EFFECTS OF POTASSIUM PERMANGANATE ON THE SAILFIN MOLLY, *Poecilia latippinna,* AT VARYING SALINITY LEVELS

By

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To my family and friends who have supported me throughout my college career.

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Abstract of Thesis Presented to the Graduate School
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Potassium permanganate ($KMnO_4$) is used in fish culture for disease treatment, water clarification, rotenone detoxification, and historically for management of oxygen depletion. Most commonly, $KMnO_4$ is used in freshwater systems at 2 mg/L to control ectoparasites, bacteria, and fungi. Effective concentrations are determined by the $KMnO_4$ demand of the water being treated. Although safe use of $KMnO_4$ in freshwater systems is well documented, its toxicity to fish in saltwater systems is less well known.

The sailfin molly, *Poecilia latipinna*, a euryhaline species, was used as a model to test the toxicity of $KMnO_4$ at varying concentrations and at different salinity levels. Target $KMnO_4$ concentrations of 0.0, 0.5, 1.0, and 3.0 mg/L plus the $KMnO_4$ demand were tested. Toxicity was tested at salinity levels of 2, 15, and 30 g/L. Mortality rates and fish behavior were monitored throughout the experiment and tissue samples for histological analysis were taken at time zero, immediately post-treatment (12 hours), and at the end of the monitoring period (7 days).

The mortality rate was significantly higher in the 30 g/L salinity, 3.0 mg/L $KMnO_4$ treatment group than in any other treatment group ($p < 0.001$). The 15 g/L salinity, 3.0 mg/L $KMnO_4$ treatment group was also found to be significantly different from the 15 g/L salinity 0.0 and 0.5 mg/L $KMnO_4$ treatment groups ($p < 0.001$). The 2 g/L salinity, 3.0 mg/L $KMnO_4$ treatment was not found to be significantly different. Dunn's multiple comparison test indicated that treatments of 2 g/L, 15 g/L, and 30 g/L salinities treated with 3.0 mg/L showed significant changes in behavior resulting in the loss of equilibrium. Dunn's multiple comparison test also indicated that treatments 15 and 30 g/L salinity, 3.0 mg/L $KMnO_4$ concentration showed significantly different gill damage as indicated by secondary lamellar fusion, lifting of epithelial cell lining by expansion of the lamellar interstitium by inflammation and edema, and necrosis.

Results from this study suggest that $KMnO_4$ at concentrations of 0.5 and 1.0 mg/L may be safe for use in water containing sailfin mollies in water of salinities of 2, 15, and 30 g/L. However, $KMnO_4$ should not be used at concentrations of 3.0 mg/L in 2, 15 or 30 g/L salinity water on the sailfin molly until further research is conducted. Toxicity of potassium permanganate increased in the higher salinity groups (15 and 30 g/L) compared to the low salinity group (2 g/L).

CHAPTER 1
LITERATURE REVIEW

Introduction

Potassium permanganate, $KMnO_4$, is an oxidizing agent that is used in fish culture for disease treatment, water clarification, rotenone detoxification, and historically for management of oxygen depletion in fish ponds. Potassium permanganate has been used to treat external pathogens including fungus, bacteria, and some parasites (Lay 1971; Masser and Jenson 1991; Noga 1996; Francis-Floyd and Klinger 1997; Plumb 1999; Carpenter et al. 2001; Stoskopf 1993; Bishop 2001; Straus and Griffin 2002; Thomas-Jinu and Goodwin 2004). $KMnO_4$ can also be used for water clarification purposes (Lay 1971) by oxidizing organic material in the water, forming precipitates that can be removed from the water by a filter. When rotenone is used in water treatments, $KMnO_4$ can be used as a counter agent to detoxify the water (Lawrence 1956). $KMnO_4$ was also used to add oxygen to aquaculture ponds in situations of low dissolved oxygen levels in water (Lay 1971). This method is no longer practiced as $KMnO_4$ did not raise oxygen levels significantly and may cause oxygen levels to decrease by killing the oxygen producing algae in the pond (Tucker and Boyd 1977).

Many different treatment regimes have been developed over the years for the different uses of $KMnO_4$ in aquaculture. However, use of $KMnO_4$ in salt water is less common than in freshwater systems so less is known about what happens when it is used as a treatment in the marine environment. There is debate over the use of $KMnO_4$ for

treatment of marine fish and concern regarding the toxicity threshold to $KMnO_4$ in marine species held in saltwater.

Marking and Bills (1975) found that $KMnO_4$ was more toxic to fish in waters with pH between 8.5 and 9.5 and total hardness of 300 mg/L as $CaCO_3$ (compared to water with a total hardness of 12 mg/L as $CaCO_3$). Stuart (1983) and Noga (1996) have suggested that $KMnO_4$ is toxic to fish in saltwater because of the higher pH typically associated with saltwater, which may cause manganese dioxide to precipitate onto the gills. Natural seawater typically has a pH between 7.8 and 8.2 (Spotte 1992), which may be higher than the pH of some freshwater systems, which are commonly between 6.8 and 7.2. In a freshwater efficacy study using $KMnO_4$ at a dosage of up to 1.5 mg/L to treat ichthyophthiriasis in channel catfish, *Ictalurus punctatus*, the pH was maintained at 8.5 +/- 0.1 (Straus and Griffin 2002), and the authors reported that no fish died in the effective treatment group (1.25 mg/L). This suggests that pH levels above those typically found in freshwater may not be the sole cause of $KMnO_4$ toxicity to fish.

Reardon and Harrell (1994) determined the $KMnO_4$ concentration that caused 50% mortality of exposed population over 96 hours, 96h LC_{50}, at different salinity levels up to 15 g/L for juvenile and larval striped bass, *Morone saxatilis*. In this study juveniles were most tolerant of $KMnO_4$ at a salinity level of 5 g/L, while larvae were most tolerant of $KMnO_4$ at a salinity level of 3 g/L. Both juvenile and larval striped bass were least tolerant of $KMnO_4$ at salinity levels of zero. The 96h LC_{50} levels that Reardon and Harrell (1994) reported are depicted in Table 1-1 (see page 10).

There was a significant decrease in the 96h LC_{50} level at salinity levels of 0, 10, and 15 g/L compared to the 5 g/L treatment for juveniles with $KMnO_4$ being most toxic

to juveniles at the 0 g/L salinity followed by the 15 g/L salinity. It was suggested that the greatest toxicity occurred in 0 g/L water because this was the salinity where the greatest osmotic imbalance occurred. This experiment showed that margin of animal safety may narrow when $KMnO_4$ is applied at higher salinities, but it did not demonstrate if the trend continued as salinity increased above 15 g/L.

The Sailfin Molly – *Poecilia latipinna*

The sailfin molly, *Poecilia latipinna,* formerly described and named *Mollienesia latipinna* by Charles Alexandre Lesueur in 1821 (Robins 2003), is from the family Poeciliidae, comprising over 190 species (Parenti and Rauchenberger 1989). The natural distribution of the sailfin molly is fresh, brackish, and salt waters of Florida, Mexico, Texas, South and North Carolina, and Virginia (Petrovicky 1988; Robins 2003; Courtenay and Meffe 1989). Non-indigenous populations are established in the western U.S. (Arizona, California, and Nevada), Hawaii, Canada, Central America, Singapore, Australia, New Zealand, Guam, and the Philippines (Courtenay and Meffe 1989). The sailfin molly prefers lowland areas such as marshes, lowland streams, swamps, and estuaries (Robins 2003).

The sailfin molly is a fusiform shaped small fish (15-53 mm total length) with a small head and upturned mouth (Robins 2003)(see Figure 1-1, page 9). The dorsal fin is greatly enlarged in mature males compared to those of mature females. The dorsal fin is used as a display to attract females for reproduction. Only dominant males display the dorsal fin. Subordinate males use the "ambush" technique for breeding (Balsano et al. 1989). The "ambush" technique refers to the chance that the dominant male becomes distracted, allowing the subordinate male to breed with the female before being chased away. Males have a modified anal fin called the gonopodium, which is used for internal

fertilization. At rest the gonopodium points caudally, but during reproduction, the gonopodium is pointed forward and is inserted into the female in a quick motion, which results in sperm being deposited into the female. The female molly can store the sperm deposited by the male. The gestation period is 3-4 weeks. Females are viviparous and give birth multiple times during the year (Robins 2003).

The sailfin molly, *Poecilia latipinna*, is a euryhaline species that can tolerate salinities as high as 70 to 80 g/L (Nordlie et al. 1992). Gustafson (1981) used short-term salinity acclimation as a method to evaluate the influence of salinity on plasma osmoregulation and routine oxygen consumption. Gustafson (1981) altered the salinity at a rate of 4.14 g/L per day for sailfin mollies of brackish water origin (mostly 10-20 g/L, but ranged from 4-35 g/L) and for sailfin mollies of freshwater origin (no information on the salinity level given). Frank Nordlie (The University of Florida, personal communication) recommended salinity level adjustments at a rate of 5 g/L every five days for proper osmoregulation balance. Sailfin mollies are highly adaptable to changing salinity ranges that are found in their natural habitat.

For aquaculture purposes, salinity up to 3 g/L is considered freshwater (Chapman, The University of Florida, personal communication), while brackish water ranges from 3.0 g/L to 29 g/L and saltwater is 30 g/L and above. Some species are more sensitive to salinity than others, therefore, it is necessary to know the limits of the species being used for experimentation.

The sailfin molly is primarily an herbivore, eating plants and algae, but is also opportunistic and will eat other food items including detritus or insect larvae and cannibalism has been reported (Meffe and Snelson 1989). The sailfin molly is a prey

item for many predators. Predators that eat this fish include reptiles, birds, other fishes, amphibians, and insects. The sailfin molly is also popular in the aquarium trade and is available in a wide variety of colors through domestication. It has also been used for research and for biological control of mosquitoes (Courtenay and Meffe 1989).

