Residue depletion and histopathological alterations in gilthead sea bream (Sparus aurata) after oral administration of oxytetracycline

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ABSTRACT
Aquaculture is a key component of the animal food industry, but intensive farming conditions increase the incidence of infectious diseases. Oxytetracycline (OTC) plays a major role for infectious diseases in fishes. Its MRLs include their 4-epimers, so in this trial, the depletion of residues of OTC and 4-epioxytetracycline in muscle and liver have been evaluated in gilthead sea bream (Sparus aurata) after oral administration. Hepatotoxicity has been investigated with histopathological effects on target tissues. A validated DAD-HPLC with SPE extraction has been applied. Residual levels in muscle and liver depleted with a similar k\text{ol} but mean retention time and t\text{ret} resulted longer in muscle than in liver because of different vascularization. The OTC concentrations were below the LMR at 48 h after dosing. No analytical peaks ascribable to 4-epi-OTC or other derivatives were detected, while histopathology of liver showed degenerated parenchymal hepatocytes, nuclear pyknosis, focal necrosis and inflammatory leucocytes infiltration. It can be concluded that the assessment of pharmacokinetic and residual depletion of antibiotics result fundamental to determine the most suitable therapeutic regime and to minimize the toxic effects in fish species.

1. Introduction
With the expansion of cultured fish and shellfish species, aquaculture has become a key component of animal food industry as one of the most promising and sustainable farming alternatives (FAO 2018; Choi et al. 2020). Gilthead sea bream (Sparus aurata) and sea bass (Dicentrarchus labrax) are among the most valuable fish species in the Mediterranean and have now become the leading species of marine aquaculture (Bronzi et al. 2001): EU consumption for sea bream amounts to 107,300 tons (2015 data) and 96% of the European supply depends on aquaculture, with Italy being the largest market, followed by Greece and Spain. The Italian gilthead sea bream aquaculture is based on two main breeding systems, sea net pens and ground systems (EUMOFA – European Market Observatory for Fisheries and Aquaculture Products 2019). The intensive farming system is usually characterized by crowding stress, poor water quality, low feed quality as well as high mortality rate of fishes. Therefore, it is necessary to use pharmaceutical and/or biological products to control possible diseases (Scarano et al. 2018). Antibiotics have been largely used in aquaculture as therapeutic and prophylactic agents, especially in developing countries where the farmers lack knowledge on the proper use of antibiotics (Chuah et al. 2016; Preena et al. 2020).

In aquaculture, farming drugs are mainly delivered through medicated feed, but this method of administration can result in not fully ingested feed and/or in many cases in poorly absorbed and metabolized drugs, with the consequence of a ubiquitous dispersal of antibiotics in aquatic systems. For this reason, measurements must be enacted to prevent subsequent harm to the environment; in fact, antibiotics are biologically active at low concentrations, able to enter the food chains, act as potential micropollutants of the environment, and are currently categorized as emerging environmental contaminants of concern (Boonsaner and Hawker 2013; Rodrigues et al. 2017; Colussi et al. 2018; Higuera-Llantén et al. 2018; Leal et al. 2019).

Constant exposure to antibiotics for the treatment of bacterial diseases in aquaculture has led to their omnipresence in the environment, with a continuous selective pressure on the microbial community, the development of an environmental reservoir of resistant mutants, the increase in the horizontal transfer of antibiotic resistance genes (ARGs) and the emergence of antimicrobial resistance in wild organisms (Girgorakis and Rigos 2011; Chuah et al. 2016; Higuera-Llantén et al. 2018; Preena et al. 2020). Considering the current situation, antibiotic resistance must be addressed from perspectives of human and animal health, and it is, according to the General Assembly of the United Nations, a priority topic, being on par with global warming (Higuera-Llantén et al. 2018). Finally, medicated feed affects the fish microbiota (Zhou et al. 2018) Microbiomes are thought to have evolved to optimize the immune response and promote homeostasis, and the disruptions of this equilibrium (dysbiosis) can lead to an increase of opportunistic pathogens and disease susceptibility (Rosado et al. 2019).
To date, only few drugs are approved in Italy for use in aquaculture such as amoxyclillin, flumequine, sulfadiazine-trimetoprim, oxytetracycline (OTC) (Agnetti et al. 2008). OTC plays a major role as first-choice antibiotic for the treatment of main fish diseases as furunculosis, vibriosis, yersiniosis, pseudotuberculosis, pseudomonas and columnarisis (Malvisi et al. 1996; Yonar 2012; Chuah et al. 2016). The OTC is a member of tetracyclines (TCs) broad-spectrum antibiotic produced by Streptomyces spp. with bacteriostatic action. It acts interfering with bacterial protein synthesis (mRNA translation) by binding to the bacterial 30S ribosomal subunit of the 70S ribosomes, compromising protein synthesis (Rigos et al. 2004). Its popularity derives from its demonstrated efficacy, low cost, and the possibility of administration via the oral route as medicated food (3.5–7.5 g/kg feed) or topical treatment of water (Agnetti et al. 2008). The OTC is poorly absorbed through the intestinal tract of fish (more than 20% of the total dosage orally administered to farmed fishes may pass unabsorbed to the environment), because of complexion with Ca²⁺ and Mg²⁺ that reduces the bioavailability, but its absorption is rapid; it is poorly metabolized or unmetabolized, with a large percentage (up to 70–80%) excreted in its active form via urine and faeces (Leal et al. 2019).

