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FLUOROQUINOLONES AND TETRACYCLINE ANTIBIOTICS IN A PORTUGUESE AQUACULTURE SYSTEM AND AQUATIC SURROUNDINGS: OCCURRENCE AND ENVIRONMENTAL IMPACT

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The growth of aquaculture over the past few years is widely recognized as one of the main sources of antibiotics, mainly fluoroquinolones (FQ) and tetracyclines (TC), in the aquatic environment, consequently, increasing the risk of the emergence of antibiotic bacterial resistance and promoting the spread of resistant genes. This study aimed to (1) develop and validate a multiresidue method for determination and quantification of ciprofloxacin (CIP), difloxacin (DIFL), enrofloxacin (ENR), norfloxacin (NOR), sarafloxacin (SARA), and oxytetracycline (OXY) in aquaculture waters and surrounding water bodies and (2) provide the first Portuguese data to utilize in assessment of risk of adverse effects. In addition, the potential environmental impact posed by these antibiotics to aquatic organisms, belonging to different trophic levels, when exposed to the studied aquaculture waters was also assessed. The analytical strategy comprised of solid-phase extraction (SPE) through Oasis HLB cartridges, and detection and quantification by liquid chromatography with tandem mass spectrometry (LC/MS^n). Method detection limits (MDL) and method quantification limits (MQL) were in the range of 0.7–3 ng/L and 2.4–10 ng/L, respectively. Recoveries varied between 57.4 and 122.8%. The method was applied to 31 water samples collected from an aquaculture and surrounding water bodies located in north of Portugal. Residues of all antibiotics, except SARA and DIFL, were detected at concentrations ranging from 3 to 75.1 ng/L. Norfloxacin was the antibiotic present at highest frequency and concentration. Regarding the environmental impact assessment (EIA), a risk quotient higher than 1 was observed for NOR.

Global population is expected to reach 9 billion by 2050, and the world food-producing sector needs to secure food and nutrition for the growing populace through increased production and reduced waste. Globally, fish currently represents approximately 16.6% of animal protein supply and 6.5% of all protein for human consumption (Food and Agriculture Organization [FAO], 2014). Fish are usually low in saturated fats, carbohydrates, and cholesterol and provide not only high-value protein but also a wide range of essential micronutrients, including various vitamins, minerals, and polyunsaturated omega-3 fatty acids (FAO, 2014).

The last 20 years have seen a four-fold growth in industrial aquaculture worldwide, and it is expected that this growth of aquaculture will rise at an even faster rate in the future, stimulated by depletion of fisheries and globalization of sources of food supply. Nearly two-thirds of the seafood ingested will be farm raised in 2030 (Cabello, 2006; Naylor and Burke, 2005). This growing industry

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encompasses a wide range of species and methods, from simple traditional systems, to intensive industrial-scale production systems (Heuer et al., 2009; Rico and Van den Brink, 2014; Tuševljak et al., 2012). Such practices may result in heavy use of antibiotics that will potentially contaminate the environment in high levels, justifying a growing concern (Boxall et al., 2003; Capleton et al., 2006).

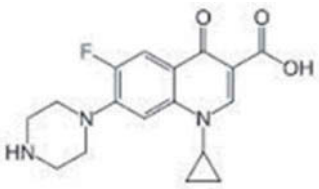
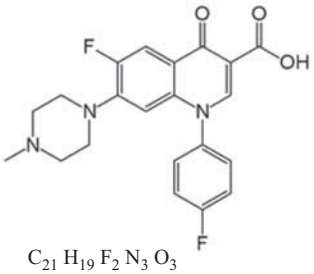
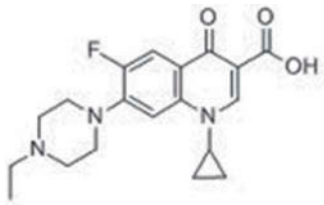
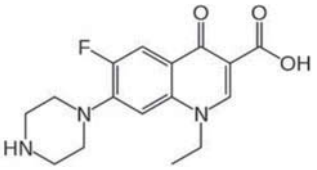
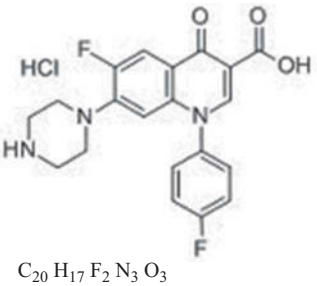
In Europe, only 14 medicinal products are authorized and approved for aquaculture, including 7 antibiotic medicinal products (amoxicillin, florfenicol, flumequine, oxolinic acid, oxytetracycline [OXY], sarafloxacin [SARA], and sulfadiazine/trimethoprim) (EAHC, 2013). Fluoroquinolones (FQ) and tetracyclines (TC), in addition to being employed in human therapy (Cabello, 2006; Heuer et al., 2009; Martinez, 2009; Naviner et al., 2011; Sapkota et al., 2008), are also widely used and effective veterinary antibiotics to prevent and treat fish diseases (FAO, 2010). Thus, FQ and TC were selected as target pharmaceuticals for the present study. In Portugal (2011), the total consumption of FQ was 22.42 tonnes with 9.02 tonnes for veterinary medicine. The higher consumption data regarding this group is mainly due to ciprofloxacin (CIP) (10.94 tonnes, human medicine) and enrofloxacin (ENR) (8.39 tonnes, veterinary medicine). For TC, only 1% of total consumption is linked to human medicine; in 2011 OXY accounted for 30% (13.33 tonnes) of total TC but in 2010 it represented 60% (46.54 tonnes) of total TC use (Almeida et al., 2014). Antibiotic release into the environment may have serious ecological impacts, since up to 90% of these antibiotics are excreted unchanged. These residues may contaminate surface waters, groundwaters, sediments, and biota (Heuer et al. 2009; Kümmerer 2009; Redshaw et al, 2013). Excessive use, and their potential to enter the environment, demand urgent attention with respect to environmental impact and risk assessment, according to the implemented guidelines (International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Pharmaceuticals [VICH SC]).