Potassium Permanganate – $KMnO_4$

Historically, the Environmental Protection Agency (EPA) (1985), registered $KMnO_4$ for use in cooling towers, evaporative condensers, air wash systems, ornamental ponds, cooling fountains, aquaria, human drinking water, poultry drinking water, for surface disinfection, sanitization, and as a deodorizer. However, $KMnO_4$ is now exempt from registration by the EPA because it does not pose unreasonable risks to public health or the environment.

Currently $KMnO_4$ is a U.S. Food and Drug Administration (FDA), investigational new animal drug (INAD) under investigation by Carus Chemical Company (Peru, IL) and Stuttgart National Aquaculture Research Center (SNARC) (Stuttgart, AR). FDA approval will allow for the legal use of $KMnO_4$ in water containing food fish. To gain approval from the FDA, Carus Chemical Company is responsible for research on product chemistry and mammalian toxicology, while SNARC is responsible for research on efficacy, human food safety, target animal safety, and environmental safety (Straus, SNARC, personal communication). Regulatory action on $KMnO_4$ has been deferred pending the outcome of current research.

As a therapeutant for fish, $KMnO_4$ has been used as an external bactericidal and fungicidal agent. Formulations of $KMnO_4$ are available as ready-to-use liquids, pellets or tablets, powder, or crystals. The active ingredient is the permanganate ion. It functions as a strong oxidizing agent that is corrosive and burns any organic material it comes into

contact with. For this reason it is effective if used in fish culture to control external disease-causing agents including bacteria, fungi, and some parasites. The oxidizing activity is also the primary problem for treated fish.

As an oxidizing agent, $KMnO_4$ is able to add oxygen, remove hydrogen, or remove electrons from an element or compound (Carus Chemical Company 2004). For example in drinking water treatments, $KMnO_4$ is able to oxidize soluble manganese and iron into manganese dioxide and iron oxide, which are insoluble and can be removed by filtration.

The effectiveness of $KMnO_4$ is related to the amount of oxidizable material in the water, i.e. organic material and other elements (inorganic) that may be oxidized. This is referred to as the $KMnO_4$ demand of the water (Tucker 1984). Engstrom-Heg (1971) developed a test, using a spectrophotometer, to determine the $KMnO_4$ demand of the water to be treated. Later, Boyd (1979) developed a quick visual test to determine the $KMnO_4$ demand of the water to be treated. He treated 1,000 mL samples of water with 0, 1, 2, 3, 4, 6, 8, 10, and 12 mg/L of $KMnO_4$ in separate containers; after fifteen minutes, the container with the lowest concentration that was still pink was considered the $KMnO_4$ demand of the water.

Potassium permanganate is rendered inactive by organic material (Tucker and Boyd 1977), therefore, in a recirculating system the biological filter may be affected because organic material, including the nitrifying bacteria, may be oxidized. However, it is unclear if $KMnO_4$ has a significant effect on the efficiency of the biofilter (Spotte 1992). One study (Levine and Meade 1976) demonstrated that a treatment of $KMnO_4$ inhibited nitrification 86%, while another study (Collins et al. 1975) demonstrated that

$KMnO_4$ had no effect on nitrification. If the filter has a build-up of organic material, it will increase the $KMnO_4$ demand of the water if the filter is left online during treatment and the biological portion of the filter may be damaged.

Tucker (1989) developed a method to estimate the required treatment of $KMnO_4$ based on the 15-minute $KMnO_4$ demand of the water. The calculation from his work is 2.5 multiplied by the value obtained in the fifteen-minute test. However, the level of $KMnO_4$ needed to control an ichthyophthiriasis outbreak in an efficacy study was 1.25 mg/L (Straus and Griffin 2002). In that study it was determined that using Tucker's method, the treatment rate indicated would be 1.0 mg/L. This implies that Tucker's method is not a "fail-safe" method for determining treatment dosages. Another recommendation for compensation of the $KMnO_4$ demand is to add 2 mg/L to the $KMnO_4$ demand of the water (Plumb 1999). Other treatment recommendations found in the literature are summarized in Table 1-2 (see page 10).

The toxicity margin of $KMnO_4$ is narrrow (1-3 mg/L) (Plumb 1999). Toxicity levels have been determined for carp fry as an LC_{50} ranging from 37.5 to 48 mg/L at 26°C and 45 to 37.5 mg/L at 32°C, at 24 and 48 hours, respectively (Ghosh and Pal 1969). The pH was maintained between 7.8 and 8.2. Studies have shown that 20 mg/L $KMnO_4$ is toxic to guppies and 3.2 mg/L $KMnO_4$ is toxic to catfish (Scott and Warren as cited by Lay 1981), however $KMnO_4$ demand or exposure time is not reported. Lawrence (1956) reported that toxicity levels of $KMnO_4$ were 3 mg/L for bluegills, 4 mg/L for largemouth bass, and 6 mg/L for goldfish. Finally Lawrence (1956) showed a toxicity level of 5 mg/L $KMnO_4$ for fathead minnows at a temperature of 68°F in a prolonged bath (no method for removing the $KMnO_4$ was employed), but restocking the

aquaria 24 hours after treatment showed that the water was no longer toxic to fish at that time. Toxicity of $KMnO_4$ decreases with increasing $KMnO_4$ demand (Tucker and Boyd 1977). Potassium permanganate tolerance is dependent on water quality, exposure time, and the species of fish.

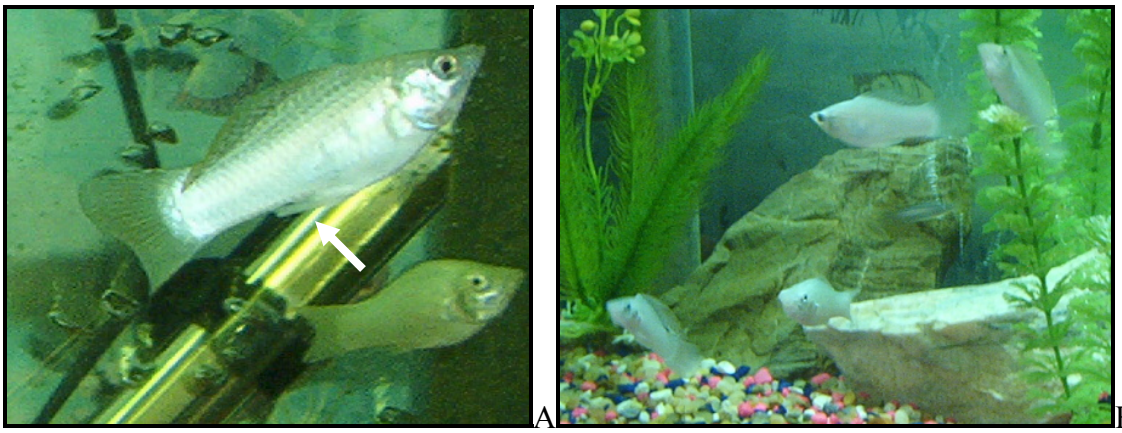
Potassium permanganate efficacy studies have been conducted with columnaris and ichthyophthiriasis. Straus and Griffin (2002) reported that a treatment of 1.25 mg/L $KMnO_4$ was effective against ichthyophthiriasis, meaning that no trophonts were found on treated channel catfish, *Ictalurus punctatus*. In that study, treatments were administered daily after a complete water change. The pH was maintained at 8.5 +/- 0.1 with a temperature of 17°C +/- 2.5 and the study was run until the control fish died. In a challenge against columnaris, $KMnO_4$ applied at a dose of 2 mg/L as an indefinite bath at a temperature of 22°C, reduced mortality to 69% compared to the 100% mortality in infected control channel catfish, *Ictalurus punctatus* (Thomas-Jinu and Goodwin 2004).

A fish that has been treated with $KMnO_4$ may show signs of tissue damage depending on the dose of $KMnO_4$. Darwish et al. (2002) found that channel catfish, *Ictalurus punctatus*, treated with $KMnO_4$ (0.438, 1.315, and 2.190 mg/L) at a pH of 7 +/- 0.2, showed microscopic lesions in the gills using a routine hematoxylin and eosin histology stain, while liver and trunk kidney showed no lesions. The fish treated with 0.438 mg/L $KMnO_4$ showed mild hypertrophy and spongiosis of the epithelium of the gill filaments and lamellae. The fish treated with 1.315 and 2.190 mg/L $KMnO_4$ showed extensive gill epithelial hyperplasia, lamellar fusion, and obliteration of the interlamellar spaces with inflammatory exudates containing necrotic epithelial cells.

Potassium permanganate is a strong oxidizer and contact with other materials may cause fire; however by itself it is stable and will keep indefinitely if stored correctly. Potassium permanganate is incompatible with the following substances: formaldehyde, powdered metals, alcohol, arsenites, bromides, iodides, phosphorous, sulfuric acid, organic compounds, sulfur, activated carbon, hydrides, strong hydrogen peroxide, ferrous or mercurous salts, hypophosphites, hyposulfites, sulfites, peroxides, and oxalates. Care should also be taken when handling potassium permanganate; it is corrosive and may cause burns to any area of contact and may be harmful if swallowed or inhaled. (Mallinckrodt Baker, Inc. 2001)

Potassium permanganate is an important chemical in aquaculture because of its availability and its various uses. The goal of this study is to determine if $KMnO_4$ creates toxicity problems for sailfin mollies held in saltwater compared to cohorts maintained in freshwater. This study will examine the effect of $KMnO_4$ on the sailfin molly maintained at different salinity levels and at different $KMnO_4$ concentrations.

Tables and Figures



A B

Figure 1-1. The sailfin molly. A. The gonopodium on this male sailfin molly is indicated by the arrow. B. A group of sailfin mollies. Photo credit: Chris Langeneck.

Table 1-1. The 96h LC_{50} levels for potassium permanganate ($KMnO_4$) determined for juvenile and larval striped bass, *Morone saxatilis*.

Juveniles (One-month old)		Larvae (18-day old)	
Salinity Level g/L	96h LC_{50} Level mg/L $KMnO_4$	Salinity Level g/L	96h LC_{50} Level mg/L $KMnO_4$
0	0.96[a]	0	1.02[a]
5	3.26[b]	3	2.11[b]
10	1.63[c]	6	1.41[ab]
15	1.48[c]	9	1.73[ab]

*Data taken from Reardon and Harrell (1994). Means with identical superscript are not significantly different at the 5% level using the SNK test.