The actual MRL for OTC in fish species have been stated by the Commission Regulation (EU) No 37/2010 of 22 December 2009. For the complete recovery of the compounds, the Commission stated that also the 4-epimer of OTC have to be determined in fished species (Rigos, Nengas et al. 2004). Its popularity derives from its demonstrated efficacy, low cost, and the possibility of administration via the oral route as medicated food (3.5–7.5 g/kg feed) or topical treatment of water (Agnetti et al. 2008). The OTC is poorly absorbed through the intestinal tract of fish (more than 20% of the total dosage orally administered to farmed fishes may pass unabsorbed to the environment), because of complexion with Ca²⁺ and Mg²⁺ that reduces the bioavailability, but its absorption is rapid; it is poorly metabolized or unmetabolized, with a large percentage (up to 70–80%) excreted in its active form via urine and faeces (Leal et al. 2019).

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2. Materials and methods

Animal care and handling were performed according to the provisions of EU Directive 2010/63 and in compliance with the 3Rs principles (replacement, reduction, refinement) for experimental protocols implying the use of live animals. The study was approved by the Bioethics Committee of the University of Bari Aldo Moro, Italy.

A total of 120 gilthead sea bream, 12 months old and 200–230 g body weight, in a known disease-free status (normal behaviour and feed consumption, absence of physical abnormalities), were divided into two experimental groups (Group A consisting of 90 subjects and Group B of 30 subjects) and maintained in two 6 m³ fibreglass tanks receiving water from deep well with abundant hot groundwater (Itrica Caldoli, Sannicandro Garganico, FG, Apulia, Italy) in RAS (Recirculated Aquaculture System) with treatment of wastewater in natural lagoon. Water was supplied to the tanks at a flow rate of 8 L/min (temperature 21.3°C, salinity 16‰). Oxygen was kept close to saturation by oxygenation systems with O₂ dissolution (Tecnos S.a.S, Chioggia, VE, Italy).

The fishes were fed daily, twice a day at a rate of 2% biomass with commercial dry pelleted feed (NutriTech, Villimpenta MN Italy): crude protein 50%, crude fat 16%, crude cellulose 1.10%, crude ash 11.5%, total carbohydrates 12.4%, phosphorus 1.50%, vitamin C 400 mg/kg, vitamin E 300 mg/kg, digestible energy 18.46 MJ/kg. The fishes were allowed to acclimate for 2 weeks before starting the trial. During the entire period the mortality was <1% (in total 6 subjects – 5 from Group A and 1 from Group B – died for accidental causes).

2.1. Animals

Group A (90 subjects) was used for the study on the residual depletion of OTC. Group B (30 subjects) was used for the test on the toxicity of OTC.

2.1.1. Residue depletion and metabolism after a single dose

After the acclimation period, the animals were starved for 2 days, then treated by oral administration of OTC medicated feed (Pescemed NutriTech, Villimpenta, MN, Italy) at 75 mg kg⁻¹ of OTC hydrochloride (Leal et al. 2017), at a rate of 2% biomass for 1 d. The animals were sacrificed at 0 (control) before administration of medicated food, and at 2, 12, 24, 36, 48, 72, 96, 144, 288, 336, 432 h, respectively. At each sampling time, five animals were sacrificed (total animals = 60). The study following the VICH (Veterinary International Conference on Harmonization) guidelines. Muscle and liver
were immediately collected, frozen at −18°C, transferred to the laboratory and analyzed for the presence of residues of OTC and 4-epi-OTC.

