COPPER UPTAKE AND TOXICITY IN TILAPIA *Oreochromis niloticus* EXPOSED TO COPPER SULPHATE

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To my wife and children,

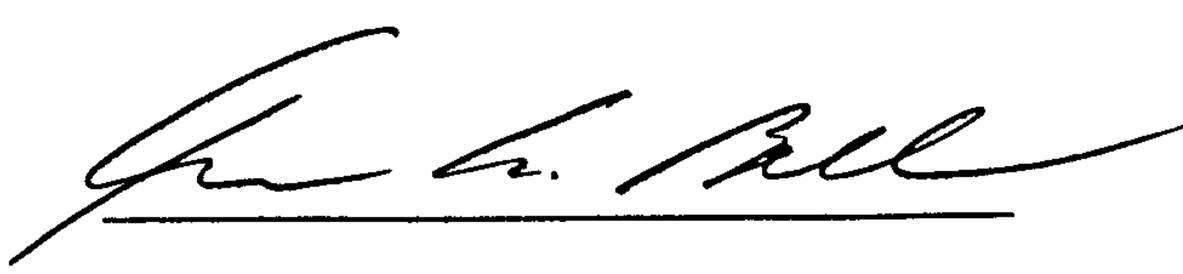
for enduring more than I in the pursuit of this degree.

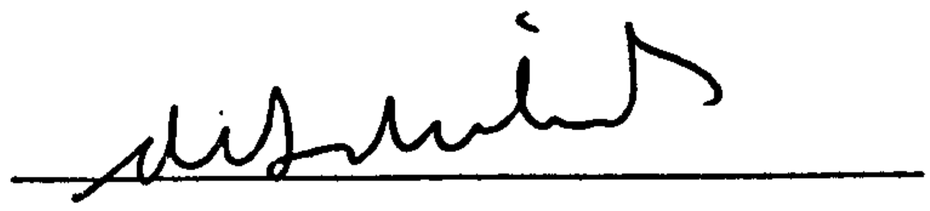
To my parents,

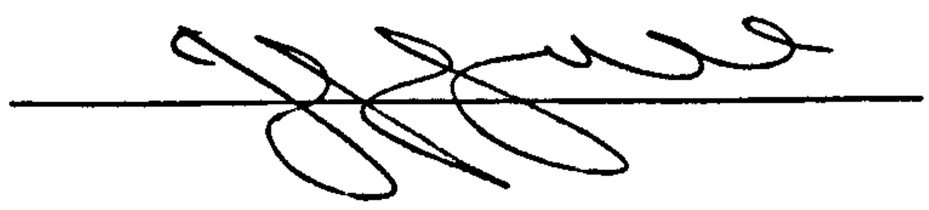
for instilling something in me that translates into a passion to strive and persevere.

DECLARATION

I hereby declare that this thesis has been composed by myself and is the result of my own investigations. It has neither been accepted nor is being submitted for any other degrees. All the sources of information have been duly acknowledged.

Candidate's signature 

Principle supervisor's signature 

Co-supervisor's signature 

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I would like to acknowledge several key people for their contributions to this work, and will do so in chronological order. They all have played an important part, without any of which, this may not have been possible. Dr. Donald Lightner provided the initial suggestion to pursue a doctorate. Dr. James Turnbull offered the University of Stirling as an alternative, novel as it may have seemed at the time. Drs. Robert Livingston, Andrew Beaulieu and Steven Vaughn provided the opportunities and incentives. Professor Randolph Richards, Dr. James Turnbull, Dr. Donald Baird, Mr. Billy Struthers, Mr. Alan Porter, and numerous others, furnished essential guidance during my initial work at Stirling. Dr. Renate Reimschuessel graciously offered words of encouragement, valuable guidance and the use of her wet-lab. Dr. Andrew Kane offered valuable and timely advice. Dr. Claude Veillon and Ms. Kris Patterson (soon to be Dr.) not only provided space to work and allowed for the use of their analytical instruments, but taught this "bucket-chemist" how to confidently and competently use those instruments. Dr. James Turnbull deserves an extra thank you for working beyond what was expected; without Dr. Turnbull's advice, this surely would not have come to fruition.

ABSTRACT

The copper uptake and toxicity of young and market-size tilapia, *Oreochromis niloticus*, exposed to copper sulphate were investigated. A series of preliminary experiments were conducted to provide requisite information for the final experiment. Preliminary experiments established uptake by the culture systems, feed and/or faeces, and the impact of fish nutritional state on uptake. Other experiments established the median lethal concentration of copper to this species, an extrapolated minimal lethal concentration, and the optimum exposure duration and concentration for copper uptake. The remaining preliminary experiments defined the relationship between toxicity and uptake in small compared to market-size tilapia, and between muscle location (within the fillet) and copper uptake. The final experiment established the amount of copper uptake in the edible tissue of market-size tilapia after a worst-case exposure to copper sulphate. The worst-case was defined as the maximum non-lethal concentration for a period significantly exceeding that encountered in commercial production systems.

The worst-case experiment comprised market-size fish (350 to 570 g) being exposed at a nominal concentration of 365 ppb copper for nine days. This study demonstrated that copper did not accumulate in the edible tissue of tilapia above that measured in non-exposed control fish. A mean level of copper in the edible muscle of non-exposed fish, as measured by atomic absorption spectometry, was 2.14 ppm (dry

weight basis), while in fish exposed to a nominal concentration of 365 ppb of copper it was found to be 2.31 ppm.

These data are suitable for incorporation into a United States Food and Drug Administration, New Animal Drug Approval application for the approval of copper sulphate use as a drug for aquatic species.

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CHAPTER I

INTRODUCTION

1 Objectives and Rationale of Study

The overall objectives of this study were two-fold.

- Determine if market-size tilapia (*Oreochromis niloticus*) accumulate copper in their muscle and attached skin, exceeding endogenous levels, after being exposed to copper sulphate at levels greater than therapeutic concentrations, but less than lethal concentrations.

- Generate this information at a level of quality and in a form which can be of value to the aquaculture industry of the United States of America (US), in its quest to obtain US federal approval for the use of copper sulphate as a drug with cultured aquatic species.

The culture of tilapia (*Oreochromis* spp.) is increasing in importance in the US (Harvey 1994). Tilapia are considered to be disease resistant compared to other species of fish (Stickney 1993), but are not disease free. There are specific drugs and chemicals which are needed by aquaculturists to optimise the production of tilapia, as well as other species of cultured fish. Copper sulphate is a chemical of potential value in the production of tilapia.

Copper sulphate (pentahydrate) is commonly used in US aquaculture for the control of algae within culture systems (Jackson 1974), and for the control of bacterial, fungal and/or protozoan epibionts of fish and crustacea (Jackson 1974; Lightner 1993; Stoskopf 1992). It is registered by the US Environmental Protection Agency (EPA) as an approved algicide for use in aquaculture systems. It is not, however, approved by the US Food and Drug Administration's (FDA) Center for Veterinary Medicine (CVM) as a drug for use on cultured aquatic species. The CVM is responsible for reviewing and approving animal drug applications in the US.

Presently, there are only five CVM-approved drugs for use in the US with food fish. Two approved drugs are antibiotics, Romet-30® and oxytetracycline, one drug is a broad-spectrum microbiocide, formalin; and one drug is an anaesthetic, tricaine methanesulfonate. The fifth approved drug is also an antibiotic, sulfamerazine, but is no longer being manufactured in the US and is not available. All of the drugs approved by CVM for use on aquatic species have product labels which restrict their use to a limited number of species and disease indications.

The growth of the aquaculture industry in the US has been significant within the past several decades, and in the rest of the world (Stickney 1994). Together with this growth has been an increase in the US consumption of seafood products

(Harvey 1994). The increase in seafood consumption has prompted the CVM to increase its attention to the safety of food derived from aquatic species. The CVM found that the aquaculture industry's needs for therapeutants, and other drugs, had far outstripped those legally available. As a result there has been significant use of drugs not approved for aquatic species. Thus began an era of increased co-operation between all interested parties, and allied efforts to obtain CVM-approvals for new animal drugs. Specifically, copper sulphate was designated by a US Federal advisory committee, and other private and public entities, as being a compound of critical importance to the aquaculture industry. Copper sulphate was thus placed on a list of drugs designated to receive the greatest efforts toward receiving CVM approval.

US private and public sector organisations are presently in the process of generating and compiling data required to complete a CVM New Animal Drug Approval (NADA) application package for copper sulphate use in fish. A NADA package must include data to support the claim that copper sulphate is both safe and effective when used as a drug with cultured aquatic species. A subset of required NADA safety data addresses safety to humans consuming food products derived from fish exposed to therapeutic levels of copper sulphate.

The CVM requires data which have been generated by well-controlled and scientifically-sound protocols. The

requirements are most stringent for data used to support claims of human food safety. Although there is a plethora of data in the literature that addresses residues of copper in fish exposed to copper sulphate, or other sources of copper, these sources of information do not alone fulfil the CVM's human food safety data requirements for copper sulphate (Brungs, Leonard and McKim 1973; Cross, Hardy, Jones and Barber 1973; McKim and Benoit 1974; Benoit 1975; Dixon and Sprague 1981; O'Neill 1981; Buckley, Roch, McCarter, Rendell and Matheson 1982; Stagg and Shuttleworth 1982; Frazier 1984; Laurén and McDonald 1987; Carbonell and Tarazona 1992; Pelgrom, Lamers, Garritsen, Pels, Lock, Balm and Wendelaar Bonga 1994). The NADA requirements for data quality are quite strict, and usually can not be satisfied solely with data contained in the public literature.

The CVM has indicated to the aquaculture community that CVM's understanding of the mass of literature to support an NADA package for copper sulphate may be nearly complete. The only exception being copper tissue residue studies from representative fish species conducted under Good Laboratory Practice (GLP). The CVM has further stated that it would be willing to accept such data from a limited number of fish species as representing that for all fish species. The aforementioned GLP quality studies had already begun on channel catfish (*Ictalurus punctatus*) and has since been

completed (Hobbs 1996). Residue studies from one additional species was necessary.

It was proposed to the CVM that tilapia be considered as the additional representative species, and the CVM agreed. Tilapia was proposed for such studies due, in part, to its tendency towards domestication and its hardiness in the laboratory. The CVM agreed with the proposal for two reasons. First, there is a paucity of information on tilapia in the literature relative to copper. Second, tilapia is rapidly expanding as a cultured species in the US and has significantly increased its presence in the US wholesale and retail market.

The CVM's requirements for human food safety include residue data from the edible tissue of the test animal. The CVM defines edible fish tissue as a fillet with attached fat and de-scaled skin. There is an exception to this definition, being fish that is normally bought and sold without the skin attached. The most notable example of this is channel catfish (*I. punctatus*).

The secondary objective of this study was to generate pivotal copper tissue residue data (definative studies acceptable to the CVM), collected under GLP or near-GLP conditions, for potential inclusion in a copper sulphate NADA. To provide a higher level of confidence it was decided that a pivotal study should be conducted in a worst-case scenario, under which the fish were allowed to maximise their uptake of copper. The

rationale was that if copper does not accumulate above endogenous levels under the worst conditions, then it would be unlikely to accumulate under normal use.

The body of literature that addresses the residues of copper in fish (see above) are in general agreement that copper does not accumulate in the muscle of fish exposed to elevated concentrations of copper in their environment. There is a possibility that tilapia will behave in a similar manner, in spite of their being a different species from those previously reported and their apparent resistance to exogenous factors. To assure that copper is being taken up by the fish and as a means of monitoring that uptake, parallel liver samples will also be assayed for copper accumulation. The livers of teleost fish are generally considered to be the organ of greatest heavy metal accumulation (Sorensen 1991).

The study objectives were further defined as being:

1. To define the worst-case test conditions.

2. To conduct a copper uptake experiment with market-size tilapia under these worst-case conditions.

3. To analyse edible tissue of such exposed fish for the presence of copper, and compare to copper levels in the edible tissue of unexposed fish.

4. To confirm copper exposure and potential for accumulation in the muscle, by monitoring copper uptake in parallel liver samples.

The test conditions, apart from copper levels *per se*, were points of major consideration. It was felt that this study should be conducted within the context of production aquaculture parameters. This was the proper approach, but due to resource and time limitations, an alternate approach was adopted. It was decided to first characterise the test system to be sure that its parameters were within the biological range of the tilapia. Second, monitor the system closely to confirm that the conditions were maintained over the course of the experiments.

The reasoning behind this decision was twofold and quite simple. First, any choice of typical test system parameters would more than likely be countered as not being typical. Second, the resources available for the conduct of this study did not allow for diluters, pumps, real-time monitoring devices, etc. required to maintain the typical production conditions.

CHAPTER II

GENERAL MATERIALS AND METHODS

1 Experimental Animals and Culture/Holding Systems

Small (10 to 30 g) and market-size (350 to 570 g) tilapia (*O. niloticus*) were obtained either from the University of Maryland Eastern Shore, Princess Anne, Maryland, USA or from Aquamar Industries, Pocomoke City, Maryland, USA. The fish were transported to the University of Maryland at Baltimore (UMAB), School of Medicine, Department of Pathology, Aquatic Pathobiology Center, Baltimore, Maryland, USA, in oxygenated or aerated insulated fish transport tanks. Maximum transport time was approximately three hours. Upon arrival at UMAB, the fish were transferred and held at low densities (less that production densities) in 150 l polypropylene (PPE) tanks and acclimated for a minimum of 14 days. The same type of tanks were used for acclimation, holding and the experiments. Heated, dechlorinated (via activated carbon) Baltimore city water was supplied to each tank at a rate of approximately 200% daily exchange. Water supplied to the tanks was consistently pH 7.0 to 7.3, with a total hardness of 80 to 85 ppm (as $CaCO_3$; calculated from the flame atomic absorption spectrophotometric, or AAS, determination of its Ca and Mg content; American Public Health Association, American Water Works Association and Water Pollution Control Federation 1981), with negligible

copper (less than 5 ppb) and a temperature of 18 to 20°C. Each tank was supplied with aeration to maintain dissolved oxygen at or near saturation.

Fish were supplied a daily, *ad libitum* ration of commercial trout feed during both acclimation and experimental periods (if feeding was part of the protocol). The trout feed was analysed for copper via atomic absorption spectrometry (AAS) and found to have (on a dry weight basis) an average copper content of 26.1 ppm (n = 6; standard deviation = 6.6 ppm).

The fish used in all experiments were of undetermined gender at the time of the trial. In some trials, in particular those conducted with small fish, the gender was never ascertained. The gender of the fish was determined at postmortem in those experiments on market-size fish, from which liver samples were collected. Fish obtained from production or research/production systems are normally from mono-sex male populations. The sex reversal process used to produce mono-sex populations was apparently not 100% effective.

The exact parentage of fish used in all experiments was impossible to determine. Both facilities acquired their original seed stock commercially. The UMES facility did not produce fish on a commercial scale, and was primarily involved in experimental-scale public demonstration projects. Hence, UMES typically produced its own seed from on-site broodstock. At the time of these experiments, Aquamar Inc.

did not maintain their own broodstock and purchased all seed from private vendors. Although fish were obtained from two separate facilities, the fish were not mixed within experiments.

Both sources of fish for these experiments claimed their fish were *O. niloticus*. No further effort was made to positively ascertain the validity of their claims. Such an investigation was beyond the resources of this study.

2 Static 50% Renewal Exposure Regime

All experiments (regardless of total duration) were conducted under a static 50% renewal exposure regime in the 150 l PPE tanks. At the end of each 24 h experiment period, and immediately following the collection of fish sample, 50% of the water was removed from each tank and replaced with new water and sufficient copper to re-establish the nominal copper concentration. Except during the 50% water exchanges, no water was intentionally added or removed from the experimental tanks. Controls were conducted following exactly the same procedures, except for the addition of copper. To achieve 50% renewal, special standpipes were constructed that permitted the water to be reduced by half.

The following comprised the standardised 50% renewal regime:

a) the water and fish samples were collected for the just-completed sampling period (see Section 3 below for specific sampling details),

b) dead fish were removed,

c) debris was siphoned from the bottom of the tank, and scum and debris were removed from the sides of the tank above the waterline,

d) the normal standpipe was quickly replaced with the modified standpipe,

e) the water was drained to the depth of the modified standpipe and the normal standpipe was quickly put in its place,

f) the tank was refilled with new, tempered (heated to 20°C), dechlorinated water, taking care not to overfill,

g) stock copper sulphate solution was pipetted into the tank in sufficient quantity to bring the new water up to the desired nominal copper concentration (see Section 4 below for stock copper sulphate preparation procedures),

h) the new water and copper sulphate were allowed to thoroughly mix for a minimum of 15 min,

i) appropriate water samples for the beginning of the new sampling period were collected (see Section 2 below for specific sampling details).

3 Sampling and Collection Procedures

3.1 Water Samples

3.1.1 Copper

As stated above (Chapter I, Section 1), the study was to be monitored as closely as possible within the limits of time and funding. A key parameter to be monitored was the amount of copper actually administered to each experimental tank. The nominal level value was not satisfactory, nor was a mere sample at the outset and termination of the experiment. It was decided that a daily analysis of actual administered copper, sampled and measured on the same day, was achievable and of significant merit.

Copper, and heavy metals in general, are normally subject to complexation with organic and inorganic materials in the environment they share (see Chapter III Section 1.2.1). Consequently, that amount of copper intentionally added to a test system may not all be bioavailable to the test animals. Sprague (1985) noted that due to the often ill-defined effects of known and unknown modifying factors, "Measurement of toxic forms of metal is better approximated by the 'dissolved'

metal (i.e., the metal able to pass through a 0.45 µm filter)...". Sprague's (1985) comments, procedures followed by Howarth and Sprague (1978), and anecdotal observations at the UMAB laboratory, suggested the following routine sampling strategy.

a) Water samples were collected in new 13 ml screw-top, high-purity polypropylene test tubes (Sarstedt Inc.; Newton, NC , USA) that had been pre-filled with 100 µl of ultrapure concentrated nitric acid (Seastar Chemicals, Seattle, WA, USA; Analytical Grade, maximum copper content of 0.02 ppb).

b) To each water sample test tube, 9.9 ml of sample water was pipetted and then capped. The final concentration of HNO_3 in the water samples was approximately 0.1M.

c) As indicated above, four general types of water samples were collected, and were defined as follows:

1) pre-exchange - collected at the end of a 24 h exposure period, just prior to initiating the 50% renewal procedures,

2) post-exchange - collected at the beginning of a 24 h exposure period, approximately 15 min following the 50% renewal procedure and the addition and mixing of the new copper,

3) non-filtered - water sampled directly from the test system, without any filtration,

4) filtered - water collected from the test system and passed through a 0.45 µm syringe filter (Gelman Sciences, Ann Arbor, MI, USA; low protein binding, non-pyrogenic, single-use) directly into the sample test tube.

d) The sample types were combined, e.g., pre-exchange filtered.

e) All water samples were stored at room temperature and analysed via AAS for copper within 24 h of collection.

Throughout these discussions, copper levels have been referred to either as nominal or actual. Nominal were defined as being that concentration added to the experimental tank, based on a mathematical ratio of copper to water. Actual were defined as being the measured concentration (by AAS) of copper in filtered water samples (as described in Section 3.1.1 above).

3.1.2 Calcium and Magnesium

Estimations of water hardness were essential to this study for water hardness directly affects the toxicity of copper to fish (Chapter III, Section 1.2.1). Hardness can be expressed as

ppm of $CaCO_3$ and can be measured in several manners. The American Public Health Association, American Water Works Association and Water Pollution Control Federation (1981) publication, *Standard Methods for the Examination of Water and Wastewater*, defines two procedure for routine determination of total water hardness. An EDTA titrimetric procedure is routinely used. However, the second method, based on elemental analysis for calcium and magnesium, is the preferred procedure. The equation for the calculation of hardness (expressed as mg equivalents of $CaCO_3$ per litre) from calcium and magnesium concentrations is as follows:

$$Hardness = 2.497 \times [Ca] + 4.118 \times [Mg]$$

where: Hardness = mg equivalents of $CaCO_3$ per litre, and [Ca] and [Mg] = concentration expressed as mg per litre or ppm

Separate samples were collected for AAS determination of calcium and magnesium by using the same sample tubes as those used for copper and processing these samples on the AAS for their respective elements. The actual AAS procedures for calcium and magnesium were similar to that for copper with the exception of AAS lamp and wavelength and a slightly modified matrix (see Sections 5.4.2, 5.4.3, 5.5.2 and 5.5.3 below).

3.1.3 Dissolved oxygen, pH, temperature and nitrites

Dissolved oxygen (DO) determinations were normally determined using a Yellow Springs Instrument (YSI) Model 57, dissolved oxygen meter (Yellow Springs Instrument Co. Inc., Yellow Springs, OH, USA) and standard DO/temperature probe (5700 Series). The meter and probe were calibrated as per manufacturer's instructions prior to each use. During those occasions when the YSI meter was not available or not functioning a LaMotte titrimetric dissolved oxygen test kit (Model EDO-AG-30; LaMotte Inc., Chestertown, Maryland, USA) was used.

The pH of water was determined using a Orion Model EA 920 meter (Orion Research Incorporated, Laboratory Products Group, Boston, MA, USA) and a standard pH probe.

Temperature was determined with either the standard DO/temperature probe on the YSI meter or with a mercury thermometer. The two devices were checked against each other and found to agree.

Nitrites levels were determined by a colorimetric procedure (American Public Health Association et al. 1981) using a Coleman Jr. II spectrophotometer (Coleman Beckman Inc., Maywood, Illinois, USA). The calculations were determined by calibration of the spectrophotometer against freshly made

nitrite standards, as per American Public Health Association et al. (1981) procedures.

3.2 Fish Samples

3.2.1 General Fish Sampling Procedures

All fish were arbitrarily, but gently netted from their respective tanks and immediately sacrificed. Small fish were pithed in the brain/anterior spinal cord with a dissecting needle, while market-size fish were sacrificed by severing the spinal cord with a serrated stainless steel knife, dorsal to the operculum. Thought was given to the use of other procedures, such as an overdose of tricaine methansulfonate. However, it was felt that pithing and spinal cord severing would have the least effect on tissue samples. All fish were immediately placed on a balance and their weight recorded. All fish were then placed either on a wax or other non-metallic surface and scales were removed from the left side with either a stainless steel scalpel or a fish scaling tool. The surface of the fish was rinsed with fresh, dechlorinated tap water to remove detached, but adhering scales. Samples were then collected; gills for histology first, then muscle and liver for copper residue analyses, followed finally by the remaining samples for histology (as described below in Sections 3.2.2 and 3.2.3).

3.2.2 Fish - Copper Residue Analysis Samples

The entire left fillet was removed either with a stainless steel scalpel blade (small fish) or a stainless steel filleting knife (market-size fish). Small fish fillet samples were transferred to new, capped scintillation vials, which had been pre-labelled (one fish sample per vial). Market-size fillet samples were transferred to new, pre-labelled plastic zip-lock bags. One fish fillet per bag. All samples were placed on ice in a transport cool box.

The left wall of the peritoneum was then removed to expose the viscera. An incision was made anteriorly from the vent to the isthmus and then dorsally just posterior to the operculum until reaching near the dorsal limits of the operculum. A second incision was then made diagonally from the vent to the dorso-anterior end of the first incision. The latter incision was made through the rib cage. Care was taken not to rupture the gall bladder.

The entire (or as much as possible) liver was removed from each small fish (exclusive of that retained for a histological sample). This sample was also transferred to a new, capped scintillation vial, which had been pre-labelled (one fish sample per vial).

The posterior-most lobe of the liver, being the most easily standardised, was the source of sample for market-size fish.

A minimum liver sample of 2.0 g from the posterior tip of the posterior lobe was excised and placed in a new, pre-labelled plastic zip-lock bags. All samples were placed on ice in a transport cool box.

All tissue samples collected for copper analyses were either immediately transported to the U.S. Department of Agriculture, Agriculture Research Service, Beltsville Human Nutrition Research Center, Beltsville Agriculture Research Center, Beltsville, MD 20705 USA (USDA), and placed in a -20°C freezer for later analyses or stored overnight in a domestic freezer (<0°C) and then transported the following day to USDA and stored in the -20°C freezer.

3.2.3 Fish - Histology Samples

Immediately following euthanasia and weighing, the outermost left gill arch was excised and placed in a pre-labelled scintillation vial (or larger glass or plastic vial), that had been pre-filled with 10% neutral buffered formalin (NBF). Following the opening of the visceral cavity and the removal of liver samples for copper residue analyses, internal organs were sampled for later histological examination. All organs sampled were placed in the same vial used for the gill arch. At most, the following organs were excised and fixed in NBF: liver, trunk kidney, left eye and left nare (in addition to the gills already noted). Other organs were not sampled due to their typically not being the site of copper-related

pathology (see Chapter VI, Section 1.2). In those experiments in which histological examination was not an intended part of the protocol, only liver and gill were sampled and fixed in NBF. All histology samples were stored in NBF in the original sample vials until processed.

4 Test Compound Preparation

A stock solution of copper sulphate pentahydrate was prepared for use with each experiment in this study. Although the stock solutions for each experiment could have differed from each other, the fact that actual daily AAS measurements were being made decreased the importance of such variations.

Reagent grade copper sulphate pentahydrate (100.7% iodometry assayed content) was used as the original source (J.T. Baker Inc., Phillipsburg, NJ, USA). The original container of reagent grade copper sulphate crystals was maintained in the normal chemical storage area of UMAB, while the stock solution was maintained in a separate area, to minimise chances for cross-contamination.

The first batch of stock solution was prepared to provide a 50 ppb final solution of copper in a 477 l PPE tank used for mixing, storage, and supply by mixing 1 ml of stock solution into the storage tank. The following equation was used to

calculate the amount of copper sulphate pentahydrate needed to be added to each 1.0 ml of stock solution.

$$\frac{mg\ CuSO_4 \cdot 5H_2O}{ml\ of\ Stock\ Soln} @ \frac{50\ ppb\ Cu}{Final\ Conc} = \frac{0.050\ mg\ Cu}{1} \times \frac{249.6\ mg\ CuSO_4 \cdot 5H_2O}{63.5\ mg\ Cu} \times 477\ L = \frac{93.9\ mg}{ml}$$

The stock solution was made the first time by weighing out 46.95 g of copper sulphate pentahydrate (= 500 x 93.9 mg) into an acid-washed, triple-rinsed (with distilled deionized water), 500 ml volumetric flask. After the addition of the copper sulphate, the flask was filled to the 500 ml mark with deionized distilled water. All uses of stock solution in other experiments was by the direct addition to the experimental tanks, and hence the amounts to be added were determined by means of simple proportional equations. The stock solution flask was labelled with name, contents, mixing procedures and stock solution concentration.

5 Analytical Procedures

5.1 Muscle Sample Preparation

All tissues were processed for AAS analysis via a dry-ashing procedure as outlined by Patterson, Holbrook, Bodner, Kelsay, Smith and Veillon (1984). Special care was used to ensure that no copper was transferred between specimens or from utensils; e.g., the Plexiglas™ cutting surface and surgical stainless steel utensils used for tissue mincing were rinsed with deionised distilled water between specimens, new

stainless steel razor blades were used for each specimen, new acid-washed borosilicate digestion tubes were used for all digestions. For all tissues, if enough tissue was available, an attempt was made to retain an equal amount of tissue in the freezer for subsequent analyses where necessary.

Muscle samples were processed in two slightly different manners, dependent upon whether they were from small or market-size fish. The CVM defines the edible tissue of fish to be skeletal muscle with interspersed fat and attached, de-scaled skin (the exception being those fish that are always marketed with the skin removed, such as channel catfish, *I. punctatus*). For small fish, the entire fillet was removed from its vial after slight thawing, placed on the cleaned and rinsed Plexiglas™ cutting board and minced into approximately 3 mm pieces with new surgical steel razor blades (each piece included the attached skin). All pieces were then randomised and approximately 1.0 g was removed and carefully placed in the bottom of a new, acid-washed, previously etched, individually weighed and recorded, 16x100 mm borosilicate test tube. The test tube and sample were then weighed together and the combined weight recorded. The tubes were placed in a rack and covered with Parafilm© and stored at room temperature until the complete set of samples (approximately 40 in number) had been prepared and weighed.

Market-size fish were treated in a very similar manner, the exception being that it was impractical to mince the entire fillet. Instead a special stainless steel cylindrical punch (approximately 1.25 cm in diameter) was used to produce three plugs of combined muscle, attached skin and fat from three predetermined areas of the fillet. The procedures used to establish which areas of the fillet to use for these samples are discussed in Chapter VII. The entire tissue plug, irrespective of weight, was carefully placed at the test tube bottom, attempting to avoid touching the test tube sides. All other procedures followed were the same as that applied to the muscle samples from small fish.

5.2 Liver Sample Preparation

The initial processing steps for liver samples were similar to those for muscle samples, with the exception that the liver tissue was not minced and that the approximate weight of the liver added to each test tube was 0.25 g.

5.3 Final Sample Preparation Procedures for All Tissue Types

All samples were weighed on a precision balance (Mettler, Model AT200). The analytical balance was periodically calibrated against reference weights traceable to the National Institute of Standards and Technology (NIST) standard weights. The balance used in this study also contains an automatic internal calibration feature and was accurate to

0.1 mg. After wet weights had been recorded for a complete set of samples, the set was placed, with a loose fitting Parafilm© cover, into a -20°C freezer for a minimum of 2 h. Once completely frozen, tissues were freeze-dried for a minimum of 8 h in a FTS Systems, Model FD-6-84-vp freeze-drier at -100°C and 50 mtorr vacuum (FTS Systems, Inc.; Stone Ridge, NY USA). Samples were removed from the freeze-drier and immediately weighed and weights recorded.

Once weights had been recorded, all tubes from a set were placed in clean 400 ml beaker and covered with a clean watch glass. The beaker was placed in a calibrated muffle furnace. The furnace was programmed for the following ashing scheme: increase (ramp-up) to 200°C for 2 h, maintain 200°C for 2 h, ramp-up to 400°C for 2 h, maintain 400°C for 96 h. The tissues were checked after 96 h, and if all tissue were not at least partially grey, the temperature was maintained for 24 h or until all tissues were at least partially grey. The tissues were removed from the muffle furnace after cooling and were transferred to a limited-access fume cupboard.

