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CONTROLLING INFECTIOUS DISEASE IN LABORATORY
ZEBRAFISH (*DANIO RERIO*)

by

Carolyn T. Chang

Department of Environmental and Forest Biology

A dissertation
submitted in partial fulfillment
of the requirements for the
Doctor of Philosophy Degree
State University of New York
College of Environmental Science and Forestry
Syracuse, New York
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Abstract

C.T. Chang, Controlling infectious disease in laboratory zebrafish (*Danio rerio*). 202 pages, 8 tables, 22 figures, 2017. CSE style guide used.

Mycobacteriosis is a bacterial disease caused by *Mycobacterium* spp. that is common in captive, wild and research fish species. The overall goal of this thesis was to investigate mycobacteriosis in laboratory zebrafish in order to increase our understanding of this disease with the intention of influencing control and management practices. First, disease prevention through embryo disinfection was investigated. The effectiveness of several disinfectants were evaluated and povidone-iodine was identified as an effective disinfectant *in vitro*, it was then evaluated *in vivo* and showed minimal effects embryo health. Second, the potential of antibiotic treatment against mycobacteriosis in zebrafish was evaluated *in vitro* where tigecycline and clarithromycin were identified as key drug candidates. The tolerance and efficacy of both antibiotics were tested *in vivo* in adult zebrafish; where treatments were well tolerated and resulted in a decreased severity in establish mycobacterial infections. Last, natural modes of transmission were examined. Transmission between tank biofilms and zebrafish was demonstrated and the role mycobacterial biofilms play as both a reservoir for and source of *Mycobacterium* spp. in zebrafish tanks was identified. Finally, the role that live feeds play as a vector of mycobacterial transmission to zebrafish was tested and common zebrafish feeds are able to transmit *Mycobacterium* spp. to zebrafish. Altogether, these studies contribute to our current knowledge of mycobacterial infections in laboratory zebrafish and inform management. These results are also of use to other fish species as well.

Key Words: antibiotics; biofilm; disinfection; mycobacteria; *Mycobacterium* spp.; surveillance; zebrafish

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Chapter 1: Literature Review and Project Summary

1.1 The Zebrafish Model

The zebrafish (*Danio rerio*) is a freshwater minnow originating from the Indian subcontinent that was first introduced in pet stores in the 1970s (Schilling and Webb 2007). This fish gained attention from the research community as it is easy to house, can be bred year-round, produces large egg clutches, and its embryos are transparent and easily manipulated. Throughout 1980-1990, the zebrafish gained popularity as an experimental model and tools were developed to perform large-scale genomic analyses (Schilling and Webb 2007). Since then the usage of the zebrafish model has increased and there are an estimated 2000 zebrafish facilities in the United States (Holland, Lawrence et al. 2013). Zebrafish are now considered the “rising stars” of model-organism research, with an almost 60% increase in R01 awards from the National Institutes of Health for zebrafish studies between 2008 and 2015 (Gaind 2016).

Zebrafish are typically housed at high density in large rack systems of tanks where design incorporates principles of industrial aquaculture, laboratory rodent housing, and research genetics (Lawrence and Mason 2012). These systems are commercially available from several vendors and many research institutes are creating large centralized zebrafish facilities to be shared amongst researchers (Lawrence and Mason 2012). The sophistication of these systems and facilities ranges from simple recirculating systems to fully-automated systems that are accessed remotely (Lawrence and Mason 2012).

Despite the increased usage of zebrafish and their elaborate housing, an underlying understanding of the biology of the laboratory zebrafish remains to be fully elucidated. Peer-reviewed publications examining the optimal and experimentally validated husbandry and environmental conditions for laboratory zebrafish are outnumbered by those that describe research discoveries using zebrafish (Kent, Feist et al. 2009). Studies of the former are only

recently being conducted enabling researchers to understand the importance of environmental factors such as lighting (Blaser and Penalosa 2011), tank complexity (Carfagnini, Rodd et al. 2009), water quality (Ramsay, Feist et al. 2009), and zebrafish health on research results. Currently, these studies are primarily focused on zebrafish reproductive maturity and fecundity, as fast generation times and large clutches are required for the high-throughput experiments. An important aspect of zebrafish biology where our understanding has lagged behind the growth in zebrafish usage is the pathogens that affect laboratory zebrafish, especially those pathogens that result in subclinical infections that are more difficult to detect but may significantly alter research results (Kent, Feist et al. 2009). Additionally, since the current morbidity and mortality rates associated with the microorganisms causing disease in zebrafish have been considered “acceptably” low; researchers have become tolerant of their presence despite repercussions of infections (Kent, Feist et al. 2009).

This lag in pathogen discovery also occurred during the development of the laboratory mouse, which is now the most widely used and best understood animal model used (Care 1984). The last 100 years of laboratory rodent usage has been previously described as three periods with regard to the struggle against pathogens infecting these animals (reviewed in (Weisbroth 1996)). The first period (1880-1950) was the period of domestication where the usage of rodents as research subjects increased, and many of these original stocks harbored a variety of indigenous pathogens. Throughout this period progress was made in preventing and controlling these infections through improvements to sanitation, nutrition, environmental control and other aspects of husbandry. The second period (1960-1985) included the derivation of gnotobiotic strains where cesarean re-derivation was used to replace infected stocks with uninfected offspring, effectively eliminating pathogens not transmitted *in utero*. The final period (1980-1996) included

the eradication of indigenous murine viruses and reduction of other pathogens through practices including serological testing of animals for pathogen-specific antibodies and cesarean re-derivation of positive colonies. Through the progress made during these periods, as well as through ongoing studies on infectious diseases affecting laboratory rodents, there is a continued steady decline in microbial contamination in laboratory colonies. Routine prevention of infection in laboratory rodents requires: an understanding of susceptibility to disease and disease processes, timely testing, appropriate housing, rapid diagnosis and control of outbreaks (Jacoby and Lindsey 1998). Importantly, there is agreement throughout the scientific community that advances in laboratory animal health have improved the reliability of rodent-based research (Jacoby and Lindsey 1998).

The zebrafish community can draw from the progress made by the laboratory rodent community and seek direction from the steps taken to minimize disease in these laboratory animals. Compared to what is now known regarding laboratory rodent pathogens, much less is known about diseases affecting captive zebrafish. This need for knowledge has recently been identified (Kent 2012, Whipps 2012) and emphasized, as it is prudent to conduct research on healthy, disease-free animals where possible.

1.2 Disease in Laboratory Zebrafish

Thus far, progress has been made to identify common zebrafish pathogens and implement basic control measures to prevent disease spread. Microsporidiosis and mycobacteriosis are the most common diseases observed in laboratory zebrafish (Kent 2012). Microsporidiosis is caused by *Pseudoloma neurophilia* and affects 74% of the facilities submitting cases to the Zebrafish International Resource Center (ZIRC) diagnostic service (Figure 1-1) (De Kinkelin 1980, Zebrafish International Resource Center (ZIRC) Health Services 2017)((Kent 2012). Mycobacteriosis is caused by *Mycobacterium* spp. and affects approximately 40% of facilities

submitting cases to ZIRC diagnostic services (Figure 1-1) (Kent, Whipps et al. 2004, Kent 2012, Zebrafish International Resource Center (ZIRC) Health Services 2017). Less frequently than microsporidiosis or mycobacteriosis, laboratory zebrafish are affected by myxozoan and helminth parasites, fungal infections, and other non-acid fast bacterial species (Figure 1-1) (Kent 2012, Collymore, Crim et al. 2016, Zebrafish International Resource Center (ZIRC) Health Services 2017). Basic disease control and management measures broadly recommended include: quarantine of fish from outside facilities, equipment disinfection, UV water sterilization, embryo disinfection, and sentinel programs. Still lacking, is a deeper understanding of the manifestation of these diseases (i.e., beyond overt clinical signs), the mode of transmission of these pathogens (between both the environment and facilities as well as between facilities in the zebrafish research community), and the optimal species-specific method to control and eradicate these pathogens.

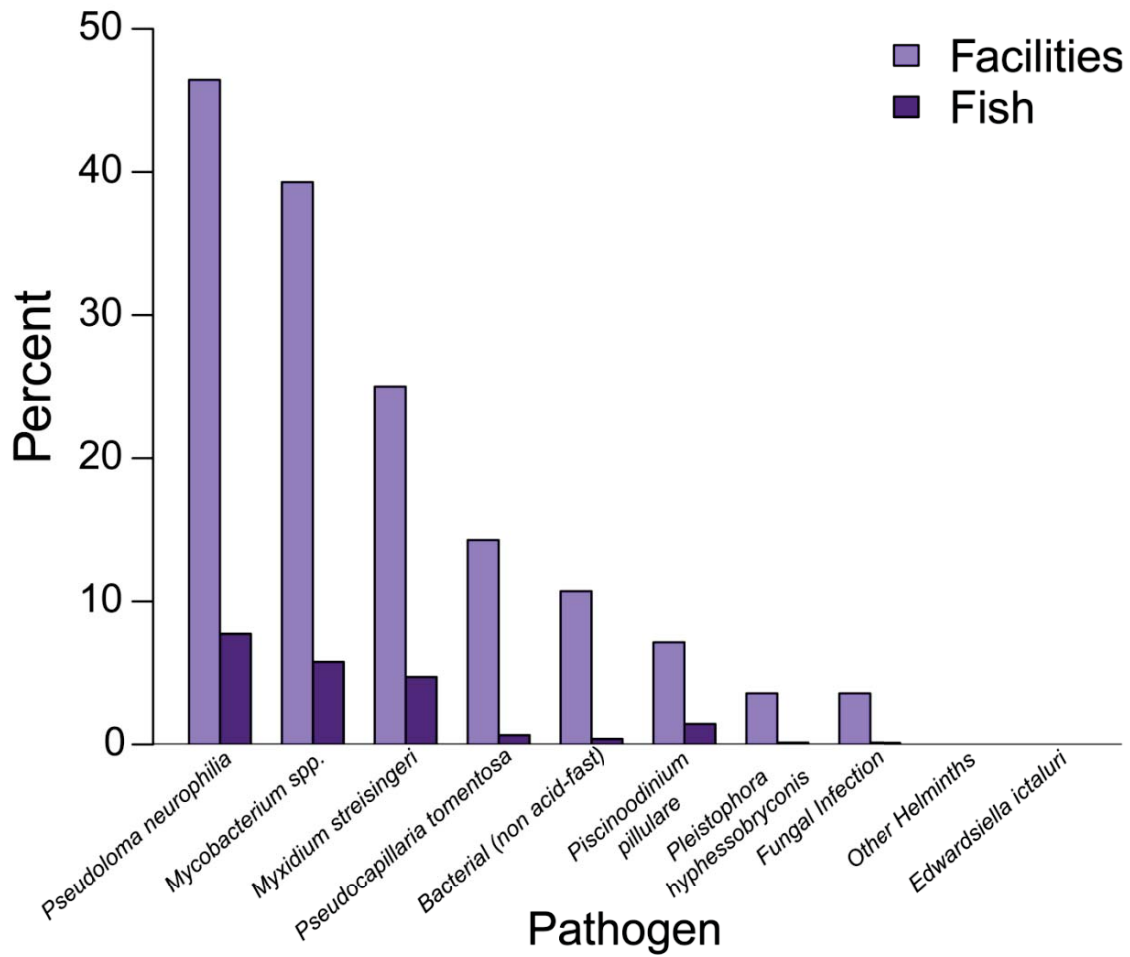


Figure 1-1. Pathogens present in laboratory zebrafish facilities based on a 2015 survey of cases submitted to the Zebrafish International Resource Center’s Diagnostic Services (Zebrafish International Resource Center (ZIRC) Health Services 2017).

1.3 The genus *Mycobacterium*

The genus *Mycobacterium* includes many medically important species that have impacted human and animal health. This genus includes the well-known *Mycobacterium tuberculosis*, the cause of tuberculosis (TB) which the World Health Organization (WHO) estimates infects almost one third of the human population (1.8 billion people) (www.who.org). Other species of *Mycobacterium* that are recognized to impact human health are *Mycobacterium leprae*, the causative agent of leprosy. Additionally, non-tuberculosis mycobacteria (NTM) are recognized to be a health threat to the health of immunocompromised individuals such as those infected with the human immunodeficiency virus (HIV). More than 185 *Mycobacterium* species have been validly published to date, according to the List of Prokaryotic Names with Standing in Nomenclature (<http://www.bacterio.net/mycobacterium.html>). Mycobacteria are Gram-positive, aerobic bacteria and the only member of the family Mycobacteriaceae in the order Actinomycetales. *Mycobacterium* species have high genomic GC content (62-70%) and a waxy, lipid-rich, cell wall that is regularly identified using acid-fast staining that allows for these acid-alcohol-fast rods to be differentiated from other bacteria.

Phylogenetically, based on 16S ribosomal gene sequences, mycobacteria are subdivided into fast/rapid and slow growing species (Saviola and Bishai 2006). The rapid growing mycobacteria (RGM) form colonies on selective media in less than 7 days compared to the slower growing mycobacteria (SGM) that require longer than 7 days. Historically there have been multiple attempts to group mycobacteria based on their ecology, first through dividing species into pathogenic or saprophytic groups (Saviola and Bishai 2006). Later, divisions into anonymous, typical or atypical, opportunist, non-tuberculosis, and other groups were proposed (Runyon 1959, Wolinsky 1979, Davidson 1981, Jenkins 1981, Grange 1991). While at first helpful, these groupings became confusing due to differences in interpretation of groups. Kazda

(2009) proposes three epithets for dividing mycobacteria: obligate pathogenic mycobacteria, potentially pathogenic mycobacteria, and environmental saprophytic mycobacteria.

Obligate pathogenic mycobacteria (OPM) are the most specialized species of *Mycobacterium* and cause disease in humans and can be transmitted to other animals (e.g., feral opossum and badger). These species exhibit a high virulence and are able to survive in a dormant form for extended periods of time; however, their survival in the environment is limited.

The environment serves as a primary source for potentially pathogenic mycobacteria (PPM) where these species persist and grow, while also being found in living hosts occupying environmental niches like mucous membranes. The PPM are able to cause mycobacteriosis in susceptible hosts (e.g., immunocompromised individuals) and serve a transitional role between OPM and environmental saprophytic mycobacteria.

Environmental saprophytic mycobacteria (ESM) together are the largest group of mycobacteria and can be isolated from a range of environments including: sphagnum and moss vegetation, surface and drinking water, and soil. These species are often identified as a contaminant in clinical material and their presence has been demonstrated to enhance the pathogenicity of leprosy bacillus.

1.4 Mycobacteriosis in Fishes

The first documental case of fish mycobacteriosis was the identification of a tuberculous-like disease in carp (*Cyprinus carpio*) (Bataillon, Dubard et al. 1897). Mycobacteriosis is now recognized as one of the most common chronic diseases of freshwater and marine fishes in temperate and tropical climate with a worldwide distribution (Pavlik and Khol 2009).

Mycobacteriosis is thought to be the most common chronic disease affecting aquarium fish (Noga 2010). Freshwater and marine aquarium fish are susceptible, particularly members of the

freshwater families Anabantidae, Characidae, and Cyprinidae (Decostere, Hermans et al. 2004) (Nigrelli and Vogel 1963) (Smith 1997, Zanoni, Florio et al. 2008). More recently mycobacteriosis has become a problem in laboratory research facilities that maintain medaka (*Oryzias latipes*) (Teska, Twerdok et al. 1993) and zebrafish (Astrofsky, Schrenzel et al. 2000, Harriff, Bermudez et al. 2007). Mycobacteriosis in aquarium fish encompasses infections with several species of *Mycobacterium* including *Mycobacterium marinum*, *Mycobacterium fortuitum*, *Mycobacterium chelonae*, *Mycobacterium gordonae*, and *Mycobacterium peregrinum* (Noga 2010).

Mycobacterial infections are also problematic in cultured food fish including European sea bass (Colorni, Ankaoua et al. 1993, Colorni, Avtalion et al. 1998), tilapia, and striped bass (Hedrick, McDowell et al. 1987). Mycobacteriosis has historically also been an issue in salmonid culture when the use of raw fish offal was used as a feed (Ross and Brancato 1959). More recently, salmonid mycobacteriosis occurs less frequently; however, asymptomatic infections have been observed to affect certain populations (Arakawa and Fryer 1984). Mycobacteriosis is a known disease of striped bass in Chesapeake Bay, USA (Rhodes, Kator et al. 2004).

It has been proposed that fish shed mycobacteria from infected skin ulcers and the intestine, and that these are a major source of infection for other fish (Noga 2010). The intestinal epithelium has been demonstrated to be a route of entry (Harriff, Bermudez et al. 2007), and ingestion is suspected to be a major source of infection (e.g., fish that have consumed dead tank mates). The swim bladder is another possible route of initial infection as zebrafish are physostomus and hence this organ is connected to the intestinal tract via the pneumatic duct. Often the swim bladder is the only organ infected, with colonization of the swim bladder epithelium and lumen (Whipps, Matthews et al. 2008). Mycobacteria have been shown to

survive in the environment for 2 years (Reichenbach-Klinke 1972). In the aquatic setting, mycobacteria persist in surface biofilms (Beran, Matlova et al. 2006, Whipps, Matthews et al. 2008). Additionally, transovarian transmission of mycobacteria has been shown in bettas (*B. splendens*) (Chinabut, Kanayati et al. 1994) and guppies (Conroy 1966) but not in other species such as salmonids (Ross and Johnson 1962).

Generally, the fish-pathogenic mycobacteria are considered atypical mycobacteria, non-tubercular mycobacteria, or environmental mycobacteria. However, some species of fish-pathogenic mycobacteria can infect humans causing localized, non-healing ulcers (fish tank granuloma/fish handler's disease/swimming pool granuloma)(Kern, Vanek et al. 1989, Wu, Chiu et al. 2012). Some of these zoonotic infections can be difficult to treat due to the antimicrobial resistance of some isolates of mycobacteria.

Mycobacteriosis in fishes was historically identified to be caused by mostly *Mycobacterium chelonae*, *Mycobacterium fortuitum*, and *Mycobacterium marinum* (Frerichs 1993). Through increased study and also more molecular diagnostic methods, at least 16 different species have been described from infections in fish (Whipps, Dougan et al. 2007, Gauthier and Rhodes 2009).

1.5 Mycobacteriosis in Zebrafish

There are 6 described species of *Mycobacterium* that have been implicated in zebrafish mycobacteriosis (Table 1-1). The first described cases of zebrafish mycobacteriosis were *M. abscessus*, *M. chelonae*, and *M. fortuitum* were isolated in zebrafish experiencing decreased survival and fecundity (Astrofsky, Schrenzel et al. 2000). Following this description, several more species were isolated from zebrafish facilities experiencing mortality events (Kent, Whipps et al. 2004, Kent, Whipps et al. 2004). For these cases, *M. peregrinum*, *M. haemophilum* were

found to be associated with severe outbreaks, while *M. chelonae* and *M. chelonae*-like bacterium were found in fish from facilities experiencing moderate to minimal outbreaks.

Table 1-1. Species of *Mycobacterium* identified to infect laboratory zebrafish (from Whipps et al. 2012).

Species	Source
<i>Mycobacterium abscessus</i>	Astrofsky et al. (2000); Watral and Kent (2007)
<i>Mycobacterium chelonae</i>	Astrofsky et al. (2000); Watral and Kent (2004); Whipps et al. (2008)
<i>Mycobacterium chelonae</i> -like	Kent et al. (2004); Whipps et al. (2007a)
<i>Mycobacterium fortuitum</i>	Astrofsky et al. (2000)
<i>Mycobacterium haemophilum</i>	Whipps et al. (2007b)
<i>Mycobacterium marinum</i>	Watal and Kent (2007)
<i>Mycobacterium peregrinum</i>	Kent et al. (2004)

The manifestation of zebrafish mycobacteriosis is variable and clinical signs are not pathognomonic (Astrofsky, Schrenzel et al. 2000, Kent, Spitsbergen et al. 2012). External signs include nonspecific dermal lesions, raised scales, swollen abdomens, and emaciation. Fish may show behavioral signs including erratic swimming or lethargy. Importantly, often fish display no signs of disease. Internal signs may include disseminated granulomas throughout, particularly on hematopoietic organs. Diffuse infections have been reported with an absence of granulomatous lesions for infections with *M. haemophilum* (Whipps, Dougan et al. 2007) and *M. marinum* (Ramsay, Watral et al. 2009). Bacteria in the ovaries (Kent, Whipps et al. 2004) and swim bladder (Whipps, Matthews et al. 2008) have also been observed. Additionally, bacteria have been observed in the intestinal epithelium within the luminal space indicating shedding across the intestinal surface and fecal excretion (Whipps, Butler et al. 2007).

Mycobacteriosis in zebrafish is thought to be transmitted through the ingestion of contaminated food or tissues (i.e., fish cannibalism) as this has been demonstrated in other fishes (Ross 1970). Invasion has been reported to occur through the intestine (Harriff, Bermudez et al.

2007), rather than through the skin or gills; however, further research is required before transmission routes are fully understood. Other potential modes of transmission are vertical transmission following reproduction (Kent, Whipps et al. 2004, Kent, Watral et al. 2016), exposure to mycobacteria that form biofilms in tank systems, and transmission from fomites and handlers.

In terms of controlling and managing this disease, disease prevention is paramount. Recent reviews of zebrafish diseases emphasize the importance of preventative measures (i.e., quarantine, disinfection, UV water sterilization, sentinel monitoring programs, regular health monitoring) (Kent et al., 2009; Whipps et al., 2012)(Collymore, Crim et al. 2016, Mason, Snell et al. 2016). In the event that an outbreak of mycobacteriosis occurs, control measures become much more invasive and include depopulation, system sterilization, and rederivation of zebrafish stocks (Whipps et al., 2012). Although these invasive measures have been demonstrated to be effective at controlling mycobacteriosis (Whipps et al., 2012) they may not always be feasible due to ongoing experiments and a lack of means to preserve valuable mutant lines, demonstrating a need for alternative methods for treating/controlling this disease in laboratory zebrafish.

1.6 Objectives

In this dissertation, I aim to increase our understanding of mycobacteriosis in laboratory zebrafish through investigation of the prevention, treatment, and transmission of *Mycobacterium* spp. in zebrafish colonies.

1. **Prevention:** Chapters 2 and 3 describe the evaluation of common disinfectants for zebrafish embryo disinfection *in vitro* and *in vivo*, respectively.

Hypothesis: I hypothesize that the species of *Mycobacterium* isolated from zebrafish will be differentially susceptible to disinfectant treatments due to previous observation of differential disinfectant susceptibilities of *Mycobacterium* species and that disinfection treatments already used in aquaculture will not result in adverse health effects on zebrafish embryos.

2. **Treatment:** Chapters 4 and 5 evaluate the potential for antibiotic treatment of zebrafish mycobacteriosis through both an evaluation of the antibiotic susceptibility of species of *Mycobacterium* isolated from zebrafish in Chapter 4 and through an evaluation of the tolerance and efficacy of antibiotics in adult zebrafish in Chapter 5.

Hypothesis: I hypothesize that the different species of *Mycobacterium* isolated in zebrafish will be differentially susceptible to different antibiotic treatments *in vitro* as *Mycobacterium* spp. have previously been observed to be differentially susceptible to antibiotic treatments as well as previous identification of genetic resistance in mycobacteria. I also hypothesize, that *Mycobacterium* spp. *in vivo* will be susceptible to antibiotic treatments observed to be effective in prior *in vitro* testing.

3. Transmission: Chapter 6 investigates the role biofilms play in zebrafish infections with mycobacteria. Finally, Chapter 7 investigates the effect live food vectors play in mycobacterial transmission.

Hypothesis: I hypothesize that transmission of *Mycobacterium* spp. occurs between tank biofilms and zebrafish because the same strains of mycobacteria have previously been identified in both fish and biofilms. I also hypothesize that *Mycobacterium* spp. can be transmitted to zebrafish through live feed vectors, and that the degree of virulence is vector-dependent because passage through a cell is known to activate virulence mechanisms.

Significance:

The overall goal of this research is to provide insight that will inform effective disease control and management practices for the research community and improve zebrafish as a model organism.

1.7 References

Arakawa, C. K. and J. L. Fryer (1984). "Isolation and Characterization of a New Subspecies of *Mycobacterium-Chelonei* Infectious for Salmonid Fish." Helgolander Meeresuntersuchungen **37**(1-4): 329-342.

Astrosfky, K. M., M. D. Schrenzel, R. A. Bullis, R. M. Smolowitz and J. G. Fox (2000). "Diagnosis and management of atypical *Mycobacterium* spp. infections in established laboratory zebrafish (*Brachydanio rerio*) facilities." Comparative Medicine **50**(6): 666-672.

Bataillon, E., R. Dubard and U. Terre (1897). "Un nouveau type de tuberculose." C. R. Soc. Biol. **49**: 446-449.

Beran, V., L. Matlova, L. Dvorska, P. Svastova and I. Pavlik (2006). "Distribution of mycobacteria in clinically healthy ornamental fish and their aquarium environment." Journal of Fish Diseases **29**: 383-393.

Blaser, R. E. and Y. M. Penalosa (2011). "Stimuli affecting zebrafish (*Danio rerio*) behavior in the light/dark preference test." Physiology & Behavior **104**(5): 831-837.

Care, C.-C. C. o. A. (1984). Laboratory Mice. Guide to the Care and Use of Experimental Animals. **2**.