Table 1-2. Dosages and target pathogens of $KMnO_4$ based on literature review.

Recommended dose of $KMnO_4$ (mg/L)	Duration of treatment	Treatment Use	References
1000	10-40 second bath	fungi, protozoa	Carpenter et al. 2001; Noga 1996; Lay 1971
100	5-10 minute bath	fish lice	Carpenter et al. 2001; Noga 1996
20	1 hour	crustaceans, protozoa	Stoskopf 1993
10	30 minutes	fungi, protozoa	Lay 1971
5	30-60 minute bath	ectoparasites, gill/skin bacterial infections	Carpenter et al. 2001; Noga 1996
2	prolonged immersion or bath, indefinite, at least 4 hours, or at least 12 hours	ectoparasites, gill/skin bacterial infections, protozoa, oxidation/ detoxification of hydrogen sulfide	Bishop 2001; Noga 1996; Thomas-Jinu and Goodwin 2004; Francis-Floyd and Klinger 1997; Plumb 1999; Masser and Jensen 1991
2-5	indefinite	crustaceans, protozoa	Stoskopf 1993
2	prolonged bath	counteract rotenone	Lawrence 1956
2.5	flush for 4 consecutive days	bacterial gill disease in salmonids	Stoskopf 1993
2	flush	ectoparasites, gill/skin bacterial infections	Noga 1996

CHAPTER 2
PILOT STUDY

Experimental Design

A pilot study was conducted at the University of Florida Department of Fisheries and Aquatic Sciences wet lab (Gainesville, Florida) to determine $KMnO_4$ treatment levels to be tested. Six, 37.85L tanks (filled to 30 L), each with one airstone, were set up at a salinity level of 5 g/L. Salinity was tested using a refractometer (Aquatic Eco-Systems, Apopka, Florida). Thirty-six sailfin mollies, *Poecilia latipinna*, (mean weight = 2.3 grams) were received from Segrest Farms (Gibsonton, Florida) and six fish were arbitrarily placed into each tank. Fish were fed once daily (1% of total biomass of tank) a flake fish food (Zeigler Tri-color flake, Gardners, Pennsylvania). Fifty percent water changes were conducted every other day in conjunction with salinity adjustments.

Saltwater was made with Instant Ocean salt mix (Aquarium Systems, Mentor, Ohio). Salinity was adjusted so that two tanks each were established at 2 g/L, 15 g/L, and 30 g/L, respectively, over a time period of fourteen days. In an effort to meet a deadline, the salinity level was altered 5 g/L every other day until the desired salinity was reached, with the exception of the 2 g/L tanks, which were adjusted by an increment of 3 g/L. The salinity level of 2 g/L was chosen over 0 g/L because captive sailfin mollies prefer to live in water that contains some salt (Petty, University of Florida, personal communication). The treatment level of $KMnO_4$ to be tested was 2 mg/L and 6 mg/L; one tank of each concentration (2 and 6 mg/L) was represented at each salinity (2, 15, and 30 g/L).

The $KMnO_4$ demand of the water was determined to be 0.20 mg/L $KMnO_4$ for 30 g/L salinity water, 0.15 mg/L $KMnO_4$ for 15 g/L salinity water, and 0.10 mg/L $KMnO_4$ for 2 g/L salinity water. Actual concentrations of $KMnO_4$ were not determined because chloride interferes with typical spectrophotometric techniques that might be used (Delfino, The University of Florida, 2004) because it reacts with $KMnO_4$. Each salinity would require its own standardization curve and the higher salinity water would produce a less reliable curve because the active concentration of $KMnO_4$ would be changing with increased contact time (some of the inorganic compounds, including chloride, of saltwater reduce $KMnO_4$).

The 16th day after fish arrival a fifty percent water change was done on each tank and one fish was sampled from each tank for histologic assessment of tissue prior to $KMnO_4$ exposure. The designated $KMnO_4$ treatment (either 2 or 6 mg/L $KMnO_4$) plus the $KMnO_4$ demand was added to the tanks and behavior and mortality was monitored for twelve hours. Behavioral observations were recorded to aid in the design of the scoring system for the larger experiment at time zero (0), 0.08, 0.16, 0.33, 0.66, 1.5, 3, 6, and 12 hours. Each tank was dosed with $KMnO_4$ and then fish were observed for behavioral alterations for 2 minutes until all tanks had been observed at this observation time. This required two people to physically monitor the tanks for the first four time periods (zero, 0.08, 0.16 and 0.33 hours). All fish were sampled for histological analysis either at the time of their death (mortalities were monitored hourly) or following twelve hours $KMnO_4$ exposure. Fish that survived the treatment were euthanized with buffered (sodium bicarbonate) tricaine methanesulfonate (1 g/L dose) (Finquel, Argent Laboratories, Redmond, WA) before fixation. Whole fish were fixed in 10% neutral buffered formalin.

After 48 hours of fixation whole fish were separated into two histology cassettes; one cassette contained complete, whole gill sections and the other cassette contained three sections of body tissue. After the gills were removed the sample was cut into three transverse sections (or steaks) – cuts were made at the posterior eye, just cranially to the pectoral fin and at the anus. Tissue was processed according to the schedule listed in Appendix A.

Results

One hundred percent mortality was observed in all tanks at all salinities (2, 15, and 30 g/L) treated with 6 mg/L $KMnO_4$. All fish from the 30 g/L salinity water treated with 6.0 mg/L $KMnO_4$ died by the third hour of observation, while the 2 g/L and 15 g/L salinity group fish treated with 6.0 mg/L $KMnO_4$ all died by the sixth hour of observation. Mortality was not observed in the three other tanks treated with 2 mg/L $KMnO_4$. Behavior observations were recorded and it was determined that fish generally exhibited the following behaviors in sequential/chronological progression 1) increased opercular movement; 2) erratic swimming; 3) intermittent loss of equilibrium or lying on the bottom resting; and 4) the complete loss of equilibrium. Microscopic examination of control tissue samples were within normal limits for the gills and other internal organs (e.g. kidney, liver, spleen, intestine). All gill tissue samples collected from $KMnO_4$ treated fish showed mucous cell infiltration and hyperplasia and inflammatory cell infiltration and hyperplasia. The 15 g/L and 30 g/L salinity treatment at 6.0 mg/L $KMnO_4$ showed severe inflammation, expansion of the lamellar interstitium by edema and inflammatory cells with lifting off of the epithelium, lamellar fusion and necrosis of the gill epithelium. The gill samples of fish from the 30 g/L salinity, 2 mg/L $KMnO_4$ treatment were also beginning to show signs of epithelial lifting from edema and lamellar

fusion. The whole body sections of $KMnO_4$ treated fish appeared to be within normal limits for this species.

Discussion

The results of this pilot study suggest that $KMnO_4$ caused more severe gill damage to fish as the salinity increased. A more thorough, replicated experiment will test this hypothesis. The fish in 30 g/L salinity group treated with 6.0 mg/L $KMnO_4$ died faster than those in the 2 and 15 g/L salinity treatments. These results suggest that fish in the 30 g/L salinity treatment group were more severely effected by the $KMnO_4$ treatment. A new experiment will be designed to test levels of $KMnO_4$ that are not toxic to 100% sailfin mollies (i.e., less than 6 mg/L $KMnO_4$) to determine if $KMnO_4$ causes more severe gill damage and possibly higher mortality at higher salinity levels.

CHAPTER 3
MATERIALS AND METHODS

Experimental Procedures

Experimental Fish and Quarantine Procedure

One thousand five hundred sailfin mollies were obtained from Segrest Farms in Gibsonton, Florida. Upon arrival at the University of Florida Tropical Aquaculture Laboratory (Ruskin, Florida) fish transport bags were placed in holding tanks for 30 minutes for temperature acclimation. Fish were exposed to a saltwater bath of 25 g/L for approximately 5 minutes (Stoskopf 1993) to remove ectoparasites. All salt used in this experiment was made with Instant Ocean salt mix (Aquarium Systems, Mentor, Ohio). Fish were weighed in groups of approximately 100 (each individual bag with water) on an electronic scale, netted out, and then placed into one of three holding tanks (680 L each, 5 g/L salinity water). The bag and water were reweighed after fish were released to determine an actual weight of fish in the bag. Fish were kept in the holding tanks until experimental salinity acclimations (described below) were complete. Any sailfin mollies that died during the salinity acclimation period were necropsied (including microscopic gill, skin, and fin examination) to ensure that the sailfin mollies were remaining as free of parasites as possible and for the early detection of other problems.

Fish in the holding tanks were acclimated to water salinities of 2 g/L, 15 g/L, or 30g/L, respectively. A reservoir tank containing no fish, but holding approximately 680L of 30 g/L salinity water was used for water changes. The water in the reservoir tank was made with Instant Ocean salt mix and well-water and was aerated for 24 hours prior to

water use (see Appendix B for typical composition). This water was added to the tanks to adjust the salinities upward. Well water was added to decrease salinity. Salinities were verified using a refractometer (Aquatic Eco-Systems, Apopka, Florida).

Fish were fed twice a day, five days a week a generic bulk tricolor flake food (Zeigler Tri-color flake, Gardners, Pennsylvania). The weight of the total fish population in each holding tank (about 500 fish) was 1247.5 grams (2 g/L salinity water), 1252.5 grams (15 g/L salinity water), and 1249.5 grams (30 g/L salinity water). The approximate average weight per animal was 2.5 grams. Based on those weights, fish were fed 12.5 grams (1% of total body weight in holding tank) of food twice per day, five days a week.

Acclimation Procedure

Sailfin mollies were divided into one of three different holding tanks (500 fish per tank) that were maintained at 5 g/L salinity. Freshwater was considered 2 g/L salinity and the holding tank being adjusted to that salinity was not adjusted until the seventeenth day of acclimation. The salinity of the other two holding tanks was raised twice a week in increments of 5 g/L salinity.