2.1.2. Toxicity after long term treatment
The second experiment started at the end of experiment 1 and the rest of gilthead sea beams (25 subjects) of Group B were used as a control group. Standard protocols of OTC include a maximum dosage of 100 mg kg⁻¹ for a maximum of 14 days of treatment (Leal et al. 2017), so Group B was treated by oral administration of OTC medicated feed (Pescemed NutriTech, Villimpenta, MN, Italy) at 100 mg kg⁻¹ of OTC hydrochloride at a rate of 2% biomass for 14 days and 10 subjects were sacrificed 24 h after treatment (360 h) for histopathological evaluation of liver. Samples of liver and muscle were immediately collected and frozen at −18°C and transferred to laboratory.

The fishes in both experiments were euthanized in accordance with AVMA (American Veterinary Medical Association) guidelines (Balko et al. 2018; Leary et al. 2020).

2.2. Evaluation of toxicity
Liver and gills are considered suitable organs for histological examination in order to determine the effects of pollution since they easily respond to environmental xenobiotics exposure. In the present trial, the system of water supply to the tanks provides 50% from deep well and 50% from lagoon, where the low depth of the recirculating waters guarantees a good photodegradation of the OTC (Grigorakis and Rigos 2011; Boonsaner and Hawker 2013; Botelho et al. 2015; Choi et al. 2020) and the presence of vegetation and seaweeds operates a conspicuous phytoremediation (Rosa et al. 2019). For these reasons, residual OTC concentration from fishes excretion and/or release from uneaten feed in the tank was considered negligible, so only the oral route of exposure was considered, and after dissection of fishes tissue specimens of liver were collected and fixed in buffered formaldehyde (4%).

2.3. Analytics
The analyses were performed according to the validate method for DAD-HPLC described by Evaggelopoulou et al. (2012) with few adaptations. Analytical standards: OTC hydrochloride was obtained from Sigma-Aldrich Chemical Co. (Milan, Italy); 4-epi-OTC was obtained from ACROS Organics (Springfield, NJ).

Solvents and water: Acetonitrile, water (both of HPLC grade), and methanol (analytical grade) were obtained from LAB-SCAN Analytical Sciences (Labscan Limited, Dublin, Ireland). Other reagents: EDTA disodium salt dihydrate, oxalic acid dihydrate, citric acid and trisodium citrate were obtained from Sigma-Aldrich Chemical Co. (Milan, Italy). All reagents were of analytical-reagent grade.

SPE cartridges Oasis R® HLB (200 mg/6 mL) were used for the removal of any endogenous interference stemming from gilthead seabream matrix. Ultrapure water provided by a Milli-Q R® purification system (Millipore, Bedford, MA, U.S.A.) was used throughout the study.

2.4. Instrumentation
HPLC system: Programmable Solvent Module mod. 126, Diode Array detector 168, autosampler 508 Beckman, and Software 32 Karat Beckman (Beckman Instruments, Inc.). HPLC column: Synergi 4 µ Hydro-RP 150 × 4.60 mm i.d. (Phenomenex, Torrance, CA).

2.5. Chromatographic conditions
The mobile phase was delivered according to a gradient curve, starting at a composition of 95% in 0.001 M Na₂EDTA and 5% in ACN, isocratic for the first 10 min, changing to 83:17 v/v (0.001 M Na₂EDTA/ACN) until 38 min and finally to 81:19 v/v (0.001 M Na₂EDTA/ACN) until 45 min. The flow rate was stable at 1.5 mL/min until the end of the curve. Caffeine (CAF) at 2.5 ng/mL was used as internal standard. The injected sample volume was 50 µL.

Mobile phases were prepared daily, filtered through a 0.45 µm filter (phase filtration apparatus with membrane filters – nylon filter 66 – 0.45 µm × 47 mm – Supelco Inc., Supelco Park, Bellefonte, PA) and degassed under vacuum. Detection was set at 355 nm.