The digestion process was begun by placing all tubes, including standard reference materials (SRM's) and processing blanks, in a heating block set at 105-110°C and adding 500 µl of deionized distilled water, followed by 500 µl of concentrated ultrapure HNO_3 (Seastar Chemicals, Seattle, WA,

USA; maximum copper content of 0.02 ppb). The tubes were allowed to heat overnight until thoroughly dry. Following overnight drying, 100 µl of HNO_3 was added to each tube, followed by the addition of 100 µl of Baker Ultrex Reagent H_2O_2 (J.T. Baker Inc., Phillipsburg, NJ, USA; copper content <0.07 ppb). Each tube was swirled, tipped and turned, or whatever other means was necessary to expose all material in the tube to the HNO_3/H_2O_2. Extreme care was exercised to avoid losing any of the material/solution during the mixing. Following the addition of these reagents, the tubes were allowed to nearly dry and repeated 50 or 100 µl doses of HNO_3 and H_2O_2 were added until the solution became clear and the remaining material in the tube, once dried, was white. If the process was not completed in any day, the last addition was allowed to thoroughly dry overnight. The process was repeated for all tubes in a set until all material, in all tubes, was white. If there were significant differences noted in the rate at which the two tissue types (liver and muscle) were digesting, these were split into two subsets, each subset retaining one processing blank, and one each of the two SRM tubes with the subset. Once the set or a subset had reached the white material and clear solution stage, 100 µl of ultrapure 6N HCl (Seastar Chemicals, Seattle, WA, USA; maximum copper content of 0.05 ppb) was added to each tube. The contents of each tube was mixed until all material was completely dissolved in the acid and it was then allowed to dry. After the content of all tubes had thoroughly dried, the tubes were

removed from the heating block, allowed to cool and covered with Parafilm© until there was sufficient time to dilute the samples and analyse on the AAS in the same day. During the entire digestion process, a record of all reagents amounts added to the set or subsets was maintained. As a general procedure, all tubes within a set or subset were always treated in exactly the same manner; whatever was added to one tube in a set or subset, was added to all other tubes within that set or subset, including processing blanks and SRM's.

When sufficient time was available to analyse a set of digested specimens, the samples were diluted with a primary, and possibly secondary or even tertiary, dilution to produce a AAS reading in the range of 100 to 1000 ppb copper. To accomplish this dilution, an estimate of the probable copper content and the actual weight of the sample had to be known. The first dilution was accomplished by adding sufficient 1.0 M HCl (Seastar Chemicals, Seattle, WA, USA; maximum copper content of 0.05 ppb), followed by deionized distilled water at a 1:10 ratio, producing specimens dissolved in a 0.1M HCl matrix. If a second or third dilution was required to bring the AAS readings into the proper range, the secondary or tertiary dilution was made with 0.1M HCl. All dilutions were accomplished using pipetors and autopipetors which had been calibrated immediately prior to use. All specimens were thoroughly vortexed after the addition of each diluent. All

operations within this protocol set were accomplished within a limited access, standard- or clean-room, wearing lab coats, safety glasses and powder-free gloves.

5.4 Standard Reference Materials (SRM's) and Processing Blanks

For each set of approximately 40 specimens, 2 test tubes (minimum) each of the following were prepared and processed with the specimen set. In several earlier experiments, other SRM's were used in addition to or in place of those listed below. Such use is noted in respective Chapters.

a) DORM-1: Dogfish (*Squalus acanthias*) Muscle SRM for Trace Metals; Marine Analytical Chemistry Standards Program; National Research Council of Canada; Environmental Measurement Science; Institute for Environmental Research and Technology; Ottawa, Ontario, Canada, K1A OR6; Bottle No. 278.

b) DOLT-2: Dogfish (*Squalus acanthias*) Liver SRM for Trace Metals; Marine Analytical Chemistry Standards Program; National Research Council of Canada; Environmental Measurement Science; Institute for Environmental Research and Technology, Ottawa, Ontario, Canada, K1A OR6.

c) Processing blanks: no specimen or other material added to tube.

Both the DORM-1 and the DOLT-2 tubes were filled with approximately 0.25 g of tissue. Weights were measured and recorded for each tube, and the tube plus contents, as was the case for test specimens. Once weights were measured and recorded, approximately 1.5 ml of deionized distilled water was added to each SRM tube and the tube was vortexed slowly to wet all SRM material.

All SRM and processing blanks were processed, from this point on, in exactly the same manner as the test specimen samples (see above, Sections 5.1 to 5.3 above).

5.4 Water Sample Preparation

5.4.1 Copper

Water samples, once acidified as noted above in Section 3.1.1, required no further processing, with one exception. If it was anticipated that the copper concentration would exceed 1.0 ppm, then the sample was diluted with a sufficient quantity of 0.1 M HNO_3 to bring the final concentration into the range of 100-300 ppb.

5.4.2 Calcium

Water samples, once acidified as noted above in Section 3.1.2, required only dilution. It was desirable to measure calcium in the sample at a level between approximately 100 and 1000 ppb, therefore the samples were diluted with a sufficient quantity of 0.1 M HCl diluent containing 0.5% La (Lanthanum). If phosphates are present calcium phosphate will be formed which is thermally stable. Hence, the amount of Ca in the sample will be underestimated. The La preferentially replaces Ca in calcium phosphate, freeing the Ca to be atomized.

5.4.3 Magnesium

Water samples, once acidified as noted above in Section 3.1.2, normally required only minimal processing, that being dilution. It was desirable to measure magnesium in the sample at a level between approximately 100 and 1000 ppb, therefore the samples were diluted with a sufficient quantity of 0.1 M HCl diluent containing 0.5% La (Lanthanum). If phosphates are present magnesium phosphate will be formed which is thermally stable. Hence, the amount of Mg in the sample will be underestimated. The La preferentially replaces Mg in calcium phosphate, freeing the Mg to be atomized.

5.5 Atomic Absorption Spectrometric Settings and Procedures

5.5.1 Copper

Analyses were conducted by flame AAS on a Perkin Elmer Model 5000 by using the following procedures and instrument settings.

a) Energy level = 66 (autosetting); Lamp# = 1 (Cu); Lamp current = 15 ma; Slit high = 0.7 mm; λ peak = 324.7 nm; t = 1.0 s; average = 3 (or 6); CV = "active"; print = "active"; hold = "active"; conc. = "active"; AA = "active"; S1 = 0.200, 0.500, or 1.000 ppm (depends on standard used).

b) The AAS was allowed to warm up for a minimum of 30 min.

c) The flame was ignited as per laboratory standard operating procedures (SOP's) and allowed to burn for a minimum of 5 min.

d) The instrument was calibrated as per AAS SOP's, using 0.1M HNO_3 as the AZ (auto-zero) for water or 0.1M HCl for tissue. Calibration of the AAS was continued with either a 0.200, 0.500 or 1.000 ppm (depending on the standard used in that run) copper standard made up in 0.1M HNO_3 (or 0.1M HCl). The calibration was

immediately checked by running the standard, as a sample, and recording the measured value. The calibration procedure was repeated until readings stabilised. Once stable, calibration was checked with as many standards as possible, which were within the calibration range (for example, if calibrated at 0 and 1.000 ppm, then calibration was also checked with the 0.200 and the 0.500 ppm standard). The deviation of measured standard values was noted from the nominal standard values.

e) Measurement of specimens was begun by keying in the AAS identification number (ID) of the first specimen. The AAS automatically continued to number each sample consecutively. The measurement set was repeated if sufficient specimen sample was available in the sample tube. The 3-reading set average (AVE), with the least coefficient of variance (CV) was accepted as the recorded results.

f) The analysis of four specimen sample sets (and replicate sets if sufficient sample remained) was conducted and followed by the measurement of a standard.

g) A copper standard (in the range of the currently measured samples) was tested as a sample and its deviation from the initial test of the same standard was noted. If the difference between the first and this

standard reading exceeded 0.005 ppm, the AAS was re-calibrated and immediately followed by a repeat reading of standards as samples.

h) After re-calibration (if necessary), the next sample AAS ID number was keyed in and the analyses of specimen samples was continued. Steps #f and #g were repeated until all specimen samples were analysed.

i) After the last specimen sample was analysed, all standards initially checked, including AZ, were analysed, as samples, and recorded.

j) All measured AAS values from the automatic data tape were recorded to the archival log book. If two measurement sets were analysed for a particular specimen, the average for the 3-sample set with the lowest CV was recorded in the log book. The same values recorded in the log book were also recorded in the appropriate computer database, from which actual values relative to dilutions were calculated.

k) If at any time AAS readings had a large CV and re-calibration did not remove the excessive deviation, the maintenance procedures in the SOP's were applied.

5.5.2 Calcium

General procedures were the same for calcium as they were for copper with the following exceptions.

- Energy level = 59 (autosetting); Lamp# = 3 (Ca); Lamp current = 10 ma; Slit high = 0.7 mm; λ peak = 422.7 nm; t = 1.0 s; average = 3 (or 6); CV = "active"; print = "active"; hold = "active"; conc. = "active"; AA = "active"; S1 = 1.000 ppm; S2 = 3.000 ppm.

5.5.3 Magnesium

General procedures were the same for magnesium as they were for copper with the following exceptions.

- Energy level = 58 (autosetting); Lamp# = 4 (Mg); Lamp current = 6 ma; Slit high = 0.7 mm; λ peak = 285.2 nm; t = 1.0 s; average = 3 (or 6); CV = "active"; print = "active"; hold = "active"; conc. = "active"; AA = "active"; S1 = 0.500 ppm; S2 = 1.000 ppm.

6 Histological Preparation Procedures

All samples collected were processed in the same manner. When needed, required tissues were removed from the NBF and needed portions trimmed as appropriate. An effort was made to retain an approximately equal portion of the sample

if needed for future examinations. Trimmed samples were assigned a UMAB Case Number and placed in labelled histological processing cassettes. Labelled cassettes were submitted to the UMAB School of Medicine Department of Pathology for routine paraffin embedding and H&E staining.

7 Statistical Procedures

All calculated copper values were corrected for incurred procedural copper readings by subtracting the copper level of parallel processing blanks from tissue values.

Corrected copper tissue values, determined via AAS and corrected as noted above, were statistically compared. Data sets were assessed for deviations from normality either graphically on normal probability scale paper and/or by D'Agostino's test (Zar 1984). Nominal data were analysed by contingency tables. When appropriate, Fisher's LSD multiple comparison testing was utilised to identify significant differences. All statistical analyses (other than the D'Agostino's tests) were conducted using the computer programs StatView® or SuperANOVA™ (Abacus Concepts, Inc.; Berkeley, California, 94704, USA; Version 1.11).

CHAPTER III

TANK, FEED AND FAECES EXPERIMENT

1. Introduction

1.1 Objectives

The overall intent of this entire study was to assay edible fish tissue for copper by subjecting the test fish to a defined level of copper sulphate. This particular experiment was primarily designed to establish what portion, if any, of the administered (nominal) concentration of copper sulphate was actually unavailable to the fish, as limited by inherent characteristics of the test system.

Due to logistic limitations, this experiment and all other experiments in this study were conducted in static systems under a daily partial water/copper exchange regime (Chapter II, Section 2). It was not known to what extent copper or copper sulphate would be rendered biologically unavailable within the system. This experiment was designed to determine if offered food, faeces generated or the tank surface, or any combination thereof, sequestered copper or copper sulphate.

A secondary, though equally important, objective of this experiment was to determine if the uptake of copper by the

fish was affected by the nutritional state of the fish during the exposure period, i.e., would feeding of the fish during the experiment affect the extent to which copper was taken up. Early pilot studies conducted at the University of Stirling suggested that the nutritional state of the fish may have affected copper uptake and/or deposition within the edible tissues. To accommodate this second objective, the study was duplicated allowing one set of tanks to received feed during the experiment, while the second set did not.

The effects that inorganic, organic, biotic or abiotic factors, within the experimental test systems, could have on the results of subsequent experiments were unknown. There was also concern about how the nutritional state of the fish, when exposed to copper sulphate, might bias experimental results in subsequent experiments. This experiment was designed to confirm the existence of, and estimate the effect of, any modifying factor(s) to copper uptake and toxicity in the test systems.

1.2 Literature Review

1.2.1 Copper Complexation

Copper, as with most heavy metals, has been observed to complex with a large variety of biotic and abiotic particles, and organic and inorganic materials (Sprague 1985). Several authors have discussed specific aspects of complexation with

copper. French and Hunt (1987) reviewed inorganic complexing, as did Pagenkopf, Russo and Thurston (1974); Andrew, Biesinger and Glass (1977); Borgmann (1983); Borgmann and Ralph (1983); and Borgmann and Charlton (1984). French and Hunt (1987) summarised their review by stating that (1) carbonates complex with copper, tending to reduce copper toxicity, and that alkalinity typically increases the level of carbonates which in turn decreases toxicity; (2) hydroxides complex with copper, but the actual impact on copper toxicity can be variable, and (3) hardness appears to affect toxicity as does alkalinity, but the toxic response may be much more rapid.

As noted above, alkalinity and/or hardness can play a major role in copper toxicity. The two properties have been frequently used interchangeably, but refer to two different water quality parameters. Alkalinity is defined by Rand and Petrocelli (1985) as "...the acid neutralizing (i.e., proton-accepting) capacity of water; the quality and quantity of constituents in water which result in a shift in the pH toward the alkaline side of neutrality." Hardness is defined by the same authors as "...the concentration of all metallic cations, except those of the alkali metals, present in water. In general, hardness is a measure of the concentration of calcium and magnesium ions in water and is frequently expressed as mg/l calcium carbonate equivalent." The roles of alkalinity

and hardness relative to toxicity are discussed further in Chapter IV, Section 1.2.2.

A number of organic substances likewise complex with copper resulting in modification of copper toxicity. Zitko, Carson and Carson (1973) found that as humic acid levels increased, the degree of copper toxicity to juvenile Atlantic salmon decreased. Giesy, Newell, and Leversee (1983) found the same to be the case in their studies with daphnids. Several groups have investigated a broad spectrum of organic compounds, ranging from sewage effluent to citric acid and EDTA, and they have likewise concluded that organics typically bind to copper and reduce copper's toxicity (Brown, Shaw and Shurben 1974; Chynoweth, Black and Mancy 1975). Still others have found that with factors such as pH maintained constant, as dissolved organics increase copper toxicity will decrease (Meador, 1991; Welsh, Skidmore, Spry, Dixon, Hodson, Hutchinson and Hickie 1993).

1.2.2 Feeding Compared to Non-feeding

The relationship between the nutritional state of fish and their heavy metal uptake or regulation has been reported by several authors. Segner (1987) compared the responses of fed and non-fed roach, *Rutilus rutilus*, to sublethal copper contamination, concluding that starved fish may be less able to regulate the inter-tissue movement of copper. The availability of dietary carbohydrates may, according to Dixon

and Hilton (1981), change the tolerance of rainbow trout (*O. mykiss*) to waterborne copper. Handy and Eddy (1990) demonstrated that the mucus and gills of starved rainbow trout accumulated more zinc than trout which were fed. More recent studies by Pelgrom et al. (1994) on tilapia fry (*Oreochromis mossambica*) supported the same general concept (but provided no explanation why) that starved fish accumulate copper more rapidly than fish which were fed.

2. Study-Specific Materials and Methods

Culture systems used were as described in Chapter II. Fish were acquired, acclimated and held prior to initiation of the experiment as described in Chapter II. All fish used in this study were young tilapia ranging in size from 10 to 30 g in weight.

Each of four, previously cleaned and flushed, standard experimental, 150 l tanks was stocked with 30 acclimated, randomly selected fish. Stocking densities, tank size and water quality were identical in the acclimation and experimental tanks. Consequently, the experimental period began after acclimation. Attempts were made to minimise transfer stress. A fifth tank was also part of the experiment, however, neither fish, nor feed were placed in this tank.

The experimental design comprised a single, unreplicated tank for each combinations of copper and feed and a single tank

with no fish. The experiment design is represented in Table 3-1 below.

Table 3-1 Treatment regimes for the Tank, Feed and Faeces Experiment

Tank Number	Copper Present	Feed Provided	Fish in Tank
1	+	-	-
2	+	-	+
3	+	+	+
4	-	-	+
5	-	+	+

All fish received feed up to within 24 h of the experiment initiation. Those fish designated as receiving feed during the study continued to receive feed as prior to the experiment. Fish designated not to received feed did not throughout the entire experiment.

The study was conducted for seven days. Four fish were collected from each tank for AAS determination of copper on each of the sample days. Sampling began on Day 0 and was repeated every 24 h up to and including Day 6. A fillet was removed as per standard procedures (Chapter II), as was the liver. Samples were stored on ice for approximately 1 to 2 h until AAS processing began. Each fish was weighed immediately prior to tissue sample collection. Tissue samples were not freeze-dried; only wet weight calculations were

determined from AAS analyses. No samples were collected for histological processing. Water samples were collected twice per 24 h period, at the beginning and at the end (pre- and post-exchange) of each period. All water samples were non-filtered.

The SRM's used for this experiment were not DORM-1 nor DOLT-2 as noted in Chapter II. Instead, U.S. National Bureau of Standards, or NBS (presently named the U.S. National Institute of Standards and Technology, or NIST) Oyster Tissue SRM No. 1566 and Bovine Liver SRM No. 1577 were used.

3. Results

3.1 Water Copper Concentrations

A two factor analysis of variance (ANOVA) was used to compare whether actual water copper concentrations were different relative to the day sampled and the tank from which they were sampled. Included in this ANOVA was a theoretical 0 ppb copper tank (Tank No. 0) and a theoretical 50 ppb copper tank (Tank No. 6). The difference in mean copper measurements between the five actual tanks (No. 1-5) and the theoretical tanks (No. 0 and 6) were statistically significant ($N = 14$, $F_{calc} = 708.519$, $P_{calc} = 0.0001$). The difference in mean copper measurements between the seven days of the experiment were statistically significant ($N = 14$, $F_{calc} = 4.594$, $P_{calc} = 0.0005$). A post-hoc multiple comparison test (at $\alpha =$

0.05) was applied to the data to elucidate differences between tanks. Table 3-2 below summarises these comparisons. All three treated tanks (Tanks 1, 2 and 3) were statistically greater than the theoretical value of 50 ppb, but did not differ from each other. The water in the two control tanks (Tanks 4 and 5) did not statistically differ from each other, nor were they statistically different (at $\alpha = 0.05$) from the theoretical value of 0 ppb. Because the controls were found not to differ from the theoretical 0 ppb tanks, they were dropped from further analyses.

The previous ANOVA indicated that there was a difference between the copper measured on different days. Therefore a second two factor ANOVA was used to compare, in the copper exposed tanks only, whether actual water copper concentrations were different relative to the day sampled and the tank from which they were sampled. The difference in mean copper measurements between Tanks 1, 2, and 3 were not statistically significant ($N = 14$, $F_{calc} = 0.107$, $P_{calc} = 0.8994$), however, the differences between copper measurements on the seven days sampled were statistically significant ($N = 6$, $F_{calc} = 8.809$, $P_{calc} = 0.0001$). A post-hoc multiple comparison test (at $\alpha = 0.05$) was applied to these data as well to point out differences between days. Table 3-2 below summarises these comparisons. The primary differences noted were between the initial 4 days and the latter 3 days.

Assuming there were no statistical differences between copper in treated tanks (as noted above), a single factor ANOVA was conducted on all treated tanks, comparing copper water concentrations taken at the beginning of the sample period with those collected at the end of the sample period. In this latter comparison, the mean differences between the two sample periods (beginning and end of 24 h periods) were not found to be statistically significant ($N = 21$, $F_{calc} = 0.226$, $P_{calc} = 0.6374$).

Table 3-2 Statistical comparison of mean measured actual copper concentrations (ppb) in water.

Tank No.	Nominal Conc.	Actual Mean	Stand. Dev.	Significance*	Day	Actual Mean	Stand. Dev.	Significance
"0"	0	0	0	a	1	65.00	9.32	a b
1	50	58.07	6.17	b	2	69.33	4.37	a
2	50	58.93	8.16	b	3	61.33	3.39	b c
3	50	58.87	8.94	b	4	55.50	5.39	c d
4	0	2.00	3.82	a	5	53.70	1.29	d
5	0	2.00	4.10	a	6	52.67	3.14	d
"6"	50	50.00	0	c	7	52.83	2.04	d

* factors with the same letter signify no statistically significant differences (at $\alpha = 0.05$)

3.2 Tissue Copper Levels

3.2.1 Muscle Copper Levels

A two factor analysis of variance (ANOVA) was used to compare the effect of either copper or no copper exposure, and food or no food, and any interaction between the two on the AAS measured muscle copper levels (Table 3-3). The convention of Line Numbers (first column Table 3-3) will be used here, and throughout the text, to clarify comments in reference to tables. The mean difference (Lines 1 and 2) between the copper levels (Day 7 sample only) in the muscle of fish exposed to copper and those not exposed were statistically significant ($N = 8$, $F_{calc} = 6.597$, $P_{calc} = 0.0246$). At the same time, the mean difference (Lines 3 and 4) between the copper levels (Day 7 sample only) in the muscle of fish fed and those not fed were statistically significant ($N = 8$, $F_{calc} = 5.880$, $P_{calc} = 0.0320$). There was no statistically significant interaction between the two factors.

The muscle in those fish which were both exposed to copper and not fed (Line 5) contained the highest levels of copper when compared to the other combinations of feed and copper (Lines 6 to 8) as determined by a Least Square Means table of a pair-wise comparison of factor combinations (Table 3-3).

Table 3-3 The actual mean measured copper concentrations in muscle.

Line No.	Factor or Combination	Sample Size	Mean Measured Cu ppm (wet wt basis)	Standard Deviation	Significance
1	Cu	8	0.43	0.06	a
2	No Cu	8	0.37	0.05	b
3	Feed	8	0.37	0.06	a
4	No Feed	8	0.43	0.05	b
5	Cu/no feed	4	0.47	0.04	a
6	Cu/feed	4	0.39	0.04	b
7	No Cu/no feed	4	0.39	0.02	b
8	No Cu/feed	4	0.36	0.06	b

3.2.2 Liver Copper Levels

A two factor analysis of variance (ANOVA) was used to compare the effect of either copper or no copper exposure, and food or no food, and any interaction between the two on the AAS measured liver copper levels (Table 3-4). The mean difference (Lines 1 and 2) between the copper levels (Day 7 sample only) in the liver of fish exposed to copper and those not exposed were not statistically significant ($N = 8$, $F_{calc} = 4.229$, $P_{calc} = 0.0622$). At the same time, the mean difference (Lines 3 and 4) between the copper levels (Day 7 sample only) in the livers of fish fed and those not fed were not statistically significant ($N = 8$, $F_{calc} = 1.568$, $P_{calc} = 0.2344$). There was no statistically significant interaction between the two factors.

The livers in those fish which were both exposed to copper and not fed (Line 5) contained the highest levels of copper when compared to the other combinations of feed and copper (Lines 6 to 8) as determined by a Least Square Means table of a pair-wise comparison of factor combinations (Table 3-4). Contrary to the statistical comparisons, an inspection of the copper liver values indicates an arithmetic difference between those values from fish exposed to copper and not fed and the fish from all other combinations of copper and feeding.

Table 3-4 The actual mean measured copper concentrations in liver.

Line No.	Factor or Combination	Sample Size	Mean Measured Cu ppm (wet wt basis)	Standard Deviation	Significance
1	Cu	8	214	61	a
2	No Cu	8	158	47	a
3	Feed	8	169	51	a
4	No Feed	8	203	67	a
5	Cu/no feed	4	237	72	a
6	Cu/feed	4	190	43	a b
7	No Cu/no feed	4	168	45	a b
8	No Cu/feed	4	147	53	b

4. Discussion

4.1 Water Copper Concentrations

The data presented in Table 3-1 supports the premise that, under the conditions tested, the presence of fish and the

addition of feed (and as a result the presence of faeces) does not significantly reduce the level of copper compared to a tank with neither fish nor feed present. The lack of differences between copper concentrations from samples collected at the beginning and the end of the sample period would suggest that even in Tank 1, where there were neither fish, feed nor faeces, the tank itself did not bind appreciable levels of copper. These data are limited to observations from small fish, held in relatively large systems and treated with relatively low concentrations of copper. Extrapolation to larger fish at higher densities (kg per unit water volume), and with higher copper concentrations should only be made with an understanding of such limitations.

In further experiments, the role of tank, feed and faeces was considered to be negligible, even in those which were conducted at considerably higher concentrations of copper. Limited resources did not allow testing for tank uptake at higher doses.

As noted in the previous paragraph, extrapolation of the findings from this experiment to those with higher concentrations of copper would be done with caution. Additional information from this experiment supports that cautionary note.

An error was made at the outset of this experiment, which caused the measured copper levels to remain above the

nominal for approximately four days. The first day's copper was mistakenly made up at 50 ppm not 50 ppb; a 1000-fold error. This error was realised on the morning following, and the experiment was restarted. Fish were not scheduled to be placed in the tanks until the second day, therefore they were not exposed to 50 ppm copper. Once the error was discovered, all tanks were thoroughly drained, flushed and rinsed. However, a small residual remained on the tanks. This would suggest two items to consider. First, this probably was a factor contributing to the differences noted in the copper in the tanks relative to day sampled (Table 3-2). Second, the tanks do have the capacity to retain copper, but it may not be detectable unless very high levels of copper are used.

4.2 Tissue Copper Concentrations

This experiment was conducted under conditions which differed considerably from what was later established as a worst-case. The intent of the experiment was not to attempt to reproduce a worst-case, but instead as a preliminary measure of system constraints and nutritional state. The experiment served its purpose adequately relative to system constraints, as noted above in Section 4.1. The interpretation of the test animals nutritional state is somewhat less clear.

The results do suggest that the movement of copper into, and within, young tilapia is not independent of external factors. A

factor such as feeding may be well within the control of the researcher.

The results of this experiment did not provide strong evidence that copper was taken up by muscle (stronger evidence for the liver) when the test fish were not fed during copper exposure. However, these data provide adequate rationale for withholding feed in the subsequent experiments, especially in the worst-case experiment. To reiterate, the purpose of the worst-case experiment was to measure copper in the edible tissue of fish which had been exposed to the highest non-lethal level of copper possible under conditions which would maximise uptake. It appeared reasonable to assume that withholding feed during the worst-case experiment would not have decreased copper uptake and may even have increased the uptake and movement into the edible tissue.

Withholding feed from all subsequent experiments also provided an additional benefit. Under the less than optimum water quality conditions of a partial renewal regime, any technique employed that reduced water quality deterioration was desirable. Reducing the organic input to the water, directly through uneaten feed and indirectly by faeces produced was of value in maintaining higher water quality. Starving fish during copper exposure studies is not without precedent; Sayer, Reader and Morris (1991) withheld feed for four days prior to and during the entire 72 h of copper

exposure studies in brown trout. The authors did so to "...minimise the formation of organo-metallic complexes from faeces or surplus feed...".

The standard procedures for SRM preparation, presented in Chapter II, Section 5.4, were not used in this experiment. Both SRM's noted in Chapter II had not yet been acquired, therefore other SRM's were used to check AAS procedural consistency.

CHAPTER IV

MEDIAN LETHAL CONCENTRATION EXPERIMENTS

1 Introduction

1.1 Objectives

The overall purpose of this study was to ascertain whether copper accumulated in edible tissue of fish exposed to copper sulphate under worst-case conditions. Worst-case has been defined for this study to include the highest non-lethal concentration. There is presently no information in the literature which addresses the toxicity of copper to any species of tilapia. Before it is possible to estimate the highest non-lethal concentration of a substance for a particular species (i.e., copper sulphate LC1 to LC10 for *O. niloticus*), at least the median lethal concentration, LC50, should be known.

The objective of this experiment was to generate the 96 h LC50 of copper sulphate for the test species, *O. niloticus*. The data used to calculate the 96 h LC50 were used to extrapolate to an approximate LC1-10. The LC1-10 concentration which was then used as the highest non-lethal concentration in the worst-case experiment.

An LC50 was determined for both market-size and young fish. Although the worst-case experiment was to be conducted on

market-size fish, there was a need, again due to system limitations, to substitute young fish for market-size fish in interim experiments. A relationship between the LC50 of young fish and market-size fish was an essential part of validating such a substitution. The conduct of this comparative experiment will be discussed in greater detail in Chapter V.

Although not necessary for the determination of this toxicity data, a secondary objective of this experiment was to minimise the number of fish used to determine the LC50. There were two reasons for this. First, the period of time and the facilities required to conduct a full-scale LC50 experiment (including a range-finding preliminary experiment) in market-size tilapia exceeded the available resources. Secondly, there was an effort made to limit the number of animals for humane reasons.

In response to the latter objective, the experiment was designed to take advantage of the lower number of animals required by using an up-and-down procedure, for at least a portion of the experiment.

1.2 Literature Review

1.2.1 Historical Review of Copper Toxicity Testing in Fish

Copper (normally as copper sulphate) has been used extensively in aquaculture and is a significant industrial and agricultural pollutant, consequently there have been numerous studies reporting its lethal limits to a variety of fish. Fish previously tested include numerous commercial and non-commercial species and are outlined in Table 4-1 below.

Table 4-1. Fish species for which copper toxicity testing has been published.

Common Name	Scientific Name	Reference Source(s)*
American eel	*Anguilla rostrata*	5
Arctic grayling	*Thymallus arcticus*	16
banded killifish	*Fundulus diaphanus*	5
blacknose dace	*Rhinichthys atratulus*	8
blue gourami	*Trichogaster trichopterus*	6
bluegill	*Lepomis macrochirus*	1, 3, 4, 8, 14
bluntnose minnow	*Pimephales notatus*	8
brook trout	*Salvelinus fontinalis*	2
brown bullhead	*Ictalurus nebulosus*	8
channel catfish	*Ictalurus punctatus*	18
coho salmon	*Oncorhynchus kisutch*	9, 16
common carp	*Cyprinus carpio*	5
creek chub	*Semotilus atromaculatus*	8
cutthroat trout	*Salmo clarki*	12
fathead minnows	*Pimephales promelas*	3, 7, 8
goldfish	*Carassius auratus*	3, 11
guppy	*Poecilia reticulata*	3
catfish (Indian)	*Mystus bleekeri*	15
orangethroat darter	*Etheostoma spectabile*	8
pink salmon	*Oncorhynchus gorbuscha*	10
pumpkinseed	*Lepomis gibbosus*	5
rainbow darter	*Etheostoma caeruleum*	8
rainbow trout	*Oncorhynchus mykiss*	13, 16
sockeye salmon	*Oncorhynchus nerka*	10
stoneroller	*Campostoma anomalum*	8
striped bass	*Morone saxatilis*	5, 17
striped shiner	*Notropis chrysocephalus*	8
white perch	*Morone americanus*	5

*Reference Sources: 1) Trama 1954; 2) McKim and Benoit 1971; 3) Pickering and Henderson 1966; 4) O'Hara 1971; 5) Rehwoldt, Bida and Nerrie 1971; 6) Roales and Perlmutter 1974; 7) Brungs, Geckler and Gast 1976; 8) Geckler, Horning, Neiheisel, Pickering, Robinson and Stephan 1976; 9) Lorz and McPherson 1976; 10) Servizi and Martens 1978; 11) Tsai and McKee 1978; 12) Chakoumakos, Russo and Thurston 1979; 13) Miller and Mackay 1980; 14) Thompson, Hendricks and Cairns 1980; 15) Gupta and Rajbanshi 1981; 16) Buhl and Hamilton 1990; 17) Reardon and Harrell 1990; 18) Straus and Tucker 1993.

