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Analysis of Disinfectants on *Mycobacterium* spp.

by

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With Honors

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Abstract

The zebrafish, *Danio rerio*, is an important model organism in the field of biomedical research. Unfortunately, these fish are negatively impacted by different species of *Mycobacterium* which cause subclinical infections, lesions on the body, and sometimes premature death. When new fish are introduced to a facility, it is typically by eggs only, and the standard disinfection practice, a treatment of 50ppm sodium hypochlorite, has unknown efficacy for killing mycobacteria. This study tested 100ppm and 150ppm bleach, as well as 1.5% and 3% hydrogen peroxide and 100ppm iodine, to determine the germicidal efficacy for use in fish facilities. Cultures of *M. gordonae* and *M. peregrinum* grown in Middlebrook broth were used. Each trial included a positive control, low concentration, and high concentration of disinfectants; iodine contained only a control and low treatment. Bleach was applied for 10 minutes and deactivated by sodium thiosulfate while hydrogen peroxide and iodine were applied for 5 minutes and removed. Plate counts were made on treatment and control groups following serial dilution and a week of growth at 30°C. Only iodine resulted in complete killing of both *Mycobacterium* species tested. Bleach treatment impacted growth of both species. The hydrogen peroxide only impacted *M. peregrinum*. ANOVA statistical analysis showed the following significant differences: 1) bleach treated cultures of *M. peregrinum* exhibited less growth than untreated.

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Acknowledgements

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Introduction

Mycobacteriosis is a chronic disease that plagues various species of freshwater and marine fish around the world. It is caused by Gram positive and acid-fast positive bacilli in the genus *Mycobacterium*. These bacteria are considered to be hydrophobic and oligotrophic since it is able to survive with low levels of dissolved carbon in the environment (Whipps, 2012). The disease can cause fish to suffer from wasting, alterations of scale coloration, deformities of the curvature of the spine as well as small stature. Skin ulcerations and lesions along the surface of the body are commonly seen with this type of bacterial infection (Decostere et al., 2004). The disease can also contribute to reproductive problems in populations, which can lead to diagnosis of the disease (Francis-Floyd, 2011).

Mycobacteria can be transferred from one fish to another through multiple routes. Horizontal occurs when bacteria are spread from one fish to another. (Francis-Floyd et al. 2011). This transfer can occur through contaminated food sources, such as a diseased carcass, the decomposition of an infected fish, and the introduction of a contaminated fish into a closed system, such as a fish facility (Decostere et al. 2004). There is also a vertical transfer of the disease that can occur from mother to eggs (Francis-Floyd et al. 2011). This is a problem in research facilities because the offspring may not survive to maturity and the eggs that are transferred to other facilities or aquariums can cause the disease to spread. It is also believed that the water itself can be the source of transmission (Manious, 2005). When an infected fish is eaten or decomposes, the bacteria are freed from the tissues and enter the surrounding water

where it is able to move and eventually thrive in the biofilms of a system (Whipps et al., 2012).

This has become a major problem in medical research due to the infection of a popular laboratory model organism *Danio rerio*, or zebrafish. This species has increased in popularity as a research model due to the immune responses produced by the fish (Whipps et al. 2012). This disease has a large impact on research because data, time, and money are lost on the fish that are killed from this disease. Once a fish is found to be infected it needs to be removed immediately, the other fish in the system are quarantined, and the entire tank needs to be broken down and disinfected to ensure that the bacteria will not cause any additional infections (Francis-Floyd et al. 2011). Once again, this takes a lot of time and resources, so it is imperative that the method of disinfection that is used on the fish and on the tanks have a high germicidal efficacy so that the bacterium does not infect the fish once they are introduced into the system. In addition to disinfecting tanks, the eggs that are introduced into the system need to be disinfected as well; the standard protocol for this is a 10 minute exposure to sodium hypochlorite, but the efficacy of this treatment is unknown (Whipps et al. 2012). Because this is still considered to be a huge problem in fish facilities, the efficacy of this surface disinfection method is in question. Further research must be done in order to determine a protocol that is effective with a high germicidal efficacy that should become the new standard for treatment of zebrafish eggs that are introduced into these closed systems.

