitroimidazole antibiotics	Metronidazole	56	$C_6H_9N_3O_3$	Ronidazole-d_3
	Metronidazole-OH*	57	$C_6H_9N_3O_4$	Ronidazole-d_3

*metabolites

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Table2. Target antibiotics, classified by their chromatographic retention time, and their optimized UPLC-QqLIT-MS/MS parameters by positive ionization mode

Antibiotics	Rt (min)	Precursor ion (m/z)	Q3	DP/CE/CXP	Q3	DP/CE/CXP
Metronidazole-OH	0.74	188 $[M+H]^+$	126	51/23/18	123	51/19/16
Amoxicillin	0.74	366 $[M+H]^+$	349	56/13/50	114	56/27/18
Ronidazole-d_3 (IS)	0.83	204 $[M+H]^+$	160	261/41/26	-	-
Metronidazole	0.84	172 $[M+H]^+$	82	56/35/10	128	51/21/10
Cefuroxime	0.87	425 $[M+H]^+$	364	81/21/16	321	76/17/14
Cefapyrin	0.87	423 $[M+H]^+$	124	81/63/12	152	81/33/14
Sulfisomidin	0.90	279 $[M+H]^+$	186	76/25/16	65	76/73/8
Lincomycin	0.96	407 $[M+H]^+$	359	96/27/12	389	96/25/12
Lincomycin-d_3 (IS)	0.96	410 $[M+H]^+$	129	56/37/14	-	-
Pipemidic acid	1.00	304 $[M+H]^+$	217	101/33/12	304	101/45/18
Sulfadiazine	1.00	251 $[M+H]^+$	156	56/23/14	92	56/37/12
Ampicillin	1.06	350 $[M+H]^+$	106	116/35/20	114	116/41/18
Ampicillin-^{15}N (IS)	1.06	351 $[M+H]^+$	107	91/39/16	-	-
Sulfathiazole	1.07	256 $[M+H]^+$	156	66/21/22	92	66/39/10
Cefalexin	1.08	348 $[M+H]^+$	158	31/15/24	106	31/43/12
Sulfapyridine	1.10	250 $[M+H]^+$	156	51/23/16	92	51/37/14
Trimethoprim	1.10	291 $[M+H]^+$	230	91/33/12	261	91/35/10
Marbofloxacin	1.11	363 $[M+H]^+$	72	86/27/10	320	86/23/12
Norfloxacin	1.14	320 $[M+H]^+$	276	106/25/12	233	106/35/34
N-acetylsulfadiazine	1.14	293 $[M+H]^+$	65	56/59/12	134	56/33/18
Ofloxacin-d_3 (IS)	1.15	365 $[M+H]^+$	321	96/27/12	-	-
Ofloxacin	1.16	362 $[M+H]^+$	318	86/27/12	261	86/39/12
Oxytetracycline	1.16	461 $[M+H]^+$	426	71/29/16	443	71/19/16
Ciprofloxacin-d_8 (IS)	1.16	340 $[M+H]^+$	296	86/27/12	-	-
Sulfamerazin	1.17	265 $[M+H]^+$	156	66/23/14	92	66/41/14
Ciprofloxacin	1.18	332 $[M+H]^+$	288	66/27/12	245	66/33/16
Cefotaxime	1.18	456 $[M+H]^+$	125	86/77/10	167	86/27/22
N-acetylsulfamerazine	1.21	307 $[M+H]^+$	65	96/67/10	134	96/35/10
Danofloxacin	1.22	358 $[M+H]^+$	340	96/35/12	314	96/27/12
Tetracyclin	1.25	445 $[M+H]^+$	410	101/27/14	154	76/37/18
Enrofloxacin	1.27	360 $[M+H]^+$	316	76/27/12	245	76/39/12
N-acetylsulfamethazine	1.29	321 $[M+H]^+$	65	81/65/10	134	81/37/14
Orbifloxacin	1.30	396 $[M+H]^+$	352	91/27/12	295	91/35/12
Sulfamethizole	1.32	271 $[M+H]^+$	156	21/21/14	92	21/41/8
Cefazolin	1.33	455 $[M+H]^+$	323	61/17/12	156	61/21/18
Sulfamethoxipyridazine	1.34	281 $[M+H]^+$	156	66/25/12	92	66/39/10
Spiramycin	1.42	843 $[M+H]^+$	174	16/47/14	43	16/129/8
Azithromycin	1.43	749 $[M+H]^+$	591	121/41/22	116	121/48/13
Azithromycin-d_3 (IS)	1.43	752 $[M+H]^+$	594	01/43/22	-	-
Chlortetracycline	1.52	479 $[M+H]^+$	444	51/31/18	260	51/77/42
Clindamycin	1.53	425 $[M+H]^+$	126	96/35/12	377	106/27/12
Doxycycline	1.59	445 $[M+H]^+$	154	76/37/18	429	76/17/14
Sulfadoxine-d_3 (surrogate)	1.60	314 $[M+H]^+$	156	51/25/12	-	-
Tilmicosin	1.63	869 $[M+H]^+$	88	26/119/16	696	26/57/26
Cinoxacin	1.64	263 $[M+H]^+$	245	56/21/10	189	56/39/16
Sulfamethoxazole-d_4 (IS)	1.64	258 $[M+H]^+$	160	101/23/18	-	-
Sulfamethoxazole	1.65	254 $[M+H]^+$	156	81/23/12	92	81/37/12
Sulfisoxazole	1.73	268 $[M+H]^+$	156	76/19/20	113	76/23/16
Ceftiofur	1.74	524 $[M+H]^+$	125	66/91/14	241	66/25/10
Oxolinic acid	1.78	262 $[M+H]^+$	160	76/51/20	216	76/41/34
Sulfabenzamide	1.86	277 $[M+H]^+$	156	56/19/24	92	56/39/14
Sulfadimethoxine-d_6 (surrogate)	1.87	317 $[M+H]^+$	162	61/33/8	-	-