As shown in Table 1, FQ and TC, with low octanol–water partition coefficients (log Kow, −2.867 to 1.283) are relatively hydrophilic and tend to have high water solubility and low bioconcentration factors for aquatic life. Nonetheless, these drugs might form divalent cationic complexes with Ca^{2+} and Mg^{2+} and also adsorb to soils and sediments, leading to environmental persistence. Regarding soil organic carbon partition coefficients (log Koc), norfloxacin (NOR) (1.964) has clearly the lowest value compared to all the other compounds that are in the range of 3.379 to 5.261. Evidently, NOR displays lower adsorption to sediment (Burridge et al., 2010; FAO, 2005; Tamminen et al., 2011; Thiele-Bruhn, 2003).

The Food and Agriculture Organization of the United Nations (FAO), World Health Organization (WHO), International Office of Epizootics (OIE), and a number of national governments raised the issue of irresponsible use of antibiotics in fish industries, with particular concern for the potential risks to public health (Executive Agency for Health and Consumers [EAHC], 2013). Aquatic contamination by these persistent antibiotics resulted in emergence of bacterial resistance and subsequent development of more resistant and virulent pathogens in the exposed environments (Tuševljak et al., 2012). Several studies documented elevated levels of bacterial antibiotic resistance in the surrounding environment of aquaculture production systems, indicating a global health problem (Gordon et al., 2007; Naviner et al., 2011; Sapkota et al., 2008). Emergence of bacterial resistance presents one of the major emerging threats to human health and is by far the highest risk for humans of having medicinal products residues in the environment (EAHC, 2013). A strong coordinated surveillance system on antimicrobial consumption in veterinary medicine would enable policymakers to decide the best way to tackle the rising threat from antimicrobial resistance.

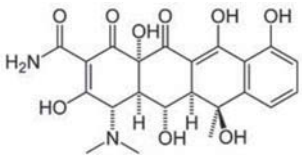
Although the fishery sector authorities indicated excessive use of FQ and TC in aquaculture as well as their consequences (FAO, 2010), a lack of information on

TABLE 1. CAS Number and Physical–Chemical Characteristics of the Selected Antibiotics

Name	CAS number	Molecular weight	pKa[a]	log k_{ow}[b]	log k_{oc}	Molecular structure (formula)
CIP	085721-33-1	331.4	6.09; 8.62	−0.001	4.785[c]	 $C_{17}H_{18}F_1N_3O_3$
DIFL	098106-17-3	399.4	5.80; 8.26	1.283	3.465[d]	 $C_{21}H_{19}F_2N_3O_3$
ENR	093106-60-6	359.4	6.09; 7.91	0.701	5.261[e]	 $C_{19}H_{22}F_1N_3O_3$
NOR	070458-96-7	319.3	6.22; 8.38	−0.306	1.964[d]	 $C_{16}H_{18}F_1N_3O_3$
SARA	098105-99-8	385.4	5.87; 8.88	1.071	3.379[d]	 $C_{20}H_{17}F_2N_3O_3$

(Continued)

TABLE 1. (Continued)

Name	CAS number	Molecular weight	pKa[a]	log k_{ow}[b]	log k_{oc}	Molecular structure (formula)
OXY	000079-57-2	460.4	3.22;7.46; 8.94	−2.867	4.680[f]	 $C_{22}H_{24}N_2O_9$

[a]Babić et al. (2007).
[b]ECOSAR v.1.11.
[c]Martins et al. (2012).
[d]PCKOCWIN v1.66.
[e]De La Torre et al. (2012).
[f]Rabølle and Spliid (2000).

aquaculture use persists, and, to our knowledge, concerning Portugal, no apparent data are available on FQ and TC residues in aquaculture systems or aquatic surroundings. This study aimed to develop and validate a simple, accurate, and sensitive multiresidue analytical method, comprising a solid-phase extraction (SPE) with Oasis HLB cartridges and liquid chromatography with tandem mass detection (LC/MS^n) analysis for determination of CIP, difloxacin (DIFL), ENR, NOR, SARA, and OXY in aquaculture waters and surrounding surface waters. In addition, the potential environmental impact posed by these antibiotics to aquatic organisms, belonging to different trophic levels, when exposed to the studied aquaculture waters, was also assessed, providing the first Portuguese data.

MATERIALS AND METHODS

Standards, Chemicals, and Materials

Standards of NOR and OXY were purchased from Sigma Chemicals Corp. (St. Louis, MO), while CIP, ENR, DIFL, and SARA were acquired from Fluka, SIGMA-Adrich (Steinheim, Germany). All antibiotics were analytical standard, with purity degree above 98%. Individual stock solutions were prepared in methanol at 0.2 mg/ml and stored at −20°C in the dark. An intermediate standard solution, of all antibiotics, was prepared in methanol at 2 μg/ml, and working solutions from 5 to 500 ng/ml were prepared in methanol:water (1:2).