The reported range of copper concentrations toxic to fish is broad. As examples of the range, the 96 h LC50 for coho salmon (*O. kisutch*) was found to be 60 to 74 ppb in water of 88 ppm total hardness (as $CaC0_3$) (Lorz and McPherson 1976). O'Hara (1971) found that bluegills (*L. macrochirus*) were very tolerant to copper; the 96 h LC50 of copper to bluegills was 2400 ppb in water with a total hardness of 35 ppm (as $CaC0_3$).

1.2.2 Modifying Factors to Toxicity

Copper toxicity is significantly affected by a variety of water quality parameters, some of which have been reviewed in Chapter III Section 1.2.1. There have been numerous studies that have characterised such relationships. The following is a brief list of the major factors which affect copper toxicity, a brief statement of the general relationship between each factor and copper toxicity, and a representative listing of literature in which the topic has been presented.

a) Alkalinity - as alkalinity increases the toxicity of copper is reduced (Chakoumakos et al. 1979; Miller and Mackay 1980; Laurén and McDonald 1986; Straus and Tucker 1993).

b) Suspended and dissolved organics - many of the reports regarding these substances suggest that they tend to bind copper and effectively reduce the toxicity of a given concentration, by directly removing it from the

water column and/or complexing with it into a form that is less toxic (Engel, Sunda and Fowler 1981; Meador 1991; Brown et al. 1974; Zitko et al. 1973; Winner 1984).

c) Dissolved oxygen (DO) - as DO decreases, the toxicity of a given concentration of copper increases; this may in part be a function of increased ventilation, which in turn increases the amount of copper presented to the absorptive gills surfaces (Lloyd 1961).

d) pH - the relationship between pH and other water quality parameters is complex, often case-specific and difficult to state in general terms. An example of a relationship , under conditions of constant copper and dissolved organic carbon, as the pH decreases the toxicity of copper increases (Waiwood and Beamish 1978; Chakoumakos et al. 1979; Miller and Mackay 1980; Engel et al. 1981; Pagenkopf 1983; Laurén and McDonald 1986; Meador 1991; Straus and Tucker 1993; Pynnönen 1995).

e) Salinity - increasing salinity tends to reduce the toxicity of copper; however, its relationship can be quite complex, especially when one considers normal physiological responses to salinity changes, such as changes in osmoregulation (Engel et al. 1981; Reardon and Harrell 1990).

f) Water hardness - although there appears to be two schools of thought as to which parameter, hardness or alkalinity, has the greatest modifying effect on copper toxicity, water hardness is important; copper toxicity decreases as hardness increases (Inglis and Davis 1972; Pagenkopf et al. 1974; Howarth and Sprague 1978; Waiwood and Beamish 1978; Chakoumakos et al. 1979; Miller and Mackay 1980; Pagenkopf 1983; Laurén and McDonald 1986; Straus and Tucker 1993; Pynnönen 1995).

g) Temperature - in two species of darters (*Etheostoma* sp.) that had been exposed to copper, Lydy and Wissing (1988) reported that the fishes' ability to withstand temperature increases (or thermal tolerance as calculated by the CTMax method) had been reduced, when compared to non-exposed controls. The authors suggested that the reduction in thermal tolerance may have been associated with reversible copper-induced anaemia and the increased oxygen demand of thermal stress.

These toxicity studies were conducted taking into account the aforementioned relationships. Even though an attempt was made to minimise some effects (complexing with organics), as noted in Chapter III, there was no attempt to reproduce conditions that might be found on a production site. Since

such conditions can vary dramatically, it would be difficult to produce representative conditions within the constraints of this study. In lieu of simulating production conditions, an attempt was made to insure that: 1) conditions remained relatively constant during the course of all related experiments and that 2) water parameters fell within the theoretical range found on commercial fish farms.

1.2.3 Up-and-Down Procedures

Median lethal dosage (LD50) or concentration (LC50) procedures were first introduced by J.W. Trevan in 1927 as a means of standardising toxicity testing of biologically relevant products (cited by DePass 1989). Median lethal dosage refers to calculations made relative to products which are provided to the test animal in a standardised dosage form, e.g., as an injectable, *per os*, as an implant, etc., whereas median lethal concentration refers to calculations made relative to products being provided via the test animal's surroundings, e.g., in the water surrounding fish. The procedures have been generally adopted by the scientific community and have, up until recently, been required testing by regulatory agencies world wide as the basis for establishing safe levels for various products (DePass 1989). The LD or LC50 procedures normally require that a significant number of animals be tested at each of multiple levels, from which there is calculated an inherently accurate estimation of the median lethal dose or

concentration. The median lethal dose or concentration is an estimated nominal concentration. Any concentration above the LC50 would be expected to produce more than 50% mortalities in a given population, and any concentration below the LC50 would be expected to produce less than 50% mortalities in the same population.

When any new compound is to be tested against a new test animal, the first step is to determine the approximate toxic dose (or concentration). This can be accomplished in several ways. The easiest being to consult the scientific literature and draw conclusion from existing related information. There may already be information related to the testing of a related product against a related test animal, or there may be information on the testing of the product in question against other, possible less related, test animals. An alternative, if such information were not available, is to conduct a range-finding trial.

A range-finding trial is merely an LD or LC50 trial, the test levels of which are quite widely separated, usually one order of magnitude apart. Like the full LD or LC50 trial, the range-finding trial is normally conducted with a reasonable numbers of animals. However, the accuracy inherent to an LD or LC50 trial is not required for a range-finding trial. The purpose of a range-finding trial is to determine the approximate toxic dose (or concentration), within an order of

magnitude, which can then in turn be divided into five-plus equally spaced levels for the actual LD or LC50 trial.

There are alternatives to standard LD and LC50 trials, which may be even more applicable to range-finding trials. Such alternative procedures are characterised by the use of considerably fewer animals. The earliest proposed alternate procedure was published in 1943 by Deichmann and LeBlanc (cited by DePass 1989). Possibly the most widely accepted alternate procedure was first published in 1948 by Dixon and Mood (cited by DePass 1989) and has since been included in standard statistical methods textbooks (Dixon and Massey 1983) and reviewed by others (Bruce 1985; DePass 1989; Dixon 1991; Yam, Reer and Bruce 1991). This procedure is a staircase design and is referred to as an up-and-down method (Dixon 1991).

In essence, the up-and-down procedure entails a series of single-animal tests, each at a pre-determined concentration. Each successive concentration being a constant incremental difference apart from immediately surrounding levels. The concentration at which each test is conducted is a function of the results of the immediately preceding test. The procedure's strength lies in its inherent focus of efforts in the region of the actual mean concentration, as opposed to the use of many animals at concentrations which are either non-toxic (produce no mortalities or toxicity) or highly toxic (produce

100% mortalities or toxicity). As a consequence, fewer animals are used. For the purposes of this study, an up-and-down procedure was considered to be ideal for use as a range-finding trial.

2 Study-Specific Materials and Methods

2.1 Systems/Procedures

Culture systems used were as described in Chapter II. Fish were acquired, acclimated and held prior to initiation of the experiment as described in Chapter II. All exposure of fish to copper sulphate was by means of the static 50% renewal system defined in Chapter II. Each segment of this experiment was conducted for 96 h only, unless all subject fish within a given segment died prior to the end of the 96 h test period. Water quality parameters were determined at the beginning and the end of each test, in particular for pH, DO, temperature and nitrites (final sample only).

2.1.1 Up-and-Down Range-Finding Experiment

This experimental subset comprised an up-and-down procedure customised to suit the facilities and time-frame available. It involved multiple tests, each potentially consisting of a different number (3 to 6) of experimental tanks (as opposed to a single-tank test described for standard up-and-down procedures). All tanks were the standard 150 l

size, and prior to use each was thoroughly cleaned and flushed. Each tank contained two airstones and was covered by a framed plastic mesh cover.

The following were the procedures followed for the up-and-down trial.

a) An estimate of the 96 h LC50 for tilapia was deduced from the literature that describes such for other fish. Only those data from fish in which water hardness was close (within approximate 120 ppm) to that of this test system (85 ppm as $CaCO_3$) were considered. Table 4-2 below summarises those other data considered in this estimate.

Table 4-2. Previously reported 96 h LC50's for copper of fish tested in freshwater near the hardness the UMAB test system.

Fish	96 h LC50 (ppb)	Water Hardness (ppm as $CaCO_3$)	Reference	Source
coho salmon, *Oncorhynchus kisutch*	60-74	~89	Lorz & McPherson 1976	
brook trout, *Salvelinus fontinalis*	100	45	McKim & Benoit 1971	
brown bullhead, *Ictalurus nebulosus*	170-190	202	Brungs et al. 1973	
fathead minnow, *Pimephales promelas*	430; 460-490	198; 200	Mount 1968; Pickering, Brungs & Gast 1977	
bluegills, *Lepomis macrochirus*	1100; 2400	45; 35	Benoit 1975; O'Hara 1971	

From the information noted in the table above, it was reasoned that 200 ppb copper was an acceptable first estimate of the 96 h LC50 for market-size tilapia.

b) From the 200 ppb estimate, two concentrations above and two below this level were determined based on the up-and-down procedural criteria. The concentrations were calculated to be approximately 0.3 natural log units apart (98, 132, 200, 267 and 361 ppb ⇒ natural log concentration 4.59, 4.89, 5.29, 5.59 and 5.89; a calculation error was made for the two lowest concentrations, which should have been 147 and 108 ppb or natural log 4.99 and 4.69).

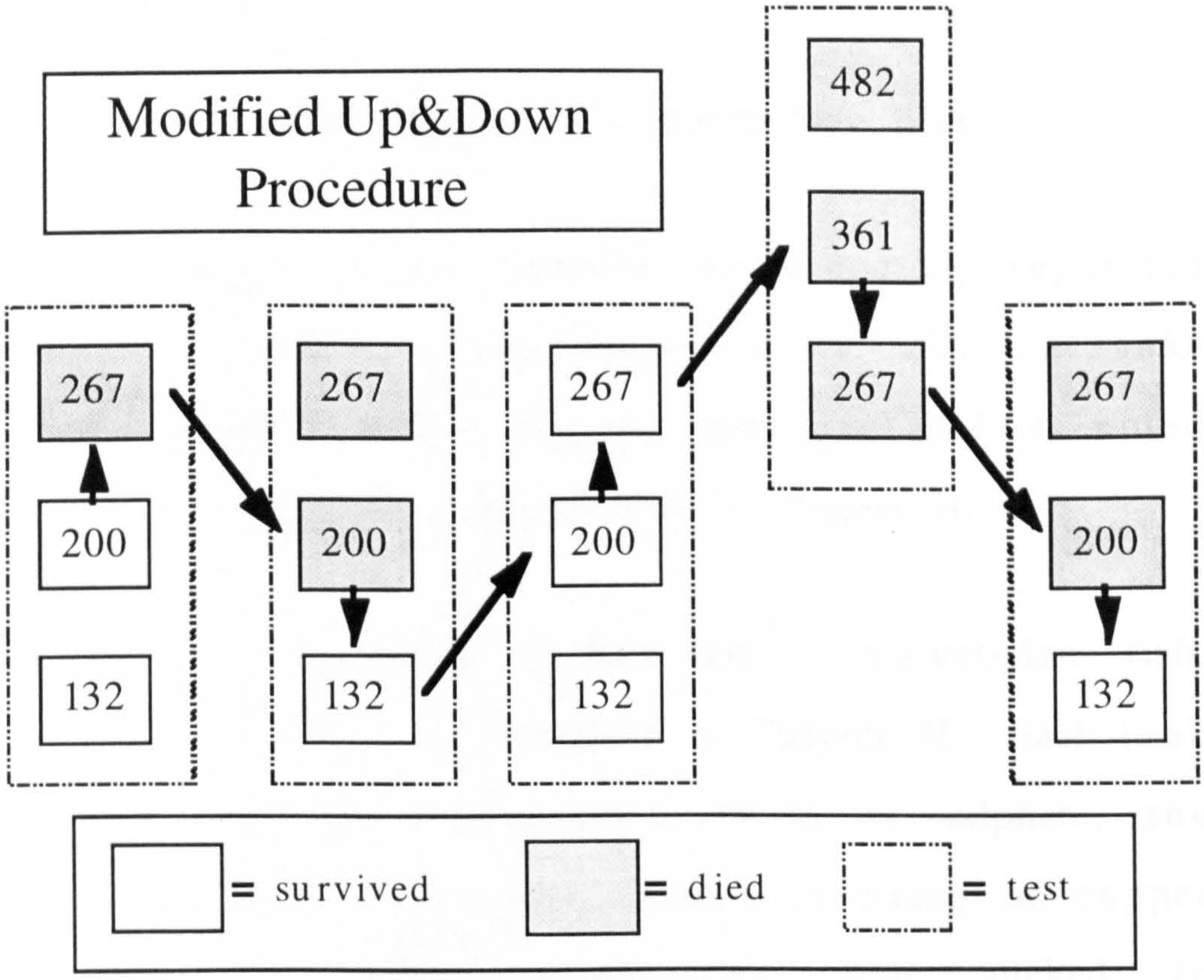
c) A test of three tanks was set-up with one fish per tank and each tested with one of three consecutive concentrations of copper sulphate. This test, and all tests, were conducted for 96 h or until all fish died, whichever occurred first. Any up and down procedure followed a simple path; if a fish tested died, the next fish to be tested would be at the next lower concentration; if the first fish lived instead, the next fish to be tested would be at the next higher concentration.

d) In a hypothetical example of the standard up-and-down trial, if the first concentration tested were 200 ppb and the fish lived, then the next concentration to be tested would be 267 ppb; if the first fish died at 200 ppb then the next concentration to be tested would be 132 ppb. In this modified up and down procedure three concentrations were used within each test in order to reduce the time required for the whole series of trials.

Using the same hypothetical example and referring to Figure 4-1 below, the mid concentration of 200 ppb was taken as the starting point for the modified up and down procedures. This fish lived, therefore the next highest concentration of 267 ppb was examined. Since the fish exposed to 267 ppb died, the starting point for the next test, or set of three concentrations, was the next lowest concentration, or 200 ppb. Given

that each test should include three concentrations, one above and one below the starting point, and that this test starts at 200 ppb there will also be included a 132 and 267 ppb exposure tank. In this second test, the fish exposed to 200 ppb died, hence the next fish examined was the 132 ppb, which lived. Considering that the last fish at 132 ppb lived, the starting point for next test should be 200 ppb, and should have one concentration above and below it included within the test. In this third test the 200 ppb exposed fish lived, which requires that the next fish to be examined is the one exposed to 267 ppb, which also lived. Given that the last fish lived when exposed to 267 ppb, the next set should include the next higher concentration of 361 ppb and a concentration above and below it. The same rationale was followed for each successive test in the hypothetical example of the modified up and down procedure followed in this experiment (Figure 4-1).

Figure 4-1. Example dosing strategy of the modified up-and-down toxicity trial



e) This trial was repeated for 7 tests. In some cases, due to perceived extreme variation in the results, a particular test may have included up to 6 tanks, each tank with a single fish. In these latter tests, the strategy for determining the concentrations of the next test was a variation of that depicted above.

f) Once all tests were completed, the resulting information was used to generate an approximate LC50 upon which

the concentrations of the full LC50 trial were to be conducted. The calculations made were as defined by Dixon and Massey (1983), in Chapter 19, Section 19-2 and Table 19-3.

2.1.2 Standard 96 h LC50 Experiment - Market-Size Fish

This experimental subset comprised two temporally separated replicate runs. Each replicate consisted of six tanks. All tanks were the standard 150 l size, and were prepared as noted above in this chapter and as outlined in Chapter II.

Each tank was arbitrarily stocked with 6 market-size fish, previously acclimated as described in Chapter II. Each tank received one of five concentrations of copper sulphate, the sixth tank was the non-treated control, receiving no copper sulphate. The five concentrations were picked to approximately straddle the estimated LC50 derived in the up-and-down trial, and were consistent with procedures outlined in American Society for Testing and Materials (1989), in particular the specification that each concentration should be at least 60% of the next higher concentration, exclusive of the 0 ppb and the highest concentration. The concentrations were randomly assigned to one of the six tanks. The nominal concentrations used were calculated from a best-fit line equation relating the actual copper levels measured and the nominal concentration administered. This equation was derived from previous water analyses data generated in

earlier experiments within this study. The nominal and expected concentrations used were as noted in Table 4-3 below.

Table 4-3. Copper concentrations (required and nominal) applied to the 96 h LC50 trial for market-size tilapia

Required Actual Copper Concentration (ppb)	Estimated Nominal Copper Concentration Applied (ppb)
330	365
474	482
765	889
1105	1326
1636	2416

As noted above, this trial consisted of two replicate runs, and due to logistic limitations it was conducted during two successive time periods. The random assignment of concentrations to respective tanks was maintained between the two tests to minimise the potential for contamination from one test to the next.

The copper sulphate and 50% of the water was renewed daily, and appropriate water samples were collected as per routine procedures defined in Chapter II. During this same period, fish were observed for any signs of toxicity. If a fish was found dead, it was removed, condition and time recorded and placed in the morgue freezer for potential later analytical sampling. If fish were found to be moribund, the

observations were recorded. Mortality checks were conducted minimally once per 24 hour period. Each replicate test was conducted over a 96 h period.

2.1.3 Standard 96 h LC50 Experiment - Small Fish

This experimental subset consisted of a single replicate test, consisting of six tanks. All tanks were the standard 150 l size, and were prepared as noted above in this chapter and as outlined in Chapter II.

Each tank was stocked with 12 arbitrarily selected small fish, previously acclimated as noted in Chapter II. Each tank received one of five concentrations of copper sulphate, the sixth tank was the non-treated control, receiving no copper sulphate. The five concentrations did not directly utilise the estimated LC50 derived in the up-and-down trial, but were otherwise as noted in the previous section. It was assumed that copper sulphate would be more toxic to younger fish, therefore the overall range of concentrations was reduced. The concentrations were randomly assigned to one of the six tanks. The nominal values used were calculated from a best-fit line equation relating the actual copper levels measured and the nominal concentration administered. This equation was derived from previous water analyses data generated in earlier experiments within this study. The nominal and expected concentrations used were as noted below in Table 4-4.

Table 4-4. Copper concentrations (required and nominal) applied to the 96 h LC50 trial for small tilapia

Required Actual Copper Concentration (ppb)	Estimated Nominal Copper Concentration Applied (ppb)
72	72
119	120
191	200
305	333
780	925

The copper sulphate and 50% of the water was renewed daily, and appropriate water samples were collected as per routine procedures defined in Chapter II. During this same period, fish were observed for any signs of toxicity. If a fish was found dead, it was removed, condition and time recorded and placed in the morgue freezer. If fish were found to be moribund, the observations were recorded. Mortality checks were conducted minimally once per 24 hour period. Each replicate test was conducted over a 96 h period.

2.2 Fish

All fish used in this study were either young tilapia ranging in size from 10 to 30 g in weight or market-size fish ranging in size from 350 to 570 g (depending upon the phase of this experiment being conducted). In all trials, the planned routine followed either of two tracks. The fish were transferred from the holding tanks to test tanks and

maintained in the latter under holding conditions (flow-through water exchange, etc.) for several days, or the fish were transferred directly to the test tanks upon initial acquisition of the fish (at slightly higher densities to allow for mortalities). On occasion, there were conditions which dictated that fish were transferred from a holding tank into a test tank, with very little test-tank acclimation allowed. This was felt to be acceptable, due to the similarity of systems. However, under no circumstances were fish ever part of an experiment without having gone through the general quarantine period.

2.2.1 Up-and-Down Range-Finding Experiment

This study used only market-size fish. The fish were being held in tanks identical to the experimental tanks, and had been acclimated as per standardised routines. The holding tanks were stocked at densities considerably higher than the stocking densities of this experimental system. Fish were stocked in holding tanks at 7 to 9 fish per tank, which equates to approximately 16 to 34 kg per cubic metre of water, whereas the test tanks, with one fish each, contained from approximately 2.3 to 3.8 kg per cubic metre of water. Although not directly comparable, the holding tanks were maintained on a flow-through water supply, while the test tanks were operated under a static 50% renewal regime. In

the latter, the test fish were considered to be in better water conditions (due to densities) during this experimental subset.

2.2.2 Standard 96 h LC50 Experiment - Market-Size Fish

This study used only market-size fish. The fish were being held in tanks identical to the experimental tanks, and had been acclimated as per standardised routines. The holding tanks were stocked at densities slightly higher than the stocking densities of this experimental system. Fish were stocked in holding tanks at 7 to 9 fish per tank which equates to approximately 16 to 34 kg per cubic meter of water, whereas the test tanks, with 6 fish each, contained from approximately 14 to 23 kg per cubic meter of water. This is not directly comparable, since the holding tanks were maintained on a flow-through water supply, while the test tanks were operated under a static 50% renewal regime. The experimental period began 24 h after stocking.

2.2.3 Standard 96 h LC50 Experiment - Small Fish

Fish for this trial were stocked from an identical holding tank into the test tanks. The holding tank contained approximately 80 fish, which were equally divided between the holding tank and five identical test tanks (12 fish each), with the remaining fish stocked into a second holding tank. The initial holding tank, which then contained 12 fish, became an experimental tank. The experimental period began 24 h after stocking.

2.3 Sampling

Fish were not routinely sampled during the LC50 trials for analytical work. Fish remaining at the end of the trials, however, were sacrificed and stored in the freezer.

Routine water samples for copper were collected during all LC50 trials, to establish true exposure concentrations. The following samples were collected from each experimental tank during every 24 h period: a pre-exchange non-filtered, a pre-exchange filtered and a post-exchange non-filtered sample as described in Chapter II.

Water samples were collected several times over the course of the entire study for hardness determinations. Temperature, pH and DO levels were determined at the beginning and end of each 96 h exposure period, and nitrite only at the end.

2.4 Analyses

Water copper, calcium and magnesium (the latter two for hardness calculations) levels were determined via AAS as described in Chapter II. Water quality parameters (pH, DO, temperature, and nitrites) were determined as per procedures defined in Chapter II.

3 Results

3.1 Up-and-Down Range-Finding Experiment

3.1.1 96 h LC50 Estimations

This trial was conducted over the course of 26 days and included 30 fish. The lowest nominal concentration tested was 132 ppb copper, while the highest tested was 2416 ppb. The estimated 96 h LC50 for nominal copper to market-size tilapia was determined to be approximately 1187 to 1217 ppb copper, while the 96 h LC50 for actual level of copper exposure was estimated to be 994 to 1018 ppb copper. The number of tested concentrations far exceeded those originally planned; increasingly higher concentrations were tested with each test in response to a lack of mortality in the previous ones tested. The actual response of the fish tested, in the modified up-and-down procedures noted above in Section 2.1.1, are as depicted in Figure 4-2 below .

Figure 4-2. Responses of fish to copper concentrations in the modified up-and-down trial.

Nominal Conc. (ppb)	Nominal Conc. (Log_e)	Approximate Sequence of Tests (time ⇒) and Response of Each Fish (O = lived, X = died)
2416	7.79	X
1620	7.39	X O
1326	7.19	X X O X O X X
1086	6.99	O X O O O X
889	6.79	O X
658	6.49	O
482	6.19	O
361	5.89	O
267	5.59	O
200	5.29	O
132	4.89	O

The responses noted in the above figure were analysed as per Dixon and Massey's (1983) procedures. There were, however, several subsets of the above data used for the calculations. The range of estimated 96 h LC50's noted in the previous paragraph represents the calculated LC50's based on three choices of observations: a) considering N´ (Nominal Sample Size defined by Dixon and Massey, 1983) to be equal to 22, which represents the entire set of observations, b) considering N´ to be 14, which is the first 14 observations, and c) considering N´ to be 17, which is the last 17 observations.

3.1.2 Actual Water Copper Concentrations

The actual water copper concentrations measured via AAS were less than the nominal copper levels administered to the respective tanks. The actual copper values were calculated as a mean of the measured filtered pre-exchange samples and the estimated values of post-exchange samples had they been filtered. Post-exchange filtered samples were not collected and measured, due primarily to the added cost of extra filters, but instead their values were estimated.

The estimations of post-exchange filtered samples were made based on several assumptions. First, it was assumed that the proportion of copper bound to filtratable material would remain relatively constant for short periods of time (15 to 30 min). Second, it was assumed that this would remain true even when the water in which the bound copper was being measured was diluted with a volume of new water and copper at the same nominal concentration. Third, it was assumed (and arguably in error) that none of the new copper had had time to bind to anything in the new water.

Given these assumptions were true, it was felt that the amount of bound copper would be the same in the tank water 15 to 30 min following the daily water/copper exchange. If the amount of copper remained constant during this length of time, then an estimate of the post-exchange copper level can be made. The post-exchange filtered samples were estimated

to be 50% of the pre-exchange filtered samples, which had been collected 15 to 30 min before. The 50% factor was used because half of the water had been removed, and replaced with new water and copper. The copper newly added had not yet had time to bind to anything.

Having made the previous assumptions, AAS measured copper values for pre-exchange filtered, pre-exchange non-filtered and post-exchange non-filtered water were used to calculate the estimated post-exchange filtered samples (Excel, Microsoft, Version 5.0 for the Macintosh). The amount of copper in the post-exchange filtered sample for any given day was estimated by the following equation

$$POST_f = POST_{nf} \times (1 - (((PRE_{nf} - PRE_f) \div PRE_{nf}) \div 2))$$

where: PRE_f = pre-exchange filtered copper, PRE_{nf} = pre-exchange non-filtered copper, $POST_f$ = post-exchange filtered copper, and $POST_{nf}$ = post-exchange non-filtered copper.

Table 4-5 below represents a summary of the nominal copper concentrations tested in this trial and the overall mean of the actual copper exposures. The latter are based on two values for each 24 h period of the 96 h trial (start of 24 h period = post-exchange, end of 24 h period = pre-exchange) for all tests of that particular concentration.

Table 4-5. Mean calculated and actual copper concentrations in the up-and-down trial.

Nominal Concentration (ppb)	Mean Actual Concentration (ppb)
132	121
200	179
267	231
365	438
482	474
658	598
889	765
1086	938
1326	1105
1620	1235
2416	1637

The actual concentration measured for the nominal concentration of 365 ppb was actually 438 ppb; there was a significant error made due to pipetting of stock copper sulphate solution into the tank on the first day of the experiment. The mistake was not discovered until after the first 24 h 50% renewal had taken place. It was then decided to continue on with the resultant concentration and a correction was made to the nominal concentration to be added from that point forward.

3.2 Standard 96 h LC50 Experiment - Market-Size Fish

The mortality patterns of the two replicate tests of this trial differed somewhat from each other, in that there were differences in the mortalities in several of the median concentrations. However, as per the basic testing criteria,

both replicates of the non-treated controls produced 0% mortalities and both replicates of the highest concentration produced 100% mortalities. The total mortalities observed during the 96 h LC50 trials are summarised in Table 4-6 below. Included in Table 4-6 are calculations of the percentage of copper being filtered from the water samples. There appears, by inspection, to be a relationship between nominal copper administered and the proportion of copper filtered from the water.

Table 4-6. Information summary of the 96 h LC50 trial for market-size tilapia.

Nominal Concentration (ppb)	0	365	482	889	1326	2416
Mean Measured Concentration, Non-filtered (ppb)	0	346	461	791	1223	2008
Mean Actual Concentration, Filtered (ppb)	0	328	427	699	987	1456
Mean Copper Reduction Due to Filtration (%)	-	5.2	7.4	11.6	19.8	27.5
Mortalities	0	0	2	2	8	12

The 96 h LC50 of this trial was calculated to be 808 ppb of actual copper with a 95% confidence interval of 674 to 978 ppb. The LC50 estimations were derived using the EPA Probit Analysis Program (Version 1.4). In addition to calculations of the LC50, estimation were also calculated for LC1 to LC99, with 95% confidence intervals. Table 4-7 below summarises the estimations.

Table 4-7. Summary of lethal concentration estimates calculated for market-size tilapia.

Lethal Concentration Point	Estimated Lethal Concentration, Actual Copper (ppb)	Lower 95% Confidence Limit (ppb)	Upper 95% Confidence Limit (ppb)
LC1	318	161	432
LC5	418	253	531
LC10	484	320	595
LC15	534	374	645
LC50	**808**	**674**	**978**
LC85	1224	1006	1786
LC90	1350	1089	2091
LC95	1562	1220	2654
LC99	2051	1498	4180

3.3 Standard 96 h LC50 Experiment - Small Fish

The mortalities of this trial were considerably less than expected, and did not fit well into an LC50 analysis. As examples, the highest concentration did not produced 100% mortalities and three concentrations produced no mortalities, at all. The total mortalities observed during this 96 h LC50 trial are as presented in Table 4-8 below. Included in Table 4-8 are calculations of the percentage of copper being filtered from the water samples. Unlike the LC50 trials with market-size fish, there appears to be no clear relationship between nominal copper administered and the proportion of copper filtered from the water. The non-filtered measured concentrations of copper in the 120 ppb nominal tank do not represent errors in calculations, in spite of their being larger than the nominal. The value of 124 ppb represents an error

in the amount of copper added at the beginning of the third 24 h period, elevating that day's level to approximately 170 ppb. No correction was made to future additions to that tank.

Table 4-8. Information summary of the 96 h LC50 trial for small tilapia.

Nominal Concentration (ppb)	0	72	120	200	333	925
Measured Concentration, non-filtered (ppb)	0	59	124	169	296	796
Actual Concentration, filtered (ppb)	0	56	122	165	287	757
Copper Reduction Due to Filtration (%)	-	5.1	1.6	2.4	3.0	4.9
Mortalities	0	0	0	0	8	11

The 96 h LC50 of this trial was calculated to be 308 ppb of copper with a 95% confidence interval of 243 to 435 ppb. The LC50 estimations were derived using the EPA Probit Analysis Program (Version 1.4). In addition to calculations of the LC50, estimation were also calculated for LC1 to LC99, with 95% confidence intervals. Table 4-9 below summarises the estimations.

Table 4-9. Summary of lethal concentration estimates calculated for small tilapia.

Lethal Concentration Point	Estimated Lethal Concentration, Actual Copper (ppb)	Lower 95% Confidence Limit (ppb)	Upper 95% Confidence Limit (ppb)
LC1	107	47	150
LC5	146	81	191
LC10	172	108	219
LC15	192	130	243
LC50	**308**	**243**	**435**
LC85	493	368	965
LC90	552	401	1179
LC95	651	454	1593
LC99	887	568	2822

4 Discussion

4.1 Up-and-Down Range-Finding Experiment

The up-and-down portion of this experiment was successful in generating the information it had been designed to provide. A workable estimate of the LC50 for copper was made, which was then in turn used to establish the doses to be used in the full LC50 trial.

There were, however, several serious negative aspects of this procedure relative to the needs of this study. In addition to the obvious need to generate an estimate of the LC50, this procedure was intended to do so with a minimum of fish and possibly in a shorter period of time.

There appears to be several reasons for its failure to meet these latter goals. Two entrance assumptions for the use of this procedure are 1) possession of a reasonably accurate estimate of the LC50 before the start and 2) limiting the time of each test to as short as possible, i.e., 24 to 48 h.