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Sulfadimethoxine	1.89	311 [M+H]+	156	71/29/14	65	71/79/10
Tylosin	1.95	916 [M+H]+	174	21/43/30	772	21/49/14
Penicillin G	2.03	335 [M+H]+	176	96/19/22	160	96/21/8
Nalidixic acid	2.12	233 [M+H]+	215	46/25/18	187	46/37/16
Clarithromycin	2.16	748 [M+H]+	158	96/37/14	590	96/27/22
Penicillin V	2.19	351 [M+H]+	160	41/17/14	114	41/49/18
Roxithromycin	2.19	837 [M+H]+	679	91/31/26	158	91/43/14
Flumequine	2.20	262 [M+H]+	244	01/29/36	202	01/43/28
Sulfanitran	2.22	336 [M+H]+	156	96/21/14	134	96/35/16
Oxacillin	2.32	402 [M+H]+	144	146/33/22	77	146/91/12

Table3. Analytical method validation parameters: recoveries obtained for target antibiotics in different water matrices (HWW: hospital wastewater; UWWI: urban wastewater influent; UWWE: urban wastewater effluent; RW: river water), instrumental detection limits (IDLs), linearity, repeatability (run-to-run analysis) and reproducibility (day-to-day analysis)

Chemical groups	**Antibiotics**	**% Recoveries (% RSD) (n=3)**				**IDL (pg inject**
		HWW	**UWWE**	**UWWI**	**RW**	
Fluoroquinolones	Ofloxacin	68 (±31.8)	115 (±18.2)	113 (±28.2)	55 (±2.8)	1.51
	Ciprofloxacin	137 (±11.9)	136 (±18.7)	163 (±10.8)	55 (±8.0)	2.50
	Enrofloxacin	68 (±3.4)	133 (±12.2)	112 (±8.0)	85 (±5.8)	0.49
	Danofloxacin	73 (±2.8)	150 (±15.4)	131 (±4.1)	108 (±17.7)	3.30
	Norfloxacin	189 (±5.4)	142 (±11.4)	153 (±11.6)	121 (±5.0)	5.45
	Orbifloxacin	30 (±2.9)	50 (±11.2)	40 (±6.4)	51 (±10.3)	0.25
	Marbofloxacin	53 (±4.8)	79 (±11.7)	70 (±1.6)	94 (±3.9)	0.66
	Cinoxacin	30 (±5.1)	55 (±10.7)	44 (±16.9)	94 (±3.9)	0.5
Quinolones	Flumequine	85 (±0.3)	119 (±11.9)	111 (±1.9)	119 (±10.4)	0.15
	Oxolinic acid	30 (±6.6)	132 (±11.9)	117 (±1.2)	119 (±10.4)	0.23
	Nalidixic acid	60 (±5.5)	110 (±11.3)	101 (±2.5)	104 (±4.5)	0.20
	Pipemidic acid	176 (±16.3)	95 (±17.4)	119 (±5.3)	75 (±15.6)	2.0
Penicillins	Amoxicillin	126 (±15.6)	20 (±9.9)	20 (±9.1)	20 (±10.4)	0.20
	Ampicillin	78 (±5.2)	80 (±11.2	94 (±11.2)	108 (±3.9)	0.05
	Penicillin G	33 (±4.3)	30 (±21.5)	30 (±16.3)	50 (±20.0)	0.25
	Penicillin V	40 (±1.1)	136 (±1.6)	111 (±20.0)	97 (±10.7)	0.10
	Oxacillin	75 (±8.5)	54 (±4.8)	40 (±7.4)	n.c.	0.50
Cephalosporines	Cefalexin	50 (±3.7)	63 (±6.9)	83 (±9.4)	69 (±13.6)	0.25
	Cefazolin	93 (±9.5)	118 (±10.8)	118 (±10.8)	131 (±2.2)	5.0
	Cefotaxime	101 (±1.0)	90 (±6.5)	125 (±7.4)	70 (±12.0)	0.10
	Cefuroxime	104 (±10.7)	127 (±17.5)	71 (±8.2)	133 (±10.2)	2.00
	Ceftiofur	40 (±3.5)	50 (±7.3)	64 (±9.4)	77 (±6.1)	0.10
	Cefapirin	129 (±2.1)	80 (±8.0)	95 (±15.7)	70 (±12.0)	0.10
Macrolides	Azithromycin	108 (±26.0)	108 (±20.0)	81 (±2.5)	60 (±5.8)	0.25
	Clarithromycin	73 (±2.3)	138 (±15.6)	115 (±13.1)	66 (±6.5)	0.30
	Roxithromycin	50 (±1.0)	133 (±1.6)	105 (±3.0)	95 (±3.4)	0.11
	Tylosin	50 (±4.5)	142 (±1.0)	137 (±1.2)	70 (±4.1)	1.04
	Tilmicosin	95 (±7.8)	156 (±1.0)	155 (±11.0)	160 (±17.1)	1.00
	Spiramycin	145 (±23.4)	73 (±2.1)	50 (±1.0)	84 (±14.5)	5.00
Tetracyclines	Tetracycline	70 (±2.9)	114 (±3.2)	117 (±12.2)	108 (±9.2)	0.30
	Chlortetracyline	104 (±5.7)	98 (±7.6)	102 (±25.1)	108 (±3.1)	0.60
	Doxycycline	50 (±7.4)	125 (±11.7)	117 (±1.84)	76 (±18.34)	5.0
	Oxytetracycline	80 (±0.2)	150 (±4.2)	180 (±4.12)	88 (±8.0)	0.25
Lincosamides	Clindamycin	88 (±7.0)	118 (±10.8)	131 (±3.2)	107 (±5.7)	0.05
	Lincomycin	70 (±1.7)	63 (±4.5)	98 (±3.7)	82 (±20.0)	0.25
Sulfonamides	Sulfamethoxazole	60 (±13.8)	63 (±4.5)	98 (±3.7)	89 (±15.0)	0.05
	Sulfadiazine	40 (±9.2)	64 (±7.0)	90 (±4.5)	50 (±18.0)	0.1
	Sulfisomidin	79 (±5.7)	50 (±4.4)	60 (±6.9)	50 (±18.6)	0.1

