ORIGINAL

Protocol Title: Field effectiveness of Aquaflor® (florfenicol) and Terramycin 200 For Fish® (oxytetracycline dihydrate) to control mortality in coolwater and warmwater finfish due to Motile Aeromonad infections.

Test Facility and Sponsor:

Upper Midwest Environmental Sciences Center (UMESC)
Biological Resources Division, U. S. Geological Survey, Department of the Interior
2630 Fanta Reed Road
La Crosse, Wisconsin 54603

Study Number: AEH-09-MAS-02

Aquaflor® INAD Number: 011-902
Terramycin 200 For Fish® INAD Number: 011-366

Proposed Experimental Start Date: June 2011
Proposed Experimental Termination Date: September 2012

Principal Investigator: Maren Tuttle-Lau

Protocol Approval

Reviewed and Approved by:

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Principal Investigator, UMESC
11Jul2011
Date

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24Jun2011
Date

Michael Jawson, Ph.D
Center Director, UMESC
6/30/11
Date

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1. PROTOCOL OBJECTIVE:
   Determine the effectiveness of Aquaflor® (florfenicol; FFC) and Terramycin 200 For Fish® (oxytetracycline dihydrate; OTC) administered separately in medicated feed to control mortality in coolwater and warmwater finfish due to Motile Aeromonad infections (MAI).

2. STUDY OBJECTIVE(S):
   2.1 Evaluate the efficacy of FFC-medicated feed offered at a dose of 15 mg/kg body weight (BW) to control mortality from MAI in cool or warmwater finfish.
   2.1.1 Null hypothesis (H_o): mortality of fish from MAI that receive FFC-medicated feed at a dose of 15 mg/kg BW for 10 days is not different from mortality in nonmedicated controls.
   2.1.2 Alternative hypothesis (H_a): mortality of fish from MAI that receive FFC-medicated feed at a dose of 15 mg/kg BW for 10 consecutive days is different from mortality of nonmedicated controls. Effectiveness will be established if treated fish have a cumulative mortality rate statistically different from and lower than that of untreated fish.
   2.2 Evaluate the efficacy of OTC-medicated feed offered at a dose of 82.5 mg/kg BW for 10 consecutive days to control mortality from MAI in cool or warmwater finfish.
   2.2.1 Null hypothesis (H_o): mortality of fish from MAI that receive OTC-medicated feed at a dose of 82.5 mg/kg BW for 10 consecutive days is not different from mortality in nonmedicated controls.
   2.2.2 Alternative hypothesis (H_a): mortality of fish from MAI that receive OTC-medicated feed at a dose of 82.5 mg/kg BW for 10 consecutive days is different from mortality of nonmedicated controls. Effectiveness will be established if treated fish have a cumulative mortality rate statistically different from and lower than that of untreated fish.

3. STUDY SCHEDULE:
   3.1 Proposed date of initiation: June 2011
   3.2 Schedule of events: A definitive study schedule cannot be developed because the study is dependent on the development of natural epizootics. It is expected that efficacy trials will be initiated in the summer of 2011. The following is the expected sequence of events that will occur once a suitable epizootic is identified:
   3.2.1 Schedule of events:
   D -10 to -1: In-life study facility owner or staff identifies mortality increase or behavioral change indicative of *Aeromonas* sp. epizootic.
   D -5 to -1: Experimental tanks are transported from UMESC to the In-life study facility.
   D -1 to D 0: Principal Investigator presumptively diagnoses *Aeromonas* sp. infection and experimental tanks are stocked with fish. Microbiological samples are collected for confirmatory diagnosis.
   D 0 to 9: Feed (nonmedicated control or medicated) is offered to appropriate tanks; mortality observed/recorded and fish samples are collected for appropriate microbiological assessment. UMESC staff weighs feed, collects data, and collects appropriate microbiological samples.. Feed samples (nonmedicated control and medicated) are collected and submitted to determine drug concentration. UMESC QA observes trial to ensure adequate data collection.
   D10 to 23: Nonmedicated control ration is offered; mortality observed/recorded and fish samples are collected for appropriate microbiological assessment. UMESC staff weighs feed, collects data and microbiological samples. . UMESC-QA returns to In-life study facility on D 23 for final data review and to observe the collection of final microbiological samples.
   D 23 to 30: UMESC personnel complete final microbiological sample processing.
   3.3 Proposed completion date: September 2012

4. STUDY DESIGN:
   4.1 Treatment Groups.
   Fish will be equally divided between a nonmedicated control group and two medicated groups. The nonmedicated control group will only be offered the nonmedicated control ration throughout the course of the trial whereas the medicated groups will be offered sufficient FFC or OTC-medicated feed as the sole ration to achieve the respective daily dose. FFC-medicated feed will be offered to achieve a dose of 15 mg FFC/kg BW for 10 consecutive days. OTC-medicated

feed will be offered to achieve 82.5 mg OTC/kg BW for 10 consecutive days. The medicated groups will be offered the nonmedicated control ration as the sole ration for the 14 day post-dose observation period.

4.2 Experimental Design.

The objective of the study is to determine the effectiveness of FFC and OTC to control mortality due to MAI in cool- and warmwater finfish. Treatment will be assigned to tank according to a randomized block design. A total of 18 tanks will be used with 6 tanks per treatment group.

4.2.1 Inclusion criteria – a fish lot will be included into a trial if: 1) clinical symptoms indicative of a MAI are observed (Appendix 1); 2) mortality increases above background population mortality; 3) the Principal Investigator or UMESC study personnel are on site to allocate fish to test tanks and initiate treatment; 4) UMESC QA or designee is available to verify trial initiation and protocol procedures are followed.

4.2.2 Exclusion criteria – fish lots will be excluded if: 1) substantial secondary disease infections are present. The presence of secondary pathogens will be documented in the study raw data and compared to a standard fish health diagnostic scheme to determine whether or not the secondary pathogen is likely to cause mortality. The presence of a secondary pathogen will not automatically exclude fish from the test unless the other pathogens are present in sufficient numbers to adversely affect the outcome of the study. (2) Water flow interruption or dewatering events (e.g. standpipe inadvertently left out) that occur for a period of time that unduly stress test fish and may result in one or more tank exclusion. If exclusion of any test unit or lot of fish is deemed necessary by the Principal Investigator or Supervisory Biologist, it will be documented in the final study report.

4.2.3 Post-treatment exclusion criteria – a tank of test fish will be excluded from analyses if 1) fish exhibit clinical signs associated with pathogens other than an *Aeromonas* sp. (e.g. *F. columnare*), 2) loss of water flow to the test tank or depletion of oxygen within the test tank water results in mortality of test fish.

4.3 Test Facilities: Public or private hatcheries/aquaculture facilities in the North Central Region; specific locations are to be determined based on MAI outbreaks.

4.4 Blocking factor(s).

The test system will consist of two ten-tank units (labeled A or B), each consisting of ten individually plumbed tanks. Influent water is provided to each tank via a main supply line that extends the entire length of the unit with each tank receiving its influent water from a valve branching off of the main water supply. Effluent from each tank is eliminated via a standpipe located at the base of each tank.

4.5 Randomization Procedures.

Random assignment codes will be generated through the use of a SAS random number generator (see Appendix 2 for sample code; actual SAS code used will be included in the study raw data).

4.5.1 Allocation of treatment to block of tanks:

The test units will be considered blocks and each test unit will have an equal number of test tanks with an equal distribution of treatments. Within a block, treatment (control, FFC-medicated, or OTC-medicated) assignment will be completely randomized.