Before beginning this trial, it was concluded that a 24 h LC50 for copper would not suffice as an estimate for a 96 h study. Experience at the UMAB lab with extreme differences in 24 and 96 h copper LC50 results (80 ppb compared to 720 ppb) in brown bullhead (unpublished) suggested that conducting 24 h LC50 trials would not be suitable as the basis for a 96 h trial. Therefore, the decision was made to conduct 96 h tests (instead of 24 h), but the projected time-frame for sequentially running several fish (estimated at 6 to 10) for 96 h each (24 to 40 days total) was considerably outside of the time allotted for this segment of the study.

To reduce the testing period, the up-and-down procedures were modified to include multiple, individually-housed fish in each 96 h test. Seven tests were conducted, taking a total time of 26 days. This was much longer than anticipated, but if the modification had not been made, and the same number of animals had been tested by standard up-and-down procedures, 120 days would have been required.

The decision to not substitute a 24 h (or even 48 h) for a 96 h trial was validated by the data. Table 4-10 below summarises

the 24 and 48 h mortality patterns of the up-and-down and standard LC50 trials combined. These data are not adequate for estimating a 24 or 48 h LC50, far less a 96 h LC50.

Table 4-10. Summary of 24 and 48 h mortality data from the up-and-down and the LC50 trial for market-size tilapia.

Nominal Concentration (ppb)	No. of Fish Tested	Observed Mortalities at Concentration within:	
		24 h	48 h*
132	1	0	0
200	1	0	0
267	1	0	0
365	13	0	0
482	13	0	0
658	1	0	0
889	**15**	**1**	**2**
1086	**9**	**0**	**3**
1326	20	0	0
1620	3	0	0
2416	**13**	**1**	**2**

* includes mortalities indicated under 24 h column

Examination of the other assumption relative to the use of an up-and-down procedure (a reasonable knowledge of the LC50 before starting the trial) and the data, produces support for the importance of this assumption. The information in Figure 4-2 in Section 3.1.1 of this chapter, suggests that the first six fish tested provided little useful information. This is verified in the calculations of the LC50 from these data (Section 3.1.1). One calculation included the first six fish and a second did not.

The resulting LC50 estimates are nearly identical (1018 ppb and 1014 ppb actual copper, respectively). The usefulness of this procedure, assuming one can start the experiment close to the LC50, is confirmed if a calculation is carried out for the nine tested fish starting with fish number six (658 ppb). The calculated LC50 estimate using these nine fish is 958 ppb (actual copper).

As further confirmation of the validity of the up-and-down procedure, the results from the 30 tested fish were entered into the EPA Probit Analysis for LC50 calculations. The resulting 96 h LC50 calculated by this procedure was 1117 ppb (actual copper).

4.2 Standard 96 h LC50 Experiment - Market-Size Fish

The calculated LC50 value (actual copper) for market-size fish is certainly much higher than the original estimate of 200 ppb. The 96 h LC50 for the market-size tilapia, when compared to the literature values for other species held in similar conditions of water hardness (see Table 4-2) is quite high. The only species more tolerant of copper is bluegills. As a rule, aquaculturists tend to consider tilapia as a very hardy fish, especially when it comes to disease and a tolerance of poor water conditions (Stickney 1993). Their tolerance to copper sulphate would support that premise. It would appear logical that if a fish is successful at adapting to a wide range of environmental conditions, as tilapia are, that it was not only

necessary that they did not die under the new environmental conditions, but that they also continue to grow, reproduce and function normally otherwise. The extreme adaptability of tilapia appears to include a high tolerance for copper, as well as other characteristics.

This experiment not only provided an estimate of the LC50 for market-size tilapia, but also allows for the estimation of the highest non-lethal concentration of copper sulphate to these same fish. The data in Table 4-7, above, allows for a best guess of the concentration to which market-size tilapia should be exposed during the worst-case experiment and provide maximum exposure (and hopefully copper uptake), while at the same time minimising mortalities during that exposure.

4.3 Standard 96 h LC50 Experiment - Small Fish

The 96 h LC50 calculated for small tilapia appears to be reasonable when compared to that of the market-size tilapia, not only with regard to absolute value but also its confidence interval. The LC50 being less than that in the market-size fish is intuitive, in spite of suggestions in the literature that tolerance to copper does not necessarily increase as a fish get older/larger. Overall the literature suggests that any such relationship is more than likely species and compound specific.

Several publications have reported on examinations of differential toxicity relative to fish size. Sprague (1985) suggests that there is no generalisation that can be applied to all fish species for a given toxicant/environment combination. Howarth and Sprague (1978) report that their studies with rainbow trout (*O. mykiss*) demonstrate a significant difference, as much as a 2.5 fold higher LC50 for 10 g fish compared to 0.7 g fish tested in the same water conditions. Anderson and Spear (1980) (cited by Sprague 1985) found no change in the LC50 of 3.9 to 176 g rainbow trout, but did find a difference in LC50's of pumpkinseed sunfish (*Lepomis gibbosus*) of 1.2 g compared to those weighing 7.6 g. Laurén and McDonald (1986) studied the relationship of copper exposure to sodium fluxes between the fish and the surrounding water. They found that juvenile rainbow trout (*O. mykiss*) under specific water conditions would die when they lost 50 to 55% of their Na^+, and this would occur at approximately 200 ppb copper. The same level of copper would only result in a 23% loss of Na^+ in adult fish, which did not relate to mortality. They further equated the higher Na^+ efflux in juveniles to the higher surface area to weight ratio in juveniles.

The use of the 96 h LC50 for small tilapia should be confined to this study. If more precise values are required, 96 h LC50 studies should be repeated with greater numbers of fish per

concentration. In essence, this reported 96 h LC50 trial should be considered as a range-finding trial.

4.4 Small Fish Compared to Market-Size Fish - Data Relevance

The 96 h LC50 for small tilapia, presented in Section 3.3 above, does not have a high degree confidence. It is however, adequate for the intended purposes. The intent, from the outset of this part of the study, was to establish if the copper sensitivity of young fish were within an order of magnitude of the copper sensitivity of older/larger fish of the same species. It was understood, that if there were no differences in toxicity exceeding an order of magnitude, and if residue studies comparing the two size groups also revealed an uptake differential of the same order, then future experiments could be conducted substituting small fish for market-size fish, when necessary and applicable. This experimental subsection confirmed at least the first part of the premise; the tolerance of small tilapia were not orders of magnitude different from that of market-size tilapia. The second part of the premise will be address specifically in Chapter V.

4.5 Filtered vs. Non-Filtered Water Samples

Filtered water samples were different from the parallel non-filtered samples and these differences are believed to be significant relative to the toxicity of the administered concentration of copper. The differences were much more

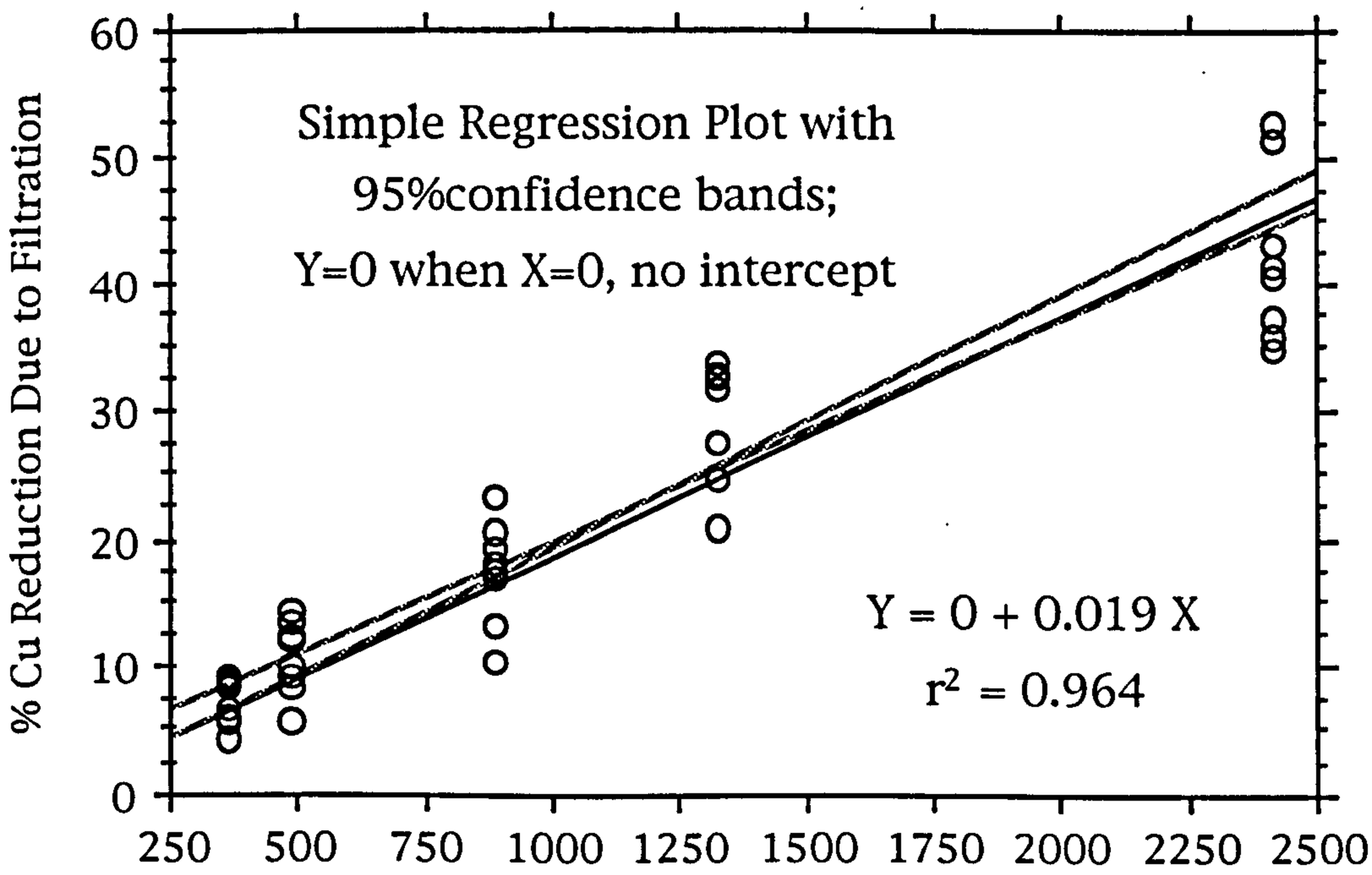
apparent in those samples collected in the market-size fish trials and even more so in the samples collected from the highest nominal doses of the market-size fish trials (see Tables 4-6 and 4-8 above). There appears to be a trend in the data from the market-size fish trials.

A simple regression analysis of the percent reduction in copper (due to filtration alone), against changes in nominal concentration of copper administered, supports the apparent relationship between the two variables. The calculated r^2 value is 0.964 for the following relationship

$$\%\ copper\ reduction = 0 + (0.019)\ x\ (nominal\ copper\ concentration)$$

The values used for this regression, were not the mean percent reduction values represented in Table 4-6 above, but were instead all of the parallel sets of filtered and non-filtered copper measurements collected for each concentration tested (and used to generate the mean values in Table 4-6). A regression plot of these data are represented in the Figure 4-3 below.

Figure 4-3. Simple regression plot of nominal copper concentration against the percent reduction of copper due to filtration of water samples



The same analysis was used to compare the percent copper reduction compared to nominal copper administered in the trials with small tilapia. The same relationship did not exist, as suggested by data presented in Table 4-8 above. A calculated r^2 value for this same regression analysis in small fish was 0.031.

As highlighted in Chapter II Section 1.2.1 and Chapter IV Section 1.2.2 there are numerous abiotic and biotic factors which complex with copper, and in some cases render large

portions of the copper biologically unavailable. The trials reported here were conducted in a manner to minimise exogenous modifying factors; there was no food provided, a minimum of faeces was produced and the tanks were prepared prior to use to remove any attached material. At the outset of each trial there appeared to be very little, if any, particulate material in the water.

As the trial progressed over the 96 h trial period, there appeared to be several concentration and time-related phenomena taking place. A scum was found adhering to the tank walls just above the water-line. In some instances, particularly in the highest test concentrations of copper, there appeared to be a concentration-related increase in blue tint to this scum. At the same time that scum appeared, there was usually a concentration and time-dependant turbidity in the water. When water samples were collected, there also seemed to be an increase in resistance to passing the water sample through the 0.45 μm filter. When all observations were considered, along with the AAS measured, concentration dependent decrease in copper noted in filtered samples, it appeared that some modifying factor was being produced within the tank which was not only proportional to the amount of copper administered, but also was responsible for rendering greater proportions of the copper biologically unavailable.

It appears that mucus was being produced by the fish in response to the copper and that its presence may have modified the toxicity of a given copper concentration. The concept of increased mucus production is consistent with observations published by various researchers. Several authors noted that exposure to heavy metals is associated with either increased mucus production and/or mucus complexation with the heavy metal to which it was being exposed (Carpenter 1930; Westfall 1945; Skidmore and Tovell 1972; Wong, Luk and Choi 1977; Kumar and Pant 1981; Lock, Cruijsen and van Overbeeke 1981; Handy, Eddy and Romain 1989; Handy and Eddy 1990; Pelgrom et al. 1994; Kirk and Lewis 1993; Wilson and Taylor 1993 (cited by Taylor, Beaumont, Butler, Mair & Mujallid, 1996); Eddy and Fraser 1982; Ultsch and Gros 1979).

The authors in at least two of the above noted publications suggest that mucus may in some way influence the toxicity of the metal by simply binding with it. Carpenter (1930) conducted studies on the common shiner (*Notropis cornutus)* where she compared the toxicity of lead nitrate to a standardised fish mass, in a standardised concentration of toxicant, but varied the volume of water. She found that there was a minimum volume of water that produced full toxic action (defined as death) and below the minimum volume, the length of time to death increased, while above it, the length remained the same. The author proposed that the

toxicant increased the mucus production, which in turn was responsible for binding the toxicant. The resultant mucus/toxicant complex was sloughed into the water and thus unavailable biologically. This appeared to be a case where due to the size of the container, the mucus production and binding with the heavy metal could physically decrease the toxicity of the metal administered by reducing the amount available.

Pelgrom et al. (1994) found that the accumulation factor for Cd decreased with increased Cd exposure, but could not explain the reason for such a decrease. Even though they offered metal binding as a possible explanation, they went on to argue that the increased mucus production of fish exposed to heavy metal, as observed by several other authors (Lock et al. 1981; Handy and Eddy 1990, 1991), may not account for the decreased accumulation factor, for there has not yet been demonstrated a concentration-dependent relationship between mucus production and concentration of metal present.

An examination of the data from this series of LC50 trials may offer some explanation for the decreased accumulation factor described by Pelgrom et al. (1994). Although, parallel residue studies were not conducted on the fish exposed in these trials, and therefore the actual accumulation factor can not be assessed relative to mucus production, the results of these

studies do support the notion that there may be a concentration-dependent relationship between heavy metal exposure and mucus production. These data also support the argument that the proportion of copper being complexed with mucus (or something produced within the system) increased at a rate relative to the amount of copper present in the system and could be filtered from the tank water.

This relationship was not apparent with the LC50 trials conducted on the small tilapia. The mucus being produced within the small tilapia tanks did not appear to be produced in substantial quantities nor in relationship to the amount of copper being administered to the tanks holding the fish.

It is possible that the same copper:mucus relationship existed in the small fish tested. However, it could not be detected due to a simple dilution of mucus by the water. The ratio of water volume to total biomass/surface area (and hence, mucus production) was significantly smaller in the market-size fish trial compared to that of the small fish trial. Consequently, the mucus produced by the small fish may have likewise been in direct relationship to the amount of copper exposure, but may have been undetectable due to its dilution with a considerably greater proportion of tank water.

CHAPTER V

SMALL AND MARKET-SIZE TILAPIA BRIDGING EXPERIMENT

1 Introduction

1.1 Objectives

The objective of this portion of the study was to determine if small tilapia could be substituted for market-size tilapia in experiments in which facility limitations would restrict the use of the latter. To meet this objective, two investigations were designed to examine whether small tilapia respond in a manner similar to market-size tilapia when exposed to copper sulphate, i.e., generate bridging information between the two sizes of fish. One investigation was to generate comparative copper toxicity information and the other was to be conducted to provide comparative copper residue information.

The former investigation, comparative toxicity, comprised a portion of the experiments discussed in Chapter IV. This experiment addresses the latter, comparative accumulation. This bridging experiment was designed to expose small and market-size tilapia to copper sulphate at the same concentration for the same length of time and to compare the resulting accumulation of copper.

1.2 Literature Review

There is very little information in the literature which compares copper (or any heavy metal) accumulation rates between large and small fish of the same species. This is the case in spite of there being numerous publications which investigate copper accumulation in several fish species, such as: rainbow trout, *O. mykiss* (Bradley, DuQuesnay and Sprague 1985; Carbonell, Cebrián and Tarazona 1992; Farag, Boese, Woodward and Bergman 1994; Pilgaard, Malte and Jensen 1994), brown bullhead, *Ictalurus nebulosus* (Brungs et al. 1973); coho salmon, *O. kisutch* (Buckley et al. 1982); channel catfish, *I. punctatus* (Hobbs 1996); tilapia, *O. mossambicus* (Pelgrom et al. 1994; Pelgrom, Lamers, Lock, Balm and Wendelaar Bonga 1995; Pelgrom, Lock, Balm and Wendelaar Bonga 1995a; Pelgrom, Lock, Balm and Wendelaar Bonga 1995b); fathead minnow, *Pimephales promelas* (Playle, Dixon and Burnison 1993a; 1993b); European flounder, *Platichthys flesus* (Stagg and Shuttleworth 1982); common carp, *C. carpio* Yamamoto, Ishii and Ikeda 1977; Wong and Kwan 1981) and general copper levels in tissues of numerous species (Harrison 1986).

Anderson and Spear (1980) specifically investigated copper uptake in the gills of pumpkinseed sunfish (*L. gibbosus*) as a function of fish size. Although the fish size did not differ to the extent of the tilapia in this study, the authors found there

was an inverse relationship between size and copper accumulation rate in the gills between fish groups averaging 3.3, 4.3, 5.4 and 6.5 g. The rate of accumulation varied from 0.0082 to 0.0066 μg Cu $(g\ fish)^{-1}h^{-1}$. They offered two possible reasons for their observation. First, they suggested an inverse relationship between gill surface area and fish weight, and second they noted that the oxygen consumption rate of pumpkinseed sunfish, as a function of unit weight, decreases as the fish grow (possibly being an inverse function of weight to gill surface area).

Cross et al. (1973) surveyed two species of marine fish for levels of copper, zinc, iron, manganese and mercury in white muscle. The authors found that while Hg increased in concentration in the white muscle of bluefish (*Pomatomus saltatrix)* with age, there was no similar relationship between weight and copper.

Tong et al. (1974) (cited by Stokes, 1979) sampled lake trout (*Salvelinus namaycush*), 1 to 12 yr of age, from Cayuga Lake, New York, USA. The authors tested for 33 trace metals. Although several metals increased with age and a few decreased, the authors found that the levels of copper were not correlated with age.

McFarlane and Franzin (1980) examined two species of fish from five lakes in Manitoba, Canada. They analysed the liver and gonads for levels of copper, zinc and cadmium. They

found that the concentration of copper increased in the livers of northern pike (*Esox lucius*) in relationship to the size of the fish. This occurred in spite of the fact that the levels of copper contamination in the lakes tested were all decreasing with time, and represented lakes with different levels of contamination.

Julshamn, Andersen, Ringdal and Brenna (1988) examined the effects of dietary copper on the levels of hepatic copper in rainbow trout (*O. mykiss*) over an 18 week period. They observed a positive linear relationship between copper provided in the diet with the level of copper in the liver. They further suggested that the age of the fish may influence the level of hepatic copper.

Felton, Grace and Landolt (1994) surveyed levels of zinc and copper in the whole bodies of coho salmon smolts (*O. kisutch*) from hatchery and wild sources. They analysed hatchery smolts ranging in size from approximately 10 to 30 g and wild smolts from approximately 11 to 46 g. They found the whole body copper levels (gdw = grams dry weight) to average 7.54 gdw (SD = 0.67) and 9.15 gdw (SD = 1.00), respectively. These values were determined to be statistically higher in wild smolts, but with no correlation between copper levels and weight in either group.

McCoy, O'Hara, Bennett, Boyle and Lynn (1995) examined channel catfish (*I. punctatus*) ranging in size from 0.85 to

3.0 kg during four months of one year (January, April, July and October). They assayed the liver and kidney for copper, zinc and cadmium. They found that copper levels in the livers were positively correlated with size of animal in the summer period (July). They suggested that this observation may be linked to historically greater use of copper sulphate as an algicide during the spring and summer, for this correlation was not noted in the other months.

Only Anderson and Spear (1980) directly investigated the levels of accumulated copper relative to size of fish under controlled conditions. The other studies primarily looked at natural levels. The findings in all the noted studies seemed to have little in common, but may well point to the fact that the phenomenon of copper uptake relative to fish size is very species and compound dependent. This information offers very little insight into what the expected relative differential uptake of copper might be in small compared to market-size tilapia.

2 Study-Specific Materials and Methods

2.1 Systems/Procedures

Culture systems used were as described in Chapter II. Fish were acquired, acclimated and held prior to initiation of the experiment as described in Chapter II. All exposure of fish to copper sulphate was by means of the static 50% renewal

system defined in Chapter II. This experiment was conducted for 12 days, although it had originally been planned to extend for 14 days.

Two 150 l experimental tanks were used for this trial. Before this experiment began, the tanks contained fish left-over from previous experiments. In both cases the fish had been non-exposed controls, and hence, were retained for this experiment. The tank water level was reduced to approximately 35 l in volume and the tank walls and bottom were scrubbed with clean cotton floss, as needed. The tanks were flushed with the equivalent of several volumes of tempered (heated to 20°C) new water. The normal standpipes were returned and the tanks were filled to the 150 l level with tempered water. The tanks were allowed to flush for several hours before the experiment began. The fish were not removed from the tanks during cleaning and flushing.

The test concentration of copper was the same for both groups of fish. The nominal concentration for this study was set at 889 ppb copper. This concentration, at the time of this experiment, was the highest concentration tested that had not caused mortalities in the up-and-down range-finding trial on market-size tilapia. This nominal concentration was back-calculated using the best-fit equation generated from nominal compared to actual copper concentrations in previous experiments. The nominal 889 ppb copper was estimated to

provide an actual copper concentration of approximately 750 ppb.

2.2 Fish

Two sizes of fish were tested in this experiment, small tilapia (10 to 30 g) and market-size (350 to 570 g) tilapia, each being consistent with previous studies. Each of the two size groups was contained in a single replicate tank. There were no non-treated control tanks for either group of fish. The market-size tank was stocked with seven fish, while the small fish tank was stocked with 41 fish. The fish had not been fed for 48 h immediately prior to the initiation of the experiment.. At the outset, it was decided that both groups of fish would not be fed during the experiment, consistent with an expected higher copper uptake in starved compared to fed fish. This experiment was scheduled to last for 14 days.

2.3 Sample Collection

2.3.1 Water

Water samples for copper were collected as described previously. A pre-exchange filtered and non-filtered sample was collected at the end of each 24 h exposure period and a post-exchange non-filtered sample was collected at the beginning of each 24 hr exposure period.

Water quality parameters, other than copper, calcium and magnesium, were to be determined at the beginning, periodically in the middle and at the end of the experiment, in particular for pH, temperature and nitrites. Calcium and magnesium samples were collected approximately six weeks prior to this experiment and were collected three weeks after this experiment.

2.3.2 Fish

Fish samples for histological examination were not scheduled for collection during this experiment.

The procedures for analytical sample collection and preparation differed somewhat from standard procedures. The only fish samples scheduled during this experiment were those to be collected on the last day of the experiment. However, the scheduled final-day muscle and livers samples could not be immediately removed from the fish due to other experiments in progress. Therefore, all fish surviving to the end of the experiment were immediately frozen whole (at <0°C) in separate labelled plastic zip-lock bags. Fish found dead during the experiment were saved in the freezer for potential copper analyses, and an estimate was made and recorded as to the approximate time of death.

The muscle and liver samples for copper analyses were removed from the collected fish eight days after the fish had

been placed in the freezer. When the analytical samples were removed, the weight and gender (market-size fish only) of each fish was also determined and recorded. Standard procedures (as described in Chapter II) were followed for all other aspects of fish sample collection.

2.4 Sample Preparation

2.4.1 Liver

All liver samples, from both small and market-size tilapia were prepared for analyses as previously described in Chapter II.

2.4.2 Muscle

All muscle samples from small tilapia were prepared for analyses as previously described in Chapter II.

The procedures for the preparation of the muscle samples from market-size tilapia were modified as follows. Two sets of muscle samples were prepared from each de-scaled fillet (with attached skin), a whole-fillet sample and a fillet-slice sample.

The fillet-slice sample comprised an entire 6 mm slice of muscle (and attached skin). The slice of muscle was taken diagonally from the ventral anterior corner to the dorsal

posterior corner of the fillet (viewed with the skin-side down). Each slice sample weighed approximately 1.0 to 1.5 g.

The whole-fillet sample was an aliquot of the entire fillet, exclusive of the previously removed slice. The fillets weighted approximately 50 to 90 g, thus the mincing/mixing procedures used for the small fish fillets were considered to be an inadequate means of collecting a random muscle sample. The entire remaining fillet was cubed into approximately 1.0 cm pieces. These pieces of fillet, after being weighed together, were blended with a Waring blender. The blending was conducted in a washed, triple-rinsed (with deionized distilled water) 1 l plastic blending container, fitted with stainless steel blades. A weighed quantity of deionized distilled water was added to each blending container with the cubed fillet. The water/fillet mixture was blended for several minutes until a slurry was formed in which the skin portion of the fillet was reduced to sufficiently small pieces (less than 1 mm). An aliquot sample of approximately 1.0 gram was transferred from the slurry to an etched, weighed standard digestion test tube via a new, disposable pipette, from which the conical tip had been removed. Samples were then weighed and wet weights recorded. The remaining procedures for these muscle samples were as described in Chapter II.

2.4.3 Standard Reference Materials (SRM's) and Blanks

The SRM's used for this experiment were DORM-1 and DOLT-1. DORM-1 was described in Chapter II. DOLT-1 is Dogfish (*Squalus acanthias*) Liver SRM for Trace Metals; Marine Analytical Chemistry Standards Program; National Research Council of Canada; Environmental Measurement Science; Institute for Environmental Research and Technology; Ottawa, Ontario, Canada, K1A OR6; Bottle No. 151.

Processing blanks were prepared and used as previously described in Chapter II.

2.5 Sample Analyses

All water and tissue samples for AAS were analysed as per procedures described in Chapter II.

3 Results

3.1 Water Samples

3.1.1 Copper

The water samples collected during the entire trial were usually analysed the day following collection, and at most three days following collection (Friday's samples analysed on the following Monday). The results of the AAS analyses, Table 5-1 below, confirmed a reduction in copper concentration

from the nominal to actual concentrations, but the reduction exceeded that expected (approximately 750 ppb). The overall mean actual copper concentrations measured in the two tanks were nearly identical, however the reduction due to filtration alone was greater in the tank for the market-size tilapia than in that for the small tilapia (see Table 5-1). There was a differences in the percent reduction due to causes other than filtration (last column). However, tests were not conducted to determine the cause for this difference, nor was this difference compared statistically.

Table 5-1. Summary results of water samples collected during bridging experiment.

Fish Size	Nominal Cu ppb	Mean AAS Measured Cu (ppb)* Beginning non-fltr'd	Ending non-fltr'd	Ending filtered	Overall Actual	% Total reduction	Filter % reduction	Other % reduction
mrkt	889	801	696	571	652	28.7	18.0	10.7
sml	889	783	672	601	663	23.2	10.6	12.6

* Beginning = mean of all samples collected at the start of each 24 h sample period; ending = mean of all samples collected at the end of each 24 h sample period; overall = grand mean of all ending and beginning filtered samples (beginning filtered were estimated, see Chapter IV, Section 3.1.2 for procedures)

3.1.2 Calcium and magnesium

The concentrations of calcium and magnesium were not measured during this experiment, but as noted above, had been determined approximately six weeks prior to this experiment. The first calculation (completed six weeks

before) was determined to be within the range of the historic hardness of Baltimore city water at the UMAB laboratory (85±2 ppm as $CaCO_3$). The calcium and magnesium determinations made by AAS were 23.82 and 6.50 ppm, respectively. The hardness calculation based on these calcium and magnesium concentrations (using the formula described in Chapter II, Section 3.1.2) resulted in a value of 86.25 ppm (as $CaCO_3$).

3.1.3 pH, Temperature and Nitrites

Water quality parameters were collected several times during the course of this experiment. Of particular concern was the holding of high number of market-size tilapia for a long period of time and the potential water quality deterioration as a result. A summary of the water quality information collected during this experiment is summarised in Table 5-2 below.

Table 5-2. Summary of water quality data collected during the bridging experiment.

Water Quality Parameter	Experimental Sample Day	Market-size Fish-tank Values	Small-size Fish-tank Values
NO_2-N	1	0 ppb	21.0 ppb
NO_2-N	4	1.2 ppb	2.0 ppb
NO_2-N	5	12.0 ppb	25.0 ppb
NO_2-N	8	>200 ppb	14.0 ppb
pH	1	7.31	7.38
pH	4	7.27	7.36
pH	5	7.29	7.35
pH	8	7.44	7.47
temperature	1	20°C	20°C
temperature	4	20°C	20°C
temperature	5	19°C	20°C
temperature	8	19°C	19°C

The turbidity of the water increased over the experimental period, especially in the tank holding the market-size tilapia, due in large part to the change in feeding regime (see below Section 3.3). DO measurements were not taken during this experiment due to a faulty YSI Oxygen Meter and the unavailability of other means.

3.2 Tissue Samples

3.2.1 Muscle

The AAS analyses were conducted on the muscle of seven fish from each of the two tanks. The fish analysed from the market-size tilapia tank did not all survive to the end of the experiment and consequently four of the seven fish were not

euthanised immediately prior to being frozen, but were already dead.

Irrespective of the time of death in the market-size tilapia, each fillet collected from the market-size tilapia was processed by two methods, the whole fillet procedure and the slice procedure as outlined in Section 2.4.2 above. The following table (Table 5-3) summarises the results of AAS muscle analyses and statistical comparisons for the various categories of muscle analysed.

Table 5-3. Summary of AAS analyses and statistical comparisons from bridging experiment muscle samples.