Carfagnini, A. G., F. H. Rodd, K. B. Jeffers and A. E. E. Bruce (2009). "The effects of habitat complexity on aggression and fecundity in zebrafish (*Danio rerio*)."
Environmental Biology of Fishes **86**(3): 403-409.

Chinabut, S., Y. Kanayati and T. Pungkachonboon (1994). Study of transovarian transmission of mycobacteria in *Betta splendens* Regan. L. Chou, A. D. Munro, T. J. Lam et al. Manila, Philippines, Asian Fisheries Society: 339-341.

Collymore, C., M. J. Crim and C. Lieggi (2016). "Recommendations for Health Monitoring and Reporting for Zebrafish Research Facilities." Zebrafish **13**: S138-S148.

Colorni, A., M. Ankaoua, A. Diamant and W. R. Knibb (1993). "Detection of mycobacteriosis in fish using the polymerase chain reaction technique." European Association of Fish Pathologists Bulletin **13**(6): 195-198.

Colorni, A., R. Avtalion, W. Knibb, E. Berger, B. Colorni and B. Timan (1998). "Histopathology of sea bass (*Dicentrarchus labrax*) experimentally infected with *Mycobacterium marinum* and treated with streptomycin and garlic (*Allium sativum*) extract." Aquaculture **160**(1-2): 1-17.

Conroy, D. A. (1966). "Observaciones sobre casos espontáneos de tuberculosis íctica. [Observations on spontaneous cases of tuberculosis in fish.]" Microbiologia Espanola **19**: 93-113.

Davidson, P. T. (1981). "International Conference on Atypical Mycobacteria. Introduction." Reviews of Infectious Diseases **3**(5): 816-818.

De Kinkelin, P. (1980). "Occurrence of a microsporidian infection in zebra danio *Brachydanio rerio* (Hamilton-Buchanan)." Journal of Fish Diseases **3**: 71-73.

Decostere, A., K. Hermans and F. Haesebrouck (2004). "Piscine mycobacteriosis: a literature review covering the agent and the disease it causes in fish and humans." Veterinary Microbiology **99**(3-4): 159-166.

Frerichs, G. N. (1993). Mycobacteriosis: nocardiosis. London, Blackwell Scientific Publications.

Gaind, N. (2016). "US grants for zebrafish studies on the rise." Nature News & Comment.

Gauthier, D. T. and M. W. Rhodes (2009). "Mycobacteriosis in fishes: a review." The Veterinary Journal **180**(1): 33-47.

Grange, J. M. (1991). "Environmental mycobacteria and human disease." Leprosy Review **62**(4): 353-361.

Harriff, M. J., L. E. Bermudez and M. L. Kent (2007). "Experimental exposure of zebrafish, *Danio rerio* (Hamilton), to *Mycobacterium marinum* and *Mycobacterium peregrinum* reveals the

gastrointestinal tract as the primary route of infection: a potential model for environmental mycobacterial infection." Journal of Fish Diseases **30**(10): 587-600.

Hedrick, R. P., T. McDowell and J. Groff (1987). "Mycobacteriosis in Cultured Striped Bass from California." Journal of Wildlife Diseases **23**(3): 391-395.

Holland, J., C. Lawrence and N. King (2013). Children's Hospital Boston Makes Zebrafish Feeding Regime Breakthrough. Innovation. Global Aquaculture Advocate: 76-79.

Jacoby, R. O. and J. R. Lindsey (1998). "Risks of infection among laboratory rats and mice at major biomedical research institutions." ILAR **39**(4): 266-271.

Jenkins, P. A. (1981). "The epidemiology of opportunist mycobacterial infections in Wales, 1952--1978." Reviews of Infectious Diseases **3**(5): 1021-1023.

Kent, M. L., S. W. Feist, C. Harper, S. Hoogstraten-Miller, J. M. Law, J. M. Sanchez-Morgado, R. L. Tanguay, G. E. Sanders, J. M. Spitsbergen and C. M. Whipps (2009). "Recommendations for control of pathogens and infectious diseases in fish research facilities." Comparative Biochemistry and Physiology Part C Toxicology and Pharmacology **149**(2): 240-248.

Kent, M. L., S. W. Feist, C. Harper, S. Hoogstraten-Miller, J. Mac Law, J. M. Sanchez-Morgado, R. L. Tanguay, G. E. Sanders, J. M. Spitsbergen and C. M. Whipps (2009). "Recommendations

for control of pathogens and infectious diseases in fish research facilities." Comparative Biochemistry and Physiology Part C Toxicology & Pharmacology **149**(2): 240-248.

Kent, M. L., J. M. Spitsbergen, J. M. Matthews, J. W. Fournie, K. N. Murray and M. Westerfield. (2012). "Diseases of Zebrafish in research facilities." Retrieved 10/25/16, 2016.

Kent, M. L., Varga, Z. (2012). "Use of Zebrafish in Research and Importance of Health and Husbandry." ILAR **53**(2): 89-94.

Kent, M. L., V. G. Watral, N. S. Kirchoff, S. T. Spagnoli and T. J. Sharpton (2016). "Effects of Subclinical *Mycobacterium chelonae* infections on fecundity and embryo survival in zebrafish." Zebrafish **13 Suppl 1**: S88-95.

Kent, M. L., C. M. Whipps, J. L. Matthews, D. Florio, V. Watral, J. K. Bishop-Stewart, M. Poort and L. Bermudez (2004). "Mycobacteriosis in zebrafish (*Danio rerio*) research facilities." Comparative Biochemistry and Physiology Part C Toxicology & Pharmacology **138**(3): 383-390.

Kent, M. L., C. M. Whipps, J. L. Matthews, D. Florio, V. Watral, J. K. Bishop-Stewart, M. Poort and L. Bermudez (2004). "Mycobacteriosis in zebrafish (*Danio rerio*) research facilities." Comparative Biochemistry and Physiology Part C Toxicology & Pharmacology **138**(3): 383-390.

Kent, M. L. S., J. M., Matthews, J. M., Fournie, J. W., Murray, K. N., Westerfield, M. (2012).

"Diseases of Zebrafish in Research Facilities." Retrieved July 10, 2014, from

<http://zebrafish.org/zirc/health/diseaseManual.php>.

Kern, W., E. Vanek and H. Jungbluth (1989). "[Fish breeder granuloma: infection caused by *Mycobacterium marinum* and other atypical mycobacteria in the human. Analysis of 8 cases and review of the literature]." Med Klin (Munich) **84**(12): 578-583.

Lawrence, C. and T. Mason (2012). "Zebrafish Housing Systems: A Review of Basic Operating Principles and Considerations for Design and Functionality." ILAR Journal **53**(2): 179-191.

Mason, T., K. Snell, E. Mittge, E. Melancon, R. Montgomery, M. McFadden, J. Camoriano, M. L. Kent, C. M. Whipps and J. Peirce (2016). "Strategies to mitigate a *Mycobacterium marinum* outbreak in a zebrafish research facility." Zebrafish **13**: S77-S87.

Nigrelli, R. F. and H. Vogel (1963). "Spontaneous tuberculosis in fishes and in other cold-blooded vertebrates with special reference to *Mycobacterium fortuitum* Cruz from fish and human lesions." Zoologica **48**: 131-144.

Noga, E. J. (2010). Fish Disease: Diagnosis and Treatment. Iowa, USA, Wiley-Blackwell.

Pavlik, I. and J. L. Khol (2009). Poikilothermic Vertebrates. The ecology of mycobacteria: impact on animal's and human's health. I. Pavlik and J. O. I. Falkinham, Springer Dordrecht Heidelberg London New York: 234-239.

Ramsay, J. M., G. W. Feist, Z. M. Varga, M. Westerfield, M. L. Kent and C. B. Schreck (2009). "Whole-body cortisol response of zebrafish to acute net handling stress." Aquaculture **297**(1-4): 157-162.

Ramsay, J. M., V. Watral, C. B. Schreck and M. L. Kent (2009). "Husbandry stress exacerbates mycobacterial infections in adult zebrafish, *Danio rerio* (Hamilton)." Journal of Fish Diseases **32**(11): 931-941.

Reichenbach-Klinke, H. H. (1972). "Some aspects of mycobacterial infections in fish." Symposia of the Zoological Society of London **30**: 17-24.

Rhodes, M. W., H. Kator, I. Kaattari, D. T. Gauthier, W. Vogelbein and C. A. Ottinger (2004). "Isolation and characterization of mycobacteria from striped bass *Morone saxatilis* from the Chesapeake Bay." Diseases of Aquatic Organisms **61**: 41-51.

Ross, A. J. (1970). Mycobacteriosis among Pacific salmonid fishes. A Symposium on Diseases of Fishes and Shellfishes. S. F. Sniesko. Washington, D.C., American Fisheries Society: 279-283.

Ross, A. J. and F. P. Brancato (1959). "*Mycobacterium fortuitum* Cruz from the tropical fish *Hyphessobrycon-Innesi*." Journal of Bacteriology **78**(3): 392-395.

Ross, A. J. and H. E. Johnson (1962). "Studies of transmission of mycobacterial infections in Chinook salmon." The Progressive Fish-Culturist **24**(4): 147-149.

Runyon, E. H. (1959). "Anonymous Mycobacteria in Pulmonary Disease." Medical Clinics of North America **43**(1): 273-290.

Saviola, B. and W. Bishai (2006). The Prokaryotes. The Prokaryotes. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer and E. Stackebrand. New York, Springer New York. **Volume 3: Archaea. Bacteria: Firmicutes, Actinomycetes:** 919-933.

Schilling, T. F. and J. Webb (2007). "Considering the zebrafish in a comparative context." Journal of Experimental Zoology Part B-Molecular and Developmental Evolution **308B**(5): 515-522.

Smith, S. A. (1997). "Mycobacterial infections in pet fish." Seminars in Avian and Exotic Pet Medicine **6**(1): 40-45.

Teska, J. D., L. E. Twerdok, J. Beaman, M. Curry and R. A. Finch (1993). "Isolation of *Mycobacterium abscessus* from Japanese Meduka." Journal of Aquatic Animal Health **9**(3): 234-238.

Weisbroth, S. H. (1996). "Post-indigenous disease: changing concepts of disease in laboratory rodents." Lab Animal **25**(9): 25-33.

Whipps, C. M., W. R. Butler, F. Pourahmad, V. G. Watral and M. L. Kent (2007). "Molecular systematics support the revival of *Mycobacterium salmoniphilum* (ex Ross 1960) sp nov., nom. rev., a species closely related to *Mycobacterium chelonae*." International Journal of Systematic and Evolutionary Microbiology **57**: 2525-2531.

Whipps, C. M., S. T. Dougan and M. L. Kent (2007). "*Mycobacterium haemophilum* infections of zebrafish (*Danio rerio*) in research facilities." FEMS Microbiology Letters **270**(1): 21-26.

Whipps, C. M., Lieggi, C., Wagner, R. (2012). "Mycobacteriosis in zebrafish colonies." ILAR **53**(2): 95-105.

Whipps, C. M., J. L. Matthews and M. L. Kent (2008). "Distribution and genetic characterization of *Mycobacterium chelonae* in laboratory zebrafish *Danio rerio*." Diseases of Aquatic Organisms **82**(1): 45-54.

Whipps, C. M., J. L. Matthews and M. L. Kent (2008). "Distribution and genetic characterization of *Mycobacterium chelonae* in laboratory zebrafish *Danio rerio*." Diseases of Aquatic Organisms **82**(1): 45-54.

Wolinsky, E. (1979). "Nontuberculous mycobacteria and associated diseases." American Reviews of Respiratory Diseases **119**(1): 107-159.

Wu, T. S., C. H. Chiu, C. H. Yang, H. S. Leu, C. T. Huang, Y. C. Chen, T. L. Wu, P. Y. Chang, L. H. Su, A. J. Kuo, J. H. Chia, C. C. Lu and H. C. Lai (2012). "Fish tank granuloma caused by *Mycobacterium marinum*." PLoS One **7**(7): e41296.

Zanoni, R. G., D. Florio, M. L. Fioravanti, M. Rossi and M. Prearo (2008). "Occurrence of *Mycobacterium* spp. in ornamental fish in Italy." Journal of Fish Diseases **31**(6): 433-441.

Zebrafish International Resource Center (ZIRC) Health Services. (2017). "Summary of Submissions to the ZIRC Diagnostic Service 2006-2016." Retrieved October 4, 2017, from https://zebrafish.org/wiki/_media/health/submission/submissions_summary_2006-2016.pdf.

Chapter 2

Evaluating the effectiveness of common disinfectants at preventing the propagation of *Mycobacterium* spp. isolated from zebrafish.

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2.1 Abstract

Mycobacteriosis is a bacterial disease that is common in captive, wild and research fish. There is no one causative agent of mycobacteriosis, as several strains and species of *Mycobacterium* have been identified in zebrafish. With increased usage and investment in wild-type and mutant zebrafish strains, considerable value is placed on preserving zebrafish health. One control measure used to prevent mycobacterial spread within and between zebrafish facilities is egg disinfection. Here we investigate the effectiveness of three disinfectants [chlorine bleach, hydrogen peroxide, and povidone iodine (PVPI)] commonly included in egg disinfection protocols for laboratory fish as well as aquaculture fish and compare the knockdown effect of these treatments on *Mycobacterium* spp. *in vitro*. Despite current usage, comparison of these disinfection regimes' abilities to prevent mycobacterial growth has not been tested. We found that the germicidal effect of different disinfectants varies by *Mycobacterium* spp.. Hydrogen peroxide was the least effective disinfectant, followed by unbuffered chlorine bleach, which is commonly used to disinfect embryos in zebrafish facilities. Disinfection with 25 ppm PVPI for 5 min was very effective, and may be an improved alternative to chlorine bleach for embryo disinfection. Results from this study can be utilized by laboratory fish facilities in order to prevent the spread of mycobacteriosis in research fish.

Key words: zebrafish, embryo disinfection, husbandry, iodine, chlorine bleach, hydrogen peroxide, mycobacteriosis, biosecurity.

2.2 Introduction

Mycobacteriosis is a chronic bacterial disease caused by *Mycobacterium* species and is common in laboratory zebrafish colonies (Astrofsky et al., 2000; Kent et al., 2004; Kent, 2012; Whipps et al., 2012). Mycobacteria are facultative pathogens that can persist both within the host and in the environment and are readily isolated from surface biofilms (Falkinham, 2009; Falkinham et al., 2001). There is no single etiological agent for zebrafish mycobacteriosis and several species, both rapid-growing and slow-growing, of *Mycobacterium* have been implicated in zebrafish infections (Astrofsky et al., 2000; Kent et al., 2004; Whipps et al., 2012; Whipps et al., 2008). The manifestation of mycobacterial infections is species-specific and variable ranging from acute, severe epizootic outbreaks with significant colony mortality to chronic, low-level infections presenting no clinical signs of disease (Watrall and Kent, 2007; Whipps et al., 2007a; Whipps et al., 2007b; Whipps et al., 2012). Thus, mycobacterial infections are detrimental to research when severe outbreaks cause population loss, but they are also concerning as a source of uncontrolled experimental variance in the case of chronic, low-level, sub-clinical infections (Kent et al., 2004; Whipps et al., 2012).

Control recommendations for mycobacteriosis in zebrafish colonies emphasize the importance of disease prevention through quarantine, disinfection, UV sterilization, and sentinel programs for monitoring disease (Kent et al., 2009; Whipps et al., 2012). Included in these recommendations is the surface disinfection of eggs through bleaching (Westerfield, 2000; Lawrence, 2007; Kent et al., 2009). Also, investigations involving the generation of gnotobiotic zebrafish include surface disinfection of embryos using immersion in bleach followed by an iodine solution (Milligan-Myhre et al., 2011). However, the efficacy of these disinfection treatments against *Mycobacterium* spp. from zebrafish is unknown (Whipps et al., 2012).

Disinfectant is a term that usually describes a chemical agent that prevents infection through the destruction of harmful microorganisms, but that may not eliminate bacterial spores (Block, 2001). The Centers for Disease Control and Prevention prescribes three main levels for disinfection: high-level disinfectants killing all microorganisms (including spores); intermediate-level disinfectants that kill vegetative cells, most viruses and some spores; and low-level disinfectants that kill vegetative cells (not including spores) (Garner and Favero, 1986). Many disinfectants currently used in aquaculture provide an intermediate-level of disinfection (Noga, 2010). Compared to sterilization, disinfection is a less lethal process as not all forms of life are destroyed (Block, 2001; Noga, 2010). The effectiveness of a disinfectant is specific to the infectious agent in question as their susceptibilities vary (Block, 2001). Therefore considerations should be given to the required application prior to the selection of a particular disinfectant (Block, 2001).

Mycobacteria are considered to be resistant to disinfection and they are considered to fall between vegetative bacteria and endospores in terms of their resistance to chemical disinfection and are generally susceptible to intermediate-to-high-level disinfectants (Block, 2001; Widmer and Frei, 2003). This degree of resistance can be attributed to the extremely resilient waxy mycobacterial cell wall that is highly hydrophobic (Russell, 1996). This hydrophobicity prevents hydrophilic antimicrobials and chemical disinfectants from penetrating the cell wall, protecting the mycobacteria from elimination (Russell, 1996; Block, 2001). In addition to this, biofilm formation has been shown to increase mycobacterial resistance to disinfection (Bardouniotis et al., 2003; Steed and Falkinham, 2006). Also, the susceptibility of mycobacteria in biofilms to disinfection is species specific (Russell, 1996; Block, 2001; Bardouniotis et al., 2003; Steed and Falkinham, 2006).

Chlorine bleaching of zebrafish embryos is already an established and accepted practice (Westerfield, 2000). Most zebrafish laboratories use concentrations of 25-100 ppm chlorine and dose embryos for up to 10 minutes (Westerfield, 2007; Harper and Lawrence, 2011; Kent et al., 2014). More recently, an increased chlorine concentration from 50 ppm to 100 ppm has been recommended to increase the killing of *Pseudoloma neurophilia* spores, another common zebrafish pathogen (Ferguson et al., 2007; Kent et al., 2014). Buffered bleach solutions have also been shown to be more effective at killing *P. neurophilia* (Ferguson et al., 2007); however, buffered bleach is more toxic to zebrafish embryos (Kent et al., 2014). It is not known if, like *P. neurophilia*, buffering bleach results in decreased mycobacterial survival compared to the currently utilized unbuffered solutions. In general, zebrafish embryo bleach disinfection involves the immersion of embryos in a 25-100 parts per million (ppm) chlorine bleach solution for up to 10 minutes followed by rinsing in either system water or embryo medium (Westerfield, 2007), neutralization in sodium thiosulfate and rinsing in embryo medium (Detrich et al., 2011), or rinsing in sterile embryo media for the derivation of gnotobiotic fish (Milligan-Mhyre et al., 2011). Hydrogen peroxide is another disinfectant that is often used for embryos of other fish species, particularly in catfish, at a concentration of 250-500 ppm in both bath and flow-through treatments (Small, 2003). For *Mycobacterium* spp., however, a higher concentration of hydrogen peroxide seems to be necessary as recommendations for using hydrogen peroxide to disinfect *Mycobacterium tuberculosis* include the usage of concentrations greater than 30,000 ppm (Noga, 2010). Iodine disinfection of embryos is a widely used and accepted practice in salmonid fisheries with a recommended immersion in 100 ppm povidone-iodine (PVPI) for 10 minutes (Wood, 1979; Alaska Department of Fish and Game, 1983, 1988; United States Fish and Wildlife Service, 2004; Wagner et al., 2008). Immersion in PVPI is also used for generating

gnotobiotic zebrafish at 100 ppm for 2 minutes (Milligan-Mhyre et al. 2011). Despite the usage of these disinfectant regimes on fish embryos, much remains to be understood about the effectiveness of these treatments on preventing the spread of fish mycobacteriosis.

Mycobacteria are documented to be susceptible to the following chemical disinfectants: alcohols, aldehydes, some alkalis, halogens (including chlorine and iodine compounds), some peroxygen compounds and some phenols (Block, 2001; Widmer and Frei, 2003; Noga, 2010). Because most investigations into the effectiveness of chemical disinfectants on mycobacteria are clinically oriented, information specific to the *Mycobacterium* spp. found in zebrafish facilities is limited to studies investigating *Mycobacterium marinum* and *Mycobacterium fortuitum* (Bardouniotis et al., 2003). Therefore, more information is needed regarding the susceptibility of zebrafish mycobacteria to disinfection and the efficacy of currently utilized disinfection practices at preventing mycobacterial spread.

The aim of this study was to investigate the susceptibility of several *Mycobacterium* spp. isolated from zebrafish research facilities in the United States to chemical disinfection *in vitro*. Chemical disinfection regimes were chosen with a focus on methods already utilized within the fish community for egg disinfection. We hypothesize that *Mycobacterium* spp. will show differential susceptibility to different disinfectants and there will be species-specific susceptibilities similar to what has been previously shown in literature for non-zebrafish mycobacteria.

2.3 Methods

Bacterial culture and growth media

Isolates of *Mycobacterium abscessus*, *Mycobacterium chelonae*, *Mycobacterium gordonae*, and *Mycobacterium peregrinum* maintained in our culture collection were used. All were isolated from zebrafish facilities in the United States and were previously identified based on *hsp65* gene sequencing as previously described (Kent et al. 2004; Poort et al. 2006; Whipps et al. 2007a; Whipps et al. 2007b). All isolates were grown at 28-30°C for seven days on solid-phase Middlebrook 7H10 (MB 7H10) agar (BD Biosciences 262710) supplemented with oleic albumin dextrose catalase (OADC, BD Biosciences 211886), prior to preparation for disinfection treatments.

Disinfection treatment and analysis

Chlorine Bleach (unbuffered) – We treated *Mycobacterium* spp. isolates with chlorine bleach at concentrations of 100 and 150 ppm chlorine bleach (Clorox®) for 10 minutes. Isolated *M. abscessus*, *M. chelonae*, *M. gordonae*, and *M. peregrinum* colonies were individually inoculated with a sterile loop into sterile culture tubes containing 3 ml of 100% Middlebrook 7H9 broth and incubated for 7 days at 30°C and 300 rpm. Following incubation, broth culture was diluted in sterile water to reach a concentration of 10^5 colony forming units (CFU) per milliliter measured using a nephelometer (Sensititre). Chlorine bleach treatment solutions (100 ppm, 150 ppm, control) were prepared by diluting Clorox® bleach in autoclaved Milli-Q® filtered water to a 1000 ppm concentration. Chlorine concentration was verified using a chlorine meter (Extech CL200). For each treatment, 1.0 mL of diluted broth culture was added to a sterile 2 ml Eppendorf tube and 1000 ppm chlorine bleach in Milli-Q® was added to bring treatment solutions to a final concentration of 100 ppm, 150 ppm chlorine bleach. For the control, sterile

Milli-Q water was added to the broth culture. The pH of the treatment solution was measured before and after treatment. Tubes were incubated for 10 minutes at room temperature with gentle mixing. Following exposure time, an equal volume of 1% sodium thiosulfate (Na_2HPO_4 , Fisher Scientific S-446) made in autoclaved Milli-Q® water was added to neutralize the chlorine with gentle mixing. Neutralizing activity of the sodium thiosulfate was confirmed using a chlorine meter. Following neutralization, serial dilutions of these solutions were prepared at 10^0 , 10^{-1} , 10^{-2} for treatment tubes and 10^{-3} , 10^{-4} , and 10^{-5} for control tubes. Finally, 100 μl of each dilution was plated onto MB 7H10 agar plates in triplicate using a sterile spreader. Plates were then incubated at 28°C for 7 days and colonies were counted. This experiment was repeated two more times.

Chlorine Bleach (buffered) - In addition to the chlorine experiment described above, we conducted another trial of chlorine disinfection in order to compare disinfection with buffered and unbuffered bleach. Cultures were prepared to 10^5 CFU/ml as above. For each treatment, 1 ml of this diluted culture was added to a sterile 2.0 ml tube and centrifuged to form a pellet. Following centrifugation the supernatant was removed and was replaced with 1 mL of treatment solutions [100 ppm, 150 ppm, or 0 ppm chlorine bleach prepared in autoclaved chlorine demand free buffer (CDFB), prepared by mixing 42% 0.05M KH_2PO_4 , and 58% 0.05M Na_2HPO_4 , or in autoclaved Milli-Q® water as a control for comparison]. The same inoculating cultures were used for both treatments. *M. chelonae* and *M. peregrinum* cultures were used for this second trial for 5 and 10 minute exposure times at 100 ppm or 150 ppm concentrations for both buffers. Following exposures, neutralization, serial dilution and plating were carried out as in the previously described trial. This experiment was then repeated two more times.

Hydrogen peroxide – We treated *Mycobacterium* spp. isolates to 15,000 ppm and 30,000 ppm hydrogen peroxide (H_2O_2 , drugstore brand) for 5 minutes. Cultures were prepared to

10^5 CFU/ml as above. For each treatment, 1 ml of this diluted culture was added to a sterile 1.5 ml tube and centrifuged to form a pellet. Following centrifugation the supernatant was removed and was replaced with 1 ml of freshly made hydrogen peroxide (H_2O_2 , drugstore brand) treatment solution (15,000 ppm or 30,000 ppm) or an equal volume of sterile water for the control treatment. Hydrogen peroxide treatment solutions were undiluted (30,000 ppm) and diluted with sterile water (15,000 ppm). Treatment tubes were vortexed to break pellet apart and tubes with incubated for 5 minutes at room temperature in the dark. Following incubation tubes were centrifuged again to re-pellet cells and the treatment/control solutions were replaced with 1 ml of sterile water. Tubes were vortexed to resuspend cells and serial dilutions of 10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} , and 10^{-3} were prepared and 100 μ l of each dilution was plated onto MB 7H10 in triplicate. These plates were incubated at 28°C for 7 days and colonies were counted. This experiment was repeated two more times.

