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RESEARCH ARTICLE

Accumulation and depletion of oxytetracycline (OTC) and oxolinic acid (OXA) in Pompano, *Trachinotus blochii*

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Accumulation and depletion including withdrawal period for oxytetracycline (OTC) and oxolinic acid (OXA) in pompano (*Trachinotus blochii*) were determined following oral administration. Pompano were cultured in 250-L fiberglass tanks in a flow-through system provided with aeration. Observed average temperature was 30°C; salinity was 30 ppt. Fish were starved for 2 days then fed with OTC medicated diet (75mg/kg fish/day) or OXA medicated diet (30 mg/kg fish/day) 3 times a day for 10 successive days at 2% body weight and thereafter switched to regular diet for 45 days. Muscle and blood samples were taken at regular intervals during and after cessation of medication. OTC residues in the muscle and blood were analysed using the high performance liquid chromatography (HPLC). Peak OTC accumulation was observed at day 10 of treatment. Higher OTC accumulation was observed in the muscle (0.88+0.27 µg/g) than in the blood (0.3+0.09 µg/ml). OXA accumulation peaked on day 5 of treatment; higher OXA accumulation was observed in the muscle (0.11+0.06 µg/g) compared to blood (0.005+0.0001 ug/ml). Withdrawal period at 30°C for OTC in pompano muscle was 19 days (570 degree-days) and 17 days (510 degree-days) in the blood. For OXA, the withdrawal period in pompano muscle and blood at 30°C temperature was 3 days (90 degree-days).

Introduction

Pompano, *Trachinotus blochii*, is one of the high-valued food fish being cultured in the Philippines and has great potential for market abroad. The fish is promoted for culture in mariculture parks in the Philippines. Pompano is cultured in marine cages and pens, or in net cages inside earthen ponds. Pompanos are fed with either trash fish or commercial pellets until they reach marketable size of 400 to 800g in 4 to 8 months. Incidence of bacterial infection in *Trachinotus blochii* has been reported (Amal et al. 2012). In the Philippines, *Aeromonas, Staphylococcus, Streptococcus*, and *Vibrio* were some of the bacteria reported to infect pompano in the year 2016-2017 (Regidor and Somga 2017). An effective method to control bacterial diseases is through the use of antibiotics. Oxolinic acid (OXA) and oxytetracycline (OTC) are some of the antibiotics used in farmed fish in most Asian countries (Arthur, Lavilla-Pitogo, and Subasinghe 2000). OTC and OXA are also 2 of the antibiotics used in aquaculture in the Philippines based on a survey done in the mid 1990's and in 2009 (Cruz-Lacierda, De la Peña, and Lumanlan-Mayo 2000; Regidor and Somga 2017).

Although antibiotics are used in aquaculture to prevent and treat diseases that affect farmed shrimp and fish, the indiscriminate use of antibiotics could lead to drug resistant strains and multiple antibiotic resistance in bacteria. Oxolinic

acid was detected in the muscles of farmed and wild fish after OXA medication (Samuelsen et al. 1992). Furthermore, Samuelsen et al. (1992) reported an elevated number of OXA resistant bacteria in mussel harvested in the area. Antibiotic resistance may reduce efficacy of antibiotic treatment for human and animal diseases. To avoid this, a proper withdrawal period should be observed. The withdrawal period is the time from the last administration of the drug to the time when residues are below the allowable limit or below the established maximum residue limit (MRL). MRL is the maximum concentration of residue of a substance that may be allowed in food of animal origin. The recommended MRL for OTC in fish muscle is 200ug/kg (FAO FNP 41/14). For OXA, the recommended MRL in fin fish is 100ug/kg (EMEA/CVMP/41090/2005).

The withdrawal period should be considered in planning the time of harvest in order to ensure that aquaculture products are safe for human consumption. Withdrawal period varies with the kind of drug, fish species and water temperature. The withdrawal period for tilapia (*Orechromis niloticus*) cultured in Brazil was determined to be 6 days (Paschoal et al. 2011). A withdrawal period of 9 days was observed in pompano (*Trachinotus blochii*) given a single dose of 60mg/kg cultured at 26-27°C; 15 days in orange-spotted grouper (*Epinephelus coioides*) and seaperch (*Lates calcarifer*) (Chen et al. 2021). Withdrawal period of OXA and OTC in *Trachinotus* cultured at 30°C has not yet been reported. It is important to establish the withdrawal period of OXA and OTC in pompano to ensure the absence of the antibiotics in harvested fish.

This paper describes the accumulation and depletion as well as the withdrawal period of OTC and OXA in pompano, *Trachinotus blochii*.

Materials and Method

Antibiotics

Oxytetracycline hydrochloride (OTC) used here was purchased from Spectrum Chemical Manufacturing Corporation. The antibiotic is 95-102% anhydrous. The common recommended dose for OTC is 75 mg/kg body weight per day for 4 to 10 days (Leal, Santos, and Esteves 2018) given orally through feeds. Thus, the dosage used in the study is 75 mg/kg body weight fish per day for 10 days.

