UNAJ #713898, AFS ID: UNAJ-2012-0039.R1

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QUERY SHEET

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Q1. Au: Note that beginning in 2013 we will be capitalizing the common names of fishes in accordance with updated AFS Special Publication 29 *Names of Fishes* . . . .

Q2. Au: in Abstract and in Results: is the actual OTC dose reported as mean ± SE, mean ± SD, or other?

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North American Journal of Aquaculture 00:1–5, 2013
American Fisheries Society 2013
ISSN: 1522-2055 print / 1548-8454 online
DOI: 10.1080/15222055.2012.713898

COMMUNICATION

A Terramycin 200 for Fish (44.09% Oxytetracycline Dihydrate) Treatment Regimen Proposed for the Fluorescent Marking of Rainbow Trout Vertebrae

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Q1 *Abstract*

We evaluated the efficacy of a Terramycin 200 for Fish (TM200; 44.09% oxytetracycline [OTC] dihydrate) treatment regimen proposed for fluorescent marking of the vertebrae of Rainbow Trout *Oncorhynchus mykiss*. Test fish weighed 36.6 ± 1.9 g (mean ± SD) at the start of treatment. The TM200-treated feed was administered to six tanks of fish at a target dosage of 82.7 mg OTC·kg fish^{-1}·d^{-1} for 10 d, while nontreated control feed was administered to three tanks of fish (20 fish/tank in each treatment group). After a 22-d posttreatment period, vertebrae were extracted from all fish and were examined for fluorescent marks with a dissecting scope and ultraviolet light. The vertebrae of all treated fish were marked, whereas vertebrae of control fish were not marked. Consequently, the probability of marking success in treated tanks was significantly different from that in control tanks. The 95% CI for the proportion of treated tanks containing one or more nonmarked fish was 0.00 to 0.39. Analysis of treated feed samples revealed that the actual OTC dose administered to treated tanks Q2 was 73.4 ± 0.1 mg OTC·kg fish^{-1}·d^{-1} (89% of target). The results indicate that the target TM200 treatment regimen administered in this study is suitable for the marking of Rainbow Trout vertebrae.

Fluorescent marking of skeletal tissue has long been used to study fish age and growth, fish movement, and the contribution of hatchery-reared fish to wild fish restoration and recovery programs and to angler catch (Parker et al. 1990; Nielsen 1992). Fluorescent marking is accomplished by administering compounds (e.g., tetracyclines, calceins, or alizarins) to fish via immersion, injection, or feed (Parker et al. 1990; Nielsen 1992; Blom et al. 1994). These compounds fuse with calcium and are deposited in bones, fin rays, scales, and other calcifying structures of growing fish (e.g., Leips et al. 2001; McFarlane and King 2001; Bashey 2004; Logsdon et al. 2004; Crook et al. 2007; Johnson et al. 2010; Chang et al. 2011). Fluorescent marks can be detected by viewing calcified structures (or sometimes whole fish) under ultraviolet to blue light (excitation range ~365–550 nm; e.g., Choate 1964; Weber and Ridgway 1967; Bilton 1986; Nielsen 1992; Brooks et al. 1994; Leips et al. 2001; Bashey 2004).

In the United States, oxytetracycline (OTC) hydrochloride ($C_{22}H_{24}N_2O_9{\cdot}HCl$) and OTC dihydrate ($C_{22}H_{24}N_2O_9{\cdot}2H_2O$) are the only compounds that are currently approved by the U.S. Food and Drug Administration (FDA) for the skeletal marking of food fishes (fish that are reared for market or for release into the wild, with the possibility of being harvested for human consumption). Oxytetracycline hydrochloride is approved for the marking of all freshwater-reared finfish fry and fingerlings via a 2–6-h immersion at 200–700 mg of OTC/L of water. Oxytetracycline dihydrate is approved for the marking of freshwater-reared Pacific salmon *Oncorhynchus* spp. via a 4-d, in-feed treatment at 250 mg of OTC·kg of fish^{-1}·d^{-1}. Use of OTC dihydrate is restricted to fish that are smaller than 30 g, and the fish must be held for 7 d posttreatment before they can be released. This in-feed treatment regimen (dosage) and its restrictions reflect the seminal work of Weber and Ridgway (1962, 1967) and Jones (1969, cited in Bilton 1986) in studies of Pacific salmon and Rainbow Trout *O. mykiss*.