2.6. Standard solutions
Methanolic stock standard solutions of OTC, 4-epi-OTC and caffeine (1 mg/mL) were prepared from reference standards, then stored at 4°C in absence of light. Working methanolic standards were prepared from stocks by the appropriate dilution at 0.1, 0.5, 1, 2, 4, 8, 10, 15, 20 and 25 ng/µL, containing IS at 2.5 ng/µL. These were freshly prepared every day from stocks. Calibration levels of TCs in gilthead seabream tissue were prepared by fortifying drug-free samples with standard solutions. The buffer solution for the leaching of antibiotics was prepared by mixing 20.5 mL of 0.1 M citric acid, 29.5 mL of 0.1 M citric tri-sodium salt and 50 mL H₂O (pH 4.0). Intermediate and working solutions were prepared daily and protected from light.

2.7. Sample preparation and extraction
The extractions were performed according to the method described by Evaggelopoulou et al. (2012) (Evaggelopoulou et al. 2012). After evaporation to dryness at 35°C under a gentle stream of N₂, the residue was reconstituted in 500 µL of MeOH. The sample was then filtered through a 0.45 µm syringe filter (Chromafil Einzelfilter type 0–45/15, 0.45 µm × 15 mm – Mackery-Nagel, Duren, Germany), protected from light and transferred to autosampler vials.

2.8. Method validation
The developed method was validated according to the performance characteristics of European Commission Decision 2002/657/EC using spiked gilthead seabream tissue. Linearity, accuracy, precision and sensitivity were examined using spiked tissue samples at various concentrations.

Linearity was checked by constructing the calibration curves using spiked drug-free gilthead seabream tissue samples at
concentration levels in the range 0.1–25 µg/kg. Calibration curves for OTC and 4-epi-OTC with the respective correlation coefficient were calculated by least-squares linear regression analysis of the peak area ratio of each analyte to IS. The calculations for the limits of detection (LOD) were based on the standard deviation of y-intercepts of regression analysis (r) and the slope (S), using the equation LOD = 3.3σ/|S|.

The selectivity of the analytical method in terms of the absence of interference from endogenous compounds for each matrix was checked by comparison of blank and spiked samples extracted with the reported procedure.

The recovery was assessed by comparing the concentrations of OTC and 4-epi-OTC in the spiked samples after extraction with the known amounts of drugs added before the extraction. For each matrix, this is expressed as a percentage and reported as the mean ± SD of 12 replicates for each concentration tested (1.0, 0.5, 0.25 µg/g or mL). The mean recovery is expressed as the mean ± SD of 36 determinations (3 concentrations/12 replicates).

Precision and accuracy expressed in terms of recovery from gilthead seabream tissue samples were studied by analyzing spiked samples at the concentrations of 0.5, 1 and 1.5 times the permitted limit according to the European Decision for OTC and epi-OTC.

Precision of the chromatographic method was expressed as the repeatability under the same operating conditions over a 1-day time interval (intra-assay precision) and over a 6-day period (inter-assay precision). It was calculated as the relative standard deviation (coefficient of variation) of the mean of the values recorded after extraction (quadruplicate) of each matrix spiked at three different OTC/4-epi-OTC concentrations over a 1-day (intra-assay precision) or 6-day (inter-assay precision) period.

To test the possibility of occurrence of 4-epi-OTC as artefact of extractive procedures, the same procedure has been applied to OTC standard in water.

The linearity of the method after extraction from spiked samples was tested by calculating the regression line with the least square method, using the data from the inter-assay evaluation.

The decision limit, CCa, was calculated by analysis of 20 samples as the mean measured concentration at the MRL and the LOQ level plus 1.64 times the SD of three consecutive measurements at these concentrations. The detection capability, Ccb, was calculated as CCa plus 1.64 times the SD of three consecutive measurements at these concentrations. Statistical analysis was performed at the 95% confidence level and the number of replicate analyses was 20. Peak identification was performed by spectral information provided by the diode array detector.

### 2.9. Pharmacokinetic analysis

OTC residues concentrations to time in muscle and liver were analyzed using a software (WinNonlin vs 4.1; Pharsight Co., Mountain View, CA, U.S.A.) that provides compartmental and non-compartmental analyses of the experimental data.

The following pharmacokinetic parameters were derived by the non-compartmental analysis of the raw time–concentration data computed by the program: the half-life value (t1/2) and the elimination rate constants (keln) in muscle and liver; the area under the curve (AUC), calculated by the trapezoidal method from the time of dosing and extrapolated to infinity (AUCinf) and the extrapolated percent from the last sample time to infinity (AUCextr/obs), the mean residence time extrapolated to infinity (MRT). Peak concentrations (Cmax) and peak times (Tmax) in muscle and liver were expressed as experimental data. Pharmacokinetic variables are expressed as means ± standard errors (SE).