Oxolinic acid (OXA) or 5,8-Dihydro-5-ethyl-8-oxo-1,3-dioxolo[4,5-g]quinoline-7-carboxylic acid used here was obtained from Sigma. The antibiotic is < 100% concentrated. OXA is administered at 10, 20 or 30 mg/kg body weight /day (Samuelsen and Bergh 2004; González, Fernández, and Martínez Vidal 2010) for 5 to 10 days. The maximum reported dosage of 30mg/kg body weight/ day for 10 days was used in the study.

Feed Preparation

Formulated feeds for pompano grow-out were mixed with antibiotic based on the commonly recommended daily dosage: 75 mg/kg fish/day for OTC and 30 mg/kg fish/day for OXA. Briefly, 3.54 g of OTC were mixed with one kilogram of dry feed ingredients, and 1.52 g for OXA. The dry feed mixture was added to pre-cooked binder (wheat flour, added to boiling water in 1:3 flour: water ratio, mixed and allowed to cool) and thoroughly mixed. The dough was extruded in a No. 6 die of manual feed extruder to produce 3 mm diameter feeds. Extruded feeds were air dried and reduced to desired size (5-10 mm) by hand crushing. The medicated feeds were stored at 4°C until use.

Experimental animals

Two hundred and thirty-four disease-free pompano (Average body weight, ABW=74.41g) purchased from a research facility were used in the study. The fish were held, at 13 individuals/ tank, in eighteen 250L - capacity fiberglass tanks, aerated and with flow through seawater system. Temperature in the tanks ranged from 29 to 31°C with an average of 30°C; average salinity of 30 ppt.

Fish were acclimatised for one week using regular commercial pompano diet, and were starved for 2 days prior to medicated-diet feeding. Fish were fed three times a day at 2% body weight. After the 2-day starvation, fish were treated by feeding with either OTC- (75mg/kg fish/day) or OXA- (30 mg/kg fish/day) medicated diet for 10 successive days, and thereafter switched to a regular diet for 45 days. Control fish were fed with pompano regular diet (drug-free). Six tanks were used for each treatment, three tanks for the evaluation of the accumulation pattern and the other three tanks for the evaluation of the withdrawal period.

Fish may not take in the same amount of food; thus, the accuracy of the dosage of antimicrobial taken in by the fish could not be ascertained.

Sample collection

Three fish samples were collected prior to treatment, daily during the 10-day treatment period, and on days 1, 2, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, and 45 after cessation of treatment. OTC and OXA residues were analysed from the blood and muscle of the samples.

A 1 mL heparinised syringe was used to withdraw blood from the caudal veins of fish samples. The withdrawn blood in 1.5 mL Eppendorf tubes were centrifuged at 4,000 rpm for 5 min. Plasma were treated according to the modified method of Elema, Hoff, and Kristensen (1996). Trichloroacetic acid (TCA) for protein precipitation were added to make 3 % effective TCA concentration. The plasma was mixed for 2 min in a vortex shaker and centrifuged at 15,000 rpm at 4°C for 5 min. The supernatant was filtered through a 0.2 μm membrane filter prior to injection in HPLC.

Table 1. High performance liquid chromatography (HPLC) analysis condition and validation parameters.

Conditions	Oxolinic Acid	Oxytetracycline
Extracting solution	Acentronlie	1% metasphoric acid with dichloromethane
Mobile phase	Aceto-MeOH-0.1M citic acid	Aceto-THF-0.025M oxalic acid
Flow rate	0.5ml/min	1.0 ml/min
Injection volume	20µL	20µL
Column	Reverse phase ODS	Reverse phase ODS
Detector	Fluorescence	UV-VIS
Retention time	5.0-5.6 min	8.0-10.0 min
Accuracy	97%	98%
Linear regression coefficients R^2	0.9996	1.0
Limit of detection (LOD)	0.02µg/g	0.05µg/g
Limit of quantification (LOQ)	0.06µg/g	0.15µg/g

The muscle was separated from the whole fish, minced and thoroughly mixed prior to obtaining a 5 g muscle sample from which the drugs were extracted as described below. All fish samples and plasma were kept in a labelled container and stored in -30°C as back-up in case the succeeding steps could not be conducted immediately.

Important water parameters such as temperature and salinity were monitored daily.

Sample analyses

The high performance liquid chromatography (HPLC) was used to detect OTC and OXA residues in the samples. For the OTC assay, the procedure described by Carignan, Carrier, and Sved (1993) was followed. The procedure described by Borlongan and Ng Poh Chuan (2004) was followed for the OXA detection. The HPLC analysis conditions are presented in Table 1.