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	Sulfathiazole	50 (±4.8)	50 (±2.0)	77 (±9.0)	70 (±19.0)	0.05
	Sulfadimethoxine	113 (±7.5)	81 (±14.0)	90 (±5.5)	50 (±16.0)	0.05
	Sulfapyridine	71 (±6.8)	50 (±14.0)	71 (±8.1)	60 (±18.0)	0.10
	Sulfamerazine	63 (±11.9)	51 (±6.3)	80 (±7.3)	72 (±15.0)	0.10
	Sulfamethizole	50 (±4.1)	50 (±4.1)	81 (±4.9)	64 (±13.3)	0.05
	Sulfamethoxypiridazine	40 (±11.0)	50 (±3.7)	74 (±8.8)	76 (±17.0)	0.05
	Sulfisoxazole	50 (±11.0)	60 (±13.3)	80 (±4.4)	50 (±16.3)	0.10
	Sulfanitran	100 (±1.1)	100 (±1.1)	95 (±3.5)	100 (±15.0)	0.50
	Sulfabenzamide	70 (±13.0)	60 (±4.0)	80 (±2.0)	71 (±13.0)	0.10
	N-acetylsulfadiazine*	83 (±2.4)	109 (±6.9)	126 (±8.4)	89 (±20.0)	0.50
	N-acetylsulfamethazine*	93 (±7.3)	105 (±1.9)	80 (±14.3)	104 (±11.4)	0.10
	N-acetylsulfamerazine*	109 (±3.6)	89 (±6.4)	64 (±7.8)	90(±3.3)	0.25
Dihydrofolate reductase inhibitors	Trimethoprim	122 (±8.5)	84 (±7.6)	74 (±6.1)	102 (±8.6)	0.05
Nitroimidazole antibiotics	Metronidazole	51 (±9.6)	55 (±3.9)	78 (±5.6)	30 (±20.0)	0.25
	Metronidazole-OH*	20 (±15.0)	20 (±15.0)	40 (±10.1)	n.c.	0.25

n.c.: value not calculated for this specific matrix; compounds with this "*" symbol are antibiotic metabolites.

Table4. Analytical method validation parameters: Method detection and quantification limits (MDL, MQL) in all matrices studied.