### 2.10. Histopathology

Samples of liver were fixed in buffered formaldehyde (4%), then dehydrated using ethanol solutions of increasing degree (70%, 80%, 90%, and 100%). This was followed by embedding in paraffin wax (56–58°C) using an automatic tissue processor. Five-micrometer sections were cut with a 2030 Biocut microtome (Reichert-Jung, Germany). The sections were then stained with standard haematoxylin and eosin stain and examined under a light microscope (Leica, DM LB2, Germany).

### 3. Results

In the described chromatographic conditions, the retention times (mean ± SE) of 4-epi-OTC and OTC were 9.35 ± 0.02 min and 10.82 ± 0.02 min, respectively. The calibration curve of the detector response was linear over the concentration range selected (0.1–25 µg/kg). The regression line equation of OTC was $y = 0.832x + 0.012$, with a correlation coefficient equal to 0.9998; for 4-epi-OTC the regression line equation was $y = 0.828x + 0.019$, with a correlation coefficient equal to 0.9998.

The analytical method proved to be specific for both the checked matrix and no interfering peaks were detected in the blanks of the matrixes at the retention times of the two analytes.

The accuracy of the recoveries and the mean recovery obtained for each matrix, as well as the precision of the different methods, expressed by the relative standard deviation (coefficient of variation) calculated over a 1-day (intra-assay precision) or a 6-day period (inter-assay precision) are reported in Table 1.

In the samples obtained from the extraction of standard solutions of OTC in water, 4-epi-OTC was not detected as artefact of OTC manipulations.

All the chromatograms of OTC spiked tissues showed no detectable peak eluting at the retention time of 4-epi-OTC, indicating that this compound was not originated as degradation derivative during sample extraction.

The method proved to be linear after the analyses of blank tissue samples fortified with OTC and 4-epi-OTC. The equations for the regression lines, correlation coefficients, LODs and LOQs for the analytes and the matrixes are reported in Table 2.
3.1. OTC residual depletion

OTC and 4-epi-OTC residues in liver and muscle of gilthead sea bream after treatment Table 3 (mean + SE) and Figure 1, while pharmacokinetic parameters are reported in Table 4.

In all the chromatograms of spiked and incurred samples, both of muscle and liver, no detectable peak eluting at the retention time of 4-epi-OTC was reported.

3.2. Histopathology

Macroscopically, no lesions were present on the skin while internal organs, but gills and muscles were pale (Figure 2).

Liver sections showed degenerated parenchymal hepatocytes, nuclear pyknosis, focal necrosis and inflammatory changes (leucocytes infiltration) (Figure 3).

4. Discussion

The validation parameters of the adopted analytical procedures are compliant with EU Decision 2002/657/EC. Thus, the procedure can be easily applied to any surveillance program of monitoring TCs residues in fish from aquaculture and proved suitable for the intended purpose of this trial.

The results obtained in the depletion residue study show higher levels of the parent drug in the liver, while it has been Table 1. Recovery accuracy and precision of OTC and 4-epi-OTC determinations in sea bream matrixes spiked with different concentrations of analytes.

| Matrix  | μg/g | Recovery % + SE | Mean recovery + SE | Accuracy | Mean reading + SE | RSD % |
|---------|------|-----------------|-------------------|---------|-----------------|-------|
| Muscle  |      |                 |                   |         |                 |       |
| 1       |  95.58 + 2.43 |              | 91.33 + 7.46      | 28.42   | 0.47 + 0.02     | 4.19  |
| 0.5     |  91.69 + 4.17 |              | 28.31             | 0.20 + 0.01 | 4.80       | 0.21 + 0.01 | 5.81 |
| 0.25    |  88.00 + 11.14 |             | 22.00             | 0.10 + 0.01 | 4.41       | 0.11 + 0.02 | 14.29 |
| Liver   |      |                 |                   |         |                 |       |
| 1       |  98.76 + 2.76 |              | 96.66 + 9.06      | 29.24   | 0.45 + 0.03     | 7.32  |
| 0.5     |  97.87 + 6.63 |              | 18.13             | 0.24 + 0.02 | 8.10       | 0.23 + 0.02 | 6.46 |
| 0.25    |  95.27 + 19.85 |             | 11.73             | 0.12 + 0.03 | 22.48      | 0.11 + 0.02 | 15.05 |

