

**STUDY PROTOCOL FOR A COMPASSIONATE AQUACULTURE
INVESTIGATIONAL NEW ANIMAL DRUG (INAD)
EXEMPTION FOR COPPER SULFATE
(INAD # 9101)**

Sponsor:

U.S. Fish and Wildlife Service, Division of Fish Hatcheries

Sponsor Signature

Date Approved

Manufacturer:

Phelps Dodge Refining Corporation
P.O. Box 20001
El Paso, Texas 79998

Facility for Coordination of Copper Sulfate INAD:

Bozeman National INAD Office
4050 Bridger Canyon Road
Bozeman, Mt 59715

Proposed Starting Date March 23, 1995

Proposed Ending Date March 22, 1996

Study Director Mr. Jim Bowker

Study Director Signature

Date

Clinical Field Trial Location and Trial Number:

Type or Print Facility Name

Trial Number

Investigator _____
Type or Print Name

Investigator Signature

Date

STUDY PROTOCOL FOR A COMPASSIONATE AQUACULTURE INVESTIGATIONAL NEW ANIMAL DRUG (INAD) EXEMPTION FOR COPPER SULFATE UNDER INAD #9101

I. STUDY ID AND TITLE

Clinical field trials to determine the effectiveness of copper sulfate in controlling external protozoan and metazoan parasites, bacterial, and fungal diseases on a variety of cultured warmwater fish species. INAD #9101

II. SPONSOR

Dr. David Erdahl, U.S. Fish and Wildlife Service, Branch Chief, Aquatic Animal Drug Approval Partnership Program, 4050 Bridger Canyon Road, Bozeman, MT 59715; Phone: 406-587-9265 x 125; Fax: 406-582-0242; Email: dave_erdahl@fws.gov

Manufacturer: Phelps Dodge Refining Corporation
P.O. Box 20001
El Paso, Texas, 79998

Study Director: Mr. Jim Bowker, U.S. Fish and Wildlife Service, Aquatic Animal Drug Approval Partnership Program, 4050 Bridger Canyon Road, Bozeman, MT 59715; Phone: 406-587-9265 x 126; Fax: 406-582-0242; Email: jim_bowker@fws.gov.

Principal Regional INAD Coordinators: See Appendix I for names and addresses.

Study Monitors for Copper Sulfate INAD: See Appendix II for names and addresses.

III. INVESTIGATORS/FACILITIES

See Appendix IIIa for names and addresses. Each facility has been assigned a trial number that reflects the INAD number (9101) and a unique number for that facility (e.g., San Marcos NFH&TC 9101- 01).

IV. PROPOSED STARTING AND COMPLETION DATES:

Proposed Starting Date: March 23, 1995

Proposed Completion Date: March 22, 1996

V. BACKGROUND/PURPOSE

A. Ectoparasitic disease

Protozoan and metazoan ectoparasites are responsible for over 40% of diseases of cultured warmwater fishes. Among these are obligate parasites that require a fish host and other, normally facultative, organisms that appear to become pathogens when poor water quality, poor health, or poor nutrition cause unacceptably high stress on fish. The genera represented in the group of protozoan ectoparasites include all Trichodinids, *Ichtyobodo*, *Abmiphya*, *Trichophyra*, *Ichthyophthirius*, *Apiosoma*, and *Chilodonella*. A few other genera combine to represent about 1% of reported protozoan diseases. Genera of metazoan ectoparasites known to cause fish diseases include *Cleidodiscus*, *Gyrodactylus*, and *Dactylogyrus*. Diagnosis is relatively simple and the different ectoparasites are easily differentiated, morphologically. When an ectoparasite infection is established, serious gill and surface damage can occur in a very short time. The outcome may be the death of the fish as a direct result of damage done by the parasites, or secondary infections may be established due to exposure of sensitive unprotected areas to water borne bacteria and fungi.

B. Columnaris disease

Columnaris disease (caused by *Flavobacteria columnare*) is an acute to chronic bacterial infection that has been reported as a mortality factor in several species of cultured catfish, bait minnows, goldfish, basses, and sunfish (Post 1987). Columnaris disease is reported to occur in 33% of warmwater diseases. Optimum temperatures for its occurrence are near 28 - 30°C, but epizootics will occur in cultured fishes at 10 - 17°C. Columnaris disease seldom occurs in waters below 10°C; thus outbreaks are the highest during the summer months. Stressors such as crowding and handling are predisposing factors, although highly virulent strains of flavobacteria can cause outbreaks even in cooler waters.

The transmission of *F. columnare* from fish to fish occurs directly through the water. Fish infected with the organism can harbor it over winter and then become sources of infection during the summer months.

F. columnare first invades the skin of the head region, including the mouth, lips, cheeks and gills or injuries to the body of the fish. The type of lesions varies with the species of fish. In scaleless fish such as channel catfish, the lesions are small and circular with gray-blue necrotic centers and red margins surrounded by a ring of inflamed tissue. In scaled fish, necrotic lesions begin at the outer margin of the fins and spread toward the body. The gills may be involved and demonstrate light-colored areas at the tips of the gill filaments. Infected areas on surface or gills may be found to contain many characteristic microscopically visible masses of bacterial growth. As the disease progresses, gill filaments are lost to advancing necrosis and sloughing of gill tissue (Bullock et al. 1986). The bacterium may invade the blood stream through a gill or skin lesion and become systemic. Columnaris disease is usually fatal within a relatively short period of time following development of a bacteremia (Post 1987).