The only OTC dihydrate product that is currently approved by the FDA for the skeletal marking of food fishes is Terramycin 200 for Fish Type A Medicated Article (TM200; 44.09% OTC; Phibro Animal Health Corp., Ridgefield Park, New Jersey). In the United States, TM200-treated feed is typically purchased from licensed feed manufacturers at 1–4% TM200 per 453.6 g of feed (2–8 g of OTC per 453.6 g of feed). However, TM200 can also be purchased over the counter and then top-coated onto feed by fish culture personnel. The TM200 skeletal marking treatment regimen currently approved by the FDA is efficacious; however, many in the U.S. aquaculture community would like the approval revised so that (1) TM200 is administered in feed

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Received April 23, 2012; accepted July 16, 2012

at 82.7 mg OTC·kg fish^{-1}·d^{-1} for 10 d to achieve the skeletal marking of all freshwater-reared salmonids less than 55 g; and (2) the fish are held for 21 d posttreatment before release. The proposed revision would increase the number of salmonid species and the mean weight of fish approved for marking while decreasing the total amount of OTC administered during each marking event from 1,000 mg/kg fish to 827 mg/kg fish. Moreover, if the revision is adopted, the skeletal marking treatment regimen for salmonids would be the same as that allowed by the FDA for several therapeutic uses of TM200 (USFWS 2011). A potential disadvantage of the revision is that the withdrawal period would increase from 7 to 21 d; however, this change is not likely to adversely affect most marking projects because of the inherent withdrawal period that is associated with treating small fish.

The objective of our study was to evaluate the efficacy of TM200 administered in feed at a target dosage of 82.7 mg OTC·kg fish^{-1}·d^{-1} over 10 d for marking vertebrae of the Rainbow Trout, a representative freshwater-reared salmonid (Mayer and Ellersieck 1986).

METHODS

Testing facility, test article, and test fish.—The study was conducted in 2009 at the U.S. Fish and Wildlife Service (USFWS) Bozeman Fish Technology Center (BFTC), Bozeman, Montana. One 20-kg bag of TM200-treated feed (1% TM200; Bio-Trout, 3.0 mm) and one 20-kg bag of nontreated control feed (Bio-Trout, 3.0 mm) were obtained from Bio-Oregon (Longview, Washington). Before any feed was administered, we collected three samples of treated feed (from the top, middle, and bottom of the feed bag) and one sample of control feed (from the top of the feed bag). The collection of one control feed sample was all that was required by our study protocol (USFWS 2008). All feed samples were sent to Eurofins AvTech Laboratories, Inc. (Portage, Michigan), for analytical determination of OTC concentrations. These concentrations were used to calculate the actual OTC doses that were administered to groups of treated and control fish.

Rainbow Trout eyed eggs were obtained from Troutlodge, Inc. (Tacoma, Washington). When the eggs arrived at the BFTC, they were disinfected in a 100-mg/L solution of iodine; however, no other chemical treatments were administered to eggs or fish before the study began. Egg incubation, egg hatching, and the rearing of fish to desired size were performed indoors. The reference population comprised 280 fish with a weight of 36.6 ± 1.9 g (mean ± SD) and an estimated mean TL of 149 mm (Piper et al. 1982). Twenty of these fish were impartially collected by dipnetting, evaluated for fish health, and characterized as disease free. A few fish had mild fin erosion, which was limited to the distal margins (<1.0 mm) of the dorsal, caudal, and anal fins; however, no bacteria or fungi appeared to be associated with the erosion. All other external and internal organs and tissues were characterized as normal. No bacteria or parasites were detected during microscopic examination of wet-mount skin-scrapes, and no bacterial colonies were evident after culturing kidney tissue samples on brain–heart infusion agar for 10 d at 22°C.