4.5.2 Allocation of animals to experimental units:

Fish will be randomly distributed to the test tanks according to a completely random assignment code provided by the Supervisory Biologist. Groups of ≤5 fish will be transferred from the appropriate source tank by UMESC study personnel into the assigned test tank according to the random distribution code until each tank has no less than 20 fish and no more than 100 fish per tank depending upon fish loading densities of the source tank. Fish in the source population will be crowded into an area and netted from that crowded population to ensure fish are indiscriminately collected.

5. STUDY PROCEDURES:

5.1 Test Animals: Representative of cool or warmwater fish species cultured in public or private aquaculture that have a natural MAI outbreak in progress will be used. Coolwater fish species tested may include muskellunge *Esox masquinongy*, northern pike *Esox lucius*, walleye *Sander*

vitreum or yellow perch *Perca flavescens*. Warmwater fish species tested may include bluegill *Lepomis macrochirus*, channel catfish *Ictalurus punctatus*, largemouth bass *Micropterus salmoides*, smallmouth bass *Micropterus dolomieu*, hybrid striped bass *Morone saxatilis x chrysops* or tilapia *Oreochromis* sp. The fish species in the lot assigned to the study will be identified using the identification key in Eddy and Underhill (1978) by hatchery personnel.

5.1.1 Description:

5.1.1.1 Age – juvenile fish will (≤1 year) be used.

5.1.1.2 Sex – fish will be used without regard to sexual maturation.

5.1.1.3 Initial body weight – fish are expected to weigh between 5 to 100 g at test initiation.

5.1.1.4 Physiological state – juvenile fish will be tested. Fish are not expected to achieve spawning condition by the termination of the study. Physiological status, beyond confirmation of disease in select fish, will not be considered.

5.1.2 Number of animals – depending on size and stocking density in the source tank, the number of fish placed in each test tank will be no less than 20 to no more than 100. Thirty fish will also be used for disease confirmation. The total number of fish used per trial will be ~2,000. We expect to conduct four trials, two with coolwater species and two with warmwater species.

5.1.3 Source of animals – lot history information (date of receipt, hatchery source, mortality, and water chemistry records) will be placed in the study raw data and summarized in the final study report.

5.1.4 Identification method – individual fish will not be tagged or have other identifying marks. Dead or moribund fish removed from tanks during the test period or at the end of the post-dosing period will be assigned a number corresponding to the tank the fish were removed from. For example, if a dead or moribund fish was removed from tank A2 and was the first mortality removed, the fish would be designated as A2-1.

5.2 Acclimation of Test Animals:

5.2.1 Duration - Since the water used to culture test fish will be from the same source, acclimation to water chemistry will not be required. Test fish will be transferred to the test tanks 2-18 h before dosing administration; dosing will be initiated as soon as microscopic identification is complete but no sooner than two hours after transfer to the test tanks. Depending on the size of fish tested, feed may or may not be withheld during test tank acclimation. If smaller fish require feeding, nonmedicated control feed will be offered.

5.2.2 Medication and/or vaccination during acclimation period – None.

5.2.3 Baseline data collected prior to initiating study – mortality (recorded per hatchery standard procedures and data forms), water temperature, dissolved oxygen, pH. Thirty fish will be collected and euthanized by an overdose of tricaine-methanesulfonate (MS-222) for disease confirmation (UMESC GEN 132). Total length, weight, and necropsy data will be collected. Necropsy and microbiological procedures are described in Appendix 1.

5.3 *Aeromonas* sp. diagnosis and identification:

5.3.1. Clinical signs: Clinical signs of an *Aeromonas* infections typically include hemorrhages and ulcerative skin lesions which may be on the surface, on organs or deep within tissues (Inglis et al. 1993). External lesions may vary from an extensive superficial reddening of the surface, often with necrosis of fins or tail (fin rot). Internally there may be an excess of ascetic fluid. The spleen is often enlarged, rounded and cherry red. The enlarged kidney will often undergo liquefactive necrosis with necrotic fluid oozing out (Inglis et al. 1993).

5.3.2 Presumptive identification: A presumptive diagnosis will be made by observation of clinical signs and observation of gram-negative rods in gram stains, or motile bacteria with a monotrichous polar flagellum in wet mounts.

5.3.3 Confirmatory identification: *Aeromonas* sp. will be isolated by inserting a bacterial loop into the posterior kidney and inoculating onto a trypticase soy agar (TSA) plate. Plates will be incubated at about 30°C for 36 to 48 hours. Further identification and characterization will be defined under a separate protocol.

5.3.4 Combined infections: Preliminary work with isolates collected during naturally occurring MAI outbreaks (study number AEH-09-MAS-01) indicate that the many MAI outbreaks are the result of combined infections of two or more *Aeromonas* sp. For example, *A. sobria* and *A. veronii* were separately isolated along with *A. hydrophila* in samples collected during our preliminary work at two facilities with a history of MAI outbreaks caused by *A. hydrophila*. Clinical signs reported for motile aeromonads infections of fish are generally indistinguishable (Austin and Austin 2007).

5.4 Blinding of study:

5.4.1 Extent of blinding, (e.g., monitor, investigator, etc.) – The Supervisory Biologist (or designee and UMESC QA) will have access to the treatment assignment code and therefore will not make any observations or record any data during the study. UMESC study personnel collecting mortality or water quality data, removing dead or moribund fish, offering feed during dosing and post-dosing periods, or conducting microbiological sample preparation/analysis will be unaware of the treatment assignment.

5.4.2 Blinding method(s) and procedure(s) – bulk feed (nonmedicated control and medicated ration) will be prepared at the In-life study facility. Treatment codes will be assigned by the Supervisory Biologist. The Supervisory Biologist or his designee will weigh the rations into bags labeled with the study number, trial number, date sampled, treatment tank number, initial weight of bag and feed, and the initials of the person weighing the feed. Nominal concentration will not be listed; however, all feed samples will be labeled with the following statement:

CAUTION: CONTAINS A NEW ANIMAL DRUG FOR USE ONLY IN LABORATORY RESEARCH ANIMALS OR FOR TESTS IN VITRO. NOT FOR USE IN HUMANS.

5.4.3 List personnel with access to treatment codes and rationale: Supervisory Biologist and UMESC-QA. The Supervisory Biologist and UMESC QA will have access to the blinding code. The Supervisory Biologist will perform the random assignment of treatment to tank for the given trial. The Supervisory Biologist will have access to the blinding code because he will weigh test feed for the given trial. The Supervisory Biologist and UMESC-QA will have access to the treatment code to ensure proper randomization of fish to the test tanks, the assignment of treatment to tank, and the collection of data.

5.5 Water chemistry:

5.5.1 Dissolved oxygen: Dissolved oxygen will be measured and recorded once daily in each test tank (UMESC AEH 394 or equivalent meter).

5.5.2 Temperature: Water temperature will be measured and recorded in each test tank with a thermometer whose calibration has been verified (UMESC AEH 903).

5.5.3 pH: The pH will be measured and recorded once daily in each test tank (UMESC AEH 310 or equivalent meter).

5.5.4 Flow rate: Flow rates will be measured and recorded weekly in each tank to ensure one full tank exchange per hour designated by size and species of fish being tested and In-life study facility procedures. Flow rates will be measured and recorded as measured flow rate and what the flow rate was adjusted to.