For the OTC assay, the procedure described by Carignan, Carrier, and Sved (1993) was followed. Briefly, 5 g muscle was homogenised using 1 % meta-phosphoric acid extracting solution with dichloromethane and centrifuged to separate tissue sediment. Excess solvent was removed by rotary evaporation and the final test solution was passed through a 0.2 µm membrane filter prior to liquid chromatographic analysis. Shimadzu High Performance Liquid Chromatograph (HPLC) LC10At system with UV detector set at 355 nm was used for quantitative determination using oxalic acid - acetonitrile – tetrahydrofuran - mobile phase system at a flow rate of 1.0 mL/min across a reversed phase octadecylsilyl (ODS) column. Oxytetracycline standard (Sigma) was used for identification and quantification.

For the OXA Assay, the procedure described by Borlongan and Ng Poh Chuan (2004) was followed. Five gram minced sample was homogenised in acetonitrile, centrifuged and filtered through a 0.2µm membrane filter. A twenty microliter clean sample was injected in Shimadzu HPLC LC10At system with fluorescence detector set at 365nm and 337nm emission and

excitation wavelengths, respectively. Acetonitrile-methanol system was used as the mobile phase at a flow rate of 0.5 mL/min across a reversed phase ODS column. Oxolinic acid standard (Sigma) was used for identification and quantification.

The method was validated for each antibiotic using spiked pompano tissue at different concentration levels in the range 0.02–2.0 μg/mg. The liquid chromatography method employing a UV detector set at 355 nm for the determination of OTC followed that described by Carignan, Carrier, and Sved (1993). The liquid chromatography method employing a fluorescence detector for the determination of OXO followed that described by Borlongan and Ng Poh Chuan (2004). The parameters included were linearity, accuracy, and limit of detection and quantification. Linearity was checked by constructing the calibration curves using spiked drug-free pompano tissue samples. The accuracy of the methods was obtained by calculating the percent recovery (%) of the analyte recovered. The limits of detection (LOD) were calculated by the equation LOD=3.3 σ/S; limits of quantitation (LOQ) were calculated by the equation LOQ=10 σ/S (European Medicines Agency, 2005). Both calculations were based on the standard deviation of y-intercepts of regression analysis (r) and the slope (S). The validation was performed three times.

Data Analysis/ Determination of the withdrawal period

The data on drug concentration was plotted versus time. Succeeding days after the first day the drug was not detected in samples were not included in the plot. Values less than 0.001 were considered zero or undetected. The withdrawal period was calculated based on the equations obtained from the regression curve. This is based on the assumption that linear regression fits the regression model to estimate the withdrawal period as the upper limit of the 95 or 99% tolerance interval (with 95% confidence) (Vranic et al. 2003).

Results

The methods used to quantify OTC and OXA in pompano muscle and blood were validated. The HPLC conditions and validation parameters are presented in Table 1. The method used were shown to be adequate for the quantification of OTC and OXA.

OTC accumulation in pompano muscle and blood during the 10-day feeding with medicated feed is shown in Figure 1. OTC accumulation fluctuated in both types of samples; highest accumulation was observed at day 10 of treatment. Higher OTC accumulation was observed in the muscle (0.88±0.27 μg/g) compared to the blood (0.3±0.09 μg/ml) (Table 2).

OXA accumulation in pompano muscle and blood during the 10-day feeding with medicated feed is shown in Figure 4. OXA accumulation was found fluctuating in both muscle and blood samples. OXA accumulation peaked on