Chemical groups	Antibiotics	MDL (ng/L)				
		HWW	**UWWE**	**UWWI**	**RW**	**HWW**
Fluoroquinolones	Ofloxacin	15,70	6,44	13,81	2,54	52,32
	Ciprofloxacin	8,95	5,55	13,58	1,71	29,84
	Enrofloxacin	7,58	7,73	8,42	0,54	25,28
	Danofloxacin	11,93	47,43	34,35	13,55	39,78
	Norfloxacin	28,75	55,11	77,71	2,53	95,84
	Orbifloxacin	1,19	2,67	3,82	0,65	3,97
	Marbofloxacin	4,31	2,23	4,02	2,50	14,37
	Cinoxacin	1,80	6,12	15,64	5,52	6,00
Quinolones	Flumequine	2,68	3,17	4,25	0,73	8,94
	Oxolinic acid	2,45	16,45	19,60	2,62	8,15
	Nalidixic acid	1,71	10,56	7,92	1,77	5,71
	Pipemidic acid	5,24	6,02	16,87	3,31	17,45
Penicillins	Amoxicillin	9,49	2,65	3,32	1,32	31,63
	Ampicillin	4,56	3,08	2,73	0,83	15,20
	Penicillin G	2,55	3,48	8,62	4,00	8,51
	Penicillin V	11,31	7,04	22,82	5,37	37,71
	Oxacillin	48,32	34,63	49,43	n.c.	161,08
Cephalosporines	Cefalexin	4,32	1,43	3,40	0,77	14,41
	Cefazolin	49.21	54,11	39,81	13.37	164.02
	Cefotaxime	22,52	5,16	19,47	2,82	75,06
	Cefuroxime	24,04	26,90	22,90	5,48	80,13
	Ceftiofur	3,84	5,32	8,31	1,26	12,80
	Cefapirin	18,54	10,70	18,49	2,37	61,80
Macrolides	Azithromycin	4.04	4,63	3,26	0,61	13.46
	Clarithromycin	2,93	9,13	16,64	0,35	9,77
	Roxithromycin	8,50	8,71	5,80	0,31	28,35
	Tylosin	11,97	28,11	34,00	2,37	39,90
	Tilmicosin	7.50	7,68	17,82	3,73	25.01

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	Spiramycin	12.16	13,20	26,41	3,99	40.53
Tetracyclines	Tetracycline	24,30	13,42	16,25	4.72	81,00
	Chlortetracyline	17,72	13,77	24,02	11,20	59,08
	Doxycycline	33,65	77,49	59,79	11,23	112,16
	Oxytetracycline	3,75	5,91	6,01	4,40	12,51
Lincosamides	Clindamycin	4,89	1,48	3,13	0,48	16,29
	Lincomycin	9,13	11,24	17,82	6,04	30,44
Sulfonamides	Sulfamethoxazole	3,78	4,78	8,07	1,39	12,60
	Sulfadiazine	5,92	11,14	11,25	3,35	19,73
	Sulfisomidin	11,76	5,16	17,58	5,49	39,21
	Sulfathiazole	8,55	6,80	14,40	2,03	28,48
	Sulfadimethoxine	16,17	9,67	11,53	0,41	53,90
	Sulfapyridine	3,05	2,79	4,33	1,82	10,15
	Sulfamerazine	19,24	8,74	16,66	2,37	64,14
	Sulfamethizole	11.76	5.16	18	5.49	39.21
	Sulfamethoxypiridazine	5,00	5,17	7,97	0,81	16,66
	Sulfisoxazole	4,42	2,57	6,62	0,87	14,75
	Sulfanitran	n.c.	10,93	16,86	5,16	n.c.
	Sulfabenzamide	3,06	1,92	2,94	0,75	10.21
	N-acetylsulfadiazine*	25,21	14,36	34,48	3,41	84,05
	N-acetylsulfamethazine*	7,45	7,67	16,19	4,72	24,84
	N-acetylsulfamerazine*	33,73	19,40	24,63	4,14	112,43
Dihydrofolate reductase inhibitors	Trimethoprim	11,50	2,35	10,26	1,71	38,34
Nitroimidazole antibiotics	Metronidazole	6,49	1,80	4,45	0,43	21,64
	Metronidazole-OH*	6,34	5,20	6,36	n.c.	21,15

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1 **Table5**. Concentrations of antibiotics, expressed in ng/L, detected in hospital
2 wastewater, in urban influent and effluent wastewaters and in river waters analyzed.
3

