The Effect of Bleach, Hydrogen Peroxide, and Iodine on *Mycobacterium* Species

by

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Abstract

The laboratory zebrafish, *Danio rerio*, is a model organism used in numerous area of biological research, and is also subject to its own diseases. A common disease in zebrafish is mycobacteriosis, caused by *Mycobacterium* species. Elimination of *Mycobacterium* spp. is crucial in both preserving research studies as well as preventing the spread of the zoonotic pathogens to humans. Because of the common practice of exchanging fish between facilities, as well as a lack of a standard protocol for disinfection, these infections have become commonplace. In order to investigate the efficacy of disinfectants, this study tested the germicidal effect of sodium hypochlorite, hydrogen peroxide, and iodine on *Mycobacterium chelonae* and *Mycobacterium abscessus*. Concentrations of 100 ppm and 150 ppm sodium hypochlorite (from bleach),1.5% and 3% hydrogen peroxide, and 100 ppm iodine were tested. Statistically significant decreases in growth were observed in the treatments of *M. abscessus* with hydrogen peroxide, sodium hypochlorite, and iodine and *M. chelonae* with hydrogen peroxide and iodine. When treated with 1.5% hydrogen peroxide, *M. abscessus* showed a 14-fold germicidal effect and *M. chelonae* showed a 6–fold germicidal effect when treated with 3% hydrogen peroxide. When treated with 150 ppm sodium hypochlorite*, M. abscessus* showed an 11–fold germicidal effect. When treated with 100 ppm iodine, both *M. chelonae* and *M. abscessus* were completely eliminated. Therefore, the current protocol for the application of bleach to zebrafish eggs cannot be relied upon for complete disinfection of mycobacteria, but the use of iodine shows promise as the basis for a new method.

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Introduction

Mycobacteriosis is a common problem in populations of zebrafish, and can be detrimental to research studies. The disease, often referred to as “fish tuberculosis,” is characterized by wasting and granulomatous inflammation in the zebrafish (Francis-Floyd, 2011). Granulomas can be visible grossly or microscopically and are characterized by a thick capsule, necrotic center, and surrounding epithelioid cells (Francis-Floyd, 2011). Often, they are found in blood filtering organs the spleen, liver, and kidney (Decostere, Hermans, & Haesebrouck, 2004), but disseminated infections involving most tissues have been reported for more pathogenic species such as *Mycobacterium haemophilum* (Whipps et al., 2007). Diagnosis of the disease is the first step to control. Mycobacteriosis can be either acute or chronic; although the acute form is more rare, it results in rapid death after the exhibition of few clinical signs (Decostere et al., 2004). The signs displayed by the more common form may emerge years after infection; zebrafish may act listless and become solitary, or show emaciation and skin ulcerations (Decostere et al., 2004). The presence of reproductive problems can be suggestive of the disease as well (Francis-Floyd, 2011).

Diagnosis is typically made with the observation of acid fast positive rods after staining of fresh tissue or histological sections, and is indicative of *Mycobacterium* species (Francis-Floyd, 2011). Culture on BD™ Middlebrook agar is also recommended for diagnosis, where mycobacteria form. In addition, the bacteria are gram positive, non-motile, and non-spore forming (Francis-Floyd, 2011). Molecular testing can be used to validate a presumptive diagnosis and has been

shown to correlate well to culture data (Whipps et al., 2008). A tough, waxy cell wall envelops the *Mycobacteria*, which enables them to survive many attempts to eliminate them from systems (Francis-Floyd, 2011). The organisms are facultative pathogens that can grow in surface biofilms, and variation among host genetics can lead to variation in disease pathology (Whipps et al., 2012). The pathogenicity of the strain of *Mycobacterium* has been correlated with environmental conditions and quality of the host (zebrafish) immune system (Whipps et al., 2012). A study conducted by Ramsay et al. (2009) showed that zebrafish with high stress levels were associated with high levels of *M. chelonae* infections and mortality.