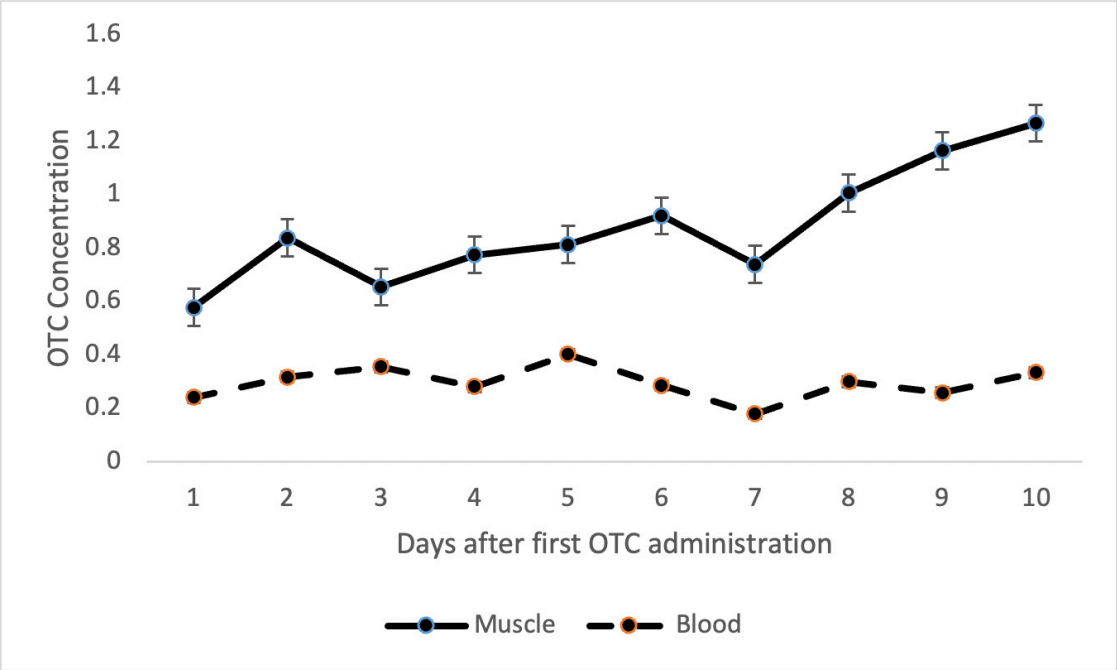

Figure 1. Oxytetracycline (OTC) accumulation in pompano muscle (µg/g) and blood (µg/ml) during drug administration at 30mg/kg .

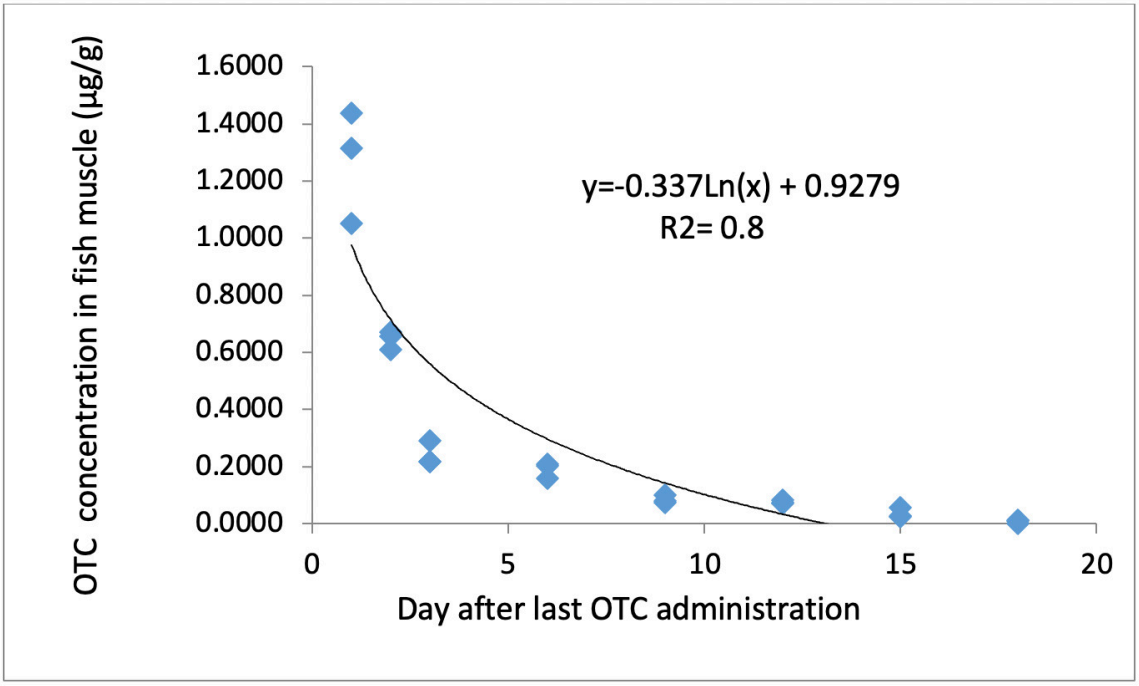

Figure 2. Oxytetracycline (OTC) depletion in pompano muscle after drug administration at 75mg/kg for 10 days.

day 5 of treatment for both samples. Higher OXA accumulation was observed in the muscle (0.11+0.06 µg/g) compared to blood (0.005+0.0001 µg/ml) (Table 3).

Antibiotic accumulation in pompano was higher in OTC than in OXA in both muscle and blood samples (Tables 2 and 3).