C. Fungal disease

The incidence of fungal diseases varies between 3 and 8% from year to year in warmwater fishes, and the severity in any year may depend largely on the severity of the winter season. Fungal species of the genera *Saprolegnia*, *Achlya*, *Aphanomyces*, *Leptomitus*, and *Pythium* have been reported to cause fish

disease, with *Saprolegnia* as the most commonly involved. When the integrity of the skin or scale barrier is broken, or the mucus layer is removed, fungal growth may begin from invading aquatic borne fungal zoospores that are always present in fish culture water. Although these pathogens are considered to be secondary invaders, once a fungal colony is established it can continue to grow and spread and can, without treatment, lead to the death of large numbers of fish.

While precise identification of fungus specimens from lesions on fish requires a considerable level of familiarity with taxonomy of the aquatic Phycomycetes, the detection of a significant level of fungal involvement does not require rigorous classification. Fungal infection is recognized as white to brown or darker patches of cottony growth on lesions on the body surface, on the fins and barbels, or in the mouth. Microscopically, individual hyphae are evident that are nonseptate and about 20 microns in diameter. Older segments of hyphae often terminate in zoosporangia containing zoospores. Once recognized, the growth of an aquatic phycomycete on lesions is difficult to confuse with any other aquatic form.

D. Control of parasites, columnaris, and fungus diseases

Copper sulfate is the preferred treatment for many surface infections involving protozoan and metazoan ectoparasitic, bacteria, and aquatic fungi of many cultured warmwater fishes. The amount of copper sulfate used for disease control in fish culture is the same level used for aquatic algae control, for which it is approved by EPA. The effectiveness of copper sulfate, either as an algae control or fish disease treatment, is related to both total alkalinity, expressed as mg/L as CaCO₃, and total hardness, also expressed as mg/L as CaCO₃. Alkalinity can be related to hardness, in the following manner:

$$\text{Alkalinity} = \text{antilog}[-4.720 + \text{pH} - 1.381\text{Log}_{10} \text{Hardness} + 0.1601 \text{pH} \text{Log}_{10} \text{Hardness} - 0.08175 \text{pH}^2 + 0.1753 (\text{Log}_{10} \text{Hardness})^2] - 1.0$$

Using this methodology, it is necessary to deal with only one mineral classification. The formula used for calculating the dosage of copper sulfate, based on total alkalinity, is:

$$\frac{\text{Total alkalinity}}{100} = \text{mg/L CuSO}_4 \bullet 5\text{H}_2\text{O}$$

There is general agreement that copper sulfate should not be applied to water of total alkalinity less than 40 mg/L as CaCO₃ because of toxicity to most species of cultured fishes at low alkalinity. At low alkalinity the toxic effects of certain Cu species (Cu⁺², CuOH, and Cu₂(OH)₂⁺²) are dramatically increased.

When copper sulfate is used to treat a static pond, the chemical is applied as a powder and can be broadcast across the surface of the water, spread from a sack pulled behind a boat moving around the pond, or applied in the wake of a paddle wheel (or other type aerator) which circulates the chemically treated water. When a raceway is treated, the chemical is applied either to the entire contents of the raceway which has had the flow stopped for the interval of the treatment or can be metered into the flow of the incoming water to gradually build up to the correct treatment level which is maintained for the necessary treatment time.

When static ponds are treated, the treatment is allowed to remain in the pond:

this is called treatment for an indeterminate time. When water in a raceway is treated, a definite time for treatment is determined in advance and after that time, the water in the raceway is discharged and replaced with fresh water. These are called timed treatments.

Special variations of treatment are recommended for certain fish diseases and the special considerations used in determining the proper treatment are included in the research protocol. However, an example will illustrate the need for these special treatments. The protozoan, *Ichthyophthirius multifiliis*, cause of "Ich" or "white spot", has a life cycle which proceeds in stages that at times do, and at times do not, involve infection of the fish host. During the stage of fish infection, treatment is ineffective; the parasite is protected by the integument. When the protozoan is in a free swimming stage, away from the fish, it can be readily killed with copper sulfate. The transformation from stage to stage takes 4 to 70 days, depending on water temperature and at temperatures above about 27°C the protozoan enters a non-infective phase in its life cycle. While temperatures remain in the range of 7°C to 26°C the protozoan repeatedly cycles from infective stage to free-swimming stage. To effectively remove the infection from a population of fish, the free-swimming stage must be treated each time it appears, so repeated treatments must be applied, at intervals determined by the water temperature, until the disease is under control.

E. Purpose

The purpose of this compassionate INAD on copper sulfate is to develop clinical field trial data that will extend the knowledge of the most appropriate treatment regimens and treatment times for controlling protozoan and metazoan parasitic, bacterial, and fungal diseases of selected cultured warmwater fishes. These data will be used to support a new animal drug application (NADA) for copper sulfate.

