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Evaluation of antimicrobials residues in farmed gilthead seabream (*Sparus aurata*) after administration through medicated feed

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ABSTRACT

The use of antimicrobials in aquaculture is a well-known fact and merits the focus of the scientific community. In the present study, five drugs (oxytetracycline, sulfadiazine, trimethoprim, oxolinic acid and flumequine) were selected to assess their retention in muscle tissues from gilthead seabream (*Sparus aurata*). Fish were placed in 150 L tanks at 18 °C, and fed for 7 days with experimental diets containing two concentrations of each antimicrobial (ranging from 5.51 to 131.16 mg kg^{-1}). Edible tissues were then analyzed through a validated multi-class quantification method (UHPLC-MS/MS). The results indicate that sulfadiazine concentrations were the highest immediately after the feeding period and decreased towards day 3. Flumequine was only detected on the first day with concentrations below the MRL. Both trimethoprim and oxolinic acid concentrations were below the MRLs 3 days after the feeding period was over (oxolinic acid was not detected in muscle samples at day 14 for prophylaxis and day 28 for both treatments). Oxytetracycline residues in muscle tissues were the highest through time, with concentrations above the MRL for 7 days (C_{day7} of 111.2 and 157.2 μg kg^{-1} for both dosages). Results suggest that these antimicrobials can be present in gilthead seabream muscle samples for longer periods than previously reported, when realistic conditions are tested. With the exception of oxytetracycline, concentrations were below the MRLs established 3 days after the feeding trial was over meaning that adverse effects related to human consumption are not likely. Nevertheless, allergic reactions or resistance to antimicrobials can be developed if low concentrations of such compounds are ingested on a frequent basis, as is the case of the Mediterranean diet.

1. Introduction

Fish continues to be one of the most traded food commodities worldwide, with increasing consumption per capita from an average of 9.9 kg in the 1960s to 19.2 kg in 2012 (FAO, 2014). In order to face the demand for seafood products and also driven by the fact that wild life stocks are reaching their limits (Goldburg & Naylor, 2005), aquaculture systems have experienced unprecedented growth (Sapkota et al., 2008), with much more intensive cultivation methods (Goldburg, Elliott, & Naylor, 2001). Concomitantly, infectious diseases pose a risk to production since they may cause problems to animal welfare as well as significant stock losses (Romero, Feijoó, & Navarrete, 2012), regardless of the hygienic conditions (Rigos, Bitchava, & Nengas, 2010). In order to prevent and solve disease outbreaks in culture ponds, the use of antimicrobials among other chemicals is characteristic in these intensified methods (Romero et al., 2012; Sapkota et al., 2008). Fish farmers tend to rely on the most cost effective method, which is oral administration through formulated feeds, with the drug either incorporated into food pellets or coated on the outside of the pellet, making this administration route the most used in aquaculture

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production.

Among the antimicrobials used in aquacultures worldwide, oxytetracycline was the most administered drug in top producing countries, followed by oxolinic acid and chloramphenicol, present in 69% of the countries examined by FAO (versus 92% that use oxytetracycline) (Sapkota et al., 2008). Oxytetracycline is part of the tetracyclines family and is considered by many authors the most used antimicrobial in aquaculture (e.g. Alday-Sanz, Corsin, Irde, & Bondad-Reantaso, 2012; Rigos & Smith, 2013). It presents broad spectrum activity effective against diseases caused by Gram-positive and Gram-negative bacteria and even against some anaerobic organisms (Burridge, Weis, Cabello, Pizarro, & Bostick, 2010; Serrano, 2005). This antimicrobial is widely prescribed in the treatment of fish, poultry, pigs and cattle, and was reported in 11 out of 15 of the top aquaculture producing countries (Sapkota et al., 2008). Flumequine and oxolinic acid were two of the most frequently administered antimicrobials in Norwegian aquaculture (Ellingsen, Midttun, Rogstad, Syvertsen, & Samuelsen, 2002), but their importance on human medicine has led to their prohibition in aquaculture in the United States, Scotland and Canada (Burridge et al., 2010). They present low minimum inhibitory concentrations for most fish pathogens and show good tissue penetration when administered orally in medicated feed (Martinsen & Horsberg, 1995; Samuelsen, 2006). Sulfonamides are another class of antimicrobials administered in aquaculture although at a lower extension, but the combination of sulfadiazine and trimethoprim (as potentiated sulfonamides) is used in one third of the main producing countries (Sapkota et al., 2008). Both compounds are heavily used as veterinary drugs due to their low cost and wide range against Gram-positive and Gram-negative bacteria (Suzuki & Hoa, 2012).