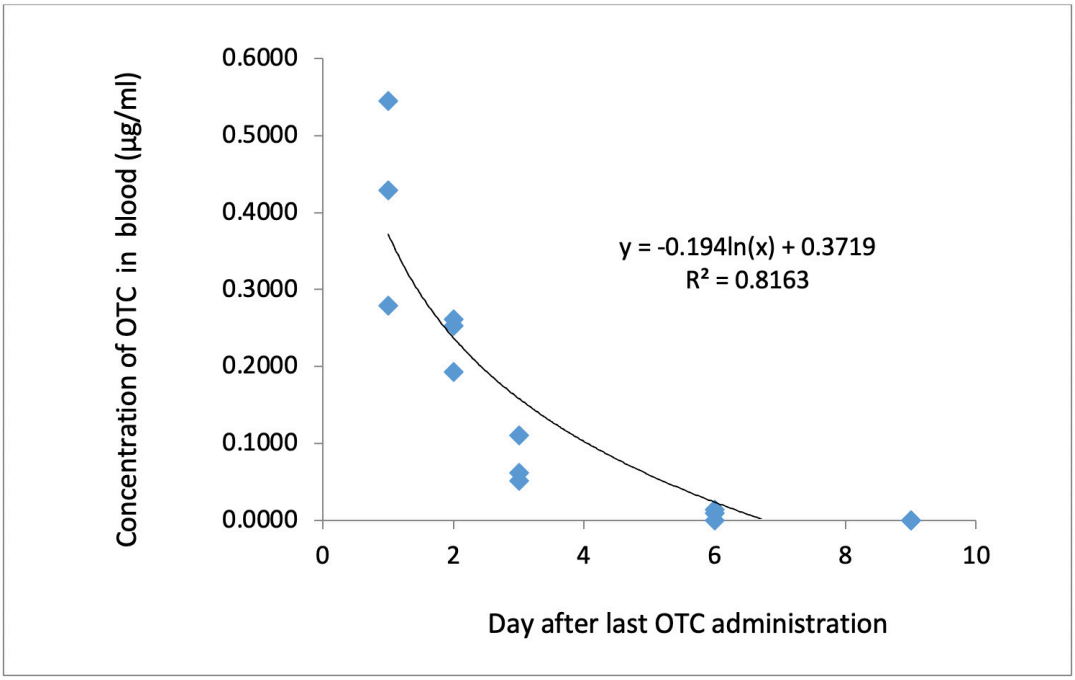

Figure 3. Oxytetracycline (OTC) depletion in pompano blood after drug administration at 30mg/kg for 10 days.

Table 2. Mean Concentration (+ standard deviation) of oxytetracycline accumulation in pompano muscle and blood during oral administration of medicated feed at 75mg/kg for 10 days

	Concentration (n=3)	
Day after first dose	Muscle (µg/g)	Blood (µg/ml)
1	0.58±0.18	0.24±0.1
2	0.84±0.05	0.32±0.04
3	0.66±0.12	0.36±0.07
4	0.77±0.05	0.28±0.04
5	0.81±0.15	0.4± 0.1
6	0.92±0.21	0.29±0.03
7	0.74±0.02	0.18±0.02
8	1.01±0.30	0.3±0.12
9	1.16±0.42	0.26±0.09
10	1.27±0.2	0.33±0.11

OTC-depletion from pompano muscle and blood over time are shown in Table 4, and Figures 2 and 3, respectively. OTC was undetected at day 18 until end of monitoring at day 30 post treatment in the muscle; days 9-30 post treatment in the blood. The regression equation obtained from the plotted OTC concentration vs. time has a coefficient (R^2) of 0.8 both for muscle and blood samples (Figures 2-3). Based on the generated equation, the withdrawal time for OTC in the muscle of Pompano was 18.75 days (approx. 19 days) and 16.80 days (approx. 17days) in the blood. Considering temperature, the withdrawal period for OTC in pompano muscle was 570 degree-days. A lower withdrawal period of 510 degree-days was observed for pompano blood.