5.5.5 Alkalinity and hardness: Alkalinity (UMESC AEH 706) and hardness (UMESC AEH 712) will be measured once during the dosing period over the 23 day study period. Data capture forms are included in the SOP's listed above.

5.6 Study Facilities:

5.6.1 In-life study facilities: In-life study facilities will include facilities that have sufficient fish with clinical signs indicative of an MAI infection to stock test tanks at appropriate loading densities.

5.6.1.1 Aeration: Air stones will be supplied to every tank.

5.6.1.2 Water supply: Water supply to the test tanks will be the culture water used at the In-life study facility. Flow rates will be a minimum equivalent of one full tank exchange per hour in each tank designated by the size and species of fish being tested.

5.6.1.3 Location of lighting: Light intensity will be measured above the test tanks using a hand-held photometer once during the dosing period (UMESC AEH 190).

5.6.1.4 Test tank dimensions: Two test units consisting of 10 individually plumbed and drained test tanks each (Figure 1) will be transported to the In-life study facility. The test units are ~81 cm x 305 cm long. Each test tank within the test unit is 62 cm long x 28 cm wide x 22 cm deep (standpipe height) and will contain ~38 L of water.

5.6.2 Space allocation per animal: Fish loading density will be maintained at 110 to 125% of the loading density of the source culture tank.

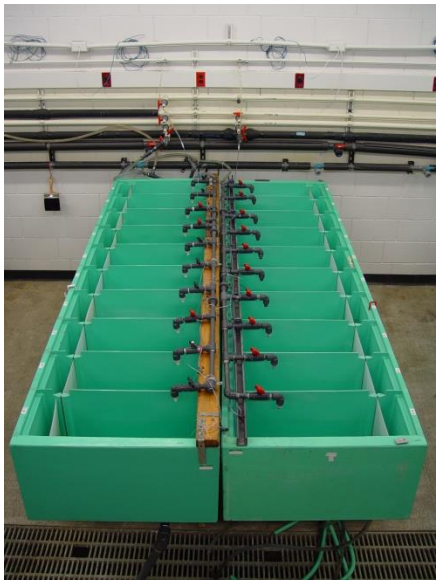

Figure 1. Experimental units (tanks) will be segregated into blocks of 10 test tanks contained within two ten-tank units. Individual tanks are separately plumbed and do not share water between tanks.

5.7 Experimental Diet(s) (if applicable):

5.7.1 Diet formulation(s): Medicated feed will be prepared as a top-coated feed. Top-coated feeds will be prepared according to the procedures described in Appendix 3.

5.7.2 Drug Concentration Assay:

5.7.2.1 Contaminant assay

5.7.2.1.1 Assay procedure – One sample (~4 kg) of the control ration used in each trial will be collected and split into two ~2 kg aliquots with one aliquot shipped to the appropriate contract assay laboratory and the other stored frozen (~-80°C) at UMESC. The control ration will be assayed to determine the levels of potential antibiotic contaminants including: FFC, OTC and Romet (sulfadimethoxine + ormetoprim).

5.7.2.2 Assayed drug concentration:

5.7.2.2.1 Drug assay: FFC and OTC concentration in the medicated ration will be confirmed at the initiation of dosing and at the completion of the dosing period for each trial. Diet samples collected will be ~600 g and will be hand mixed after collection then hand-split into two ~300 g aliquots and double bagged in Ziploc™ or equivalent bags. Samples will be labeled according to UMESC AEH 011 to include: Study Number, sample number, compound name, nominal concentration, sample interval, date sampled, and storage conditions (<-20°C). One aliquot will be submitted to Eurofins for FFC and OTC determination and the other reserved at ~-80 °C at UMESC. Feed samples will be frozen at ~-20°C then shipped in coolers via overnight delivery to Eurofins according to UMESC chain of custody procedures (UMESC ECO 513); sample temperature may exceed >-20°C during shipment.

Initiation of dosing: One sample will be collected from the medicated bag(s) in which feed is withdrawn from for the given trial. Samples will be collected no earlier than two days before but no later than the first dosing day. Concurrent with medicated ration sampling, a similar sample will be collected from the nonmedicated control bag used for the nonmedicated controls.

Completion of dosing: One sample will be collected from the medicated bag used for the last dosing day concurrent with the last dosing period that feed was withdrawn. Example, if feed for dosing day 9 was withdrawn on dosing day 5 and stored at the In-life study facility under ambient conditions, then the completion of dosing sample would also be withdrawn on dosing day 5, placed in a labeled sample bag and stored under conditions similar to the feed withdrawn for dosing day 9. Concurrent with medicated ration sampling, a

similar sample will be collected from the first control ration bag used for feeding during the post dosing period.

5.7.2.2.2 Anticipated analytical variation and assay limits: Samples submitted for FFC or OTC analysis will be analyzed in duplicate. Acceptance criteria for the FFC-medicated feed are: no FFC detected in the nonmedicated control feed and FFC level in the medicated diet within 80-110% of the target feed concentration. Acceptance criteria for the OTC-medicated feed are: no OTC detected in the nonmedicated control feed and OTC level in the medicated diet within 65-135% of the target feed concentration. .

5.7.2.2.3 Analytical method: Concentrations will be determined according to Eurofins procedure number SP 025290-123A-007-01.05 for FFC and PHB-109.02 for OTC. One sample of the control ration will be collected and split in two ~ 2 kg aliquots with one aliquot shipped to the contract assay laboratory and the other stored frozen (~-80°C) at UMESC. The control ration will be assayed to determine the levels of potential antibiotic contaminants including FFC, OTC, and Romet. The absence of interfering compounds will be confirmed by analysis of the control ration concurrent with medicated ration assay. The Principal Investigator will be contacted if interfering compounds (those with a peak greater than the LOQ) are identified. FFC or OTC feed assay analyses will conform to GMP regulations (21CFR225). Any remaining feed at Eurofins following analysis will be disposed of according to Eurofins standard procedures. Any feed remaining at UMESC will be incinerated after written approval from the Principal Investigator and verification by the Study Monitor or UMESC-QA (UMESC GEN 007).

5.7.2.3. Analytical laboratories:

5.7.2.3.1.1 Facility
Eurofins AvTech Laboratories (FFC assays)
6859 Quality Way
Kalamazoo, MI 49002
Tel: (269) 323-3366

5.7.2.3.1.2 Contact
PJ Newland
6859 Quality Way
Tel: (269) 323-3366
Kalamazoo, MI 49002
Email: patricianewland@eurofinsus.com

5.7.2.3.1.3 Facility
Eurofins Scientific Inc. (OTC and Romet assays)
3507 Delaware Avenue
Des Moines, IA 50313

5.7.2.3.1.4. Contact
Jody Bejarano, Project Manager
Tel: (515) 265-1461
Fax: (515) 266-5453
Email: jodybejarano@eurofinsus.com

5.7.3 Feed form: The feed type, manufacture, and size will be recorded in the study raw data.

5.7.4 Feeding program, (i.e. schedule for feeding experimental diets): Once transferred to the test tanks, fish will be offered either the nonmedicated control ration, or the FFC-medicated ration, or the OTC-medicated ration as the sole feed during the 10-d dosing period (Day 0 to Day 9). If fish must be held in the test tanks for more than 18 h before dosing is initiated then the nonmedicated control ration may be offered as the sole ration until the disease has been presumptively identified. Thereafter, fish will be fed either the nonmedicated control or medicated feed according to the dosing schedule. Feed will be offered at up to 10% BW/day depending on the fish size at dosing. During the post-dosing observation period (Day 10 to Day 23) the nonmedicated control ration will be offered as the sole ration. Feed will be offered either by hand or by an automatic feeder (e.g., Zeigler Brothers, Inc., Gardners, Pennsylvania, USA). Regardless of how offered, feed will not be offered sooner than 1 hour after tank maintenance. The exact method used to feed fish and the feeding schedule will be recorded in the study raw data.