In order to accomplish the efficacy of disinfection, this thesis explores the standard disinfectant sodium hypochlorite, as well as two additional disinfectants; hydrogen peroxide and iodine. Higher concentrations of sodium hypochlorite than typically used in a zebrafish facility, 100ppm and 150ppm, will be tested. Because the standard protocol requires only 50ppm, it is possible that a higher concentration of the compound will provide a higher germicidal efficacy. Hydrogen peroxide can be commonly found in drugstores at a concentration of 3%. This initial concentration will be tested, as well as a lower concentration of 1.5%. Iodine, which is typically used in the salmon aquaculture industry, will be used at a concentration of 100ppm with a pH of between 6.5 and 7. This will ensure that the treatment is not overly basic or acidic which can harm the eggs that are being treated. The treatments will be performed on cultures of 2 species that are commonly found to infect zebrafish; *Mycobacterium gordonae* and *Mycobacterium peregrinum*.

Methods

Mycobacterium gordonae and *Mycobacterium peregrinum* were grown in 3 mL of Middlebrook on a shaker incubator at 400rpm and 28°C for one week. A McFarland Standard was then used to determine the approximate concentration of colony forming units (CFU)/mL. The cultures were then diluted to a concentration of 10^4 CFU/mL; in a 3mL volume. The rationale for using this starting concentration is to approximate the number of bacteria one might find in the spawn water of a heavily infected fish (Virginia Watral, Oregon State University, pers. comm.). The culture was then separated into 3 microfuge tubes containing 1 mL of culture in each; a positive control, low treatment, and high treatment.

For the bleach treatment, the stock solution of 82,500 ppm sodium hypochlorite was diluted down to a 1000 ppm, the working stock solution. A 1 ppm solution of sodium hypochlorite was made from the 1000 ppm stock and a chlorine meter was used to determine the exact concentration. There were 3 trials done to ensure accuracy and the average of the trials was used as the exact concentration of the working stock. A relative volume equation was then used in order to determine the volume of stock solution to be added to the cultures to produce 100 ppm and 150 ppm treatments; ie. $1000 \text{ ppm } (V_1) = 100 \text{ ppm } (1000 \text{ } \mu\text{L} - V_2)$. The positive control received an equal volume of water as the 100 ppm treated received of sodium hypochlorite. The cultures are then exposed for 10 minutes, after which equal volumes of sodium thiosulfate were then added in order to deactivate the sodium hypochlorite. The positive control was then diluted to 10^3 , 10^2 , and 10^1 while the treated cultures were diluted to 10^3 and 10^2 and

plated in triplicates on Middlebrook agar. The cultures were incubated at 28°C until colonies were visible and able to be counted.

The hydrogen peroxide treatment followed a different protocol. The cultures were diluted to a slightly higher concentration of 10^6 CFU/mL, separated into 3 microfuge tubes, and the cells were pelleted in a centrifuge at 3000 g for 10 minutes; the higher concentration ensured that a visible pellet would form. Hydrogen peroxide treatments at 3% and 1.5% were used. The supernatant was removed from the pellets and the treatments were added directly to the cells; 1 mL of water for the positive control, 1 mL of 1.5% H_2O_2 to the low treatment sample, and 1 mL of 3% to the high treatment sample. The cells were suspended by vortexing and exposed for 5 minutes. After exposure, the cells were pelleted at 5000 g for 30 seconds. The supernatant was removed and the cells were re-suspended in water. The cultures were diluted to 10^5 and 10^4 , each dilution was plated in triplicate on Middlebrook agar and the plates were incubated at 28°C until colonies were observed.

The same protocol was used for the iodine treated samples. The only change was that there were 2 samples, a positive control and a treatment. The stock solution contained 1% iodine. A 100 ppm solution was made by combining 4.95 mL of water with 50 μ L of iodine. The same protocol was performed as above and the cultures were diluted, plated in triplicates, and incubated.

Plate counts of each treatment of each species were entered into a spreadsheet. The counts were taken in CFU/0.1mL and had to then be converted to CFU/mL. The

averages of the triplicate plates were taken and the standard error was determined. The data was then exported into MINITAB® where One-Way ANOVA using Tukey post tests comparison and Kruskal-Wallis analysis was performed.