Because of unsanitary handling procedures among workers as well as the lack of proper upkeep of fish tanks, *Mycobacterium* species spread not only between fish, but to humans and other species as well. It has been found that humans and zebrafish can be infected by the same strain of *Mycobacterium* (Whipps et al., 2012). Mycobacteriosis generally occurs in animals that have weakened immune systems, have been treated regularly with corticosteroids, or that have been raised in substandard conditions (Francis-Floyd, 2011). In humans, this zoonotic disease, called "fish handlers' disease", results in localized granulomas (Mainous & Smith, 2005). It generally occurs in zebrafish handlers with broken skin that have been exposed to infected fish (Francis-Floyd, 2011). The disease rarely progresses to a systemic state, or to forming lesions within muscles and tendons (Francis-Floyd, 2011). Humans infected with Mycobacteriosis can combat the infection with antibiotics, but are instructed to avoid corticosteroids (Francis-Floyd, 2011). The diagnosis of disease in humans can be difficult to obtain; even when a culture is

taken, many times the mycobacteria will not grow because the hospital incubators are set at 37°C, higher than the optimal growing temperature for these species (Decostere et al., 2004). In response to these challenges, a PCR based technique has been developed for the identification of these strains (Decostere et al., 2004). *M. marinum* has been treated in vitro in humans by ethambutol, rifampicin, streptomucin, trimethoprim-sulfamethoxazole, tetracyclines, and more, with no treatment showing significantly more success than the others (Decostere et al., 2004).

The zoonotic potential of mycobacteria alone makes study of their growth, disinfection, and pathogenesis essential, but it is only one of several reasons to prioritize these pathogens (Whipps et al., 2012). Zebrafish are model organisms for cancer research and developmental biology, and are useful to these fields because of their regenerative properties, cheap cost, and human-like immune system. An outbreak of Mycobacteriosis in a zebrafish research laboratory can be crippling to research studies. The fish will either be killed or be immunologically compromised by the disease; in either case, the results that would have been obtained using the fish are lost. The loss of these results and zebrafish means a loss of time and economic investment.

There is no available vaccine for treatment of Mycobacteriosis, rendering quarantine, disinfection, and euthanasia as the most likely treatment options (Francis-Floyd, 2011). Recirculation of water, high organic loads, and crowding of fish in holding tanks enhances the longevity of the disease, in turn making it difficult to eradicate (Francis-Floyd 2011). The species grow optimally in conditions found

in zebrafish systems: at warm temperatures with low dissolved oxygen content, low pH, and high levels of soluble zinc, fulvic acid and humic acid (Francis-Floyd, 2011). The spread of Mycobacteriosis occurs through different agents; a main, problematic source of the spread of infection involves the decomposition of infected fish in which the bacteria are released from the internal organs (Francis-Floyd, 2011). Likewise, the disease can be spread from mother to offspring, meaning the practice of removing infected fish from the system is vital to limiting its spread (Francis-Floyd, 2011). The consumption of contaminated feed or detritius and the cannibalism of infected fish are also agents of epidemiology for the disease (Decostere et al., 2004). Previous studies have also shown that other invertebrate species act as reservoirs and are able to transmit mycobacteria to zebrafish in enclosed environments (Decostere et al., 2004).

Various species of *Mycobacterium* appear in zebrafish, and are summarized by Whipps et al. (2012). Species include *M. marinum, M. fortuitum,* and *M. chelonae. Mycobacterium marinum* is highly pathogenic in zebrafish populations (showing mortality rates from 30 to 100%), and the other strains vary in pathogenicity, reinforcing the importance of strain identification in the management of Mycobacteriosis (Francis-Floyd, 2011). The mechanisms of action of the pathogens are not well known, but it has been found that *M. marinum* exhibits mucinase activity while *M. chelonae* and *M. fortuitium* exhibit lipase and RNAse activity (Decostere et al., 2004). In addition, *M. marinum* is able to replicate in macrophages, a characteristic shared with *M. tuberculosis*, implying that this strain could possibly be used as a model pathogen for research (Decostere et al., 2004). *M. chelonae* and

M. abscessus have been associated with a moderate mortality rate and low-level, chronic disease (Whipps et al., 2012). *M. chelonae* is mostly an opportunistic pathogen, but, like *M.abscessus*, varies in its pathogenicity (Whipps et al., 2012).