Gilthead seabream (*Sparus aurata*) is according to the Federation of European Aquaculture Producers the most farmed species in the Mediterranean region, with an estimated production of 129,000 tons (Valente et al., 2011). Together with seabass, these species account for the gross production in Southern Europe, representing 94% of marine fish aquaculture production, attaining a value of 300,000 tons in 2015 (FEAP, 2016). Several diseases such as pasteurellosis or the most common vibriosis are common in the production of these species and, are in most cases, treated with antimicrobials. Improving production conditions in terms of hygiene and good disinfection can help prevent such problems, but antimicrobials are still commonly used.

The present work aimed to increase the available information on carcass retention of gilthead seabream in response to antimicrobials supplemented in manufactured diets, using a validated multi-residue detection method for oxytetracycline (OTC), sulfadiazine (SDZ), trimethoprim (TRI), oxolinic acid (OXO), and flumequine (FLU); and to increase the knowledge on dose dependency, by using both prophylactic and therapeutic concentrations.

2. Materials and methods

2.1. Materials and reagents used

Oxytetracycline, sulfadiazine, trimethoprim, oxolinic acid and flumequine used in formulation of experimental diets were purchased from Sigma-Aldrich (Madrid, Spain). All reagents used in the extraction method were of analytical grade (or HPLC grade for solvents used in the mobile phase). The internal standards used were demethyltetracycline for tetracyclines, sulfameter for sulfonamides and trimethoprim, and lomefloxacin for quinolones,; also provided by Sigma-Aldrich.

An UHPLC system coupled to a triple quadrupole tandem mass spectrometer was used for chromatographic separation and MS detection for the quantification in muscle samples. The column used in the system was a reverse-phase Acquity HSS T3 2.1 × 100 mm, 1.8 μm particle size. Mobile phase consisted in ultrapure water acidified with 0.1% formic acid (solvent A) and acetonitrile (solvent B). The gradient used flowing at a rate of 0.45 mL min^{-1} was as follows: 97%–40% (A) in the initial 5 min, from 5 to 9 min the solvent A was reduced to 0%, maintained for 1 min and then back up to 97% (A) in 1 min; from 10 to 12 min the system maintained the original composition, with 97% of solvent A and 3% of solvent B. The mass spectrometer detector was a Xevo TQ MS – Acquity UPLC system (Waters, Milford, MA, USA), and data were analyzed using the Masslynx 4.1 software also by Waters.

For antimicrobial quantification in feed, an Agilent 1100 Series LC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a triple quadrupole tandem mass spectrometer Sciex API 2000 (Applied Biosystems, Foster City, CA, USA) was used. Data were analyzed by Sciex Analyst software. Chromatographic separation was achieved with an Agilent Zorbax XDB C18 2.1 × 100 mm, 3.5 μm particle size column with an Agilent Zorbax XDB C8 4 × 2.1 mm, 5 μm guard column. Mobile phase composition was the same, with the gradient going from 97% (A) to 10% and then finishing again at 97%, with a total run time of 11 min.

2.2. Experimental diets

A control diet was formulated according to the nutritional requirements of juvenile seabream. Main ingredients were ground (<250 μm), mixed in a horizontal helix ribbon mixer (Mano, 100 L capacity, CPM, San Francisco, USA) and dry pelleted using a laboratory pellet press (CPM, C-300, San Francisco, USA) with a 2.4 mm die. Diets were stored at 5 °C until posterior utilization. Samples of each diet were taken for proximate composition. Two composite samples of each experimental diet were taken and chemical analyses performed according to the method described by AOAC (1990). Samples were then pooled and moisture content was determined (105 °C for 24 h). Samples were analyzed for crude protein (N x 6.25, Leco Nitrogen analyser, Model FP-528, Leco Corporation, St. Joseph, USA), crude lipid content by petroleum ether extraction (Soxtherm Multistat/SX PC, Gerhardt, Koenigswinter, Germany; 40–60 °C), gross energy in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany). The basal diet was formulated to be isonitrogenous, isolipid and isoenergetic and presented 96.9% dry matter (DM), 43.8 (% DM) crude protein, 16.9 (% DM) crude fat, and 22.4 MJ kg^{-1} DM gross energy.