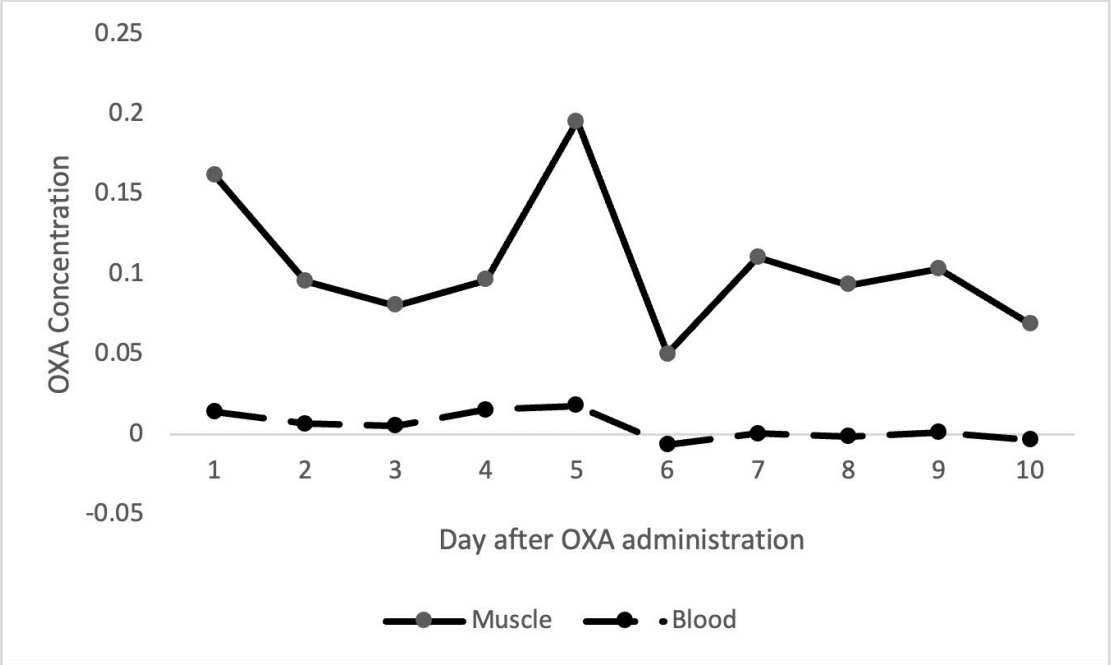

Figure 4. Oxolinic acid (OXA) accumulation in pompano muscle (µg/g) and blood (µg/ml) during drug administration at 30mg/kg .

Table 3. Mean Concentration (+ standard deviation) of oxolinic acid accumulation in pompano muscle and blood during oral administration of medicated feed at 30mg/kg for 10 days

	Concentration (n=3)	
Day after first dose	Muscle (µg/g)	Blood (µg/ml)
1	0.16+0.09	0.01+0.02
2	0.10+0.03	0.01+0.01
3	0.08+0.03	0.01+0.02
4	0.10+0.10	0.02+0.01
5	0.20+0.06	0.02+0.01
6	0.05+0.01	-0.01+0.0
7	0.11+0.05	0.00±0.00
8	0.09+0.03	0.00±0.00
9	0.10+0.06	0.00±0.00
10	0.07+0.04	0.00±0.01

OXA-depletion from pompano muscle and blood over time are shown in Table 5 and Figures 5 and 6, respectively. OXA was undetected from day 2 until day 30 post treatment in the blood of pompano. The regression equation obtained from the plotted OXA concentration vs. time has a coefficient (R^2) of 0.7 and 0.8668 for the muscle and blood, respectively (Figures 5-6). Based on the generated equation, the withdrawal time for OXA in the muscle of pompano was 2.6 days (approx. 3 days) and 2.45 days (approx. 3 days) in the blood. Considering temperature, the withdrawal period for OXA in both the muscle and blood of pompano was 90 degree-days.

Table 4. Mean Concentration (+ standard deviation) of oxytetracycline depletion in pompano muscle and blood after last dose of medicated feed administered at 75 mg/kg for 10 days.

	Concentration (n=3)	
Day after last dose	Muscle (µg/g)	Blood (µg/ml)
1	1.27+0.2	0.42+0.13
2	0.64+0.03	0.24+0.04
3	0.24+0.04	0.07+0.03
6	0.19+0.03	0.01+0.01
9	0.08+0.01	0.00±0.00
12	0.08+0.01	0.00±0.00
15	0.04+0.02	0.00±0.00
18	0.01+0.01	0.00±0.00
21	0.00±0.00	0.00±0.00
24	0.00±0.00	0.00±0.00
27	0.00±0.00	0.00±0.00
30	0.00±0.00	0.00±0.00

Table 5. Mean Concentration (+ standard deviation) of oxolinic acid depletion in pompano muscle and blood after last dose of medicated feed administered at 30mg/kg for 10 days

	Concentration (n=3)	
Day after last dose	Muscle (µg/g)	Blood (µg/ml)
1	0.07+0.04	0.00+0.01
2	-0.01+0.00	-0.01+0.01
3	-0.01+0.00	-0.02+0.0
6	-0.01+0.00	-0.02+0.00
9	-0.01+0.00	0.00±0.00
12	-0.01+0.00	0.00±0.00
15	-0.01+0.00	0.00±0.00
18	-0.02+0.00	0.00±0.00
21	-0.02+0.00	0.00±0.00
24	-0.02+0.00	0.00±0.00
27	-0.02+0.00	0.00±0.00
30	-0.02+0.00	0.00±0.00

Discussion

Accumulation of OTC in the muscle and blood of pompano are high and with fast absorption. The concentration is double the MRL for OTC a day after the medicated feed was given and steadily increased during the 10-day medication period. This suggests that OTC was not metabolised and excreted by the fish during the period the OTC-medicated feed was given. It has been reported that OTC is poorly metabolised or unmetabolised by fish (Leal, Santos, and Esteves 2018).