5.8 Drug Administration:

5.8.1 Dosing regime (dose(s), frequency, and duration): Three dose levels will be used. Fish will be assigned to either a nonmedicated control group, OTC-medicated group, or an FFC-medicated group. Fish in the FFC-medicated group will be offered FFC-medicated feed at a dose rate of 15 mg/kg BW/d for ten days. Fish in the OTC-medicated group will be offered OTC-medicated feed at a dose rate of 82.5 mg/kg BW/d for ten days. Fish in the medicated group will be offered the medicated feed for one ten-day period only and will receive nonmedicated control feed for the remaining post-dosing observation period.

5.8.2 Route of administration: Oral.

5.8.3 Investigational withdrawal period: Not applicable. All fish used will be euthanized by an overdose of MS-222 after the post-dosing period (UMESC GEN 132) except that surviving control fish may be returned to the providing facility if requested by the In-life study facility owner.

5.9 Removal of Subject(s) from the study:

5.9.1 Criteria for removal of subjects from the study: Only dead or moribund fish will be removed from the study. Moribund fish are those that have lost equilibrium and do not respond to physical stimulus.

5.9.2 Procedures for removal of subjects from the study: Dip nets will be used to remove dead test fish from test tanks. Net(s) will be disinfected before and after each use (UMESC GEN 132). Nets will be rinsed with well water before removing a dead or moribund fish from the tank.

5.9.3 Fate of removed study animals: During the trial, dead or moribund fish will be removed and sampled. If a fish dies in the test tanks before the first treatment administration, the fish will be subtracted from the original total number of fish in the tank. Dead or moribund fish not selected for microbiological assessment will be disposed of according to test site procedures.

5.10 Concurrent/Concomitant Medications/Therapies: There will be no concurrent/concomitant medication/therapy administered during the course of the study.

5.11 General management practices: Test tanks will be cleaned once daily when live fish are present before fish are fed. Tanks will be cleaned by draining or siphoning out uneaten feed and fecal matter. Surviving control fish will be returned to the In-life study facility at the conclusion of the study at the discretion of the In-life study facility manager. Surviving medicated fish will be counted and euthanized by an overdose of MS-222 (UMESC GEN 132) and disposed of according to hatchery procedures.

5.12 Provisions for necropsy and disposal of expired test subjects: Weight, length and observations will be recorded. Necropsy procedures such as gill clips and skin scrapes will be performed on no more than 2 dead or moribund fish per tank per day during the study period. Necropsy procedures are detailed in Appendix 1.

6. SPECIFICATION OF VARIABLES:

6.1 Variable(s) to be measured for evaluating labeled claim: Mortality; we intend to evaluate product effectiveness using the primary outcome measure of cumulative mortality in each tank on the last day of the study (Day 23) divided by the number of fish in each tank at study initiation. Moribund fish will be considered a mortality in this study. Cumulative moribund fish and cumulative morality will be summed together on Day 23 to represent the study cumulative mortality for statistical purposes.

6.1.1 When variable will be assessed: Twice daily during treatment period and continuing through 14 d after the last dose administration. The cumulative mortality in each tank on Study Day 23 will be calculated as the total cumulative mortality on Study Day 23 divided by the number of total number of fish in each tank at study initiation (described in section 14.2).

6.1.2 Procedures for assessing variable: Visual observation of lack of opercular movement and lack of response to stimuli of caudal peduncle, counting of dead fish.

6.1.3 Equipment used to assess variable: Not applicable

6.2 Other variables to be recorded during the study:

6.2.1 When variable(s) will be assessed

Feed consumption: Once daily from Day -1 through Day 23 concurrent with tank cleaning.
Body weight/length: During disease confirmation (Day -1; 30 fish) and at completion of the post-dosing period (Day 23; 10 fish/test tank or surviving fish, whichever is less).
Isolation of motile Aeromonads: Thirty (30) fish pre-treatment. Only dead or moribund fish will be collected for isolations of motile Aeromonad which will not exceed ≤10 fish/test tank during the dosing period, with no more than 5 fish collected per test tank per 5-d period during dosing (two sample periods within 10 d; D 0 to 4 and D 5 to 9); ≤10 fish/test tank during the post-dosing observation period, with no more than 5 fish collected per test tank per 7-d period during dosing (two sample periods within 14 d; D 10 to 16 and D 17 to 23); and 5 fish/test tank at study termination. Mortalities or moribund fish that exceed the number of fish to be sampled for isolations of motile Aeromonads will be weighed, measured, observed and recorded. Disposal of mortalities will follow the In-life study facility procedures.
Fish Behavior: General fish behavior will be observed in the test tanks daily concurrent with the first mortality observation.

6.2.2 Procedures for assessing variable
Feed consumption: Visual assessment of the amount of uneaten feed remaining in each test tank will be recorded as a (1) if >75% of feed remains, (2) if > 50% of feed remains, (3) if >25% of feed remains or (4) if >0% of feed is remaining. The presence or absence of fecal material in a tank will be recorded as present or absent. Feed consumption will be recorded 1 hour post-dosing.
Body weight/length: weight (wet) and length (total length) will be measured according to UMESC AEH 606.
Isolation of motile Aeromonads: Initial samples will be streaked for isolation on Tryptic Soy Agar (TSA) plates. Identification and characterization will be defined under a separate protocol.
Fish Behavior: Behavior will be noted as normal or abnormal. Abnormal behavior observed will be described on the fish behavior recording form.

6.2.3 Equipment used to assess variable
Feed consumption: Not applicable.
Body weight/length: weight – Sartorius BP 3100S balance (S/N 12907582; UMESC AEH 338) or equivalent; length – metric ruler or fish measuring board.

7. DATA ANALYSIS

7.1 Define the experimental unit: The experiment unit will be the test tank.

7.2 Define the number of replicates per treatment: six.

7.3 Define statistical methodology to be used. Cumulative tank level mortality on Study Day 23 will be analyzed using a generalized linear mixed model assuming a binomial distribution. The model will include a fixed treatment effect and a random tank within treatment effect to adjust for over-dispersion among study tanks. The model will include a fixed treatment effect, a random block effect and a random tank within treatment effect to adjust for over-dispersion among study tanks. We intent to evaluate product effectiveness using the primary outcome measure of cumulative mortality in each tank on the last day of the study (Day 23) divided by the number of fish in each tank at study initiation.

7.4 Define how the statistical results will be used to draw conclusions about the study's objectives. Statistical significance will be declared at $p \leq 0.05$ using a two-sided test to evaluate effectiveness. The effectiveness of oxytetracycline-medicated feed will be evaluated using only data collected from the oxytetracycline treated and the control tanks. The effectiveness of florfenicol-medicated feed will be evaluated using only data collected from the florfenicol treated and control tanks.

7.5 Other data analyses. Statistical methods for other study data collected (weight, length, etc.) will include calculation of means, standard deviations, and coefficients of variation of drug assay results. Other statistical methods as required will be documented in the study data and described in the final report.