Iodine –We treated *Mycobacterium* spp. isolates to 12.5-100 ppm PVPI for 5 minutes. Cultures were prepared to 10^5 CFU/ml. For each treatment, 1 ml of this diluted culture was pelleted in a sterile 1.5 ml tube. Following centrifugation the supernatant was removed and was replaced with 1 ml of freshly made iodine treatment solution made in sterile Milli-Q® water. Initially, *M. chelonae* was tested at four concentrations (12.5 ppm, 25 ppm, 50 ppm, or 100 ppm) of PVPI (10%, drugstore brand). Following this trial, 25 ppm PVPI was chosen as the optimal treatment to be tested on *M. abscessus*, *M. goodsonae*, and *M. peregrinum*. Control treatments consisted of an equal volume of sterile water. All treatment solution concentrations were verified using iodine test paper (LaMotte). Treatment tubes were vortexed to break pellet apart and tubes with incubated for 5 minutes at room temperature in the dark. Following incubation tubes were centrifuged again to re-pellet cells and the treatment/control solutions were replaced with 1 ml of

sterile water. Tubes were vortexed to resuspend cells and serial dilutions of 10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} , and 10^{-3} were prepared, and 100 μ l of each dilution was plated onto MB 7H10 agar plates in triplicate. Plates were incubated at 28°C for 7 days and colony counts conducted. This experiment was repeated two more times.

Statistics

The following analysis was carried out to compare differences between species for the same disinfectant treatment and also to compare different disinfectant treatments for each species separately. The same statistical method was utilized for all analyses using R 3.1.0 (R Core Team, 2013) and R Studio (R Studio, 2012). Colony count data were entered into a spread sheet where percent survival for each treatment was determined by comparing treatment counts (CFU/mL) to control counts (CFU/mL); Percent Survival = [(Treatment Count/Control Average Count)*100]. Data were then sorted by treatment or species and saved as individual text files for analysis. For each data file descriptive statistics were obtained using the “psych” package (Revelle, 2014). Data were also checked for normality equal variances using the “stats” package (R Core Team, 2013) and “car” package (Fox and Weisberg, 2011) respectively. Since all data sets were found to have non-normal distributions ($p < 0.05$) and unequal variances ($p < 0.05$) the non-parametric Kruskal-Wallis rank sum test was used to compare percent survival values using the “stats” package (R Core Team, 2013). In the case of a significant result, indicating differences between disinfection treatments, post-hoc tests for pairwise multiple comparisons of the ranked data were performed using the “PMCMR” package (Pohlert, 2015). Data were then visualized as a clustered bar graph using the “sciplot” package (Morales et al., 2012).

2.4 Results

Chlorine bleach disinfection

Treatment of *M. abscessus*, *M. chelonae*, *M. gordonae*, and *M. peregrinum* with 100ppm Clorox® chlorine bleach diluted in Milli-Q® water resulted in a minimum average survival of 2.94% for *M. gordonae*, followed by 18.89% for *M. peregrinum*. Average survival of *M. abscessus* and *M. chelonae* was 27-40% (Figs 1-4). There was no significant difference between species for this treatment ($p>0.05$). Treatment with 150 ppm Clorox® chlorine bleach diluted in Milli-Q® water resulted in a similar trend in survival with a minimum average survival of less than 1% for *M. gordonae*. *Mycobacterium chelonae* had an average survival of 14.59%, *M. abscessus* had a 20.21% average survival, and *M. peregrinum* showed the greatest survival at 32.47%. Unbuffered treatment solution pH values are as follows: 100 ppm solutions ranged from 11.67-14.10, 150 ppm ranged from 7.98-9.81, and control solutions ranged from 9.0-10.60. The difference in average survival between species was significant (Kruskal-Wallis; $\chi^2(3)=35.5966$, $p<0.0001$) with *M. abscessus* and *M. peregrinum* equivalent to each other, with *M. peregrinum* having significantly higher survival than *M. chelonae* and *M. gordonae*, but *M. abscessus* only having higher survival over *M. chelonae*. *Mycobacterium gordonae* was significantly different from *M. chelonae* as well as *M. peregrinum* ($p<0.05$). When comparing 100 ppm and 150 ppm chlorine bleach to one another by species, there was no significant difference between concentrations ($p<0.05$) (Figures 2-1 - 2-4).

Additional Clorox® chlorine bleach treatment trials were completed using a CDFB as the diluent with *M. chelonae* and *M. peregrinum*, and compared to chlorine bleach diluted in Milli-Q® water. For *M. chelonae* bleach disinfection, there were significant differences between treatments (Kruskal-Wallis; $\chi^2(7)=48.3911$, $p<0.0001$), specifically, the 10 minute treatments in

Milli-Q® were different from all others (Figure 2-2, significantly different groupings are indicated by group labels, $p < 0.05$). Trials with CDFB resulted in less than 1% average survival for all concentration and treatment durations (not shown); whereas, only 150 ppm Clorox® chlorine bleach diluted in Milli-Q® resulted in this degree of knock-down (Figure 2-2). Trials with *M. peregrinum* resulted in similar outcomes (Figure 2-4); with less than 1% average survival for CDFB trails (not shown). For comparison, less than 1% average survival of *M. peregrinum* following unbuffered Clorox® chlorine bleach treatment was only observed for 5 minute, 100 ppm treatments (Figure 2-4). Buffered treatment solution pH values are as follows: 100 ppm solutions ranged from 5.07-5.1, 150 ppm ranged from 4.90-5.07, and control solutions ranged from 9.0-10.60.

Hydrogen peroxide disinfection

Treatment of *M. abscessus*, *M. chelonae*, *M. goodnae*, and *M. peregrinum* with 15,000 ppm hydrogen peroxide resulted in a minimum average survival of 12.87% for *M. abscessus*, 45.26% survival for *M. chelonae* and 100% or more survival for *M. goodnae* and *M. peregrinum* (Figures 2-1 - 2-4). This treatment did differ significantly in its effectiveness as significant differences in mycobacterial survival are observed between species (Kruskal-Wallis; $\chi^2(3)=12.0656$, $p < 0.05$). Post-hoc comparisons revealed that *M. abscessus* and *M. chelonae* had a significantly lower survival than *M. peregrinum* ($p < 0.01$). Results for 30,000 ppm hydrogen peroxide were similar to the lower 15,000 ppm treatment (Figures 2-1 - 2-4). *Mycobacterium abscessus* had the lowest resulting average survival of 13.60%, followed by *M. chelonae* with 19.28% resulting average survival. Average survival of *M. goodnae* was 47.41%, and *M. peregrinum* had the highest resulting average survival of 76.01%. This treatment did differ significantly in its effectiveness between species (Kruskal-Wallis; $\chi^2(3)=10.2262$, $p < 0.01$). Post-

hoc comparisons revealed that *M. abscessus* had a significantly lower survival than *M. peregrinum* ($p < 0.01$). When comparing these two treatment concentrations to one another by species, there was no significant difference between concentrations ($p < 0.05$) (Figures 2-1 - 2-4).

Iodine disinfection

Initial PVPI disinfection treatments were tested on *M. chelonae* at a range of concentrations (12.5 ppm, 25 ppm, 50 ppm, and 100 ppm) for 5 minutes. Percent survival for treatments were significantly different between treatment concentration (Kruskal-Wallis; $\chi^2(3) = 32.5721$, $p < 0.0001$). Post-hoc comparisons revealed that there was no significant difference between the 25-100 ppm treatments (average survival less than 1%); however, the 12.5 ppm concentration resulted in a significantly higher percent survival (average survival of 51.25%) of *M. chelonae* ($p < 0.05$). Further PVPI testing was narrowed down to testing 25 ppm treatment as this was the lowest concentration found to be equally as effective as 50 and 100 ppm. Treatment of *M. abscessus*, *M. chelonae*, *M. gordonae*, and *M. peregrinum* with 25 ppm PVPI resulted in all species having less than 10% (Figures 2-1 - 2-4), with *M. chelonae* having the most survival at an average of $5.53\% \pm 18.79\%$. There was no significant difference found between species (Kruskal-Wallis; $\chi^2(3) = 8.25$, $p = 0.05$). Prepared iodine solutions were evaluated for iodine concentration using iodine test strips immediately after they were prepared and used in these trials, as well as 24 hours later. There was a noticeable decrease in concentration (Figure 2-5).

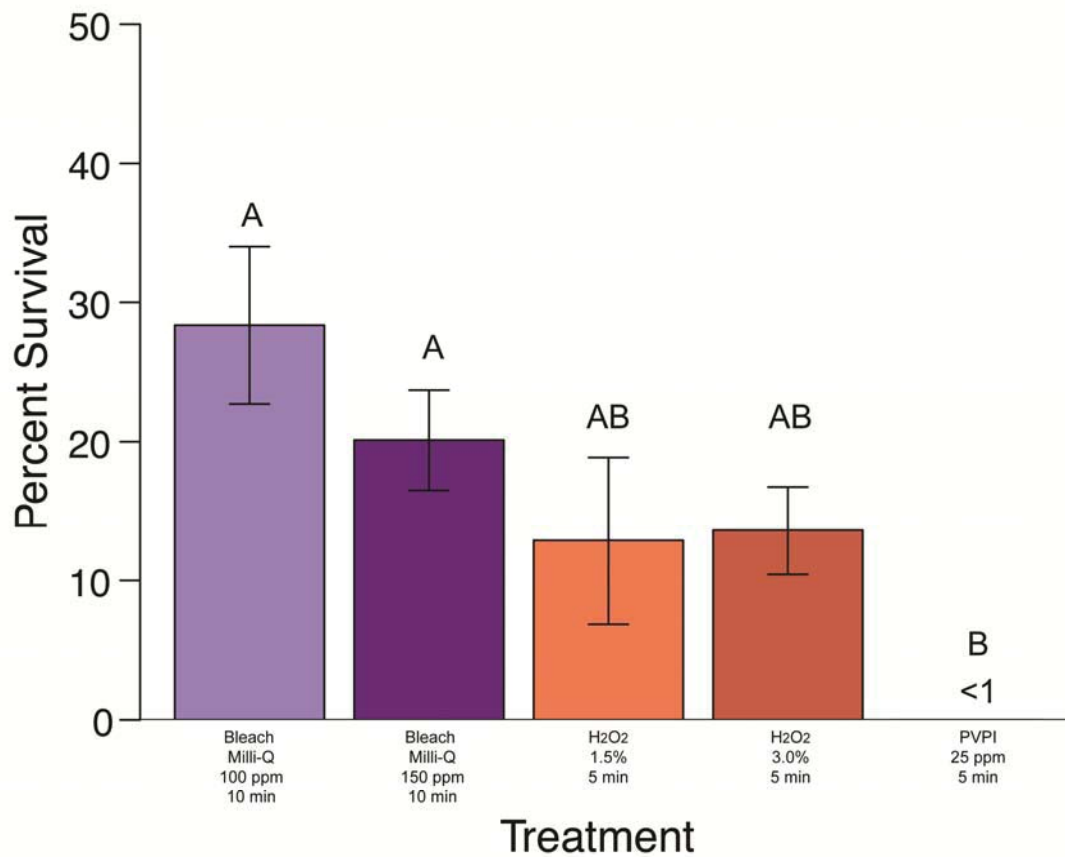


Figure 2-1. *Mycobacterium abscessus* percent survival effect of disinfectant treatments (Clorox® bleach in Milli-Q®, hydrogen peroxide, and PVP-I). Group labels (A or B) identify treatments that differ significantly [$\chi^2(4)=33.38$, $p < 0.05$]. Treatments resulting in survival values less than 1% are indicated (<1).

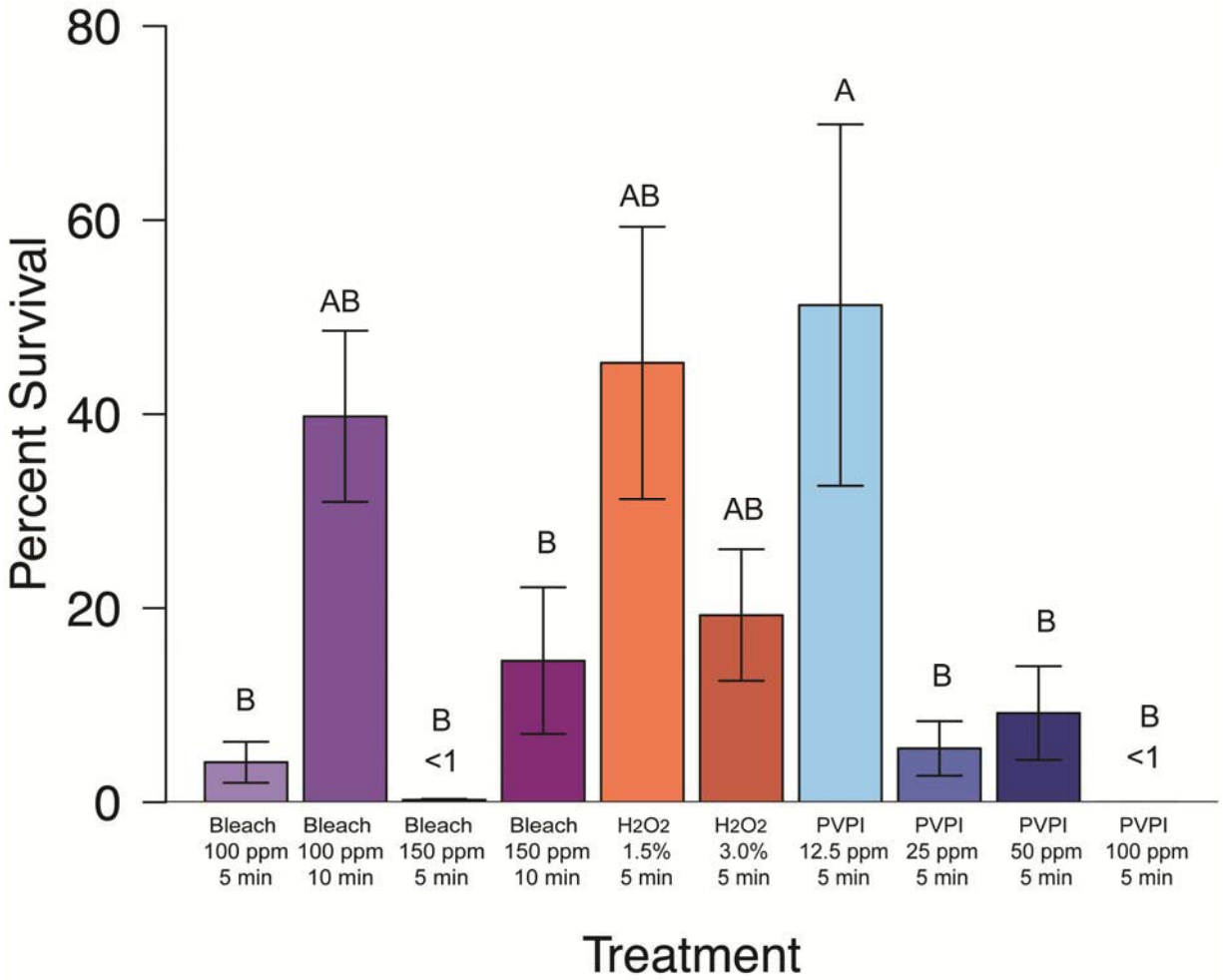


Figure 2-2. *Mycobacterium chelonae* percent survival effect of disinfectant treatments , (Clorox® bleach in Milli-Q®, hydrogen peroxide, and PVPI). Group labels (A or B) identify treatments that differ significantly [$\chi^2(13)=99.55$, $p < 0.05$]. Treatments resulting in survival values less than 1% are indicated (<1).

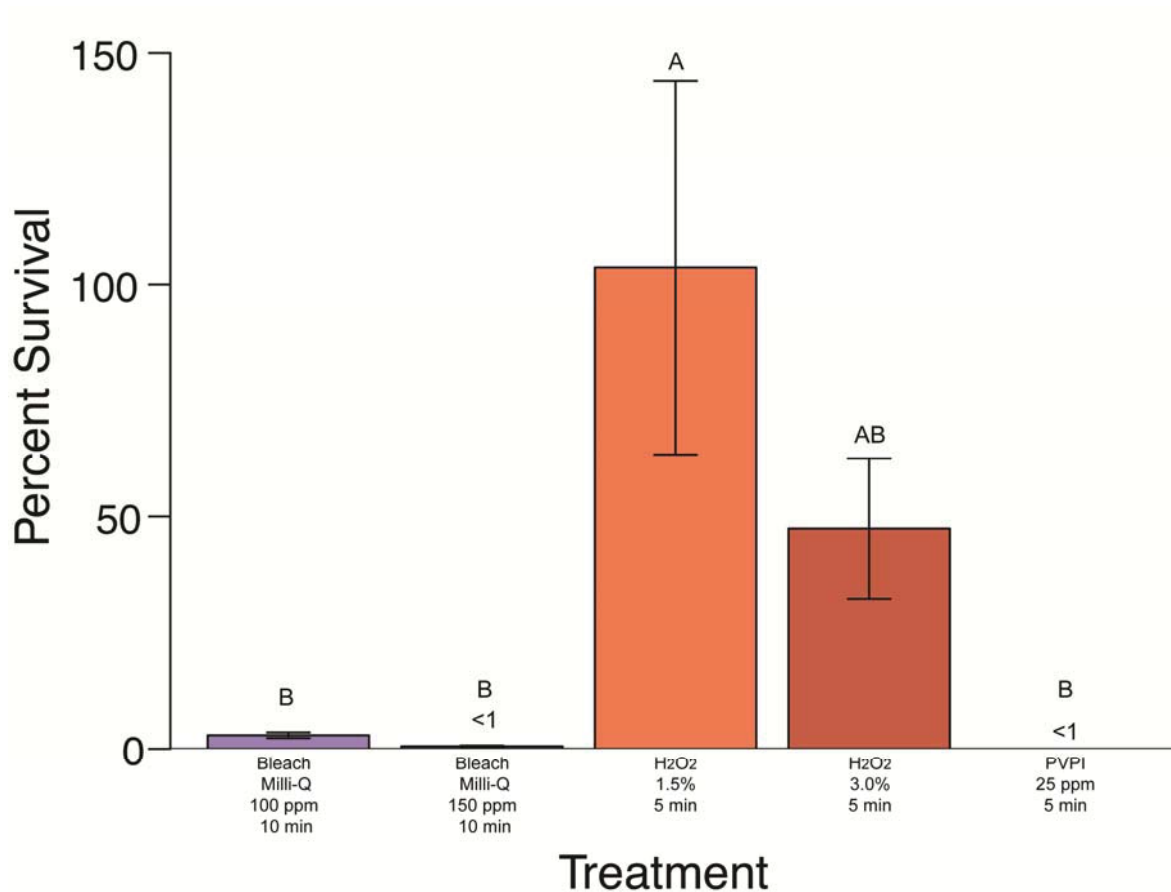


Figure 2-3. *Mycobacterium gordonae* percent survival effect of disinfectant treatments (Clorox® bleach in Milli-Q®, hydrogen peroxide, and PVP-I). Group labels (A or B) identify treatments that differ significantly [$\chi^2(4)=17.71$, $p < 0.05$]. Treatments resulting in survival values less than 1% are indicated (<1).

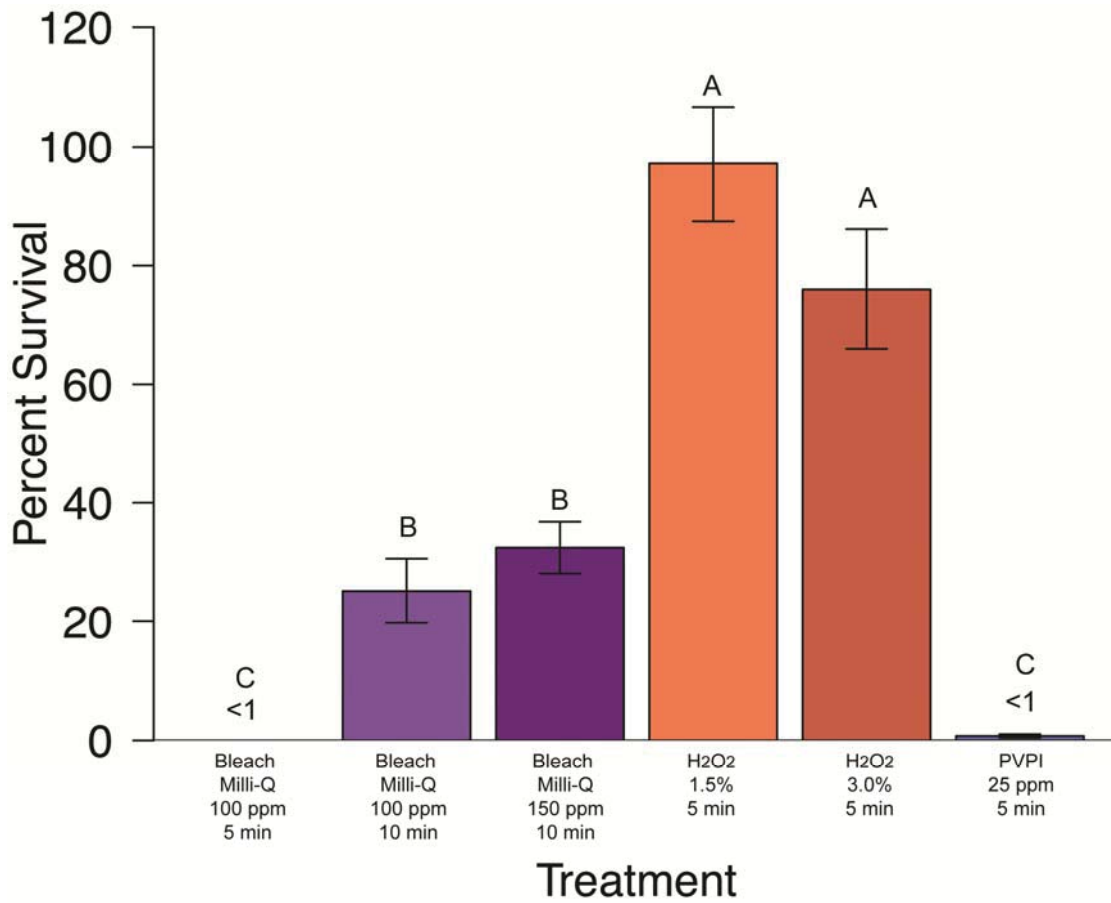


Figure 2-4. *Mycobacterium peregrinum* percent survival effect of disinfectant treatments (Clorox® bleach in Milli-Q®, hydrogen peroxide, and PVP-I). Group labels (A, B, or C) identify treatments that differ significantly [$\chi^2(7)=67.23$, $p < 0.05$]. Treatments resulting in survival values less than 1% are indicated (<1).

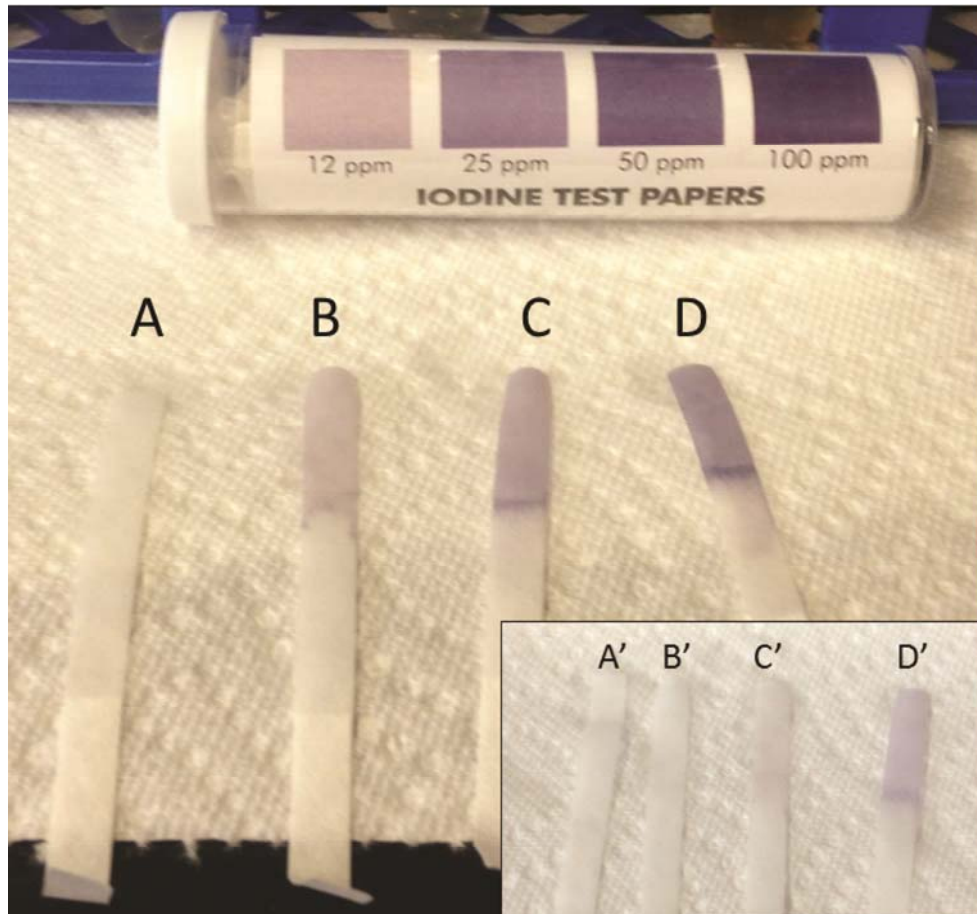


Figure 2-5. Iodine test strips verifying the concentration of (A) 0 ppm, (B) 12.5 ppm, (C) 25 ppm, and (D) 50 ppm PVP-I solutions immediately following solution preparation. The concentration of the same solutions 24h later (A', B', C', D') has decreased and solutions are no longer usable.