This basal mixture was used as the basis to create 8 experimental diets. The same formulation was used but antimicrobials (Oxytetracycline, OTC; Oxolinic Acid, OXO; Flumequine, FLU; Sulfadiazine, SDZ; Trimethoprim, TRI) were added as premixes in the form of powders during the manufacturing process. These diets differ from each other in concentration (two) and type (four) of antimicrobial incorporated into each, corresponding to either prophylactic (P) or therapeutic (T) dosages (Table 1).

Experimental diets were formulated taking into account the most used antimicrobials in aquaculture and dosages set accordingly, and duplicates of each one were analyzed for antimicrobial exact concentration.

2.3. Experimental conditions

The experimental trial was conducted by trained scientists following category C recommendations from the Federation of European Laboratory Animal Science Associations (FELASA) (Guillen, 2012) and the European Directive 2010/63/EU of European Parliament and of the Council of European Union on the protection of animals used for scientific purposes.

Table 1
Formulation of diets used in the experiment. Ingredients are expressed in percentage of the total formulation and antimicrobial concentration in mg kg^{-1} (mean values ± SE). The following dosages were prepared, for prophylactic and therapeutic treatments, respectively: Diet CTRL – no antimicrobial; Diet OTC – 37.5 mg OTC kg^{-1} (P) and 75 mg OTC kg^{-1} (T); Diet MIX – 110 mg SDZ kg^{-1} (P) and 22 mg TRI kg^{-1} (P), 220 mg SDZ kg^{-1} (T) and 44 mg TRI kg^{-1} (T); Diet OXO – 6 mg OXO kg^{-1} (P) and 12 mg OXO kg^{-1} (T); Diet FLU – 6 mg FLU kg^{-1} (P) and 12 mg FLU kg^{-1} (T). Please refer to footnote for information on components.

Ingredients (%)	Diet								
	CTRL	OTC		MIX		OXO		FLU	
		P	T	P	T	P	T	P	T
Fishmeal 70 LT	10								
Fishmeal 65	20								
Corn gluten	11								
Soybean meal	16								
Rapeseed meal	7								
Sunflower meal	5								
Wheat meal	6								
Pea starch	6								
Fish oil	15								
Vit & Min Premix[1]	1								
Lutavit E50[2]	0,1								
Hilyses[3]	0,5								
Betaine	0,5								
Soy lecithin	0,5								
Binder[4]	0,5								
Antioxidant[5]	0,2								
L-Lysine	0,5								
DL-Methionine	0,2								
Antibacterials (mg kg^{-1})									
Oxytetracycline	–	5,51 (0,43)	16,49 (1,12)	–	–	–	–	–	–
Sulfadiazine	–	–	–	70,02 (2,58)	131,16 (5,52)	–	–	–	–
Trimethoprim	–	–	–	20,96 (1,05)	39,76 (1,67)	–	–	–	–
Oxolinic Acid	–	–	–	–	–	7,04 (0,03)	14,06 (1,39)	–	–
Flumequine	–	–	–	–	–	–	–	6,38 (1,07)	17,91 (0,83)

[1] Mineral and vitamins premix. Covered nutritional requirements of seabream (Supplied by SPAROS Lda. Olhão, Portugal).
[2] Vitamin E acetate; Premix, Portugal.
[3] Hydrolyzed Yeast; Premix, Portugal.
[4] Guar gum; Sorgal, Portugal.
[5] Rosamox – rosemary extract, Kemin; Italy.