The study was conducted indoors in circular fiberglass tanks (90 L of water per tank), each of which was supplied with first-pass water at a rate of 3.8 L/min. Thus, fish were held at a density index (DI) of 0.09 and a flow index (FI) of 0.28 (Piper et al. 1982). Water temperature was maintained at 10.3 ± 0.3°C, and dissolved oxygen concentration was maintained at 8.2 ± 0.5 mg/L. Water hardness and alkalinity (as $CaCO_3$) were 293 ± 56 and 195 ± 9 mg/L, respectively; pH was 7.9 ± 0.0. All of these water quality characteristics were suitable for the rearing of Rainbow Trout. The overhead lights in the tank room were on for 8–10 h of each day.

Experimental design and data collection.—Completely randomized designs were used to allocate 180 fish from the reference population to nine tanks (6 treated tanks and 3 control tanks; 20 fish/tank) and to assign a treatment condition (TM200 treated versus nontreated control) to each tank (Bhujel 2008). The in-life phase of the study comprised a 1-d acclimation period, a 10-d treatment period, and a 22-d posttreatment period. The length of the posttreatment period approximated the proposed withdrawal period and allowed the deposition of new, unmarked bone, which allows for easier detection of OTC marks (Weber and Ridgway 1967). No feed was administered during the acclimation period. During the treatment period, we administered TM200-treated feed to the treated tanks and control feed to the control tanks at 1.875% of initial mean fish body weight per day. The feeding rate was dictated by the percentage of TM200 in the treated feed (USFWS 2011). During the posttreatment period, control feed was administered to all tanks. Feed was administered twice daily by hand (one-half daily ration in both the morning and the afternoon). Data that were collected daily included mortality, general fish behavior (normal or abnormal), and fish feeding behavior (aggressive, semiaggressive, or nonaggressive; Bowker et al. 2012). Personnel that fed the fish and collected the data did not know the treatment conditions that were assigned to the tanks.

At the end of the in-life phase, all fish were collected and euthanized in a solution of tricaine methanesulfonate (TRICAINE-S; Western Chemical, Inc., Ferndale, Washington), were individually bagged by fish and tank number, and were then frozen (Trojnar 1973; Bilton 1986). One month later, all fish were removed from the freezer and thawed. For each fish, the two vertebrae immediately anterior to the dorsal fin were extracted and cleaned to remove all soft tissue. The centrum of each vertebra was examined for a fluorescent mark with a dissecting microscope (8–40 × magnification; StereoZoom 5; Bausch & Lomb, Rochester, New York) and ultraviolet light (365 nm; Blak-Ray Model B-100A Longwave Ultraviolet Lamp; Mineralogical Research Co., San Jose, California). Mark quality was graded as 0 (no mark), 1 (faint and incomplete mark circle), 2 (faint and complete mark circle), or 3 (bright and complete mark circle).

The individual who evaluated mark quality did not know the treatment conditions that were assigned to the tanks.

Statistical analysis.—Preliminary inspection of the marking data revealed no variation in mark response among fish (observational units) within tanks (experimental units). Each tank was therefore classified as a treatment success or failure, and Fisher's exact test ($\alpha = 0.05$; two sided) was used to test the null hypothesis that the probability of marking success was equal between treatment groups (Zar 2010; SYSTAT 2011). Additionally, we calculated a 95% CI for the proportion of treated tanks containing one or more nonmarked fish given six independent trials (i.e., $n = 6$ tanks). The lower confidence limit was assumed to be zero, and the upper confidence limit was estimated as $1 - \alpha^{(1/n)}$ (Jovanovic and Levy 1997). The upper confidence limit represented the worst case scenario for studies conducted in a manner identical to ours.

RESULTS

All treated tanks were classified as treatment successes because all vertebrae extracted from treated fish were graded as 3 (bright and complete mark circle). In contrast, all control tanks were classified as treatment failures because all vertebrae extracted from control fish were graded as 0 (no mark). Consequently, the probability of marking success in treated tanks was significantly ($P = 0.0119$) different from that in control tanks. The 95% CI for the proportion of treated tanks that contained nonmarked fish was 0.00 to 0.39. During the study, general fish behavior was characterized as normal and fish feeding behavior was characterized as aggressive in all tanks; there were no mortalities in any of the tanks.

