2 Withdrawal Periods of Antibiotics, Oxytetracycline, and Oxolinic Acid, in Fish Species Cultured in the Tropics

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Food safety is one of the major concerns of products derived from aquaculture. Farm inputs, e.g. drugs and agrochemicals, introduced whether intentionally or unintentionally during culture, may contaminate and remain in the product and become a hazard to the consumers. Chemical hazards in aquaculture products, among them drugs used for the chemotherapy of bacterial infection in fish and other cultured aquatic animals present a negative impact in aquaculture. Fish farmers often result to this treatment in order to save their cultured stock when threatened with infection, although a general conception nowadays is the discouragement of its use, being considered only as the "last recourse." Drugs, specifically antibiotics, have a long history of successful use in aquaculture (Alderman, 1980).

One of the most widely used antibacterial agents for therapy of systemic bacterial infections in farmed fish is oxytetracycline (OTC). It is a pale yellow to tan crystalline powder, MW 460.44, molecular formula $C_{22}H_{24}N_2O_9$ and chemical structure as shown in Figure 2.1. As an antibacterial, OTC's mode of action is by inhibiting the ability of bacteria to produce proteins. Without these proteins, bacteria cannot grow and multiply, therefore, it stops the spread of infection and the remaining bacteria are killed by the immune system or eventually die. Oxytetracycline free base is highly insoluble in water but readily forms soluble salt. One commonly used salt in aquaculture medicated feeds is the form oxytetracycline hydrochloride. Oxytetracycline can be given orally thru feeds and the typical dosage is 75 mg/kg body weight fish per day for 4 to 10 days. The recommended maximum residue limit (MRL) is 0.2 mg/kg for fish muscle (FAO FNP 41/14).

Another antibacterial agent also being used is the oxolinic acid (OXA), a white crystalline powder, and has a structure shown in Figure 2.2. OXA is a synthetic antimicrobial agent belonging to the group 4-quinolone. Quinolones are bactericidal which inhibit the bacterial enzyme DNA-gyrase resulting in breaks in the bacterial DNA coiling. OXA is efficacious against gram negative bacterial pathogens of fish. It can be administered orally through feeds with daily dosages of 12-20 mg/kg, or 30 mg/kg BW for 5 to 10 days. In fin fish, the maximum residue limit (MRL) is 100 ug/kg (EMEA/CVMP/41090/2005).

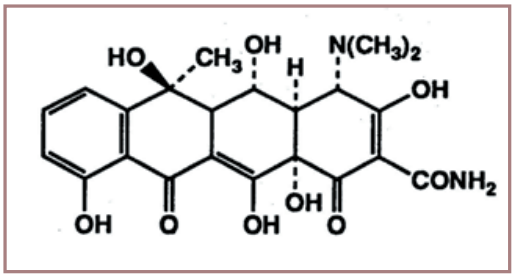

Figure 2.1. Structural formula of oxytetracycline

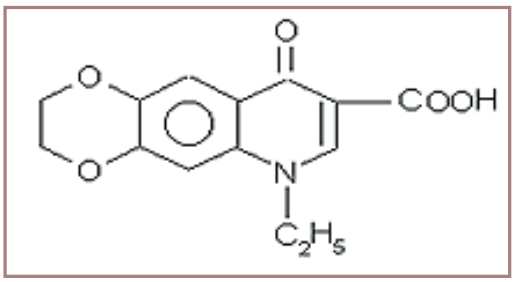

Figure 2.2. Structural formula of oxolinic acid

One of the draw backs in the use of antibiotics is the development of antibiotic resistant bacteria that could compromise the treatment of human infection. Although it cannot be assumed that there is a causal relationship between antibiotics and antibiotic-resistant bacteria, the overwhelming evidence has made this theory widely accepted among scientists (Mortazavi, 2014).

Residues of antibiotics present in fish may pose a risk to the health of the consumers. As mentioned by Mortazavi, humans who were exposed for long to antibiotic residues may disrupt their natural intestinal flora and could become susceptible to entering pathogenic bacteria.

For food safety reasons, cultured aquatic animals must have withdrawn all the residues or even at least, to acceptable levels before they are harvested for consumption, in order to prevent the entry of these contaminants from the "farm to fork" food chain, although cooking may reduce the residual concentration of OTC in fish (Sharafati, Chakeshtori, et al. 2013). Drug excretion is influenced by the temperature, the elimination time generally expressed in degree-days, which is the product of the temperature in ^{o}C and the withdrawal period in days (Inglis, 1996). Most previous studies reported results only in withdrawal periods for specified temperature or temperature ranges.