This trial was performed at the Experimental Research Station (Vila Real, Portugal) at the University of Trás-os-Montes e Alto Douro (UTAD) facilities. Juveniles of *S. aurata* presented an average weight of 75.5 ± 1.1 g and were randomly distributed into 9 fiberglass tanks (1 for CTRL diet + 4 for OTC, MIX, OXO, FLU prophylactic diet + 4 for OTC, MIX, OXO, FLU therapeutic diet) of 150 L water capacity each, with 26 fish in each tank. The tanks are part of a circulating saltwater system unit with partial renewal of water and temperature control. Water oxygen, temperature and quality were regularly monitored. Mean water temperature was 17.8 ± 0.6 °C during the experimental feeding period and oxygen saturation was over 90%. Ammonia, pH, nitrites, and nitrates were maintained within the recommended limits for the species. A 12:12 h light:dark cycle was applied during the trial. Fish were acclimated to experimental conditions for 15 days, during which time all fish received the control-based (CTRL) diet to apparent visual satiety.

During a 7-day feeding period, fish were fed manually with experimental diets twice a day (9:00 and 17:00), and received a similar daily portion, which varied form 1.3%–1.5% body weight per day. After this feeding period with medicated diets, fish were fed with CTRL diet during the rest of the experiment time. Three fish from each tank were sacrificed with a sharp blow to the head, and muscle samples (dorsal area) were collected from each dietary treatment at days 0, 3, 5, 7, 14 and 28. Samples of muscle tissue were frozen in liquid nitrogen and stored at −80 °C until further use.

2.4. Antimicrobial analyses

The extraction and analyses of antimicrobials in muscle tissues was conducted according to the validated multi-class quantification method developed by our group (Freitas et al., 2014). Limits of detection and quantification for each compound were determined as $X_0+K.\sigma_0$ and $X_0+10\sigma_0$, respectively (Relacre, 2000). *Sparus aurata* muscle samples from the dorsal area were weighed (2.0 ± 0.09 g) and homogenized in 15 mL Falcon tubes. A solution of internal standards with 10 μg mL^{-1} was added (20 μL), vortex mixed and allowed to stand in the dark for at least 10 min. Afterwards, a simple solvent extraction was performed by vortex mixing and shaking the sample with 5 mL of acetonitrile and 1 mL of 0.1 M EDTA using a Reax shaker for 20 min, followed by a centrifugation at 3100*g* for 15 min. The supernatant was transferred to a new tube and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The residue was dissolved with 400 μL of 0.1% formic acid, filtered through a 0.45 μm PVDF Mini-uniprep™ and injected into the UHPLC-MS/MS under MRM-optimized conditions for each compound (for details on the Multiple Reaction Monitoring acquisition conditions for each antimicrobial used, see Freitas et al., 2014). Quantification of antimicrobials concentrations in feed were performed by weighing ~1.0 g of sample, followed by a simple extraction with 50:50 water:acetonitrile. Confirmation was made running triplicate samples in the UHPLC system described above, and the measured concentrations can be found in Table 1.

2.5. Data treatment

The results of the antimicrobial concentrations in gilthead seabream for muscle tissues were reported as mean ± SE. The elimination time curve was analyzed by a non-linear regression analysis (Microsoft® Excel Analysis Toolpak), assuming a first-order

kinetics with equation $C(t) = C_0e^{-\beta t}$. The elimination half-life ($t_{1/2}$) of antimicrobials for muscle was calculated by $t_{1/2} = \ln 2/\beta$, where β is the elimination rate constant, obtained from the elimination curve equations (Baggot, 1997). Area under the curve (AUC) was determined following the trapezoidal rule, and was extrapolated to infinity (Ritschel, 1986).

3. Results

Mean concentrations of sulfadiazine, trimethoprim, flumequine, oxolinic acid and oxytetracycline retained in fish muscle tissues for three replicates at each sampling day and for both concentrations used are present in Table 2. Antimicrobial analyses were performed after experimental conditions were finished.