Compounds	Hospital wastewater		WWTP1		WWTP2			WWTP3	
	HW_A November 2011	HW_B December 2011	Influent	Effluent	Influent	Effluent Biological treatment	Effluent tertiary treatment	Influent	Efflu
Ofloxacin	10368	2978	524	67	162	96	101	73	6
Ciprofloxacin	7494	5329	613	nd	445	133	147	185	5
Enrofloxacin	nd	nd	nd	nd	58	<MQL	52	nd	n
Danofloxacin	nd	nd	nd	nd	nd	nd	nd	nd	n
Norfloxacin	327	nd	nd	nd	385	149	107	nd	n
Orbifloxacin	nd	nd	nd	nd	nd	nd	nd	nd	n
Marbofloxacin	nd	nd	nd	nd	39	16	96	nd	n
Cinoxacin	nd	nd	nd	nd	nd	nd	nd	nd	n
Flumequine	nd	<MQL	nd	<MQL	<MQL	nd	nd	<MQL	<M
Oxolinic acid	nd	nd	nd	nd	nd	nd	nd	nd	n
Nalidixic acid	<MQL	<MQL	nd	nd	nd	nd	nd	nd	n
Pipemidic acid	nd	nd	nd	nd	nd	<MQL	<MQL	nd	n
Amoxicillin	<MQL	218	nd	nd	nd	216	258	nd	n
Ampicillin	nd	nd	nd	nd	nd	nd	nd	nd	n
Penicillin G	nd	nd	nd	nd	nd	nd	nd	nd	n
Penicillin V	nd	nd	nd	nd	nd	nd	nd	nd	n
Oxacillin	nd	nd	nd	nd	nd	nd	nd	nd	n
Cefalexin	nd	nd	nd	nd	nd	nd	nd	nd	n
Cefazolin	nd	nd	nd	nd	nd	nd	nd	nd	n
Cefotaxime	nd	89	nd	nd	nd	nd	nd	nd	n
Cefuroxime	nd	nd	nd	nd	nd	nd	nd	nd	n
Ceftiofur	nd	nd	nd	nd	nd	nd	nd	nd	n
Cefapirin	nd	nd	nd	nd	nd	nd	nd	nd	n
Azithromycin	113	85	437	403	nd	225	184	299	59
Clarithromycin	113	973	632	172	185	201	194	241	22
Roxithromycin	nd	nd	nd	nd	<MQL	nd	nd	<MQL	n
Tylosin	nd	nd	nd	nd	nd	nd	nd	nd	n
Tilmicosin	nd	nd	nd	nd	nd	nd	nd	nd	n
Spiramycin	nd	nd	nd	nd	nd	nd	nd	nd	n
Tetracycline	nd	nd	<MQL	<MQL	nd	nd	nd	<MQL	n
Chlortetracyline	nd	nd	nd	nd	nd	nd	nd	nd	n
Doxycycline	nd	nd	nd	nd	nd	nd	nd	nd	n
Oxytetracycline	nd	nd	nd	nd	nd	nd	nd	nd	n
Clindamycin	1465	184	37	57	31	21	22	14	1
Lincomycin	119	nd	nd	nd	nd	81	100	nd	n
Sulfamethoxazole	65	200	528	198	43	32	27	180	1
Sulfadiazine	nd	nd	nd	nd	nd	nd	nd	nd	n
Sulfisomidin	nd	nd	nd	nd	nd	nd	nd	nd	n
Sulfathiazole	nd	nd	nd	nd	nd	nd	nd	nd	n
Sulfadimethoxine	nd	nd	nd	nd	nd	nd	nd	nd	n
Sulfapyridine	<MQL	nd	32	59	<MQL	nd	nd	159	9
Sulfamerazine	nd	nd	nd	nd	nd	nd	nd	nd	n
Sulfamethizole	nd	nd	nd	nd	nd	nd	nd	nd	n
Sulfamethoxypiridazine	nd	nd	nd	nd	nd	nd	nd	nd	n
Sulfisoxazole	nd	nd	nd	nd	nd	nd	nd	nd	n
Sulfanitran	nd	nd	nd	nd	nd	nd	nd	nd	n
Sulfabenzamide	nd	nd	nd	nd	nd	nd	nd	nd	n
N-acetylsulfadiazine*	nd	nd	nd	nd	nd	nd	nd	nd	n
N-acetylsulfamethazine*	nd	nd	nd	nd	nd	nd	nd	nd	n

N-acetylsulfamerazine*	nd	nd	nd	nd	nd	nd	nd	nd	nd
Trimethoprim	216	50	178	108	<MQL	<MQL	<MQL	67	69
Metronidazole	643	67	316	58	28	20	17	72	8[illegible]
Metronidazole-OH*	887	150	454	177	nd	nd	nd	127	99

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1 Highlights
2 Simultaneous determination of 53 antibiotics and some metabolites.
3 High sample throughput (fast sample preparation and chromatographic separation).
4 High sensitivity by using advanced mass spectrometry
5 Good analytical performances for ß-lactam antibiotics.
6 Analysis of antibiotics in hospital wastewaters.
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Figure1. Recoveries obtained for selected antibiotics in Milli-Q-water using Oasis HLB cartridges, with and without sample pH adjustment, and Oasis MCX cartridges.