The actual OTC dose administered to treated tanks was 73.4 ± 0.1 mg·kg fish^{-1}·d^{-1} (89% of the target), resulting in a total dose of 734 mg OTC/kg fish for the 10-d treatment. A small amount of OTC was detected in the control feed sample; consequently, the OTC dose administered to control tanks was 0.33 mg·kg fish^{-1}·d^{-1}, totaling 3.3 mg OTC/kg fish for the 10-d treatment. This relatively small amount of OTC was insufficient to induce marks that would be visible with the detection equipment used in our study.

DISCUSSION

In our study, TM200-treated feed that was administered at an actual dosage of 73.4 mg OTC·kg fish^{-1}·d^{-1} for 10 d induced a bright and complete mark circle on the vertebrae of all treated Rainbow Trout. This result met the FDA's requirement for demonstrating the efficacy of the target dosage (82.7 mg OTC·kg fish^{-1}·d^{-1} for 10 d; USFWS 2008). Our study was conducted with disease-free fish held at DI and FI levels that were well below the upper limits (DI = 0.5; FI = 1.5) recommended for rearing of salmonids at 10°C at the BFTC's elevation (1,463 m above mean sea level; Piper et al. 1982). However, in repeated identical studies, we estimated that up to 39% of treated tanks could contain one or more nonmarked fish. The magnitude of this estimate reflects the small number of tanks used in our study but illustrates that treatment might not be 100% efficacious in all tanks on all occasions. Typically, treated feed will be administered to tanks, raceways, or ponds holding a few hundred to a few thousand fish. For reasons associated with fish health and feeding hierarchy (Duston and Cusack 2002; Storey 2005; Samuelsen 2006), some fish will probably not eat enough treated feed to produce a visible mark.

It is not yet known whether the target OTC dosage administered in our study is efficacious for marking Rainbow Trout otoliths (not collected or evaluated in our study; USFWS 2008) or the calcified structures of other fish species. If so, potentially many fish species could be marked at a total OTC dose less than those previously reported as efficacious. With respect to salmonids, Trojnar (1973) marked Rainbow Trout fry at 100 mg tetracycline·kg fish^{-1}·d^{-1} for 4 d (total dose = 400 mg/kg fish); however, consistent deposition of tetracycline occurred only in the anterior ribs. Although Trojnar (1973) speculated that the minimum effective daily dose was less than 100 mg/kg fish, he noted that mark intensity increased with daily dose and duration up to 700 mg·kg fish^{-1}·d^{-1} for 8 d (total dose = 5,600 mg/kg fish). In studying Rainbow Trout and Pacific salmon (Coho Salmon *O. kisutch*, Sockeye Salmon *O. nerka*, Chinook Salmon *O. tshawytscha*, and Chum Salmon *O. keta*), Weber and Ridgway (1967) concluded that the most satisfactory and consistent marks were obtained by administering 250 mg tetracycline·kg fish^{-1}·d^{-1} for 4 d (total dose = 1,000 mg/kg fish). Johnson et al. (2010) double marked the vertebrae of Chinook Salmon fingerlings by administering two regimens of 250 mg OTC·kg fish^{-1}·d^{-1} for 4 d separated by 1 d of no treatment (total dose = 1,000 mg/kg fish for each mark). Odense and Logan (1974) found that administering 250 mg OTC·kg fish^{-1}·d^{-1} for 5 d (total dose = 1,250 mg/kg fish) was needed to produce a stable mark on the vertebrae of Atlantic Salmon *Salmo salar*. Choate (1964) reported that administering a total dose of 4,000 mg OTC/kg fish over 4–7 d produced recognizable marks in Brook Trout *Salvelinus fontinalis* fry and fingerlings; however, his observations of fluorescence were made on living fish that were held in a pan of shallow water. Bilton (1986) marked the vertebrae of Chum Salmon fry in 7 d with OTC-treated feed that was prepared at the same concentration used in our study (2 g of OTC per 453.6 g of feed); however, the daily and total doses were not determined in that study because the fish were fed to satiation.