The USFWS anticipates requesting that the U.S. Food and Drug Administration (FDA) grant an extension of the copper sulfate INAD for at least an additional 2 years at the end of the first treatment season. The USFWS feels that data from at least 3 treatment seasons will be required in order to adequately assess the effectiveness of copper sulfate for controlling protozoan and metazoan parasitic, bacterial, and fungal diseases of selected cultured warmwater fishes.

VI. SPECIFIC OBJECTIVES

- A. Collect scientific data necessary to establish the effectiveness of copper sulfate for treatment and control of protozoan and metazoan ectoparasitic, bacterial, and fungal diseases of selected cultured warmwater fishes.
- B. Provide an opportunity for USFWS fish culturists to legally use copper sulfate to control protozoan and metazoan ectoparasitic, bacterial, and fungal diseases of cultured fishes and maintain healthy stocks of fish during the period of time necessary for collection of data on temporal effectiveness that will be used to support an NADA for the use of copper sulfate on fish.

VII. MATERIALS

A. Test and control articles

1. Identity

a. Active ingredient

Trade Name: Copper sulfate

Chemical Name: Cupric sulfate pentahydrate

Structural formula: $[\text{Cu} \bullet (\text{H}_2\text{O})_4] \text{SO}_4 \bullet \text{H}_2\text{O}$

Appearance: Large, blue or ultramarine, triclinic crystals or blue granules or light-blue powder.

(See Appendix IV for further information on nomenclature, as well as chemical and physical properties of the pure chemical).

b. Strength and dosage form

Copper sulfate is 25.5% copper; the copper portion of the molecule [either as the free Cu^{+2} ion or as the complexed CuOH^+ or $\text{Cu}_2(\text{OH})_2^{+2}$], and not the sulfate radical, is the active species of this chemical. In application, powdered copper sulfate is dissolved in water at a rate determined by the alkalinity or hardness of the water. Alkalinity can be related to hardness, in the following manner:

$$\text{Alkalinity} = \text{antilog}[-4.720 + \text{pH} - 1.381\text{Log}_{10} \text{Hardness} + 0.1601 \text{pH} \text{Log}_{10} \text{Hardness} - 0.08175 \text{pH}^2 + 0.1753 (\text{Log}_{10} \text{Hardness})^2] - 1.0,$$

so that it is necessary to deal only with one mineral classification. The formula used for calculating the dosage of copper sulfate, based on total alkalinity, is:

$$\frac{\text{Total alkalinity (mg/l as CaCO}_3\text{)}}{100} = \text{mg/L CuSO}_4 \bullet 5\text{H}_2\text{O}$$

There is general agreement that copper sulfate should not be applied to water of total alkalinity less than 40 mg/L as CaCO_3 because of toxicity to most species of cultured fishes at low alkalinity. At low alkalinity the toxic effects of certain Cu species (Cu^{+2} , CuOH , and $\text{Cu}_2(\text{OH})_2^{+2}$) are dramatically increased.

When copper sulfate is used to treat a static pond, the chemical is applied as a powder and can be broadcast across the surface of the water, spread from a sack pulled behind a boat moving around the pond, or applied in the wake of a paddle wheel (or other type aerator) which circulates the chemically treated water. When a raceway is treated, the chemical is applied either to the entire contents of the raceway which has had the flow stopped for the interval of the treatment or can be metered into the flow of the incoming water to gradually build up to the correct treatment level which is maintained for the necessary treatment time.

When static ponds are treated, the treatment is allowed to remain in the pond: this is called treatment for an indeterminate time. When water in a raceway is treated, a definite time for treatment is determined in advance and after that time, the water in the raceway is discharged and replaced with fresh water. These are

called timed treatments.

c. Manufacturer, source of supply

Phelps Dodge Refining Corporation
P.O. Box 20001
El Paso, Texas 79998

Contact: David Fisher
Phone: 915-775-8853
Fax: 915-775-8350

2. Verification of drug integrity and strength

The manufacturer (Phelps Dodge Refining Corporation) will provide the analytical data necessary to establish purity of each lot of copper sulfate supplied. The lot number and date of manufacture for each batch of copper sulfate will be placed on the label of each container. The lot number and date of manufacture will also be recorded on Form 1 "Guide for Reporting Investigational New Animal Drug Shipments for Poikilothermal Food Animals" that will be used to notify FDA of drug orders/shipments. If the integrity of the copper sulfate is compromised (e.g., by spilling or contamination of the stock container) the event will be carefully recorded, dated, and signed in the Chemical Use Log (Form 2). The Study Monitor assigned to the Investigator involved will be immediately notified and the remaining material will be returned to the Study Monitor along with copies of Forms 1 and 2.

3. Storage conditions

Copper sulfate will be stored in the original container supplied by the manufacturer with the appropriate investigational label attached. The container will be stored in a cool, dry location away from direct sunlight. The container will NOT be stored in a refrigerator since opening a cold container can cause condensation of moisture on copper sulfate. Copper sulfate should be stored in a secure location such as in a locked cabinet.

4. Handling procedures

Each Study Monitor and Investigator will be required to have a current copy of the Material Safety Data Sheet (MSDS) for copper sulfate (Appendix IVa). Each person involved with the study and each person who may be present during the use of copper sulfate shall be required to read the MSDS. Safety precautions as outlined in the MSDS will be followed at all times when working with copper sulfate. Standard laboratory equipment such as gloves, lab coats or aprons, eye protection, etc., will be worn at all times.