Except for FLU, antimicrobials showed similar patterns of presence and degradation through time, with first-order elimination kinetics best describing the antimicrobials tested (R^2 values for prophylactic and therapeutic dosages, respectively, are 0.6161 and 0.5531 for TRI, 0.5606 and 0.6449 for SDZ, 0.7537 and 0.5828 for OXO, 0.9163 and 0.8654 for OTC). Flumequine was only detected immediately after the feed administration period, with an average concentration of 13.6 μg kg^{-1} for prophylactic and 36.0 μg kg^{-1} for therapeutic dosages at day 0. Such concentrations are below MRL limits established for fin fish (600 μg kg^{-1}), and only corresponded to 0.2% of the initial concentration present in the administered pellets. Trimethoprim also presented very low percentages of antimicrobial retention in muscle tissues, with 0.48 and 0.71% for prophylactic and therapeutic dosages, respectively. Oxolinic acid concentrations in muscle samples at day 0 corresponded to 2.00 and 2.11% of the initial concentrations present in feed. Sulfadiazine and oxytetracycline presented the highest percentages of carcass retention on day 0, with 5.20 and 6.57% of the feed concentration for SDZ and 4.63 and 6.22% for OTC, for prophylactic and therapeutic doses, respectively.

Sulfadiazine concentration in edible tissues remained higher than the MRL (100 μg kg^{-1}) for at least 3 days after the feeding period for the therapeutic dose, and the mean concentrations were the highest detected among all antimicrobials. The other compound tested – trimethoprim – only presented concentrations higher than the MRL established (50 μg kg^{-1}) on the last day of feed administration, decreasing 4 and 8 times their concentration from day 0–3, for prophylactic and therapeutic doses, respectively. Oxolinic acid presented the same behavior, significantly decreasing from 140.7 μg kg^{-1} (prophylactic) and 296.5 μg kg^{-1} (therapeutic) to 5.6 μg kg^{-1} and 11.1 μg kg^{-1} from day 0 to day 3. Oxytetracycline presented concentrations higher than the MRL established for at least one week after medicated feed administration, with concentrations higher than 100 μg kg^{-1} for both dosages tested. As expected, AUC from zero to infinity of SDZ was the highest of the antimicrobials tested with a value of 14601.3 μg kg^{-1} d^{-1}, since the feed pellets were the most concentrated (versus 10876.5 μg kg^{-1} d^{-1} for OTC in therapeutic dose; see Table 3). Considering the prophylactic treatment, OTC presented the highest values of AUC from zero to infinity, despite having lower initial concentrations than SDZ present on the medicated feed (7051.2 and 6492.9 μg kg^{-1} d^{-1}, respectively). Such values are due to OTC high retention in muscle samples through time. Despite presenting values lower than the MRL established for most of the days sampled, on the last sampling day, only FLU and OXO on the prophylactic dose were below the limit of detection. As expected, fish fed with CTRL diet did not present any antimicrobial concentrations in muscle tissues in all samples analyzed.

4. Discussion

Although feeds were formulated to have dosages ranging from 6 to 220 mg kg^{-1}, we observed that measured concentrations of antimicrobials in feed ranged from 5.51 to 131.16 mg kg^{-1}. The highest differences were observed in SDZ and OTC diets, where concentrations were 70.02 and 131.16 mg kg^{-1} for 110 and 220 mg kg^{-1} dosage, respectively, for SDZ and 5.51 and 16.49 mg kg^{-1} for 37.5 and 75 mg kg^{-1} dosage, respectively, for OTC. Diets were formulated in order to present the best homogeneity possible, therefore adding the drug in the beginning of the mixing process. Heat and humidity involved in the pelleting process could possibly degrade some compounds to a certain degree (Daniel, 2009, pp. 85–94). This process allowed nevertheless the correct mixture of the compounds, and despite concentrations being lower than expected, concentrations were similar in all replicates analyzed for confirmation.

Flumequine was only present in the initial sampling time (day 0), with very low concentrations in gilthead seabream muscle. The concentration administered was too low to establish the pharmacokinetics of the antimicrobial through time, both for prophylactic and therapeutic dosages. Plakas et al., (2000) detected lower residue concentrations of FLU in channel catfish muscle in comparison to liver, where concentrations were ~4x higher, which could indicate higher concentrations also in liver in the present study. Moreover, concentration peaks in all major tissues analyzed were observed 12–24 h after administration, and half-lives of 22 h were registered for oral dosing. The results obtained were in accordance with other studies for FLU pharmacokinetics in seawater species, with the antimicrobial rapidly decreasing to half of its initial concentration on Atlantic salmon muscle tissues during the first hours of administration (e.g. 17.6–21.3 h and 1.3 h following oral administration (Elema, Hoff, & Kristensen, 1994; Rogstad, Ellingsen,

Table 2
Antimicrobial concentrations in gilthead seabream (*S. aurata*) muscle samples (μg kg^{-1}). Concentrations obtained following oral administration for Prophylactic and Therapeutic treatments (mean values ± SE, n = 3). Values in bold indicate concentrations above the MRL established by the Commission Regulation (EU) No 37/2010 (100, 50, 600, 100 and 100 μg kg^{-1} for SDZ, TRI, FLU, OXO and OTC, respectively).