Due to the ability of *Mycobacterium* spp. to persist in aquaculture, disinfection procedures have been tested with varying levels of success. It is standard protocol to use chlorine bleach to disinfect not only equipment and tanks, but zebrafish and eggs as well. If an already infected population is being maintained, there should be no transfers of fish into or out of the system, equipment should be completely disinfected, and environmental conditions should be kept at an optimum for the fish, not the bacteria (Francis-Floyd, 2011). A study by Mainous and Smith (2005) investigated the efficacy of seven common disinfectants against mycobacteria through disinfection of the water, aquatic surfaces, and aquarium equipment. Due to the high cell wall lipid content and hydrophobicity, *mycobacteria* are considered second only to bacterial spores in measures of resistance (Mainous & Smith, 2005). The impacts of the disinfectants were effected by temperature, pH, contact time, and disinfectant concentration (Mainous & Smith, 2005). Ethyl alcohol (50% and 70%), Lysol, and Clidox-S were found to be the most potent disinfectants (Mainous & Smith, 2005).

The objectives of this study were to establish a standard protocol for complete elimination of mycobacteria from zebrafish populations, determine the germicidal efficacies of bleach, hydrogen peroxide, and iodine in doing so, and determine a reproducible procedure for testing the effectiveness of disinfectants. It was expected that the addition of 100 ppm and 150 ppm bleach to isolates of

mycobacteria would result in a statistically significant decrease in growth in *M. chelonae* and *M. abscessus*. Likewise, it was hypothesized that the addition of 1.5% and 3% hydrogen peroxide to isolates would result in a statistically significant decrease in growth, as would the addition of 100 ppm iodine.

Methods

Cultures of *M. chelonae* and *M. abscessus* were maintained on BD™ Middlebrook agar plates supplemented with OADC at 28°C prior to being transferred into 3 mL of BD™ Middlebrook broth supplemented with OADC approximately a week before testing. The isolates tested were *M. chelonae* and *M. abscessus*. McFarland standards were used to obtain a rough estimate of the colony forming units (CFU) per mL of the isolate in the broth. Using this estimate, the broth culture was diluted to an approximate concentration of either 10^4 CFU/ mL (Hydrogen peroxide and iodine treatments) or 10^6 CFU/ mL (Bleach treatments) in 3 mL of sterilized water. The diluted culture was used to prepare three 1 mL aliquots in separate 1.5 mL collection tubes. Disinfectant testing was performed at room temperature.

Bleach Treatment- Bleach concentration was measured using an ExStik® chlorine meter. A working concentration of roughly 1,000 ppm sodium hypochlorite was prepared in sterilized water immediately before use. Water was used as a solvent, to reflect the common practice used in zebrafish facilities.

The samples at a starting concentration of 10^4 CFU/ mL were tested independently; the first sample acted as a positive control, the second sample was treated with 100 ppm bleach, and the third sample was treated with 150 ppm

bleach. Using a relative volume calculation, the amount of bleach necessary for each treatment was determined. The bleach was added directly to the 100 ppm and 150 ppm tubes using a micropipette, and BD™ Middlebrook broth was added to the positive control. After 10 minutes of exposure, the same amount of sodium thiosulfate was added to the samples as the bleach and broth previously added.

The control sample was diluted in sterile water to concentrations of 10^3, 10^2, and 10^1 CFU/ mL in sterile water, and 100 µL of each dilution was plated in triplicate on BD™ Middlebrook agar plates. The 100 ppm and 150 ppm treatments were diluted to concentrations of 10^3 and 10^2 CFU/mL, and the original 10^4 CFU/mL concentration sample as well as the dilutions were plated in triplicate. All plates were stored in an incubator at 28°C. After one week, the number of CFU per plate was counted.

In order to test the efficacy of the bleach further, we also conducted trials using 500 ppm bleach. To two 1.5 mL collections tubes, 0.5 mL of undiluted broth culture of *M. chelonae* was added. One tube was treated with a final concentration of 500 ppm bleach, and broth was added to the tube acting as the positive control. From each tube, 100 µL of the samples were plated on BD™ Middlebrook agar using a sterile spreader and the plates were incubated at 28°C and colonies counted after 1 week.