Results

Bleach

When treated with 100ppm or 150ppm bleach, cultures of *M. gordonae* did not show a significant difference in growth versus controls (Fig. 1). One-Way ANOVA, for *M. gordonae* showed no difference between untreated, 100ppm, and 150ppm groups, $F(2, 24) = 2.74$, $p = 0.084$. The untreated group ($M = 38889$, 95% CI [42788, 155435]) did not show a statistically significant difference when compared to the 100ppm sample ($M = 1712$, 95% CI [666, 2421]). These groups did not show a statistically significance when compared to the 150ppm sample ($M = 288$, 95% CI [185, 675]) (Fig 1). The Kruskal-Wallis Test ($H = 3.91$, $p = 0.142$) resulted in a median rank of 17.8 for 100ppm, 13.8 for 150ppm, and 10.4 for the untreated samples.

The ANOVA testing of the first treatment of *M. peregrinum*, $F(2, 24) = 39.89$, $p = 0.000$, there was a statistically significant difference between the untreated sample ($M = 31789$, 95% CI [7580, 27538]) and the 100ppm sample ($M = 5264$, 95% CI [1334, 4848]), as well as a difference between the untreated and the 150ppm sample ($M = 5264$, 95% CI [1255, 4561]) (Fig. 1). The Kruskal-Wallis test ($H = 17.39$, $p = 0.000$) showed that that data rejected the null hypothesis and there is a significant difference between the untreated and treated samples. The median ranking showed 23.0 for untreated, 9.8 for 100ppm, 9.2 for 150ppm. For the second treatment of *M. peregrinum*, the ANOVA test, $F(2, 24) = 17.09$, $p = 0.000$ showed that there was a statistically significant difference between the untreated ($M = 21444$, 95% CI [3118, 11326]) and 100ppm sample ($M = 12649$, 95% CI [2909, 10571]) and the untreated and 150ppm sample ($M = 10118$, 95%

CI [1872, 6801]) (Fig. 1). The null hypothesis was rejected in the Kruskal-Wallis test ($H = 15.74$, $p = 0.000$), showing that there was a statistically significant difference between the untreated and treated samples with median rank of 22.2 for untreated, 12.2 for 100ppm, and 7.7 for 150 ppm.

Hydrogen Peroxide

The growth of *M. gordonae* was not statistically significantly different when treated with 1.5% and 3% hydrogen peroxide (Fig. 2). The One-Way ANOVA, $F(2,24) = 1.68$, $p = 0.207$, showed no statistically significant difference between the untreated ($M = 65.56$, 95% CI [40, 144]), 1.5% ($M = 133.33$, 95% CI [91, 30]), and 3% ($M = 56.67$, 95% CI [34, 123]) samples (Fig. 2). The Kruskal-Wallis Test ($H = 1.12$, $p = 0.572$) showed that the data failed to reject the null hypothesis and there was no statistical significance between the untreated and treated samples. The median rank showed 16.1 for 1.5%, 13.8 for 3%, and 13.8 for untreated.

M. peregrinum also did not show a statistically significant difference in growth when treated with 1.5% and 3% hydrogen peroxide (Fig. 2). The results of the One-Way ANOVA for *M. peregrinum*, $F(2, 24) = 1.05$, $p = 0.364$, showed that there was no statistically significant difference between the untreated ($M = 2760$, 95% CI [841, 3055]), 1.5% ($M = 2602$, 95% CI [461, 1677]), and 3% ($M = 2098$, 95% CI [519, 1888]) samples (Fig. 2). The data failed to reject the null hypothesis, showing that there was no statistically significant difference between the untreated and treated samples in the

Kruskal-Wallis test ($H = 5.63$, $p = 0.060$). The median rank showed 16.9 for untreated, 16.2 for 1.5%, and 8.9 for 3%.