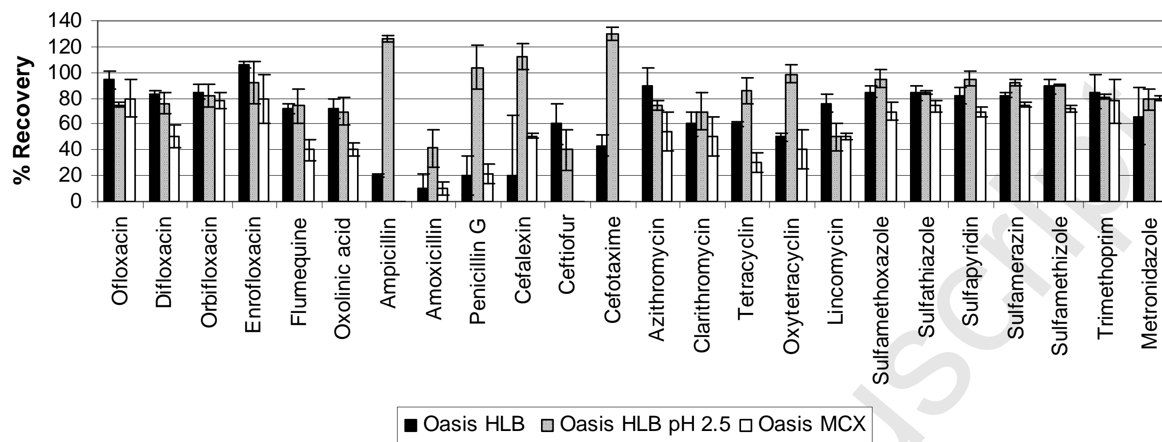

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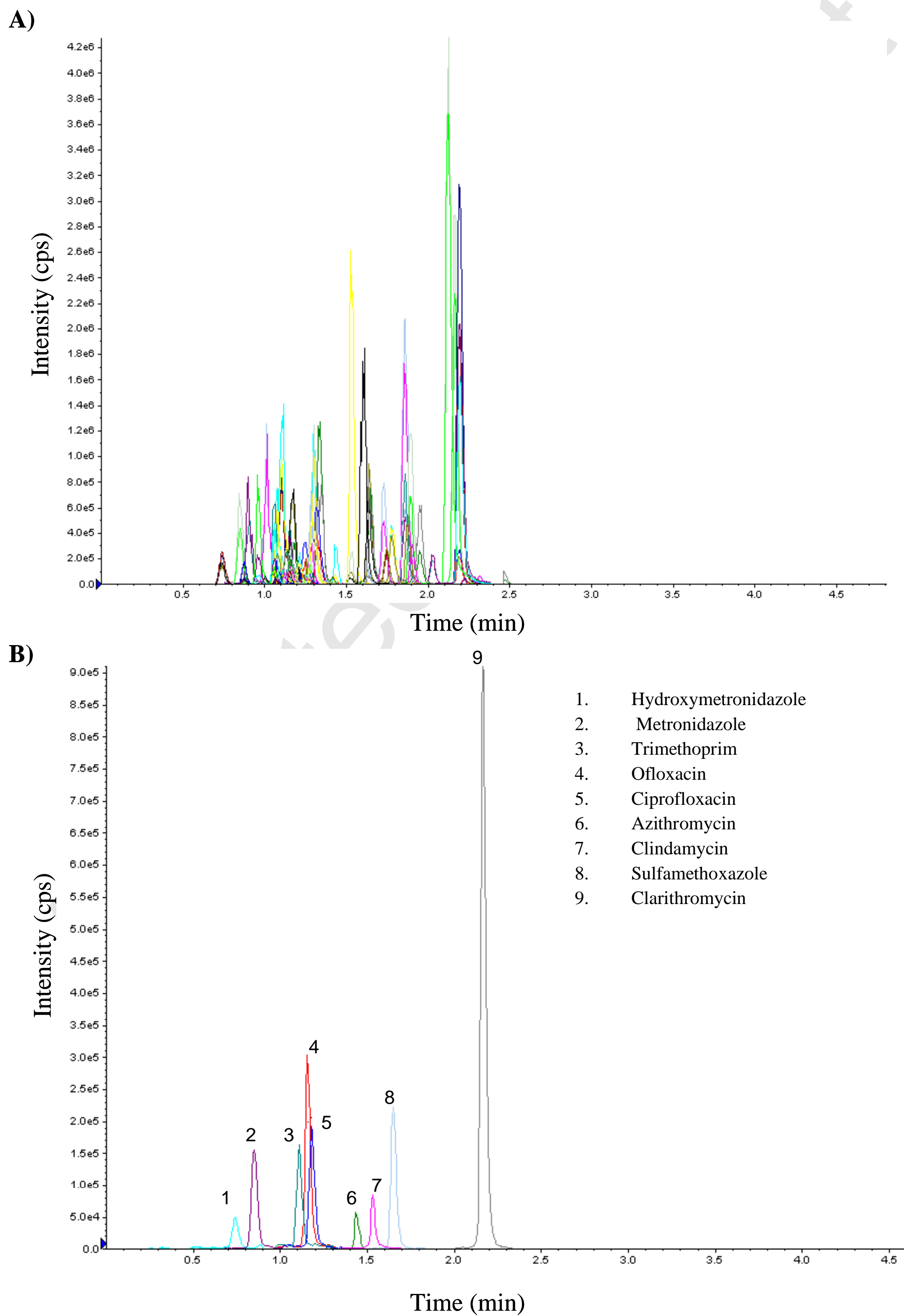
Figure2. Representative total ion current (TIC) chromatograms of (A) a 50ng/mL standard mixture of antibiotics and chromatograms showing some of the most ubiquitous compounds detected in (B) an urban influent wastewater and (C) a river water sample.