Table 2. Linearity parameters of the method after extraction of OTC and 4-epi-OTC from liver and muscle of gilthead sea bream.

| Tissue | Regression line equation | R | LOD (μg/kg) | LOQ (μg/kg) | Regression line equation | R | LOD (μg/kg) | LOQ (μg/kg) |
|--------|--------------------------|---|-------------|-------------|--------------------------|---|-------------|-------------|
| Muscle | y = 1.931x + 0.0666       | 0.961 | 17.5 | 37.9 | y = 2.024x – 0.0228     | 0.965 | 9.8 | 28.9 |
| Liver  | y = 2.003x + 0.0085       | 0.9821 | 19.6 | 54.2 | y = 1.972x + 0.0416     | 0.9962 | 11.7 | 35.2 |

Table 3. Concentrations of OTC and 4-epi-OTC in liver and muscle of gilthead sea bream after oral administration of 75 mg/kg.

| Time (h) | OTC | Epioxytetracycline |
|---------|-----|-------------------|
|         | Mean | SE    | Mean | SE    |
| 0       | 0    |       | 0    |       |
| 2       | 49.18 | 7.75  | b.d.l. |       |
| 12      | 63.97 | 23.38 | b.d.l. |       |
| 24      | 82.12 | 25.12 | b.d.l. |       |
| 36      | 130.88 | 25.69 | b.d.l. |       |
| 48      | 83.30 | 2.72  | b.d.l. |       |
| 72      | 50.45 | 8.26  | b.d.l. |       |
| 96      | 36.04 | 2.14  | b.d.l. |       |
| 144     | b.d.l. |       | b.d.l. |       |
| 288     | b.d.l. |       | b.d.l. |       |
| 336     | b.d.l. |       | b.d.l. |       |
| 432     | b.d.l. |       | b.d.l. |       |

Table 4. Pharmacokinetic parameters of OTC and 4-epi-OTC in liver and muscle of gilthead sea bream.

| Time (h) | OTC | Epioxytetracycline |
|---------|-----|-------------------|
|         | Mean | SE    | Mean | SE    |
| 0       | 0    |       | 0    |       |
| 2       | 488.13 | 47.88 | b.d.l. |       |
| 12      | 655.19 | 35.17 | b.d.l. |       |
| 24      | 752.97 | 96.29 | b.d.l. |       |
| 36      | 581.78 | 130.14 | b.d.l. |       |
| 48      | 382.25 | 111.96 | b.d.l. |       |
| 72      | 366.98 | 61.79 | b.d.l. |       |
| 96      | 113.62 | 24.19 | b.d.l. |       |
| 144     | 44.28 | 11.58 | b.d.l. |       |
| 288     | 32.70 | 8.75  | b.d.l. |       |
| 336     | 197.38 | 88.71 | b.d.l. |       |
| 432     | b.d.l. |       | b.d.l. |       |

SE: standard error.
not possible to detect analytical peaks ascribable to 4-epi-OTC or to other degradation derivatives of OTC.

After the oral administration, absorption and distribution begin very precociously, OTC residues being detectable in both matrixes at the first sampling time (2 h), but they reach the Cmax only at 20 and 36 h after dosing for muscle and liver, respectively, suggesting a slow absorption/distribution phase.

The available literature on OTC pharmacokinetics in gilthead sea bream following a single intravascular injection of OTC (40 mg/kg) in 100 g fishes kept at 20°C, reported a distribution half-life ($t_{1/2}$) in plasma of 2 h. This resulted in a slow distribution from the blood compartment to the tissues of sea bream, longer than those published for sea bass at 22 or 13°C (0.2–1 h) and kept at 18°C (1 h) (Rigos et al. 2003). In the same study, the apparent volume of distribution of OTC at a steady state (Vd(ss)) (3.72 l/kg) indicated an adequate distribution throughout the body from the blood compartment of this species. This can be considered as a favourable kinetic profile of OTC when treating bacterial pathogens localized in poorly vascularized areas such as skin or muscle (Rigos et al. 2003).