8. PERSONNEL: All study personnel will be identified in the final study report; curriculum vitae are on file at UMESC.

8.1 Supervisory Biologist: Mark P. Gaikowski, M.A.

8.1.1 Address: Upper Midwest Environmental Sciences Center, 2630 Fanta Reed Rd, La Crosse, Wisconsin 54603

8.1.2 Contact: Tel: (608) 781-6284, Fax: (608) 783-6066, Email: mgaikowski@usgs.gov

8.1.3 Training and experience: CV on file at UMESC.

8.2 Quality Assurance Unit

8.2.1. Acting Quality Assurance Officer: Jane E. Rivera,

8.2.2. Address: Upper Midwest Environmental Sciences Center, 2630 Fanta Reed Road, La Crosse, Wisconsin 54603

8.2.3. Contact: Tel: (608) 781-6214; Fax: (608) 783-6066, Email: jerivera@usgs.gov

8.3 Principal Investigator: Maren T. Tuttle-Lau, M.S.

8.3.1 Address: Upper Midwest Environmental Sciences Center, 2630 Fanta Reed Rd, La Crosse, Wisconsin 54603

8.1.2 Contact: Tel: (608) 781-6243, Fax: (608) 783-6066, Email: mttuttle@usgs.gov

8.1.3 Training and experience: CV on file at UMESC.

9. COLLECTION AND RETENTION OF SOURCE DATA.

All data generated in the study at the in-life facility or the microbiology test facility (LFHC) will be recorded in bound laboratory notebooks or kept in file folders (UMESC GEN 008) at the respective facility; the analytical and contract laboratories will record data according to their standard procedures. All data sheets, file folders, laboratory notebooks and computer disks will be encoded with the study number when the data are generated and stored in secure files (UMESC GEN 008). Completion reports and other study associated data received at UMESC from contract laboratories will be encoded with the study number and stored in secure files. Upon completion of data collection for a given trial, exact copies will be retained by the In-life facility and original raw data transferred to UMESC for storage in secure, fire-proof cabinets. Raw data, laboratory notebooks and electronic files generated by the in-life facility and LFHC and contract laboratory reports will be filed in the UMESC archives (UMESC GEN 023) before the Principal Investigator signs the final report. The final report will then be signed and archived. Each test facility (In-life study facility) will be provided with a copy of the final study report to retain with the study data developed by their facility in their facility archive.

The UMESC-QA will review the study protocol. Study activities performed and records developed at the In-life facility or FHC will be periodically examined by UMESC-QA. The final report will be prepared by the Principal Investigator and inspected by the Supervisory Biologist and UMESC-QA.

10. GOOD CLINICAL PRACTICE

Data collection, storage and retrieval procedures for the study will be conducted in compliance with FDA regulations for Good Clinical Practices (GCP; FDA 2001). The study protocol and progress of the study will be reviewed at the start of the study and periodically throughout the study by the Study Monitor. The Principal Investigator has the responsibility of ensuring that all procedures used in conjunction with the study conform with GCP. Any data that is generated that does not conform to GCP principles will be identified in the final study report. Standard Operating Procedures (SOP's) to be used in this study are listed in Section 17 of the study protocol.

11. AMENDMENTS/DEVIATIONS TO THE PROTOCOL:

11.1 Protocol amendments: A signed copy of the Study Protocol will be retained on-site at the in-life facility during the pre-dosing, dosing, and post-dosing periods. Proposed amendments

to the protocol shall be brought to the attention of the Supervisory Biologist, Center Director and UMESC QAU. When the Principal Investigator, Supervisory Biologist and Center Director agree verbally, the study can proceed with the change. As soon as possible, the Principal Investigator will then prepare a written protocol amendment that is signed by the Principal Investigator, UMESC Center Director, and UMESC-QA. The Animal Owner or UMESC Animal Care and Use Chair may also sign as needed. The amendment then becomes an official part of the protocol.

11.2 Protocol deviations: All deviations from this approved protocol will be documented and reviewed by the Principal Investigator. The Principal Investigator will make a judgment on the impact of the deviations. The Principal Investigator will notify the Supervisory Biologist and UMESC-QAU as soon as possible, in writing, of any deviations to the protocol, including their impact on the study.

12. INVESTIGATIONAL DRUG AND CONTROL:

12.1 Test Substance(s): Aquaflor® 50% Type A Medicated Article (50% florfenicol)

- 12.1.1 Chemical name: R-(R*,S*)-2,2-dichloro-N-[1-(fluoromethyl)-2-hydroxy-2-[4-(methylsulfonyl)phenyl)ethyl] acetamide
- 12.1.2 Trade name: Aquaflor®
- 12.1.3 Active/inactive ingredients: Aquaflor® is the final formulation. Florfenicol is the sole active ingredient. Inactive ingredients are lactose & polyvinylpyrrolidone.
- 12.1.4 Dosage form (solution, powder, granules, etc.): Feed premix
  - 13.1.4.1 Manufacturing site: Bray, Ireland
  - 13.1.4.2 Lot Number: to be determined upon receipt
  - 13.1.4.3 Batch Number: The batch number will be recorded in the study raw data and included in the final study report.
  - 13.1.4.4 Expiration/Recertification Date: to be determined upon receipt
- 12.1.5 Dose(s) to be tested (mg/gram, mg/mL, etc.): 0 or 15 mg FFC/kg BW/day for 10 consecutive days
- 12.1.6 Packaging: Foil packet
- 12.1.7 Drug storage during study: Aquaflor® premix will be stored in a locked cabinet under ambient environmental conditions at UMESC. A subsample of the test chemical will be archived according to UMESC Standard Operating Procedures.
- 12.1.8 Material Safety Data Sheet (MSDS): The MSDS will be on file at each facility handling either the pre-mix or the medicated diet (UMESC APP 054). Study staff will follow all safety procedures listed in the MSDS.

12.2 Test Substance(s): Terramycin 200 For Fish® Type A Medicated Article

- 12.2.1 Chemical name: mono-alkyl (C8-C18) trimethyl ammonium oxytetracycline
- 12.2.2 Trade name: Terramycin 200 For Fish®
- 12.2.3 Active/inactive ingredients: Type A medicated Article feed pre-mix that contains approximately 220 g oxytetracycline/kg premix
- 12.2.4 Dosage form (solution, powder, granules, etc.):
  - 12.2.4.1 Manufacturing site: Phibro Animal Health
  - 12.2.4.2 Lot Number: to be determined upon receipt
  - 12.2.4.3 Batch Number: The batch number will be recorded in the study raw data and included in the final study report.
  - 12.2.4.4 Expiration/Recertification Date: to be determined upon receipt
- 12.2.5 Dose(s) to be tested (mg/gram, mg/mL, etc.): 0 or 82.5 mg OTC/kg BW/day for 10 days
- 12.2.6 Packaging: 50 lb bag
- 12.2.7 Drug storage during study: Terramycin 200 For Fish® premix will be stored in a locked cabinet under ambient environmental conditions at UMESC. A subsample of the test chemical will be archived according to UMESC Standard Operating Procedures.
- 12.2.8 Material Safety Data Sheet (MSDS): The MSDS will be on file at each facility handling either the pre-mix or the medicated diet.

12.3 Control: The nonmedicated control ration will serve as the experimental control diet.

13. ADVERSE EVENTS: Any adverse event will be recorded in the study logbook and the Supervisory Biologist will be notified. A description of the vent will be recorded on the adverse event recording form.