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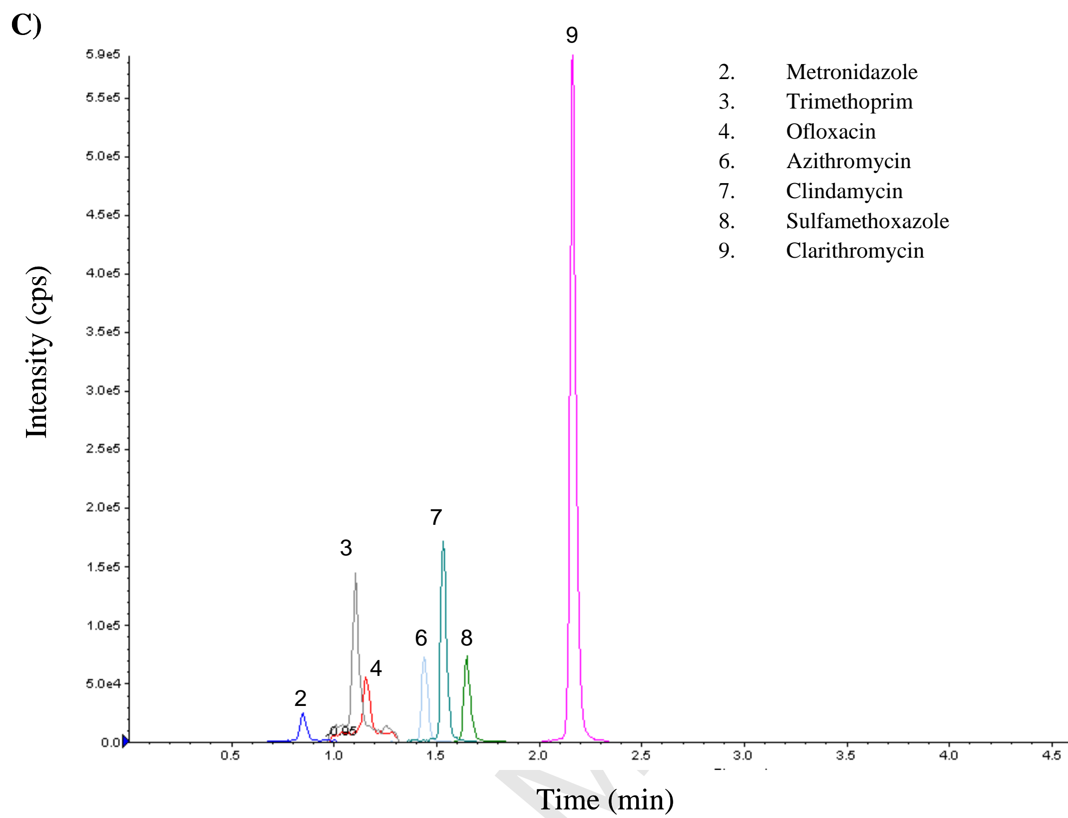

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Figure3. Matrix effects evaluation. Bars show the percentage of signal reduction (ionization suppression) for some representative antibiotics in (A) hospital wastewater and (B) urban influent wastewater.