Line No.	Muscle Type	Fish Size	No. of Samples	Mean Cu (ppm) dry weight	Standard Deviation	Significantly different (at $\alpha = 0.05$)
1	alive	market	6	2.82	1.43	no
2	dead	market	8	6.12	5.38	(F = 3.65; P = 0.11)
3	whole	market	7	7.25	5.16	yes
4	slice	market	7	2.20	0.54	(F = 10.31; P = 0.03)
5	whole	small	7	5.30	1.86	no
6	whole*	market	7	7.25	5.16	(F = 0.88; P = 0.36)

* included the muscle samples from both alive and dead fish

The results of the market-size tilapia samples were first compared by a repeated measures ANOVA (Lines No. 3-4), in which sliced and whole preparation procedures were considered to be repeated measures. The slice procedure is not independent from the whole tissue procedure, they are

merely different procedures applied to the same tissue. Consequently, they can not be tested as totally unrelated levels of a treatment factor, but instead must be compared for within factor differences. This is unlike the samples collected from dead fish or living fish which are quite independent observations, other than being in the same tank and exposed to the same concentration of copper. The mean difference between the copper levels in the muscle from alive fish (Line 1) and those in the muscle from dead fish (Line 2) were not statistically significant ($F_{calc} = 3.65$, $P_{calc} = 0.11$). This comparison could did not statistically reject the hypothesis that there was no difference between whether the fish was alive (sacrificed) or already dead when the samples were collected. At the same time, however, the mean difference between the copper levels in the muscle from whole-fillet (Line 3) and those in the muscle from slice-fillet preparation (Line 4) were statistically significant ($F_{calc} = 10.31$, $P_{calc} = 0.03$). The results of this latter comparison rejects the hypothesis that the whole preparation procedure produces the same results as the slice procedure.

The final comparison was made of whole muscle samples between market-size and small tilapia. The previous repeated measures ANOVA (Lines 1 to 4) allowed for all seven market-size tilapia samples to be compared (irrespective of whether the fish was alive or dead when sampled). However, because only whole samples of the small tilapia were

processed, the only samples which these could be compared to were the whole market-size tilapia samples. Lines 5 and 6 summarise the results of a single factor ANOVA used to compared the AAS measured copper levels in the whole processed muscle samples of market-size and small tilapia.

The mean difference between the copper levels in the whole muscle from small fish (Line 5) and those in the muscle from market-size fish (Line 6) were not statistically significant ($F_{calc} = 0.88$; $P_{calc} = 0.36$). The statistical analyses did not allow for the null hypothesis to be rejected, that is, copper levels measured in the muscle of small tilapia were not different from copper levels measured in the muscle of market-size tilapia. Therefore, it must be assumed, from the data collected within this experiment, that small tilapia when exposed to a nominal concentration of copper equal to 889 ppb did not accumulate copper in the muscle differently from market-size tilapia exposed under the same conditions.

3.2.2 Liver

The AAS analyses were conducted on the livers of seven fish from each of the two tanks. The fish analysed from the market-size tilapia tank did not all survive to the end of the experiment and consequently four of the seven fish were not euthanised immediately prior to being frozen, but were already dead.

Unlike the muscle samples from the same fish, there was only one procedure used for processing the livers of both the market-size tilapia and the small tilapia. These procedures were outlined in Chapter II Section 5.2. The following table (Table 5-4) summarises the results of AAS liver analyses for copper and the statistical comparisons for the various categories of liver analysed.

Table 5-4. Summary of AAS analyses and statistical comparisons of liver samples from the bridging experiment.

Line No.	Liver Type	Fish Size	No. of Samples	Mean Cu (ppm) dry weight	Standard Deviation	Significantly different (at $\alpha = 0.05$)
1	alive	market	3	889	379	no
2	dead	market	4	2230	1236	(F = 3.16; P = 0.14)*
3	alive & dead	market	7	1656	1150	no
4	alive	small	7	1251	760	(F = 0.60; P = 0.45)#

* = significantly different at $\alpha = 0.14$

\# = significantly different at $\alpha = 0.46$

The results of the market-size tilapia samples were first compared by single factor ANOVA (Lines No. 1 and 2), in which copper in the liver samples collected from fish which had died prior to sampling (dead) were compared with those from fish which had been euthanised immediately before sampling (alive). The mean difference between the copper levels in the liver from alive fish (Line 1) and those in the liver from dead fish (Line 2) were not statistically significant

($F_{calc} = 3.16$, $P_{calc} = 0.14$). The statistical analyses did not allow for the rejection of the null hypothesis that the liver copper levels were the same in the two groups of fish. Therefore, it can be assumed that the fish which died prior to sampling had not accumulated copper in their liver differently than had the livers in market-size tilapia which had been euthanised immediately before samples were collected. Consequently, the next statistical procedure utilised liver samples from both dead and alive fish combined.

Lines 3 and 4 summarise the single factor ANOVA used to compare the copper in livers of small tilapia with the copper in livers collected from market-size tilapia (both dead and alive). The mean difference between the copper levels in the liver from market-size fish (Line 3) and those in the liver from small fish (Line 4) were likewise not statistically significant ($F_{calc} = 0.60$; $P_{calc} = 0.45$). In a similar manner, the ANOVA did not allow for the rejection of the null hypothesis that the liver copper levels were the same from the two size groups of fish. Therefore, one must assume from the data collected within this experiment, that small tilapia when exposed to a nominal concentration of copper equal to 889 ppb did not accumulate copper differently in the liver from market-size tilapia exposed under the same conditions.

3.3 Deviations from Protocol

A major change in feeding protocol was initiated on Day 4 of the experiment. As early as Day 3 the small tilapia appeared exceptionally emaciated and dark in colour. These clinical signs were accompanied by chronic low level mortalities. Feeding was begun on Day 4 in an attempt to decrease mortalities. The market-size tilapia were noted to be neither emaciated nor discoloured. However, to maintain some semblance of consistency between the two tanks, the market-size tilapia were also provided food beginning on Day 4. The feeding was begun in spite of concern regarding reduced water quality due to high fish density in the market-size tilapia tank.

The feeding regime was again modified on Day 8. The turbidity in the market-size tilapia tank was extremely high, and the measured nitrite (NO_2-N) and the pH were likewise elevated considerably. It was decided to suspend feeding until the tanks were re-evaluated.

3.4 Gross Observations

The following table (Table 5-5) documents the gross observations noted in the two experimental tanks during the bridging experiment.

Table 5-5. Summary of gross observations made during the bridging experiment.

Fish Group	Day of Observation	Observations and Comments
market-size	3	1 fish was noted to be somewhat darker in colour
market-size	4	Started to provide feed
market-size	8	Several fish noted with small areas of petechial haemorrhaging near the base of all fins, otherwise all fish looked normal; stopped providing feed
market-size	9	1 fish was noted to have lost equilibrium and was often swimming inverted; others with small areas of petechial haemorrhaging
market-size	11	1 mortality removed (previous moribund fish); 1 fish with similar loss of equilibrium; remaining fish with some petechial haemorrhaging, slight overall darkening and an exaggeration of stripes
market-size	12	3 mortalities removed (1 removed at 0900 and 2 at 1530 h); terminated study due to mortalities, remaining fish looked quite good, all frozen including mortalities
small	2	2 mortalities removed
small	3	3 mortalities removed; some fish were observed to be very dark in colour and emaciated
small	4	8 mortalities removed; started to provide feed
small	5	2 mortalities removed
small	6	3 mortalities removed; fish began to lighten in colour and lose their emaciated appearance
small	7	4 mortalities removed
small	8	Some turbidity; 1 fish near death with no equilibrium and dark in colour; stopped providing feed
small	9	2 mortalities removed
small	11	1 mortality removed; remaining somewhat emaciated
small	12	1 mortality removed; 15 fish remaining emaciated; experiment terminated; all fish frozen

4 Discussion

4.1 Gross Observations

It was apparent from the outset of this experiment that especially the small tilapia were being severely stressed by either the copper and/or lack of feed. The darkened coloration and accentuated stripes did not appear to be a function of copper alone. Such colour changes often accompany fish that are compromised in any way, be it via poor nutrition, infectious or non-infectious disease or poor water quality.

The market-size tilapia did not appear to be as severely affected by the tank conditions as did the small tilapia. In part, this may have been purely a function of the normal physiological state of market-size fish compared to that of small fish. During dissection procedures for tissue sampling, the amount of visceral fat deposits in market-size tilapia far exceeded that of small tilapia, there was often no fat in the latter, even in uncompromised fish. This was also the case, in other experiments within this study, even where market-size tilapia had been starved for considerable periods of time.

This experiment was begun before the LC50 experiment range-finding trials (Chapter IV) had been completed. The test concentration selected for the bridging study was based

on preliminary information from the LC50 ranging finding study, which in hindsight, was extremely high, nearly approaching the subsequently determined 96 h LC50 for market-size tilapia. The mortality and toxicity patterns noted in the bridging experiment were not unusual, given the current understanding of toxicity of copper sulphate to small and market-size tilapia.

4.2 Deviations from Protocol

It had been decided after considering the results of the Tank, Feed and Faeces Experiment (Chapter III) that the remaining experiments within the study would be conducted under a non-feeding regime. This approach was taken to maximise copper uptake. The non-feeding regime was adopted for this experiment, but was quickly abandoned due to excessive mortalities in the small tilapia. This was decided to be preferable to further excessive mortalities.

The decision to begin feeding took into account the potentially serious negative consequences. The number of market-size tilapia being held within the test tank was cause for concern relative to water quality. The change to a feeding regime increased the probability of water quality problems, but the alternative of no data was unacceptable. The decision was made to change to a feeding regime for both tanks to maintain consistency between treatments.

The results of the water quality monitoring confirmed the concern regarding feeding during the experiments. The nitrite levels, in spite of 50% daily water renewal, had increased in the market-size tilapia tank to potentially detrimental levels by Day 8. Although the fish did not appear to be overtly stressed at that time, the decision was made to return to the non-feeding regime. In spite of the fact that the appearance of the small tilapia did improve after feeding began, there was no noticeable reduction in their mortality rate. The attempt to reduce the mortality rate in the small tilapia tank may not have been successful, and a return to the non-feeding regime was considered to be not as important as originally thought. Even with the present knowledge of toxicity levels to tilapia, the respective contributions of non-feeding and copper toxicity could not be determined.

Water hardness is important relative to copper toxicity, and presumably accumulation as well. The decision was made not to sample water for hardness for several reasons, many of which were resource related. Historical information collected over many years at the UMAB lab indicated that the hardness of the Baltimore city water delivered to the lab was extremely consistent. Initial (and subsequent) hardness determinations during this study have been consistent with the previous laboratory findings.

4.3 Water

The levels of actual copper in the two test tanks were similar in all respects with possibly one exception, filterable copper. Statistical analyses were not conducted on these data. However, by inspection, there appears to be a difference between the measured levels of copper in the tank with small tilapia compared to that with market-size tilapia. The largest arithmetic difference is noted between the amounts of copper removed by filtration. This may be another example (cf. LC50 Experiment, Chapter IV, Section 3.2) in which the amount of copper filtered out of the water was in proportion to the exposure level and the biomass of fish, and this in turn directly influenced the amount of mucus production. The mucus, as suggested by several researchers (see Chapter IV, Section 4.5 for references), is believed to be in response to the elevated copper, and as suggested by some of the same researchers, copper is bound to the mucus and remains bound after the mucus has sloughed off the fish. When the level of copper reduction due to filtration in this experiment is compared to that in the LC50 trial tank with approximately the same actual concentration (699 ppb) (Chapter IV, Section 4.5), it appears that the percentage of copper reduction is higher in this trial. A possible explanation for this might be: a) this trial contained 7 fish (17% higher) compared to 6 in the LC50 trial, the higher number (and biomass) would be expected to produce more mucus, b) during a portion of this

trial the fish were being fed, which also provided the potential for more copper binding sites, and c) the increased ammonia followed by nitrites within the tank may have been an additional irritant causing increased mucus production (Mallatt 1985).

The other aspects of water quality remained reasonably stable throughout the experiment, with the exception of nitrites and pH in the market-size tilapia tank during the last half of the experiment. Although the fish in the elevated nitrites tank did not appear to be abnormally stressed, the levels of nitrites (>200 ppb) did exceed that generally considered to be a maximum safe level (20 ppb) in water with minimal chloride ions present (Branson 1993). Switching to a non-feeding regime, after discovery of the elevated nitrites, reduced their levels from that point on. No further investigations were made into the potential role the elevated nitrites may have had in this experiment.

4.4 Tissue

The statistical analyses conducted on both the muscle and livers from the experimental fish demonstrated that there were no differences between the measured copper of the small tilapia and the market-size tilapia (muscle or liver). Arithmetic comparisons of the measured values in these two groups of fish may suggest otherwise (see Table 5-6 below).

Table 5-6. Summary of AAS muscle and liver sample analyses from bridging experiment.

Line No.	Fish Size	Tissue	Analytical Procedure	Alive or Dead	No. of Samples	Mean AAS Actual Cu (ppm dry wt)	Standard Deviation
1	market	muscle	whole	dead	4	10.1	5.1
2	market	muscle	whole	alive	3	3.5	1.8
3	market	muscle	whole	all	7	7.2	5.2
4	market	muscle	slice	dead	4	2.2	0.5
5	market	muscle	slice	alive	3	2.2	0.7
6	market	muscle	slice	all	7	2.2	0.5
7	small	muscle	whole	alive	7	5.3	1.9
8	market	liver	whole	dead	4	2230	1236
9	market	liver	whole	alive	3	890	379
10	market	liver	whole	all	7	1656	1151
11	small	liver	whole	alive	7	1252	760

The levels of copper measured in the tissues collected from the dead fish are arithmetically different, in spite of the fact that statistical tests failed to reject the null hypothesis that there were no differences between the means of the different tissue types. With the exception of the slice samples, the samples from the dead fish were 2-3 fold that of the living fish. These arithmetic differences not only suggest that the dead fish had accumulated more copper that the alive fish, but they may have died due to toxic copper accumulation.

The full relationship of copper (or other heavy metal) accumulation and toxicity is not fully understood, but several apparently key components have been studied in depth and reviewed (Klaverkamp, Macdonald, Duncan and Wagemann 1984; Hodson 1988; Hogstrand and Haux 1991; Roesijadi 1992;

Roesijadi and Robinson 1994). The actual sequence of events may be to some degree fish and metal specific.

Copper enters the body either via the gills or the intestine, depending on its form (Roesijadi and Robinson 1994). Published studies (Laurén and McDonald 1985) have indicated that the gills, rather than internal organs, are the initial site for metal toxicity and as such may even reduce its impact on the other organs. The fish in this experiment were not examined histologically for signs of pathology. However, the samples for the dead fish would have been of limited value due to post-mortem changes.

Within the gills, copper can induce metallothionein (MT) synthesis. Metallothioneins are a specific group of metal-binding proteins found in vertebrates and invertebrates within various organ systems (Roesijadi and Robinson 1994). The gill MT's can sequester copper, but they do not appear to facilitate actual copper uptake (Roesijadi 1992). The copper, once it has passed the gills and irrespective of toxicity there, moves throughout the body via the bloodstream. The exact components involved in this movement are not understood, but it is thought that copper may be bound either to serum MT, ceruloplasmin, serum albumin or other plasma proteins or any combination (Roesijadi and Robinson 1994). It is clear, however, that metal-binding proteins, in particular MT's, are responsible for

the regulation and detoxification of copper and other metals (Roesijadi 1992), and that the MT's are induced by metal exposure. It has been speculated (Roesijadi 1992) that MT's detoxify copper (and other metals) by first initially binding with that copper which is free within the cells and secondly by actually dislodging copper bound to other sites and binding to the dislodged copper. It is the latter role that is of greatest importance, for some of the other cellular sites are those associated with toxicity.

The dislodging of copper from sites associated with toxicity apparently is the short-term action which detoxifies potentially toxic levels of copper that have entered the body. A second major point, for which there is disagreement, is the mechanism of toxicity. The sequence proposed by Roesijadi (1992), suggests that there is no differential uptake of copper by the MT's, compared to the other intracellular binding sites. Once the MT's are saturated and the body exceeds its capacity to produce more MT (and remove the copper from the other sites), overt toxicity will occur. An alternate, and earlier, proposal by Brown and Parsons (1978) (cited by Hodson 1988), suggests a similar scenario and referred to it as the spillover hypothesis. In this latter hypothesis, the primary difference appears to be a preferential binding of copper to the MT's, and when saturated, the copper spills over to other sites where toxicity ensues.

The statistical analyses of copper levels in the livers of dead compared to alive fish, did not allow the null hypothesis of equal means to be rejected. The inability to reject the null hypothesis further implies that the differences noted (i.e., 2230 vs. 890 ppb) do not represent samples from two different populations of samples, each with a different mean copper levels. Instead, they are merely two sets of samples from the same population, and due to the small sample size, they do not accurately represent the true population mean.

It is proposed that the two liver copper means (Lines 8 and 9) actually represent samples from two different populations (dead fish that have higher liver copper and died as a result of it, and alive fish which did not). The previous paragraphs would appear to support this premise. Assuming that the fish represented in Lines 1 and 8 died as a result of copper toxicity, then it would be expected to see copper at elevated levels within some organ of their body, and most likely the liver which normally accumulates the highest levels. Additionally, it would be expected to see mortalities in this experiment since the exposure concentration (approximately 650 ppb) was nearly equal to the subsequently determined 96 h LC50 for market-size tilapia (808 ppb, Chapter IV Section 3.2).

4.5 Overall Conclusion

Due to limited facilities, this experiment was conducted to determine if small tilapia could be substituted for market-size tilapia in experiments. In particular this experiment was designed to compare the resulting accumulation of copper when both small and market-size tilapia were exposed under the same conditions.

The data support the claim that the two groups of fish were exposed to copper under the same conditions. The results of tissue analyses by AAS, and the statistical comparison of these results, provide adequate information to claim that small and market-size tilapia do not accumulate copper significantly different from each other.

The results of this experiment, and the comparative examination of 96 h LC50's from small and market-size tilapia documented in Chapter IV, fulfil the requirements of the study plan. The LC50's of the two size groups are within an order of magnitude of each other (approximate 2 to 3 fold difference) and the resulting residues in the muscle of the two size groups are within a standard deviation of each other. Small tilapia were subsequently used in at least one preliminary experiment within this study, but their use was restricted to a situation where facility constraints excluded the use of market-size tilapia.

CHAPTER VI

OPTIMUM CONCENTRATION/DURATION EXPERIMENT

1 Introduction

1.1 Objectives

This experiment was designed to combine several smaller experiments, each with a small but specific goal, into one experiment. The objectives of this experiment were defined as follows.

1) Test three concentrations of copper sulphate for differential uptake of copper. The concentrations to be tested had all been previously estimated (Chapter IV) to be near a maximum non-lethal concentration.

2) Establish how long it will take for the levels of copper in the liver to stabilise during exposure. The liver was used as the tissue most responsive to copper exposure.

3) Extrapolate from the information generated in this experiment with small tilapia to market-size tilapia. This information was subsequently used in the final experiment of this study, conducted with market-size tilapia.

The role of copper toxicity/pathology is not completely understood as discussed in Chapter V. This experiment was initiated to address the general lack of understanding, and in particular that of gill toxicity and pathology. The goal of this entire study is to test copper uptake under worst-case conditions. Worst-case was defined as including the highest non-lethal concentration. Further, it has been defined within the framework of this study that the highest non-lethal concentration will be approximately equal to the extrapolated LC1. Due to the potential impact of gill toxicity/pathology, which could have either increased or decreased copper uptake, it was decided that several concentrations of copper near the LC1 should be tested to confirm the assumption that a higher concentration actually would have given a higher uptake. Accepting the lack of information regarding gill toxicity and pathology, it is conceivable that a higher concentration could have produced a lower copper uptake due to gill toxicity and pathology which may have restricted copper uptake.

1.2 Literature Review

Copper dissolved in the surrounding water, as with many metals or toxicants in general, can cause some degree of pathology in the gills of fish, as well as other organs (Mount 1968; Baker 1969; Eisler and Gardner 1973; Gardner and LaRoche 1973; Péquignot 1975; Gardner 1975; Wong et al.

1977; Schreck and Lorz 1978; Gupta and Rajbanshi 1981; Bodammer 1981, 1985, 1987; Kumar and Pant 1981; Sultan and Khan 1982; Benedetti, Albano and Mola 1989; Enesco, Pisanti and Totaro 1989; Baatrup 1991; Khangarot 1992; Roncero, Durán, Soler, Masot and Gómez 1992; Kirk and Lewis 1993; Saucier and Astic 1995; Sola, Isaia and Masoni 1995). The lesions described from gills, although somewhat varied, usually fell into a few general categories, and typically were not pathognomonic for copper, but were common to other heavy metals and toxicants.

Mallatt (1985) reviewed 135 studies, published up to 1985, that had described gill lesions stemming from either natural or experimental exposures to toxicants. The types of lesions were then statistically compared and tabulated. The author found that, irrespective of toxicant, the most common lesions in lethal (acute and chronic) and sublethal (chronic) studies (in approximate order of proportion reported) were: lifting of the epithelium, epithelial necrosis, lamellar fusion, epithelial hypertrophy, epithelial hyperplasia, loss of epithelial integrity (rupture), excessive mucus secretion, lamellar aneurysms (telangiectasis or clavate lamellae), lamellar congestion, mucous cell proliferation, early chloride cell damage, chloride cell proliferation, leukocyte infiltration, and sinus dilation or constriction. Eight studies were reviewed by Mallatt (1985) that examined the effects of copper. The following table

(Table 6-1) characterises the prevalence of the various lesions noted in the copper studies reviewed by the author.

Table 6-1. Number of studies reporting specific gill lesions as a result of lethal (acute or chronic) or sublethal (chronic) exposure to copper (from Mallatt 1985).

Pathology	Acute Lethal	Chronic Lethal	Chronic Sublethal
lifting of the epithelium	1		1
epithelial necrosis	3	1	
lamellar fusion	2	1	
epithelial hypertrophy	1		
epithelial hyperplasia	3		
loss of epithelial integrity (rupture)	1	2	
excessive mucus secretion	3		
lamellar aneurysms (clavate lamellae)			
lamellar congestion		1	
mucous cell proliferation			
early chloride cell damage			
chloride cell proliferation		1	1
leukocyte infiltration	1		
sinus dilation or constriction.		1	

As a net result of any one or combination of these lesion types, there is a probability of gill dysfunction and an increase or decrease in copper uptake.

2 Study-Specific Materials and Methods

2.1 Systems/Procedures

Culture systems used were as described in Chapter II. Fish were acquired, acclimated and held prior to initiation of the experiment as described in Chapter II. All exposure of fish to copper sulphate was by means of the static 50% renewal system defined in Chapter II. The experiment was designed to be conducted for 24 days, but was terminated after 23 days. Water quality parameters were not measured during this experiment.

Three 150 l experimental tanks were used for this trial. Before this experiment began, the tanks were cleaned and flushed with several volumes of heated (to 20°C) new water and filled to the 150 l level with heated water. The tanks were allowed to flush for one day before the test fish were added to the tanks.

The experiment comprised one tank each for the three copper concentrations. The three nominal concentrations of copper were 208, 273 and 315 ppb. These concentrations represented the estimated nominal concentrations needed to provide the 96 h LC1, LC5 and LC10 for small tilapia. The LC1, LC5 and LC10 were calculated from preliminary data generated in the 96 h LC50 experiment conducted on small

tilapia (Chapter IV, Section 3.3). These data were generated prior to the completion of the 96 h LC50 experiment for small fish was completed. The LC values generated in Chapter IV were actual measured values. Consequently, a best-fit equation for actual concentrations compared to nominal concentrations was determined from previous studies with small tilapia. This equation was used to back-calculate a series of estimated nominal concentrations needed to provide the actual concentrations determined in the 96 h LC50 experiment.

2.2 Fish

The fish tested in this experiment were all small tilapia within the size range previously noted. Thirty six fish were arbitrarily added to each tank and allowed to acclimate for 3 days before the experiment started. The fish had been fed before the experiment began. Prior to the experiment, it was decided that the fish would be fed for its duration. The experience gained during the Bridging Experiment (Chapter V), relating to feeding and mortalities in small fish, and the fact that this experiment was scheduled to extend for 24 days precluded the non-feeding regime.

2.3 Sample Collection

2.3.1 Water

Water samples for copper were sampled consistent with previous procedures. A pre-exchange filtered and non-filtered sample was collected at the end of each 24 h exposure period and a post-exchange non-filtered sample was collected at the beginning of each 24 h exposure period.

Water quality parameters, other than copper, were not collected during this experiment.

2.3.2 Fish

Six fish from each treatment concentration (tank) were collected for analyses on Days 1, 4, 10 and 23 (final day). Only two fish were collected from each tank on Day 0. The latter provided a total of six fish which represented fish which had not been exposed to copper prior to the experiment.

Fish samples for histological examination were collected during this experiment. A standard sample of gills was removed from each fish collected for copper analyses as per standard collection procedures defined in Chapter II, Section 3.2.3.

The procedures for analytical sample collection and preparation were as defined in the standard procedures (Chapter II). The weight of each fish was determined and recorded at the time of the collection.

2.4 Sample Preparation

2.4.1 Histology

All procedure for histological sample preparation were followed as described in Chapter II, Section 6.

2.4.2 AAS

2.4.2.1 Liver and Muscle

All samples for AAS analyses were processed as per the procedures described in Chapter II, Section 5.3.

2.4.2.2 SRM's and Blanks

All samples for AAS analyses were processed with SRM's and processing blanks. The SRM's used for this experiment were DORM-1 and DOLT-1. DORM-1 was described in Chapter II. DOLT-1 is Dogfish (*Squalus acanthias*) Liver SRM for Trace Metals; Marine Analytical Chemistry Standards Program; National Research Council of Canada; Environmental Measurement Science; Institute for Environmental Research

and Technology; Ottawa, Ontario, Canada, K1A OR6; Bottle No. 151.

Processing blanks were prepared and used as previously described in Chapter II.

2.5 Sample Analyses

2.5.1 Histology

Gill samples collected were surveyed for pathology using standard light microscopy. Lesions noted were recorded and if the nature of the lesion allowed for quantification of its prevalence a statistical comparison of observations was performed.

2.5.2 AAS

All water and tissue samples, including SRM's and blanks, for AAS were analysed as per procedures described in Chapter II.

3 Results

3.1 Water

The water samples collected during the entire trial were usually analysed the day following collection, and at most three days following collection (Friday's samples analysed on the following Monday). The results of the AAS analyses, Table

6-2 below, confirmed a reduction in copper concentration from the nominal to actual concentrations, and the estimated reduction was nearly equal to the expected reduction for all three concentrations. The reduction due to filtration alone may have been slightly greater in the two highest concentrations. The differences in reduction from other causes was likewise higher in the two highest concentrations. Tests were not conducted to determine the cause for these difference, nor were these differences compared statistically.

Table 6-2. Summary results of water samples collected during the optimum concentration and duration experiment.

		Mean AAS Measured Cu (ppb)*						
Nominal Cu, ppb	Intended Cu, ppb	Beginning non-fltr'd	Ending non-fltr'd	Ending filtered	Overall Actual	% Total reduction	Filter % reduction	Other % reduction
208	173	195	181	164	175	10.3	9.4	0.9
273	227	260	242	215	231	17.3	11.2	6.1
315	261	282	257	231	249	23.2	10.1	8.0

* Beginning = mean of all samples collected at the start of each 24 h sample period; ending = mean of all samples collected at the end of each 24 h sample period; overall = grand mean of all ending and beginning filtered samples (beginning filtered were estimated, see Chapter IV, Section 3.1.2 for procedures)

3.2 Tissue

3.2.1 Muscle

The AAS analyses were conducted on the muscle of six fish from each of the three tanks on each of the five sample days.

Each fish muscle sample, once processed for analyses, was analysed three consecutive times on the AAS as per Chapter II, Section 5.5.1. Hence, for the entire muscle evaluation there were 270 data points entered into an Excel spreadsheet (3 concentrations x 5 days x 6 fish x 3 AAS measurements).

The data were compared statistically in two steps. The first step comprised a completely nested or hierarchical designed ANOVA where copper levels on given days were compared for a given concentration. This procedure also analysed for differences between fish within a given sample period. All three AAS measurements were considered within the analyses, but were not analysed for differences.

The following table (Table 6-3) summarises the results of three AAS muscle analyses (one for each copper concentration; Lines 1-5, 6-10, and 11-15, respectively). The null hypothesis that copper levels within fish collected on a given sample day are the same and that the copper levels are the same in fish irrespective of the day sampled had to be rejected (at $\alpha = 0.05$). Hence, it can be assumed that the fish did have different copper levels in muscle relative to the number of days exposed and that fish within a given concentration and sampled on one occasion did not accumulate copper at the same level. A multiple comparison of the former is represented in Table 6-3. The last column indicates that at both the 208 and the 315 ppb nominal concentration,

the levels of copper measured on each day were different from levels measured on every other day. The measured copper levels for fish exposed to 273 ppb copper were slightly different from the other two concentrations. The measured copper levels were statistically different for all days sampled except Days 1 and 23, which were not statistically different from each other, but differed from levels on all other days.

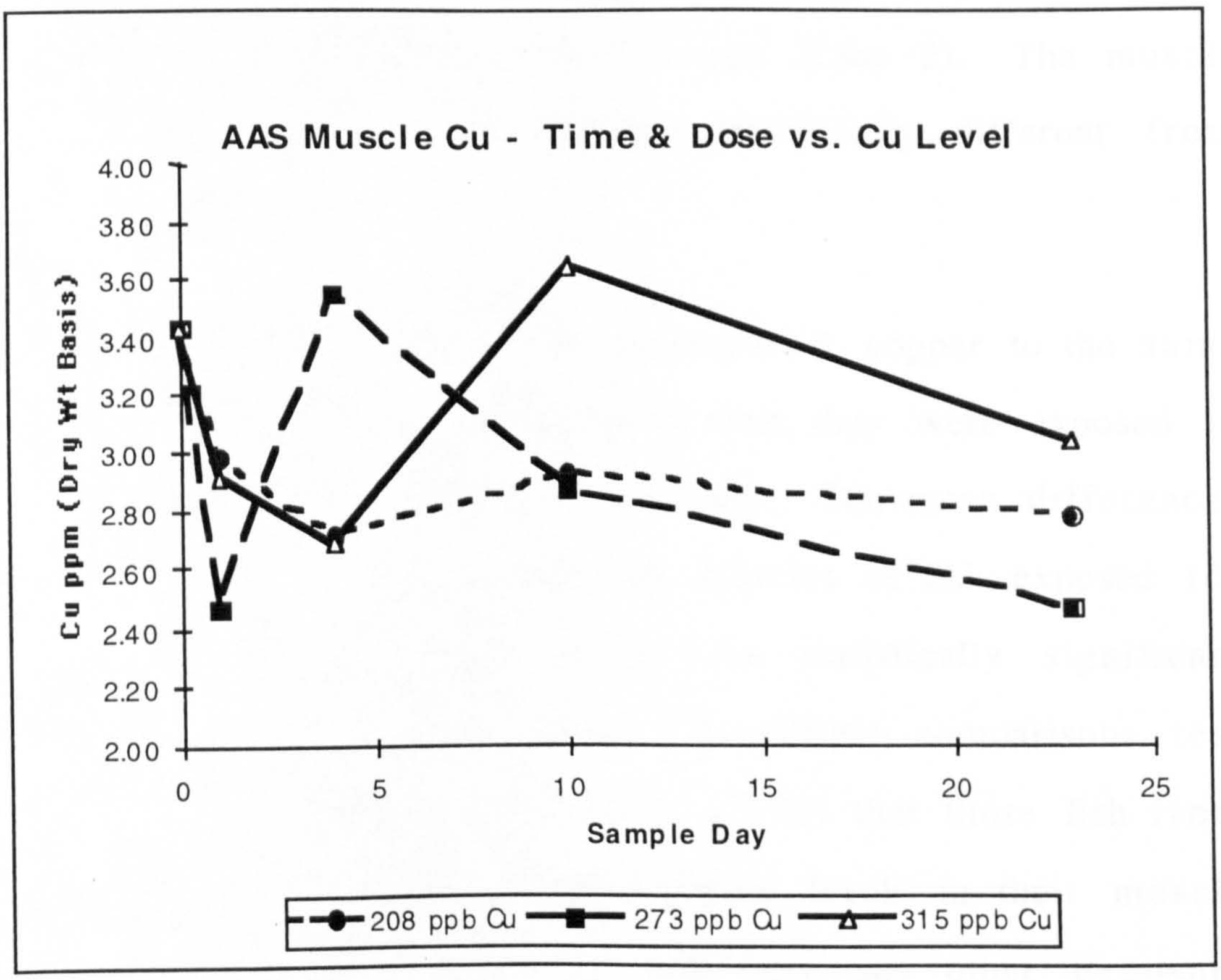
Table 6-3. Summary of AAS analyses and statistical comparisons from optimum concentration and duration experiment muscle samples.