14. DRUG DISPOSITION/ANIMAL ACCOUNTABILITY/FEED DISPOSITION/FEED ACCOUNTABILITY: The accountability and disposition of all unused drug supplies, unused feed, and animals will be documented.

14.1 Drug disposition: Upon arrival at UMESC, a bound logbook will be prepared for each packet of Aquaflor® or bag of Terramycin 200 For Fish® received to document use during the study. At completion of the study, an original drug packet label will be retained in the study raw data. The Principal Investigator will authorize, with UMESC-QA approval, the destruction of the remaining Aquaflor® or Terramycin 200 For Fish® at the conclusion of the study (UMESC GEN 007).

14.2 Animal accountability: Animal inventory in tanks at the In-life study facility will be accomplished according to the facility standard practices. Mortality in the source culture tanks will be recorded as per In-life study facility procedures. Upon entry into the study, a Record of Vertebrate Transfer (UMESC GEN 133) will be prepared to document study use. Daily mortality after transfer to the study will be recorded in the study raw data. Fish selected for microbiological testing will be processed at the In-life study facility by UMESC study personnel. At completion of the post-dosing observation period the number of fish remaining in each tank will be hand counted and recorded after euthanasia by an overdose of MS-222 (UMESC GEN 132). Fish will be hand counted at the end of the study to ensure no mortalities were missed during the course of the study. The number of live fish counted out of the study and the cumulative mortality/morbidity will be summed. This number should equal the number of fish added to the test tank. If the sum of these two numbers is less than the initial number of fish placed in the test tank, the difference will be added to the cumulative mortality (assumes that one or more dead fish was not recovered). If the sum of these two numbers is greater than the number of fish added to the tank, the difference will be added to the initial number added to the tank (assumes the initial number of fish was greater than initially recorded). Nonmedicated control fish may be hand counted at the end of the post-dosing observation period and returned to the facility owner if requested.

14.3 Feed disposition/accountability: Upon preparation, a feed log will be prepared to document medicated feed use during the study. Feed withdrawn will be documented in the log and will be stored in sealed, labeled containers at room temperature in a secure storage area. Withdrawn feed will be tracked on an individual tracking sheet that will be retained in the study raw data. Upon completion of the dosing and post-dosing period, unused feed will be inventoried by the Principal Investigator and returned to UMESC where the inventory will be certified by UMESC-QA. The Principal Investigator, with UMESC-QA review, will authorize the destruction of the remaining feed (UMESC GEN 007) in an approved landfill or by incineration at the conclusion of the study.

15. BIOSECURITY: Research will be conducted in such a manner as to minimize the risk of spread of pathogens and invasive species. Staff will have read and understood UMESC APP 075. All equipment and boots that come in contact with water or fish in the study area will remain in the study area and be properly treated or sterilized before removal or disposal. All study material (tanks, nets, etc.) will be disinfected prior to leaving UMESC, disinfected again before leaving the In-life study facility and again when the study materials return to UMESC. Hazard Analysis Critical Control Point (HACCP) procedures will be developed to identify and mitigate potential biosecurity risks associated with study activities to the UMESC facilities. Fish samples collected during the course of the study and transported to laboratories within UMESC will be contained in secondary containment apparatus reducing any biosecurity risk either facility. Samples will be held in refrigerators or freezers. Carts used to transport samples or equipment from the study area to other laboratories, the exterior of secondary containers, and equipment from the study area will be disinfected before being brought into other laboratories.

16. REFERENCES.

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Popoff, M. 1984. Genus III *Aeromonas*. Pages 545-548 *in* N. R. Krieg and J. G. Holt, editors. Bergey's Manual of Systematic Bacteriology. Williams and Wilkins, Baltimore, MD.

Shotts, E.B. Jr., and R. Rimler. 1973. Medium for the isolation of *Aeromonas hydrophila*. Applied Microbiology 26:550-553.

Wakabongo, M., E. Bortey, F.A. Meier, and H.P. Dalton. 1992. Rapid identification of motile *Aeromonas*. Diagnostic Microbiology and Infection Disease 15: 511-515.

Wang, G., C.G. Clark, C. Lui, C. Pucknell, C.K. Munro, T.M.A.C. Kruk, R. Caldeira, D.L. Woodward, and F.G. Rodgers. 2003. Detection and characterization of the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR. Journal of Clinical Microbiology 41:1048-1054.

17. STANDARD OPERATING PROCEDURES

UMESC GEN 008 - Maintenance of Data Recording of Raw Data for Regulated Studies
UMESC AEH 011 - Procedures for Labeling Chemicals & Specimens
UMESC GEN 023- Archives management for clinical studies intended to support Investigational New Animal Drug (INAD) exemptions sponsored by the UMESC
UMESC APP 045 – Project Plan Development, Review, Approval and Science Information System (SIS) Inputs
UMESC APP 054 – Laboratory Safety Devices
UMESC APP 075 – Procedures to Minimize the Risk of Transfer of Pathogens and Invasive Species
UMESC GEN 132 – Care, Maintenance & Disposal of Aquatic Vertebrates
UMESC GEN 133 – Receipt, Distribution, Care & Maintenance of Aquatic Vertebrates
UMESC AEH 310 – Hanna pH meter, Model H1991001, SN 370973
UMESC AEH 338 – Sartorius Balance, Model BP3100s, SN 12907582
UMESC AEH 394 – YSI Handheld Dissolved Oxygen Meter, Model 55/12FT, Serials 94C17261 & 97F0837AG
UMESC AEH 416 – Determination of oxytetracycline in fish feed
UMESC ECO 513 - Chain-of-Custody Procedures for Environmental & Experimental Samples
UMESC AEH 602 – Dissection and Weighing Procedures for Test Fish
UMESC AEH 605 - Procedures for Anesthetizing Experimental Animals
UMESC AEH 606 - Methods Used to Weigh, Measure & Mark Test Animals
UMESC AEH 706 - Determination of Total Alkalinity by the Titrimetric (pH 4.5) Method
UMESC AEH 712 - Determination of Total Hardness

Equivalent equipment may be used with its respective SOP, which will be identified in study data.

18. APPENDICES:

18.1 Necropsy, microbiological procedures and isolation recording form

18.2 Sample SAS randomization code to randomly assign treatment to test tanks.

18.3 Procedures to prepare Aquaflor® or Terramycin 200 For Fish® medicated feed

Appendix 1. Necropsy and microbiological procedures for *Aeromonas* sp. sample collection and presumptive identification.

1. Clinical signs: Clinical signs of MAS typically include hemorrhages and ulcerative skin lesions which may be on the surface or organs or deep within tissues (Inglis et al. 1993). External lesions may vary from an extensive superficial reddening of the surface of a large area of the body, often with necrosis of fins or tail (fin rot). Internally there may be an excess of ascetic fluid. The spleen is enlarged, rounded and cherry red. The enlarged kidney will often undergo liquefactive necrosis and necrotic fluid oozes out (Inglis et al. 1993).

2. Sample collection: The surface of the fish will be disinfected by swabbing with 70% ethanol (avoiding the anus and any skin lesions). After the surface dries, the body wall will be cut longitudinally following the ventral midline and the kidney exposed using aseptic techniques. A 1-µL sterile bacterial loop will be inserted into the posterior kidney and used to inoculate a trypticase soy agar (TSA) plate. Plates will be incubated at 30°C for 36 to 48 hours.