5. Investigational labeling

Copies of the labels to be attached to each container are provided in Appendix V. It is the responsibility of the Investigator to ensure proper labeling of containers

6. Accountability

Phelps Dodge Refining Corporation will ship copper sulfate to USFWS facilities in pre-weighed packets of 25 and 120 kg. Each USFWS Investigator will notify FDA prior to any shipment of copper sulfate for use under this INAD. Immediately upon placing an order with the approved supplier for copper sulfate, the investigator will complete Form

1, "Guide for Reporting Investigational New Animal Drug Shipments for Poikilothermic Food Animals" and send it to his/her Study Monitor. The Study Monitor will then send the original plus two copies to the FDA. Both the Investigator and the Study Monitor are required to sign Form 1. The Study Monitor will also send a single copy of Form 1 to the Study Director at the Bozeman National INAD Office. The Investigator will keep one copy of the completed Form 1 for the facility's INAD file. Arrangements should be made between Investigators and Study Monitors to insure completed Form 1s are received by the FDA within 7 days of the date an order was placed.

A Chemical Use Log (Form 2) will be supplied to each Investigator. Each time any copper sulfate is used, the weight of the chemical and container will be recorded and compared with the weight of chemical and container recorded after the previous use. The person weighing copper sulfate will also record the weight of chemical removed, the name and number of the study involved, his/her signature, and the date. After weighing out the amount of copper sulfate needed, the container and remaining chemical will be weighed and recorded. At the conclusion of the study, all remaining copper sulfate will be shipped to the Study Monitor along with the properly recorded Chemical Use Log (Form 2). The Study Monitor will then verify the Chemical Use Log against the quantity of copper sulfate remaining. All remaining copper sulfate will then be returned to the Manufacturer.

B. Items needed for sample collection, observations, etc.

Sampling and diagnostic equipment should include scissors, clean microscope slides, cover slips, a compound microscope, and a dissecting microscope.

When the Study Protocol has been approved and treatments are scheduled, the Investigator at each facility covered by the copper sulfate INAD will need to complete several forms:

- Form 1. Guide for reporting investigational new animal drug shipments for poikilothermic food animals.
- Form 2. Chemical use log for clinical field trials on copper sulfate under INAD #9101.
- Form 3. Diagnosis and treatment record for clinical field trials on copper sulfate under INAD #9101.
- Form 4. Disposal record for animals from clinical field trials on copper sulfate under INAD #9101.

Copies of these forms are attached to this Study Protocol.

VIII. EXPERIMENTAL UNIT

The experimental unit in this clinical field trial will consist of a contained or isolated group of fish. This could be a group of fish contained in a tank, raceway, or pond.

IX. ENTRANCE CRITERIA

- A. A list of the number and species to be treated under this INAD is presented in Appendix VI.

B. Diagnosis of disease

1. Protozoan and metazoan ectoparasites

a. Initial indications that ectoparasites may be present on fish will involve changes in behavior and gross appearance. Most of the diagnosis of the disease will be provided by the Investigator. If there is any question concerning the diagnosis, the Study Monitor will confirm the diagnosis. Descriptions of fish behavior and appearance suggesting the presence of ectoparasites may include the following:

- 1) Flashing, scratching, or rubbing against objects in the pond or raceway. Twitching, darting, or convulsing.
- 2) "Topping" or "piping" at the water surface; appear to be gasping for air.
- 3) Failure to feed.
- 4) Gills appear swollen, discolored, mottled bright red, may bleed freely when touched.
- 5) Areas of excess mucus. Nodules, white spots, or pustules appear on surface, fins, or gills.
- 6) Frayed fins or tail, eroded tail.

b. Fish selected for use as diagnostic samples should represent the diseased portion of the population. If such a collection is not possible, the second choice will be a random sampling of the fish. Selection techniques that produce the most healthy and vigorous fish, such as hook and line, should not be used. The same sampling techniques used for selecting diagnostic subjects should be used for selecting subjects for evaluating the results of the treatment.

c. Definitive diagnosis should be determined from microscopic examination of excised gill filaments, fin clippings, or mucus scrapings for the presence of identifiable parasites. A minimum of 5 fish should be selected and examined. Fish can be anesthetized with MS-222 to reduce trauma and to make the fish easier to handle. From each fish at least 5 gill filaments, clipped close to the gill arch should be wet-mounted on a clean slide, spread apart, and cover slipped. Terminal areas of fin rays should be clipped from each fish and wet-mounted. Mucus samples from the head and from the skin or scale surface (near the mid region along the lateral line) should be taken, placed on a slide and cover slipped.