Residue depletion studies have been conducted on various cold water fishes, such as rainbow trout, *Salmo gairdneri* (Bjorklund and Bylund, 1990). Temperature related absorption and excretion studies done on different temperature ranges on rainbow trout *Onchorynchus mykiss* (Bjorklund, Eriksson and Bylund, 1992), and on *Salmo gairdneri* (Jacobsen, 1989). Hybrid striped bass, *Morone saxatilis*, tilapia, pure strain *Oreochromis niloticus* (Chen, et al. 2005 and Paschaol), were among those few studies on warm water fish.

The Southeast Asian Fisheries Development Center, Aquaculture Department, with the financial support from the Government of Japan, has conducted studies on the withdrawal periods of different fish species cultured in the tropics. These studies aim to estimate the depletion of two commonly used antibiotics, OTC and OXA, from several species cultured in the tropics: milkfish, *Chanos chanos*, hybrid red tilapia (*O. mossambicus*-hornorum hybrid X *O. niloticus*); mangrove red snapper, *Lutjanus argentimaculatus;* and orange-spotted grouper*, Epinephelus coioides.*

In the absence of withdrawal studies on specific species and temperature, fish farmers often follow antibacterial's manufacturer's recommended withdrawal period. The specificity of this study in terms of fish species, culture and temperature range is geared towards a more reliable estimation of the withdrawal period in tropical aquaculture.

Drug administration

The antibiotics were given through feeds which were mixed with SEAFDEC/AQD formulated diet for grow-out culture of species being studied. For every kilogram of diet formulation, 4.54 g of OTC was added. In contrast, OXA-mixed diet was added with 1.52 g active ingredient per kilo of the formulated diet. The antibiotic-mixed diets were given to the fish for ten days with the daily dose of the drugs equivalent to 75 mg/kg body weight for OTC and 30 mg/kg body weight for OXA. The daily feeding ration was about 2% of body weight. The test animals were given non-medicated feeds prior to and post antibiotic-mixed diet feeding.

Fish samples were either kept in 250L capacity fiber glass tanks with flow-through water system or large capacity concrete tanks compartmentalized by hapa nets. Water temperature and salinity were monitored daily.

After the 10-day antibiotic-mixed diet feeding, triplicate samples were retrieved at every collection period done regularly on a 3-day interval. The drug residues were extracted and analyzed using the procedure described below.

The experimental data of concentration was plotted versus time and the decay curve was evaluated by regression analysis. Equations for the time expressed in days, and the concentration in parts per million, were obtained from the regression curve. The withdrawal period can be estimated when the concentration is assumed zero.

Drug residue analysis

a. Oxytetracycline (OTC). The residues of OTC from the muscles of milkfish were analyzed using the high performance liquid chromatograph (HPLC) following the procedure of Carignan, et al. (1993). Briefly, residues are extracted using dichloromethane and 1% metaphosphoric acid solution, homogenized and centrifuged. Solvent removal was done by rotary evaporation and the final solution was filtered through membrane filter and injected in HPLC. The mobile phase of the HPLC system is composed of 0.025M oxalic acid-acetonitrile-terahydrofuran added with octane sulfonic acid sodium salt, and pumped at a rate of 1.0 mL/min, across a reversed phase octadecylsilyl (ODS) column, 5 µm, 4.6 X 150 mm. UV detector was set at 355 nm with 20 µL sample volume injected. Quantitation was based on calibration curves prepared from OTC standard solutions.

b. Oxolinic Acid (OXA). HPLC method was likewise employed for the evaluation of oxolinic acid. The procedure of Ng Poh Chuan et al. (2014) for the detection of oxolinic acid was followed. Accordingly, the residues were extracted with acetonitrile, homogenized and filtered into a separatory funnel containing acetonitrile-hexane solution. The solvent with the extract was drained and the solvent removed by rotary evaporation. Final extract layer from solvent-solvent extraction was pipetted out and filtered in membrane filter prior to HPLC injection. Twenty microliter of the clean sample was injected in an HPLC system with fluorescence detector set at 337 nm excitation and 365 nm emission wavelengths. The mobile phase system consisting of acetonitrile – methanol - 0.1M citric acid system was used and pumped at a rate of 0.5 mL/min. across a reversed phase octadecylsilyl (ODS) column, 5 µm, 4.6 X 150 mm. Quantitation was based on calibration curves generated from prepared standard OXA solutions.