2.5 Discussion

Our study has demonstrated that not all disinfectants are effective at preventing mycobacterial growth and that species-specific differences in susceptibility exist. The standard procedure for zebrafish embryo disinfection is currently bleaching in a 25-100 ppm bleach solution prepared in system water for a total of 10 minutes (Westerfield, 2000). We found that

this method (100 ppm bleach in Milli-Q®) results in a decrease in mycobacterial survival; however, an average survival of 2.94-28.36% was observed and this was significantly variable between *Mycobacterium* spp. tested. This mycobacterial persistence post-bleaching emphasized the need for alternative disinfectant treatments to be considered. Additionally, because effectiveness varied between species, control and management recommendations are complicated as generally diagnostics are not performed prior to preventative measures. We did test bleaching further, this time using a CDFB as a diluent. We found that using a demand-free buffer does increase the effectiveness of bleach disinfection significantly, resulting in less than 1% mycobacterial survival. The difference between these two bleach treatments was not surprising as it has been previously shown that the germicidal properties of chlorine are reduced as pH increases above 7.5, and the toxicity of bleach to microorganisms doubles as pH changes from 9 to 7 (Clark et al. 1989; Health Canada 2004). A shift in pH from 7 to 9 results in a drastic decrease of the germicidal form of chlorine, hypochlorous acid (HOCl), as chlorine exists predominantly in the less-active hypochlorite (OCl⁻) form (Clark et al. 1989). Important considerations when assaying chlorine compounds is to make sure reaction buffers are rendered chlorine-demand free. Otherwise, HOCl can react with the buffer and subsequently decrease the amount available to reach with target molecules (Pizza, 2002). For our experimental trials, the pH of the bleaching solution made in Milli-Q® water ranged from 7.98 to 14.10 during the treatment; whereas, the bleach solution prepared in CDFB was 4.9-5.1. This difference in pH and subsequent effect on chlorine availability explains the difference in germicidal activity on the *Mycobacterium* spp. tested. Despite this increased effectiveness, recommendations for using this buffered bleach treatment for embryos may not be ideal. First, this protocol for preparing the CDFB bleach solution would not be practical with the large-scale and frequent treatments used in

many zebrafish facilities (Kent et al. 2014). Second, buffered chlorine bleach treatments on zebrafish embryos were previously shown to be more toxic to embryos corresponding to higher mortality and malformations than unbuffered (and currently utilized) protocols (Kent et al. 2014). Finally, throughout this study we experienced preparation of the bleaching solutions to be quite involved, requiring a chlorine meter to determine chlorine concentrations as calculations based on the concentration of chlorine in Clorox® resulted in solutions with varying amounts of actual measured chlorine. Due to this variability a more reliable and straight-forward treatment should be considered.

We then considered hydrogen peroxide disinfection as a candidate for preventing mycobacterial spread. This disinfectant is already used for controlling pathogens in other aquatic species (e.g., catfish) and is regularly used in bath and flow-through set-ups to treat eggs at a concentration of 250-500 ppm (Small and Wolters, 2003). Recommendations for *Mycobacterium* spp. include the usage of a much more concentrated solution of 30,000 ppm hydrogen peroxide (Noga, 2010). We tested hydrogen peroxide at both 15,000 ppm and 30,000 for 5 minute treatments and found both treatments resulted in very little bacterial killing for all species of *Mycobacterium* tested (Figures 2-1 – 2-4). A longer duration of treatment may result in an increased germicidal effect, as bath treatments used in other fisheries are longer than five minutes (Small and Wolters, 2003). However, germicidal effect was poor even at very high concentrations which are unlikely to be safe for fish, and we do not recommend hydrogen peroxide disinfection as an alternative to bleach.

Finally, we considered iodophor disinfection using PVPI. Iodine is already an established embryo disinfectant in salmonid culture (Wood 1979, Game 1983, 1988, Service 2004, Wagner et al. 2008). We first tested PVPI disinfection on *M. chelonae*, a frequent zebrafish pathogen, at

multiple concentrations (12.5 ppm, 25 ppm, 50 ppm, and 100 ppm) for a 5 minute duration. We found 25-100 ppm treatments resulted in a significant decrease of *M. chelonae* survival as well as no significant difference between these treatment concentrations. We then choose 25 ppm as the concentration to test additional *Mycobacterium* spp. as it was the lowest concentration with a significant effect on bacterial survival. This treatment was also effective for *M. abscessus*, *M. gordonae* and *M. peregrinum* resulting in less than 1% average survival for all of these species. This treatment is comparable to the buffered chlorine bleach treatment, but requires much less preparation. During these trials we found that preparing PVPI disinfection solutions was straightforward and concentration calculated from the original solution consistently produced treatment solution concentrations, verified by iodine test strips. Importantly, we did find that these PVPI treatment solutions need to be prepared shortly before treatment, as the concentration of iodine in these solutions decreased over time (Figure 2-5). We recommend making stock solutions immediately prior to use and not to be stored longer than a day. Results from the PVPI disinfection experiments identify iodophor disinfection at 25 ppm for 5 minutes as an effective alternative from chlorine bleach for killing mycobacteria from zebrafish. Toxicity of this disinfection treatment on zebrafish embryos is still unknown but is currently under investigation.

Additionally, many factors influence the effectiveness of disinfectants including temperature, time of contact, pH, concentration as well as the presence of organic matter (Mainous and Smith, 2005). It is important to consider these factors. For example, embryos should be rinsed well to remove excess organic matter prior to disinfection. Rinsing solutions should be free of pathogens as rinsing with a contaminated solution following embryo disinfection could negate the efforts of disinfection. Adequate storage and preparation of disinfectants is important in order to ensure germicidal activity. Depending on the environmental

conditions (e.g., temperature, lighting) within a zebrafish facility, storage long-term storage of disinfectants may not be appropriate and alternative storage is necessary (e.g., refrigeration) in order to ensure disinfectant integrity. Also, as previously discussed, working solutions of disinfectants should be prepared shortly prior to use and concentrations verified. As successful as these treatments are at preventing the spread of microorganisms, they will not inhibit intraovum pathogens (e.g., *P. neurophilia*) (Sanders and Kent, 2013). Additional disease prevention and monitoring measures should be used in addition to regular embryo disinfection.

The usage of disinfectants in zebrafish facilities is an important disease control measure that all facilities should consider, especially when introducing embryos from an outside facility. Here we showed that the germicidal effect of different disinfectants on *Mycobacterium* spp. varies by species, and that the currently used unbuffered chlorine bleach does have a germicidal effect, but 25 ppm PVPI for 5 min may be an improved alternative, once *in vivo* testing determines it is safe for embryos.

2.6 Acknowledgements

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2.7 References

Alaska Department of Fish and Game, 1983. Fish culture manual, Juneau, Alaska.

Alaska Department of Fish and Game, 1988. Safer chemical use in Alaskan Aquaculture, Juneau, Alaska.

Astrosky, K.M., Schrenzel, M.D., Bullis, R.A., Smolowitz, R.M., Fox, J.G., 2000. Diagnosis and management of atypical *Mycobacterium* spp. infections in established laboratory zebrafish (*Brachydanio rerio*) facilities. *Comparative Medicine* 50, 666-672.

Bardouniotis, E., Ceri, H., Olson, M.E., 2003. Biofilm formation and biocide susceptibility testing of *Mycobacterium fortuitum* and *Mycobacterium marinum*. *Current Microbiology* 46, 28-32.

Block, S.S., 2001. *Disinfection, Sterilization, and Preservation*, 5th ed. Lippincott Williams & Wilkins, Philadelphia.

Clark, R.M., Eleanor, R.J., Hoff, J., 1989. Analysis of inactivation of *Giardia lamblia* by chlorine. *J Environ. Eng.* 115:80-90.

Detrich III, H.W., Westerfield, M., Zon, L.I., 2011. The zebrafish: genetics genomics and informatics 3rd Ed. *Methods in Cell Biology* 104, 471-473.

Falkinham, J.O., 3rd, 2009. Surrounded by mycobacteria: nontuberculous mycobacteria in the human environment. *Journal of Applied Microbiology* 107, 356-367.

Falkinham, J.O., 3rd, Norton, C.D., LeChevallier, M.W., 2001. Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other mycobacteria in drinking water distribution systems. *Applied Environmental Microbiology* 67, 1225-1231.

Ferguson, J.A., Watral, V., Schwindt, A.R., Kent, M.L., 2007. Spores of two fish microsporidia (*Pseudoloma neurophilia* and *Glugea anomola*) are highly resistant to chlorine. *Diseases of Aquatic Organisms* 76, 205-214.

Fox, J., Weisberg, S., 2011. An {R} Companion to Applied Regression. Sage, Thousand Oaks, CA, USA.

Garner, J.S., Favero, M.S., 1986. Cdc Guideline for Handwashing and Hospital Environmental-Control, 1985. *Infection Control and Hospital Epidemiology* 7, 231-243.

Harper, C., Lawrence, C., 2011. The laboratory zebrafish. CRC Press, Boca Raton, FL.

Health Canada., 2004. Guidelines for Canadian drinking water quality: supporting documentation - Protozoa: *Giardia* and *Cryptosporidium*. Water Quality and Health Bureau, Healthy Environments and Consumer Safety Branch, Health Canada, Ottawa.

Kent, M.L., Feist, S.W., Harper, C., Hoogstraten-Miller, S., Law, J.M., Sanchez-Morgado, J.M., Tanguay, R.L., Sanders, G.E., Spitsbergen, J.M., Whipps, C.M., 2009. Recommendations for control of pathogens and infectious diseases in fish research facilities. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology* : CBP 149, 240-248.

Kent, M.L., Whipps, C.M., Matthews, J.L., Florio, D., Watral, V., Bishop-Stewart, J.K., Poort, M., Bermudez, L., 2004. Mycobacteriosis in zebrafish (*Danio rerio*) research facilities. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology* : CBP 138, 383-390.

Kent, M.L.S., J.M., Matthews, J.M., Fournie, J.W., Murray, K.N., Westerfield, M., 2012. Diseases of Zebrafish in Research Facilities, Zebrafish International Resource Center.

Kent, M. L. S., Buchner, C., Barton, C., Tanguay, R. L., 2014. Toxicity of chlorine to zebrafish embryos. *Diseases of Aquatic Organisms* 107, 235-240.

Lawrence, C., 2007. The husbandry of zebrafish (*Danio rerio*): A review. *Aquaculture* 269, 1-20.

Mainous, M.E., Smith, S.A., 2005. Efficacy of Common Disinfectants against *Mycobacterium marinum*. *Journal of Aquatic Animal Health* 17: 284-288.

Milligan-Myhre, K., Charette, J.R., Phennicie, R.T., Stephens, W.Z., Rawls, J.F., Guillemin, K., Kim, C.H., 2011. Study of Host-Microbe Interactions in Zebrafish. *Methods in Cell Biology* 105, 87-116.

Morales, M., with code developed by the R Development Core Team, with general advice from the R-help listserv community and especially Duncan Murdoch, 2012. *sciplot: Scientific Graphing Functions for Factorial Designs.*, R package version 1.1-0. ed.

Noga, E.J., 2010. *Fish Disease: Diagnosis and Treatment*, 2 ed. Wiley-Blackwell, Iowa, USA.

Pizzi N. *Water Treatment Operator Handbook* 2nd ed. 2002 American Water Works Association.

Pohlert, T., 2015. *PMCMR: Calculate Pairwise Multiple Comparisons of Mean Rank Sums.*, 1.1 ed. R package.

Poort, M.J., Whipps, C.M., Watral, V. G., Font, W. F., Kent, M. L., 2006. Molecular characterization of *Mycobacterium* species in non-native poeciliids in Hawaii using DNA sequences. *Journal of Fish Diseases* 29, 3: 181-185.

R Core Team, 2013. *R: A language and environment for statistical computing.* . R Foundation for Statistical Computing, Vienna, Austria.

R Studio, 2012. *R Studio: Integrated development environment for R.*, 0.96.122 ed. R Studio, Boston, MA, USA.

Revelle, W., 2014. *psych: Procedures for Personality and Psychological Research*, Version = 1.4.8 ed. Northwestern University, Evanston, Illinois, USA.

Russell, A.D., 1996. Activity of biocides against mycobacteria. *Journal of Applied Bacteriology* 81, S87-S101.

Small, B.C., Wolters, W. R., 2003. Hydrogen peroxide treatment during egg incubation improves channel catfish hatching success. *North American Journal of Aquaculture* 65, 314-317.

Steed, K.A., Falkinham, J.O., 3rd, 2006. Effect of growth in biofilms on chlorine susceptibility of *Mycobacterium avium* and *Mycobacterium intracellulare*. *Applied Environmental Microbiology* 72, 4007-4011.

United States Fish and Wildlife Service, 2004. Iodophor Disinfection of Fish Eggs, Handbook of Aquatic Animal Health Procedures and Protocols. United States Fish and Wildlife Service, 1-7.

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.

Watrall, V., Kent, M.L., 2007. Pathogenesis of *Mycobacterium* spp. in zebrafish (*Danio rerio*) from research facilities. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology* : CBP 145, 55-60.

Westerfield, M., 2000. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (*Danio rerio*), 4th ed. University of Oregon Press, Eugene.

Westerfield, M., 2007. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (*Danio rerio*), 5th ed. University of Oregon Press, Eugene.

Whipps, C.M., Butler, W.R., Pourahmad, F., Watral, V.G., Kent, M.L., 2007a. Molecular systematics support the revival of *Mycobacterium salmoniphilum* (ex Ross 1960) sp nov., nom. rev., a species closely related to *Mycobacterium chelonae*. International Journal of Systematic and Evolutionary Microbiology 57, 2525-2531.

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

Whipps, C.M., Lieggi, C., Wagner, R., 2012. Mycobacteriosis in Zebrafish Colonies. Ilar J 53, 95-105.

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.

Widmer, A.F., Frei, R., 2003. Decontamination, Disinfection, and Sterilization, in: P.R. Murray, E.J. Baron, J.H. Jorgensen, M.A. Pfaller, R.H. Tenenbaum, R.M. Tenenbaum (Eds.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, DC, USA, 77-108.

Wood, J.W., 1979. *Diseases of Pacific Salmon: Their Prevention and Treatment*, 3rd ed. State of Washington Department of Fisheries, Hatchery Division.

**Chapter 3: Zebrafish embryo disinfection with povidone iodine: evaluating an alternative
to chlorine bleach**

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3.1 Abstract

Mycobacteriosis is a common bacterial infection in laboratory zebrafish caused by several different species and strains of *Mycobacterium* including both rapid- and slow-growers. One control measure used to prevent mycobacterial spread within and between facilities is surface disinfection of eggs. Recent studies have highlighted the effectiveness of povidone-iodine (PVPI) on preventing propagation of *Mycobacterium* spp. found in zebrafish colonies. We evaluated the effect of disinfection using 12.5-50 ppm PVPI (unbuffered and buffered) on zebrafish exposed at 6 or 24 h post-fertilization (hpf) to determine if this treatment is suitable for use in research zebrafish. Our results show that 6 hpf embryos are less sensitive to treatment as fewer effects on mortality, developmental delay and deformity were observed. We also found that buffered PVPI treatment results in a greater knockdown of *Mycobacterium chelonae* and *Mycobacterium marinum*, as well as results in decreased harmful effects on embryos. Treatments of shorter (2 min vs. 5 min) duration were also more effective at killing mycobacteria in addition to resulting in fewer effects on embryo health. Additionally we compared the efficacy of a rinsing regime to rinsing and disinfecting. Based on the findings of this study, we recommend disinfecting embryos for 2 min with buffered PVPI at 12.5-25 ppm.

3.2 Introduction

Disease prevention in laboratory zebrafish and the maintenance of healthy stocks is a primary interest of the zebrafish research community. Diseases in laboratory zebrafish not only directly affect research due to the loss of valuable wild-type and mutant genetic lines, but can also have indirect effects as chronic subclinical infections are a potential confounding source of uncontrolled experimental variance (Kent, Whipps et al. 2004, Whipps 2012).

Mycobacteriosis is a common bacterial disease that affects zebrafish (Astrofsky, Schrenzel et al. 2000, Kent, Whipps et al. 2004, Kent 2012, Whipps 2012). The Zebrafish International Resource Center's (ZIRC) Diagnostic Services report that over 40% of facilities submitting specimens between 2006-2010 had fish diagnosed with mycobacteriosis (Kent 2012). Several species of *Mycobacterium* have been implicated in outbreaks in zebrafish (Astrofsky, Schrenzel et al. 2000, Kent, Whipps et al. 2004, Whipps, Matthews et al. 2008, Whipps 2012). Manifestation of mycobacterial infections in zebrafish is species-specific (Watrall and Kent 2007, Whipps, Butler et al. 2007, Whipps, Dougan et al. 2007, Whipps 2012). For example, infection with *Mycobacterium chelonae*, the most common species found in laboratory zebrafish, typically results in chronic and low-to-subclinical infections (Watrall and Kent 2007). Alternatively, *Mycobacterium marinum* causes acute and severe outbreaks that often result in obvious mortalities (Whipps, Burton et al. 2006). Both of these manifestations have the potential to be devastating to research.

Recommendations for mycobacterial disease management in zebrafish highlight the importance of disease prevention, as established infections are difficult and time-consuming to eradicate (Whipps 2012). As a measure of biosecurity, facilities are encouraged to implement "eggs-only" policies, introducing only disinfected embryos from outside facilities.

Embryo surface disinfection is a common practice in fisheries and the standard disinfectant for zebrafish eggs is currently rinsing embryos with chlorine bleach. However, povidone iodine (PVPI) has been shown to be a promising alternative as *in vitro* studies show PVPI is effective at killing *Mycobacterium* spp. that commonly infect zebrafish (Chang, Colicino et al. 2015).

Before PVPI can be recommended for use in zebrafish an evaluation of the effect of disinfection on the health of zebrafish embryos is required. To accomplish this, we evaluated both unbuffered and buffered povidone iodine on wild-type zebrafish embryos at two developmental time-points [6 hours post-fertilization (hpf) and 24 hpf]. We also tested the effect of these treatments on the survival of planktonic *Mycobacterium chelonae* and *Mycobacterium marinum* cultures.

3.3 Methods

Fish

All embryos used in the exposure studies were bred using AB and AB/Tübingen zebrafish obtained from both the zebrafish facility at the SUNY-ESF Center for Integrated Teaching and Research in Aquatic Sciences as well as from the Amack Lab at SUNY Upstate Medical University. Embryos were obtained from both paired and large group spawns and held in E2 Embryo Medium(Westerfield 2000) (pH 7.4-7.5) at 28.5°C while development was monitored and staged (Kimmel, Ballard et al. 1995).

Exposure

Ninety-six eggs per treatment (3 replicates of 32) were used for each concentration of PVPI [unbuffered(drugstore brand) or buffered Ovadine ®(Western Chemical)] and were designated to treatment groups randomly. For all treatment solutions, iodine concentration was

verified using iodine test strips (LaMotte). The PVPI solutions were prepared fresh within 30 min of each treatment in autoclaved Milli-Q® water. A method for disinfectant exposure similar to a previous study evaluating chlorine bleach disinfection was utilized¹². Embryos were exposed at either 6 hpf or 24 hpf as embryo disinfection usually occurs following egg collection and screening (6 hpf) or following shipment of embryos to another facility (24 hpf) (Kent, Buchner et al. 2014).

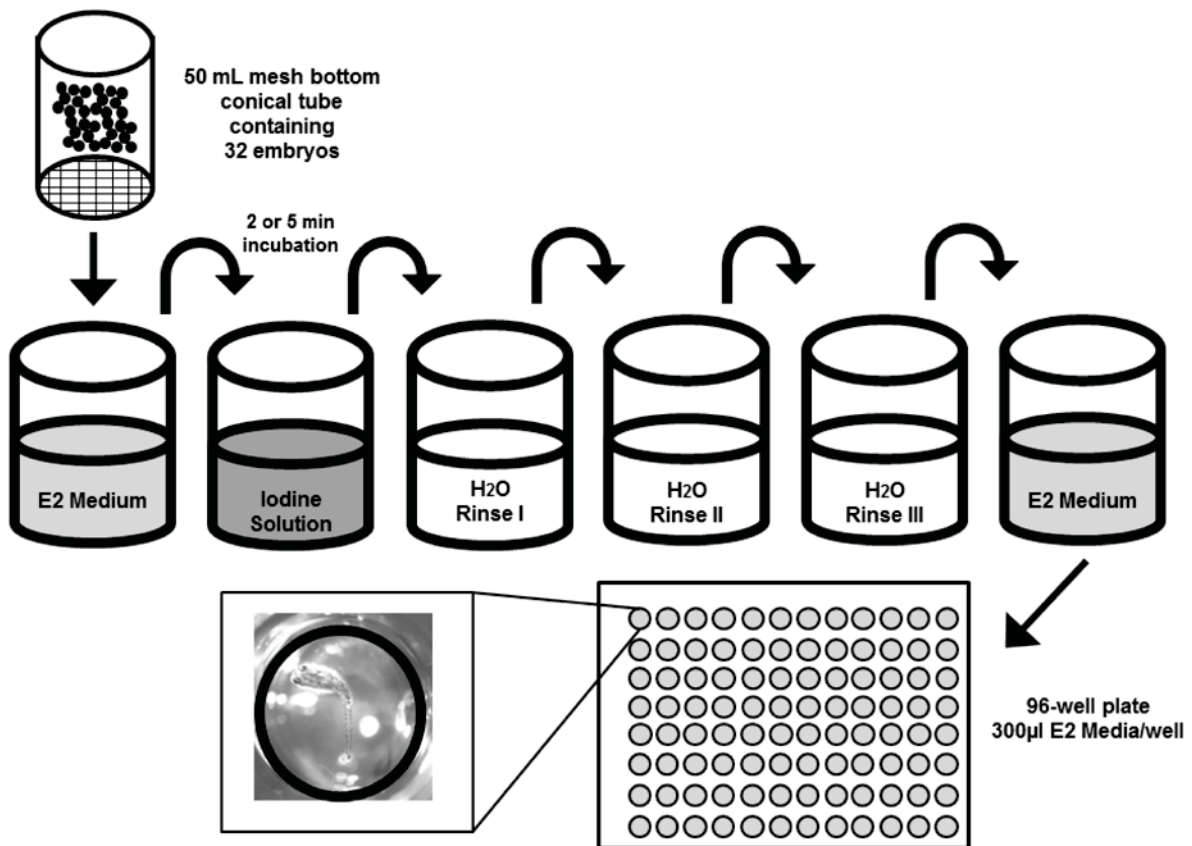


Figure 3-1. Schematic of the embryo disinfectant exposure experiment.

Embryo exposures were carried out by placing 32 embryos at 6 hpf or 24 hpf in a 50 mL conical tube where the bottom was replaced with a 50 µm mesh screen held in E2 Medium. This

tube was transferred to treatment and rinse solutions as illustrated (Figure 3-1). For each treatment solution (unbuffered PVPI: 12.5 ppm, 25 ppm, and 50 ppm; buffered PVPI: 12.5 ppm, 25 ppm, and 50ppm) three treatment groups were run concurrently with embryos from the same clutch: 2 minute exposure, 5 minute exposure and 5 minute control (no iodine) exposure. During each exposure the 32 embryos in the conical tube were transferred from the E2 medium, to a PVPI treatment solution for 2 or 5 minutes, to three separate sterile Milli-Q® water rinses (conical tubes were fully lowered into beakers containing 35 mL of sterile Milli-Q® and lifted out of solution, total rinse time lasted approximately 5 seconds) and to fresh E2 Medium (pH 7.4-7.5).

Toxicity Evaluation

Following exposures, embryos were individually loaded into wells containing 300 µl of E2 Medium (pH 7.4-7.5) in a sterile 96-well plate so that embryos from the three concurrent exposures were incubated in the same 96-well plate. Following exposures, 6 hpf embryos were monitored at 30 minutes post-exposure and daily up to 5 dpf. Twenty-four hpf embryos were monitored at 30 min and 5 hours post-exposure as well as daily up to 5 dpf. Monitoring included observation of mortality, developmental delay, and deformity as in previous studies (Kent, Buchner et al. 2014) (Truong, L et al. 2010). Once embryos reached 5 dpf they were euthanized in a solution of 300 mg/L MS222 buffered to a pH of 7.5.

Culture

An *in vitro* exposure of *M. chelonae* and *M. marinum* to the PVPI treatment solutions used in this study was also carried out. These exposures were carried out in triplicate following a previously described method (Chang, Colicino et al. 2015) for *M. chelonae* cultures and similarly for *M. marinum*; however, for *M. marinum* the initial suspension of cells was prepared by

inoculating sterile water with *M. marinum* freshly cultured on solid-phase Middlebrook 7H10 (MB 7H10) agar supplemented with hemin. Also, following exposures, solid-phase MB 7H10 agar supplemented with hemin was used, and plates were incubated for 14 days prior to colony counts.