The slides should be examined within 5 minutes of preparation. Mounted specimens should be scanned at dissecting scope range (25-40X), low power (100X), and high power (400-450X). Protozoan and metazoan parasites can be identified to the genus level by reference to numerous guides for identification of fish parasites.

d. The decision to apply copper sulfate should be based on the presence and density of identifiable protozoan and metazoan parasites. Prophylactic treatments unsupported by microscopically observed and recorded protozoan or metazoan parasites are not to be conducted under this research protocol. The decision to treat with copper sulfate may be reached in a number of ways, but any decision must be documented. Circumstances vary and parasite loads and

distributions will differ. However, if the decision to apply a treatment is made, it is necessary to have a basis for treatment and to have a record of the kinds and numbers of parasites present before treatment, to make it possible to later evaluate the success or failure of the treatment. The following are examples of observations that would warrant treatment:

- 1) An average of 25 or more trichodinids per filament in a wet mount of excised gill tissue in 3 of 5 fish examined. For fry and small fingerlings (less than 3"), 4 or more trichodinids per filament.
- 2) An average of 50 or more trichodinids per field (25-40X) in a wet mount of a clipped terminal fin ray in 3 of 5 fish examined. In fry or small fingerlings, 10 or more per field.
- 3) An average of 25 or more *Chilodonella sp.* per filament in a wet mount of excised gill tissue in 1 of 5 fish examined. Treat if any are found on small fingerlings or fry.
- 4) An average of 20 or more mixed protozoans per microscopic field (100X) in mucus samples from 5 of 5 fish examined.
- 5) An average of 1 *Ichthyophthirius* per fish for 3 of 5 fish examined. In cooler temperatures, treat whenever seen in fry and young fingerlings.
- 6) An average of 50 or more *Ambiphyra* per gill filament in 3 of 5 fish examined. In fry or small fingerlings, 10 or more per filament.
- 7) An average of 25 or more *Trichophrya* per gill filament in 3 of 5 fish examined. In fry or small fingerlings, 10 or more per filament.
- 8) An average of 25 or more *Costia sp.* per filament in 1 of 5 fish examined. A lower level if seen in 4 of 5 fish examined and any level seen in fry and young fingerlings.
- 9) An average of 40 or more *Apiosoma sp.* per gill filament or per field of view (40X).
- 10) An average of 50 or more *Dactylogyrus* on fingerlings less than 5.0 cm in length.

Note: Do not use copper sulfate to treat fry less than 3 weeks old. If the toxicity of copper sulfate for a species is not known, a preliminary exposure of a few fish to the chemical at the proposed rate and time should be done.

These examples are not intended to restrict or limit the freedom of managers in deciding when treatment is needed, as there are many other sets of observations that would support a decision to treat.

2. Fungal diseases

Fish most often have fungal infections during periods of cool to cold water temperatures (45 to 70°F); however, malnourishment and infections caused by other microorganisms may result in secondary fungal infections at any time of the year. The infection initially appears as white to dirty brown cottony patches that may be confined to the site of some other disease lesion or injury or may be spread over a larger portion of the animal.

Microscopic examination of excised gill filaments, scrapings from skin or scales, and fin clippings that reveal non septate hyphae that terminate in sporangia containing zoospores is sufficient evidence to warrant consideration of treatment. The presence, in a pond or raceway of fish, of 5% or more of the total population displaying visible signs of fungal infections that prove positive microscopically, or in 2 of 5 selected fish, is sufficient to warrant treatment of the pond or raceway with copper sulfate.

3. Columnaris disease

Lesions characteristic of columnaris disease initially occur on the body, head, fins, or gills as white to off-white spots that may be slightly eroded. Lesions on the skin usually become shallow ulcers that can progress deeply into the tissue and even expose underlying bone. On fins there is a loss of tissue from fin rays and the fins and tail take on a tattered appearance. Lesions often appear to start on the back near the dorsal fin and progress around the sides and under the abdomen giving a saddle-back appearance. Surface lesions involving skin or fin surfaces may have a yellowish coloration. Lesions on gills consist of areas of necrosis that start at the distal end of filaments and progresses toward the gill arch. Several filaments may be involved and the lesion may spread laterally as well as becoming more penetrating.

a. The presumptive diagnosis of CD is based on the presence of long, thin, bacterial rods in necrotic lesions on the surface of the skin or gills. This condition is sufficient to warrant treatment. Microscopic examination of gills or other necrotic tissues will reveal piles of the rod-shaped bacterium in a "haystack" appearance in wet mount preparations. For this observation, the wet mount should be prepared and a period of 15 minutes allowed to lapse before examination. This delay gives time for the "haystack" characteristic to develop.

b. Definitive diagnosis of CD requires isolation of the bacterium on cytophaga medium or slide agglutination test using *F. columnare* antiserum. The growth of *F. columnare* on solid media is yellow-green, with flat, rough, spreading colonies which adhere to the media.

c. Another definitive method involves the biochemical and cultural characteristics that are unique to *F. columnare*. Four characteristics can be observed directly on the original isolation plate: (1) growth in the presence of neomycin sulfate and polymyxin B; (2) color and colonial morphology consistent with typical *F. columnare*; (3) production of a diffusible, gelatin-degrading enzyme; and (4) binding of aqueous Congo red dye in the surface secretions of the suspect colony. The last characteristic would be observed by using a brief test for the presence of chondroitin AC lyase activity in an agar block excised from the isolation plate. If *F. columnare* is present, a diffusible enzyme that degrades chondroitin sulfate will be produced. Specific identification of *F. columnaris* can be made in minutes after visible growth appears on primary isolation plates.