Hydrogen Peroxide and Iodine Treatments- The 1.5 mL collection tubes containing the positive control sample and low and high treatment samples at a concentration of 10^6 CFU/ mL were centrifuged at 3,000 G for 10 minutes. Following the centrifugation, the supernatant was discarded from the tubes. To the positive

control tube, 1 mL of sterile water was added, to the low treatment tube, 1 mL of 1.5% hydrogen peroxide was added, and to the high treatment tube, 1 mL of 3% hydrogen peroxide was added. The original hydrogen peroxide solution was at a concentration of 3%, and the 1.5% hydrogen peroxide solution was prepared in sterilized water immediately before use. When testing iodine, rather than the addition of hydrogen peroxide to the treatment tubes, 1 mL of 100 ppm iodine was added (there was only one treatment group). The 100 ppm iodine solution was prepared by diluting a 10,000 ppm iodine solution in sterilized water, and recording the pH of the disinfectant after the addition. Once the disinfectants were added, the tubes were vortexed briefly. After an exposure time of 5 minutes, the samples were centrifuged at 5,000 G for 30 seconds, and the resulting supernatant was again discarded. The pellet formed was suspended in 1 mL of water.

The control and treatment samples were diluted to concentrations of 10^5 and 10^4 CFU/mL in sterile water, and 100 µL the original 10^6 CFU/mL concentration sample as well as the dilutions were plated in triplicate after a brief vortexing. All BD™ Middlebrook plates were stored in an incubator at 28°C. After one week, the number of CFU/ .1 mL was counted.

Statistical Analysis- Statistical tests were performed using the plate counts after the treatments to determine if growth differences between control and treatment groups were statistically significant. One-way ANOVA was first used to test for significance using Minitab® software. Using the same software, further analysis using the Kruskal-Wallis test was performed. The Kruskal-Wallis test does not assume a normal distribution of residuals, and was used to confirm ANOVA results.

Lastly, the germicidal efficacy of each disinfectant on each *Mycobacterium* isolate was found by comparing the amount of growth between the control and treatment groups.

Results

The treatment using bleach resulted in a statistically significant decrease in growth between the treated and untreated groups of one trial of *M. abscessus* ($F(2,24)= 5.14$, $p=0.014$), but showed no statistically significant decrease in growth for *M. chelonae* ($F(2,24)= 2.14$, $p=0.139$) and a second trial of *M. abscessus* ($F(2,24) = 1.10$, $p=0.348$) (Figure 1). When treated with 150 ppm sodium hypochlorite*, M. abscessus* showed an 11–fold germicidal effect in the trial that showed a significant decrease in growth. The results of a Kruskal-Wallis test were significant for one trial of *M. abscessus* ($H=7.75$, 2 d.f., $p=0.021$), and not significant for *M. chelonae* ($H=3.47$, 2 d.f., $p=0.177$) and the second trial of *M. abscessus* ($H=.16$, 2 d.f., $p=0.924$) (Table 1).

The hydrogen peroxide treatment resulted in a statistically significant decrease in growth between the treated and untreated groups of *M. chelonae* ($F(2,24)= 3.77$, $p=0.038$) and *M. abscessus* ($F(2,24)=11.61$, $p= 0.00$) (Figure 2). *M. chelonae* showed a 6–fold germicidal effect when treated with the 3% solution. When treated with 1.5% hydrogen peroxide*, M. abscessus* showed a 14-fold germicidal effect. The results of a Kruskal-Wallis test were significant for *M. chelonae* ($H=6.74$, 2 d.f., $P=0.034$) and *M. abscessus* ($H=18.23$, 2 d.f., $P=0.00$) (Table 2).

The last disinfectant tested was a 100 ppm iodine solution. Statistically significant decreases in growth were observed between the treated and untreated

groups for *M. chelonae* (F(1,16)= 12.84, p=0.002) and *M. abscessus* (F(1,16)= 849.99, p=.000) (Figure 3). The treatment of the two strains with the iodine solution resulted in complete killing of the *mycobacteria*. The results of a Kruskal-Wallis test were significant for *M. chelonae* (H=8.09, 1 d.f., P=0.004) and *M. abscessus* (H=14.61, 1 d.f., P=0.00) (Table 3).