In contrast to OTC, OXA accumulation is low and unstable. OXA concentration was a little above its MRL a day after OXA-medicated feed was given to the fish; the concentration fluctuated below the MRL, 2 days after medication. This suggests that OXA absorption by pompano is weak and the

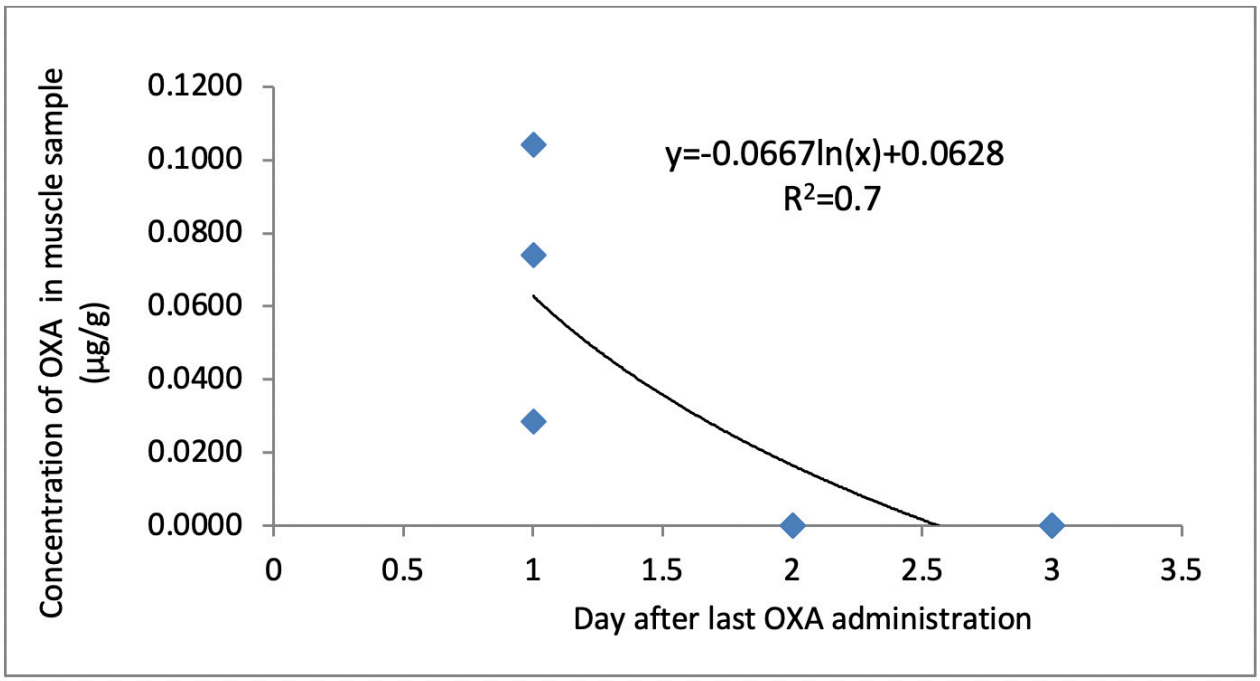

Figure 5. Oxolinic Acid (OXA) depletion in pompano muscle over time.

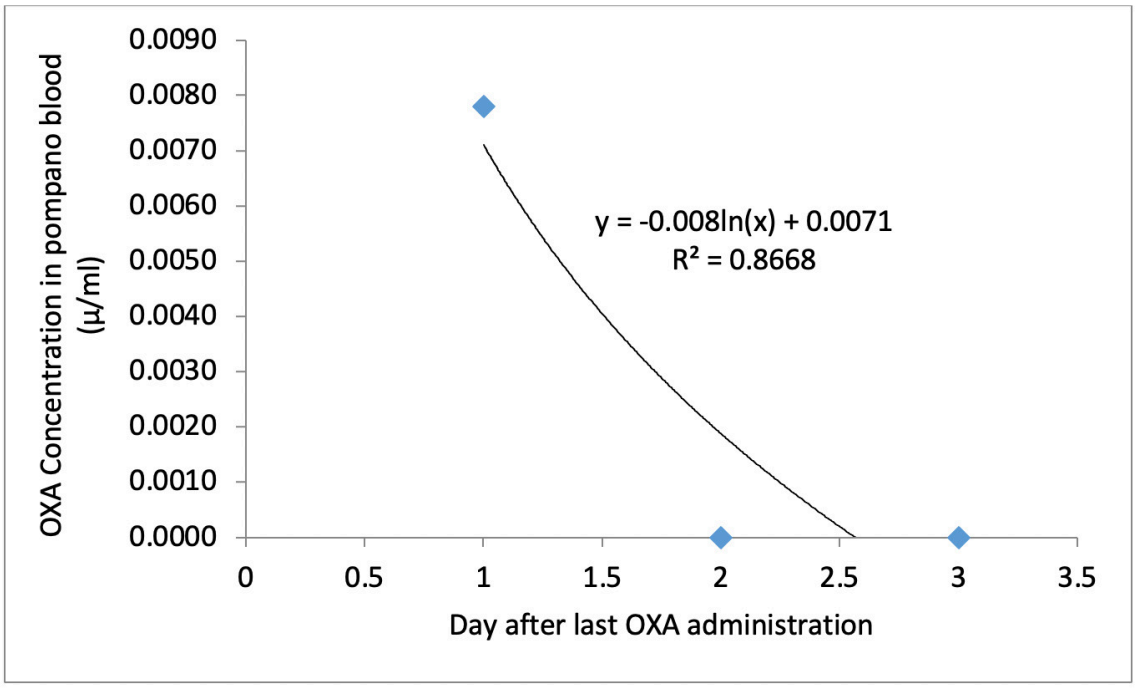

Figure 6. Oxolinic acid (OXA) depletion in pompano blood over time.