Estimation of withdrawal periods

a. Milkfish. Milkfish is the most commonly cultured species in the Philippines and is now becoming a popular export aquaculture commodity. Its commercial production is one of the major concerns of the aquaculture industry in the country. Most often, monoculture techniques is used however, polyculture system is also being practiced and the milkfish-shrimp polyculture is quite common. Occurrence of milk fish mortalities has been reported and these were attributed to pathogenic bacteria.

Milkfish samples (BW 85±15 g, mean±SD) were kept in six hapa nets measuring 0.5m x 0.5m x 1m, suspended in an 8-ton capacity canvass-lined concrete tank with a flow-through seawater system. Temperature and salinity (mean±SD) during treatment were 29.6±1.0°C and 33±1.0 ppt, respectively and during withdrawal experiment, temperature and salinity were 30.4±0.55°C and 32±1.0 ppt

Regression analysis of the decay curve estimated the time to wash out OTC residues from the muscle of milkfish to be 22 days. In contrast, the value obtained for OXA was 27 days.

b. Hybrid red tilapia. Tilapia is also commonly cultured in the Philippines. It is considered a disease-resistant species, however, intensified tilapia culture may lead to development of bacterial infections.

Tilapia samples (BW 120.32 ±32 g, mean± SD) were kept in 250L capacity fiber glass tanks with flow-through water system. The experiment was conducted using freshwater and tank water temperature was 29.2°C±0.64 (mean ± SD).

Results showed that the estimated withdrawal period of OTC administered to tilapia at a dose of 75mg/kg fish per day was 26 days while that of oxolinic acid (OXA) given at a dose of 30 mg/kg fish per day, was 17 days.

c. Mangrove red snapper. Mangrove red snapper is one of the high value fish food species being cultured in the Philippines and in other Southeast Asian countries. Incidence of bacterial infection has been reported and chemotherapy is sometimes employed.

Snapper fish samples (86±15.6 g body weight) were stocked at 13 pcs per tank in 250L capacity fiber glass tanks with a flow-through seawater system. Water temperature during experimentation was 27.7°C ± 0.77 (mean ± SD).

When the concentration versus time data were plotted with regression analysis, the estimated withdrawal periods from mangrove red snapper, were 21 and 18 days for OTC and OXA, respectively.

d. Orange- spotted grouper. Groupers are also among fish food species that are of high economic value. They are being cultured in the Philippines and in other countries in Asia. Infectious agents, have caused diseases on cultured groupers, which lead them to be treated with antibiotics.

In 250L capacity fiberglass tanks, grouper juveniles with body weights 164± 25.91 g (mean ± SD), were stocked at 13 pcs/tank. Water temperature during the experiment was 28°C ± 1 (mean ± SD). From the decay curve of the experimental data, the estimated time to eliminate OTC residues from the muscle of orange spotted grouper was 21 days. In contrast, the value obtained for OXA was 17 days.

e. Black tiger shrimp. Black tiger shrimp, is a marine crustacean which is also widely cultured in the Philippines and in other Asian countries.

P. monodon samples, average BW 50.88 g, were kept in aerated, seawater flow through 250L capacity fiber glass tanks stocked at 3 pcs/tank. Water temperature during the experiment ranged from 29-31°C and salinity of 28-32 ppt. OTC was added to shrimp feed formulation in a ratio of 5 g/kg feeds while in OXA treatment 4.5 g/kg of feed OXA was added. Shrimps were fed with antibiotic-mixed diet for seven days with a daily ration of the feeds at 3% of BW, spread into three times a day.

The estimated withdrawal period of OTC was found to be 17 days while 19 days for OXA.

Table 2.1 Summary of results

Species	Withdrawal period, days		Temperature, oC
	Oxyteracycline (OTC)	Oxolinic acid (OXA)	
Milkfish	22	27	28.7 - 31.5
Hybrid red tilapia	26	17	28 - 30
Mangrove red snapper	21	18	26 – 29.5
Orange- spotted grouper	21	17	25 – 29.5
Black tiger shrimp	17	19	29-31

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