Discussion

The untested, yet widely used protocol of bleaching zebrafish eggs for 10 minutes at 50 ppm had not been verified as a viable solution for disinfection (Whipps et al., 2012). This study found bleach and hydrogen peroxide to be useful for only sometimes statistically significant decreases in growth of *mycobacteria*, but not complete disinfection. The low concentration of bleach used (100ppm and 150 ppm) in addition to a relatively short contact time (10 minutes) are precautions taken to ensure the viability of the zebrafish eggs after treatment, but unfortunately are unable to curb growth of *M. chelonae* and *M. abscessus*. Prior research found that chlorine compounds required an exposure time of 10 to 30 minutes to effectively kill the bacteria, while the other disinfectants varied in their efficacy (Mainous & Smith, 2005). The sodium hypochlorite required 10 minutes of exposure to reduce the concentration of bacteria, and 20 minutes to eliminate the species (Mainous & Smith, 2005).

Hydrogen peroxide, at 1.5% and 3% treatment concentrations with a 5 minute contact time, had the same shortcomings. In a separate study, cod and haddock eggs were treated with either hydrogen peroxide (3%), polyvinylprrolidine iodine (1%), sodium hypochlorite (0.1%), or an antibiotic solution (0.005%) (Peck

et al., 2004). The study showed that treatment of eggs with these disinfectants correlated with a greater hatch rate for the antibiotic solution and PCP iodine, but treatment with hydrogen peroxide or PVP iodine had variable results (Peck et al., 2004). Bacterial growth was exhibited on all media plates containing treated eggs, meaning no disinfectant completely eliminated bacteria from the eggs, although the bleach treatment resulted in the least amount of growth (Peck et al., 2004).

Previous research has examined the modes of action of the three disinfectants. The treatment of microbial species with bleach is most likely to kill the organism because of causing a reaction that damages multiple cellular components (Gray et al., 2013). The cause of death of bacterial species in unknown, and may vary based on species differences (Gray et al., 2013). In bleach treatments that are effective in killing bacteria, it has been hypothesized that the death was the result of loss of ATP and DNA replication as well as lack of transport of proteins across inner membranes in the bacteria (Gray et al., 2013). Meanwhile, hydrogen peroxide acts as an oxidant when coming into contact with bacterial species, and produces hydroxyl free radicals that attack lipids, proteins, and DNA (McDonnell & Russell, 1999). The hydrogen peroxide may more readily seek out double bonds and sulfhydryl groups to react with (McDonnell & Russell, 1999). It is likely that the bleach and hydrogen peroxide treatments were not effective because of the low concentration of the disinfectants used during treatment.

Iodine is effective as a disinfectant because it penetrates into bacteria and attacks proteins, amino acids like cysteine and methionine, nucleotides, and fatty acids (McDonnell & Russell, 1999). Iodophors link free iodine with a carrier

molecule, so the reactive nature of iodine is maintained (McDonnell & Russell, 1999). The success of iodine in the treatment of bacterial species has been seen in organisms other than mycobacteria. In a study by Berkelman et al. (1982), diluted povidine-iodine solutions (i.e., a 1% solution compared to a 10% solution) were bactericidal after just seconds of exposure against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas cepacia*, and *Streptococcus mitis*. In an additional study that examined the efficacies of commonly used disinfectants on the bacteria of Rainbow Trout eggs, iodine was found to be a more effective treatment than hydrogen peroxide, salt, and formalin (Wagner et al., 2008). It was also noted that increasing the concentration of the iodine for treatments did not result in a dramatic decrease in bacterial growth (Wagner et al., 2008). In conjunction with determining the germicidal effects of the iodine, the study showed that iodine treatments would not harm the fish eggs after water hardening (Wagner et al., 2008).