Methanol liquid chromatography–mass spectroscopy (LC-MS) analyzed reagent was acquired from JTBaker (Deventer, The Netherlands), formic acid (50%) was purchased from Fluka (Buchs, Switzerland), and phosphoric acid (85%), hydrochloride acid (37%), ammonia (25%), citric acid, ethylenediamine tetraacetic acid (EDTA), and sodium hydroxide were obtained from Merck (Darmstadt, Germany). Ultrapure Milli-Q water was obtained through a Millipore (Molsheim, France) equipment.

Polyamide membrane filters (0.45 and 0.2 μm) were obtained from Whatman (Dassel, Germany) and the SPE cartridges used, Oasis MCX (150 mg, 6 ml), Oasis MAX (150 mg, 6 ml), and Oasis HLB (200 mg, 6 ml), were from Waters Corp. (Milford, MA).

Sample Collection and Pretreatment

Thirty-one samples were collected from November 2010 until May 2012 in a trout aquaculture system located in the north of Portugal. The freshwater farm, which is tank based and located near the River Caima using its water, carries out intensive trout monoculture, with an average production of 40 tonnes/yr, and uses commercial pellet fish feed. River freshwater is conducted through the

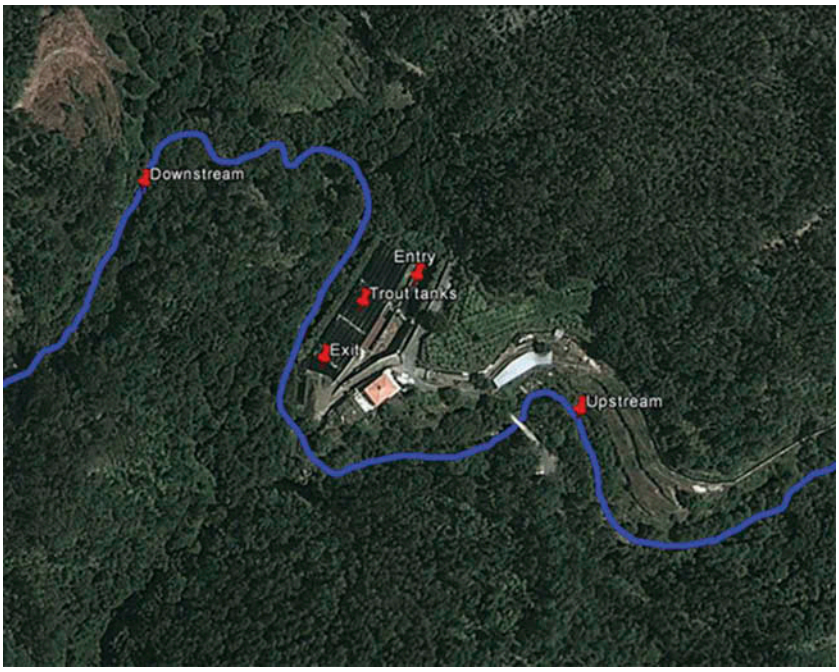


FIGURE 1. Map of the studied area and sample site locations.

entire aquaculture, passing through all the fish tanks, at controlled volume, and then is delivered directly into the river basin. Several sites of interest were identified along the stream, as shown in Figure 1. Grab water samples (1 L) were obtained from 5 different points as follows: in the river, 250 m upstream from the aquaculture system (5 samples); at the entrance of the aquaculture (7 samples); in a trout tank (9 samples); at the exit of the aquaculture (7 samples); and 250 m downstream from the aquaculture system (3 samples). After collection into sterilized plastic bottles, samples were refrigerated during transportation, and on arrival at the lab the samples were stored at 4°C until analysis. Before SPE, samples were subsequently vacuum filtered through polyamide membrane filters, 0.45 μm and 0.2 μm, and afterward, to 300 ml was added of 54 mg of EDTA, and this was acidified to pH 2 with phosphoric acid.

Experimental Procedure

SPE was performed through Oasis HLB (200 mg, 6 ml) cartridges, previously conditioned with 2 ml methanol, followed by 2 ml Milli-Q water. After sample load (300 ml), the cartridges were washed with 2 ml Milli-Q water, left to dry for 15 min, and eluted with 2 ml methanol. Finally, the eluate was evaporated to dryness under a stream of nitrogen, at 45°C.

For liquid chromatography with tandem mass spectrometry (LC/MS^n) analysis, the dried eluate was taken in 300 μl methanol:water (1:2). A liquid chromatography tandem mass spectrometry (LC-MS/MS) system, composed of two Varian 210 high-performance liquid chromatography (HPLC) pumps and a ProStar 410 autosampler, all from Varian (Walnut Creek, CA), was assembled with an analytical column, Pursuit UPS C18 (2.1 mm ID × 50 mm, 2.4 μm), from Varian, and a guard column (2.1 mm ID × 10 mm, 3 μm) of the same characteristics. The equipment was fitted with a 10-μl sample loop, and separation was achieved at a flow rate of 300 μl/min, using a gradient of methanol and 10 m*M* formic acid in Milli-Q water: 25% methanol, rising to 50% methanol in 8 min, then to 100% methanol at 9 min and holding until 10 min; at the end of the chromatographic run the column

was reequilibrated to the initial conditions in 1 min and stabilized for 8 min. For detection, a Varian 500 MS ion trap mass spectrometer with electrospray ionization (ESI) was used. Equipment parameters were properly adjusted to obtain the maximum amount of precursor ion entering the ion trap: retention time, precursor ion, MS^2 product ion, ionization mode, capillary voltage, radio frequency (RF) loading, and collision-induced dissociation (CID) voltage (Table 2). The software used for data processing was the Varian MS Workstation version 6.9.1. Confirmation of positive results in real samples was made by comparison of the MS/MS spectra against authentic standards, and by setting two to three qualifiers and 20% tolerance criteria. The quantification of each antibiotic was based on the main characteristic MS^2 precursor/product ion transition (Table 2).