Line No.	Day of Sample	Nominal Cu ppb	No. of Samples	Mean Cu dry weight (ppm)	Standard Deviation	Significance (at $\alpha = 0.05$)
1	0	208	6*	3.43	1.20	a
2	1	208	6	2.97	0.28	b
3	4	208	6	2.70	0.63	c
4	10	208	6	2.92	0.30	d
5	23	208	6	2.77	0.39	e
6	0	273	6*	3.43	1.20	b
7	1	273	6	2.46	0.39	a
8	4	273	6	3.54	1.12	c
9	10	273	6	2.87	0.40	d
10	23	273	6	2.47	0.31	a
11	0	315	6*	3.43	1.20	a
12	1	315	6	2.91	0.67	b
13	4	315	6	2.69	0.31	c
14	10	315	6	3.64	1.43	d
15	23	315	6	3.03	0.24	e

* the two fish samples collected on Day 0 from each tank were combined into six and used to represent the Day 0 level of copper in the fish sampled in all three tanks.

The results of the three sets (concentrations) of analyses can be better visualised in graphic form, especially as it relates to the time-course of copper uptake. Table 6-3 data are plotted below in Figure 6-1. Error bars for each data point (each representing the muscle from the 6 fish sampled) were not included because the large standard deviation between fish. The error bars overlapped to the extent that information could not be obtained from the graph. As an alternative, the standard deviation of mean data are noted in Table 6-3 above.

Figure 6-1. Mean muscle copper levels from the optimum concentration and duration experiment.



The second statistical comparison was made of copper in muscle samples relative to concentration tested and period of exposure (sample period). A two factor ANOVA with nesting of fish within the day of sample was conducted.

The null hypothesis that copper levels in the muscle of fish exposed to three different concentrations of copper were the same was rejected (at $\alpha = 0.05$). The mean differences between the copper levels in the muscles of fish exposed to the three different concentrations (Lines 1 to 3) were statistically significant ($F_{calc} = 4.470$, $P_{calc} = 0.013$). A multiple comparisons test (last column) demonstrated (at $\alpha = 0.05$) that those fish exposed to 315 ppb had higher copper levels in their muscle (Line 3), than did either the fish exposed to 208 (Line 1) or 273 ppb (Line 2). The muscle levels of the latter two were not statistically different from each other.

The null hypothesis that fish accumulated copper to the same extent regardless of the period of time they were exposed to copper was also rejected (at $\alpha = 0.05$). The mean differences between the copper levels in the muscles of fish exposed for various periods (Lines 4 to 8) were statistically significant ($F_{calc} = 18.917$, $P_{calc} = 0.0001$). A multiple comparisons test (last column) demonstrated (at $\alpha = 0.05$) that those fish from the Day 0 samples had higher copper levels in their muscle (Line 4), than did either the fish from any other exposure

period. The muscle samples from Days 4 and 10 did not differ from each other and were higher than those from Days 1 and 23, which likewise did not differ from each other.

The ANOVA also demonstrated that the mean differences between the copper levels measured in the muscle of fish within concentration/day samples groups were statistically significant ($F_{calc} = 18.927$, $P_{calc} = 0.0001$).

Table 6-4. Summary of AAS analyses and statistical comparisons of optimum concentration and duration experiment muscle samples.

Line No.	Factor	Factor Level	No. of Samples	Mean Cu (ppm) dry weight	Standard Deviation	Significance (at $\alpha = 0.05$)
1	concentration	208	30	2.96	0.69	a
2	concentration	273	30	2.95	0.90	a
3	concentration	315	30	3.14	0.95	b
4	sample day	0	18	3.43	1.18	c
5	sample day	1	18	2.78	0.52	a
6	sample day	4	18	2.98	0.85	b
7	sample day	10	18	3.14	0.93	b
8	sample day	23	18	2.75	0.39	a

3.2.2 Liver

The AAS analyses were conducted on the liver of six fish from each of the three tanks on each of the five sample days. Each liver sample, once processed for analysis, was analysed three consecutive times on the AAS as per Chapter II, Section 5.5.1. Hence, for the entire liver evaluation there were 267 data

points (the digest test tube for the liver sample from fish #5, 208 ppb copper, Day 4, broke in the muffle furnace) entered into an Excel spreadsheet (3 concentrations x 5 days x 6 fish x 3 AAS measurements).

The data were compared statistically in two steps. The first step comprised a completely nested or hierarchical designed ANOVA where copper levels on given days were compared for a given concentration. This procedure also analysed for differences between fish within a given sample period. All three AAS measurements were considered within the analyses, but were not analysed for differences.

The following table (Table 6-5) summarises the first-step results of three AAS liver analyses (one for each copper concentration; Lines 1-5, 6-10, and 11-15, respectively).

The null hypothesis that copper levels in the livers of fish are the same irrespective of the day sampled had to be rejected (at $\alpha = 0.05$) for all three of the concentrations tested separately (208 ppb - Lines 1 to 5, 273 ppb - Lines 6 to 10, and 315 ppb - Lines 11 to 15). The mean differences between the copper levels in the livers of fish exposed for various periods were statistically significant ($F_{calc} = 24988.0$, $P_{calc} = 0.0001$; $F_{calc} = 79.514$, $P_{calc} = 0.0001$; $F_{calc} = 178.617$, $P_{calc} = 0.0001$, respectively). Hence, it can be assumed that the fish had different copper levels in their livers relative to the number of days exposed. A multiple comparisons analysis

of the liver concentrations on each sample day is represented in Table 6-5. The last column indicates that at both the 208 and the 273 ppb nominal concentration, the levels of copper measured on each day were different from levels measured on every other day. The measured copper levels for fish exposed to 315 ppb copper were nearly the same as the other two concentrations, with the exception of that measured on Day 0 and Day 1. The copper levels in the livers of these fish were not statistically different from each other and were both statistically less than that of the longer exposures.

The null hypothesis that copper levels within fish collected on a given sample day are the same was rejected (at $\alpha = 0.05$) for all three of the concentrations tested separately. The mean differences between the copper levels in the livers of fish collected on the sample day were statistically significant ($F_{calc} = 1760.775$, $P_{calc} = 0.0001$; $F_{calc} = 23.368$, $P_{calc} = 0.0001$; $F_{calc} = 11.267$, $P_{calc} = 0.0001$, respectively). Hence, it can be assumed that fish within a given concentration/time sample period did not accumulate copper at the same level. No further analyses were conducted on the fish within concentration data.

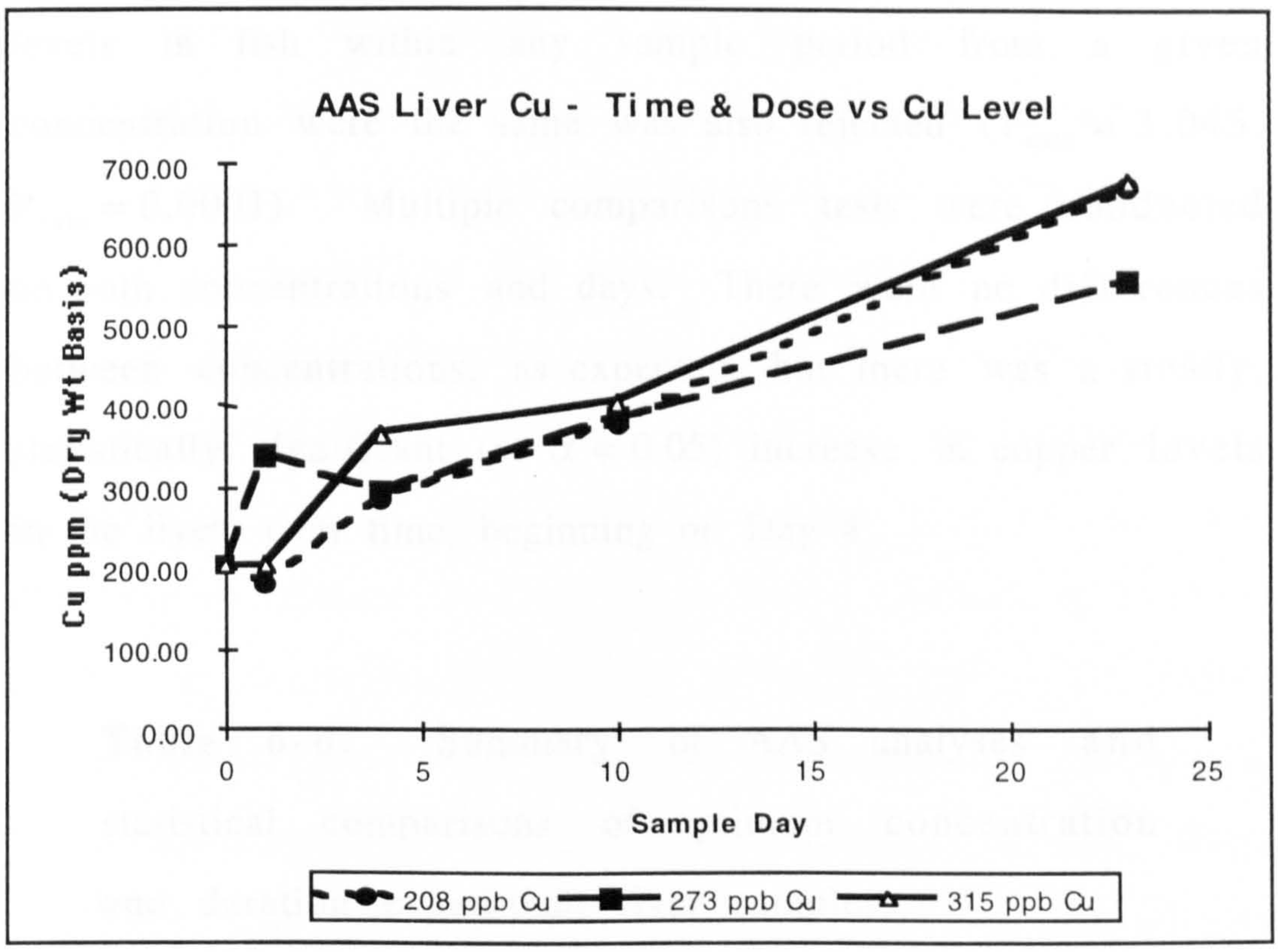
Table 6-5. Summary of AAS analyses and statistical comparisons from optimum concentration and duration experiment liver samples.

Line No.	Day of Sample	Nominal Cu ppb	No. of Samples	Mean Cu dry weight (ppm)	Standard Deviation	Significance (at α = 0.05)
1	0	208	6*	203	124	a
2	1	208	6	178	39	b
3	4	208	5	285	69	c
4	10	208	6	378	124	d
5	23	208	6	672	181	e
6	0	273	6*	203	124	a
7	1	273	6	339	89	b
8	4	273	6	295	126	c
9	10	273	6	386	278	d
10	23	273	6	553	87	e
11	0	315	6*	203	124	a
12	1	315	6	203	121	a
13	4	315	6	364	98	b
14	10	315	6	406	151	c
15	23	315	6	676	68	d

* the two fish samples collected on Day 0 from each tank were combined into six and used to represent the Day 0 level of copper in the fish sampled in all three tanks.

The results of the three sets (concentrations) of analyses can be better visualised in graphic form, especially the uptake of copper over time. Table 6-5 data are plotted below in Figure 6-2. Error bars for each data point (each representing the liver from the 6 fish sampled) were not included because of the large standard deviation between fish. The error bars overlapped to the extent that information could not be obtained from the graph. The standard deviation of mean data are noted in Table 6-5 above.

Figure 6-2. Mean liver copper levels from the optimum concentration and duration experiment.



The second statistical analysis was conducted on the copper levels in liver samples relative to concentration tested and period of exposure (sample day). A two factor ANOVA with nesting of fish within the day of sample was used for this comparison. The results of this statistical analysis permitted (at $\alpha = 0.05$) the acceptance of the null hypothesis that the copper in livers of fish exposed to three different concentrations of copper were the same ($F_{calc} = 1.573$, $P_{calc} = 0.2097$).

However, the null hypotheses that copper levels were the same regardless of how long the fish were exposed was rejected ($F_{calc} = 85.081$, $P_{calc} = 0.0001$), and that copper levels in fish within any sample period from a given concentration were the same was also rejected ($F_{calc} = 3.045$, $P_{calc} = 0.0001$). Multiple comparisons tests were conducted on both concentrations and days. There were no differences between concentrations, as expected, but there was a steady, statistically significant (at $\alpha = 0.05$) increase in copper levels in the livers over time, beginning on Day 4.

Table 6-6. Summary of AAS analyses and statistical comparisons of optimum concentration and duration experiment liver samples.

Line No.	Factor	Factor Level	No. of Samples	Mean Cu (ppm) dry weight	Standard Deviation	Significance (at $\alpha = 0.05$)
1	concentration	208	29	345	217	a
2	concentration	273	30	355	193	a
3	concentration	315	30	370	208	a
4	sample day	0	18	203	122	a
5	sample day	1	18	240	113	a
6	sample day	4	17	317	106	b
7	sample day	10	18	390	193	c
8	sample day	23	18	633	133	d

3.3 Histology

The primary lesion noted, regardless of exposure concentration, was fusion of the gill secondary lamellae. Uniformly, lamellar fusion appeared to be time-related. Several other lesions were also observed. These lesions were most often observed in fish which were exposed for the longer periods and were typically noted after Day 4. These latter lesions included: 1) increased numbers of eosinophilic granulocytes in the most distal secondary lamellae and near the base of primary lamellae, 2) increased numbers of mucous cells, and 3) increased numbers of rodlet cells. The latter three lesion types were not easily quantifiable, and hence, were not compared between concentrations or over time. However, lamellar fusion was quantified and was statistically analysed.

The quantification of lamellar fusion was accomplished by counting the total number of primary lamellae on the gill arch sectioned and the total number of primary lamellae that were fused. To make the decision whether or not to count a primary lamellus and whether or not it was fused, the following definitions were used.

a) A primary lamellus was counted in the calculations if an estimated 50% or more of its secondary lamellae had been sectioned.

b) A primary lamellus was considered to be fused if 50% of its secondary lamellae were fused from 75 to 100% of their length.

The results of the histological examination were initially expressed as the proportion of lamellae fused divided by the total number of lamellae observed. Proportions are not normally distributed, therefore the proportion data generated in this experiment were transformed using Anscombe's transformation, the results of which are considered to be normal in distribution. This particular transformation is applicable in observations where proportions are near either end of the distribution curve, near 0 or 1.000 (Zar 1984), which these data were.

The transformed lamellar fusion data were then compared between days and between concentrations by a two factor ANOVA, with fish nested within days. The null hypothesis that lamellar fusion did not differ on the fish sampled from different concentrations could not be rejected (at $\alpha = 0.05$). The mean differences between the lamellar fusion levels from the fish from different concentrations were not statistically significant ($F_{calc} = 0.042$, $P_{calc} = 0.959$). Hence, it can be assumed that fish exposed to the three tested concentrations did not develop fused lamellae to any greater extent when exposed to the higher concentrations of copper.

The null hypothesis that lamellar fusion did not differ on the fish sampled on various days of the experiment was rejected (at $\alpha = 0.05$). The mean differences between the lamellar fusion levels from the fish from different sample days were statistically significant ($F_{calc} = 19.538$, $P_{calc} = 0.0001$). Hence, it can be assumed that fish developed different degrees of fused lamellae as a function of the time they were part of the experiment. Multiple comparison tests identified those fish which were exposed for 23 days as having significantly higher (at $\alpha = 0.05$) levels of lamellar fusion than fish at any other length of exposure, the latter of which did differ from each other. The following table (Table 6-7) summarises the statistical comparisons.

Table 6-7. Summary of gill lamellar fusion prevalence and statistical comparisons from optimum concentration and duration experiment.

Factor	Factor Level	No. of Samples	Mean % fused lamellae	Standard Deviation	Significance (at $\alpha = 0.05$)
concentration	208 ppb	30	3.1	9.4	a
concentration	273 ppb	30	3.3	9.8	a
concentration	315 ppb	30	3.5	9.4	a
sample day	0	18	0.4	2.2	a
sample day	1	18	0	0	a
sample day	4	18	0	0	a
sample day	10	18	2.4	5.9	a
sample day	23	18	33.3	9.0	b

3.4 Deviations from Protocol

3.4.1 Feeding Regime

It was decided that the fish in this experiment had to be fed to avoid substantial mortalities noted in the small tilapia exposed in the bridging experiment (Chapter V). The level of feed provided began as *ad libitum*, but was reduced considerably by approximately Day 16 due to lack of appetite.

3.4.2 Water Quality Parameters

Water quality measurements, other than copper, were not made due to limited resources.

3.5 Gross Observations

The following table (Table 6-8) documents the gross observations noted in the three experimental tanks during the optimum concentration/duration experiment.

Table 6-8. Summary of gross observations made during the optimum concentration and duration experiment

Day of Observation	Observations and Comments for Fish Exposed to: 208 ppb	273 ppb	315 ppb
0	Feeding started	Feeding started	Feeding started
9	1 mortality removed, all others normal		
10	1 mortality removed, all others normal	1 mortality removed, all others normal	
11	2 mortalities removed, all others normal		
13			1 mortality removed, all others normal
14	1 mortality removed, all others normal		
15		2 mortalities removed, all others normal	1 mortality removed, all others normal
16	Concentration-related lethargy noted, general lack of appetite	Concentration-related lethargy noted, general lack of appetite	1 mortality removed, concentration-related lethargy noted, general lack of appetite
17	Continuation of behaviour as yesterday	1 mortality removed, 1 moribund fish noted, continuation of behaviour as yesterday	Continuation of behaviour as yesterday
18	Appetite even less than before, reduced feed level	1 mortality removed, appetite even less than before, reduced feed level	2 mortalities removed, appetite even less than before, reduced feed level
20	Fish continue to eat very little	2 mortalities removed, fish continue to eat very little	2 mortalities removed, fish continue to eat very little
21		1 mortality removed	
22		1 mortality removed	1 mortality removed
23	Terminated study one day early due to mortality rates, 10 alive today prior to samples.	Terminated study one day early due to mortality rates, 7 alive today prior to samples.	2 mortalities removed, terminated study one day early due to mortality rates, 6 alive today prior to samples

4 Discussion

4.1 Gross Observations

The fish in this experiment did not seem to be as stressed as did those fish in the bridging experiment (Chapter V). The most likely reason for this being that the copper concentrations in this experiment were one third to one half of that to which the small fish in the bridging experiment had been exposed. An additional contributing factor to overall health and, a reduction in stress may have been that the fish in this experiment were fed from the beginning.

The reduction in appetite noted two-thirds of the way through the experiment may have been indicative of copper related stress. Although the concentrations of copper were near the estimated LC1 to LC10, there may have been some toxic effects other than lethality. The LC1 to LC10 estimated concentrations were also based on measurements of lethality after 4 day (96 h) exposure, whereas the fish in this experiment were exposed for 23 days.

4.2 Deviations from Protocol

It was felt that feeding the fish during this experiment would not cause any increased or decreased pathology and/or copper accumulation. Twenty three days without food and with

limited stores of body-fat could, if nothing else, increase mortality rates to a point where insufficient fish would remain alive to complete the gill, muscle and liver samples in the latter portions of the experiment. The chronic mortality rates noted in this experiment were certainly of concern; just enough fish remained in the highest concentration for a complete final sample to be collected, albeit one day early. It would appear that any mortality rate greater than that noted would have seriously compromised the outcome of this experiment.

Previous experiments in this study had provided adequate information to suggest that water quality would not deteriorate to the point of compromising this experiment. The density of fish per tank was low at the outset, and within the first four days was quickly reduced by 38% due to the scheduled removal of samples. All tanks were the same size, at the same ambient temperature, provided sufficient aeration to maintain DO at saturation; it was assumed that temperature, pH, DO and nitrites would remain at acceptable levels.

4.3 Water

The levels of AAS-measured copper in the three test tanks were very close (within 5%) to target levels for desired actual concentrations. The accuracy of the estimation technique has probably improved as more samples have been collected from

which to generate a best-fit equation of nominal concentration compared to actual concentration.

The reduction due to filtration, as previously noted in Section 3.1 above, appeared to increase slightly with an increased copper concentration. Although these observations were not compared statistically, nor were there further tests conducted to determine the cause for these apparent differences, they seem to be consistent with the mucus/copper concentration hypothesis presented in Chapter IV, Section 4.5.

4.4 Tissue

The AAS analysis results of the muscle from the fish exposed to three copper concentrations near the estimated 96 h LC1 represents what appears to be greater copper levels in the muscle of fish exposed to the highest concentration (Table 6-4, Lines 1-3). However, this may be deceiving. Further examination of Table 6-4 and Figure 6-1 indicates that copper levels are statistically the highest on Day 0 (for all concentrations tested) and either continued to decrease after that, or were reduced and stayed at the same reduced level over the course of the experiment. The copper levels in muscle of fish exposed to the highest copper concentrations appear to have merely been reduced less than the other concentrations.

The AAS analysis results of the livers from the same group of fish indicated a somewhat different trend than the parallel muscle samples. The liver copper levels, regardless of which of the three copper concentrations the fish were exposed to, were shown to be statistically the same (Table 6-6, Lines 1 to 3). However, the levels of copper were shown to be statistically higher at each successive sample period (Table 6-6, Lines 4-8), indicating a continuing accumulation of copper throughout the entire experiment. These trends are also represented in Figure 6-2.

The copper tissue AAS results can be represented in a somewhat different manner. Table 6-9 below compares the highest and lowest levels of copper measured for each concentration tested, the percent change from lowest to highest and the general trend in values. In general, irrespective of the concentrations of copper tested, the fish accumulated copper starting from their first exposure to elevated concentrations and continuing throughout the 23 days of the study. The increase in accumulated copper throughout the study was only reflected in the liver, while the levels in muscle may even have decreased.

Table 6-9. Summary of AAS tissue analyses from the optimum concentration and duration experiment.

Tissue	Nominal Cu ppb	Minimum Mean Measured Cu ppm	Maximum Mean Measured Cu ppm	% Change	Trend
muscle	208	2.70	3.43	27	↔ or ↓
muscle	273	2.46	3.54	44	↔ or ↓
muscle	315	2.69	3.64	35	↔ or ↓
liver	208	178	672	278	↑
liver	273	203	553	172	↑
liver	315	203	676	233	↑

Copper is understood to enter the body either via the gills or the intestine, depending on its form (Roesijadi and Robinson 1994). Consequently, it can be assumed that the increasing levels of accumulated liver copper, noted in this experiment, were the result of copper passing from the surrounding water to the blood via the gills. This movement of copper did not appear to be concentration-related, for there were no differences noted between accumulated liver copper, as a function of the concentration of copper to which the fish were exposed.

There appeared, at the same time that copper was accumulating in the liver, to be a simultaneous trend of increasing prevalence of gill lesions. Gill pathology, of the type and to the extent observed in this experiment, did not restrict the movement of copper into the body. This occurred in spite of the fact that increasing copper accumulation and

increasing pathology were taking place simultaneously, and that gills are the primary route of copper movement into the body. It would appear that the severity of gills lesions noted in this experiment were not sufficient, nor of the type, to restrict copper movement across the gills.

These data also address the issue of optimum duration for exposure. Ideally these data could have provided an estimation of the time for copper equilibrium to be reached within the body. However, that does not appear to have been the case, for any of the concentrations tested. Exposure to copper, within the range of tested concentrations, resulted in continuing accumulation in the livers, and a point of equilibrium could only be speculated. The underlying purpose of this experiment, and the preceding experiments, was to assure that during the worst-case experiment the muscle of the exposed fish had been presented with the maximum opportunity to accumulate copper. The data in this experiment, in spite of an inability to predict a equilibrium point, would suggest that extending the duration of exposure beyond 23 day would provide no added opportunity for copper accumulation in edible tissue. The data even suggest that extending it beyond 23 days might even result in a decrease in copper in the edible tissue if the observed trends were to continue.

4.5 Overall Conclusion

The objective of this experiment was to determine if any gill pathology, in response to sublethal concentrations of copper, would hinder the accumulation of copper in fish. More specifically, the objective was to ascertain which of the three concentrations estimated to be near the maximum non-lethal level (LC1, LC5 or LC10) would result in the highest accumulation of copper within the body. The highest body accumulation of copper would presumably maximise the chances for accumulation of copper within the edible tissues.

The interpretation of results of this experiment suggest that exposure of fish to either an LC1, LC5, LC10 or any LC in between would result in the same body accumulation of copper. This accumulation could be verified in the liver, and irrespective of the concentration exposed to (within the LC1 to LC10 range), would provide equal opportunity for copper to accumulate in the edible tissues.

The added knowledge of copper accumulation dynamics gained in this experiment, along with the information from the previous studies, should provide adequate information to conduct the worst-case experiment in market-size tilapia.

CHAPTER VII

WORST-CASE COPPER EXPOSURE AND RESIDUE EXPERIMENT

1 Introduction

1.1 Objectives and Rationale of Study

This experiment was designed to combine all of the information gained up to this point of the study and apply it to the task of meeting one objective. That objective was to

determine if tilapia (*Oreochromis niloticus*) accumulated copper in their muscle and attached skin, exceeding endogenous levels, after being exposed to copper sulphate under worst-case conditions.

The majority of experiments conducted to this point were designed to, and succeeded in, providing information necessary to define the simulated worst-case conditions. The worst-case was defined as:

1) Fish tested were equivalent to those in a commercial production facility and having the least opportunity to deplete any copper acquired as a result of therapeutic treatment with copper sulphate. This would, in essence,

be fish which could theoretically be harvested for sale immediately following a therapeutic treatment with copper sulphate.

2) The concentration of copper sulphate to which the fish were exposed was the highest estimated non-lethal concentration the fish could be exposed to within the exposure period.

3) The period of time to which the fish were exposed was sufficient for copper accumulation to reach a plateau within the edible tissue.

4) The fish were not fed immediately prior to, nor during, the entire experiment to maximise copper uptake.

5) The concentration/duration combination exceeded any such combination which might be applied in commercial fish production for therapeutic purposes, and would not be expected to cause overt toxicity in the production environment.

6) The concentration of copper sulphate exposure applied through the water was verified by an analytically accurate and precise procedure.

7) The levels of copper in the edible tissue were measured with an analytically accurate and precise procedure.

8) The exposure to copper sulphate was not only verified as being in the water, but was confirmed to have actually been taken up by the fish by measurement of tissues which are historically more liable to accumulate copper.

1.2 Literature Review

There are numerous publications in the public literature which address copper levels in tissues of various fish, with many of the publications indicating different rationales for conducting the studies. As a consequence, it is extremely difficult to compare one with the other, for they seldom seem to have been conducted in the same manner, or used the same techniques, or reported results in the same units, and in essence, seldom conformed to any type of experimental conduct or reporting conventions.

In spite of the lack of literature uniformity, attempts have been made by several authors to review the information. Harrison (1986) conducted an extremely comprehensive review of the current literature addressing the impact of increased copper concentrations in freshwater ecosystems. The emphasis of the author's review was on copper accumulation in natural systems. The information in Table 7-1 below has been excerpted from Harrison's (1986) Table 13, and summarises those studies in which copper was measured in the muscle and/or liver.

Table 7-1. Previous studies reporting copper accumulation levels (ppm, dry weight basis) in liver and muscle of fish collected under field conditions (cited by Harrison, 1986).

Fish	Source and Collection Conditions	Cu ppm Liver	Cu ppm Muscle	Literature Source
Salvelinus fontinalis	River, polluted, Germany	71.8	0.397	Abo-Rady 1979
"	River, non-polluted, "	70.5	0.405	"
Salmo trutta	River, USA	44.2		Goettl, Sinley and Davies 1974
"	Below dam, USA	610		Grizzle 1981
Oncorhynchus mykiss	Great Lake, USA	21-28		Lucas, Edgington and Colby 1970
"	River, USA	135		Grizzle 1981
Coregonus lavaretus	Lakes, Czech.	22.8	4.0	Drbal and Budejovice 1976
C. peled	"		4.4	"
Cyprinus carpio	"	16.3	4.9	"
Tinca tinca	"	39.5	5.7	"
Abramis brama	"	13.6	5.3	"
Rutilus rutilus	"	17.9	8.4	"
Carassius caras	"		8.2	"
C. carpio	Hong Kong		15.0	Wong and Kwan 1981
Erimyzon succetta	River, USA	9.3-14.8	1.3-2.5	Leed and Belanger 1981
Scardinius erythrophtalmus	Reservoir, Belgium alive	6.9	0.7	van Hoof and van San 1981
"	Reservoir, Belgium dead	11.0	2.7	"
Puntioplites proctozysron	Polluted river, Thailand		0.77-3.73	Polprasert 1982
Puntius gonionotus	"		0.10-21.59	"
Esox lucius	Lake, Czech.	11.8	5.7	Drbal and Budejovice 1976
E. niger	Pond, USA	27.8	0.29	Wiener and Giesy 1979
Stizostedion luci	Lakes, Czech.	26.0	5.2	Drbal and Budejovice 1976
Perca fluviatilis	"	25.8	7.7	"
P. flavescens	Below dam, USA	9		Grizzle 1981
"	Great Lakes, USA	3		Lucas et al. 1970
Stizostedion vitreum	"	<4		"

Table 7-1. (con't) Previous studies reporting copper accumulation levels (ppm, dry weight basis) in liver and muscle of fish collected under field conditions (cited by Harrison, 1986).