This study tested the disinfectants on strains on *M. chelonae* and *M. abscessus* regrown onto agar plates, and the treatments were directly applied to the species growing in broth. Therefore, the methods for treatment used in this experiment have not been tested on zebrafish eggs, zebrafish themselves, or on aquatic systems. As a result, future studies should examine the effects of the disinfectants, and in particular the iodine, in practical applications of zebrafish research.

Conclusions

This study showed that due to limited germicidal effects, the current protocol for bleaching zebrafish eggs cannot be relied upon for complete disinfection. Likewise, hydrogen peroxide, having similarly limited effects to bleach, is not a

suitable substitute for bleach in the treatment of mycobacteria. The iodine solution was the most successful disinfectant, resulting in complete disinfection of both *Mycobacterium* spp. using an easily reproducible and efficient protocol. Despite its low concentration (100 ppm) and short contact time (5 minutes), it shows promising results for future disinfection treatments.

Sources Cited and Consulted

Berkelman, R.L., Holland, B.W., & Anderson, R.L. (1982). Increased Bactericidal Activity of Dilute Preparation of Povidone-Iodine Solutions. *Journal of Clinical Microbiology*, 15: 635-639. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC272159/pdf/jcm00153-0121.pdf

Decostere, A., Hermans, K., & Haesebrouck, F. (2004). Piscine mycobacteriosis: a literature review covering the agent and the disease it causes in fish and humans. *Veterinary Microbiology*, 99: 159-166, doi: 10.1016/j.vetmic.2003.07.011

Francis-Floyd, R. (2011). Mycobacterial infections of fish. *Southern Regional Aquaculture Center*, Publication 4706.

Gray, M.J., Wholey, W., & Jakob, U. (2013). Bacterial Responses to Reactive Chlorine Species. *Annual Review of Microbiology, 67:* 141-60, doi: 10.1146/annurev-micro-102912-142520

Mainous, M.E., & Smith, S. A. (2005). Efficacy of Common Disinfectants against *Mycobacterium marinum*. *Journal of Aquatic Animal Health,* 17: 284-288, doi: 10.1577/H04-051.1

McDonnell, G., & Russell, A.D. (1999). Antiseptics and Disinfectants: Activity, Action, and Resistance. *Clinical Microbiology Reviews,* 12(1): 147-149. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC88911/

Peck, M.A., Buckley, L.J., O'Bryan, L.M., Davies, E.J., & Lapolla, A.E. (2004). Efficacy of egg surface disinfectants in captives pawning Atlantic cod *Gadus morhua* L. and haddock *Melanogrammus aeglefinus* L. *Aquaculture Research,* 35: 992-996, doi: 10.1111/j.1365-2109.2004.01119.x

Ramsay, J.M., Watral, V., Schreck, C.B., Kent, M.L. (2009). Husbandry stress exacerbates mycobacterial infections in adult zebrafish, *Danio rerio* (Hamilton). *Journal of Fish Diseases* 32:931-941.

Wagner, E.J., Arndt, R.E., Billman, E.J., Forest, A., & Cavender, W. (2008). Comparison of the Efficacy of Iodine, Formalin, Salt, and Hydrogen Peroxide for Control of External Bacteria on Rainbow Trout Eggs. *North American Journal of Aquaculture,* 70: 118-127, doi: 10.1577/A06-068.1

Whipps, C.M., Dougan, S.T., & Kent, M.L. (2007). *Mycobacterium haemophilum* infections of zebrafish (*Danio rerio*) in research facilities. *FEMS Microbiology Letters* 270:21-26.

Whipps, C.M., Matthews, J.L., & Kent, M.L. (2008). Distribution and genetic characterization of *Mycobacterium chelonae* in laboratory zebrafish (*Danio rerio*). *Diseases of Aquatic Organisms* 82:45-54.