Statistical Analysis

Complete statistical analyses were performed using GraphPad Prism (6.01, GraphPad Software, Inc., San Diego, CA). To test whether the data sets were of Gaussian distribution, the D'Agostino–Pearson normality test was used. Since most of the data sets were not normally distributed, with nonhomogeneous variances, nonparametric tests were applied. For the comparison between each and the total of antibiotics in the different sampling locations, the Kruskal–Wallis test with Dunn's posttest was used. The statistical significance level was set at $p < .05$ (Magdeburg et al., 2012).

Environmental Impact Assessment

Evaluation of environmental impact assessment (EIA) is based on the risk quotient (RQ) approach, which, in our study, is the ratio of the measured environmental concentration in surface water ($MEC_{surfacewater}$) and the predicted no-effect concentration (PNEC) on non-target organisms using three different trophic levels representatives of the aquatic ecosystem (algae, daphnids and fish). The $MEC_{surfacewater}$ values, using the worst-case scenario, were accounted for by the maximal individual concentrations of pharmaceuticals found in all of the samples. PNEC values were calculated by dividing the lowest short-term toxicity endpoints available in the literature, for the *Daphnia* immobilization test (EC_{50}) and fish acute toxicity test (LC_{50}), by an assessment factor (AF) of 100 or 1000, respectively. Regarding algae, growth inhibition tests (EC_{50}) were selected and since limited experimental values were available, a factor of 1000 was selected (European Medicines Agency [EMA], 2006). When no experimental values were available, $L(E)C_{50}$ values estimated with ECOSAR 1.11 were used. If RQ is equal or above 1 there is a potential environmental risk situation, whereas when values are lower than 1, no risk is expected.

RESULTS AND DISCUSSION

Sample Pretreatment and SPE Optimization

Due to the low levels of pharmaceuticals that might be present in an environmental sample, extraction and cleanup are compulsory in

TABLE 2. MS/MS Parameters Used for the Analysis of the Antibiotics

Antibiotic	Rt (min)	Percursor ion (m/z)	MS^2 product ion (m/z)	Ionization mode	Capillary voltage (V)	RF loading (%)	CID voltage (V)
NOR	4.27	320.0	276.1	ESI +	78.0	86	0.75
CIP	4.71	332.0	288.1	ESI +	65.0	88	1.30
OXY	4.91	461.0	426.0	ESI +	68.0	69	0.90
ENR	5.23	360.0	316.0	ESI +	135.0	85	1.47
DIFL	5.62	400.0	356.0	ESI +	90.0	63	2.00
SARA	5.88	386.0	342.1	ESI +	90.0	94	1.50

Note. RF loading: radiofrequency loading. CID voltage: collision-induced dissociation voltage.

order to eliminate interference and preconcentrate analytes, enabling the desired levels of analytical sensitivity (López-Serna et al., 2012). The greatest difficulty with the multiresidue analysis of pharmaceuticals concerns the choice of the best SPE sorbent giving an acceptable recovery for all compounds characterized by different physicochemical properties (Kasprzyk-Hordern et al., 2007). An analytical compromise had to be made when choosing the extraction conditions, for analysis of the six antibiotics in aquaculture and surface waters, in a single step.

Different pretreatment procedures were attempted to obtain the most suitable extract to apply onto the SPE columns. To avoid complex formation with divalent ions such as calcium, magnesium, and metal ions (Seifrtová et al., 2008; Blesa et al., 2012), different amounts of EDTA (0, 36, 54, and 72 mg) were tested at a fortification level of 125 ng/L, and best recoveries obtained when 54 mg was used.

FQs, in addition to a carboxylic group that makes these compounds acidic, have an amino group in the heterocyclic ring (namely, piperazinyl). Thus, FQs have two dissociation constants. The reported values of pKa1 and pKa2 are in the 5.8–6.2 and 7.9–8.9 range, respectively, and thus, the intermediate form is a zwitterion (Table 1). At acidic conditions, they are in a cationic form, which is important for their retention during the SPE on cationic exchange cartridges. At basic conditions, the anionic species are less retained in comparison to cationic, zwitterionic, and neutral species on the polymeric Oasis HLB column, but they may be retained on anionic exchange cartridges. TCs show three pKa values of approximately 3, 7, and 9. Throughout the range of pH, TCs always possess a local charge, and they are zwitterionic in the approximate range of pH 3–9. Thus, pH adjustment is an important step in the analysis of TCs. The pH is usually adjusted to value $\leq$3 to assume that TCs will be in cationic form, which is critical for optimal extraction (Seifrtová et al., 2010).

Bearing this in mind, different sample pH adjustments at 1, 2, and 3.5 with HCl were evaluated; however, better results were obtained when the samples were acidified with H_3PO_4 to pH 2, below the pKa of all antibiotics (Table S1, Supplemental Data). Under this conditions all compounds were extracted in the cationic water soluble form (Zhou et al., 2012; Sturini et al., 2009; Pena et al., 2010; Choi et al., 2008).