Fish	Source and Collection Conditions	Cu ppm Liver	Cu ppm Muscle	Literature Source
Lepomis macrochirus	River, USA	3.9-182.1	nd*-10.3	Leed and Belanger 1981
"	Pond, USA	4.8	0.62	Wiener and Giesy 1979
L. gulosus	"		0.61	"
Micropterus salmoides	"	24.8	1.1	"
"	River, USA	2.7-44.1	nd-13.6	Leed and Belanger,1981
Pomoxis nigromaculatus	"	10.0-110	1.3-4.7	"
Morone chrysops	Great Lake, USA	≤ 4.0		Lucas et al. 1970
Pangasius siamensis	Polluted river, Thailand	0.23-4.76		Polprasert 1982
P. sutch	"	0.78-4.51		"
P. larnaudii	"	0.41-6.17		"
Cerataglanis scleronema	"	1.35		"
Tachysurus sp.	"	1.68		"
Morulus shrysophekadion	"	0.1-0.4		"
Cyclocheilichthys enoplos	"	nd-16.9		"
Ophiocephalus striatus	"	0.20-0.22		"
O. lucius	"	0.19		"
Kryptopterus bleeker	"	0.13-1.50		"
Notopterus notopterus	"	nd-2.02		"
Cirrhinus jullieni	"	nd		"
Heterogagrus bocourli	"	1.47		"
H. sp.	"	nd-3.16		"
Polynemus paradiscus	"	0.42-12.10		"

Table 7-1. (con't) Previous studies reporting copper accumulation levels (ppm, dry weight basis) in liver and muscle of fish collected under field conditions (cited by Harrison, 1986).

Fish	Source and Collection Conditions	Cu ppm Liver	Cu ppm Muscle	Literature Source
Liza dussumieri	Polluted river, Thailand		nd-11.1	Polprasert 1982
	Mean =	54.5	3.73	
	Range =	nd-610	nd-21.59	
	Standard Deviation =	122.6	3.38	

* nd = not detected, detection limits unknown.

The data in Table 7-1 were extracted directly from Harrison's work and consequently there was no effort to confirm the numbers in the author's Table 13. The mean, range and standard deviation of the reported numbers (many of which are a mean already) listed at the bottom of Table 7-1 was not in Harrison's (1986) original Table 13, but is part of this compilation.

There have also been several publications documenting original research conducted on the experimental exposure of fish to elevated levels of copper. This information is not as extensive as the field studies, but is as varied in its form.

Table 7-2 below represents a compilation of those studies in which there were some similarities in procedures and reporting methods. Again the variability between species is quite large. In a manner similar to that in Table 7-1, a mean and standard deviation of the reported values (many of which are the means of observations) have been provided.

Table 7-2. Previous studies reporting exposure of fish to copper, and analyses for copper accumulation.

		Measured Cu, ppm±SD (range), dry weight*				
Fish	[Cu] (ppb): Tested	Control Liver	Exposed Liver	Control Muscle	Exposed Muscle	Source
Cyprinus carpio	100	48.8± 13.6*	110.4± 20.4*	2.8± 0.8*	2.8± 1.6*	Yamamoto, Ishii and Ikeda 1977
Noemacheilus barbatulus	120 -760	53 (0.2-156)	56-163 (0.2-921)	5.1 (2.6-7.7)	8.2-53 (3-322)	Solbé and Cooper 1976
Rutilus rutilus	80	27.6± 8.0	40.0± 8.0			Segner 1987
Platichthys flesus	15	157.8± 56.4	295.8± 64.8	1.8±0.54	4.89±2.83	Stagg and Shuttleworth 1982
Tinca tinca	18.8 ppm	3.23	12.51-23.45			Roncero, Durán, Soler, Masot and Gómez 1992
Oncorhynchus mykiss#	500	14.3± 3.0	28.4± 4.0	2.0± 0.1	2.0± 0.1	Pilgaard, Malte and Jensen 1994
Salvelinus fontinalis	9.4	239.1± 109.1	238± 89.5	15.3± 6.3	17.0± 6.6	McKim and Benoit 1974
Ictalurus punctatus	900	9.952± 2.100*	85.82± 55.74*	2.128± 0.580*	1.372± 0.536*	Hobbs 1996
Lepomis macrochirus	162	28± 2.0*	1920± 1138*			Benoit 1975
Oncorhynchus kisutch#	70 140	60 60	180 350			Buckley, Roch, McCarter, Rendell and Matheson 1982
Ictalurus nebulosus	104	23 (13-35)	116 (79-181)			Brungs, Leonard and McKim 1973
Mean =		60	290	4.85	9.77	
Standard Deviation =		70	524	5.26	11.73	

* assumed to be wet weight and thus converted to dry weight values based on estimated 75% moisture

\# estimated values from graph

2 Study-Specific Materials and Methods

2.1 Systems/Procedures

2.1.1 Preliminary Fillet-Segment Copper Analyses Experiment

The bridging experiment (Chapter V) required that the fillets of small and market-size tilapia be processed and the accumulated copper compared. The procedures for processing the fillet from the small fish were straightforward, only requiring that the fillet be minced and mixed by hand, with an aliquot removed (usually representing 25 to 50% of the total fillet) for digestion. The process of hand-mincing and mixing a large fillet (50 to 100 g each) and then removing an aliquot was not only difficult and time-consuming, but subject to sampling errors. The procedures were modified for the bridging experiment, whereby the whole fillet was diced into chunks and blended. Although it may have produced a more uniform sample than hand-mincing and mixing would have, the procedure would not have been practical if applied to this experiment. Therefore, a new procedure was developed for this experiment.

In this experiment the fish fillet was visually divided into nine approximately equal segments (a 3 x 3 array), each segment being assigned a number. A diagram was drawn and used as a template for each fillet that was tested. From the

approximate centre of each segment, a 1.25 cm diameter plug of tissue (muscle and skin) was removed by means of a sharpened stainless steel pipe. The plug of muscle/skin was placed into a pre-weighed digestion test tube, and processed in the same manner as other tissue. The results of this study allowed for the choice of three tissue plugs, from specifically defined fillet-segments to be used from each fillet, instead of the homogenisation/aliquot procedure as used previously.

2.1.2 Worst-Case Experiment

Culture systems used were as described in Chapter II. Fish were acquired, acclimated and held prior to initiation of the experiment as described in Chapter II. All exposure of fish to copper sulphate was by means of the static 50% renewal system defined in Chapter II. The experiment was conducted for 9 days.

Eleven 150 l experimental tanks were used for this experiment. Four days before this experiment began, the tanks were scrubbed with clean cotton floss. The market-size fish in this experiment had been arbitrarily assigned to the tanks and had acclimated in the same experimental tanks for 34 days prior to the start of the experiment.

The experiment comprised six replicate tanks for copper exposure and five tanks used for non-exposed controls. The control tanks were divided into two groups, a two-tank set for

Day 0 samples and a three-tank set for Days 3, 6 and 9 samples. The stocking and sampling scheme used for the 11 tanks is represented in Table 7-3, Section 2.3.2. The six, replicated exposure tanks were provided with a nominal concentration of 365 ppb copper. This concentration represented the estimated nominal concentration needed to provide approximately the 96 h LC1 for market-size tilapia. The LC1 was calculated from data generated in the 96 h LC50 experiment conducted on market-size tilapia (Chapter IV, Section 3.2, Table 4-7). The LC values generated in Chapter IV represented actual measured values. Consequently, a best-fit equation for actual concentrations compared to nominal concentrations was calculated using data from previous studies with market-size tilapia. This equation was used to back-calculate an estimated nominal concentration needed to provide the actual concentration determined in the 96 h LC50 experiment.

The 150 l experimental tanks at UMAB were arranged in two banks of six tanks each (an a- and b-bank). The a-bank was located directly above the b-bank of tanks. It was decided that the controls would all be placed in the a-bank tanks to avoid potential contamination of copper-water into control-water.

2.2 Fish

The fish tested in this experiment were all market-size tilapia within the size range previously noted in Chapter II. Either seven or eight fish were arbitrarily added to each tank and allowed to acclimate for 34 days before the experiment started. The fish had been fed up to four days before the experiment began.

Prior to the experiment, it was decided that the fish would not be fed for its duration. The experience gained during previous experiments with market-size tilapia suggested that starving the fish for the four days prior to, and the nine days of, the experiment would not result in excess mortalities and the uptake of copper should be enhanced during starvation.

2.3 Sample Collection

2.3.1 Water

Water samples for copper were collected consistent with procedures from Chapter II. A pre-exchange filtered and non-filtered sample were collected at the end of each 24 h exposure period and a post-exchange non-filtered sample was collected at the beginning of each 24 h exposure period.

Water quality parameters (other than copper) including pH, DO, temperature and nitrites were determined during this experiment on Days 0, 1, 3, 4, 6 and 9.

2.3.2 Fish

Twelve fish were collected for analyses from the six copper-exposed tanks (two fish per tank) on Days 3, 6, and 9.

Twelve fish were collected for analyses from two control tanks (six fish per tank) on Day 0. Six fish were collected for analyses from three control tanks (two fish per tank) on Days 3, 6 and 9. The fish stocking and sampling scheme is represented in Table 7-3 below.

Table 7-3. Stocking and fish sampling scheme for worst-case experiment.

		Action taken on:		Samples Collected on:			
Tank	Copper?	Day -34	Day -4	Day 0	Day 3	Day 6	Day 9
1a	no	initial stocking	changed to 8, stopped feed	6	-	-	-
2a	no	"	"	6	-	-	-
3a	no	"	"	-	2	2	2
4a	no	"	changed to 7, stopped feed	-	2	2	2
5a	no	"	"	-	2	2	2
1b	yes	"	"	-	2	2	2
2b	yes	"	"	-	2	2	2
3b	yes	"	"	-	2	2	2
4b	yes	"	"	-	2	2	2
5b	yes	"	"	-	2	2	2
6b	yes	"	"	-	2	2	2

Fish samples for histological examination were collected during this experiment. A standard sample of gills was removed from each fish collected for copper analyses as per standard collection procedures defined in Chapter II, Section 3.2.3.

The procedures for analytical sample collection were as defined in the standard procedures (Chapter II). The weight of each fish was determined and recorded at the time of the collection.

2.4 Sample Preparation

2.4.1 Histology

All procedure for histological sample preparation were followed as described in Chapter II, Section 6.

2.4.2 AAS

2.4.2.1 Liver

All samples for AAS analyses were processed as per the procedures described in Chapter II, Section 5.2 and 5.3.

2.4.2.2 Muscle

The fish fillets used for the preliminary fillet-segment copper analyses experiment consisted of five collected from the

control fish and five collected from the exposed fish. The ten fish used were all from the Day 9 samples.

In the worst-case experiment, three muscle plug samples were removed from each fillet. The plug samples were removed from fillet Segment 1 (anterior dorsal region), Segment 4 (anterior mid-lateral region) and Segment 6 (posterior mid-lateral region).

In all other respects, samples for AAS analyses were processed as per the procedures described in Chapter II, Section 5.1 and 5.3.

2.4.2.3 SRM's and Blanks

All samples for AAS analyses were processed with SRM's and processing blanks. The SRM's used for this experiment were DORM-1 and DOLT-2, as described in Chapter II Section 5.4.

Processing blanks were prepared and used as previously described in Chapter II.

2.5 Sample Analyses

2.5.1 Histology

Gill samples collected were surveyed for pathology using standard light microscopy. Lesions noted were recorded and, if the nature of the lesion allowed for quantification of its

prevalence, a statistical comparison of observations was performed.

2.5.2 AAS

All water and tissue samples for AAS, including SRM's and blanks, were analysed as per Chapter II procedures.

3 Results

3.1 Water

3.1.1 Copper

The water samples collected during the entire trial were usually analysed the day following collection, and at most three days following collection (Friday's samples analysed on the following Monday). The results of the AAS analyses, Table 7-4 below, confirmed a reduction in copper concentration from the nominal to the actual concentrations. The estimated overall reduction was only slightly lower than expected. The percent reduction due to filtration alone was similar to that noted in Chapter IV, Section 3.2, Table 4-6 for the same nominal concentration of 365 ppb (8.9% in this experiment compared to 5.2% in the LC50 experiment). The control samples, although representing actual AAS measurements, were below the levels of AAS detection (3 times the standard deviation of the processing blanks = 3 x 3 = 9 ppb).

Table 7-4. Summary of results of water samples collected during the worst-case experiment.

Mean AAS Measured Cu (ppb)*								
Nominal Cu ppb	Intended Cu ppb	Beginning non-fltr'd	Ending non-fltr'd	Ending filtered	Overall Actual	% Total reduction	Filter % reduction	Other % reduction
365	322	347	316	288	309	17.0	8.9	8.1
0	0	0	1	1	1	n a	n a	n a

* Beginning = mean of all samples collected at the start of each 24 h sample period; ending = mean of all samples collected at the end of each 24 h sample period; overall = grand mean of all ending and beginning filtered samples (beginning filtered were estimated, see Chapter IV, Section 3.1.2 for procedures).

3.1.2 Temperature, pH, DO, Nitrites and Hardness

Water quality parameters were collected several times during the course of this experiment. Considering the water quality results of previous experiments with market-size tilapia, the holding of several market-size tilapia for a long period of time was of considerable concern, and water quality and fish response was monitored closely. A summary of the water quality information collected during this experiment is summarised in Table 7-5 below.

The values of pH, DO and temperature were all within acceptable ranges. The calculations of hardness based on calcium and magnesium AAS determination were within the historical range of Baltimore city water delivered to the UMAB laboratory. The values of nitrites were, however, well above expected tolerance and toxic levels of market-size tilapia. The

large differential between nitrites measured in copper exposure tanks (1b-5b) and the control tanks (3a-5a) on Day 9 were of considerable interest. The results of the Day 9 nitrite values suggested that the presence of copper may have been associated with the lack of nitrite formation.

Table 7-5. Summary of water quality data collected during the worst-case experiment.

Sample Day	Tank#, Cu (ppb)	pH	DO (ppm)	NO_2-N (ppm)	Hardness (ppm)	Temp (°C)
0	2a, 0	7.28	7.8	530@	83.71	20.5
0	4a, 0	7.22	6.9	358@	85.18	20.0
0	5b, 365	7.26	7.4	85	86.14	20.0
1	2a, 0	7.44	8.1	66	-	20.0
1	4a, 0	7.23	7.1	240@	-	20.5
1	5b, 365	7.30	7.3	604@	-	20.0
3	4a, 0	7.23	7.2	475@	-	20.0
3	5b, 365	7.45	7.8	5	-	20.0
6	4a, 0	7.10	8.0	810@	-	19.0
6	5b, 365	7.35	8.3	5	-	19.5
9	3a, 0	7.37	10.0	190@	-	20.0
9	4a, 0	7.34	9.6	625@	-	20.5
9	5a, 0	7.37	9.8	510@	-	20.0
9	1b, 365	7.50	10.0	25	-	20.5
9	2b, 365	7.56	10.4	15	-	20.0
9	3b, 365	7.60	10.8	12	-	20.0
9	4b, 365	7.49	9.4	7	-	20.0
9	5b, 365	7.55	9.9	5	-	20.5
9	6b, 365	7.51	10.0	7	-	20.0
9*	3a, 365*	7.37	10.0	200@	-	20.0
9*	4a, 365*	7.34	9.6	625@	-	20.5
9*	5a, 365*	7.37	9.8	515@	-	20.0

@ All nitrite calculated values were derived from a graph of absorbency plotted against ppb-nitrites, using freshly-made 10 ppb and 100 ppb nitrite standards. The graph extends to approximately 180 ppb. These values exceeded the graph, and thus were diluted 1:4 and re-read and then re-calculated (X5).

* The Day 9 nitrite values for Tanks 1b-6b were extremely low compared to values from Tanks 3a-5a. Assuming that the presence of copper was the cause, an amount of copper was added to additional samples from Tanks 3a-5a equivalent to that which was in the water in Tanks 1b-5b, and allowed to incubate for 5 min. Following incubation, the water was processed and absorbencies read.

3.2 Tissue

3.2.1 Muscle

3.2.1.1 Preliminary Fillet-Segment Copper Analyses Experiment

The AAS analyses were conducted on the nine muscle segment plugs sampled from each of five control fish fillets and five exposed fish fillets, all of which were collected on Day 9 of the experiment. Each of the plug samples was measured six times by the AAS. Previous experiments within this study have demonstrated that the variability between the six AAS measurements is negligible, and therefore the AAS instrument-generated mean measurements were used for all statistical analyses.

The data were compared statistically by a completely nested or hierarchical designed ANOVA, where copper levels were compared relative to their dependence on where in the fillet they were sampled from (fillet-segment number) and whether the fish had been exposed to copper or not. This procedure also analysed (nested factor) for differences between fish within a treatment (copper-exposed or control).

The following table (Table 7-6) summarises the results of the AAS muscle analyses and statistical analyses for the fillet-segments analysed as noted in the previous paragraph.

Table 7-6. Summary of AAS analyses and statistical comparisons from fillet-segment preliminary experiment.

Line No.	Day of Sample	Segment No.	Nominal Cu ppb	No. of Samples*	Mean Cu dry weight (ppm)	Standard Deviation	Significance (at $\alpha = 0.05$)
1	9	all	0	45	1.99	0.92	a
2	9	all	365	45	2.05	0.82	a
3	9	1	0 & 365	10	1.57	1.22	a b
4	9	2	0 & 365	10	1.52	0.32	a b
5	9	3	0 & 365	10	1.82	0.29	b c
6	9	4	0 & 365	10	2.04	0.30	c
7	9	5	0 & 365	10	2.65	0.41	d
8	9	6	0 & 365	10	3.78	0.43	e
9	9	7	0 & 365	10	1.70	0.31	a b c
10	9	8	0 & 365	10	1.39	0.29	a
11	9	9	0 & 365	10	1.70	0.18	a b c

* each sample represents an instrument-generated mean of six AAS measurements per sample

The mean difference between the copper levels in the muscle of control fish (Line 1) and those in the muscle of exposed fish (Line 2) were not statistically significant ($F_{calc} = 0.381$, $P_{calc} = 0.5390$). The null hypothesis that the copper levels in the muscle are the same, irrespective of the fish having been exposed to copper or not (at $\alpha = 0.05$), could not be rejected. Hence, it must be assumed that the muscle of the fish did not have different copper levels relative to their exposure to copper (with all fillet-segments factored into the analyses).

However, the same ANOVA demonstrated that the mean difference between the copper levels in the fillet-segments (Lines 3 to 11) were statistically significant ($F_{calc} = 30.621$,

$P_{calc} = 0.0001$). The results of a multiple comparisons test (at $\alpha = 0.05$) of the fillet-segments is also represented in Table 7-6. The last column indicates that Segment 6 (posterior mid-lateral) and Segment 5 (central mid-lateral) were statistically different from each other and statistically higher than any other segment. It also indicates that Segment 4 (anterior mid-lateral) was statistically higher than either Segment 2 (central dorsal) or Segment 8 (central ventral). The remaining segments were statistical the same as indicated by the same letter in the last column.

The results of this experiment lead to the choice of three fillet-segments to be used exclusively in all the remaining fillet sample analyses that were part of the worst-case experiment. It was decided to use Segments 1, 4 and 6, for they represented the approximate range of copper that might be encountered in the fillet, and one of the segments may be useful as a representative of the entire fillet (Segment 4). The segments, in addition, had the following characteristics.

1) Segment 1 - a segment with one of the lowest copper content, a very high proportion of edible muscle to skin (thick muscle) and it contained only white muscle.

2) Segment 4 - a segment with medium copper content (the closest to the grand mean of all segments, which was 2.01 ppm), a high proportion of edible muscle to

skin, but also with a significant portion of red muscle (plug was centred on the red muscle region).

3) Segment 6 - a segment with the highest copper content of all tested, a low proportion of edible tissue to skin (thin muscle) and red muscle being a high percentage of the total muscle.

The same ANOVA demonstrated that the mean difference between fish within their respective concentrations (control or exposed) were statistically significant ($F_{calc} = 3.464$, $P_{calc} = 0.0022$). These latter data were not analysed further.

3.2.1.2 Worst-Case Experiment

The AAS analyses were conducted on the muscle of 2 fish from each of 9 tanks on each of the last three sample days, plus six fish from each of two tanks on Day 0, for a total of 66 fillet samples. Three plug samples were processed from each fish fillet muscle sample, and these in turn were analysed six consecutive times on the AAS as per Chapter II, Section 5.5.1. Hence, for the entire muscle evaluation there were 1188 data points entered into the computer spreadsheet (66 fish fillets x 3 plugs x 6 AAS measurements = 1188).

The data were compared statistically by a three factor, full interaction ANOVA. The three factors being: 1) copper exposure or control, 2) exposure period and 3) fillet-segment.

The factors of fish within tanks and tanks within treatment (copper exposure or control) were also simultaneously tested as nested factors. The results of the ANOVA are summarised in Table 7-7 below.

The mean difference between the copper levels in the muscle of control fish (Line 1) and those in the muscle of exposed fish (Line 2) were not statistically significant ($F_{calc} = 0.177$, $P_{calc} = 0.675$). The null hypothesis that the copper levels in the muscle are the same irrespective of the fish having been exposed to copper or not (at $\alpha = 0.05$) can not be rejected. Hence, it must be assumed that the muscle of the fish exposed to the approximate LC1, or the highest estimated non-lethal concentration of 365 ppb copper, did not accumulate copper above endogenous levels, the latter represented by the levels of copper in the control samples.

The same ANOVA demonstrated that the mean difference between the copper levels in the muscle of fish sampled on the various days of the experiment (Lines 3-6) were statistically significant ($F_{calc} = 6.821$, $P_{calc} = 0.0002$). A multiple comparisons test (at $\alpha = 0.05$) of the exposure days is also represented in Table 7-7. The last column indicates that the copper in the muscle samples collected on Days 3, 6 and 9 were all statistically greater than those collected on Day 0 (at $\alpha = 0.05$). At the same time, there were statistical similarities in these data; the copper in muscles of Days 3 and 6 samples

were not different, nor were there any differences between the copper in the muscles of Day 6 and Day 9 samples.

The same ANOVA demonstrated, and not surprisingly after the results of the preliminary fillet-segment experiment, that the mean difference between the copper levels in the fillet-segments (Lines 7 to 9) were statistically significant ($F_{calc} = 650.315$, $P_{calc} = 0.0001$). A multiple comparisons test of the fillet-segments (at $\alpha = 0.05$) is also represented in Table 7-7. The last column indicates that Segment 6 (posterior mid-lateral), Segment 4 (anterior mid-lateral), and Segment 1 (anterior dorsal) were statistically different from each other.

The latter nested ANOVA also demonstrated that the mean differences between fish within their respective tanks were not statistically significant ($F_{calc} = 0.444$, $P_{calc} = 0.9577$), nor were the mean differences between tanks within concentrations (exposed or control) ($F_{calc} = 1.745$, P_{calc}-=-0.1602).

Table 7-7. Summary of AAS analyses and statistical comparisons of muscle samples from the worst-case experiment.

Line No.	Factor	No. of Samples*	Mean Cu dry weight (ppm)	Standard Deviation	Significance (at α = 0.05)
1	0 ppb control	90	2.14	1.11	a
2	365 ppb exposed	108	2.31	1.16	a
3	Day 0	36	1.97	1.00	a
4	Day 3	54	2.18	1.14	b
5	Day 6	54	2.29	1.17	b c
6	Day 9	54	2.40	1.18	c
7	Segment 1	66	1.10	0.52	a
8	Segment 4	66	1.96	0.24	b
9	Segment 6	66	3.63	0.48	c

* each sample represents an instrument-generated mean of six AAS measurements per sample

3.2.2 Liver

The AAS analyses were conducted on the livers of two fish from each of the nine tanks on each of the last three sample days, plus six fish from each of two tanks on Day 0, for a total of 66 liver samples. Each liver sample was analysed six consecutive times on the AAS as per Chapter II, Section 5.5.1. Hence, for the entire liver evaluation there were 396 data points entered into the computer spreadsheet (66 fish livers x 6 AAS measurements = 396).

The data were compared statistically by a two factor, full interaction ANOVA. The two factors being: 1) copper exposure or control and 2) exposure period. The factors of fish within

tanks and tanks within treatment (copper exposure or control) were also simultaneously tested as nested factors. The results of the ANOVA are summarised in Table 7-8 below.

Table 7-8. Summary of AAS analyses and statistical comparisons of liver samples from the worst-case experiment.

Line No.	Factor	No. of Samples*	Mean Cu dry weight (ppm)	Standard Deviation	Significance (at $\alpha = 0.05$)
1	0 ppb control	30	425	178	a
2	365 ppb exposed	48	464	194	a
3	Day 0	24	375	148	a
4	Day 3	18	512	223	a
5	Day 6	18	495	163	a
6	Day 9	18	436	199	a

* each sample represents an instrument-generated mean of six AAS measurements per sample

The mean difference between the copper levels in the livers of control fish (Line 1) and those in the livers of exposed fish (Line 2) were not statistically significant ($F_{calc} = 0.574$, $P_{calc} = 0.462$). The null hypothesis that the copper levels in the livers are the same, irrespective of the fish having been exposed to copper or not (at $\alpha = 0.05$), could not be rejected. Hence, it must be assumed that the livers of the fish exposed to the approximate LC1, or the highest estimated non-lethal concentration of 365 ppb copper, did not accumulate copper above endogenous levels, the latter represented by the levels of copper in the control samples.

The same ANOVA likewise demonstrated that the mean difference between the copper levels in the livers of fish sampled on the various days of the experiment (Lines 3-6) were not statistically significant ($F_{calc} = 0.569$, $P_{calc} = 0.645$). A multiple comparisons test (at $\alpha = 0.05$) of the exposure days is also represented in Table 7-8, although it was not necessary. The last column indicates that the copper in the liver samples collected on all days did not differ from each other (at $\alpha = 0.05$). The mean differences between the copper levels in the livers of fish sampled within tanks were not statistically significant ($F_{calc} = 2.701$, $P_{calc} = 0.105$), nor were the mean differences between the copper levels in the livers of fish in tanks sampled within treatments (exposed or controls) statistically significant ($F_{calc} = 0.765$, $P_{calc} = 0.749$).

3.3 Histology

A gill arch was collected and fixed in 10% NBF from each of the 66 fish sampled during this experiment. Due to limited resources, not all of the gill arches were actually sectioned and stained. All copper exposed fish on Days 3, 6 and 9 were processed, while only 3 of 12 control fish from Day 0 were processed and only 3 of 6 control fish from each of Days 3, 6 and 9 were processed.

Several types of pathology were observed in the samples examined, nearly all of which were also noted in the optimum concentration and duration experiment, Chapter VI. The

following were observed in this experiment: secondary lamellar fusion, mucous cell hypertrophy, epithelial cell hyperplasia, distal extension of the basal interlamellar epithelium ("creeping epithelium"), increased numbers of eosinophilic granulocytes (EGC's), and increased numbers of rodlet cells. The results of the histological examinations and the statistical analyses conducted are summarised in Tables 7-9 and 7-10 below.

An attempt was made to expand on the system of analyses, described in Chapter VI Section 3.3, for lamellar fusion pathology. However, the attempt failed and the data were not convertible to allow the same type of comparisons made in Chapter VI. An attempt to quantify one other lesion noted during this experiment was successful.

One lesion type was quantified and compared statistically, the number of rodlet cells within a 40x field of view on a compound light microscope (Table 7-9). A two factor ANOVA test for differences between concentration (exposed or control) and days of exposure were analysed against the means of rodlet cell counts.

Table 7-9. Summary of rodlet cell prevalence and statistical comparisons from the worst-case experiment.

Line No.	Factor	No. of Samples	Mean Number of Rodlet Cells per 40x field	Standard Deviation	Significance (at $\alpha = 0.05$)	
1	control	12	19.3	12.2	a	
2	exposed	36	23.2	13.5	a	
3	Day 0	3	5.7	3.5	a	
4	Day 3	15	20.5	12.6	a	b
5	Day 6	15	26.1	13.6		b
6	Day 9	15	23.4	12.4		b

The ANOVA demonstrated that the mean difference between the rodlet cell counts in the gills of fish sampled from control fish (Line 1) and fish exposed to copper (Line 2) was not statistically significant ($F_{calc} = 0.802$, $P_{calc} = 0.3802$). However, the ANOVA did demonstrate that the mean differences between the rodlet cell counts in the gills of fish sampled over various days of the experiment (Lines 3 to 6) were statistically significant ($F_{calc} = 3.153$, $P_{calc} = 0.0452$). A multiple comparisons test (at $\alpha = 0.05$) of the exposure days is also represented in Table 7-9. The last column indicates that the rodlet cells in the gills of fish collected on Days 6 and 9 were both statistically greater than those collected on Day 0, but did not differ from each other (at $\alpha = 0.05$) or from those collected on Day 3. At the same time, the rodlet cells in fish collected on Days 0 and 3 were not different (at $\alpha = 0.05$).

Four different categories of pathology were graded on nominal scales, which allowed for statistical comparison by means of contingency table analysis (Table 7-10 below). The four lesion types graded were:

a) epithelial cell hyperplasia, both the presence of (yes or no) and the extent of hyperplastic lesions (none, diffuse, multifocal, or focal),

b) the presence of creeping epithelium (yes or no),

c) the extent of EGC's (grade 0 = none, grade 1 = approximately 25% or less of lamellar region with EGC's, grade 2 = approximately 25% to 50% of lamellar region with EGC's, grade 3 = approximately 50% to 75% of lamellar region with EGC's or grade 4 = approximately 75% to 100% of lamellar region with EGC's,) and

d) the presence of mucous cell hypertrophy (yes or no).

The results of histological and statistical examination of these latter lesion types are represented in Table 7-10 below.

Table 7-10. Statistical comparisons of the presence and/or extent of other gill lesions from the worst-case experiment.

	Statistically Significant Association (at $\alpha = 0.05$) Between				
	Extent of:		Presence of:		
Factor	Eosinophilic Granulocytes	Epithelial Hyperplasia	Epithelial Hyperplasia	"Creeping" Epithelium	Mucous Cell Hypertrophy
Day of Experiment	no $\chi^2_{CALC} = 13.68$; $\chi^2_{12} = 21.03$; $P_{CALC} = 0.32$	no $\chi^2_{CALC} = 15.46$; $\chi^2_{9} = 16.92$; $P_{CALC} = 0.08$	no $\chi^2_{CALC} = 0.74$; $\chi^2_{3} = 7.81$; $P_{CALC} = 0.87$	yes $\chi^2_{CALC} = 20.81$; $\chi^2_{3} = 7.81$; $P_{CALC} = 0.0001$	no $\chi^2_{CALC} = 6.89$; $\chi^2_{3} = 7.81$; $P_{CALC} = 0.08$
Exposed or Control	no $\chi^2_{CALC} = 0.52$; $\chi^2_{4} = 9.49$; $P_{CALC} = 0.97$	no $\chi^2_{CALC} = 5.82$; $\chi^2_{3} = 7.81$; $P_{CALC} = 0.12$	no $\chi^2_{CALC} = 0.99$; $\chi^2_{1} = 3.84$; $P_{CALC} = 0.32$	no $\chi^2_{CALC} = 0.04$; $\chi^2_{1} = 3.84$; $P_{CALC} = 0.83$	no $\chi^2_{CALC} = 0.12$; $\chi^2_{1} = 3.84$; $P_{CALC} = 0.73$

The contingency table analyses demonstrated that there are no statistically significant associations (at χ^2_{CALC} and probability values listed in Table 7-10) between: 1) the extent of EGC's and either the day of the experiment the fish were collected or whether or not the fish were exposed to copper, 2) the presence of epithelial hyperplasia and either the day of the experiment the fish were collected or whether or not the fish were exposed to copper, 3) the extent of epithelial hyperplasia and either the day of the experiment the fish were collected or whether or not the fish were exposed to copper, 4) the presence of mucous cell hypertrophy and either the day of the experiment the fish were collected or whether or not the fish were exposed to copper, and 5) the presence of creeping epithelium and whether or not the fish were exposed to copper. There was, however, a statistically significant

association between the presence of creeping epithelium and the day of the experiment the fish were collected (at χ^2_{CALC} = 20.81, χ^2_3 = 7.81, P_{CALC} = 0.0001).