During acclimation an active, air-driven, sponge biofilter was maintained at each of the salinity levels that the fish would be held (10, 15, 20, 25, and 30 g/L) during the acclimation period as a replacement or back-up filter. Filters were started 3 weeks before the sailfin mollies arrived; the filters were fed daily with ammonia until they were needed in the fish holding tanks. When the salinity was increased in the holding tanks, the "old" biofilters were replaced with a "new" active biofilter that corresponded with the new salinity of the holding tank. Seventeen days after fish arrival the desired salinities of 2 g/L, 15 g/L, and 30 g/L, respectively, were reached. Fish were maintained in the holding

tanks for five days more before being distributed to the treatment tanks. Salinity was monitored daily using a refractometer throughout the acclimation period and salinity was adjusted as needed.

After acclimation to the appropriate salinity group, the fish were divided into the thirty-six, 75.7-L treatment tanks filled to 68 liters. Twelve tanks were randomly assigned to each of the three salinities being tested (2, 15, and 30 g/L). Fish were monitored in the 75.7-L tanks for three days before being exposed to $KMnO_4$. Each tank contained an air-driven sponge filter, which also provided aeration. Each tank was stocked with 37 fish, an average biomass of 86.4 grams per tank (fish were reweighed with an electronic scale prior to dispersal). Treatments were assigned to individual tanks using the complete randomized block method (Ott RL and Longnecker M 2001). A reservoir was available for each of the three salinity treatments so that water changes could be done when needed.

Response Variables

Tanks were checked twice daily for dead fish and results were recorded. Behavior was observed at the following times post $KMnO_4$ treatment: time zero, 0.08, 0.16, 0.33, 0.66, 1.5, 3, 6, 12, 24, 48, 96, and 168 hours. Due to physical constraints, observations for all 36 tanks could not all be made at the same time so a two-minute delay was instated. Specifically in applying $KMnO_4$, the first tank was dosed at 9:00 a.m., the second at 9:02 a.m., and so on until all tanks had been dosed in succession. Five trained observers were used to score behavior of the experimental fish. Behavior scores were categorized as follows:

1. within normal limits – fish swimming normally in midwater,
2. slight increase in opercular movement,

3. marked increase in opercular movement, fish behavior mixed between resting and actively swimming (usually erratically and repeatedly into the bottom and sides of the tank),

4. beginning to lose the ability to maintain equilibrium or just laying on the bottom of the tank, with obvious, labored respiration,

5. loss of equilibrium, floating throughout the tank with the current, very slow opercular movement, fish on the verge of death.

Tissues for histologic processing were collected at three different time points: one hour prior to $KMnO_4$ treatment for controls, twelve hours after initiation of treatment, and seven days post $KMnO_4$ treatment exposure. Six whole fish per tank were taken for each of the three histological sampling times. Prior to tissue collection, fish were euthanized using a 1 g/L dose of buffered (sodium bicarbonate) tricaine methanesulfonate (Finquel, Argent Laboratories, Redmond, WA) dissolved in water of corresponding salinity, either 2, 15, or 30 g/L. Following euthanasia, a small opening was cut in the fish's coelom and whole fish were submerged in 10% neutral buffered formalin for at least 48 hours before processing.

Following fixation, all of the gill tissue was excised from each fish and placed in individual cassettes for processing. The body of the fixed fish was also cut into three transverse sections (or steaks, as in the pilot study – Chapter 2) and placed in individual cassettes for processing. All cassettes were then placed in decalcification solution (Cal-EX, Fisher Scientific, Pittsburgh, PA) to remove any calcified material that could hinder the microtomy of the tissue. The tissue was rinsed in tap water for four hours prior to processing.

The tissues underwent a fourteen-hour tissue processing including alcohol (7 hours), xylene (3.5 hours), and paraffin wax (3.5 hours) – see Appendix A for detailed processing schedule. The tissue was embedded in paraffin. Tissue in the paraffin block

was cut at 5 micrometers and stained using a standard hematoxylin and eosin stain. Slides were ready for viewing by light microscopy. Analysis consisted of scoring each slide by scanning at 100X and examining in detail at 400X looking at approximately 50 gill filaments total using the following system:

1. Normal, includes background environmental damage (inflammation and mucous cell infiltration) at the tip of the gill
2. Secondary lamellar damage including fusion; mucous cell and inflammatory cell infiltration and hyperplasia, damage beginning to extend further from the tip of the gill
3. Expansion of lamellar interstitium by edema and inflammatory cells with lifting off of epithelium as well as fusion of lamellae
4. Necrosis and expansion of lamellar interstitium by edema and inflammatory cells in 50% or more of the lamellae

To limit observer bias the labels of each slide were covered with a slide label with an arbitrarily assigned number. After all of the slides were scored and recorded, the stickers were removed so that the scores could be matched with the slide identification.

Experimental Design

$KMnO_4$ Treatment

The experimental design consisted of exposing sailfin mollies in each of the three salinities (2, 15, and 30 g/L) to three different concentrations of $KMnO_4$, and a control group with no $KMnO_4$ treatment (Table 3-1). The dosages listed in Table 3-1 are calculated doses. Each treatment was replicated three times, making up 36 treatment tanks.

Table 3-1. Experimental treatment combinations of $KMnO_4$ concentrations and salinity levels.

Salinity (g/L)	$KMnO_4$ Treatment (mg/L)
2	0, 0.5, 1.0, 3.0
15	0, 0.5, 1.0, 3.0
30	0, 0.5, 1.0, 3.0

Experimental Procedure

Salinity was monitored daily using a refractometer. Fish were not fed during the experiment. During this time 25% water changes were done daily.

Potassium permanganate demand was determined for each salinity level using water from randomly selected tanks one and two days prior to the $KMnO_4$ treatment. The resulting six numbers were used to calculate an average $KMnO_4$ demand for all tanks at the corresponding salinity (Table 3-2).

Table 3-2. Calculated $KMnO_4$ demand for each salinity level. The mean $KMnO_4$ demand was used to calculate the actual $KMnO_4$ dosage for all treatments.

Salinity Level g/L	Mean $KMnO_4$ demand mg/L n=6
2	0.34
15	0.36
30	0.41

Three days after the fish had been transferred to the treatment tanks, water chemistry parameters (salinity, pH, TAN, NO_2) were tested in each tank. Water chemistry was measured using a HACH Fish Farming kit (model FF-1A, HACH Chemical Company, Loveland, CO) for the 2 g/L salinity tanks and a HACH Marine kit (model FF-3, HACH Chemical Company, Loveland, CO) for the 15 and 30 g/L salinity tanks. Water temperature was measured with a mercury thermometer. Water hardness was only tested in make-up water; six 190-L vats of make-up water were made using Instant Ocean saltmix and well water run through a reverse osmosis system (mixing was done with aeration).

One hour before $KMnO_4$ treatment, each sponge filter was removed and control histological tissue samples were collected. Aeration was continued in each tank throughout the $KMnO_4$ treatment period. Observations were recorded for each replicate

at the following times: time zero, 0.08, 0.16, 0.33, 0.66, 1.5, 3, 6, 12, 24, 48, 96, and 168 hours. Following $KMnO_4$ treatment (12 hours), a 50% water change was done on each tank, followed by the addition of 5 mL of Kordon AmQuel (San Francisco, CA) to all tanks (including control tanks) to deactivate the $KMnO_4$ (as indicated by the AmQuel label). Sponge filters were then replaced. Daily 25% water changes were done thereafter.

Statistics

Descriptive statistics of water chemistry parameters were derived using Minitab version 14.1 (State College, Pennsylvania). A one-way analysis of variance (ANOVA) was performed on the nitrite and unionized ammonia values by the salinity level followed by Tukey's HSD multiple comparison procedure. Mortality rate data was analyzed by salinity and the $KMnO_4$ concentration using a two-way ANOVA. The mortality rate data was then analyzed by the salinity, $KMnO_4$ concentration combination using one-way ANOVA followed by Tukey's HSD multiple comparison procedure to find which treatment concentrations were significant. An arcsin square root transformation of percentage data was performed prior to analysis. Behavioral and histological scoring data by the salinity, $KMnO_4$ concentration combination were analyzed using Kruskal-Wallis tests followed by Dunn's non-parametric multiple comparison test. Minitab version 14.1 (State College, Pennsylvania) was used for all statistical analysis except Dunn's multiple comparison test (Hollander and Wolfe 1973). A Type-I error rate (α) of 0.05 was used for all analyses except for Dunn's MCP. A Type-I error rate (α) of 0.15 was used, as recommended by Hollander and Wolfe (1973), for Dunn's MCP because of the conservative nature of the test.

CHAPTER 4
RESULTS

Water Quality

Water chemistry parameters varied in each treatment tank. Water quality measurements were taken prior to the $KMnO_4$ application (Table 4-1). The nitrite levels in the 15 g/L salinity treatment group were found to be significantly higher when compared to the 2 and 30 g/L salinity treatment groups ($F = 19.45$, $DF = 2$, $p < 0.001$). The unionized ammonia levels in the 2 g/L salinity treatment group were found to be significantly higher when compared to the 15 and 30 g/L salinity treatment groups ($F = 50.35$, $DF = 2$, $P < 0.001$). Water hardness levels in the reservoir tanks were 256.6 mg/L for 2 g/L salinity, 2,716 mg/L for 15 g/L salinity, and 5,480 mg/L for 30 g/L salinity ($n = 1$ for each salinity level). The temperature was 25°C in all tanks ($n = 36$).

Table 4-1. Select water chemistry values for experimental tanks ($n = 12$) prior to $KMnO_4$ treatments.

Salinity (g/L)	pH	Unionized Ammonia (mg/L)	Nitrite (mg/L)
2	8.33 / 0.0888	0.12 / 0.0435	0.14 / 0.0812
15	8.12 / 0.0389	0.02 / 0.01084	0.89 / 0.363
30	8.15 / 0.0522	0.03 / 0.01311	0.36 / 0.361

Data given in the format of mean / standard deviation.

Behavior

Fish behavior was affected by exposure to $KMnO_4$. There were significant differences among treatments ($H = 30.48$, $DF = 11$, $P = 0.001$) (Table 4-2). Fish that were significantly impacted showed signs of distress including increased opercular

movement, erratic swimming, beginning loss of equilibrium, and total loss of equilibrium.