Sampling day	SDZ		TRI		FLU		OXO		OTC	
	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
	1.20	4.00	0.10	0.21	0.70	2.37	0.74	2.45	5.0	8.0
	P	T	P	T	P	T	P	T	P	T
0	**3640.4 ± 810.0**	**8616.6 ± 3441.3**	**101.5 ± 1.9**	**281.6 ± 46.2**	13.6 ± 9.2	36.0 ± 10.5	**140.7 ± 29.8**	**296.5 ± 116.1**	**255.3 ± 36.8**	**1026.4 ± 47.5**
3	94.2 ± 26.0	**246.0 ± 123.8**	25.2 ± 7.2	33.2 ± 11.7	<LOD	<LOD	5.6 ± 1.29	11.1 ± 0.8	**216.8 ± 26.2**	**395.0 ± 64.8**
5	15.6 ± 1.1	65.7 ± 8.5	15.7 ± 1.3	48.9 ± 16.8	<LOD	<LOD	1.5 ± 0.1	2.5 ± 1.0	**141.7 ± 32.6**	**232.4 ± 81.0**
7	19.3 ± 1.0	18.4 ± 6.9	18.3 ± 2.0	13.5 ± 2.5	<LOD	<LOD	1.4 ± 0.6	0.7 ± 0.3	**111.2 ± 34.2**	**157.2 ± 27.5**
14	9.4 ± 1.2	15.0 ± 2.3	18.2 ± 1.7	18.7 ± 4.0	<LOD	<LOD	<LOD	0.7 ± 0.3	37.8 ± 8.0	63.8 ± 8.2
28	4.2 ± 0.3	4.4 ± 0.5	8.1 ± 0.2	10.0 ± 2.2	<LOD	<LOD	<LOD	<LOD	23.0 ± 1.0	34.6 ± 10.6
	MRL 100		MRL 50		MRL 600		MRL 100		MRL 100	

Table 3
Calculated pharmacokinetic parameters of orally administered antimicrobials in gilthead seabream (*S. aurata*) at 18 °C.

	SDZ		TRI		FLU		OXO		OTC	
	P	T	P	T	P	T	P	T	P	T
Dosage (mg kg^{-1})	110	220	22	44	6	12	6	12	37,5	75
Feed concentration (mg kg^{-1})	70,02	131,16	20,96	39,76	6,38	17,91	7,04	14,06	5,51	16,49
C day0 (μg kg^{-1})	3640,4	8616,6	101,5	281,6	13,6	36,0	140,7	296,5	255,3	1026,4
Antibiotic day0 (%)	5,20	6,57	0,48	0,71	0,21	0,20	2,00	2,11	4,63	6,22
$AUC_{0-\infty}$ (μg·h mL^{-1})	6492,9	14601,3	3450,1	3264,2	–	–	253,1	526,6	7051,2	10876,5
Clearence (mL h·g^{-1})	0,0108	0,0090	0,0061	0,0122	–	–	0,0278	0,0267	0,0008	0,0015
Elimination rate constant	0,1730	0,2060	0,0640	0,0860	–	–	0,3360	0,1860	0,0890	0,1100
$t_{1/2}$	4,01	3,36	10,83	8,06	–	–	2,06	3,73	7,79	6,30