3.4 Deviations from Protocol

The only deviation from protocol occurred on Day 2 of the experiment. The first set of water samples of the experiment (Day 1 beginning-water-samples), collected 15 to 30 min after the initial concentration of copper sulphate, were exceptionally variable and low. It was felt that the only possible reason was a growth of algae or accumulation of organic debris on the tank sides or bottom, and that this had occurred in the interim from when the tanks were cleaned with cotton floss and the start of the experiment (four days). During this period there was no feeding.

Therefore, it was decided on Day 2 to clean each tank again with floss and completely flush and replace 100% of the water and copper. The analyses of the Day 1 ending-water-samples (which were not analysed until after the 100% exchange was accomplished) verified that there was a large amount of copper loss during the preceding 24 h period; the overall mean actual copper concentrations (for the 6 treated tanks) was 159 ppb. The remaining water samples appeared to be within reason.

3.5 Gross Observations

The fish, under these experimental conditions, appeared quite normal. There were no signs of gross pathology as there were in previous experiments; the petechial haemorrhaging normally observed with the higher concentrations of copper were not observed in this experiment. This lack of gross pathology or any signs of stress was striking, given the exceptionally high levels of nitrites observed.

There was one mortality observed for the 80 fish stocked in the 11 tanks (80 fish included one or two extra fish per tank to make up for possible mortalities during the experiment). This fish died on Day 9 in one of the 365 ppb copper tanks. Although it was not possible to confirm the cause of death, gross lesions on both sides of the fish indicated mechanical abrasion as the result of courting activity; copper toxicity was not suspected. At the time the fish died, there were only 3 fish remaining in the tank, and the other two fish were males. The gender of the dead fish was not determined.

4 Discussion

4.1 Deviations from Protocol

It was felt that that the extremely low and highly variable copper concentration in the water during the first 24 h of the experiment did not impact significantly on the outcome of the

experiment. Table 7-11 below chronicles the levels of copper in the water of the six tanks, in part demonstrating the potential impact of the presumed unknown material in the tanks at the outset of the experiment.

Table 7-11. Summary of mean copper concentrations (six tank means) in the water of the worst-case experiment.

Day of Experiment	Daily Mean Cu ppb	Difference between Start & End Cu ppb	Cumulative Mean of the Difference between Start & End Cu ppb	Standard Deviation of the Cumulative Mean of the Difference between Start & End Cu ppb
1	228	138	-	-
2	318	57	98	57
3	306	50	82	49
4	310	21	67	50
5	313	21	57	48
6	320	23	52	45
7	328	19	47	43
8	329	16	43	41
9	327	35	42	39

If one were to only look at the total (for all nine days) mean difference between the start concentration and end concentration of each 24 h period (column 4) and its standard deviation (column 5), it would appear that there was a very large change in concentration from the time copper was added until it was measured again at the end of all 24 h periods.

However, if one were to look instead at the actual difference between start and end measurements on each day (column 3),

a different conclusion might be drawn. Although the difference was very large for the first three days, it was reduced significantly for the remainder of the experiment. The same conclusion might be made by examining the daily mean concentrations (column 2). Here again, the values changed rapidly from a low value to a considerably higher value within the first two days, and from there on remained within 12 ppb of the expected actual value of 322 ppb.

It would appear that the copper variation at the outset of the experiment should not have affected the fish significantly. However, if any fish were affected, the chances would have been greatest for those fish exposed for only three days.

An examination of the copper accumulation level data from muscle and liver would support the argument that the copper water concentrations had no impact on the accumulation of copper. In spite of the fact that there was differences in copper accumulation over time, these differences were noted in both fish exposed to copper and the non-exposed controls.

4.2 Water

4.2.1 Copper Measurements

A discussion of the measured actual copper concentration was provided in the previous section (Section 4.1) and will not be

discussed further here, other than to reiterate and expand on a point made in Section 3.1.1 regarding filterable copper.

Referring to Table 7-4, it is noted that the percent reduction due to filtration alone in this experiment was similar to that noted in Chapter IV, Section 3.2, Table 4-6 for the same nominal concentration of 365 ppb (8.9% in this experiment compared to 5.2% in the LC50 experiment).

The value of 8.9% represents the overall mean reduction for the nine days of the experiment. However, there was a general decreasing trend in these values over the course of the experiment; ranging from a high of 19.2% on Day 1 to a low of 6.8% on Day 7 with a precipitous drop after Day 3. Following Day 3 there were three less fish and on Day 6 the population was again reduced by 3 fish (both sampling days). The decreasing percentage in copper reduction due to filtration would be expected as fish densities are reduced, if the assumption is valid that a key component of the filtered material is mucus with bound copper. In the other experiment in which the same nominal concentration was tested (Chapter IV, Section 3.2), there was a stable percent reduction due to filtration over the course of the four day experiment; the number of animals was not reduced in the tanks over the experiment, since none died at this concentration.

4.2.2 Temperature, pH, Hardness and DO

The measured water quality within this experiment, other than nitrites, were quite acceptable and should not have been a factor which significantly modified copper uptake.

The hardness of water, determined only at the beginning of this experiment, was found to be within the same narrow range of historical Baltimore city water.

The mean of the entire set of dissolved oxygen values collected during this experiment was 8.7 ppm, which represents approximately 96% of oxygen saturation in freshwater at sea level and at 20°C (Ross 1985). It would appear that these fish were supplied with adequate oxygen.

The temperature of the test water was very stable over the entire experiment. The mean of the complete set of temperatures collected was 20.00±0.37°C. Although this was understood to be less than the ideal temperature for tilapia, it did not approach lower lethal limits and remained constant. Facility limitations precluded the temperature from being increased closer to the optimum range of tilapia. It is possible that temperatures this low, may have slowed the processing of copper by the fish and may have reduced the amount of copper uptake. However, it is as likely that this same low temperature may have slowed down the depletion of accumulated copper. The role temperature played in this

experiment and the other preliminary experiments was not investigated further.

The mean pH of the systems during the experiment, likewise remained stable. The mean pH was 7.4±0.1. The optimum pH for these tilapia was not known at the time the experiment was conducted. However, the pH of the production facility water in transport cool boxes (used in these experiments) after 3 h of transport with the tilapia was approximately 6.4 at a salinity of 4.0 ppt. As in the case of the temperature, it is probable that pH 7.4 is not the optimum pH for tilapia, however, the impact of this parameter on copper uptake was not determined.

An original underlying objective in this study, as stated in Chapter I, was not necessarily to emulate a production system, but instead to make sure that the conditions were within the range of tolerance of the fish, and could theoretically be found in a production facility. Additionally, that these conditions were maintained and verified during the experiment. These conditions were met. The test conditions were admittedly not optimum, but it is believed that the conditions were adequate for reasonable fitness and growth to take place.

As confirmation and an example, a population of market-size tilapia (approximately 20 fish total) had been maintained in the same room in a larger tank for over six months. These fish were siblings of some of the first fish used in the early

experiments. The fish were maintained at greater densities than fish in experimental tanks. Mean weights of these fish were neither determined at the beginning nor at the end of their residence. There were no mortalities over the entire period of captivity, and the fish increased roughly 20 to 30% in weight. All other conditions of culture were the same as those fish in the experimental tanks.

4.2.3 Nitrites

The elevated levels of nitrites observed may have affected other observations made in this experiment. In particular, they may have been involved in gill pathology and will be discussed further in Section 4.3 below.

A particularly perplexing observation was the difference between nitrite levels measured in those tanks with copper compared to those tanks without copper. A two factor ANOVA with full interaction was conducted to at least confirm what appeared to be obvious. The ANOVA analysed nitrite levels relative to whether the tank contained copper or not, and relative to the number of fish in the tank at the time of the measurement. The results of the ANOVA are summarised in Table 7-12 below.

Table 7-12. Summary of nitrite measurements and statistical comparisons from the worst-case experiment.

Line No.	Factor	No. of Samples	Mean measured NO_2-N ppm	Standard Deviation	Significance (at $\alpha = 0.05$)
1	control	12	429	220	a
2	exposed	10	77	187	b
3	3 fish	10	235	290	a
4	4 fish	2	195	7	a
5	5 fish	2	408	569	a
6	7 fish	6	295	229	a
7	8 fish	2	298	328	a

The mean difference between the nitrite concentrations in the water of control tanks (Line 1) and those in the water of exposed tanks (Line 2) were statistically significant ($F_{calc} = 27.953$, $P_{calc} = 0.0001$). A multiple comparison test was unnecessary; the nitrite concentrations associated with the control tanks were higher than the exposed tanks. The null hypothesis that the nitrite concentrations in the tanks are independent of the presence of copper (at $\alpha = 0.05$) must be rejected. Hence, it can be assumed that the copper in the experimental tanks was associated with the low measured levels of nitrites.

The same ANOVA also demonstrated that the mean differences between the nitrite concentrations in the water of tanks with different numbers of fish at the time of the measurements (Lines 2 to 7) were not statistically significant ($F_{calc} = 2.636$, $P_{calc} = 0.0787$). The null hypothesis that the

nitrite concentrations in the tanks are independent of the number of fish present (at $\alpha = 0.05$) can not be rejected. Hence, it can be assumed that the number of fish in the experimental tanks were not responsible for the different concentrations of nitrites measured.

A small test was performed on Day 9 water samples to determine if copper in the water had reduced the nitrite levels by a simple chemical reaction. The procedure was described in the footnotes of Table 7-5. The addition of an equivalent amount of copper to samples of the high nitrite control water did not reduce the measured nitrite concentrations. This would suggest that the relationship of copper to low nitrite levels observed in this experiment, was not simply a matter of rapid chemical reactions. This relationship was not investigated further.

4.3 Histology

The results of the histological examination of gills from the fish in this experiment were presented in Section 3.3, Table 7-9 and 7-10. The statistical analyses of the lesions revealed very little relative to relationships between lesions noted and factors in the experiment.

The difference between rodlet cells counts was shown not to be different relative to the presence of copper, but there were greater numbers as a function of time. There was also shown

to be a statistically significant association between the number of fish with creeping epithelium and sample period (more noted the longer the fish were in the experiment). There were however, no other associations demonstrated to be statistically significant (at $\alpha = 0.05$) between lesions and either day of experiment or presence of copper.

It appears from the information generated, that if there is any relationship of pathological gill lesions in this experiment to any other factor, the highest probability would be with time, not copper. Assuming this is the case, it would suggest that whatever was causing an increase in lesions with time, would have to be common to both exposed and control tanks. Nitrite levels might initially seem to be associated with the gill lesions. However, the difference in nitrite levels measured between the two sets of tanks, exposed and controls, would support a strong argument against nitrites causing the gill pathology.

Without further investigations, it will have to be assumed that very little of the pathology noted in this experiment was due to the measured variables, with the possible exception of length of time within the experiment. There was no statistical basis for associating copper uptake with pathology, and in turn, linking this to copper in the water.

4.4 Tissue

4.4.1 Muscle

The AAS analyses and statistical comparisons of copper concentrations in the muscle (and attached skin) of fish exposed to an actual measured concentration of 309 ppb copper (nominally 365 ppb copper provided as copper sulphate pentahydrate) demonstrated that the fish did not accumulate copper in the edible tissue above endogenous levels. The endogenous levels were determined from samples collected from parallel non-exposed control fish analysed by the same techniques.

The significance of these findings go beyond a simple comparison of tissue levels of exposed fish compared to control fish. The conditions of the experiment, referred to as a worst-case experiment, were intended to simulate a production-type scenario where fish ready to be harvested had been treated with copper sulphate:

a) at the highest concentration possible without causing overt toxicity (primarily death),

b) for a period considerably longer than the normal one-day exposure,

c) for a period of time sufficient to allow any accumulation of copper to reach a plateau within the edible tissue, and

d) were harvested immediately following exposure, before any depletion of accumulated copper.

Fish farmers, who have ascertained that their fish require a therapeutic treatment of copper sulphate, typically have needed only one other item of information to proceed with the treatment. Historically they have simply measured the hardness (or alkalinity) of their water and applied a simple formula to calculate the maximum amount of copper sulphate that could be added to their systems, without causing toxicity to the fish. The formula they have used was to divide the water alkalinity (which was often substituted with hardness) by 100 and the resulting value would be the maximum amount of copper sulphate to add, expressed in ppm (Straus and Tucker 1993).

If this equation was applied to the water of this test system, which was approximately 85 ppm total hardness, the resulting maximum amount of copper sulphate which could be safely added would be 0.85 ppm or 850 ppb. This experiment was conducted at a mean copper concentration of 309 ppb, which is equal to 1217 ppb copper sulphate, a concentration approximately 1.4 times the level a farmer would have applied as a maximum based on water hardness.

The data support the conclusion that copper sulphate, when applied as therapeutic treatment to market-size tilapia, even if applied at the highest concentration possible without causing fish mortalities, does not result in an accumulation of copper in the muscle with attached skin (edible tissue).

The values of copper measured in the muscle of fish within this experiment compare favourably with those from other published studies (see Tables 7-1 and 7-2). A very rough approximation was calculated for the means and standard deviations of the various literature values for other fish. Although, not of any statistical significance, a comparison of those values and the values generated in this experiment is of value. Table 7-13 below summarises such a comparison.

Table 7-13. Summary of copper concentrations in the muscle of fish from various sources and the worst-case experiment.

Mean Measured Cu ± SD ppm (dry weight)				
	Controlled Studies (Table 7-2)		Worst-Case Experiment	
Field Studies (Table 7-1)	Control Fish	Exposed Fish	Control Fish	Exposed Fish
3.73 ± 3.38	4.85 ± 5.26	9.77 ± 11.73	2.14 ± 1.11	2.31 ± 1.16

It is certainly understood that such a crude mathematical manipulation, as depicted above in Table 7-13, has not considered the tremendous diversity of species, exposure

concentrations, exposure period or any other variable which could, and probably does, affect the outcome of the work. The exercise is merely to look for gross differences, those in the order of ten-fold or greater. Such differences do not exist, and hence it might be fair to assume that the levels of copper measured in the experimental tilapia in this study do not reflect any gross errors in calculation or analyses.

A review of measurements of copper in the muscle of all experiments in this study is summarised in Chapter VIII.

The results of this experiment also provide further insight into the potential for variation in the reported literature. The fillet-segment portion of this experiment demonstrated that the copper is not evenly distributed within a given fillet, at least not for market-size tilapia fillets that include the skin. It is apparent that samples other than complete fillets or aliquots of thoroughly homogenised whole fillets could be significantly skewed either side of the value for the entire fillet. At the very least, such lack of detail in the sampling procedure, would make the interpretation of results difficult.

Cross, Hardy, Jones and Barber (1973) indicated that they had tested, in a preliminary study, for differences between segments in fish fillets, and found no differences. They did, however, limit their samples to white muscle, without skin, scales or red muscle.

The worst-case experiment was conducted on fillets with attached skin. It is assumed, but not stated, that literature reported values for copper in fish muscle do not include skin. A very rough estimation of the respective contributions of skin and muscle were conducted during preliminary experiments in this study. The following were the conditions and results of that investigation.

The left fillet of a single small tilapia (10-30 g) was divided into skin and muscle. The proportions of skin and muscle were 19.0% and 81.0% wet weight, respectively. The AAS measured copper in the skin and muscle were 1.781 ppm and 0.632 ppm wet weight, respectively. The composite copper concentration was derived mathematically to be 0.850 ppm. As an example, a 1.0 g sample of fillet, would contain 0.190 g of skin and 0.810 g of muscle. The copper concentration in the skin would be 1.781 µg Cu/g skin and that in the muscle would be 0.632 µg Cu/g muscle. Therefore, there would be 0.339 µg Cu contributed by the skin and 0.511 µg Cu contributed by the muscle, for a total of 0.850 µg Cu in a 1.0 g muscle sample. Skin contributes approximately 40% of the copper, while muscle contributes approximately 60% of the copper.

The results of the worst-case experiment are not directly comparable to values reported in the literature, but may be

converted for approximate comparisons with the following assumptions.

a) There is validity to the trial described in the previous two paragraphs.

b) That the proportions of skin and muscle remain the same for dry weight as they were for wet weight.,

c) That the proportions of skin and muscle remain the same for market-size fish as they were for small tilapia (10 to 30 g).

To convert the copper levels measured in the edible tissue samples from the worst-case experiment to approximate levels in muscle only, the edible tissue values should be multiplied by 0.60 (60%).

4.4.2 Liver

The AAS analyses and statistical comparisons of copper concentrations in the liver of fish exposed to an actual measured concentration of 309 ppb copper (nominally 365 ppb copper provided as copper sulphate pentahydrate) demonstrated that the fish did not accumulate copper in the liver above endogenous levels. The endogenous levels were determined from samples collected from parallel non-exposed control fish analysed by the same techniques.

The significance of these findings differ from those for muscle from the same fish. The primary intent for analysing parallel liver samples was to use the accumulation of copper in the liver as confirmation of actual uptake of copper by the fish, and as an indicator of either reaching equilibrium or at least showing that copper was continuing to be taken up by the fish. The results of liver analyses failed to provide such confirmation. They did, however, provide comparative data and some understanding of the tolerance of tilapia to copper.

A comparative table, like that generated for muscle, was compiled for liver. Table 7-14 below, summarises published copper accumulation data from the livers of fish collected during field studies, experimental exposure trials and during this worst-case experiment.

Table 7-14. Summary of published copper concentrations in the livers of fish from various sources and the worst-case experiment.

Mean Measured Cu ± SD ppm dry weight (range)				
	Controlled Studies (Table 7-2)		Worst-Case Experiment	
Field Studies (Table 7-1)	Control Fish	Exposed Fish	Control Fish	Exposed Fish
55 ± 123 (nd to 610)	60 ± 70 (3.23 to 239)	290 ± 524 (12.5 to 1920)	425 ± 178 (180 to 921)	464 ± 194 (220-1056)

The information in Table 7-14 suggests, at the very least, that the levels of copper which can be measured in the livers of

fish can vary tremendously, as reflected in the large standard deviations noted in Table 7-14. These can vary for a number of reasons, species variations being one of the most obvious (see Table 7-1 and 7-2). The ranges indicated in Table 7-14 for the field and experimental studies reported in the literature represent the range of means noted in the publications. The range listed for the worst-case experiment are those of individual fish.

It appears that levels of copper in the livers of tilapia fall within the range of copper levels noted for other fish, but appear to be capable of storing considerably more copper than most. Only a few species accumulated levels near that of tilapia, European flounder, *Platichthys flesus*, mean levels were as high as 295 ppm (Stagg and Shuttleworth 1982); brook trout, *Salvelinus fontinalis*, had levels recorded up to a mean of 238 ppm (McKim and Benoit 1974); brown trout, *Salmo trutta*, had levels measured up to 610 ppm (Grizzle 1981, cited by Harrison 1986).

There are two species which have been reported to store more than the levels reported for this worst-case experiment with tilapia. Bluegills, *Lepomis macrochirus*, appear to be capable of accumulating very high levels of copper in the liver, with mean levels of 1920 ppm being measured under experimental exposures (Benoit 1975). White perch, *Morone americana*, collected from unpolluted waters have been found to

accumulate levels of 2795 ppm copper (wet weight), which is nearly 1000X that (3.5 ppm) of the closely related striped bass, *M. saxatilis*, from unpolluted water (Frazier 1984).

Tilapia appear to be very tolerant to copper, as noted in Chapter IV, and this tolerance may be in part a function of their superior ability to accumulate copper in the liver. Additional information, generated within this study, on the ability of tilapia to accumulate hepatic copper is summarised in Chapter VIII.

4.5 Overall Conclusions

The objective of this experiment was to determine if tilapia (*Oreochromis niloticus*) accumulated copper in their muscle and attached skin, exceeding endogenous levels, after being exposed to copper sulphate under worst-case conditions.

The results of this study support the argument that tilapia do not accumulate copper in their edible tissue (muscle and attached skin) above endogenous levels.

CHAPTER VIII

FINAL DISCUSSION, CONCLUSIONS AND SUGGESTED FURTHER WORK

The toxicity and uptake of copper by tilapia (*O. niloticus*) exposed to elevated concentrations of waterborne copper sulphate were investigated in a series of experiments. The overall objective of the study was to determine if tilapia accumulated copper in their muscle and attached skin, above endogenous levels, after being exposed to copper sulphate under worst-case conditions.

All experiments within the study, except the final experiment, were designed to define the worst-case conditions to be used in the final experiment. The final experiment exposed market-size tilapia (350 to 570 g) to copper sulphate under worst-case conditions of concentration and exposure duration.

The following section (Section 1) summarises the experiments conducted during this study. Section 2, 3 and 4 are general discussion sections.

1 Summary of Experiments

1.1 Tank, Feed and Faeces Experiment

1.1.1 Objectives

To establish what portion of the administered (nominal) concentration of copper sulphate was actually available to the fish, as limited by inherent characteristics of the test system. The characteristics, or factors, which were evaluated were tank surface, faeces produced, feed offered and the nutritional state of the fish at the time of exposure. The studies were conducted on small tilapia (10 to 30 g) exposed to 50 ppb copper.

1.1.2 Results

The presence of fish, the addition of feed and the presence of faeces did not significantly reduce the level of copper in the water compared to a tank with neither fish nor feed present. The tank surface did not bind appreciable levels of copper.

The measured levels of copper in the muscle of fish exposed to copper and not fed were higher than those in fish exposed to copper and fed, control fish fed and control fish not fed.

The measured levels of copper in the livers of fish exposed to copper and not fed were higher than those in control fish

which had been fed. However, the former were the same as the copper levels in the livers of fish exposed to copper and fed and control fish which had not been fed.

1.1.3 Application of Results

The tank surfaces, amounts of feed provided and faeces generated in this experiment were not considered to be significant binding sites for copper. It was assumed in subsequent experiments in this study that these factors were negligible.

The nutritional state of the fish appeared to affect the uptake of copper. Therefore, wherever possible feed was withheld from fish in subsequent experiments, to maximise the uptake of copper. Withholding feed was considered a component of the worst-case condition.

1.2 Median Lethal Concentration Experiments

1.2.1 Objectives

The objective of this experimental set was to generate the 96 h LC50 of copper sulphate for small and market-size *O. niloticus*. The data used to calculate the 96 h LC50's were used to extrapolate to an estimated LC1 to LC10. The LC1 to LC10 concentration was to then be used as the highest non-lethal concentration in the worst-case experiment.

1.2.2 Results

The estimated 96 h LC50 for market-size tilapia was determined to be 808 ppb of actual copper with a 95% confidence interval of 674 to 978 ppb. The LC1 to LC10 was extrapolated from the data to be 318 and 484 ppb copper.

The estimated 96 h LC50 for small tilapia was determined to be 308 ppb of actual copper with a 95% confidence interval of 243 to 435 ppb. The LC1 to LC10 was extrapolated from the data, but was not required for future studies.

An apparent relationship was observed between the nominal concentration of copper to which market-size tilapia were exposed and the proportion of copper removed in 0.45 µm filtered samples of tank water.

1.2.3 Application of Results

The approximate LC1 to LC10 value estimated from the data for market-size tilapia was used as the highest non-lethal concentration in the worst-case condition.

1.3 Small and Market-Size Tilapia Bridging Experiment

1.3.1 Objectives

The objective of this experiment was to determine if small tilapia could be substituted for market-size tilapia in

experiments in which facility limitations would restrict the use of the latter. Market-size and small tilapia were exposed to the same concentrations of copper sulphate for the same period of time.

1.3.2 Results

The levels of copper measured in the muscle of the small fish and the market-size fish were not found to differ from each other.

The levels of copper measured in the livers of the small fish and the market-size fish were not found to differ from each other.

1.3.3 Application of Results

The results of this experiment and the results of the median lethal concentration experiment (Section 1.2 above) provided sufficient information for small fish to be substituted for market-size fish in upcoming experiments in which facilities were not sufficient to accommodate large numbers of market-size fish.

1.4 Optimum Concentration/Duration Experiment

1.4.1 Objectives

Using small fish as a substitute for market-size fish, to establish if pathology affects uptake of copper and compare this between three concentrations of copper sulphate. The concentrations, all near the maximum non-lethal concentration, had been estimated in the median lethal concentration experiments (Section 1.2 above). The study was conducted for 23 days.

A secondary objective of this experiment was to establish how long it would take for the levels of copper in the liver to stabilise during exposure. The liver was used as the tissue most responsive to copper exposure and was being used as an indicator of equilibrium.

1.4.2 Results

The level of copper measured in the muscle of fish exposed to the highest concentration of copper was higher than the levels in muscle of fish exposed to the lower two concentrations. The latter two were not different from each other. There were also differences in copper in the muscle relative to the length of time held in the experiment, although there was a tendency for the levels to decrease with time.

The levels of copper measured in the livers of fish exposed to the three concentrations of copper were not different from each other. There were, however, differences in copper in the livers relative to the length of time the fish were held in the experiment; the longer the fish were held, the more copper was measured in the livers.

Gill pathology was noted in fish from this experiment. The prevalence of the lesions was found to be unrelated to the concentration of copper to which the fish were exposed. Lesions were, however, found to be greatest in those fish sampled during the last day of the experiment.

1.4.3 Application of Results

The results suggested that when the test fish were exposed to several concentrations of copper close to the estimated maximum non-lethal concentration, there was very little, if any, differences in the amount of copper accumulated by the edible tissue. Therefore, any of the three concentrations would be appropriate for the worst-case conditions.

The measured liver concentration suggested that copper was being accumulated at the same rate for all three exposure concentrations tested. Copper equilibrium was not reached within the liver, and considering the lack of copper uptake in the muscle, an approximate mid-point duration (nine days)

was selected as the length of exposure for the worst-case conditions.

1.5 Worst-Case Experiment.

1.5.1 Objective

Determine if market-size tilapia (*Oreochromis niloticus*) accumulated copper in their muscle and attached skin, exceeding endogenous levels, after being exposed to copper sulphate under worst-case conditions.

1.5.2 Results

Market-size tilapia exposed to copper under worst-case conditions did not accumulate more copper in their muscle than parallel control fish which were not exposed to copper. The concentration to which they were exposed was approximately 1.4 times the maximum level a farmer would have applied in water of similar hardness. The period of exposure far exceeded that which would be used in commercial aquaculture production.

The levels of copper measured in the livers of fish exposed to copper were not different from those measured in the livers of non-exposed control fish. Additionally, there were no measured differences in liver copper as a function of time.

2 General Comments

All experiments within this study used commercially available Standard Reference Materials (SRM's) and processing blanks to verify the preparation and analysis of tissues by Atomic Absorption Spectometry (AAS). The measured concentrations of copper in the SRM's used in all experiments were within the published acceptable tolerances.

The copper levels measured in the processing blanks from all experiments were below the level of detection for the AAS procedure, and therefore, it was not necessary to include these values in the calculations of copper measured in tissue and water samples.

3 General Discussion and Conclusions

3.1 Tabular Summary of Analytical Results

The analytical results of this study have been summarised in the Table 8-1 below. The data represent a broad range of test conditions, which have been indicated on the table. There does not appear to be a detectable trend in the measured copper values in either the muscle or the liver, possibly due to the conditions of the experiments not being identical.

The first major increases in measured copper within either muscle or liver appeared at exposure concentrations

exceeding non-lethal levels, i.e., at approximately 650 ppb. This suggested that tilapia may be able to process copper extremely well, especially compared to other fish species.

Table 8-1. Summary of copper concentrations in the muscle and livers of fish from all experiments within this study.

Fish Size	Tissue	Cu Exposure Conc. ppb	Duration	Measured Cu dry weight ppm	Standard Deviation
small	muscle	0	7 days	1.44*	0.24*
"	"	59	"	1.88*	0.16*
"	"	175	23 days	2.96	0.69
"	"	231	"	2.95	0.90
"	"	249	"	3.14	0.95
"	"	663	12 days	5.30	1.86
market-size	"	0	9 days	2.14	1.11
"	"	309	"	2.31	1.16
"	"	652	12 days	7.25	5.16
small	liver	0	7 days	588*	212*
"	"	59	"	948*	288*
"	"	175	23 days	345	217
"	"	231	"	355	193
"	"	249	"	370	208
"	"	663	12 days	1251	760
market-size	"	0	9 days	425	178
"	"	309	"	464	194
"	"	652	12 days	1656	1150

* dry weight values were estimated from measured wet weight values by assuming 75% moisture, which was the approximate measured moisture content from other experiments completed in this study.

3.2 Discussion and Conclusions

The results of this study support the position that market-size tilapia do not accumulate copper in their edible tissue (muscle and attached skin) above endogenous levels when exposed to copper under the defined worst-case conditions.

The data in this study were generated under conditions which should allow for confident extrapolation to other conditions. It would appear that commercially produced tilapia could be therapeutically treated under production conditions with very little, if any, probability of copper accumulation in their muscle and attached skin.

The information generated in this study may be of value to the US aquaculture industry. These data, which demonstrate a lack of copper residues in the edible tissue of tilapia exposed to elevated copper concentrations, should be appropriate for inclusion within an application for a US New Animal Drug Approval for the therapeutic use of copper sulphate in fish.

4 Suggested Further Work

Several questions raised by this research, remained unanswered. The most important topic which was not adequately addressed was the role of pathology in the toxicity and accumulation of copper. Additional investigations should be conducted at exposure concentrations exceeding those used

in the final experiment of this study. It appears that the concentration of copper to which these tilapia were exposed was not only non-lethal, as intended, but may also have been very close to a maximum non-toxic concentration.

The role of mucus production as a means of defence was only touched upon by this study. An increase in mucus production, in direct response to copper exposure as noted in this study, may have allowed the tilapia to tolerate higher copper concentrations. Additional investigations should be conducted to better quantify mucus production. These studies could possibly be complemented with histological studies to document mucous cell changes. These data offered a hint that changes in the mucous cells of the gills may be linked to concentrations and/or periods of exposure to elevated copper.

The role copper apparently played in the lower levels of nitrites in experimental tanks should be investigated further. The nitrites levels in the copper exposure tanks of the worst-case experiment were significantly less than those in tanks which had been subjected to the same conditions except the presence of copper.

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