With respect to nonsalmonids, Wahl and Stein (1987) marked the vertebrae of young-of-the-year Tiger Muskellunge (Northern Pike *Esox lucius* × Muskellunge *E. masquinongy*) by administering 500 mg OTC·kg fish^{-1}·d^{-1} for 6 d (total dose = 3,000 mg/kg fish) or by administering two such treatments separated by 7 d (total dose = 6,000 mg/kg fish). Pedersen and Carlsen (1991) reported that a dose of 500 mg tetracycline·kg fish^{-1}·d^{-1} for 10 d (total dose = 5,000 mg/kg fish) produced marks on the vertebrae of seawater-reared juvenile Atlantic Cod *Gadus morhua* but did not achieve marks on otoliths. Nordeide et al. (1992) obtained good vertebral marks in seawater-reared

juvenile Atlantic Cod by administering OTC-treated feed in a regimen of 8 d at 500 mg OTC·kg fish^{-1}·d^{-1} (total dose = 4,000 mg/kg fish), 5 d of no treatment, and 10 d at 500 mg OTC·kg fish^{-1}·d^{-1} (total dose = 5,000 mg/kg fish). Hendricks et al. (1991) induced single, double, or triple marks on the sagittae of fingerling American Shad *Alosa sapidissima* by administering feed prepared at a dosage of 88 g tetracycline/kg feed for 3 d at 5- or 7-d intervals. However, Hendricks et al. (1991) did not report the daily or total tetracycline doses administered. Most recently, Chang et al. (2011) marked Black Porgy *Acanthopagrus schlegeli* by use of OTC-treated feed, but those authors did not report the daily or total doses administered or the methods used to evaluate efficacy.

Our study was not designed to evaluate long-term retention of the vertebral marks produced by the target OTC dosage administered (USFWS 2008). However, it is well known that OTC chelates to calcium, which is taken up by bone—a tissue in which calcium turnover is slow (Koenings et al. 1986). Thus, if the vertebral OTC marks that are induced via in-feed treatment are initially characterized as "clear" (Bilton 1986; Hendricks et al. 1991; Pedersen and Carlsen 1991), "good" (Weber and Ridgway 1967), or otherwise readily visible (Odense and Logan 1974), then they should be detectable for several years. For example, Weber and Ridgway (1967) found no diminution of vertebral OTC marks in Sockeye Salmon that were released into the Columbia River system and recovered 3.5 years later when they returned to spawn. Odense and Logan (1974) detected vertebral OTC marks in Atlantic Salmon that were released into the River Phillip (Nova Scotia, Canada) and recaptured 2 years later when they returned to spawn. Pedersen and Carlsen (1991) observed vertebral OTC marks in Atlantic Cod after 1.2 years of rearing the marked fish in seawater cages. Nordeide et al. (1992) identified vertebral OTC marks in Atlantic Cod after 3 years of rearing the marked fish in seawater net-pens; however, those authors noted that the intensity of the marks gradually declined over time. Johnson et al. (2010) stocked OTC-marked Chinook Salmon into Lake Huron (United States and Canada) from 2000 to 2003, and vertebral marks were evident in fish that were recaptured between 2002 and 2005. Finally, Chang et al. (2011) released OTC-marked Black Porgy into the coastal waters of northwestern Taiwan during July–September 2005, and vertebral marks were detected in fish that were caught in the area between June 2006 and December 2008.

In conclusion, the results of our study support FDA approval of administering TM200 in feed at 82.7 mg OTC·kg fish^{-1}·d^{-1} for 10 d to achieve fluorescent marking of Rainbow Trout vertebrae. Future research should focus on testing the efficacy of this treatment regimen for marking Rainbow Trout otoliths and the calcified structures of other fish species and should evaluate long-term mark retention.

ACKNOWLEDGMENTS

Miranda Dotson, Bonnie Johnson, and Molly Bowman (USFWS Aquatic Animal Drug Approval Partnership Program) conducted the in-life phase of the study. Ken Peters (USFWS Bozeman Fish Health Center) performed fish health evaluations. Miranda Dotson extracted and cleaned vertebrae, and Jim Peterson (Montana Fish, Wildlife and Parks [MFWP], retired) evaluated the fluorescent marks. Ken Staigmiller (MFWP) provided the mark evaluation guidelines, dissecting scope, and ultraviolet lamp. Paul Duquette (Phibro Animal Health Corp.) paid for the analysis of OTC in feed samples. Comments from two anonymous reviewers greatly improved the manuscript.

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