Iodine

The treatment resulted in the complete killings of CFU/mL of both *M. gordonae* and *M. peregrinum* (Fig. 3). The One-Way ANOVA test for *M. gordonae*, $F(1, 16) = 15.56$, $p = 0.001$, showed that there was a significant difference between the untreated control ($M = 352.2$, 95% CI [162, 541]) and the 100ppm sample ($M = 0$, 95% CI [0,0]) (Fig. 3). The Kruskal- Wallis test ($H = 7.74$, $p = 0.005$), failed to reject the null hypothesis and there is no statistically significant difference between the untreated and 100ppm samples. The median rank showed 13.0 for untreated and 6 for treated. The ANOVA testing for *M. peregrinum*, $F(1, 16) = 3.85$, $p = 0.067$, showed that there was no statistically significant difference between the untreated control ($M = 25.56$, 95% CI [-2, 53]) and the 100ppm sample ($M = 0$, 95% CI [0,0]) (Fig 3). The Kruskal- Wallis test ($H = 1.42$, $p = 0.223$), showed that the null hypothesis was rejected and that there was a statistically significant difference between the samples. The median rank showed 11.0 for the untreated sample and 8.0 for the 100ppm sample.

Discussion

The sodium hypochlorite treatment showed that there was a no statistically significant difference observed between the control and the treatments for *M. gordonae*. There as a 64-fold germicidal efficacy shown for the 100ppm treatment and a 230-fold germicidal efficacy for the 150ppm treatment of the first *M. peregrinum* trial. There was also a 2-fold germicidal efficacy seen for the 100ppm treatment and a 3-fold germicidal efficacy seen for the 150ppm treatment of the second *M. peregrinum* trial. Unfortunately, this is not a very effective treatment since there was little impact on the CFU/mL of *M. gordonae*. There was also no complete killing observed, which would have been ideal.

A study done by Gray et al. (2013), showed that there are mechanisms in bacterial cells that protect against the breakdown of a cell by compounds that contain chlorine. They concluded that there is a chaperone protein, Hsp33, in the cell that can help to protect the cell against chlorine damage. This protein, along with phosphate chaperones, are able to aid in the refolding of proteins after a period of stress has seized, allowing the cell to survive (Gray et al. 2013). It is possible that this is how the bacteria is able to survive the higher concentrations of bleach treatment. The treatment would be more effective if it was applied for longer than 10 minutes. At this low a concentration, complete killings can be observed after an hour of exposure (Mainous et al. 2005). However, fish eggs cannot be exposed for longer than 10 minutes, and as such, bleach is not the effective treatment and is not recommended as a disinfection protocol if complete elimination of mycobacteria is the goal. It is worthwhile noting that

there was some germicidal effect and in absence of a better method, bleach provides some reduction in bacterial load.

The hydrogen peroxide treatments did not result in any germicidal efficacy. Some *Mycobacterium* species have been reported as catalase positive. That is, they possess the enzyme catalase, which can break down peroxide. Although there was no catalase reaction observed (as evidenced by the formation of bubbles when treated with peroxide), further analysis should be done in order to determine whether or not there is another metabolic factor preventing the efficacy of the hydrogen peroxide. A protein in the human pathogen *Mycobacterium tuberculosis* called *oxyR* is responsible for helping the bacteria respond to the stress of hydrogen peroxide (Sherman, 1995). Despite the differences between the species, it is possible that the fish-species of *Mycobacterium* contain this protein, or a homologue of the protein, which is responsible for the lack of germicidal efficacy observed. The protein would have a similar effect on the bacteria as catalase and would be able to help restore the cell after the treatment (Sherman et al. 2013).

Iodine proved to be the most effective disinfectant against *M. gordonae* and *M. peregrinum*. The treatment resulted in total killings of the bacteria on each of the dilution sets, which none of the other treatments were able to achieve. Although complete killings were observed, the statistical analysis of the *M. peregrinum* stated that there was no statistical significance observed. An additional trial must be performed with this species in order to confirm that complete killings are in fact observed and to determine if a statistically significant difference can be achieved. Errors

in determining the initial concentration of bacteria may have occurred, which resulted in low plate counts for the untreated control as well.