SPE is an important step for the analysis of antibiotic residues in environmental samples. Different attempts were made to adjust the following parameters: type of cartridge, washing and elution solvents, and volumes. Three different SPE cartridges were evaluated: Oasis HLB (200 mg, 6 ml), Oasis MCX (150 mg, 6 ml), and Oasis MAX (150 mg, 6 ml) (Table S1, Supplemental Data). Oasis HLB is a polymeric resin with hydrophilic–lipophilic affinities. MCX consists of an HLB polymeric resin added with a cation-exchange sulfonic group, while the MAX adsorbent consists of an HLB polymeric resin added with an anionic-exchange quaternary ammonium group (Waters, 2013). Due to the different chemical interaction possibilities offered, these three columns are good candidates for the development of a method when analyzing the selected antibiotics (Blesa et al., 2012). All the tests were made with spiked samples at 125 ng/L.

The first attempted approach for SPE was with Oasis MCX (Blesa et al., 2012). Nonetheless, with this sorbent, recoveries were generally low, with the best recovery results (46–58%) obtained after activation through an acid aqueous solution at pH 3, and elution with a basic methanol with 5% NH_3 (Table S1, Supplemental Data). It was also observed that an acidic wash using water at pH 3 did not improve recoveries (45–57%). Unexpectedly, a methodology based on that reported by Blesa et al. (2012), for the quantification of CIP and ENR in pork meat, enabled recoveries as high as 21%.

In contrast, since Oasis MAX cartridges adsorb anions, the pH of the antibiotics extract and that of the column activation solution need to be higher than the pKa of the studied compounds, to ensure that these are in their anionic form, favoring adsorption. For eluting, the pH needs to be changed so that

the analytes can change into their cationic form, preventing electrostatic interactions that would favor retention. However, at a sample pH of 9 (higher than all the pKa), activation with 0.5 *M* NaOH and elution with 0.2 *M* HCl in methanol did not allow for improved recoveries (<10%). These results indicate that probably the hydrophilic/lipophilic interactions are favored to the detriment of pH-dependent electrostatic interactions.

Finally, different assays were attempted using Oasis HLB cartridges that allowed for significantly improved recoveries. With activation of the cartridges using 2 ml methanol, followed by 2 ml water, a washing step with 2 ml water, and elution with 2 ml methanol, recoveries ranged between 68 and 80% for CIP and DIFL, respectively. Based on the results of previous studies (Blesa et al., 2012), a wash with 5% citric acid solution at pH 4 was attempted, with 2 and 4 ml. For the higher volume, low recoveries, less than 26%, were achieved; 2 ml was found to be more effective with recoveries as high as 59%, for which this attempt was abandoned. No washing attempts were made with methanol in water on Oasis HLB, because previous experiences in our lab showed that it strongly reduces recovery percentage (Pena et al., 2010). Finally, regarding elution, 100% methanol was found to be a good eluent, confirming a clear hydrophilic/lipophilic interaction.

Oasis HLB cartridges are widely used for these compounds and generally reliable recoveries are achieved (Batt and Aga, 2005; Choi et al., 2007; Himmelsbach and Buchberger, 2005; Luo et al., 2010; Pena et al., 2010; Ye et al., 2007; Yiruhan et al., 2010; Zhou et al., 2012). Accordingly, for the selected antibiotics, the study of Goncalves et al. (2013) used JT Baker H_2O-Philic columns, with results similar to those reported herein with Oasis HLB.

LC-MS/MS Optimization

Given the challenge of analyzing the already-mentioned antibiotics in the same run, several LC-MS/MS parameters had to be optimized, and in the following only the most critical aspects are briefly discussed. Instrumental optimization began by setting up the appropriate ESI source parameters, MS/MS conditions, and the chromatographic gradient to analyze a mixture of acidic antibiotics at maximal sensitivity and resolution. The ESI source parameters (ionization polarity, drying gas temperature, needle voltage, and capillary voltage), the detector storage, and fragmentation conditions (RF loading voltage and CID voltage) were optimized on a per-analyte basis by infusion of authentic standards. The purpose was to accumulate the maximal amount of precursor ion in the detector, followed by CID fragmentation until a few percent of precursor ion remained.

Aqueous mobile phases buffered at the pH of 2.9, 3.2, 3.7, and 4.6 were tested using formic acid and ammonium formate as additives. A mobile phase composed of formic acid at 10 m*M* in water (pH 2.9) was revealed to be essential for obtaining good peak shape and resolution (Figure S2, Supplemental Data). In addition, formic acid was found to be a more effective additive for compounds determined in the positive mode (Sousa et al., 2011). Positive electrospray ionization was selected, since it yielded better sensitivity in this instrument and ionization was favored under the acidic mobile phase.

Validation Results

After sample pretreatment, SPE, and LC/MS^n optimization, full validation was performed, encompassing sensitivity, linear range, accuracy, precision, and matrix effect features that are summarized in Table 3. Linearity was studied using Milli-Q water-matched calibrations at 5 concentration levels: 5, 10, 50, 150, and 300 ng/L ($n = 3$). Linearity, achieved for every compound, in the working standard solutions, was reliable as shown by the fact that the correlation coefficients (r^2) ranged from 0.991 to 1.