An additional evaluation of disinfection *in vitro* was carried out to simulate an actual zebrafish embryo disinfection event. For this, N=32 embryos at 6 hpf were placed in a sterile 30 mL culture flask in E2 Medium (pH 7.4-7.5). This flask was then inoculated with enough broth culture to result in a final concentration of 1.0×10^6 colony forming units (CFU) /mL of *M. chelonae*. This was intended to simulate an embryo shipment to another facility, in spawn water containing bacteria. This flask was then incubated overnight at 28.5°C at 50 rpm on a shaker incubator to emulate courier transport and bacterial incubation during this period. An additional, un-inoculated, flask was prepared and incubated as a control.

The following morning at 10:30 am (a time-point when an overnight express courier option would be received) the contents of the flask were emptied into a 50 mL conical tube with the bottom replaced with 50 µm mesh and were taken through the disinfection exposure similar to the embryos undergoing a five minute exposure to either 25 ppm unbuffered PVPI or a control of sterile water. Samples (1mL) from the original E2 Medium (pH 7.4-7.5) from the flask and final E2 Medium (pH 7.4-7.5) solutions were taken and prepared for plating. Samples were diluted to 10^{-1} , 10^{-2} , and 10^{-3} . One hundred µl of each dilution was plated in triplicate on Middlebrook agar plates using a sterile spreader and incubated at 28.5°C for 7 days. Following incubation, colony counts were performed. This experiment was performed in triplicate.

Statistics

To determine the difference between disinfection treatments on embryo health at different developmental stages the following analysis was carried out using R 3.1.0 (R Core Team 2013) and R Studio (R Studio 2012). Embryo mortality, developmental delay and deformity up to 5 dpf following disinfection treatment was recorded and entered in a spreadsheet and saved as text files for analysis. Descriptive statistics were obtained using the “psych” package (Revelle 2014) and data normality and equal variances were assessed using the “stats” package (R Core Team 2013) and “car” package (Fox and Weisberg 2011) respectively.

In the case of data with a normal distribution and equal variances, an analysis of variance (ANOVA) was performed to separately compare mortality, developmental delay or deformity between disinfection treatments using the “stats” package (R Core Team 2013). In the scenario of a significant ANOVA results ($p < 0.05$) Tukey comparison post-hoc testing was performed using the “agricolae” package (De Mendiburu 2009). If data was non-normally distributed or had unequal variance a Kruskal-Wallis rank sum test was used to compare mortality, developmental delay or deformity between disinfection treatments using the “stats” package (R Core Team 2013). In the case of a significant result ($p < 0.05$), post-hoc testing for pairwise multiple comparisons of the ranked data were performed using the “PMCMR” package (Pohlert 2015). Visualization of data was then carried out as a clustered bar graph using the “sciplot” package (Morales, with code developed by the R Development Core Team et al. 2012).

Analyses of PVPI disinfection on *M. chelonae* and *M. marinum* were analyzed using R 3.1.0 (R Core Team 2013) and R Studio (R Studio 2012). Descriptive statistics were obtained using the “psych” package (Revelle 2014) and data normality and equal variances were assessed using the “stats” package (R Core Team 2013) and “car” package (Fox and Weisberg 2011)

respectively. A one-way analysis of variance (ANOVA) was performed using the “stats” package and Tukey post-hoc comparisons were carried out using the “agricolae” package (De Mendiburu 2009). Data was visualized as a clustered bar chart using the “sciplot” package (Morales, with code developed by the R Development Core Team et al. 2012).

To compare the effect of PVPI disinfection to a rinsing regime, resulting colony counts on serial dilution plates were entered into a spreadsheet and the average colony count from the original flask as well as the final E2 solution were calculated for each replicate for both the disinfection treatment as well as the control treatment. A percent survival was calculated for each replicate as $[(E2 \text{ average colony count}/\text{Flask average colony count}) * 100]$. These survival values were then analyzed using R 3.1.0 (R Core Team 2013) and R Studio (R Studio 2012).

Descriptive statistics were obtained using the “psych” package (Revelle 2014) and data normality and equal variances were assessed using the “stats” package (R Core Team 2013) and “car” package (Fox and Weisberg 2011) respectively. An independent 2-group t-test was carried out to compare the survival between the disinfection treatment and control treatment using the “stats” package (R Core Team 2013).

3.4 Results

Effects of PVPI Disinfection on Embryo Health

Results from the disinfectant embryo exposures show that for 6 hpf embryos disinfected with unbuffered PVPI, a significant increase in embryo mortality was only observed for the 50 ppm, 5 min PVPI treatment compared to the control (0 ppm) embryos [$F(12,41) = 4.46, p < 0.001$] (Figure 3-2). There was no significant effect of unbuffered PVPI treatment on developmental delay or deformity for any of the treatments at 6 hpf.

Disinfection treatments with buffered Ovadine® resulted in no significant difference in mortality, developmental delay or deformity for any of the treatments tested compared to 6 hpf control embryos (Figure 3-2). When comparing PVPI treatments of the same concentration and duration between unbuffered and buffered PVPI solutions for 6 hpf embryos, there was significant difference in embryo mortality for the 50 ppm-5 min treatment [$F(12,41) = 4.46$, $p < 0.001$] (compare Figure 3-2A and 3-2C).

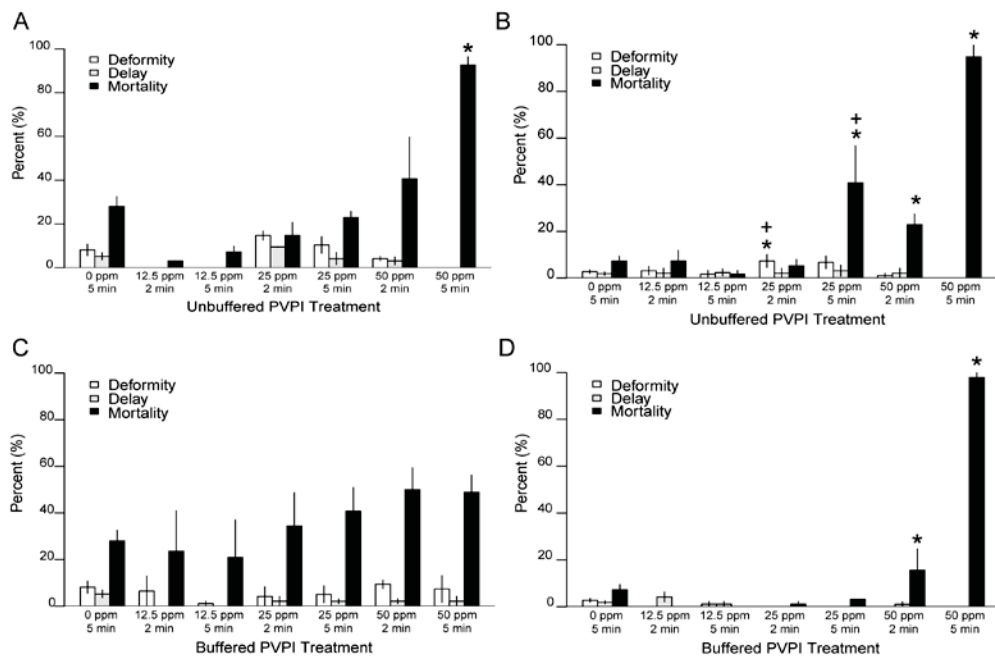


Figure 3- 2. Results of the embryo disinfectant exposures displaying the percent deformity (white bars), developmental delay (grey bars) and mortality (black bars) for (A) 6 hpf embryos exposed to unbuffered PVPI, (B) 24 hpf embryos exposed to unbuffered PVPI, (C) 6 hpf embryos exposed to buffered PVPI and (D) 24 hpf embryos exposed to buffered PVPI. Significant differences between treatment concentration/durations are indicated by an asterisk (*). Significant differences between PVPI solutions for the same treatment are indicated by a plus-sign (+).

For disinfection treatment of 24 hpf embryos, effects on embryo health were observed for 25 and 50 ppm treatments relative to controls (Figure 3-2). Unbuffered PVPI treatments resulted in a significant increase in mortality for 25 ppm-5 min, 50 ppm-2 min, and 50 ppm-5 min treatments compared to control 24 hpf embryos [$\chi^2(12) = 34.24$, $p < 0.001$] (Figure 2). Also, there was significantly greater embryo deformity observed for the 25 ppm-2 min treatment compared to control 24 hpf embryos [$F(12,47) = 2.148$, $p < 0.05$]. There was no significant effect on developmental delay observed for any of the unbuffered PVPI treatments compared to 24 hpf control embryos. Buffered PVPI treatments of 24 hpf embryos resulted in significantly greater mortality for both 50 ppm treatments (2 and 5 min) compared to control 24 hpf embryos [$\chi^2(12) = 34.24$, $p < 0.001$] (Figure 2). There were no significant developmental delay or deformity effects for buffered PVPI treatment of 24 hpf embryos. When comparing PVPI treatments of the same concentration and duration between unbuffered and buffered PVPI solutions for 24 hpf embryos, there was significant difference in embryo health effects for 25 ppm treatments. Specifically, the unbuffered PVPI solution resulted in significantly higher embryo deformity for the 25 ppm-2 min treatments [$F(12,47) = 2.148$, $p < 0.05$], as well as significantly higher embryo mortality for the 25 ppm-5 min treatment compared to the buffered treatments [$\chi^2(12) = 34.24$, $p < 0.001$] (compare Figure 2b and 2d). No other significant differences were observed when comparing the two PVPI solutions.

Effect of PVPI Disinfection on *Mycobacterium* spp.

All PVPI treatments of *M. chelonae* and *M. marinum* in culture resulted in lower survival than control treatments. Generally, knock-down of survival increased as PVPI concentration increased and knockdown of *M. chelonae* was more variable than *M. marinum* (Figure 3-3).

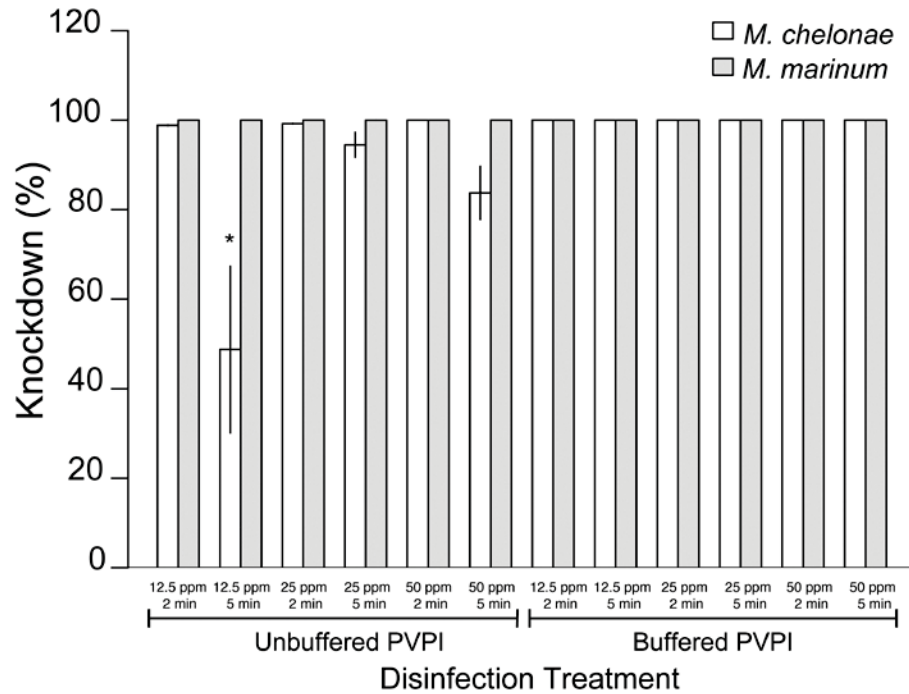


Figure 3-3. Results from the *in vitro* PVPI disinfection displaying the percent knockdown of *M. chelonae* (white) and *M. marinum* (grey). Significant differences between treatments is indicated by an asterisk (*).

For *M. chelonae* exposures, unbuffered (drugstore brand) PVPI treatments were most effective for 2 min exposures compared to 5 min exposures. Based on average bacterial knockdown, the most effective treatments were 50 ppm-2 min, 25 ppm-2 min, and 12.5 ppm-2 min (less than 2% survival) which were more effective than 5 min at the same concentration. The next most effective treatments were 25 ppm-5 min and 50 ppm-5 min (less than 20% survival). The least effective treatment, resulting in the greatest bacterial survival, was the 12.5 ppm-5 min treatment which resulted in significantly more survival of *M. chelonae* than all the other unbuffered PVPI treatments [F(23,466) = 4.012, p< 0.05].

All buffered (Ovadine®) treatments were equally effective (less than 0.1% survival for all treatments) as there was no significant difference between treatment concentrations or durations ($p>0.05$). All PVPI treatments of *M. marinum* resulted in less than 0.1% survival and there were no significant differences found between treatment concentrations or durations ($p>0.05$).

When unbuffered and buffered (Ovadine®) PVPI solutions are compared, the only difference observed was for the *M. chelonae* treatment of 12.5 ppm for 5 min ($p<0.05$), which was previously shown to be the least effective PVPI treatment of *M. chelonae*. This treatment was also the only treatment where, after comparing *M. chelonae* and *M. marinum* survival for the same PVPI treatment, differences were observed between species ($p<0.05$).

Embryo Mock-Disinfection Results

For the embryo mock-disinfection, prior to disinfection, the flasks, both control and treatment, originally inoculated and incubated overnight with *M. chelonae* contained an average $1.8 \times 10^5 - 2.0 \times 10^5$ CFU/ml. Following treatments (rinsing and PVPI disinfection, or rinsing alone) the amount of *M. chelonae* remaining in the E2 medium containing embryos decreased. For the control treatment, which underwent 3 rinses in sterile water, the resulting E2 medium contained 3.0×10^3 CFU/ml *M. chelonae* based on plate counts. The disinfection treatment, which consisted of a 25 ppm unbuffered PVPI for 5 min treatment, the resulting E2 medium contained 0 CFU/ml (SD= 0 CFU/ml) *M. chelonae* based on plate counts.

3.5 Discussion

The results from this study show that zebrafish embryos can tolerate disinfection with PVPI at concentrations/durations that are effective at killing mycobacteria. We found that similar to our previous study (Chang, Colicino et al. 2015), PVPI is effective at killing mycobacteria at

12.5-50 ppm PVPI at both 2 and 5 min treatment durations. Generally results from our evaluation of PVPI disinfection on embryo health show that effects on embryo health occur at higher concentrations and/or treatment durations of PVPI for both 24 hpf embryos and 6 hpf embryos.

The use of a buffered PVPI solution (Ovadine®) resulted in more consistent knockdown of planktonic *M. chelonae* and *M. marinum* in medium, as well as less embryo mortality and deformity compared to unbuffered treatments. This result is intriguing as buffering of chlorine bleach solutions has also been shown to enhance disinfection; although, this buffering has been shown to have a negative effect on embryo health (Kent, Buchner et al. 2014).

The pH of both buffered and unbuffered 50 ppm PVPI solutions were subsequently measured. Unbuffered PVPI had a pH of 5.97, 5.78, and 5.60 for 12.5 ppm, 25 ppm, and 50 ppm solutions, respectively. Unbuffered PVPI was unstable requiring 3 repeats of measurements. Buffered PVPI had a pH of 6.27, 6.01, and 5.70 for 12.5 ppm, 25 ppm, and 50 ppm solutions, respectively, and did not require multiple measurements. The stability of pH as well as acidity may have influenced the impact of PVPI solutions on zebrafish embryo health. The less variable knockdown of *M. chelonae* and *M. marinum* following buffered PVPI treatment was likely due to the different additives and composition of the PVPI solution and their iodine-complexing properties and the subsequent effect on the concentration of free molecular iodine available for binding (Paulson 2014).

Similar to our previous study (Chang, Colicino et al. 2015), we found that shorter durations of disinfection resulted in more consistent decrease in survival of *M. chelonae*. This could be attributed to the amount of available bactericidal iodine [e.g., hydrated iodine (I_2), hypiodous acid (HOI), and iodine cation (H_2OI^+)], as there are several potential reactions of

molecular iodine in water (e.g., hydrolysis, dissociation, protonation, complex formation, and disproportionation) that could limit availability over time as more reactions are able to occur (Cooper 2007).

Shorter durations of PVPI exposures had less of an impact on embryo health as embryo mortality was increased significantly for 6 hpf embryo treatment with unbuffered 50 ppm-5 min compared to 50 ppm-2 min as well as 24 hpf embryo treatment of 25 ppm-2 min compared to 25ppm-5 min. An exception to this pattern was with 24 hpf embryos treated with unbuffered 25 ppm PVPI for 2 minutes. Here we observed an increase in embryo deformity compared to controls and this was not observed for 5 min treated embryos; although this may be due to more 5 min embryos dying compared to 2 min.

While 6 hpf and 24 hpf embryos cannot be compared directly as they are undergoing different developmental processes and subsequent variable natural early mortality, for 24 hpf embryos instances of significantly increased mortality compared to 24 hpf control embryos occurred at 25-50 ppm (unbuffered PVPI). For buffered PVPI at 24 hpf, 25 ppm treatments are an option as increased embryo mortality was only observed for 50 ppm treatments. Whereas for 6 hpf embryos, instances of significantly higher mortality compared to control 6 hpf embryos occurred only at 50 ppm (unbuffered PVPI). This is similar to Kent et al. (2014), supporting the recommendation to, when possible, treat embryos at an earlier time-point.

We also evaluated the role that rinsing embryos has in preventing mycobacterial spread through a “mock-disinfection” experiment. We found that rinsing embryos that were incubated with *M. chelonae* resulted in a decrease in planktonic bacteria; however an average 3.0×10^3 CFU/ml bacteria persisted in the embryo medium. Although rinsing reduced the amount of planktonic bacteria dramatically, they were not eliminated and could still pose an infection risk.

The rinsing followed by disinfection resulted in no culturable mycobacteria in the embryo water. Thus, the diluting effect of rinsing coupled with disinfection with PVPI is very effective at reducing the risk of mycobacterial contamination in embryo cultures.

Generally we recommend disinfection with at least 12.5 ppm PVPI. Similar to Kent et al. (2014) we recommend disinfecting embryos from an outside facility or from a population with a known infection with higher (25-50 ppm) disinfectant concentration as potential biosecurity risk would outweigh the health effects on the first generation. Following generations could then be treated with a lower concentration. Although the potential effects of PVPI disinfection on fine developmental or physiological processes are not known, caution might be warranted in toxicological and behavioral studies until additional studies are conducted.

We only evaluated the effects of PVPI disinfection on wild-type and AB/Tübingen embryos that were not dechorionated. Also, the regular practice of pronase-mediated chorion removal may also influence the effect of disinfection on embryo health. Other genetic lines of zebrafish may be more or less sensitive to disinfection with PVPI. These factors as well as facility water hardness, pH, and conductivity should all be considered when adopting a disinfection protocol.

Also, in order to be consistent with our previous culture studies (Chang, Colicino et al. 2015), we utilized Milli-Q water for diluting our PVPI concentrates as well as for rinse solutions. The usage of alternative rinse solutions (i.e. E2 Medium, autoclaved system water, etc) may result in different embryo health effects and future studies should consider evaluating these solutions. Finally, as previously discussed (Kent et al., 2014; Chang et al., 2015) these methods contribute to good biosecurity and, as such, are likely to reduce the spread of microorganisms. However, they are less likely to prevent the spread of intra-ovum pathogens (e.g., *Pseudoloma*

neurophilia)(Sanders, Watral et al. 2013) and a holistic approach including additional disease prevention and control measures should be taken in addition to embryo disinfection.

In conclusion, we have demonstrated that PVPI is effective for killing *Mycobacterium* spp. in media and these same concentrations and durations that are not harmful to zebrafish embryo health. We also demonstrate that while the practice of rinsing embryos results in decreased bacteria counts but disinfection is much more effective. We also showed that treatment with buffered PVPI (Ovadine®) relative to unbuffered result in greater bacterial knockdown, as well as increased embryo survival.

3.6 Acknowledgements

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3.7 References

- Astrofsky, K. M., M. D. Schrenzel, R. A. Bullis, R. M. Smolowitz and J. G. Fox (2000). "Diagnosis and management of atypical *Mycobacterium* spp. infections in established laboratory zebrafish (*Brachydanio rerio*) facilities." Comparative Medicine **50**(6): 666-672.
- Chang, C. T., E. G. Colicino, E. J. DiPaola, H. J. Al-Hasnawi and C. M. Whipps (2015). "Evaluating the effectiveness of common disinfectants at preventing the propagation of *Mycobacterium* spp. isolated from zebrafish." Comparative Biochemistry Physiology Part C: Toxicology Pharmacology **178**: 45-50.
- Cooper, R. A. (2007). "Iodine revisited." International Wound Journal **4**: 124-137.
- De Mendiburu, F. (2009). Una herramienta de analisis estadistico para la investigacion agricola., Universidad Nacional de Ingenieria (UNI-PERU).
- Fox, J. and S. Weisberg (2011). An {R} Companion to Applied Regression. Thousand Oaks, CA, USA, Sage. **2nd**.
- Kent, M. L., C. Buchner, C. Barton and R. L. Tanguay (2014). "Toxicity of chlorine to zebrafish embryos." Diseases of Aquatic Organisms **107**(3): 235-240.

Kent, M. L., C. M. Whipps, J. L. Matthews, D. Florio, V. Watral, J. K. Bishop-Stewart, M. Poort and L. Bermudez (2004). "Mycobacteriosis in zebrafish (*Danio rerio*) research facilities."

Comparative Biochemistry Physiology Part C Toxicology Pharmacology **138**(3): 383-390.

Kent, M. L. S., J. M., Matthews, J. M., Fournie, J. W., Murray, K. N., Westerfield, M. (2012).

"Diseases of Zebrafish in Research Facilities." Retrieved July 10, 2014, from

<http://zebrafish.org/zirc/health/diseaseManual.php>.

Kimmel, C. B., W. W. Ballard, S. R. Kimmel, B. Ullmann and T. F. Schilling (1995). "Stages of Embryonic-Development of the Zebrafish." Developmental Dynamics **203**(3): 253-310.

Morales, M., with code developed by the R Development Core Team and with general advice from the R-help listserv community and especially Duncan Murdoch (2012). *sciplot: Scientific Graphing Functions for Factorial Designs*.

Paulson, D. S. (2014). Topical Antimicrobials Testing and Evaluation, Second Edition. Boca Raton, FL, USA, CRC Press.

Pohlert, T. (2015). PMCMR: Calculate Pairwise Multiple Comparisons of Mean Rank Sums., R package.

R Core Team (2013). *R: A language and environment for statistical computing*. . Vienna, Austria, R Foundation for Statistical Computing.

R Studio (2012). R Studio: Integrated development environment for R. Boston, MA, USA, R Studio.

Revelle, W. (2014). psych: Procedures for Personality and Psychological Research. Evanston, Illinois, USA, Northwestern University.

Sanders, J. L., V. Watral, K. Clarkson and M. L. Kent (2013). "Verification of Intraovum Transmission of a Microsporidium of Vertebrates: *Pseudoloma neurophilia* Infecting the Zebrafish, *Danio rerio*." Plos One **8**(9).

Truong, L., H. S. L and R. L. Tanguay (2010). "Evaluation of Embryotoxicity Using the Zebrafish Model." Drug Safety Evaluation **691**: 271-279.

Watal, V. and M. L. Kent (2007). "Pathogenesis of *Mycobacterium* spp. in zebrafish (*Danio rerio*) from research facilities." Comparative Biochemistry Physiology Part C Toxicology Pharmacology **145**(1): 55-60.

Westerfield, M. (2000). The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (*Danio rerio*). Eugene, University of Oregon Press.

Whipps, C. M., T. Burton, V. G. Watal, S. St-Hilaire and M. L. Kent (2006). "Assessing the accuracy of a polymerase chain reaction test for *Ichthyophonus hoferi* in Yukon River Chinook salmon *Oncorhynchus tshawytscha*." Diseases of Aquatic Organisms **68**(2): 141-147.

Whipps, C. M., W. R. Butler, F. Pourahmad, V. G. Watral and M. L. Kent (2007). "Molecular systematics support the revival of *Mycobacterium salmonophilum* (ex Ross 1960) sp. nov., nom. rev., a species closely related to *Mycobacterium chelonae*." International Journal of Systematic Evology Microbiology **57**(Pt 11): 2525-2531.

Whipps, C. M., S. T. Dougan and M. L. Kent (2007). "*Mycobacterium haemophilum* infections of zebrafish (*Danio rerio*) in research facilities." FEMS Microbiology Lett **270**(1): 21-26.

Whipps, C. M., Lieggi, C., Wagner, R. (2012). "Mycobacteriosis in zebrafish colonies." ILAR **53**(2): 95-105.

Whipps, C. M., J. L. Matthews and M. L. Kent (2008). "Distribution and genetic characterization of *Mycobacterium chelonae* in laboratory zebrafish *Danio rerio*." Diseases of Aquatic Organisms **82**(1): 45-54.