Table 4-2. Kruskal-Wallis analysis of six-hour behavior scores by treatment combinations (salinity – $KMnO_4$ concentration) (N = 3 tanks per treatment).

Treatment Combination: Salinity (g/L) – $KmnO_4$ Concentration (mg/L)	Median (see footnote)	Average Rank	Z
2 – 0.0	0	9.3	-1.57
2 – 0.5	2	13.7	-0.83
2 – 1.0	3	20.0	0.26
2 – 3.0	4	31.5	2.23
15 – 0.0	0	4.0	-2.49
15 – 0.5	3	16.3	-0.37
15 – 1.0	3	23.8	0.92
15 – 3.0	4	31.5	2.23
30 – 0.0	0	5.7	-2.20
30 – 0.5	2	14.7	-0.66
30 – 1.0	3	20.0	0.26
30 – 3.0	4	31.5	2.23

H = 30.48 DF = 11 P = 0.001 (adjusted for ties)
Median key: 0 = within normal limits; 2 = marked increase in opercular movement, erratic swimming; 3 = beginning loss of equilibrium, periods of rest on bottom of tank; 4 = total loss of equilibrium.

The Dunn's multiple comparison test demonstrated that the 2 g/L salinity, 3.0 mg/L $KMnO_4$ treatment (mean rank 27.5), the 15 g/L salinity, 3.0 mg/L $KMnO_4$ treatment(mean rank = 27.5), and the 30 g/L salinity, 3.0 mg/L $KMnO_4$ treatment (mean rank = 27.5) were significantly different indicating that the sailfin mollies in those treatment groups lost normal equilibrium and were highly effected by the $KMnO_4$ treatment (Table 4-3). All other treatment groups did not show significance, indicated by a critical value less than 26.3231. Behavior that was affected by exposure to $KMnO_4$ treatment returned to normal within 24 hours after exposure, including fish exhibiting extreme change.

Table 4-3. Dunn's multiple comparison test for behavior scores shows significant treatment groups.

Treatment Combination: Salinity (g/L) + $KMnO_4$ Concentration (mg/L)	Difference in Mean Rank
2 + 3.0	27.5
15 + 3.0	27.5
30 + 3.0	27.5

*A mean rank above the critical value (26.3231) indicates significance when compared to the 15 g/L + 0.0 mg/L $KMnO_4$ concentration (the least effected treatment or most normal). The values were found using Dunn's multiple comparison test. All other treatments did not show significance.

Histology

The slides of affected gill tissue showed secondary lamellar damage including fusion and mucous and inflammatory cell infiltration and hyperplasia (Figure 4-1). More severely affected gill tissue showed expansion of the lamellar interstitium by edema and inflammatory cell infiltration with lifting off of the epithelium as well as secondary lamellar fusion. The most severely affected gill tissue showed severe necrosis as well as expansion of the lamellar interstitium by edema and inflammatory cells with lifting off of the epithelium and secondary lamellar fusion.

There were significant differences among treatments ($H=51.06$, $DF=11$, $P<0.001$) (Table 4-4). Significance in the 15 g/L salinity, 3.0 mg/L $KMnO_4$ indicates expansion of the lamellar interstitium by edema and inflammatory cells with lifting off of the epithelium as well as secondary lamellar fusion (Figure 4-2). Significance in the 30 g/L salinity, 3.0 mg/L $KMnO_4$ treatment indicates necrosis of the gill tissue as well as expansion of the lamellar interstitium by edema and inflammatory cells with lifting off of the epithelium and secondary lamellar fusion (Figure 4-3). Slide scores of treated fish returned to normal limits by the 168^{th} hour.

Table 4-4. Kruskal-Wallis analysis of the slide score by the salinity/$KMnO_4$ treatment combinations.

Treatment Combination Salinity (g/L) – $KmnO_4$ Concentration (mg/L)	N=	Median	Average Rank	Z
2-0	54	0	252.9	-2.66
2-0.5	54	1	268.9	-1.98
2-1.0	54	1	318.7	0.11
2-3.0	54	1	318.7	0.11
15-0	54	1	327.6	0.49
15-0.5	54	1	281.9	-1.44
15-1.0	54	1	348.1	1.37
15-3.0	54	1	391.9	3.20
30-0	54	0	240.5	-3.18
30-0.5	54	1	325.8	0.41
30-1.0	54	1	313.4	-0.11
30-3.0	36	2	446.3	4.41

$H = 65.34$ $DF = 11$ $P < 0.001$ (adjusted for ties)
Median key: 0 = within normal limits; 1 = secondary lamellar damage including fusion, mucous and inflammatory cell infiltration and hyperplasia; 2 = secondary lamellar damage including fusion, mucous and inflammatory cell infiltration and hyperplasia.
The 30 g/L salinity, 3.0 mg/L $KMnO_4$ treatment groups shows n=36 due to mortality.
This table includes scores from all tissue collection times (zero, 12 hours, and 168 hours).

Table 4-5. Dunn's multiple comparison test for histology slide scores shows significantly different treatments.

Treatment (g/L salinity – mg/L $KMnO_4$)	Significantly Different From: (g/L salinity – mg/L $KMnO_4$)	Difference in Mean Rank	Critical Value
15 – 3.0	2 – 0.0	139.0	116.535
15 – 3.0	2 – 0.5	123.0	116.535
15 – 3.0	30 – 0.0	151.4	116.535
30 – 3.0	2 – 0.0	193.4	130.290
30 – 3.0	2 – 0.5	177.4	130.290
30 – 3.0	15 – 0.5	164.4	130.290
30 – 3.0	30 – 0.0	205.8	130.290
30 – 3.0	30 – 1.0	132.9	130.290

A mean rank above the critical value (116.535) indicates significance when compared to the treatment indicated. All other treatments did not show significance.

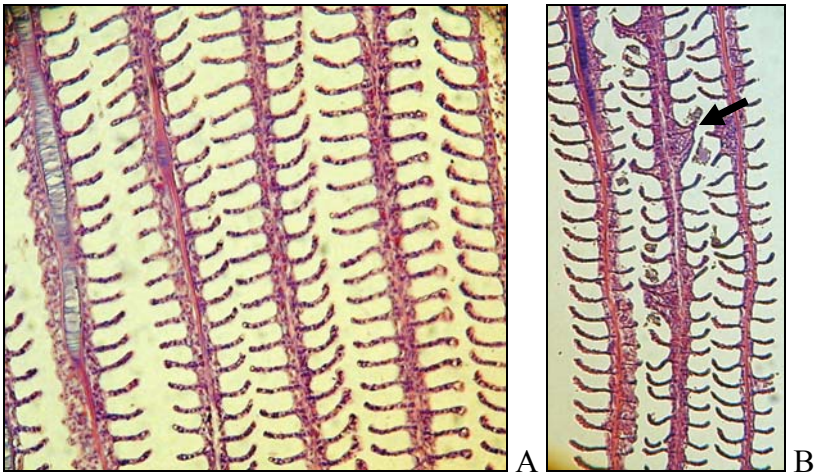


Figure 4-1. Gills from sailfin mollies in 2 g/L salinity water treated with $KMnO_4$. A(200X, 0.0 mg/L $KMnO_4$). Secondary lamellar structure is well-maintained (Slide Score = 0). B(200X, 3.0 mg/L $KMnO_4$). Secondary lamellar fusion, as indicated by the arrow, is commonly seen in this treatment group (Slide Score = 1). Stained by H&E.

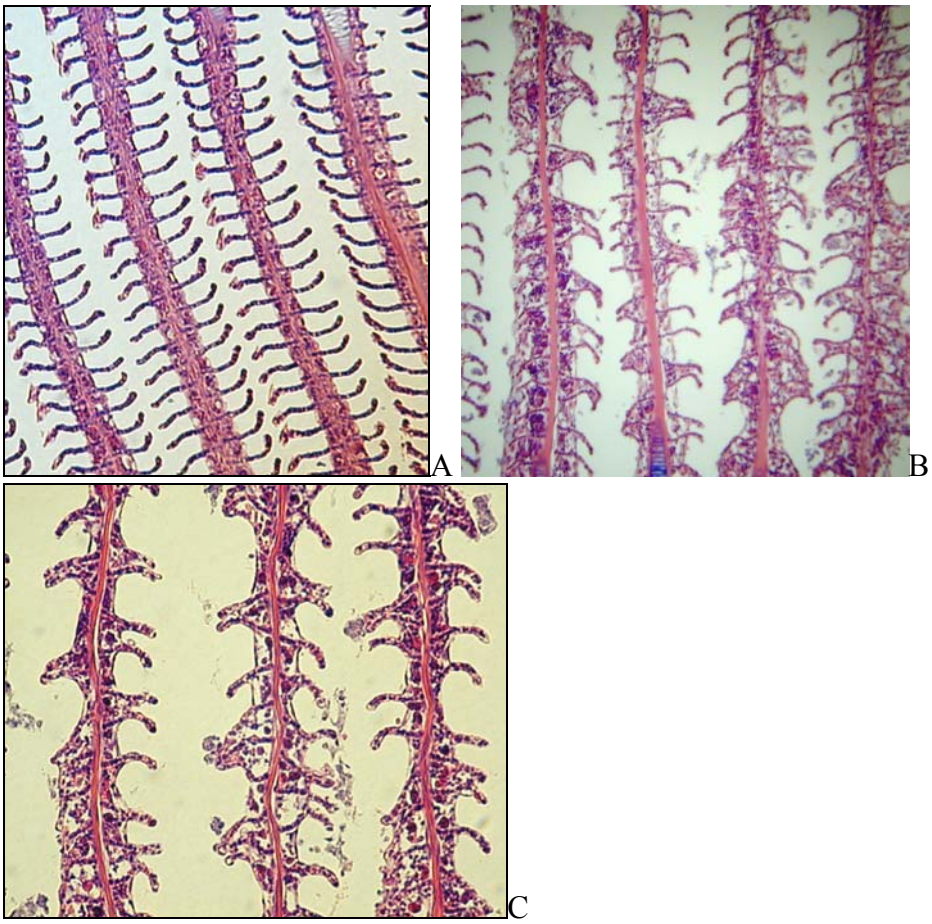


Figure 4-2. Gills from sailfin mollies in 15 g/L salinity water treated with $KMnO_4$. A (200X, 0.0 mg/L $KMnO_4$). The secondary lamellar structure is well-maintained (Slide score = 0). B(200X) and C(400X) (3.0 mg/L $KMnO_4$). There is expansion of the lamellar interstitium by edema and inflammatory cells with lifting off of the epithelium as well as fusion of the lamellae (Slide score = 2). Stained by H&E.