The method detection (MDL) and quantification limits (MQL) were estimated as the concentration giving a signal-to-noise (S/N) ratio of

TABLE 3. Validation Data Obtained for the Studied Antibiotics in Spiked Samples

Antibiotic	MDL (ng/L)	MQL (ng/L)	Matrix matched linearity (r^2)	Spiking level (ng/L)	Recovery (%)	RSD within day (%)	RSD between days (%)
NOR	0.7	2.4	.989	10	102.3	9.8	5.1
				50	92.5	12.2	3.4
				100	65.3	3.5	14.3
				300	76.7	10.5	4.2
CIP	0.9	2.9	.995	10	63.8	16.3	4.3
				50	67.2	15.6	2.9
				100	57.4	1.9	12.8
				300	66.1	0.3	7.6
OXY	3.0	10.0	.990	10	58.5	14.3	13.2
				50	66.4	15.1	16.4
				100	82.6	12.7	1.2
				300	122.8	4.6	13.1
ENR	1.0	3.4	.997	10	80.8	6.7	15.2
				50	74.8	4.7	13.7
				100	70.1	1.7	10.1
				300	67.5	0.2	8.6
DIFL	1.6	5.3	.99	10	77.4	9.5	12.7
				50	76.1	6.3	16.6
				100	87.0	7.7	12.3
				300	74.2	6.7	7.5
SARA	1.5	5.0	.996	10	72.8	5.4	7.9
				50	73.1	2.1	1.7
				100	71.2	2.0	13.1
				300	74.2	6.7	2.6

3 and of 10, respectively. MDL and MQL values ranged from 0.7 to 3 ng/L and from 2.4 to 10 ng/L, respectively. These values are considered reliable, according to other methods developed for the same purpose (Batt and Aga, 2005; Himmelsbach and Buchberger, 2005; Choi et al., 2007; Ye et al., 2007; Luo et al., 2010; Payán et al., 2011; Rodríguez et al., 2011; Gros et al., 2013; Zhou et al., 2012; Yudthavorasit et al., 2010).

Matrix effects were accessed comparing recoveries in spiked Milli-Q water and spiked samples ($n = 3$). OXY presented a significant matrix effect (51%), while CIP, NOR, ENR, SARA, and DIFL showed 1.77, 6.91, 14.17, 23.49, and 29.81%, respectively. This is probably explained by to co-extracted matrix components that suppressed analyte signal.

Accuracy and precision were tested at 4 fortification levels: 10, 50, 100, and 300 ng/L ($n = 3$). Recovery rates for all antibiotics were above 57.4%. Values for intraday (repeatability) and interday (intermediate precision) were in the range of 0.2 to 15.6% and 1.2 to 16.6%, respectively.

Application to Real Samples

The analytical method was successfully developed and validated for detection of NOR, CIP, OXY, ENR, DIFL, and SARA in aquaculture water samples. In order to evaluate the contamination of the surrounding environment, samples were collected in the river upstream and downstream of the aquaculture location, at the entry of the fish tanks, from the fish tanks, and in the water stream flowing out directly from the fish tanks (effluents). Although the upstream and entry sampling was performed to better evaluate possible aquatic environmental contamination in this river, the fish tank and exit samplings were intended to control the antibiotic use in this aquaculture; finally, with the downstream sampling it was intended to verify antibiotic use impact on the river basin.

Table 4 outlines a summary of the frequency and detected concentrations of the selected antibiotics in the studied samples. The results showed that four out of six antibiotics were detected, namely, NOR, CIP, OXY, and ENR, in 87% of samples contaminated with 2 or

TABLE 4. Frequency (%) and Detected Concentrations (ng/L) in the Studied Samples

Sample site	NOR	CIP	OXY	ENR	DIFL	SARA
Upstream 1	7.4	n.d.	n.d.	<MQL	n.d.	n.d.
Upstream 2	23.0	n.d.	n.d.	<MQL	n.d.	n.d.
Upstream 3	11.4	3.9	n.d.	<MQL	n.d.	n.d.
Upstream 4	69.7	4.9	n.d.	4.9	n.d.	n.d.
Upstream 5	n.d.	n.d.	n.d.	<MQL	n.d.	n.d.
Frequency	80.0	40.0	—	100	—	—
Mean	27.9	4.4	—	1.8	—	—
Entry 1	14.1	n.d.	n.d.	4.6	n.d.	n.d.
Entry 2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Entry 3	13.3	3.9	n.d.	4.3	n.d.	n.d.
Entry 4	32.4	n.d.	<MQL	5.3	n.d.	n.d.
Entry 5	23.0	<MQL	<MQL	4.4	n.d.	n.d.
Entry 6	22.9	3.7	n.d.	23.2	n.d.	n.d.
Entry 7	17.7	5.4	n.d.	n.d.	n.d.	n.d.
Frequency	85.7	57.1	28.6	71.4	—	—
Mean	20.6	3.5	3.0	8.4	—	—
Trout tank 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Trout tank 2	6.3	<MQL	<MQL	4.8	n.d.	n.d.
Trout tank 3	27.4	<MQL	n.d.	5.2	n.d.	n.d.
Trout tank 4	34.3	n.d.	<MQL	<MQL	n.d.	n.d.
Trout tank 5	5.4	<MQL	n.d.	n.d.	n.d.	n.d.
Trout tank 6	75.1	n.d.	n.d.	9.3	n.d.	n.d.
Trout tank 7	51.5	4.0	n.d.	n.d.	n.d.	n.d.
Trout tank 8	11.4	n.d.	n.d.	n.d.	n.d.	n.d.
Trout tank 9	21.1	n.d.	n.d.	<MQL	n.d.	n.d.
Frequency	88.9	44.4	22.2	55.6	—	—
Mean	29.1	1.7	3.0	4.3	—	—
Exit 1	23.3	<MQL	n.d.	n.d.	n.d.	n.d.
Exit 2	38.8	3.0	11.9	4.0	n.d.	n.d.
Exit 3	8.6	n.d.	n.d.	8.2	n.d.	n.d.
Exit 4	10.2	5.4	n.d.	<MQL	n.d.	n.d.
Exit 5	8.2	3.7	n.d.	4.8	n.d.	n.d.
Exit 6	27.8	<MQL	n.d.	<MQL	n.d.	n.d.
Exit 7	26.4	11.7	n.d.	n.d.	n.d.	n.d.
Frequency	100	85.7	14.3	71.4	—	—
Mean	20.5	4.3	11.9	3.8	—	—
Downstream 1	30.6	n.d.	n.d.	8.0	n.d.	n.d.
Downstream 2	24.2	19.1	9.1	<MQL	n.d.	n.d.
Downstream 3	24.4	n.d.	n.d.	<MQL	n.d.	n.d.
Frequency	100	33.3	33.3	100	—	—
Mean	26.4	19.1	9.1	3.3	—	—
Total frequency	90.3	54.8	19.4	74.2	—	—
Total mean	24.6	4.4	5.5	4.4	—	—