Chapter 4

Activity of antibiotics against *Mycobacterium* species commonly found in laboratory zebrafish

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4.1 Abstract

The zebrafish (*Danio rerio*) is a popular vertebrate model organism used in a wide range of research fields. Importance is placed on zebrafish health and the maintenance of disease-free laboratory fish so that experimental studies are not inadvertently impacted. Mycobacteriosis is a common infection of laboratory zebrafish that is caused by several *Mycobacterium* species. Little is known regarding the potential of antibiotic treatment for zebrafish mycobacteriosis; however, treatment of infected zebrafish may be appropriate to maintain valuable strains. Here, we investigate the antibiotic susceptibility of both rapid and slow growing zebrafish *Mycobacterium* spp. isolates *in vitro*. Antibiotic testing was carried out using a commercially available 96-well microtiter plate format. Results indicate that some but not all antibiotics tested are effective at inhibiting mycobacterial growth and that susceptibility varies among species and strains. Tigecycline, tobramycin, clarithromycin and amikacin were most effective at broad inhibition of rapid-growing mycobacteria; whereas, amikacin, clarithromycin, and rifampin were effective at inhibiting all slow-growing *Mycobacterium marinum* strains tested. Results support the potential for targeted antibiotic treatment of zebrafish infected with mycobacteria, but additional testing should be carried out *in vivo*.

4.2 Introduction

The zebrafish (*Danio rerio*) has become one of the most prominently used vertebrate model organisms (Dahm and Geisler 2006; Phillips and Westerfield 2014). Initially, zebrafish research was conducted in the fields of genetics and development with embryo and larval end points (Schilling and Webb 2007). More recently, adult zebrafish have become popular in the fields of oncology (Feitsma and Cuppen 2008), toxicology (Truong et al. 2011), aging (Gerhard 2003), and behavior (Wong et al. 2010). Laboratory colonies of zebrafish typically consist of both specialized mutant strains and wild-type strains used for breeding (Westerfield 2007). Due to the increased usage of adult zebrafish in biological research and the value placed on maintaining healthy mutant and wild-type stocks, laboratory zebrafish health is of utmost concern. Incidences of background infections are concerning for multiple reasons, for example: infections can result in fish mortality or decreased reproductive output (Kent et al. 2012a). Additionally, research can also be indirectly affected, as subclinical infections may be a source of uncontrolled experimental variance (or non-protocol variation). As such where possible, measures are taken in order to prevent, control and manage these diseases.

A summary report of cases submitted to the Zebrafish International Resource Center's (ZIRC) Diagnostic Services from 2006-2010 indicates that over 40% of facilities submitting specimens had fish diagnosed with mycobacteriosis (Kent et al. 2012b). Mycobacteriosis is caused by *Mycobacterium* spp. and is frequently found in wild and captive fishes (Chinabut 1999), including ornamental marine and freshwater fishes (Noga 2010). There is no single agent of zebrafish mycobacteriosis as at least six species as well as several strains of *Mycobacterium* spp. have been identified (Astrofsky et al. 2000; Kent et al. 2004; Whipps et al. 2008). In particular, *Mycobacterium marinum*, *Mycobacterium chelonae*, *Mycobacterium abscessus*,

Mycobacterium peregrinum, *Mycobacterium haemophilum* and *Mycobacterium fortuitum* are frequently associated with zebrafish mycobacteriosis (Whipps et al. 2012). Severity of infection varies between species and strains of *Mycobacterium* spp. ranging from high levels of mortality with *M. marinum* and *M. haemophilum*, and little to no observed mortality with *M. abscessus* and *M. chelonae* (Watrall and Kent 2007; Whipps et al. 2007a; Whipps et al. 2007b; Whipps et al. 2012). Morbidity due to infection is also variable and includes external signs such as skin lesions, emaciation, raised scales, swollen abdomen, and irregular or lethargic swim behavior (Astrofsky et al. 2000; Kent et al. 2012b). Internally, infection can be observed as granulomas, particularly on the spleen, kidneys and liver (Whipps et al. 2012). Diagnosis may be further complicated because signs of disease are often not observed in subclinical infections (Kent et al. 2004; Whipps et al. 2012).

Recent reviews of zebrafish diseases, including mycobacteriosis, highlight the importance of preventative measures such as quarantine, regular disinfection of eggs and surfaces, UV sterilization of water, and sentinel programs in zebrafish facilities (Kent et al. 2009; Whipps et al. 2012). Once mycobacteriosis is established in a facility control and management of the disease becomes a major challenge and involves invasive measures such as depopulation, facility sterilization and re-derivation of zebrafish populations. Although these measures have been demonstrated to be effective at controlling mycobacteriosis, such intensive measures may not always be feasible if this disease becomes established during an ongoing experiment or in a valuable zebrafish mutant line (Whipps et al. 2012). Alternative methods for controlling and treating zebrafish mycobacteriosis such as targeted use of antibiotics should be considered.

Antibiotic treatment of non-tuberculosis mycobacteriosis in humans is routine (Griffith et al. 2007; Wu et al. 2012), but similar treatments in fish have not been investigated thoroughly.

Treatment of fish destined for human consumption with antibiotics is not generally considered feasible as treatments are expensive, long in duration and there are concerns regarding the use of pharmaceuticals in fish for human consumption (Whipps et al. 2012). A limited number of studies investigating antibiotic treatment of fish infected with *M. marinum* have been previously conducted and include treatment of both food fish [yellowtail (*Seriola quinqueradiata*): Kawakami and Kusuda 1990; striped bass (*Morone saxatilis*): Hedrick et al. 1987; sea bass (*Dicentrarchus labrax*): Colorni et al. 1998] and hobby fish [gouramis (*Trichogaster trichopterus*): Santacana et al. 1982; firemouth cichlid (*Cichlasoma meeki*): Boos et al. 1995; Congo tetra (*Phenacogrammus interuptus*): Boos et al. 1995; guppies (*Lebistes reticulatus*): Conroy & Conroy 1999]. The results from these studies are highly variable and range from an observed elimination of infection to no decrease in the disease; however, these studies all used different treatment methods (i.e., antibiotic added to food, in water bath, in intraperitoneal injection), antibiotic doses, and different experimental end-points to determine the effectiveness of antibiotic treatment. In addition, the susceptibility of fish mycobacteria to antibiotics has not yet been evaluated *in vitro*. Thus, the efficacy of antibiotics as a potential treatment method for zebrafish mycobacteriosis cannot be extrapolated from these previous studies. An evaluation of antibiotic susceptibility of *Mycobacterium* spp. isolated from infected zebrafish is required in order to determine the potential for antibiotic treatment of mycobacteriosis in zebrafish.

Here we investigate the *in vitro* antibiotic susceptibility of rapid and slow growing *Mycobacterium* spp. isolated from infected fish from different zebrafish facilities in the United States. Antibiotic susceptibility will be evaluated through determination of the minimum inhibitory concentration (MIC) of antibiotic required to inhibit bacterial growth in culture. We utilized a commercially available microtiter panel system that is commonly used for drug

susceptibility testing of clinical *Mycobacterium* spp. infections. We hypothesized that the *Mycobacterium* species and strains examined in this study display antibiotic MICs consistent with those already identified for human isolates.

4.3 Methods

Bacterial Strains

Isolates maintained in our culture collection are described in Table 1. All organisms have previously been identified based on *hsp65* gene sequencing as described previously (Kent et al. 2004; Poort et al. 2006; Whipps et al. 2007a; Whipps et al. 2007b). In addition to these isolates, reference cultures of rapidly growing *Mycobacterium salmoniphilum* (ATCC13758) and slow growing *M. marinum* (ATCC927) are also included in this study. All isolates were grown on solid-phase Middlebrook 7H10 (MB 7H10) agar (BD Biosciences 262710) supplemented with oleic albumin dextrose catalase (OADC, BD Biosciences 211886) at 28-30°C for seven (rapid-growing) or 14 (slow-growing) days prior to MIC testing.

TABLE 4-1. Isolate list for all cultures used for antibiotic susceptibility testing.

Isolate ID	Species	Location	Host	Reference (source)
ESF35	<i>M.chelonae</i>	Pennsylvania, USA	Zebrafish	This study
ZF-48	<i>M.chelonae</i>	Oregon, USA	Zebrafish	Whipps et al. (2008)
ZF-55	<i>M.chelonae</i>	Oregon, USA	Zebrafish	Whipps et al. (2008)
H11-27-1	<i>M.chelonae</i>	New York, USA	Zebrafish	This study
H11-05	<i>M.chelonae</i>	North Carolina, USA	Zebrafish	Whipps et al. (2014)
JAN1	<i>Mycobacterium</i> sp. ^a	Oregon, USA	Zebrafish	Kent et al. (2004)
KC1	<i>M. abscessus</i>	Pennsylvania, USA	Zebrafish	Kent et al. (2004)
ATCC13758	<i>M. salmoniphilum</i>	Washington, USA	Chinook salmon	ATCC13758
ESF36	<i>M. fortuitum</i>	Pennsylvania, USA	Zebrafish	This study
SM4	<i>M. peregrinum</i>	Washington DC, USA	Zebrafish	Kent et al. (2004)
MA-1	<i>M.marinum</i>	Massachusetts, USA	Zebrafish	This study
AR103K	<i>M.marinum</i>	Arkansas, USA	Zebrafish	This study
OR932	<i>M.marinum</i>	Oregon, USA	Zebrafish	This study
ATCC927	<i>M.marinum</i>	Pennsylvania, USA	Salt water fishes ^b	ATCC927
TG19	<i>M.marinum</i>	Oregon, USA	Zebrafish	This study
OSU214	<i>M.marinum</i>	Oregon, USA	Zebrafish	Ostland et al. (2007)

^aOriginally characterized as *M. chelonae* by Kent et al. (2004), subsequently recognized as a distinct entity, but member of the *M. chelonae* complex by Whipps et al. (2007a)

^bFirst isolated and identified by Aronson (1926) from three saltwater fish species: sergeant major (*Abudufduf mauritii*); croakers (*Micropogon undulates*); sea bass (*Centropristes striatus*).

MIC Testing

MIC testing was performed using the commercially available Sensititre rapid- and slow-growing *Mycobacterium* MIC panels (TREK Diagnostics). For rapid-growing isolates the Sensititre RAPMYCO panel was used according to the instructions of the manufacturer (described in Cavusoglu et al. 2012), with the exception of the use of cation adjusted Mueller Hinton broth (CAMHB, Teknova M5887) without TES buffer as recommended in the CLSI M24-A guidelines (CLSI 2011). The inoculated RAPMYCO panel was incubated for three days at 28-30°C. Slow-growing isolates were tested using the Sensititre SLOMYCO panel following the CLSI 24-A (CLSI 2011) and the manufacturer's guidelines. An inoculum was prepared by sweeping a confluent portion of growth from the MB 7H10 agar plate and emulsifying it in 5 ml of sterile water. The inoculum density was adjusted to 0.5 McFarland standard using a nephelometer (Sensititre). Fifty µL of the inoculum was diluted in 11 mL CAMHB supplemented with 5% volume of OADC enrichment. The inoculated broth suspension was vortexed and then 100 µL was added to each well of the SLOMYCO MIC panel. The inoculated panel was incubated for seven days at 28-30°C. Additional positive control plates were prepared by inoculating MB 7H10 agar plates with 1µL of the positive control well contents. Serial dilutions of the inoculated CAMHB were prepared to verify density of 1.0×10^5 - 1.0×10^6 colony forming units (CFU)/ml for the panel inoculum.

Following incubation, the panels were scored daily for three consecutive days to ensure consistent scoring. Growth was scored following the CLSI M24-A guidelines for interpretation of broth microdilution MIC end points (CLSI 2011). The MIC for each antibiotic agent tested was determined based on these scores; in the scenario of growth cessation at different antibiotic concentrations between daily readings, the highest MIC was chosen. MIC panels were run in

triplicate for each species of *Mycobacterium* included in this study. There are no published breakpoints for MIC values for zebrafish mycobacteria; however, values are available for clinical isolates from humans. These available breakpoints were used to classify MICs as susceptible, intermediate, or resistant.

4.4 Results

All species grew successfully in the broth medium and growth endpoints were easily determined. Growth was observed in the positive control well of all panels, as well as on the positive control agar plates. Growth counts of the serial dilution plates confirmed an inoculating density of 1.0×10^5 - 1.0×10^6 CFU/ml.

Rapidly growing Mycobacterium spp.

A summary of MIC results for five rapid-growing species of *Mycobacterium* spp. tested with 15 different antibiotic treatments is given in Table 2. Species and strain differences in antibiotic susceptibility are observed as well as variation in the effectiveness of different treatments. Using the CLSI M24-A MIC break-points for rapidly growing *Mycobacterium* spp., strains were categorized as susceptible, intermediate, or resistant to treatment. Species in the *M. fortuitum* group (*M. fortuitum* and *M. peregrinum*) and *M. salmoniphilum* (ATCC13758) are susceptible to amikacin treatment; however *M. abscessus* and *M. chelonae* strains exhibited intermediate susceptibility. *Mycobacterium chelonae* strains and *M. salmoniphilum* show resistance to cefoxitin. *Mycobacterium abscessus* and *M. fortuitum* show intermediate cefoxitin susceptibility while *M. peregrinum* displays susceptibility. Ciprofloxacin results show resistance or intermediate susceptibility in all strains except the *M. fortuitum* group species which are susceptible to treatment. Susceptibility to clarithromycin is observed in *M. chelonae* species, *M. salmoniphilum* and *M. peregrinum*, but *M. abscessus* and *M. fortuitum* are resistant. Resistance

TABLE 4-2. MICs for rapidly growing mycobacterial strains in the microdilution broth system. Each isolate culture was tested in triplicate; a single MIC is shown when all replicates resulted in the same MIC. Reference susceptibility (S), intermediate (I) and resistance (R) breakpoints for antibiotics are indicated. Bolded results indicate MICs indicating antibiotic susceptibility.

Antibiotic Agent (S:I:R)	<i>Mycobacterium</i> sp.									
	JANI	ESF35	ZF-48	ZF-55	H11-27-1	H11-05	KCI	ATCC13758	<i>M. fortuitum</i>	<i>M. peregrinum</i>
Amikacin (≤16; 32; ≥64)	64	32	32, 64, 64	16, 32, 32	32, 32, 64	32, 64, 64	16, 32, 64	16, 16, 32	<1, 8, 16	<1
Amoxicillin ^v / clavulanic acid ^a	64/32, >64/32, 64/32	>64/32	>64/32	>64/32	>64/32	>64/32	>64/32	64/32, >64/32, >64/32	8/4, 8/4, 16/8	16/8
Cefepime ^a	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
Cefoxitin (≤16; 32-64; ≥128)	>128	>128	>128	>128	>128	>128	<32	>128	<16, <32, <32	<16
Ceftriaxone ^a	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
Ciprofloxacin (≤1; 2; ≥4)	>4	4	>4	4, >4, >4	>4	>4	>4	1, 2, 4	<0.12	<0.12
Clarithromycin (≤2; 4; ≥8)	2	0.5, 0.5, 1	1, 1, 2	2, 4, 4	1	2	4, >16, >16	1, 1, 2	0.5, >16, >16	0.25, 0.25, 0.5
Doxycycline (≤1; 2-8; ≥16)	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16
Imipenem (≤4; 8; ≥16)	4, 8, 8	32	8, 16, 16	8, 16, 16	8, 16, 16	8, 16, 16	4, 8, 8	8, 8, 8	<2	<2
Linezolid ^a	32	16, 32, 32	<2, >32, >32	32, 32, >32	16, 32, 32	32, 32, >32	>32	16	2, 8, 8	2
Minocycline ^a	>8	<1	>8	>8	>8	>8	>8	>8	>8	2, 4, >8
Moxifloxacin ^a	>8	<4, <8, <8	>8	<8, >8, >8	>8	>8	>8	<2, <2, <4	<0.25	<0.25
Tigecycline ^a	0.25	0.12, 0.12, 0.24	0.25, 0.25, 0.5	0.25, 0.25, 1	0.12, 0.25, 0.25	0.5	0.5, 2, 2	0.25	<0.015, 0.06, 0.12	0.03, 0.5, 2
Tobramycin (≤4; 8; ≥16)	8, 8, 16	4, 4, 8	4, 4, 8	4	4, 8, 8	4, 4, 8	16, 16, >16	4, 4, 8	8, 16, 16	8
Trimethoprim ^v / sulfamethoxazole (≤32; -; ≥64)	>8/152	8/152	>8/152	>8/152	>8/152	>8/152	>8/152	2/38, 4/76, 8/152	>8/152	8/152, >8/152, >8/152

^a No breakpoints indicating susceptibility, intermediate or resistance to antibiotic agent available for this antibiotic in the CSLI M24-A2 standard (CSLI, 2011)

to doxycycline is observed in all strains tested with the exception of one *M. chelonae* strain (ESF35) that was susceptible to treatment. Interestingly, this same strain of *M. chelonae* independently susceptible to doxycycline is the only strain that exhibits resistance to imipenem, while the other strains tested showed intermediate to complete susceptibility. Tobramycin was effective against growth as results show intermediate to full susceptibility among all strains tested. Based on breakpoints listed for sulfamethoxazole all strains tested are resistant to the trimethoprim/sulfamethoxazole treatment.

Of the antibiotic treatments tested without CLSI breakpoints listed, no MIC was observed for treatment with cefepime or ceftriaxone. Amoxicillin/clavulanic acid treatment resulted in no MIC for *M. chelonae* and *M. abscessus* strains and a MIC of 8/4 µg/mL and 16/8 µg/mL for *M. fortuitum* and *M. peregrinum* respectively. No MIC was observed for minocycline treatment except for the previously observed unique *M. chelonae* strain. Moxifloxacin and linezolid treatment resulted in high or no MIC for all strains except *M. fortuitum* and *M. peregrinum*. Tigecycline was effective at inhibiting growth for all species with a MIC range of 0.12-2.0 µg/mL.

Slow growing Mycobacterium spp.

A summary of MIC results for six slow-growing species of *M. marinum* tested with 12 different antibiotic treatments is given in Table 3. Some strain differences in antibiotic susceptibility are observed as well as variation in the effectiveness of different treatments. Using the CLSI M24-A MIC breakpoint indicating resistance for *M. marinum*, strains were categorized as resistant to treatment. All strains of *M. marinum* tested were susceptible to amikacin and clarithromycin treatments. Resistance to trimethoprim/sulfamethoxazole treatment was observed for all strains tested. Variation in susceptibility between strains was observed for ciprofloxacin,

doxycycline, ethambutol, moxifloxacin, rifabutin and rifampin treatments. No CLSI resistance breakpoints are available for ethionamide, isoniazid, linezolid or streptomycin. All strains show MICs for ethionamide at lower test concentrations. Variation in MICs for isoniazid and linezolid were observed across strains, and streptomycin MICs were at the high end of the range of concentrations tested.

TABLE 4-3. MICs for slow growing *M. marinum* strains in the microdilution broth system. Each isolate culture was tested in triplicate; a single MIC is shown when all replicates resulted in the same MIC. Reference resistance (R) breakpoints for antibiotics are indicated. Bolded results indicate MICs indicating antibiotic susceptibility.

Antibiotic Agent (R)	MIC (µg/ml) for SGM isolates					
	MA-1	AR103K	OR932	ATCC927	TG19	OSU214
Amikacin (>32)	<1, 2, 2	<1, 2, 2	2, 2, 4	2	2, 2, 4	2
Ciprofloxacin (>2)	1, 4, 4	4, 2, 8	4, 4, 8	4, 4, 8	8, 8, 8	2, 2, 4
Clarithromycin (>16)	2, 4, 4	2, 2, 4	4	2	2, 4, 4	2, 4, 4
Doxycycline (>4)	2, 4, 4	4, 4, >16	4, 4, 8	2	2, 4, 4	8
Ethambutol (>4)	1, 8, >16	4, 8, 16	4, 4, >16	2, 2, 4	4, >16, >16	8
Ethionamide ^a	<0.3	<0.3	0.6, 0.6, 1.2	0.6, 0.6, 2.5	0.6	0.6, 0.6, 1.2
Isoniazid (>16)	0.5, 2, 1	1	1, 2, 2	4, 4, >8	4	8
Linezolid ^a	1, 8, 1	8, 8, 32	<1, <1, <1	<1, <1, 2	8, 8, 8	<1, <1, <2
Moxifloxacin (>2)	1, 4, >8	1, 1, >8	2, 4, >8	4, 2, 2	2, >8, >8	4, 2, 2
Rifabutin (>2)	2, 4, 4	4, 8, >8	4, 8, 8	4, 4, 8	8	4, 4, 8
Rifampin (>1)	1, >8, >8	2, >8, >8	2, 4, >8	4, >8, >8	2, >8, >8	4
Streptomycin ^a	8, 16, 16	8	16	16, 16, 8	16	8
Trimethoprim/ sulfamethoxazole (>2/38)	0.5/9.5, >8/152, >8/152	8/152, 8/152, >8/152	8/152, 8/152, >8/152	>8/152	8/152, >8/152, >8/152	>8/152

^a No breakpoints indicating resistance to antibiotic agent available for this antibiotic in the CSLI M24-A2 standard (CSLI, 2011)

4.5 Discussion

Overall, the results from this study indicate that there are differences in the effectiveness of each antibiotic tested and that variable antibiotic susceptibilities are observed across different species and strains of mycobacteria. Due to these differences, the type of *Mycobacterium* spp. causing infection should be identified before considering a particular antibiotic treatment.

Antibiotic susceptibilities of different Mycobacterium spp.

Rapid growers. – Infection of *M. chelonae* and *M. abscessus* is common in zebrafish, and infections often present as subclinical with little to no external signs of infection (Watrall and Kent 2007; Whipps et al. 2007a; Whipps et al. 2007b; Whipps et al. 2012). These subclinical infections are extremely concerning as they can be a source of uncontrolled experimental variance (Kent et al. 2004; Whipps et al. 2012). For human clinical *M. chelonae* infections tobramycin is the first recommended treatment, followed by clarithromycin and linezolid as second choices (Cavusoglu et al. 2012; Griffith et al. 2007). Our results show that *M. chelonae* isolates from zebrafish are similarly susceptible to tobramycin and clarithromycin, as well as tigecycline. We also observed resistance to cefoxitin in addition to amoxicillin/clavulanic acid, ciprofloxacin, doxycycline, minocycline, and trimethoprim/sulfamethoxazole. Interestingly, of the *M. chelonae* strains tested, one strain (ESF35) was uniquely susceptible to doxycycline and resistant to imipenem treatments indicating that there is some variability in drug susceptibility testing amongst different strains of *M. chelonae*.

Results from human clinical research indicate that *M. abscessus* is uniformly resistant to standard antituberculosis agents, and drug-susceptibility testing is highly recommended prior to treatment (Griffith et al. 2007). Recommended antibiotic treatments include clarithromycin, amikacin and cefoxitin; however, clinical strains of *M. abscessus* have been shown to have acquired mutational resistance to clarithromycin and amikacin (Cavusoglu et al. 2012; Griffith et al. 2007). We showed that zebrafish *M. abscessus* is: susceptible to tigecycline; shows intermediate susceptibility to amikacin, cefoxitin and imipenem; and was resistant to all other antibiotic tested. Drug susceptibilities are very similar when comparing *M. chelonae* and *M.*

abscessus isolates, which is not surprising as they phylogenetically group within the *M. chelonae* complex (Kent et al. 2004).

Mycobacterium salmoniphilum is a *M. chelonae*-like bacterium isolated from salmon (Ross, 1960; Whipps et al. 2007a). Phylogenetically, it is nested within the *M. chelonae* complex (*M. chelonae* and *M. abscessus*) (Whipps et al. 2007a). Thus, similarities in antibiotic susceptibility to *M. chelonae* are not surprising. One difference is that *M. salmoniphilum* is also susceptible to amikacin whereas *M. chelonae* is not. This pathogen has never been isolated from zebrafish, but has been reported in salmonids, and knowledge of the antibiotic susceptibility of this species might be of interest to that industry.

Infection with *M. fortuitum* and *M. peregrinum*, both members of the *M. fortuitum* complex, are less frequently observed in zebrafish; however, *M. fortuitum* is ubiquitous in water (Galassi et al. 2003). Zoonotic transmission to humans from fish has also been previously reported for *M. fortuitum* (Astrofsky et al., 2000). Clinical research indicates that *M. fortuitum* and *M. peregrinum* are susceptible to many antibiotic treatments, and doxycycline, minocycline, clarithromycin, linezolid and sulfonamides are recommended for treatment in humans (Cavusoglu et al. 2012; Griffith et al. 2007). It should be noted that *M. fortuitum* has previously displayed macrolide (i.e., clarithromycin) resistance. Interestingly, our results show different susceptibilities compared to human isolates; we observed susceptibilities to amikacin, ciprofloxacin, imipenem and tigecycline. In that study, *M. peregrinum* infections in zebrafish have been reported as low-severe (Kent et al. 2004). In this study, *M. peregrinum* shows similar antibiotic susceptibilities as what we observe for *M. fortuitum* with the addition of ceftiofur and clarithromycin susceptibilities.