Additional studies must be performed with the iodine treatments on the eggs themselves. There have not been many studies performed on the effects of the treatment on the eggs. A study was performed by Peck (2004) using the eggs of Atlantic cod and haddock, but not zebrafish. The iodine treatment, held at a pH between 7 and 9, did not result in statistically significant differences in the percentage of eggs that hatched after the treatment was applied when compared to untreated eggs (Peck, 2004). This provides promising results to determine if it is possible to use iodine as a treatment for *Mycobacterium*. In addition, more treatments should be performed at lower concentrations of iodine in order to decrease the exposure to the chemical without compromising the efficacy of the results.

Sodium hypochlorite only had a germicidal effect on *M. peregrium* in this experiment. Hydrogen peroxide did not have a germicidal efficacy on either of the species used in this study. Unfortunately, neither of the treatment methods provided complete killings of CFU in the treated sample, so these are not efficient methods for disinfection in fish facilities. The iodine treatment not only had a germicidal efficacy on both of the species, but there were complete killings of CFU. As a result, more testing should be done in order to determine the feasibility of this treatment in fish facilities, such as treatment on eggs and tanks, in order to determine if this is the standard protocol for the disinfection of *Danio rerio*.

List of Figures

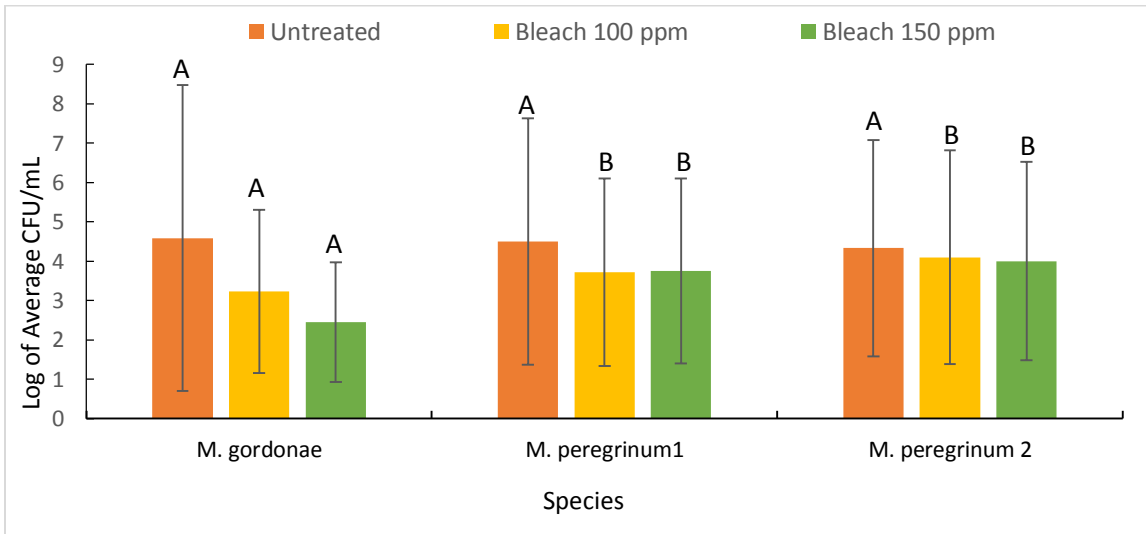


Figure 1. Bar Graph representing the log of average CFU/ml from each bleach treatment of *M. gordonae* and *M. peregrinum*. Error bars showing SE of each data set with a confidence interval of 95%. One-way ANOVA testing compared the untreated and treated samples of each species. Post-hoc Tukey analysis is represented by the letters “A” and “B”.