& Syvertsen, 1993)), and 3.1 h following intravenous administration (Martinsen & Horsberg, 1995). Furthermore, Rogstad et al. (1993) stated that more than 98% of the administered dose of FLU was distributed within a period of 10 h. Nevertheless, these authors have detected FLU up to 5 days after administration, contrarily to the results obtained in the present work, which could be explained by the fact that they used oral doses 4 times higher than in the present study. Also, Tyrpenou, Kotzamanis, and Alexis (2003) obtained half-lives of 22.14 and 21.43 h at 18 and 24 °C, respectively, but the presence of FLU was detected 168 h after feed administration, which again might be related to the concentrations used (~6x higher than prophylactic dosage used). This rapid depletion in fish muscle was previously described, for gilthead seabream (Malvisi, Rocca, Anfossi, & Giorgetti, 1997), with FLU concentrations below limit of quantification 48 h after the end of medication. These results were also observed in other study on gilthead seabream, with FLU reaching levels below the limit of detection at the 2nd and 4th day after administration, for 19.5 and 14 °C, respectively (Romero González, Fernández, Vidal, Muros, & Garrido Frenich, 2010). It has also been suggested that skin and bones can act as sink for quinolones, slowly releasing it to other tissues during longer periods of time (Malvisi et al., 1997). Our results indicate that, considering FLU, shorter sampling times should be considered in order to determine the pharmacokinetics of this chemical. Changing the sampling times might also increase the R^2 values of the first-order kinetics model since we could observe a more gradual decrease in residues concentration through time.

Regarding oxolinic acid, it was detected up to day 7 for prophylactic and day 14 for therapeutic dosages. Despite presenting initial concentrations similar to flumequine, oxolinic acid was present in fish tissues during longer periods, although in concentrations much lower than the MRL established by the Commission Regulation 37/2010. The highest values detected in fish muscle were similar to previous studies on gilthead seabream (Rigos, Nengas, Alexis, Tyrpenou, & Troisi, 2003). In a study comparing the pharmacokinetics after intravenous and oral administration, Rigos et al., (2002) did not detect OXO in the 128 h after oral administration. However, concentrations were significantly different considering administration routes (AUC values of 134.99 and 26.75 μg h^{-1} mL^{-1} for intravenous and oral, respectively), with OXO being detected in muscle only when i.v. administration was followed. The decrease in OXO concentration from day 0 to day 3 followed the same accentuated decrease, even after a multiple 10 day in-feed administration, as in other work on the same species (Rigos, Nengas, Alexis et al., 2003). Furthermore, the presence of OXO in muscle tissues might be conditioned by the unfavorable pH in the digestive tract of marine fish (Daniel, 2009, pp. 85–94; Rigos et al., 2002).

Levels of sulfadiazine remained above the MRL for 3 days, contrarily to a previous study following a multiple dosing for 5 days (Rigos et al., 2013). According to Rigos et al., (2013), sulfadiazine did not present an accumulative drug profile, decreasing its concentration even during the medication period, as reported in a similar work assessing the presence of sulfadimethoxine and ormetoprim residues in gilthead seabream (Papapanagiotou, Batzias, Iossifidou, & Psomas, 2002). The realistic concentrations used in the present work were sufficient to maintain relatively high SDZ levels, while previous studies showed a much faster depletion of SDZ in edible tissues of gilthead seabream (below limit of quantification after 1 (Papapanagiotou et al., 2002) and 4 days (Rigos et al., 2013) following administration). Trimethoprim is frequently used together with SDZ as potentiated sulfonamides, acting in synergy and blocking two sequential steps in the synthesis of bacterial folic acid (Romero et al., 2012). For this reason, studies on the depletion of trimethoprim *per se* in fish tissues are scarce, but resistance to this antimicrobial is present in wastewaters and sewages sludge all over the world (Kümmerer, 2009). Also, TRI concentrations are usually measured in association with other sulfonamides, lacking specific information on the retention of this antimicrobial in edible fish tissues.

Results obtained with trimethoprim and oxytetracycline might indicate that concentrations measured in muscle samples at the 1st day depended on the initial dosage administered (therapeutic concentrations led to ~40% higher percentage of retention at day 0, while no differences were found in FLU and OXO treatments for example). Studies addressing only one concentration in gilthead seabream (Malvisi, Rocca, Anfossi, & Giorgetti, 1996; Rigos, Nengas, Tyrpenou, Alexis, & Troisi, 2003, 2011; Tyrpenou et al., 2003) are not sufficient for comparison, since our study suggests that higher dosages can lead to higher percentages of antimicrobial retained in edible tissues and should therefore increase the withdrawal times for these antimicrobials. A recent review made by Rigos and Smith (2013) stated that such dose dependency, although easily found in crustaceans, is not well established for fish, taking into account existing studies. However, pharmacokinetic parameters can vary according to dose regimen, with multi or single oral dosage presenting different parameters, and even among seabream species differences can be registered (Rigos & Smith, 2013; Rigos, Nengas, Tyrpenou, et al., 2003, 2004). Further studies on the depletion of antimicrobials in fish muscle tissues should be addressed using different dosages in order to establish the existence of such correlation.