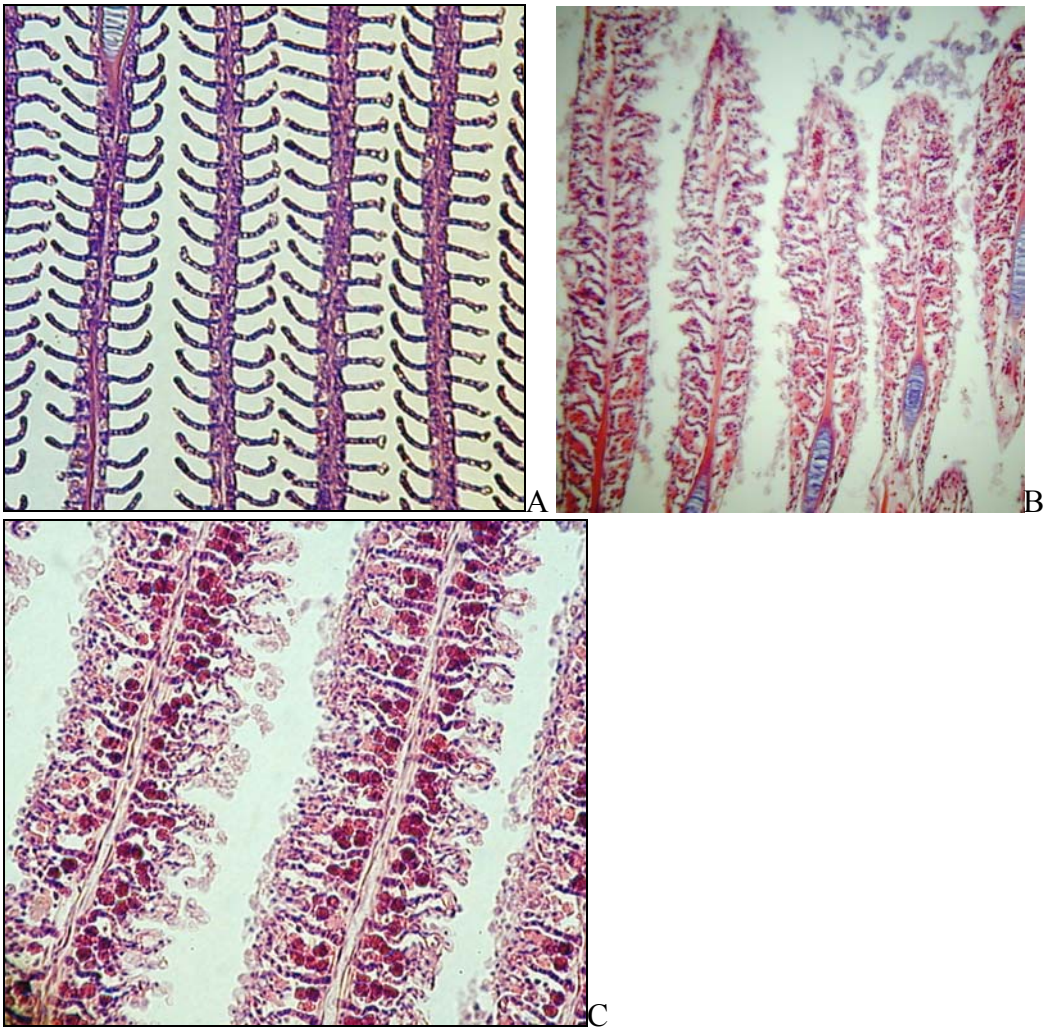


Figure 4-3. Gills from sailfin mollies in 30 g/L salinity water treated with $KMnO_4$. A. (200X, 0.0 mg/L $KMnO_4$). Secondary lamellar structure is well-maintained (Slide Score = 0). B(200X) and C(400X) (3.0 mg/L $KMnO_4$). Necrosis of the lamellae, fusion of the secondary lamellae, as well as lifting of the epithelial layer of cells by expansion of the lamellar interstitium by inflammation and edema was common in this treatment group (Slide score = 3). Stained by H&E.

Mortality

Salinity and $KMnO_4$ concentration were found to have some significantly different treatment groups (Table 4-6). Certain treatment combinations were also found to be significantly different (Table 4-7). The 30 g/L salinity, 3.0 mg/L $KMnO_4$ treatment had significantly higher mortality than all other treatments, with 100-percent mortality (Figure 4-7). The 15 g/L salinity, 3.0 mg/L $KMnO_4$ treatment had significantly higher

mortality than the 15 g/L salinity, 0.0 and 0.5 mg/L $KMnO_4$ treatments, with 36.33-percent mortality (Figure 4-6). The 2 g/L salinity, 3.0 mg/L $KMnO_4$ treatment was not found to be significantly different from other treatments, but that treatment had 11.33-percent mortality (Figure 4-5). Other treatment groups demonstrated very low, non-significant mortality over the observation period (168 hours) (see Figures 4.4, 4.5, 4.6, and 4.7).

Table 4-6. Two-way ANOVA of the total percentage mortality by the salinity and the $KMnO_4$ concentration.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-test	P-value
Salinity	2	3566.2	1783.08	12.57	< 0.001
$KMnO_4$ Concentration	3	15327.2	5109.06	36.02	< 0.001
Interaction	6	9085.4	1514.23	10.68	< 0.001
Error	24	3404.0	141.83		
Total	35	31382.8			

Table 4-7. One-way ANOVA analysis of the total mortality percentage by the treatment combinations.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-test	P-value
Treatment Combinations	11	6.6463	0.6042	18.62	< 0.001
Error	24	0.7790	0.0325		
Total	35	7.4253			

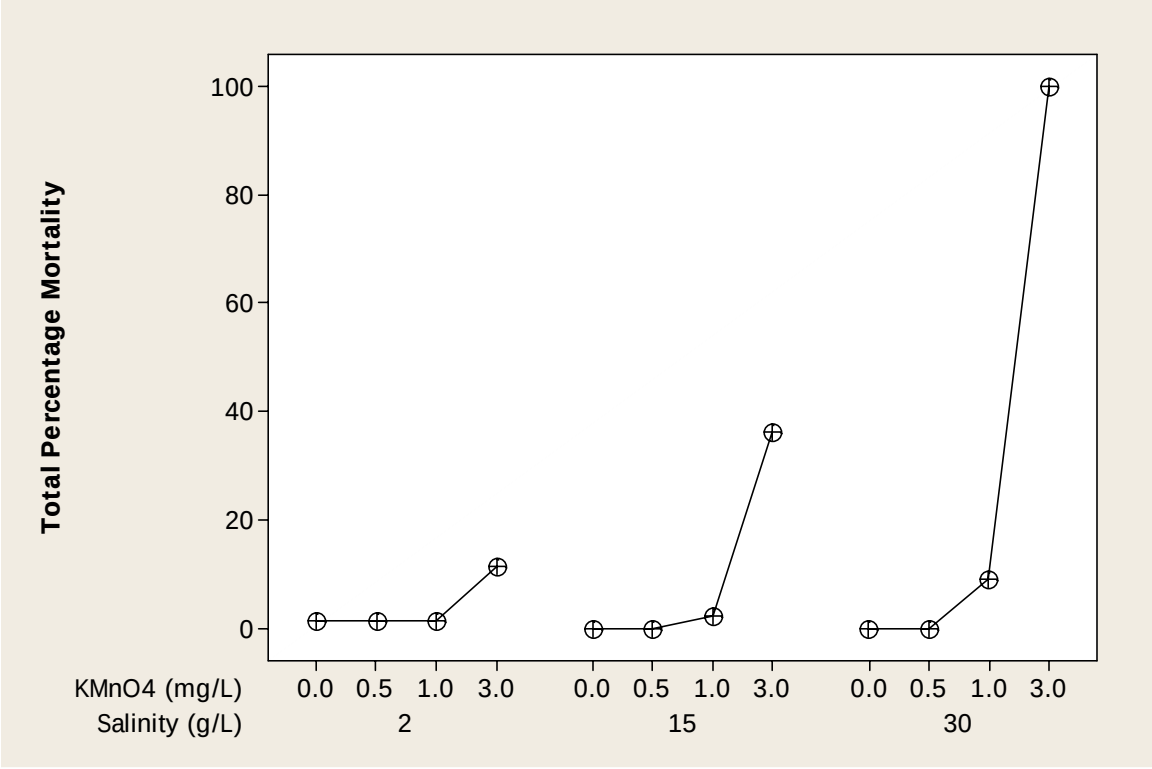


Figure 4-4. Cumulative Total Percentage Mortality Over 7 days by Salinity (fresh 2 g/L, brackish 15 g/L, and salt 30 g/L) and KMnO4 Concentration (0.0, 0.5, 1.0, and 3.0 mg/L). Connected data points = mean.