Note. n.d., Not detected. MQL, method quantification limit. $\underline{n} = 3$. RSD < 4.2%.

more antibiotics. DIFL and SARA were never detected. NOR, exclusively used in human medicine, was unexpectedly found in 90.3% of total samples, with an overall average concentration of 24.6 ng/L and up to 75.1 ng/L in a sample collected from a trout tank. In fact, in the trout tanks, the average concentration of NOR (29.1 ng/L) was numerically higher than in upstream site sampling (27.9 ng/L) and also higher than levels found in samples at the entry of the aquaculture (20.6 ng/L). The presence of NOR in the upstream samples might be attributed to a wastewater discharge or misuse in animal production. ENR, exclusively used in veterinary medicine, was the second most prevalent antibiotic (74.2%), with an overall average level of 4.4 ng/L. Although CIP is only licensed for human medicine, this drug is the major metabolite of ENR after its deethylation. Accordingly, our results showed that CIP was

present in 54.8% of sample, with an overall average of 4.4 ng/L. Finally, OXY was found in 19.4% of samples, with an overall average of 5.5 ng/L. Since the antibiotics with higher consumption are CIP, OXY, and ENR, the fact that NOR was found with higher frequencies and concentrations is probably related to the low log Koc values, promoting higher concentrations in the water compartment.

Generally, although there were high frequencies of contamination found, the concentration levels were consistently low. Further, the concentrations of antibiotics, both individually and in total, were observed to be relatively stable between different sampling locations and were not found to be markedly different. No statistical significance was observed during the different sampling campaigns.

These preliminary results indicate that probably there is a contamination upstream of the aquaculture location, possibly due to a wastewater discharge or an animal farm. Moreover, FQ and TC, due to their physical–chemical properties, are able to chelate with metal ions and adsorb to sediments (Thiele-Bruhn, 2003). Marked water-level variability may promote sediment resuspension and transport, with consequent remobilization of these antibiotics, promoting a broader and unexpected contamination of the surrounding aquatic environment (Petrovic et al., 2011). Since no apparent data from the use of antibiotics in this aquaculture were available to better assess the use of antibiotics, further studies are mandatory, specifically for incidence in sediments.

Data on aquaculture antibiotic usage are scarce. In most countries of North America and Europe, licensing and regulation of the use of antibiotics in aquaculture are strictly enforced, and their use is guided by veterinary professionals. However, a large proportion of the global aquaculture production (90%) takes place in countries insufficiently regulated and with limited enforcement for authorization of antimicrobial agents usage in animals (Heuer et al., 2009; Rico and Van den Brink, 2014).

Although few studies reported the use of FQ and TC in aquaculture, Cháfer-Pericás et al. (2010), analyzed 9 samples of marketed fish acquired in Spain and OXY residues were detected in 4 samples, and in 1 of these samples the concentration reached 60 μg/kg. In Thailand, 20% of the 35 shrimp samples analyzed contained FQ; these results were not unexpected, since Dufresne and Fouquet (2007) reported that these compounds were among the most commonly used antibiotics in shrimp farms in Thailand. In 2007, the Food and Drug Administration of the United States had to temporarily block the sales of five aquaculture products from China because they contained, among other residues, FQ (Burridge et al., 2010).

Emergence of antibiotic resistance is a major European and global societal problem, involving many different sectors, including medicine, veterinary medicine, animal husbandry, agriculture, environment, and trade. It cannot be successfully tackled through isolated, sectorial efforts. Risk mitigation measurements, related to a risk identified for a veterinary medicinal product used in fish farms, would be effective only in countries where local authorities monitor the use of products and the discharges from aquaculture facilities (EAHC, 2013).