3. External Examination:

3.1 Skin biopsy, scraping: A skin scrape will be collected by gently scraping the edge of a clean microscope slide or scalpel along the fish's body. The area sampled will include external lesions, if present. The collected material will be transferred to a glass microscope slide, mixed with a drop of water then covered with a cover slip. The skin scrape wet mount will be viewed using standard light microscopy techniques to identify potential pathogens present (Noga 1996).

3.2 Skin biopsy, fin clip: A small (0.5 cm) piece of the caudal fin will be removed and placed on a clean microscope slide. A drop of water will be added to the fin clip then covered with a microscope slide. If the caudal fin has eroded such than the erosion precludes sampling then another fin will be sampled and the fin collected will be recorded. The fin clip we mount will be viewed using standard light microscopy techniques to identify potential pathogens present (Noga 1996).

3.3 External examination (gill biopsy): Before biopsy, gills will be examined grossly for overall appearance and color. Attention should be given to not confuse postmortem changes with gill coloration (gills quickly become pale pink after death due to the drainage of blood from the gills). A pair of scissors will be inserted into the gill chamber to remove the tips of several primary lamellae. Lamellae will be transferred to a microscope slide with drops of water on a cover slip (Noga 1996). The gill biopsy wet mount will be viewed using standard light microscopy techniques to identify potential pathogens present (Noga 1996).

4. Identification Procedures for *Aeromonas* sp.

4.1 Presumptive identification: Presumptive diagnosis will be based on observation of clinical signs and observation of gram-negative rods in gram stains or observation of motile bacteria with a monotrichous polar flagellum in wet mounts.

4.2 Confirmatory identification: Confirmatory diagnosis will be made based on the results of from the biochemical reactions listed in Table 1. Isolates which are determined to not be one of the species in Table 1 will be reported as *Aeromonas* sp.

5. Minimum Inhibitory Concentration and Zone of Inhibition testing.

5.1 Minimum Inhibitory Concentration will be conducted according to CLSI standard M49-A.

5.2 Zone of Inhibition testing will be performed according to CLSI standard M42-AE.

Appendix 2. Sample SAS randomization code to randomly assign treatment to test tanks.

```
options date number pageno=1;
/*****************************************************************************
*SAS ver 9.1 Randomizied assignment date:        Page ___ of ___     *
*                  Analysis prepared by:                                    *
*                          Verified by:  _______ (Date:_______)             *
*****************************************************************************/
/*Randomized assignment of treatment to tank*/
/* block 1 = Block A, block 2 = Block B*/
data trtassign;
 do block = 1 to 2 by 1;
  do tank = 1 to 9 by 1;
   /*do fish = 1 to 15 by 1;*/
    x = ranuni(-1);
    output;
   /*end;*/
  end;
 end;
run;
data trtasign; set trtassign;
 if block = 1 and tank = 1 then tankn = 'A1'; if block = 1 and tank = 2 then tankn = 'A2'; if block = 1 and tank = 3 then
tankn = 'A3'; if block = 1 and tank = 4 then tankn = 'A4'; if block = 1 and tank = 5 then tankn = 'A5'; if block = 1 and
tank = 6 then tankn = 'A6'; if block = 1 and tank = 7 then tankn = 'A7'; if block = 1 and tank = 8 then tankn = 'A8'; if
block = 1 and tank = 9 then tankn = 'A9';
if block = 2 and tank = 1 then tankn = 'B1'; if block = 2 and tank = 2 then tankn = 'B2'; if block = 2 and tank = 3 then
tankn = 'B3'; if block = 2 and tank = 4 then tankn = 'B4'; if block = 2 and tank = 5 then tankn = 'B5'; if block = 2 and
tank = 6 then tankn = 'B6'; if block = 2 and tank = 7 then tankn = 'B7'; if block = 2 and tank = 8 then tankn = 'B8'; if
block = 2 and tank = 9 then tankn = 'B9'; run;
proc sort data=trtasign;
 by block x;
run;
proc print;
data asigndtrt; set trtasign;
if _n_ = 1 then trt = 'control'; if _n_ = 2 then trt = 'control'; if _n_ = 3 then trt = 'control'; if _n_ = 4 then trt = 'control'; if
_n_ = 5 then trt = 'control'; if _n_ = 6 then trt = 'active'; if _n_ = 7 then trt = 'active'; if _n_ = 8 then trt = 'active'; if _n_ =
9 then trt = 'active'; if _n_ = 10 then trt = 'active'; if _n_ = 11 then trt = 'control';
if _n_ = 12 then trt = 'control'; if _n_ = 13 then trt = 'control'; if _n_ = 14 then trt = 'control'; if _n_ = 15 then trt =
'control'; if _n_ = 16 then trt = 'active'; if _n_ = 17 then trt = 'active'; if _n_ = 18 then trt = 'active'; if _n_ = 19 then trt =
'active'; if _n_ = 20 then trt = 'active';

proc sort data=asigndtrt;
 by block x;
run;

proc print data=asigndtrt;
title1 h=2 'Efficacy of;
title2 h=1.5 'Study Number X05XXX';
title3 h=1 'Random assignment of treatment to tanks';
title4 h=1 'SAS ver 9  Random number generation date:       prepared by:    ';
title5 h=1 'BLINDED DATA - FOR STUDY MONITOR USE ONLY ';
title6 h=1 'BLINDED DATA - UNTIL AFTER COMPLETION OF DOSING';
title8 h=1 'BLINDED DATA';
run;
```

Appendix 3. Procedures to prepare medicated feed

1. Aquaflor®

1.A. Precautions

1.A.1. Aquaflor®,
-Read and be familiar with the Material Safety Data Sheet.
-Wear appropriate protective clothing and other safety equipment (e.g., lab coat or uniform; latex, rubber, or other gloves; plastic goggles or safety glasses) when handling bulk Aquaflor® powder.
-Avoid inhalation, skin or eye contact. If inhalation should occur, remove to fresh air. If breathing is difficult, seek medical attention. Wash contact area with soap and water if skin contact occurs. If eye contact occurs, flush with water for 15 minutes, and seek medical attention.

1.A.2. Feed Mixer. Before preparing Aquaflor®-medicated feed, read and understand the feed mixer operating instructions.

1.B. PROCEDURE:

1.B.1. Clean the mixer using Liquinox and hot water. Flush with copious amounts of well water. After flushing, rinse with ethanol or acetone followed by final flush with well water. Dry mixer completely before use. Immediately before use, mix a minimum of ~5 kg nonmedicated feed in the mixer for a minimum of 5 min then discard the feed.

1.B.2. Weigh all ingredients (fish feed [nearest 1.0 g], Aquaflor® [nearest 0.01 g], and fish oil [0.01 g]) prior to mixing.

1.B.3. Add appropriate amount (~20 kg) of feed to mixer.

1.B.4. Turn on mixer, and slowly add Aquaflor® near the center of the mixer (requires ~30 - 60 seconds).

1.B.5. Run for approximately 1 minute after adding Aquaflor®.

1.B.6. Heat fish oil for $\leq$30 s in microwave to improve spray delivery (25 seconds will heat ~50 mL of fish oil to allow it to be easily sprayed from the spray bottle). Spray fish oil as uniformly as possible over the feed using a hand held spray bottle (requires ~1-2 min).

1.B.7. Run mixer for 1-2 min after oil has been added.

1.B.8. Remove feed from mixer. Feed samples should be collected to represent the top, middle and bottom of the feed mass within the mixer. Place feed into labeled bags and store according to the study protocol.