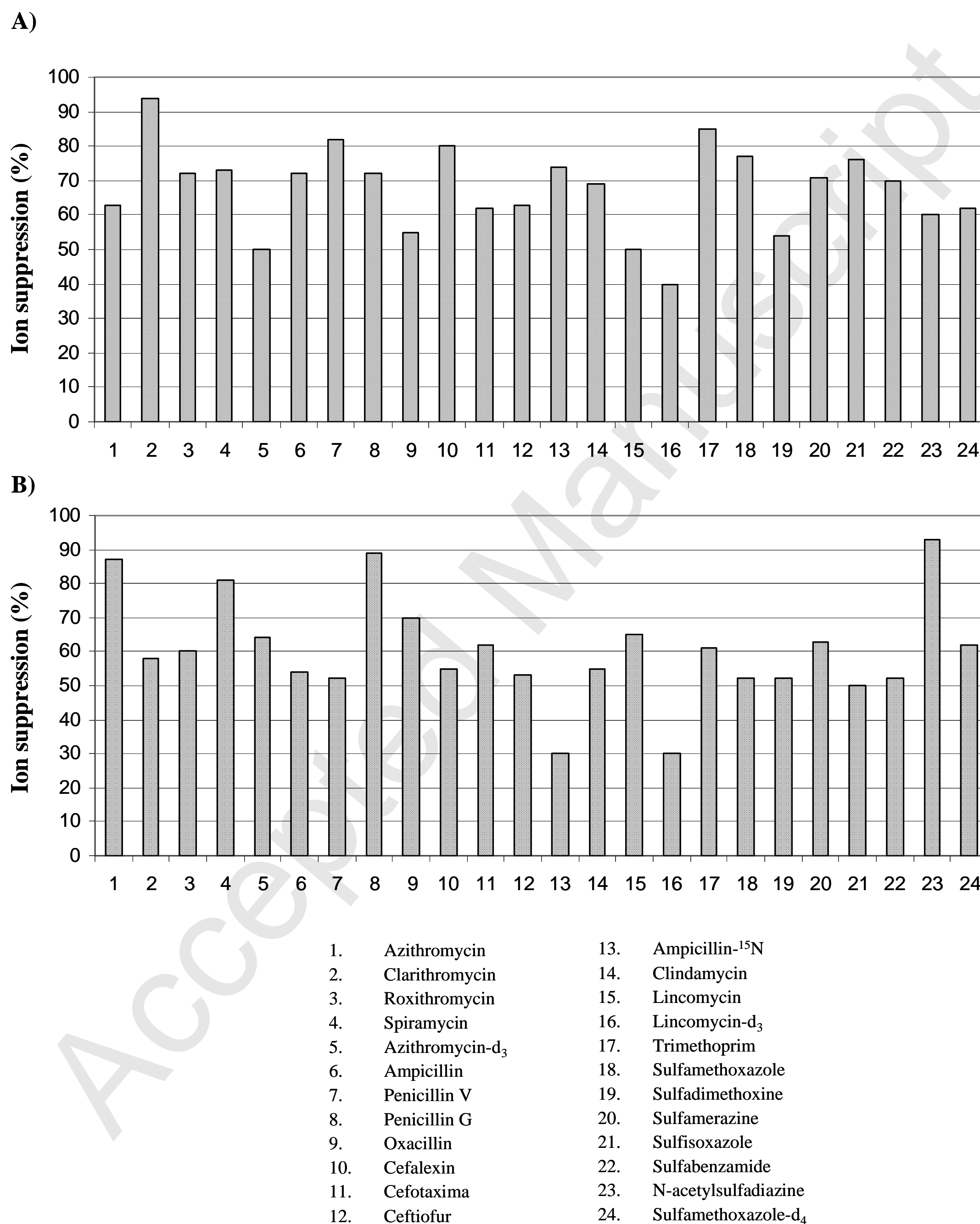

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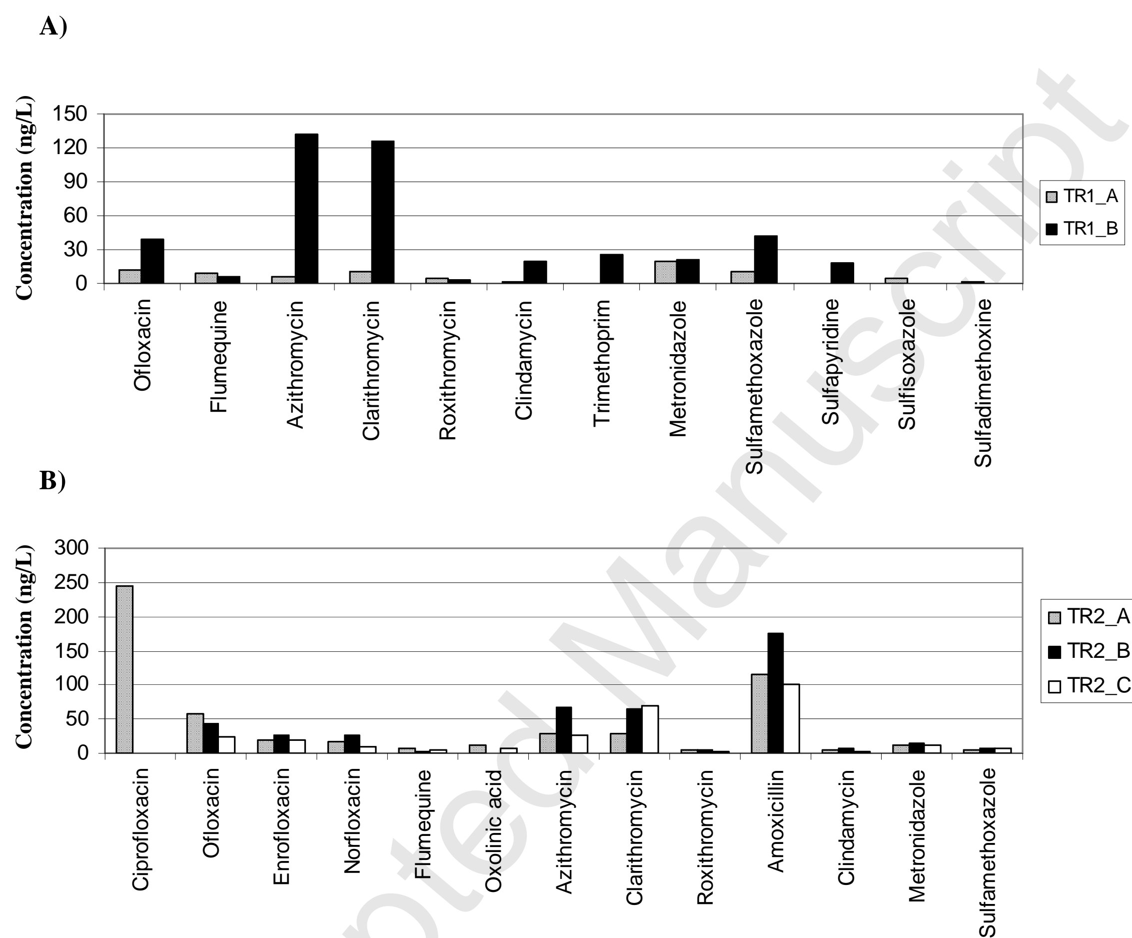
Figure4. Concentration levels of the antibiotic residues detected above the method limits of quantification in the river Ter (A) at the sampling locations before and after WWTP1 and (B) at the sampling points before and after WWTP2 and 1km downstream.



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