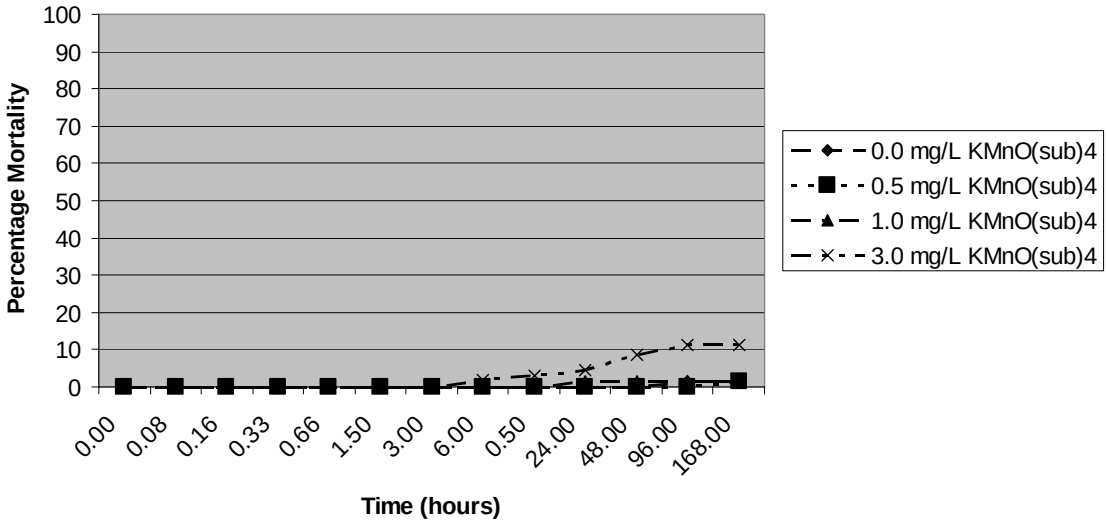


Figure 4-5. The cumulative total percentage mortality shown over time for the 2 g/L treatments.

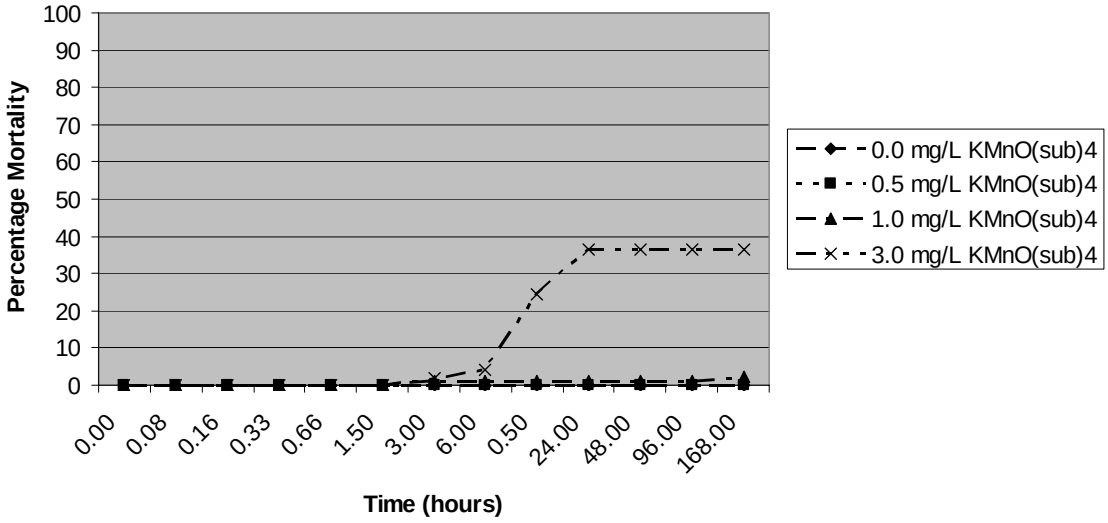


Figure 4-6. Cumulative total percentage mortality shown over time for the 15 g/L treatments.

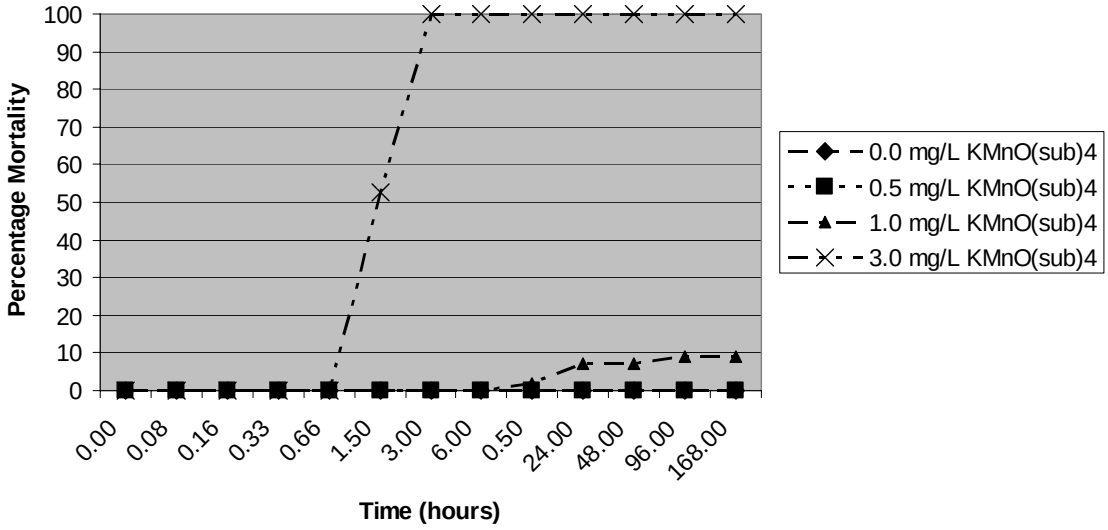


Figure 4-7. Cumulative total percentage mortality shown over time for the 30 g/L treatments.

CHAPTER 5
DISCUSSION AND CONCLUSIONS

Salinity/$KMnO_4$ Treatments

Potassium permanganate use in freshwater aquaculture is common. The concentration at which toxic effects are seen is affected by a variety of factors such as water quality and species specific sensitivity. The effectiveness of $KMnO_4$ is related to the amount of oxidizable material in the water, i.e. organic and inorganic material, which comprises the $KMnO_4$ demand of the water (Tucker 1984). It has also been suggested that water of high pH can cause manganese dioxide to precipitate (Stuart 1983 and Noga 1996). The purpose of this study was to determine if $KMnO_4$ creates toxicity problems for sailfin mollies held in saltwater compared to cohorts maintained in freshwater. This study examined the effect of $KMnO_4$ on the sailfin molly maintained at different salinity levels and at different $KMnO_4$ concentrations.

Tanks treated with 2 g/L salinity, 3.0 mg/L $KMnO_4$ demonstrated 11.33% cumulative mortality (Figure 4-4) throughout the entire 168-hour experiment. Histological results in this experimental group showed only minimal secondary lamellar fusion and mucous and inflammatory cell infiltration and hyperplasia in this same treatment group (Figure 4-1). Sailfin mollies in the 2 g/L salinity water treated with 3.0 mg/L $KMnO_4$ lost equilibrium in the water, demonstrating that they were negatively affected by the $KMnO_4$ treatment. The 2 g/L treatments also had a high mean ammonia level. Mortality rate in this group may have been affected by the added stress from the high ammonia levels in the water. The 3.0 mg/L $KMnO_4$ concentration is higher than

that of the usual recommended dose of 2.0 mg/L $KMnO_4$. It is possible that the 3.0 mg/L $KMnO_4$ dose is above the safety range of $KMnO_4$ treatment for sailfin mollies kept in 2 g/L salinity water causing minimal mortality.

Tanks treated with 3 mg/L $KMnO_4$ compared to control tanks treated with 0.0 mg/L $KMnO_4$, were visually different due to $KMnO_4$ concentration (the higher the $KMnO_4$ concentration the more purple the color of the water). This may cause observers to approximate the $KMnO_4$ concentration level, potentially creating bias. The behavior scoring system was made in an attempt to allow the observer to judge the fish solely on their behavior without factoring in the color of the water. The combined results of behavior, mortality rate, and histological analysis suggests that $KMnO_4$ treatment may not be a safe application for the 2 g/L salinity, 3.0 mg/L $KMnO_4$ even though it is not significantly different from other treatments.

Use of $KMnO_4$ in 15 g/L salinity water is a higher risk treatment than it would be in 2 g/L salinity water. The histology scoring system revealed expansion of the lamellar interstitium by edema and inflammatory cells with lifting off of the epithelium as well as fusion of the lamellae. This damage was consistently observed in this treatment group (Figure 4-2). The lifting of the epithelium away from the blood vessels presumably results in decreased efficiency of gas exchange and ammonia excretion, and therefore increases the risk of mortality. As the epithelial layer of the lamellae is moved further from the blood vessels more space is created and it is more difficult for gas (oxygen, CO_2) and other metabolites to move through the epithelium into the blood vessel and vice versa. This effect will at least slow gas exchange and at severe levels may stop gas exchange thus killing the fish.

Sailfin mollies in the 15 g/L salinity, 3.0 mg/L $KMnO_4$ demonstrated 36.33% mortality. Mortality variability was high (minimum = 0 %, maximum = 79 %), indicating some inconsistency between populations housed in different tanks within the same treatment group. This may indicate water chemistry problems (high nitrite levels in the 15 g/L salinity treatments) or that there is variability in how individual fish react to the chemical. A 3 mg/L $KMnO_4$ dose is higher than the recommended dose possibly contributing to toxicity. Further testing with increased replication would be necessary to clarify why there was so much variation in mortality among fish populations in the 15 g/L salinity water, 3.0 mg/L $KMnO_4$ treatment group.

As in the 2 g/L salinity, 3.0 mg/L $KMnO_4$, the 15 g/L salinity, 3.0 mg/L $KMnO_4$ treatment groups demonstrated loss of equilibrium during the treatment period. In conjunction with high mortality and the gill trauma observed, this suggests that the 15 g/L salinity water, 3.0 mg/L $KMnO_4$ treatment holds a higher risk for sailfin mollies. $KMnO_4$ should probably not be considered as a treatment choice in 15 g/L salinity water without conducting a small-scale animal safety test on the species being considered.

The 30 g/L salinity, 3.0 mg/L $KMnO_4$ treatment was not safe for sailfin mollies in this experiment. As in the 2 and 15 g/L salinity water, 3.0 mg/L $KMnO_4$ treatment groups, the 30 g/L salinity water, 3.0 mg/L $KMnO_4$ treatment group fish lost equilibrium during the treatment. The maximum behavior score of 4 indicated a loss of equilibrium, floating throughout the tank with the current with very slow opercular movement. Loss of equilibrium at this point may indicate that the fish is directing all of its energy towards respiration, letting equilibrium take second priority, or is so severely debilitated and has lost the ability to maintain its equilibrium.