1.C. CALCULATIONS: See section 7.2 of the study protocol

1.C.1. Information needed before starting calculations:
- Total number of fish
- Average fish weight (g/fish)
- Feed rate (% body weight)

1.C.2. Determine the total mass of fish to be treated - see Figure 2 of study protocol)

1.C.3. Determine the amount of Aquaflor® premix required - see Figure 2 of study protocol)

1.C.4. Determine the amount of fish oil required:

Example: 20,000 g fish feed oiled at 1% w/w fish oil

20,000 g feed x 0.01 = 200 g fish oil

Appendix 3. Aquaflor®-medicated feed preparation data sheet

1. Fish information:	Total number of fish	_____ fish	Date/Initials
	x average fish mass	x_____ kg/fish	
	= Fish mass	= _____ kg	_____
2. Aquaflor®-medicated feed required day	Fish mass	_____ kg	Date/Initials
	x feed rate (to be determined, TBD)	x ___ %	
	=required daily medicated feed (kg)	= _____ kg	_____
3. Target feed FFC concentration	Dose (mg FFC/kg BW)	15 mg/kg	Date/Initials
	÷ feed rate (TBD)	÷ % BW	
	= Target feed FFC concentration	= mg/kg	_____
4. Amount of Aquaflor® premix required:	Feed mass to be prepared (~20 kg)	_____ kg	Date/Initials
	x target feed FFC concentration	x mg/kg	
	x 1 g / 1000 mg	x 1 g / 1000 mg	
	x 2 (Aquaflor® premix is 50% FFC)	x 2	
	= mass of Aquaflor® premix required	= _____ g Aquaflor	_____
5. Amount of fish oil required:	Mass medicated feed prepared	_____ g	Date/Initials
	x oil rate (0.5-2% w/w)	x _____ % w/w	
	Total oil required	_____ g	_____

6. Ingredient masses	Ingredient	Mass (g)	Date/Initials
	Fish feed (balance _____)		
	Aquaflor® premix (balance _____)		
	Fish oil (balance _____)		

7. Feed preparation	Feed preparation step	Complete (Y/N)	Date/Initials
	Mixer cleaned		
	Mixer flushed with control feed		
	Control diet added to mixer (time:_____)		
	Aquaflor® premix added to mixer while running (start time:_____ / end time:_____)		
	Feed and premix mixed for 1 min (start time:_____ / end time:_____)		
	Fish oil sprayed on feed while mixing (start time:_____ / end time:_____)		
	Oiled feed mixed for 1-2 min (start time:_____ / end time:_____)		
	Feed removed from mixer and samples collected		

Comments: _____
_____
_____
_____
_____

Appendix 3. Procedures to prepare medicated feed

2. Terramycin 200 For Fish®

2.A. Terramycin 200 For Fish®

-Read and be familiar with the Material Safety Data Sheet.
-Wear appropriate protective clothing and other safety equipment (e.g., lab coat or uniform; latex, rubber, or other gloves; plastic goggles or safety glasses) when handling bulk Terramycin 200 For Fish® powder.
-Avoid inhalation, skin or eye contact. If inhalation should occur, remove to fresh air. If breathing is difficult, seek medical attention. Wash contact area with soap and water if skin contact occurs. If eye contact occurs, flush with water for 15 minutes, and seek medical attention.

2.A.2. Feed Mixer. Before preparing Terramycin 200 For Fish® medicated feed, read and understand the feed mixer operating instructions.

2.B. PROCEDURE:

2.B.1. Clean the mixer using Liquinox and hot water. Flush with copious amounts of well water. After flushing, rinse with ethanol or acetone followed by final flush with well water. Allow mixer to dry for 24 h before use. Immediately before use, mix a minimum of ~5 kg nonmedicated feed in the mixer for a minimum of 5 min then discard the feed.

2.B.2. Weigh all ingredients (fish feed [nearest 0.5 g], Terramycin 200 For Fish® [nearest 0.01 g], and fish oil [0.01 g]) prior to mixing.

2.B.3. Add appropriate amount (~20 kg) of feed to mixer.

2.B.4. Turn on mixer, and slowly add Terramycin 200 For Fish® near the center of the mixer (requires ~30 - 60 seconds).

2.B.5. Run for approximately 1 minute after adding Terramycin 200 For Fish®

2.B.6. Heat fish oil for $\leq$30 s in microwave to improve spray delivery (25 seconds will heat ~50 mL of fish oil to allow it to be easily sprayed from the spray bottle). Spray fish oil as uniformly as possible over the feed using a hand held spray bottle (requires ~1-2 min).

2.B.7. Run mixer for 1-2 min after oil has been added.

2.B.8. Remove feed from mixer. Feed samples should be collected to represent the top, middle and bottom of the feed mass within the mixer. Place feed into labeled bags and store according to the study protocol.

2.C. CALCULATIONS: See section 7.2 of the study protocol

2.C.1. Information needed before starting calculations:

- Total number of fish
- Average fish weight (g/fish)
- Feed rate (% body weight)

2.C.1. Determine the total mass of fish to be treated - see Figure 2 of study protocol)

2.C.2. Determine the amount of Terramycin 200 For Fish® premix required - see Figure 2 of study protocol)

2.C.3. Determine the amount of fish oil required:

Example: 20,000 g fish feed oiled at 1% w/w fish oil

20,000 g feed x 0.01 = 200 g fish oil

Appendix 3. Terramycin 200 For Fish® medicated feed preparation data sheet

1. Fish information:	Total number of fish	_____ fish	Date/Initials
	x average fish mass	x_____ kg/fish	
	= Fish mass	= _____ kg	_____
2. Terramycin 200 For Fish® medicated feed required day	Fish mass	_____ kg	Date/Initials
	x feed rate to be determined (TBD)	x % BW	
	=required daily medicated feed (kg)	= _____ kg	_____
3. Target feed OTC concentration	Dose (mg FFC/kg BW)	82.5 mg/kg	Date/Initials
	÷ feed rate (TBD)	÷ % BW	
	= Target feed OTC concentration	= mg/kg	_____
4. Amount of Terramycin 200 For Fish® premix required:	Feed mass to be prepared (~20 kg)	_____ kg	Date/Initials
	x target feed OTC concentration	x mg/kg	
	x 1 g / 1000 mg	x 1 g / 1000 mg	
	/ 0.44 (Terramycin 200 For Fish® premix is 44% OTC)	/ 0.44	
	= mass of Terramycin 200 For Fish® premix required	= _____ g OTC	_____
5. Amount of fish oil required:	Mass medicated feed prepared	_____ g	Date/Initials
	x oil rate (0.5-2% w/w)	x _____ % w/w	
	Total oil required	_____ g	_____

6. Ingredient masses	Ingredient	Mass (g)	Date/Initials
	Fish feed (balance _____)		
	Terramycin 200 For Fish® premix (balance _____)		
	Fish oil (balance _____)		

7. Feed preparation	Feed preparation step	Complete (Y/N)	Date/Initials
	Mixer cleaned		
	Mixer flushed with control feed		
	Control diet added to mixer (time:_____)		
	Terramycin 200 For Fish® premix added to mixer while running (start time:_____ / end time:_____)		
	Feed and premix mixed for 1 min (start time:_____ / end time:_____)		
	Fish oil sprayed on feed while mixing (start time:_____ / end time:_____)		
	Oiled feed mixed for 1-2 min (start time:_____ / end time:_____)		
	Feed removed from mixer and samples collected		

Comments: _____
_____
_____
_____