Whipps, C. M., Lieggi, C., & Wagner, R. (2012). Mycobacteriosis in Zebrafish colonies. *Oxford Journals,* 53(2). Retrieved from http://ilarjournal.oxfordjournals.org/

Appendix

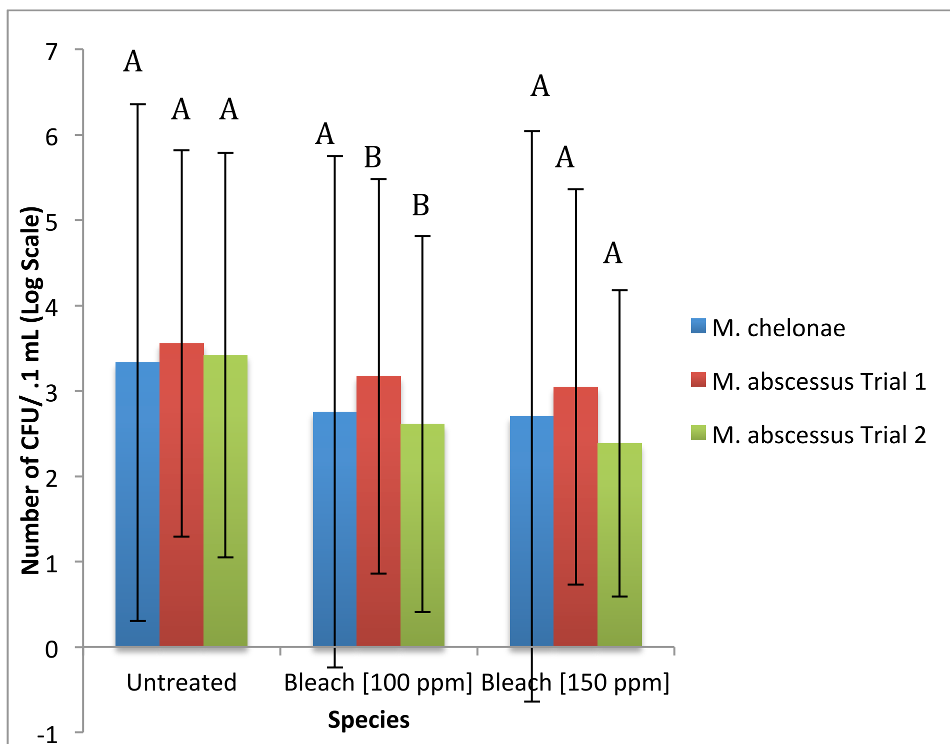

Figure 1. Statistically significant decreases in growth were seen in the treatments of one trial of *M. abscessus* with sodium hypochlorite. ANOVA: *M. chelonae* [F(2,24)= 2.14, p=.139], *M. abscessus* Trial 1 [F(2,24) = 5.14 , p=.014], *M. abscessus* Trial 2 [F(2,24) = 1.10, p=.348.] Bars are standard error.

Table 1. The results after analysis by the Kruskal-Wallis test. The null hypothesis stated that the distribution of colony forming units was the same among the treatments, while the alternative hypothesis stated that there was at least one difference in the distribution of the colony forming units among the treatments. The null hypothesis was rejected one trial of *M. Abscessus*, and there was a failure to reject the null hypothesis for *M. chelonae* and one trial of *M. abscessus*.

Species	P-value	Null Hypothesis
M. chelonae	0.177	Failure to reject
M. Abscessus	0.021	Rejected
M. Abscessus 2	0.924	Failure to reject