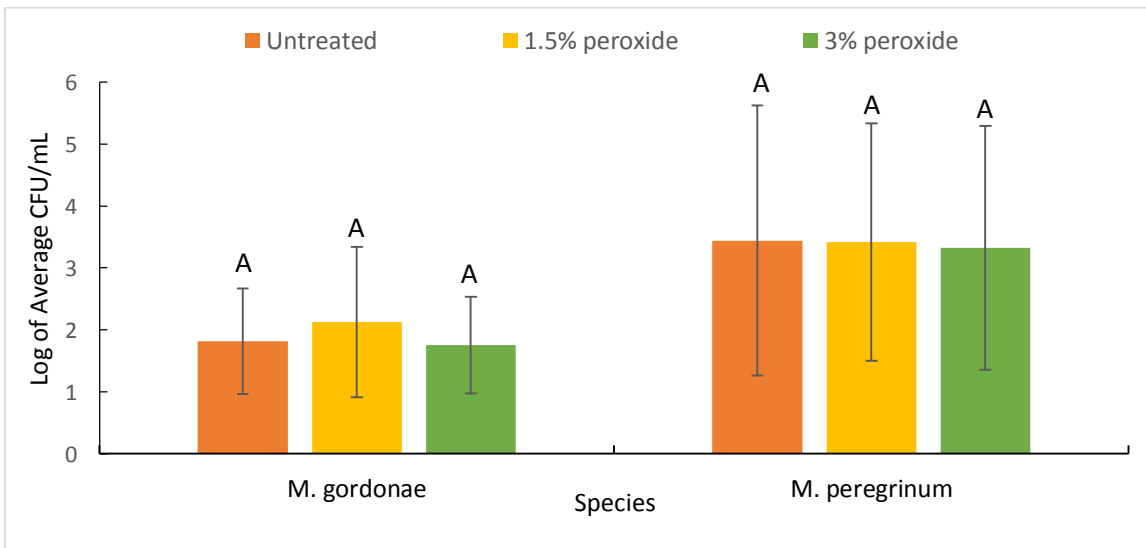


Figure 2. Bar Graph representing the log of average CFU/ml from each H₂O₂ treatment of *M. gordonae* and *M. peregrinum*. Error bars show SE with a confidence interval of 95%. One-way ANOVA compared treatment of each species. Post-hoc Tukey results are indicated by letters “A” and “B”.

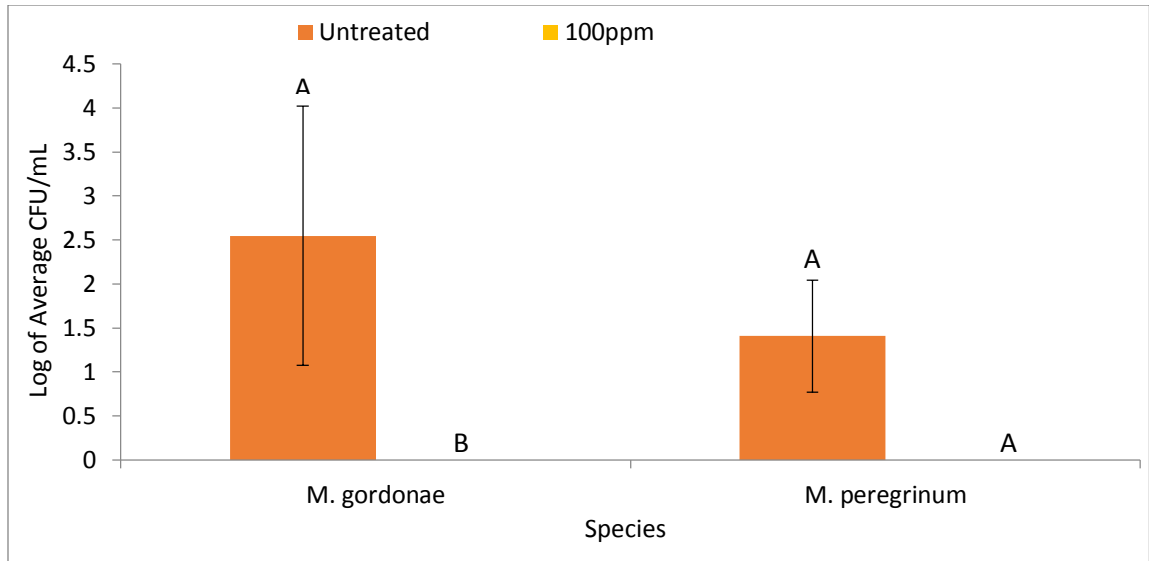


Figure 2. Bar Graph representing the log of average CFU/ml from each Iodine treatment of *M. gordonae* and *M. peregrinum*. Error bars showing SE with a confidence interval of 95%. One-way ANOVA compared the treated and untreated samples of each species. Post-hoc Tukey results are indicated by letters “A” and “B”.

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