- C. Ability of investigator to fulfill all the requirements of the Study Protocol--See Appendix IIIb for example of knowledge required of hatchery managers (i.e., Investigators)

X. TREATMENT GROUPS:

Separately confined, untreated, controls will not be required in the tests conducted to determine the effectiveness of copper sulfate. Fish from a group will first be examined to determine if treatment with copper sulfate is required. When treatment is underway or has been completed, fish from the same group will be examined to determine the effect of the treatment on the parameters used to sanction the treatment initially. This may consist of determining the percent reduction in the number of parasites per gill filament or per field of view and data will be recorded in these terms.

Although untreated control groups are not a required element of treatment under this INAD exemption and are at the discretion of the Investigator, separately confined untreated controls are strongly encouraged whenever circumstances permit. Control groups are extremely important to not only document disease virulence and disease response to treatment, but also to validate potential adverse reactions in treated animals. Use of control groups will ensure that results of efficacy studies provide useful information that will support a NADA.

XI. TREATMENT SCHEDULES:

A. Routes of administration, volume and dosage calculations, and treatment times

Chemical preparations will differ according to the type of containment in which the fish are to be held and the exposure method needed to accomplish the treatment. Static ponds, static raceways, and flowing raceways are the types of containment addressed here, with exposure methods including indeterminate and timed.

For static pond treatments, the volume of the pond or raceway should be first determined. It is most convenient to express this volume in liters. The amount of copper sulfate required for treatment can be determined by the following formula:

$$\frac{\text{Total alkalinity, mg/L as CaCO}_3}{100} = \text{mg/L CuSO}_4 \bullet 5\text{H}_2\text{O}$$

The proper amount of dry chemical can be broadcast evenly over the surface of the water, dissolved from a bag containing the chemical as the bag is pulled through the water, or applied as dry chemical into the wake of a paddle wheel aerator that circulates the treated water throughout the container. No provision is made for replacement of the water when treatment is applied to a static pond.

For static raceway treatments, the volume of the raceway should be first determined and recorded. The calculation of the amount of needed chemical is performed as above (for static pond treatment). The proper amount of dry chemical can be broadcast over the surface of the water in the raceway, dissolved from a bag containing the chemical as the bag is pulled through the water, or dissolved in a minimum volume of water and evenly applied, in liquid form, through the water. The standard exposure time for a static raceway treatment is 1 hour, or for less time if fish begin to show signs of stress due to oxygen depletion, or chemical toxicity. Air stones or agitators should be employed to maintain oxygens at adequate levels during the treatment. At the end of the treatment time, the flow in the raceway is restarted and the chemical is allowed to flush from the raceway as the treatment water is replaced by fresh water.

For flowing raceway treatments, the volume of the raceway should first be determined and recorded. The flow of freshwater into the raceway should then be determined and recorded. From the raceway volume and the flow rate, the turnover rate should be calculated and recorded. The standard exposure time for a flowing raceway treatment is 1 hour. The time needed to replace untreated water with treated water and to

replace treated water with fresh water is added to the treatment time of 1 hour. The amount of copper sulfate to be applied to the incoming water can be calculated as follows: (see next page)

$$\begin{aligned} \text{Total CuSO}_4 \text{ to be added to incoming water} &= \\ &[\text{Turnover rate}^a \times \text{mg/l CuSO}_4^b \times \text{Raceway volume}] \\ &+ [1 \text{ raceway volume} \times \text{mg/L CuSO}_4] \\ &^a \text{In raceway volumes/hour} \\ &^b \frac{\text{Total alkalinity, as CaCO}_3}{100} = \text{mg/L CuSO}_4 \bullet 5\text{H}_2\text{O} \end{aligned}$$

The length of time during which the total amount of chemical will be added is 1 hour plus the time needed to initially replace untreated water with treated water.

B. Dosing interval and repetition

A single treatment with copper sulfate is usually adequate to control an outbreak of protozoan parasites, fungal diseases, or bacterial columnaris disease, so a second or subsequent treatment is usually not required. Copper sulfate may be used again on the same population of fish, but in most cases the disease incidents will be unrelated.

Special variations of treatment are recommended for certain fish diseases and the special considerations are used in determining the proper treatment. The most notable exception to the "one disease:one treatment" rule is in the treatment of Ichthyophthiriosis. The protozoan, *Ichthyophthirius multifiliis*, cause of "Ich" or "white spot", has a life cycle which proceeds in stages that at times do, and at times do not, involve infection of the fish host. During the stage of fish infection, treatment is ineffective; the parasite is protected by the integument. When the protozoan is in a free swimming stage, away from the fish, it can be readily killed with copper sulfate. The transformation from stage to stage takes 4 to 70 days, depending on water temperature and at temperatures above about 27°C the protozoan enters a non infective phase in its life cycle. While temperatures remain in the range of 7°C to 26°C the protozoan repeatedly cycles from infective stage to free-swimming stage. To effectively remove the infection from a population of fish, the free-swimming stage must be treated each time it appears, so repeated treatments must be applied, at intervals related to water temperature, until the disease is under control.