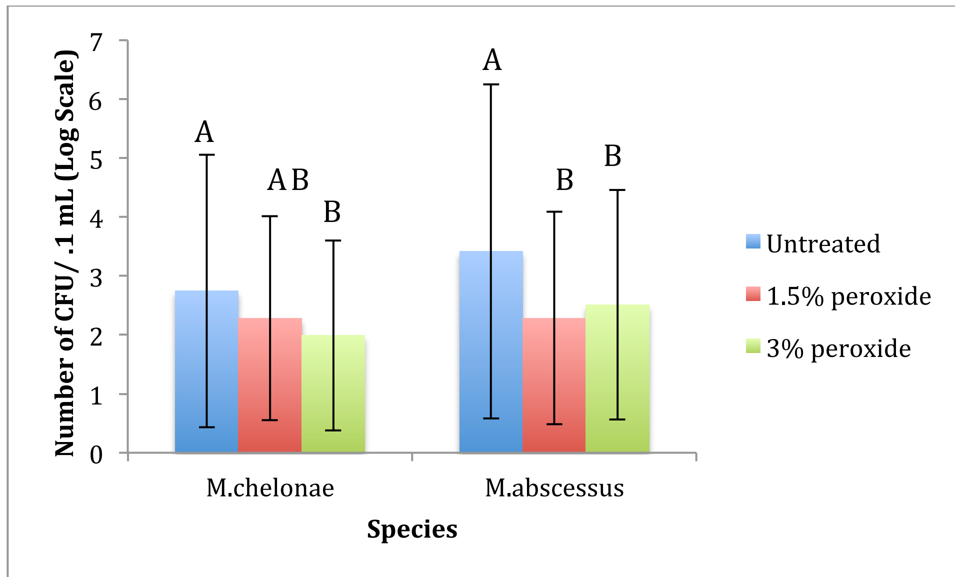

Figure 2. Statistically significant decreases in growth were seen in the treatments of *M. chelonae* and *M. abscessus* with hydrogen peroxide. ANOVA: *M. chelonae* [F(2,24)= 3.77, p=.038], *M. abscessus* [F(2,24)=11.61, p= 0.00.]. Bars are standard error.

Table 2. The results after analysis by the Kruskal-Wallis test. The null hypothesis stated that the distribution of colony forming units was the same among the treatments, while the alternative hypothesis stated that there was at least one difference in the distribution of the colony forming units among the treatments. The null hypothesis was rejected for *M. chelonae* and *M. abscessus*.

Species	P-value	Null Hypothesis
M. chelonae	0.034	Rejected
M. abscessus	0.00	Rejected

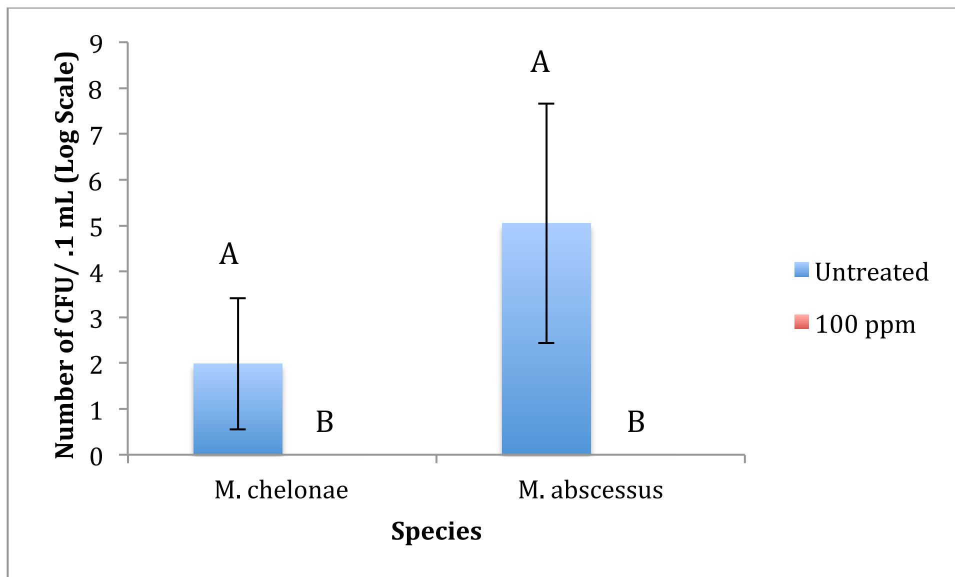

Figure 3. Statistically significant decreases in growth were seen in the treatments of *M. chelonae* and *M. abscessus* with iodine. ANOVA: *M. chelonae* [F(1,16)= 12.84, p=.002], *M. abscessus* [F(1,16)= 849.99, p=.000]. Bars are standard error.

Table 3. The results after analysis by the Kruskal-Wallis test. The null hypothesis stated that the distribution of colony forming units was the same among the treatments, while the alternative hypothesis stated that there was at least one difference in the distribution of the colony forming units among the treatments. The null hypothesis was rejected for *M. chelonae* and *M. abscessus*.

Species	P-value	Null Hypothesis
M. chelonae	0.004	Rejected
M. abscessus	0.00	Rejected