Slow growing M. marinum. – Out of all the *Mycobacterium* spp. included in this study, zebrafish *M. marinum* infections are the most severe (Watrall and Kent 2007; Whipps et al. 2007a; Whipps et al. 2007b; Whipps et al. 2012). Also, zoonotic transmission of *M. marinum* (i.e., fish handlers' disease) is a major concern as treatment is lengthy and could require debridement (Wu et al. 2012). Clinical recommendations for treatment in humans include multiple antibiotics which are commonly used in combination and include: rifampin, rifabutin, ethambutol, clarithromycin, sulfanomides, trimethoprim/sulfamethoxazole, doxycycline and minocycline (Griffith et al. 2007). In addition, clinical testing has shown resistance to isoniazid (Griffith et al. 2007). Previous studies in yellowtail fish infected with *M. marinum* show rifampin, streptomycin and erythromycin to be effective antibiotic treatments; however, a *Mycobacterium* sp. was re-isolated from surviving fish following treatment in this study suggesting complete elimination of infection was not successful (Kawakami and Kusuda 1990). Our results show that all six strains of *M. marinum* tested were susceptible to amikacin, clarithromycin, and rifampin. Variation in susceptibilities is observed for the other antibiotics tested, and some strains of *M. marinum* are more susceptible to treatment.

Additional considerations for antibiotic treatment of Mycobacterium spp.

Although *Mycobacterium* spp. isolated from zebrafish responded similarly *in vitro* to different antibiotic treatments compared to human clinical isolates, further *in vivo* testing is required in order to confirm susceptibilities due to the following considerations. First, species of *Mycobacterium* have been reported to have different antibiotic susceptibilities depending on their environment. This difference is thought to be due to differences in gene regulation when mycobacteria are in stationary metabolic phases within biofilms or active phases within a host (reviewed in van Ingen et al., 2012). For example, *M. abscessus* has smooth and rough colony

types due to differences in the expression of cell wall glycolipid content (regulated by *mtrAB* expression) that confers natural resistance to antibiotics through glycolipid-rich cell walls in rough colonies (Cangelosi et al. 1999; Cangelosi et al. 2006). In addition to this, the route of drug administrations should be taken into account. Common antibiotic administration techniques used for fish are medicated feed, bath, and injection (Toutain et al. 2010; Yanong 2013). Of these, medicated feed is the most common. When antibiotics are administered through feed they are absorbed into the gut epithelium and enter systemic circulation following a hepatic first-pass (Toutain et al. 2010). Loss of drug commonly occurs during this first-pass and depends on the amount of catabolism occurring in the liver. Although this metabolic pathway is similar to humans, drug bioavailability has been reported lower in fish compared to humans (Martinsen and Horseberg 1995). Similar concerns arise with bath treatments, where drugs enter through the gills and undergo a renal-pass (Toutain et al. 2010). Water-quality issues also arise for topical bath treatments (Yanong 2013). Treatment dose and duration also require further investigation as details regarding pharmacokinetics remain to be elucidated in fish (Yanong 2013). Additionally, zebrafish susceptibility to antibiotic treatment may be strain specific and different genetic lines of fish may respond differently to antibiotic treatment, as variation in strain susceptibility to mycobacterial infections has been previously observed (Whipps et al. 2012).

Results from this study also indicate that zebrafish mycobacteria are less resistant to antibiotics compared to clinical isolates. This is likely due to less frequent exposure of these zebrafish isolates to antibiotics and therefore weaker selection for resistance. However, antibiotic resistance is common in bacteria (including mycobacteria) isolated from ornamental fish, an industry where antibiotic use is common (Rose et al. 2013). These ornamental fish bacteria have been shown to possess plasmids that may carry resistance genes and acquisition of antibiotic

resistance has been shown in to be transferable in fish isolates of *Pseudomonas* spp. and *Aeromonas* spp. between resistant and susceptible strains (Rose et al. 2013). Thus, high potential for zebrafish mycobacteria to acquire additional antibacterial resistance exists and diligence should surround the usage of antibiotics in laboratory zebrafish. Additionally, because some species of zebrafish mycobacteria are zoonotic and treatment of these infections in humans can be long in duration and require multiple antibiotics (Wu et al. 2012) the potential consequence of antibiotic resistance for fish handlers is of great concern. Considering these risks, it is important to emphasize that antibiotic use in zebrafish be used judiciously. A specific example would be to treat a valuable or rare line of brood fish prior to breeding. We emphasize that infection would need to be diagnosed and the *Mycobacterium* species identified so that the appropriate antibiotic could be used. It would not likely be practical or wise to treat large groups of fish.

In conclusion, *Mycobacterium* spp. isolated from laboratory zebrafish do exhibit susceptibility to some, but not all antibiotics tested. Antibiotic susceptibilities show variation that coincides with phylogenetic groupings of *Mycobacterium* spp. highlighting the importance of species identification to determine the most effective antibiotic treatment for inhibiting mycobacterial growth. Similarities in drug susceptibilities are observed between zebrafish and human isolates; however, zebrafish isolates were resistant to fewer antibiotics, most likely due to the low usage of antibiotics currently in zebrafish compared to humans. More research is required in order to test antibiotic susceptibility of these isolates *in vivo* due to environmentally regulated changes in bacterial gene expression as well as the influence pharmacokinetics has on antibiotic availability.

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4.7 References

Aronson, J. D. 1926. Spontaneous tuberculosis in salt water fish. Journal of Infectious Diseases **39**: 315-320.

Astrofsky, K. M., M. D. Schrenzel, R. A. Bullis, R. M. Smolowitz and J. G. Fox. 2000. Diagnosis and management of atypical *Mycobacterium* spp. infections in established laboratory zebrafish (*Brachydanio rerio*) facilities. Comparative Medicine **50(6)**: 666-672.

Boos, S., H. Schmidt, G. Ritter and D. Manz. 1995. Effectiveness of oral rifampicin against mycobacteriosis in tropical fish. Berl Munch Tierarztl Wochenschr **108(7)**: 253-255.

Cangelosi, G. A., C. O. Palermo, J. P. Laurent, A. M. Hamlin, and W. H. Brabant. 1999. Colony morphotypes on Congo red agar segregate along species and drug susceptibility lines in the *Mycobacterium avium*-intracellulare complex. Microbiology **145**: 1317-1324.

Cangelosi, G. A., J. S. Do, R. Freeman, J. G. Bennett, M. Semret and M.A. Behr. 2006. The two-component regulatory system *mtrAB* is required for morphotypic multidrug resistance in *Mycobacterium avium*. Antimicrobial Agents and Chemotherapy **50**: 461-468.

Cavusoglu, C., T. Gurpinar and T. Ecemis. 2012. Evaluation of antimicrobial susceptibilities of rapidly growing mycobacteria by Sensitire RAPMYCO panel. New Microbiologica **35(1)**: 73-76.

Chinabut, S. 1999. *Mycobacteriosis and Nocardiosis*. Pages 319-340 in *Fish Diseases and Disorders, Viral, Bacterial and Fungal Infections*, Vol. 3. CAB International, New York, NY.

Colorni, A., R. Avtalion, W. Knibb, E. Berger, B. Colorni and B. Timan. 1998. Histopathology of sea bass (*Dicentrarchus labrax*) experimentally infected with *Mycobacterium marinum* and treated with streptomycin and garlic (*Allium sativum*) extract. Aquaculture **160**: 1-17.

Conroy, G., and D. A. Conroy. 1999. Acid-fast bacterial infection and its control in guppies (*Lebistes reticularus*) reared on an ornamental fish farm in Venezuela. Veterinary Record **144**: 177-178.

CLSI. Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard-Second Edition. CLSI document M42-A2 (ISBN 1-56238-746-4) Wayne, PA: Clinical and Laboratory Standards Institute; 2011.

Dahm, R. and R. Geisler. 2006. Learning from Small Fry: The Zebrafish as a Genetic Model Organism for Aquaculture Fish Species. Marine Biotechnology **8**: 329–345.

Feitsma, H. and E Cuppen. (2008). Zebrafish as a Cancer Model. Molecular Cancer Research **6**: 685–694.

Galassi, L., R. Donato, E. Tortoli, D. Burrini, D. Santianni and R. Dei. 2003. Nontuberculosis mycobacteria in hospital water systems: application of HPLC for identification of environmental mycobacteria. Journal of Water and Health **1**: 133-139.

Gerhard, G. S. 2003. Comparative aspects of zebrafish (*Danio rerio*) as a model for aging research. Experimental Gerontology **38**: 1333–1341.

Griffith, D. E., T. Aksamit, B. A. Brown-Elliott, A. Catanzaro, C. Dalet, F. Gordin, S. M. Holland, R. Horsburgh, G. Huitt, M.F. Iademarco, M. Iseman, K. Olivier, S. Ruoss, C. F. von Reyn, R. J. Wallace Jr. and K. Winthrop. 2007. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculosis mycobacterial diseases. American Journal of Respiratory and Critical Care Medicine **175**: 367-416.

Hedrick, R. P., T. McDowell and J. Groff. 1987. Mycobacteriosis in cultured striped bass from California. Journal of Wildlife Diseases **23(3)**: 391-395.

Kawakami, K. and R. Kusuda. 1990. Efficacy of rifampicin, streptomycin and erythromycin against experimental *Mycobacterium* infection in cultured yellowtail. Nippon Suisan Gakkaishi **56(1)**: 51-53.

Kent, M. L., C. M. Whipps, J. L. Matthews, D. Florio, V. Watral, J. K. Bishop-Stewart, M. Poort and L. Bermudez. 2004. Mycobacteriosis in zebrafish (*Danio rerio*) research facilities. Comparative Biochemistry and Physiology - Part C Toxicology & Pharmacology C **138**: 383-390.

Kent, M. L., S. W. Feist, C. Harper, S. Hoogstraten-Miller, J. M. Law, J. M. Sánchez-Morgado, R. L. Tanguay, G. E. Sanders, J. M. Spitsbergen and C. M. Whipps. 2009. Recommendations for control of pathogens and infectious diseases in fish research facilities. Comparative Biochemistry and Physiology - Part C Toxicology & Pharmacology **149**: 240-248.

Kent, M. L., C. Harper and J. C. Wolf. 2012a Documented and potential research impacts of subclinical diseases in zebrafish. Institute of Laboratory Animal Resources Journal. **53**: 126-134.

Kent, M. L., J. M. Spitsbergen, J. M. Matthews, J. W. Fournie, K. N. Murray and M. Westerfield 2012b. Diseases of Zebrafish in Research Facilities. Zebrafish International Resource Center. <http://zebrafish.org/zirc/health/diseaseManual.php>. Accessed 29 Sept 2014.

Martinsen, B. and T. E. Horsberg. 1995. Comparative single-dose pharmacokinetics of four quinolones, oxalinic acid, flumequine, sarafloxacin, and enrofloxacin, in Atlantic salmon (*Salmo salar*) held in seawater at 10°C. Antimicrobial Agents and Chemotherapy **39**: 1059-1064.

Noga, E. 2010. Fish Disease: Diagnosis and Treatment 2nd ed. Wiley-Blackwell. Ames, IA.

Ostland, V. E., V. G. Watral, C. M. Whipps, F. Austin, S. St. Hilaire, M. E. Westerman, and M. L. Kent, 2007. Biochemical, molecular, and virulence studies of select *Mycobacterium marinum* strains in hybrid striped bass (*Morone chrysops* x *M. saxatilis*) and zebrafish (*Danio rerio*). Diseases of Aquatic Organisms, **79**,107-118.

Phillips, J. and M. Westerfield. 2014. Zebrafish models in translational research: tipping the scales toward advancements in human health. Disease Models & Mechanisms **7**: 739-743.

Poort, M. J., C. M. Whipps, V. G. Watral, W. F. Font. and M. L. Kent. 2006. Molecular characterization of a *Mycobacterium* species in non-native poeciliids in Hawaii using DNA sequences. Journal of Fish Diseases **29**: 181-185.

Rose, S., R. Hill, L. E. Bermudez and T. Miller-Morgan. 2013. Imported ornamental fish are colonized with antibiotic-resistant bacteria. Journal of Fish Diseases **36**: 533-542.

Ross, A. J. 1960. *Mycobacterium salmoniphilum* sp. nov. from salmonid fishes. American Review of Respiratory Diseases **81**: 241-250.

Santacana, J. A., D. A. Conroy, M. E. Mujica, C. Marín and N. De López. 1982. Acid-fast bacterial infection and its control in three-spot gouramies, *Trichogaster trichopterus* Pallus. Journal of Fish Diseases **5**: 545-547.

Schilling, T. F. and J. Webb. 2007. Considering the zebrafish in a comparative context. Journal of Experimental Zoology **308B**: 515–522.

Toutain, P., A. Ferran and A. Bouquet-Mélou. 2010. Species differences in pharmacokinetics and pharmacodynamics. Pages 19-48 in *Comparative and Veterinary Pharmacology, Handbook of Experimental Pharmacology* 199. Springer-Verlag Heidelberg , New York, New York.

Truong, L., S. L. Herper and R. L. Tanguay. 2011. Evaluation of embryotoxicity using the zebrafish model. Methods in Molecular Biology **691**: 271-279.

van Ingen, J., M. J. Boeree, D. van Soolingen and J. W. Mouton. 2012. Resistance mechanisms and drug susceptibility testing of nontuberculous mycobacteria. Drug Resistance Updates **15(3)**: 149-161.

Watrall, V. and M. L. Kent. 2007. Pathogenesis of *Mycobacterium* spp. in zebrafish (*Danio rerio*) from research facilities. Comparative Biochemistry and Physiology - Part C Toxicology & Pharmacology **145**: 55-60

Westerfield, M. 2007. The zebrafish book, 5th Edition; A guide for the laboratory use of zebrafish (*Danio rerio*). University of Oregon Press; Eugene, OR.

Whipps, C. M., W. R. Butler, F. Pourahmad, V. G. Watral and M. L. Kent. 2007a. Molecular systematics support the revival of *Mycobacterium salmoniphilum* (ex Ross 1960) sp. nov., nom. Rev., a species closely related to *Mycobacterium chelonae*. International Journal of Systematic and Evolutionary Microbiology **57**: 2525-2531.

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

Whipps, C. M., J. L. Matthews and M. L. Kent. 2008. Distribution and genetic characterization of *Mycobacterium chelonae* in laboratory zebrafish (*Danio rerio*). Diseases of Aquatic Organisms **8**: 45-54.

Whipps, C. M., C. Lieggi and R. Wagner. 2012. Mycobacteriosis in zebrafish colonies. ILAR Journal **53(2)**: 95-105.

Whipps, C. M., L. G. Moss, D. M. Sisk, K. N. Murray, D. M. Tobin and J. B. Moss. 2014. Detection of autofluorescent *Mycobacterium chelonae* in living zebrafish. Zebrafish. **11**: 76-82.

Wong, K., M. Eegante, B. Bartels, S. Elkhayat, D. Tien, S. Roy, J. Goodspeed, J. Suci, J. Tan, C. Grimes and A. Chung. 2010. Analyzing habituation responses to novelty in zebrafish (*Danio rerio*). Brain Research **208**: 450-457.

Wu, T-S., C-H. Chiu, C-H. Yang, H-S. Leu, C-T. Huang, Y-C. Chen, T-L. Wu, P-Y. Chang, L-H. Su, A-J. Kuo, J-H. Chia, C-C. Lu, and H-C. Lai. 2012. Fish tank granuloma caused by *Mycobacterium marinum*. PLOS One **7(7)**: e41296

Yanong, R. P. E. 2013. Use of Antibiotics in Ornamental Fish Aquaculture. Circular 84. Series from the Department of Fisheries and Aquatic Science, Florida Cooperative Extension Service, Institute of Food and Agricultural Science, University of Florida, Gainesville, FL.

Chapter 5

Antibiotic treatment of zebrafish mycobacteriosis: tolerance and efficacy of treatments with tigecycline and clarithromycin

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5.1 Abstract

Zebrafish (*Danio rerio*) are a popular model organism used in a growing number of research fields. Maintaining healthy, disease-free laboratory fish is important for the integrity of many of these studies. Mycobacteriosis is a chronic bacterial infection caused by several *Mycobacterium* spp. and is the second most common disease found in laboratory zebrafish. Current mycobacteriosis control measures recommend the removal of infected fish and in severe outbreaks, depopulation. These measures can be effective, but less disruptive measures should be assessed for controlling mycobacteriosis, particularly when valuable and rare lines of fish are affected. Here, the *in vivo* efficacy two drug candidates, tigecycline (1 µg/g) and clarithromycin (4 µg/g), was tested in adult zebrafish experimentally infected with *Mycobacterium chelonae*. We assessed both short (14 day) and long-term (30 day) treatments and evaluated fecundity and pathological endpoints. Fecundity and histology results show that zebrafish tolerated antibiotics. Antibiotic treatments did not significantly impact the prevalence of acid-fast granulomas; however, the severity of infections (acid fast granuloma intensity) was significantly decreased following treatments.

Keywords: zebrafish, mycobacteriosis, antibiotic treatment, clarithromycin, tigecycline, *Mycobacterium chelonae*.

5.2 Introduction

The Zebrafish (*Danio rerio*) is a popular vertebrate model organism used in a wide range of research fields (Dahm & Geisler 2006; Phillips & Westerfield 2014). Zebrafish are currently considered rising stars of model-organism research with almost a 60% increase in National Institutes of Health R01 awards from 2008-2015 (Gaird 2016). Zebrafish are used in an ever-increasing range of disciplines including, but not limited to, toxicology (Truong, Harper & Tanguay 2011), aging (Gerhard 2003), oncology (Feitsma & Cuppen 2008), and behavior (Wong, Elegante, Bartels, Elkhayat, Tien, Roy, Goodspeed, Suci, Tan, Grimes, Chung, Rosenberg, Gaikwad, Denmark, Jackson, Kadri, Chung, Stewart, Gilder, Beeson, Zapolsky, Wu, Cachat & Kalueff 2010). Mycobacteriosis is the second most common disease in laboratory zebrafish (Kent, Spitsbergen, Matthews, Fournie, Murray & Westerfield 2012), caused by several *Mycobacterium* spp. (Astrofsky, Schrenzel, Bullis, Smolowitz & Fox 2000; Kent, Whipps, Matthews, Florio, Watral, Bishop-Stewart, Poort & Bermudez 2004; Kent 2012; Whipps, Lieggi & Wagner 2012). Several species and strains of *Mycobacterium* have been implicated in zebrafish mycobacteriosis including both rapid-growing species (e.g. *Mycobacterium chelonae*, *Mycobacterium abscessus*, *Mycobacterium peregrinum*, *Mycobacterium fortuitum*) and slow-growing species (e.g. *Mycobacterium marinum* and *Mycobacterium haemophilum*) (Astrofsky et al. 2000; Kent et al. 2004; Whipps, Matthews & Kent 2008). The severity of mycobacteriosis in zebrafish varies among species and can range between high levels of mortality with *M. marinum* and *M. haemophilum* to little observed mortality with *M. abscessus* and *M. chelonae* (Watal & Kent 2007; Whipps, Dougan & Kent 2007; Whipps et al. 2012). Infection related morbidity is also variable and includes external signs such as skin lesions, emaciation, raised scales, swollen abdomen, and irregular/lethargic

swimming behavior, as well as internal signs like granulomas, especially on haematopoietic organs (Astrofsky et al. 2000; Kent 2012; Whipps et al. 2012). Additionally, signs of disease may not be present in the case of subclinical infections (Kent et al. 2004; Whipps et al. 2012). Mycobacterial infections in zebrafish are detrimental to research both when severe outbreaks result in high levels of mortality and also when subclinical infections persist undetected in populations as a source uncontrolled experimental variance (Kent et al. 2004; Whipps et al. 2012).

Control recommendations for zebrafish mycobacteriosis focus on disease prevention through quarantine, disinfection, UV disinfection, and sentinel programs for health monitoring (Kent, Feist, Harper, Hoogstraten-Miller, Mac Law, Sanchez-Morgado, Tanguay, Sanders, Spitsbergen and Whipps 2009; Whipps et al. 2012; Chang, Colicino, DiPaola, Al-Hasnawi & Whipps 2015). Mycobacteria are facultative pathogens and persist environmentally in surface biofilms (Falkinham 2009; Falkinham, Norton & LeChevallier 2001). Thus, established mycobacterial infections in zebrafish facilities substantially complicate disease control and management and involve invasive management steps such as depopulation, facility sterilization, and rederivation of zebrafish populations (Whipps et al. 2012). These measures have been demonstrated to be effective at controlling zebrafish mycobacteriosis outbreaks, but such extreme measures may not always be feasible for research facilities (Whipps et al. 2012). For example, if an outbreak occurs in during an ongoing experiment or in a valuable mutant line depopulation and rederivation may not be an option (Whipps et al. 2012). This challenge is also true in the case of mycobacteriosis infections in other fish species such as valuable zoo collections (Strike, Feltrer, Flach, Macgregor & Guillaume 2016). There is a need to explore alternative methods for disease management, such as antibiotic treatment.

The treatment of non-tuberculosis mycobacteriosis in humans is routine (Griffith, Aksamit, Brown-Elliott, Catanzaro, Dalet, Gordin, Holland, Horsburgh, Huitt, Iademarco, Iseman, Olivier, Ruossvon, Reyn, Wallace Jr & Winthrop 2007; Wu, Chiu, Yang, Leu, Huang, Chen, Wu, Chang, Su, Kuo, Chia, Lu & Lai 2012), but similar treatments in fish have yet to be thoroughly investigated (Chang & Whipps 2015). We previously evaluated the *in vitro* susceptibility of several rapid- and slow-growing strains of *Mycobacterium* spp. isolated from infected zebrafish from different facilities in the United States (Chang & Whipps 2015). We observed differential susceptibility to the antibiotic treatments across species and drugs that coincided to phylogenetic groupings of *Mycobacterium* spp. and were also similar to those observed in human isolates (Chang & Whipps 2015). We were able to highlight key antibiotic candidates that demonstrated *in vitro* effectiveness against *Mycobacterium* species that commonly cause disease in zebrafish, as well as determine the minimum inhibitory concentration (MIC) for these antibiotics (Chang & Whipps 2015). Two candidates identified in this study were tigecycline and clarithromycin, which were effective at very low concentrations against several species of *Mycobacterium* (Chang & Whipps 2015).

In this study we evaluate the *in vivo* efficacy of tigecycline and clarithromycin treatments of *M. chelonae* infection in adult zebrafish, the most common *Mycobacterium* species found in laboratory zebrafish. We evaluate treatment using these drugs at the MIC determined in our previous study (Chang & Whipps 2015), which also corresponds to the dosages recommended for treatment of NTM infections in humans (Muralidharan, Micalizzi, Speth, Raible & Troy 2005; Kim, Chi, Oh, Kim, Kim, Lim, Kim & Kwon 2011). Both 14-day and 30-day treatments will be evaluated for both drugs. Our first study goal is to evaluate the safety of these treatments and evaluate effects on fish health and fecundity. We hypothesize that because treatments are at a

dose similar to what is currently used for human treatments, there will be no effects on morbidity, mortality or fecundity for both tigecycline and clarithromycin treatments. Our second study goal was to evaluate the effectiveness of these treatments in eliminating *M. chelonae* from infected zebrafish. We hypothesize that these treatments will result in a significant decrease in mycobacteria, with longer-term (30-d) treatments being more effective than short-term (14-d) treatments.

5.3 Methods

Fish

All fish used in this study were bred and maintained in the zebrafish facility at the SUNY-ESF Center for Integrated Teaching and Research in Aquatic Science. Adult AB wild type line zebrafish (n=576; 288 male and 288 female; age = 6 months), originally obtained from the SARL at Oregon State University (Corvallis, OR) and bred for two generations at SUNY-ESF, were utilized in this study. Animals were housed at a density of 6-10 fish/liter in either 1.8 L (tolerance experiment) or 2.6 L (efficacy study) tanks on a timed, flow-through housing system (Aquanearing, San Diego, CA). The housing system included ultraviolet disinfection of dechlorinated (carbon filter) municipal tap water maintained to pH 7.6, a conductivity of 600-700 $\mu\text{S}/\text{cm}^2$, and a temperature of 28.5°C, and ammonia levels ranging from 0-0.25 ppm. The zebrafish facility maintained a 14:10 light:dark photoperiod. Fish were fed a commercial feed for zebrafish (Gemma, Skretting) twice daily on weekdays and once daily on weekends during periods they were not being fed a treatment gelatin feed. Prior to the experiment, routine cleaning of all equipment (e.g. tanks, lids, baffles, nets, and tubing) consisted of bi-weekly washing and scrubbing in warm water with a new soft sponge and bleaching in 1000 ppm chlorine bleach for 30 minutes, followed by rinsing three times in dechlorinated water and

drying. All tanks, lids, and baffles were also autoclaved using a program specified by the tank manufacturer that reaches a temperature of 105-110°C for 15 minutes. During experiments this tank cleaning procedure occurred when the placement of fish in a new tank is mentioned in the methods below. All animal work was approved by the SUNY-ESF Institutional Animal Care and Use Committee, protocol #151001.

Medicated Feed Preparation

Antibiotics were delivered orally through a commercially developed gelatin feed (Gelly Belly, Florida Aqua Farms). This gelatin feed has previously been previously shown to be comparable to other zebrafish diets (Sciarra, Tyler & Kolb 2014). Prior to preparing medicated feeds, concentrated stock solutions of each antibiotic were prepared as followed. Tigecycline (Sigma Aldrich) was initially dissolved in DMSO to reach a concentration of 3 mg/mL as per manufacturer's recommendation. This dilution was then further diluted further to 1.5 mg/mL in Hanks Buffered Salt Solution as recommended in (Adekambi & Drancourt 2004) and stored at -20°C to minimize drug loss through storage. Clarithromycin (Sigma Aldrich) was dissolved in DMSO to reach a final concentration of 1 mg/ml as per manufacturer recommendations, and stored at -20°C. Medicated Gelly Belly feed was prepared with the addition of clam juice as in (Sciarra, et al. 2014). Medicated Gelly Belly was weighed and minced following this method as well. Food aliquots for each tank were stored in sterile culture tubes at -20°C until immediately prior to feeding. Tigecycline feed was prepared so that fish would receive a target dosage of 1 µg/g of body weight in each feeding. Clarithromycin feed was prepared so fish received a target of 4 µg/g of body weight in each feeding. The average body weight was 600 mg/fish. Control feeds were prepared with the addition of no antibiotics; sterile water was used in the place of any

medication. Once medicated and control feeds were prepared they were re-labelled A, B, or C by a third party so that further observations were blinded.