Histological changes to the gill in the 30 g/L salinity water, 3.0 mg/L $KMnO_4$ treatment were severe and given the maximum histological score of 3. Lesions included necrosis and expansion of lamellar interstitium by edema and inflammatory cells in 50% or more of the lamellae. Gill damage observed in this group was much more severe than that observed in other treatment groups. The response of the gill to $KMnO_4$ treatment, as described above, may have reduced gas exchange to the point of death by suffocation in fish from this treatment group.

Mortality of sailfin mollies in the 30 g/L salinity water, 3.0 mg/L $KMnO_4$ treatment group was significantly different from all other treatment groups tested in this experiment. One-hundred percent mortality occurred within 6 hours of exposure to 3.0 mg/L $KMnO_4$ in all replicate groups. The behavior, histology, and mortality results from this experiment indicate that $KMnO_4$ is toxic to sailfin mollies from the 30 g/L salinity, 3.0 mg/L $KMnO_4$ treatment.

Water Chemistry Parameters

Water chemistry analyses among treatment groups were highly variable. Salinity change can cause biological filtration activity to decrease or even discontinue (Hovanec and DeLong 1996). Although an attempt was made to maintain consistent water quality by adding a conditioned biofilter to each of the different salinities encountered by the fish, water quality was variable, especially at the lower salinities. The differences in the nitrification process may have been due to the differences in salinity affecting the rates of bacteria infiltration of the filter. However, the highest mean UIA levels were observed in the 2 g/L salinity and the highest NO_2 levels were observed in the 15 g/L salinity. This may have contributed to mortality data collected. The stress of coping with the water

chemistry problems added to the caustic $KMnO_4$ treatment may have caused mortality in some fish creating variability in the results.

Although the 15 g/L salinity, 3.0 mg/L $KMnO_4$ treatment group had 36.33% mortality, the highest mortality was seen in the 30 g/L salinity, 3.0 mg/L $KMnO_4$ treatment. The 30 g/L salinity treatment group exhibited the most normal water quality (in reference to the UIA and NO_2 levels), showing that mortality in this treatment group was probably not related to UIA or NO_2 levels.

In this experiment the mean pH was 8.33 for 2 g/L salinity, 8.12 for 15 g/L salinity, and 8.15 for 30 g/L salinity, demonstrating low variability. Previous studies (Noga 1996, Stuart 1983) suggested that the high pH of saltwater was responsible for mortality of fish because manganese dioxide may precipitate onto the gills. In this study the lack of variation in pH between treatment tanks does not support this hypothesis.

Ideally pH should have been measured electronically instead of colorimetrically. The colorimetric tests lack precision, leading to increased variability. In the future more comprehensive water quality analyses and other methods for parameter detection should be used that do not involve colorimetric tests.

In this experiment only a calculated dose of $KMnO_4$ was reported. The actual dose of $KMnO_4$ was not tested because the spectrophotometric methods available are not reliable when testing water containing chloride or saltwater (Delfino, University of Florida, personal communication). There was no feasible option to determine the actual $KMnO_4$ concentration in all three salinities tested (2, 15, and 30 g/L, respectively). Technology or methodology should be developed to determine the actual dose of $KMnO_4$ in water regardless of salinity.

Future Research

The objective of this experiment was to determine toxicity of $KMnO_4$ (0.0, 0.5, 1.0, and 3.0 mg/L) at varying salinity levels (2, 15, and 30 g/L respectively) using the sailfin molly as a model. The study demonstrated that a concentration of 3.0 mg/L $KMnO_4$ in water of low organic content was not safe for the sailfin molly in 2, 15 or 30 g/L salinity water as indicated by mean mortality rates of 11.33, 36.33 and 100 percent, respectively. This data suggests that $KMnO_4$ is not safe for sailfin mollies at concentrations of 3.0 mg/L. The data in this experiment also suggests that $KMnO_4$ is a higher risk treatment in water with salinity of $\geq$ 15 g/L.

The cause of observed $KMnO_4$ related mortality can be speculated to include an unknown reaction between $KMnO_4$ and the salinity, oxidation-reduction by-products that are toxic, or attributes of the sailfin molly that may not be present in other fish. Many elements other than sodium and chloride are present in saltwater. Potassium permanganate may be interacting with one of these elements present in Instant Ocean salt mix (Aquarium Systems, Mentor, Ohio) producing a new compound that causes a toxic reaction in the fish. As indicated by Mallinckrodt Baker (2001), $KMnO_4$ is incompatible with bromides, iodides, ferrous salts, and arsenites, all of which are contained in Instant Ocean salt mix (Aquarium Systems, Mentor, Ohio) in various forms (see Appendix B). The consequences of these interactions are unknown in fish, but could be potential hazards. This should be looked into further to rule out other chemical reactions that would result in the fish being exposed to another compound other than $KMnO_4$.

One example of a chemical interaction that could be taking place in saltwater involves $KMnO_4$ and bromide. $KMnO_4$ causes an oxidation-reduction reaction, similar to that of ozone (Camel and Bermond 1998). When ozone is used in saltwater systems it

can produce hypobromous acid, hypobromite ion, and then bromate through the oxidation of bromide (Tango and Gagnon 2003). These compounds may be toxic to fish in the water by way of a pH change. It is possible that the oxidation-reduction reaction produced by $KMnO_4$ could have similar byproducts that cause toxicity. In future experiments, pH and oxidation-reduction potential should be measured throughout to monitor change.

The sailfin molly used in this experiment is a euryhaline species that is able to adapt to differing salinities well. The sailfin mollies' ability to adapt to fluctuating salinities and ion concentrations may have affected the results of this experiment. The addition of $KMnO_4$ may change the ionic balance of the water compared to the blood of the fish, impacting osmoregulation. Osmotic homeostasis must be maintained in the fish to maintain good health. Sailfin mollies may be better suited to living in fresh to brackish water environments as that is more of their natural distribution. It is possible that at higher salinities even though the sailfin molly is able to survive, it is not able to flourish. A sailfin molly in saltwater may be under more environmental stress. Therefore, when challenged with a treatment such as $KMnO_4$, the fish is less able to cope with the added stress. Experiments should be completed to see if this possible cause of mortality and gill damage is unique to $KMnO_4$ treatment or if it encompasses other types of chemical treatment or stressors as well.

Conclusions

Gill damage such as expansion of lamellar interstitium by edema and inflammatory cells with lifting off of epithelium as well as fusion of lamellae and in severe cases necrosis was seen in the 15 and 30 g/L salinity, 3.0 mg/L $KMnO_4$ treatment groups. Mortality was 11.33% in the 2 g/L salinity, 3.0 mg/L $KMnO_4$ treatment group, 36.33%

in the 15 g/L salinity treatment group, 3.0 mg/L $KMnO_4$ treatment group, and 100% in the 30 g/L salinity, 3.0 mg/L $KMnO_4$ treatment suggesting that $KMnO_4$ at the highest concentration tested was not safe for sailfin mollies in this experiment. Results also suggest that as the salinity of the water increases, the toxicity of $KMnO_4$ to sailfin mollies also increases.

APPENDIX A
PROCESSING SCHEDULE FOR THE SHANDON EXCELSIOR AUTOMATIC TISSUE PROCESSOR

Table A-1. Processing schedule for the shandon excelsior automatic tissue processor

Reagent Used:	Time in hours:
Alcohol – Ethyl 50-75%	1:00
Alcohol – Ethyl	1:00
Alcohol – Ethyl	1:00
Alcohol – Ethyl	1:10
Alcohol – Ethyl	1:10
Alcohol – Ethyl 75-100%	1:30
Xylene	1:00
Xylene	1:15
Xylene	1:15
Wax – Paraffin	1:00
Wax – Paraffin	1:15
Wax – Paraffin	1:15

APPENDIX B
TYPICAL COMPOSITION OF INSTANT OCEAN SALT

Table B-1. Typical composition of instant ocean salt

Ion	Solution at Approximate Salinity of 35ppt Instant Ocean (ppm)	 Seawater* (ppm)
Chloride	19,290	19,353
Sodium	10,780	10,781
Sulfate	2,660	2,712
Magnesium	1,320	1,284
Potassium	420	399
Calcium	400	412
Carbonate/bicarbonate	200	126
Bromide	56	67
Strontium	8.8	7.9
Boron	5.6	4.5
Fluoride	1.0	1.28
Lithium	0.3	0.173
Iodide	0.24	0.06
Barium	less than 0.04	0.014
Iron	less than 0.04	less than 0.001
Manganese	less than 0.025	less than 0.001
Chromium	less than 0.015	less than 0.001
Cobalt	less than 0.015	less than 0.001
Copper	less than 0.015	less than 0.001
Nickel	less than 0.015	less than 0.001
Selenium	less than 0.015	less than 0.001
Vanadium	less than 0.015	less than 0.002
Zinc	less than 0.015	less than 0.001
Molybdenum	less than 0.01	0.01
Aluminum	less than 0.006	less than 0.001
Lead	less than 0.005	less than 0.001
Arsenic	less than 0.004	0.002
Cadmium	less than 0.002	less than 0.001
Nitrate	None	1.8
Phosphate	None	0.2

* Data for seawater values taken from *An Introduction to the Chemistry of the Sea*. 1998. M.E.Q. Pilson

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BIOGRAPHICAL SKETCH

Emily N. Marecaux grew up in Ashland, Maine. After graduating from high school she attended college at The University of Findlay in Findlay, Ohio. While at The University of Findlay she pursued a Bachelor of Science degree majoring in biology, pre-veterinary science, with a minor in chemistry. After graduating in May of 2002, Emily came to The University of Florida to work on a Master of Science degree from the Department of Fisheries and Aquatic Sciences with a focus on fish health. Emily has accepted a position at the University of Arkansas at Pine Bluff as a fish health extension associate.