C. Detailed procedures for drug administration

Standard laboratory equipment such as gloves, lab coats or aprons, eye protection, etc. will be worn at all times when working with copper sulfate. The chemical will be accurately weighed for each treatment, immediately prior to application.

D. Permissible concomitant therapy

Because efficacy data are being collected during this INAD process, there should be no other concomitant therapy. Preferably, there will be no other therapy during a period extending 1 week before treatment to 1 week after copper sulfate treatment. If concomitant therapy is required to protect stocks of fish, the treatment should be fully documented and appropriately identified so that data obtained during concomitant treatment will not be included in efficacy data for copper sulfate.

XII. TREATMENT RESPONSE PARAMETERS

A. Primary parameter

The primary parameter for evaluating the effectiveness of copper sulfate in controlling external protozoan and metazoan parasites, fungal infections, and bacterial columnaris disease, will be the change in the number or density of the microorganisms that were observed before the treatment was conducted and which provided the basis for deciding to perform the treatment.

Fish will be examined at 1 hour, 4 hours, and 18-24 hours post-treatment, or at times as near as possible to these intervals (as scheduling permits). Post-treatment examinations will be conducted following exactly the same protocol used for pre-treatment evaluations.

Data will be recorded in the same quantitative terms that were employed when fish were examined before treatment (e.g. number of parasites per gill filament, number of parasites per field of view at 40X, etc.). A thorough description of before and after treatment conditions of fish should be the objective of the examinations.

B. Secondary parameters

Secondary parameters that should be documented include non-quantitative observations of behavior or gross appearance.

C. Adverse reactions

Any adverse reaction to treatment should be recorded. This might include changes in water quality, adverse responses of the fish, or hazards to the applicator. Adverse reactions should be reported to the Study Monitor, who will in turn notify the Study Director.

Note: Investigators are strongly encouraged to record observations/comments with respect to all phases of treatment. This may include a description of events before, during, and post-treatment. All extenuating or mitigating treatment circumstances need to be described in detail. Such information is imperative so that accurate study/data analysis can be performed.

XIII. FORMS FOR DATA COLLECTION

When the Study Protocol has been approved and treatments are scheduled, the Investigator at each facility covered by the copper sulfate INAD will need to complete the following forms: (see next page)

- Form 1. Guide for reporting investigational new animal drug shipments for poikilothermal food animals.
- Form 2. Chemical use log for clinical field trials on copper sulfate under INAD #9101.
- Form 3. Diagnosis and treatment record for clinical field trials on copper sulfate under INAD #9101.

Form 4. Disposal record for animals from clinical field trials on copper sulfate under INAD #9101.

Copies of these forms are attached to this Study Protocol.

XIV. RECORD KEEPING PROCEDURES

The data should be recorded in permanent ink (preferably black). The data should be recorded on the official data record forms at the time the observations are made. The raw data should be original, i.e., they should be the first recording of the observations, rather than a transcription of original observations to another data sheet. Each original data sheet should be legibly signed and dated by the person making the observation and recording the entry. If more than one person makes and records the observations, entries should be properly attributed to each person. The data should be accurate and legible. If a mistake is made, it should be crossed out using a single strike-through and the correct data should be recorded next to it; each change to the raw data should be initialed and dated by the person making the change, and a statement should be provided explaining why the change was made. If the data sheet needs to be copied, all data should be transferred, including the properly noted changes; the original record should be retained and submitted with the revised copy, along with a memo explaining the reason for the copying.

XV. DISPOSITION OF INVESTIGATIONAL ANIMALS

Animals that die during treatment will be disposed of in an approved manner. Treated animals will be maintained in culture facilities for at least 7 days, for observation following treatment. Fish may be released at any time after this observation period. This recommendation is based on the work reported by Benoit (1975) in which copper residues in muscle tissue from fish exposed to 162 microgram Cu/L for 22 months did not differ significantly from residues of control fish. Similar results have been reported in other publications (Miller, et al. 1980; Weiner and Giesey 1979; Wilson et al. 1980), all of which support the general conclusion of Phillips and Russo (1978) that there is no significant

accumulation of copper in muscle tissue following exposure to high levels of environmental copper. The Investigator must record the disposition of all treated fish on Form 4.

XVI. DISPOSITION OF INVESTIGATIONAL DRUG

Copper sulfate will be used only in the manner and by the individuals specified in the Study Protocol. Any copper sulfate remaining at the end of the study must be returned to the Study Monitors who will verify the use records and the quantity of material remaining and then return copper sulfate to the manufacturer. The investigational drug may not be redistributed to others not specified by the protocol and may not be retained by the Investigator after completion of the study.

XVII. DATA HANDLING, QUALITY CONTROL, MONITORING, ADMINISTRATIVE RESPONSIBILITIES

A. Drug distribution

See Section VII.A.6. Accountability (pages 7 and 8) for information and details.

B. Study Monitors

The Study Monitors are generally fish health professionals with experience in diagnosing and treating fish diseases. There is one Study Monitor assigned for each facility within

the USFWS that is covered by the copper sulfate INAD. A list of Study Monitors, along with addresses and phone numbers, can be found in Appendix II. The Study Monitors are responsible for supervision of the trials, adherence of the Investigator to the Study Protocol, and inspection of the site.