Approximately 20 years after the start of industrial aquaculture, evidence emerged of the transfer of antibiotic resistance determinants between aquatic bacteria, including fish pathogens, and human pathogens. Historical evidence appears to indicate that in terrestrial animal husbandry this process took a longer time, suggesting that in the aquatic environment, resistance might be acquired more rapidly, posing even a greater problem for use of antibiotics in aquaculture (Cabello, 2006). One of the main concerns surrounding FQ and TC release into surface waters is their potential bioaccumulation in soils/sediments and biota, delaying their biodegradation and prolonging the direct contact with microorganisms, with concentrations increasing the emergence of bacterial resistance even at subinhibitory levels (Cabello, 2006; FAO/WHO/OIE, 2004; Ohlsen et al., 1998). These facts are highlighted by the results of a concurrent study, showing

that this aquaculture is a reservoir/vehicle for antibiotic-resistant including FQ- and TC-resistant pathogenic bacteria of relevance for human and animal health (Antunes et al., 2012).

Environmental Impact Assessment

The already-mentioned data regarding occurrence and fate of antibiotics are crucial in order to improve EIA in a way to evaluate health, ecological, and economic consequences. The majority of prioritization lists of pharmaceuticals are based on the concept of EIA, which takes into account the potential effect of a given pharmaceutical and its exposure level (Ginebreda et al., 2010). For that, RQ might be a useful tool, as previously found (Giger et al., 2003; Ginebreda et al., 2010; Golet et al., 2002). However, the replacement of PEC for MEC allows evaluating risks posed by pharmaceuticals in a more realistic scenario (Pereira et al., 2015; Santos et al., 2013; Silva et al., 2014; Vazquez-Roig et al., 2012).

It should be taken into account that the choice of data obviously affects the outcome. PNEC values (together with assessment factors used) and RQ deemed for each analyte are shown in Table 5. According to our results, CIP presented RQ higher than 1 (2.851) regarding algae; therefore, risk might be expected for this trophic level. In addition, a certain risk might be expected for enrofloxacin with an RQ calculated for algae between 0.1 and 1, more precisely, 0.473. Algae appeared to be the most sensitive trophic level. As far as it is known, scarce information is available on individual ecotoxicity of these compounds; however, it is noteworthy that, given their environmental presence in mixtures, and given their similar pharmacological mechanisms, additive or even synergistic effects may occur and thus the real hazard may be greater than that calculated.

This risk evaluation has its limitations given the lack of toxicological studies available; nonetheless, such evaluation, according to EMA guidelines (EMA, 2006), represents a contribution to assess the ecotoxicological risk posed by these pharmaceuticals to aquatic organisms.

CONCLUSIONS

An analytical methodology based on the use of SPE/LC-MS^n, developed and validated for determination and quantification of NOR, CIP, OXY, ENR, DIFL, and SARA, was shown to be sensitive, accurate, and reliable for estimation in aquaculture waters and surrounding surface waters. According to our preliminary results in Portuguese aquaculture, antibiotic concentrations decreased in the following order: NOR, ENR, CIP, and OXY. NOR presented an overall average concentration of 24.6 ng/L. Since NOR is exclusively for human

TABLE 5. Maximum Environmental Concentrations (MEC), PNEC, and RQ for Algae, Daphnids, and Fish for the Studied Antibiotics

Pharmaceutical	MEC (ng/L)	PNEC (ng/L) algae	RQ algae	PNEC (ng/L) daphnids	RQ daphnids	PNEC (ng/L) fish	RQ fish
Ciprofloxacin	19.1	6.7[a, (Yang et al. 2008)]	2.851	653,000[a, (Martins et al. 2012)]	0.000	13,131,424[a, b]	0.000
Difloxacin	n.d.	242,410[a, c]	—	2,490,990[a, b]	—	2,271,464[a, b]	—
Enrofloxacin	23.2	49[a, (Robinson et al. 2005)]	0.473	567,000[a, (Park and Choi 2008)]	0.000	4,922,627[a, b]	0.000
Norfloxacin	75.1	10,400[a, (Eguchi et al. 2004)]	0.007	298,800[a, (Isidori et al. 2005)]	0.000	20,081,000[a, b]	0.000
Sarafloxacin	n.d.	16,000[a, (Lützhøft et al	—	3,228,030[a, b]	—	3,016,736[a, b]	—
Oxytetracycline	11.9	170[a, (Isidori et al. 2005)]	0.070	226,400[a, (Isidori et al. 2005)]	0.000	110,100[a, (Park and Choi 2008)]	0.000

Note. n.d., Not detected.
[a]AF = 1000.
[b]LC50 was estimated with ECOSAR.
[c]EC50 was estimated with ECOSAR.

use, its presence might be attributed to a wastewater discharge or misuse in animal production.

Algae was the trophic level more sensitive to these antibiotics, and CIP presented RQ greater than 1 (2.851), suggesting this trophic level might be at risk. Since data on the use of antibiotics in aquaculture are scarce, it is of great importance to continue these studies in other aquaculture systems, in the surrounding aquatic environment, sediments and fish, to better assess contamination in aquaculture fish and its environmental impact in Portugal. This will enable minimization of growing pressures due to the increase of this important economic activity. Even if at trace levels, emergence of antibiotic resistance is a reality and a major public health issue, and risk mitigation measurements would only be effective if monitoring programs are implemented. These results will contribute, at the European Union (EU) level, for promotion of prudent use of antimicrobials.

SUPPLEMENTAL DATA

Supplemental data for this article can be accessed at http://dx.doi.org/10.1080/15287394.2015.1036185

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