The elimination half-life ($t_{1/2}$) from sea bream muscle has been reported to be longer (38 h) than in Chinook salmon (15 h) and sea bass (16–24 h) (Albedini et al. 1998; Rigos et al. 2002), suggesting a need for longer withdrawal periods prior to use dosed fishes for human intake. In the present trial, muscle residues were found to be below the MRL at 48 h, while liver residues, even if this is not to be considered as an edible tissue in fish species, were below the MRL at the 4 d post-dosing.

Concentration levels of liver showed a biphasic time–concentration curve, with a second peak detected on the 14th days after dosing, suggesting the possibility of an enterohepatic circulation of the drug, as in mammalian species (Prescott 2000).

The total residue levels in both tissues depleted with a similar $k_{el}$ (0.02 ± 0.01 and 0.028 ± 0.01 L/h for muscle and liver, respectively), but the mean retention time (MRT) (495.45 ± 19.19 and 267.06 ± 6.25 h, respectively) and $t_{1/2\beta}$ (43.84 ± 15.89 and 25.12 ± 2.76 h, respectively) were considerably longer in muscle than in liver. This suggested that the elevated and persistent OTC residual levels in liver could be caused by a rapid turnover of the drug, due to its broad vascularization, instead of accumulation or storage processes. On the other way, muscle residues are more slowly eliminated due to the scarce vascularization of this tissue.

As far as 4-epi-OTC is concerned, although the limited knowledge of the real toxicological importance of epimers, many authors have contrasting results and theories on their origin. The TCs are traditionally considered to undergo minimal, if any, metabolism in man and animals, and the 4-epimers are supposed to be only degradation products, artefacts/breakdown results of extractive procedures. The parent TCs compounds can rapidly isomerize at the C-4 dimethylamino group under acidic conditions (pH 2–6) in aqueous solutions and are reported to be in reversible equilibrium with the resulting 4-epimers (Blanchflower et al. 1997; Croubels et al. 1998; Kennedy et al. 1998). It has also been postulated the transformation of OTC in its 4-epimer in rainbow trout muscle during prolonged (30–60 days) freezing periods,
because of the slightly acidic pH of fish muscle (Tantillo et al. 1997). In the present trial, the absence of detectable peak eluting at the retention time of 4-epi-OTC in all the chromatograms of OTC spiked tissues indicated that 4-epi-OTC does not originate from sample manipulation as artefact of extractive procedures. Moreover, its absence also in all the incurred tissues samples suggests the absence of in vivo formation as a residue of metabolic origin in fish species.

Histopathology findings of liver slices showed degenerated parenchymal hepatocytes, nuclear pyknosis, focal necrosis and inflammatory changes (leucocytes infiltration). Pyknotic nucleus refer to an irreversible condensed form of chromatin material in the nucleus, which usually indicates highly severe and irreversible damage of liver tissue (Rodrigues et al. 2017). It is well known that TCs induce pyknosis by triggering cellular apoptosis mediated through the release of pro-apoptotic factors from the mitochondria, resulting in chromatin condensation and nuclear disassembly. However, pyknotic nucleus are considered reversible in fish species if the toxic agent is removed (Rodrigues et al. 2017). In fact, TCs induce pyknosis by activating calpains and consequently trigger cellular apoptosis by releasing pro-apoptotic factors (Ca2+-dependent intracellular cysteine proteases) from the mitochondria resulting in chromatin condensation and nuclear disassembly (Guerra et al. 2016). The observed hepatocellular degeneration process can be associated with oxidative stress, where lipid peroxidation is a clear source of membrane bilayer susceptibility (Ribeiro Oliveira et al. 2005). Leukocyte infiltration is a morphological disturbance commonly found in liver of individuals exposed to pollutants. It represents a first line of immunological response due to cell death, associated with hepatocellular degeneration processes and later necrosis (Rodrigues et al. 2017). It is worth mentioning that in this trial the animals were exposed to OTC in therapeutic dosages and times, with the route of administration currently applied in aquaculture practices: toxicity of OTC is generally considered low, while on the contrary it must not be underestimated to ensure the well-being of farmed species.

4. Conclusion
In the present trial, OTC was administered as a single dose in order to evaluate its tissue distribution and elimination parameters. However, to fix the correct suspension times for sea bream, the evaluation should be carried out with repeated administration according to the recommended dosage, so further studies are recommended. The evaluation of toxic effects of OTC was successfully confirmed by histopathological examinations, resulting in a suitable method to validate the consequences of exposure to contaminants. Moreover, it allowed to describe the lesions occurring in fish key organs.

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