C. Special equipment and materials

Most of the equipment and materials required for this study (with the exception of copper sulfate itself) are already available at each fish hatchery. Diagnosis and treatment of diseases of fish is a common occurrence at most fish hatcheries. Fish hatchery managers (i.e., Investigators) are well trained and well equipped to handle these situations (Appendix IIIb). If any additional equipment or materials are required, they will be provided by the Study Monitors (See Section VII.B. Items needed for sample collection, observations, etc., page 8).

D. Administrator of the drug

Copper sulfate will be administered directly by the assigned Investigator (fish hatchery manager) or under the Investigator's direct supervision (see Appendix IIIa for names). Copper sulfate will be maintained in a secure location and only the Investigator or a person under his/her direct supervision will have access.

E. Drug accountability records

See Section VII.A.6. Accountability (pages 7 and 8) for details and Forms 1-4 for actual forms to be used in the study.

F. Recording observations

The Investigator or a person under his/her direct supervision will be responsible for implementing the Study Protocol, making observations, collecting samples, and recording data during the clinical field trials. After the data have been collected and recorded on the forms, the Investigator sends the data to the Study Monitors who ensure that all the information is provided. The Study Monitors in turn send the data to the Study Director. The Study Director will analyze and summarize the data and prepare an annual report that will be submitted to the FDA.

G. Data storage

The Investigator is responsible for complete and accurate data collection. The Investigator is also responsible for archiving a complete set of all original data (with the exception of Form 1, in which case the original is forwarded to FDA through the Study Monitor, See Section VII.A.6. Accountability pages 7 and 8 for complete details). Original raw data on Forms 2 and 4 will be retained by the Investigator until completion of the study, at which time copies will be sent to the Study Monitors. Copies of Form 3 will be sent to the Study Monitors on a quarterly basis. The Study Monitors will carefully check each set of data for accuracy and completeness. If there are any discrepancies in the data, the Study Monitor will contact the Investigator immediately to rectify the problem. After review, Study Monitors will forward all data to the Study Director. As stated above, the complete set of raw data will be archived by the Investigator. All data should be stored in a secure place. Another complete data set (copies) will be archived by the Study Director.

XVIII. PLANS FOR DATA ANALYSIS

Data analysis will be completed by the Study Director located at the Bozeman National INAD Office. Data from the treatment year will be summarized through tabulation and appropriate statistical analysis. An annual report will be prepared for submission to the Sponsor who will in turn submit the report to the FDA. This submission will probably include a request for an extension of the INAD based on the data collected during that year. When sufficient data are collected, the entire INAD data set will be summarized in a final report for submission to support a full NADA.

XIX. PROTOCOL AND PROTOCOL AMENDMENTS

A signed copy of the Study Protocol must be retained by each Investigator. At any time before the study begins, desired changes in the Study Protocol should be brought to the attention of the Study Director. The desired changes will be fully described in the form of an amendment along with the reason for the change. The amendment will be signed by the Sponsor (or its representative). Copies of the signed amendment will be attached to each copy of the Study Protocol. Investigators will be liable for non-compliance violation if drugs are used without a Study Protocol or differently than specified in the Study Protocol, if forms are not filed on time, or if the study data are not properly collected, maintained, and reported. The Study Monitor is responsible for determining if all the INAD procedures are being followed as defined by the Study Protocol.

XX. PROTOCOL DEVIATIONS

Deviations from the established Study Protocol occasionally cannot be avoided. If deviations occur, the Study Monitor should be contacted immediately for advice. Protocol deviations should be fully documented and should be accompanied by a written explanation of what happened, why, and what steps were taken to mitigate the deviation. Deviation statements should be signed and dated. These statements should be forwarded to the Study Monitor along with the quarterly data summaries and ultimately be submitted to the Study Director.

PERTINENT LITERATURE RELATED TO COPPER SULFATE INAD 9101

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LIST OF APPENDICES

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- Appendix IIIa: USFWS Facilities, and Names of Investigators for Copper Sulfate INAD #9101
- Appendix IIIb: Sample of Knowledge Required for Position of USFWS Hatchery Managers (i.e., Investigators)
- Appendix IVa: Material Safety Data Sheet for Copper Sulfate Pentahydrate
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Appendix IIIb
Sample of Knowledge Required for Position
of USFWS Hatchery Manager
(i.e. Investigators)

Professional knowledge of all facets of fishery biology as well as the ability to apply new scientific findings, developments, and advances toward the resolution of critical propagation problems involving the rearing a variety of fish species under a variety of water quality conditions, water temperatures, water chemistry, etc.

Knowledge of general bacteriology, parasitology, and water chemistry sufficient to treat fish for various diseases.

Skill in interpreting biological observations and ability to draw sound conclusions from available data.

Skill in developing and coordinating available resources to ensure effective management and utilization of manpower, equipment, and funds relative to established priorities and needs.

Skill in coordination of sometimes divergent resource issues to obtain common objectives, including interaction with other Federal and State agencies.

Knowledge of USFWS policy, programs, and organizational structure in order to be able to modify and adapt standard techniques/processes and to devise new strategies and plans necessary to overcome resource problems.

Knowledge of and skill in the use of effective management and supervisory techniques to provide support, guidance, and motivation to hatchery staff.