Sparus aurata plays a major role in the economics of Mediterranean countries such as Portugal, Spain and Greece, and information on the carcass retention of antimicrobials is imperative. Furthermore, it has been demonstrated that antimicrobials presence in edible tissues can change if we consider different fish species, sizes, temperatures, freshwater or seawater and experimental protocols such as administration routes and dosage regimen (e.g. Hansen & Horsberg, 2000; Ishida, 1992; Rigos &

Smith, 2013; Rigos et al., 2002, Rigos, Nengas, Tyrpenou, et al., 2003, 2004, 2011, 2013; Samuelsen, 2006), being of vital importance to address the parameters for such high-value species, and following the administration method most common in aquaculture. In a similar work, Romero González et al., (2010) addressed the depletion of antimicrobials in gilthead seabream. Overall, our results are in accordance with previous studies, but concentrations of OTC and OXO were detected in muscle tissues for longer periods. Also, the study of different concentrations used is of vital importance in order to understand alterations of the pharmacokinetic parameters.

Legislation is not always available some countries, and withdrawal times for different antimicrobials are scarce. Potentiated sulfonamides are part of the approved aquaculture drugs with respective withdrawal times, where Romet-30®, a combination of sulfadimethoxine/ormetoprim is labeled with an withdrawal time of 42 days for salmonids and 3 days for catfish. Despite giving shorter withdrawal times for catfish due to skin removal, these periods are not sufficient for residues degradation. According to our results, sulfadiazine when used for therapeutic treatment is still present in edible tissues in concentrations higher then the MRL established by the Commission Regulation (EU) No 37/2010.

Oxytetracycline, as one of the most used antimicrobials worldwide for aquaculture, has some indications from the US Food and Drug Administration Agency, stating that "*Withdrawal times vary with indication as follows: for marking skeletal tissue in Pacific salmon, 7 days; for disease control in salmonids, 21 days; catfish, 21 days; lobster, 30 days*" – information on Terramycin® 200 for Fish. Although without information for basses, our results suggest that even following the withdrawal times prescribed, oxytetracycline residues will be present up to fish sale or consumption. Moreover, the Pacific salmon indication of withdrawal time is 7 days, which according to the present results show levels of OTC above the maximum residue limit allowed by legislation.

Oxytetracycline concentrations in feed were the lowest of the prophylactic dosages and second lowest of the therapeutic dosages. Nevertheless, concentrations detected in muscle samples were above the MRL up to 7 days after the end of the feeding period, with a clearance value of 0.0008 and 0.0015 $\mu g \cdot h\ mL^{-1}$ for prophylactic and therapeutic dosages, respectively. Even when concentrations in muscle samples are below the MRLs established, drug residues can still be detected through longer periods of time.

5. Conclusion

The present study provides updated and reliable data on the retention of antimicrobials, with conditions similar to aquaculture practices, since most studies addressing oral administration use forced feeding, which can reduce oral bioavailability and loss of appetite, as well as stress in animals. The relatively low concentrations used in the medicated feed were chosen according to the real dosages used in medicated pellets. With the exception of FLU, concentrations were detected and measured in edible tissues for longer periods than previously reported in other studies. MRLs are established to prevent consumer intake to exceed the toxicological values of chemicals used in feed and food products, but the presence of residues in foodstuff can generate problems of allergy and resistance in humans. Accordingly, withdrawal times are set so that residues are not present above the MRLs when products are sold or consumed. In areas such as the Mediterranean, where fish consumption is a major source of animal protein, caution should be taken in order to minimize exposure to antimicrobials. Understanding the pharmacokinetics of each individual species with the most used feeding method in aquaculture is vital to understand exposure pathways to these contaminants.

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