drug is easily metabolised by the fish. OXA is a weak acid with a pK value of 6.9, as such, most of the drug will be in an ionised form at seawater pH of 8.1 and thus, less available for absorption (Samuelsen et al. 1992). OXA accumulation and depletion in pompano observed in this study is consistent with the activity of OXA in sharpsnout sea bream wherein a rapid distribution and elimination, moderate tissue penetration, and low absorption of the drug was reported (Rigos et al. 2003). The observed OXA concentration in the

depletion study in pompano muscle in this study is comparable to the observation of Chen et al. (2021) given a higher dose of OXA at 60mg/kg/day for 5 days.

Higher accumulation of both OTC and OXA was observed in the muscle compared to the blood. This indicates that both OTC and OXA are well distributed in the pompano. Higher levels of drugs in the muscle compared to in the blood is an indication of the good distribution of the drug (Björklund, Eriksson, and Bylund 1992). Higher OXA concentration measured in the muscle compared to blood was also observed in rainbow trout and Atlantic salmon (Björklund, Eriksson, and Bylund 1992; Samuelsen et al. 2000). The same was observed for OTC in rainbow trout (Rogstad et al. 1991). In contrary, higher drug concentration was observed in the blood than in the muscle of pompano administered with a single oral dose of amoxicillin at a dose of 40mg/kg and in those that received the medication for five consecutive days (Wang et al. 2015).

OXA was eliminated much faster than OTC. This could be attributed to the higher OTC accumulation compared to OXA. Furthermore, OXA started to regress after it reached the peak concentration of 0.20μg/g at day 5 during the period medicated feed was given and continued to decline until the cessation of feeding with medicated feed. This implies that OXA in pompano is easily metabolised by pompano. This is also the reason for the limited data point to allow computation of the withdrawal period. The observed depletion of OXA in pompano even during the time they are receiving 30mg/kg of OXA is contrary to the observation of Chen et al. (2021).

OTC and OXA concentration in the pompano muscle decreased by half on the second day of cessation of feeding with medicated diet. Withdrawal period for OTC was computed using the model generated from the plotted OTC concentration versus time. The regression equation obtained from the plotted OTC concentration vs. time has a coefficient (R^2) of 0.8 both for muscle and blood samples. Regression coefficient of 0.8 and below are reported in OXA withdrawal studies in the muscle of orange-spotted grouper (0.79) and in the serum of giant sea perch (0.62) (Chen et al. 2021). The obtained R^2 of 0.8 both for muscle and blood samples allowed the use of the regression equation derived from the curve fitting to compute for the withdrawal period of OTC in pompano. Based on the equation, the withdrawal time for OTC in the muscle of Pompano was 18.75 days (approx. 19 days) and 16.80 days (approx. 17days) in the blood. Fish are poikilothermic, and the degree of antibiotic degradation and excretion are affected by temperature, so it is necessary to compute the withdrawal period for degree-days. Observed withdrawal periods in pompano in the present study were 570 degree-days for muscle and 510 degree-days for blood, calculated by multiplying temperature with the withdrawal time. Obtained withdrawal times for OTC in pompano are close to the recommended degree-day withdrawal period of 500 degree-days,

recommended as a rule of thumb used in drugs (Sekkin and Kum 2011). Longer OTC withdrawal period was observed in the muscle sample compared to blood. This could be attributed to the higher OTC accumulation in the muscle than in the blood.

The regression equation obtained from the plotted OXA concentration vs. time has a coefficient (R^2) of 0.7 and 0.8668 in the muscle and blood, respectively. These are comparable to the results of Chen et al. (2021) who reported regression coefficients of 0.85 and 0.83 for OXA in pompano muscle and blood, respectively. The computed withdrawal period of 3 days at 30°C or 90 degree days for OXA in the Pompano muscle and blood is shorter than the 9 days at 26-27°C reported by Chen et al. (2021) in the same fish. Antibiotic depletion occurs more rapidly at higher temperature (Gonzalez et al. 2010), this explains the shorter withdrawal period observed in this study compared to the observation of Chen et al. (2021).

Conclusion

The withdrawal period for OTC in pompano is 570 degree days; 90 degree days for OXA. Pomano cultured at 30°C treated with OTC can be sold or eaten 19 days after treatment; and 3 days after treatment with OXA.

The computed withdrawal period for OXA in the pompano blood is doubtful considering that there is only one concentration data point. This concern could have been avoided if the concentrations were measured in nanogram/g and the OXA accumulation study is for 5 days only. Nevertheless, the results of the OXA accumulation and depletion studies serve as baseline information in the treatment of fish using OXA.

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