Tolerance Study Design

Experimental groups (n=36/group) were organized as follow into feed treatment: untreated 14-d (no antibiotic feed), untreated 30-d (no antibiotic feed), treated tigecycline treatment 14-d (1 $\mu\text{g/g}$ tigecycline/day), treated tigecycline treatment 30-d (1 $\mu\text{g/g}$ tigecycline/day), treated clarithromycin treatment 14-d (4 $\mu\text{g/g}$ clarithromycin/day), treated clarithromycin treatment 30-d (4 $\mu\text{g/g}$ clarithromycin/day). For each experimental group fish were divided evenly into 12 fish/tank replicated three times. Each tank had an equal number of male and female fish.

Fish were measured prior to the start of the study so that average growth could be evaluated. For measurements, fish fasted for 12-h were anaesthetized in a bath treatment of 0.15 g/L MS222 buffered to a pH of 7.5. Once anesthetized, the sex and the standard length (snout to the end of the caudal peduncle) of each fish were measured to the nearest millimeter. Once measured, fish recovered in fresh zebrafish system water and were returned to the appropriate study tank. This measurement procedure was also carried out for all groups post-treatment (14-d and 30-d) as well as 11-weeks post 14-d or 30-d treatment.

Two weeks prior to the start of treatment feeding, background breeding was conducted weekly to determine a baseline for fecundity values. Each tank was bred overnight in its own breeding chamber (Aquaneering). The following morning fish were given 3 hours to breed following the beginning on the light photoperiod. Following breeding, fish were returned to new clean (autoclaved) tanks. The number of embryos collected as well as the 24-hour embryo mortality for each tank was determined. Following 24-hour mortality data collection, all embryos

were euthanized in 0.3g/L MS222 buffered to a pH of 7.5. Following euthanasia, all embryo waste was bleached at an estimated concentration of 1000 ppm prior to disposal. This same breeding procedure was repeated weekly from 2-11 weeks post-treatment.

Antibiotic feed treatments were administered daily from 9:30-10:30 am for either 14 days or 30 days. Prior to feeding all tanks were cleaned to remove any feces and/or detritus from tanks. At feeding, pre-prepared (described above) gelatin-based food was distributed to each tank. Fish were allowed 20 minutes of eating time, following that excess food was removed by siphoning. Observations (changes in skin color, irregular swimming, lethargy, mortality) of fish in all tanks were made at 5 min, 30 min, 1 hour, and 2 hours post-feeding. Following tigecycline, clarithromycin, and control treatments (14-d and 30-d) standard length measurements were collected again using the method described above. There were no timed water exchanges during the pre-feeding cleaning and feeding periods. Following the removal of excess food after the feeding period, a timed water exchange was carried out replacing approximately 20 percent of the tank water.

At 11 weeks post-treatment all fish were measured as described above and euthanized in 0.3 g/L MS222 buffered to a pH of 7.5. Euthanized fish were fixed in Davidson's solution for 48h, decalcified in 0.5M EDTA for 5 days, rinsed, and dehydrated to 70% EtOH for histology through a dehydration series (25% EtOH for 30 min, 50% EtOH for 30 min, 70% EtOH to store). Three representative fish from each replicate tank were sectioned and stained with hematoxylin and eosin (H&E).

Statistics - Tolerance

The analysis described below was used to compare differences between treated tigecycline, treated clarithromycin, and untreated treatments for the same duration (14-d or 30-d)

and also to compare differences between durations (14-d and 30-d) for the same treatment (tigecycline, clarithromycin, or untreated controls). The same statistical method was used for all analyses and was carried out in R 3.1.0 (R Core Team 2013) and R Studio (R Studio 2012). Length data, embryo count data, and 24-hour embryo mortality data were organized in a spreadsheet. Growth was calculated for each replicate tank by subtracting average standard length measured before treatment, from post-treatment and 11 weeks post-treatment length (e.g. $\text{Growth} = \text{average standard length at 11 weeks post-treatment} - \text{average standard length at pre-treatment}$). Percent embryo survival was calculated as follows $\{\text{Percent Survival} = [1 - (\text{24-hour embryo mortality} / \text{Total Number of Embryos Collected})] * 100\}$. Data were sorted into separate spreadsheets by time-point and treatment and saved as individual text files for analyses. Prior to each statistical analysis, data were checked for normality and equal variances using the “stats” package (R Core Team 2013) and “car” package (Fox and Weisberg 2011) respectively. Residuals were also plotted using the “stats” package to further evaluate data normality. If data had a normal distribution ($p > 0.05$) and equal variances ($p > 0.05$), parametric ANOVA test was performed using the “stats” package (R Core Team 2013). Post-hoc analyses pair-wise analyses were conducted using t-tests using the “foreign” and “car” packages. Non-normally distributed data and data with unequal variances ($p < 0.05$) were evaluated with the Kruskal-Wallis rank sum test using the “stats” package (R Core Team 2013). Post-hoc testing used the Nemenyi-tests for multiple comparisons of rank sums using the “PMCMR” package (Pohlert 2015). Descriptive statistics were determined using the “psych” package. Results were organized into bar plots using the “sciplot” package.

Efficacy Study Design

Experimental groups (N=60) were organized as follows: untreated (no antibiotic feed) sham injected, untreated (no antibiotic feed) *M. chelonae* injected, treated tigecycline (1 µg/g) sham injected, treated tigecycline (1 µg/g) *M. chelonae* injected, treated clarithromycin (4 µg /g) sham injected, treated clarithromycin (4 µg /g) *M. chelonae* injected. For each experimental group, fish were divided evenly into two tanks of 30 fish. Each tank had an equal number of male and female fish.

Prior to the set-up of experimental groups, mycobacterial infections or sham injections were established in the fish. *Mycobacterium chelonae* injected fish (N=180 fish) were injected intraperitoneally (IP) with *Mycobacterium chelonae* (H1E2), and sham injected fish (N=180) injected with sterile saline, following the method described in Watral et al. (2007). The concentration of the bacterial inoculum was determined using a nephelometer (Sensititre) and confirmed with colony counts following culture on Middlebrook 7H10 agar plates. After IP injection, fish were placed in new tanks and maintained on the flow-through system for 8 weeks in order to allow for the development of infections in bacterium-injected fish. During this 8 week period fish were fed a commercial feed (Gemma, Skretting) twice daily on weekdays and once daily on weekends. Tanks were siphoned daily following feeding during this period.

Following the 8-week incubation period, fish were arranged in their respective experimental group in new tanks. *Mycobacterium chelonae* injected fish (N=180) were randomly divided into six tanks of N=30 (15 males, 15 females). Sham injected fish (N=180) were also divided into six tanks of N=30 (15 males, 15 females). At this point, each tank was fed either untreated Gelly Belly feed (prepared as described previously) or treated tigecycline or clarithromycin feed (prepared as described previously). Prepared tubes of thawed and minced

gelatin feed were emptied into each tank using the same method described above for the tolerance study. Timed water exchanges were carried out similar to the tolerance study above. Fish were fed daily for 14 days. On the 15th day, following a 12-h fasting period, half of the fish from each tank were euthanized in 0.3g/L MS222 buffered to a pH of 7.5. Once euthanized, fish were prepared for histology as described above. The remaining fish in each tank continued experimental feeds daily up until day 30. Following the 30th feed, fish were fasted overnight for 12-h, then euthanized, fixed, decalcified and prepared for histology as described above. Sections from each specimen were stained with both hematoxylin and eosin (H&E) or Kinyoun's acid fast stain.

Following staining, slides were examined, recording the number and location of granulomas in H&E sections. For acid fast-stained sections, the presence of granulomas containing acid fast bacilli (AFB) was recorded. The number and location of granulomas containing AFB was recorded, and the location of AFB not contained in granulomas was also recorded. AFB not within granulomas were considered to be internally located if they were located inside the epithelial boundary (e.g. not in the gut lumen or on the outside of the skin/scales). The prevalence of granulomas, granulomas containing AFB, and free AFB was calculated. The intensity of granulomas containing AFB was also calculated (Intensity = number of granulomas containing AFB/number of fish with the presence of granulomas containing AFB).

Statistics - Efficacy

For each experimental group, a Chi-squared analysis with a Monte Carlo simulation method using 20,000 replications was used to compare replicate tanks to determine whether data from different tanks can be pooled for further group comparisons. Results from this test showed

no significant differences between replicate groups. Replicate data for each experimental group was pooled. Wilson's 95% confidence intervals and prevalence values were determined using the "epitools" package (Aragon, Fay & Wollschlaeger 2012). There was no occurrence of acid-fast granulomas in sham injection fish and subsequent statistical analyses excluded these sham groups. Fisher's exact test for count data was used to compare prevalence between treatment groups using the "stats" package (R Core Team 2013). Data was analysed comparing experimental groups for both sexes, with males or females only, and between sexes for each group. Results were organized into bar plots using the "sciplot" package (Morales 2012).

5.4 Results

Tolerance Study - Behavior

The prepared gelatin-based feed was eaten by all the fish without hesitation and as noted in Sciarra et al. (2014), a feeding frenzy occurred. The fish generally consumed most of their food within the 20-minute period; however, any remaining food was removed as it deteriorated water quality quickly. Observations at 5 min, 30 min, 1 hour and 2 hours post-feeding included no changes in skin color, no irregular swimming, no lethargy or mortality. The only observation noted, is that female fish would become very full and sometimes have a distended belly from consuming the gelatin feed. There was no resulting morbidity or mortality.

Tolerance Study - Embryos

Generally, all experimental groups spawned on every breeding occasion throughout this study and 24-hour embryo mortality ranged from 0-42%. When embryo counts and 24-hour embryo mortality were compared (Table 5-1), there were only two instances of significant differences between groups. First, for average embryo count, there was a significantly higher average embryo count for both 14- and 30-d tigecycline treatments at 4 weeks post-treatment

Table 5-1. Average total egg count and 24-hour mortality (brackets) per treatment group per week before and after 14-d and 30-d antibiotic treatment with tigecycline (1 µg/g), clarithromycin (4 µg/g), or control feed. Bolded values indicate those that differ significantly between treatment groups for the same week. Bolded and italicized values indicate those that differ for between weeks for the same treatment.

<i>Medication</i>	<i>Duration</i>	<i>-2</i>	<i>-1</i>	<i>Tx</i>	<i>Week</i>										<i>2-10 Avg</i>
					<i>2</i>	<i>3</i>	<i>4</i>	<i>5</i>	<i>6</i>	<i>7</i>	<i>8</i>	<i>9</i>	<i>10</i>	<i>11</i>	
Control	14 Days	76(5)	44(6)	N/A	113(3)	113(1)	50(42)	81(4)	68(6)	44(4)	55(2)	55(0)	81(3)	84(3)	74(7)
Control	30 Days	44(13)	49(9)		153(3)	153(3)	106(5)	209(2)	74(3)	25(1)	80(3)	45(16)	8(0)	48(2)	90(4)
Clarithromycin	14 Days	93(4)	55(15)		198(7)	198(7)	92(5)	85(5)	71(4)	53(8)	41(1)	128(3)	106(5)	79(2)	105(5)
Clarithromycin	30 Days	39(0)	76(7)		171(8)	171(8)	96(2)	73(8)	85(7)	93(2)	95(4)	133(5)	105(3)	59(4)	108(5)
Tigecycline	14 Days	8(12)	72(3)		226(20)	226(20)	248(6)	130(3)	111(3)	61(11)	162(5)	107(3)	127(6)	105(3)	150(8)
Tigecycline	30 Days	106(3)	56(3)		202(2)	202(2)	255(2)	131(9)	143(6)	38(0)	104(5)	120(5)	110(5)	74(3)	138(4)

compared to 14-d clarithromycin, untreated, and 30-d clarithromycin treatment [$F(5)=7.627$, $p=0.002$] (Table 1). Second, there was significantly higher percent mortality at both 2 and 3 weeks post-treatment for the 14-d tigecycline treatment group compared to 30-d tigecycline and both 14- and 30-d untreated groups [week 2: $F(5)=4.162$, $p=0.02$; week 3: $F(5)=4.509$, $p=0.0152$] (Table 5-1). When comparisons were made for the same experimental group over time, there was only one instance where embryo count differed significantly between weeks. For the 14-d tigecycline treatment, the average embryo count at 7-weeks post-treatment (61) was significantly lower than counts at weeks two and three (226) [$F(1)=5.37$, $p=0.03$] (Table 5-1).

Tolerance Study - Growth

When post-treatment growth between experimental groups was compared immediately following treatment, there was no significant difference between treated and untreated groups ($\chi^2(5)=6.05$, $p=0.30$)(Figure 5-1). However; when comparing growth 11 weeks post treatment, there was a significant difference between groups ($\chi^2(5)=13.17$, $p=0.02$)(Figure 5-1). For this time-point, the 30-d clarithromycin and untreated groups resulted in significantly less growth than the other groups.

Treatment Efficacy – H&E Granuloma Prevalence

Granulomas were mainly located in the reproductive tissues of both male and female fish. For the sham injected groups, granulomas were restricted to the ovaries, testes, and swim bladder. For the *M. chelonae*-injected tigecycline and clarithromycin treated groups the granulomas were located in additional types of tissues in addition to the ovaries, testes, and swim bladder, including: intestine, liver, pancreas, and adipose. The *M. chelonae* injected untreated group had granulomas in more tissue types (ovaries, testes, swim bladder, intestine, liver,

pancreas, adipose, and kidney). compared to the *M. chelonae* injected tigecycline and clarithromycin treated groups.

The prevalence of granulomas in H&E stained slides varied between experimental groups and sex (Appendix Table 1). Sham-injected groups displayed prevalence ranging from 0-0.13 (Figure 5-2). *Mycobacterium chelonae*-injected groups display a granuloma prevalence ranging from 0.38-0.56 (Figure 5-2). There was no significant difference between the prevalence of H&E stained granulomas between experimental groups when comparing groups including both sexes, only males, and only females (Figure 5-2). However, when granuloma prevalence for males and females were compared for each group the *M. chelonae* injected 14-d tigecycline treated females had a significantly higher prevalence compared to males ($p=0.01718$) (Figure 5-2).

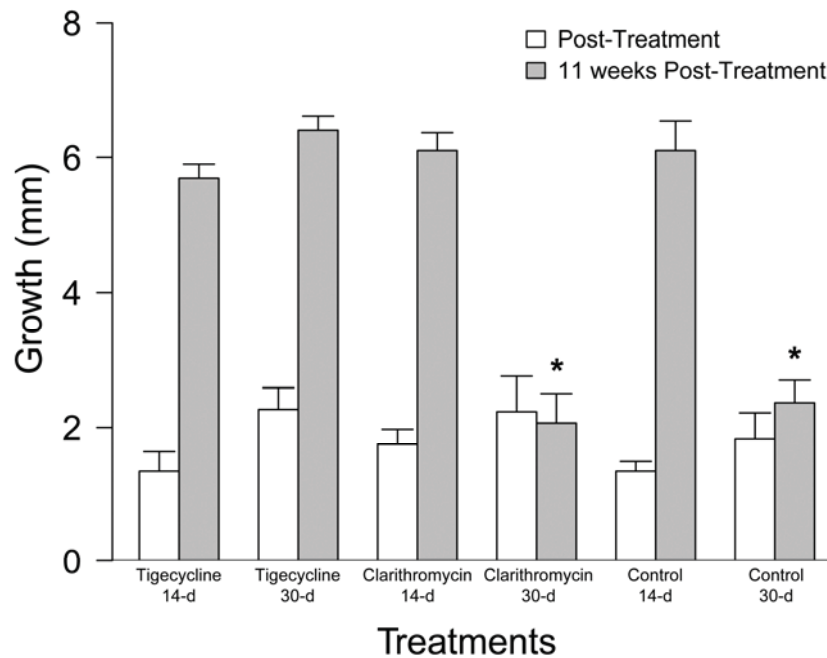


Figure 5-1. Tolerance Study Growth Results. Growth (mm) in standard length (SL) immediately following 14-d and 30-d antibiotic treatment with tigecycline (1 $\mu\text{g/g}$), clarithromycin (4 $\mu\text{g/g}$), or control feed (white bars) and at 11-weeks post-treatment (grey bars). Significant differences are indicated by an asterisk (*).

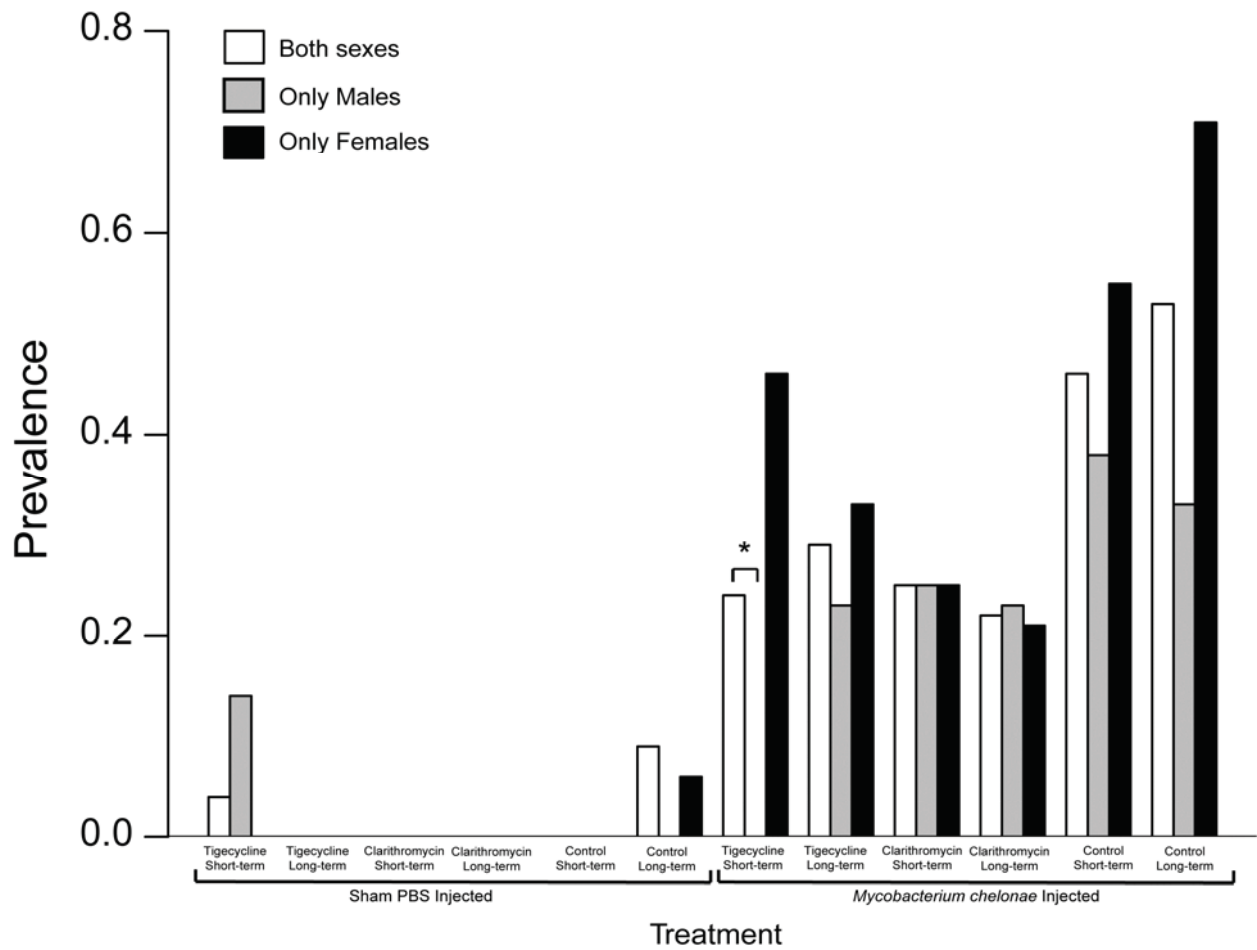


Figure 5-2. Barplot displaying prevalence of H&E granulomas for both sexes (white bars), males only (grey bars), and females only (black bars) for both sham-injected and *M. chelonae*-injected zebrafish treated with 14-d or 30-d tigecycline (1 $\mu\text{g/g}$), clarithromycin (4 $\mu\text{g/g}$), or control feeds. A significantly higher prevalence is observed for females compared to males for the 14-d tigecycline treatment (*).

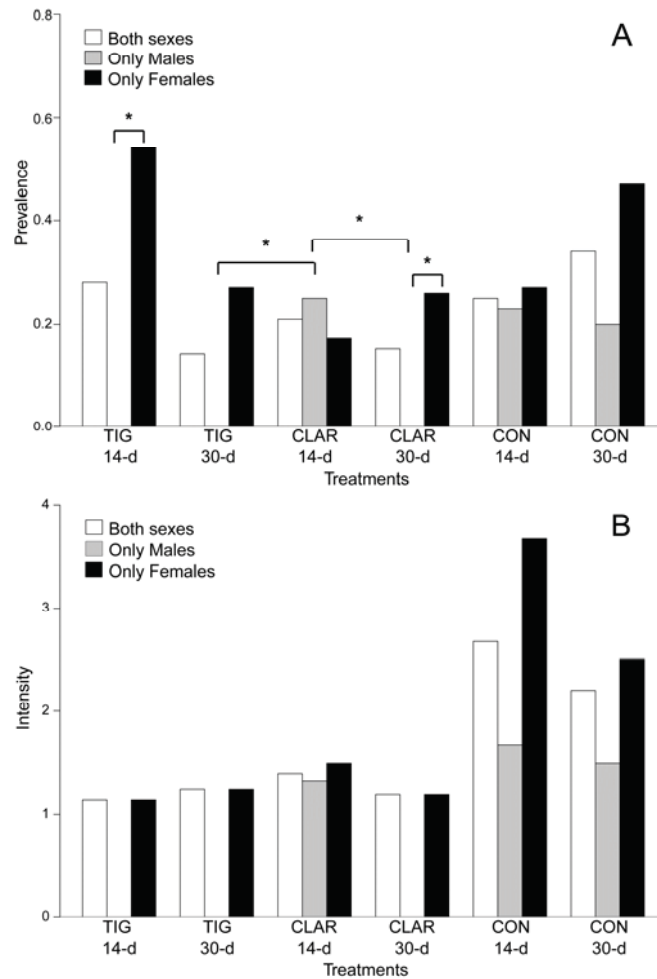


Figure 5-3. Barplots displaying the (A) prevalence and (B) intensity for both sexes (white bars), males only (grey bars), and females only (black bars) of acid fast positive granulomas for *M. chelonae*-injected zebrafish treated with 14-d or 30-d tigecycline (1 $\mu\text{g/g}$), clarithromycin (4 $\mu\text{g/g}$), or control feeds. Significant differences in prevalences are indicated by asterisks (*).

For males only a significantly higher prevalence of AFG is observed for 30-d tigecycline and clarithromycin treatments compared to 14-d clarithromycin treatment. Higher prevalence was observed for females compared to males from the same treatment for 14-d tigecycline an

30-clarithromycin treatments. A significant difference between intensity (B) for treatment groups of both sexes was also observed with control groups having the highest intensities.

Treatment Efficacy - AF Granuloma Prevalence

When comparing the prevalence of acid-fast granulomas to granulomas observed in H&E stained sections, there was generally a lower prevalence of acid-fast positive granulomas compared to H&E stained counts. Two exceptions to this were observed for female granuloma prevalence of the *M. chelonae* tigecycline 14-d treated group and the *M. chelonae* 30-d clarithromycin treated group where a higher prevalence for granulomas in the ovaries were observed in acid-fast stained sections compared to H&E (Compare Figure 5-2 to Figure 5-3A).

The prevalence of acid-fast positive granulomas (Appendix Table 1). differed between experimental groups and sexes (Figure 5-3A). There were no acid-fast positive granulomas in sham-injected groups. When comparing the prevalence of acid-fast positive granulomas between *M. chelonae* injected experimental groups, there was no significant difference between treatments when considering groups including both sexes. However, significant differences were observed when single-sex groups were compared. A significantly higher prevalence was observed for males in the 14-d clarithromycin treated group compared to both the 30-d tigecycline and 30-d clarithromycin treated groups ($p=0.04634$) (Figure 5-3A). Males had a significantly lower acid-fast granuloma prevalence compared to females for the 14-d tigecycline treated group ($p=0.004577$) as well as 30-d clarithromycin treated group ($p=0.06413$) (Figure 5-3A).

Treatment Efficacy - AF Granuloma Intensity

For individual fish with acid-fast positive granulomas, the intensity of acid-fast positive granulomas was determined and varied between experimental groups and sexes (Figure 5-3B). When intensity was compared between experimental groups there was significant difference ($p < 0.1$) between groups [$F(5) = 3.631$, $p = 0.0739$], with the untreated groups having a significantly higher number of granulomas (*) (Figure 5-3B).

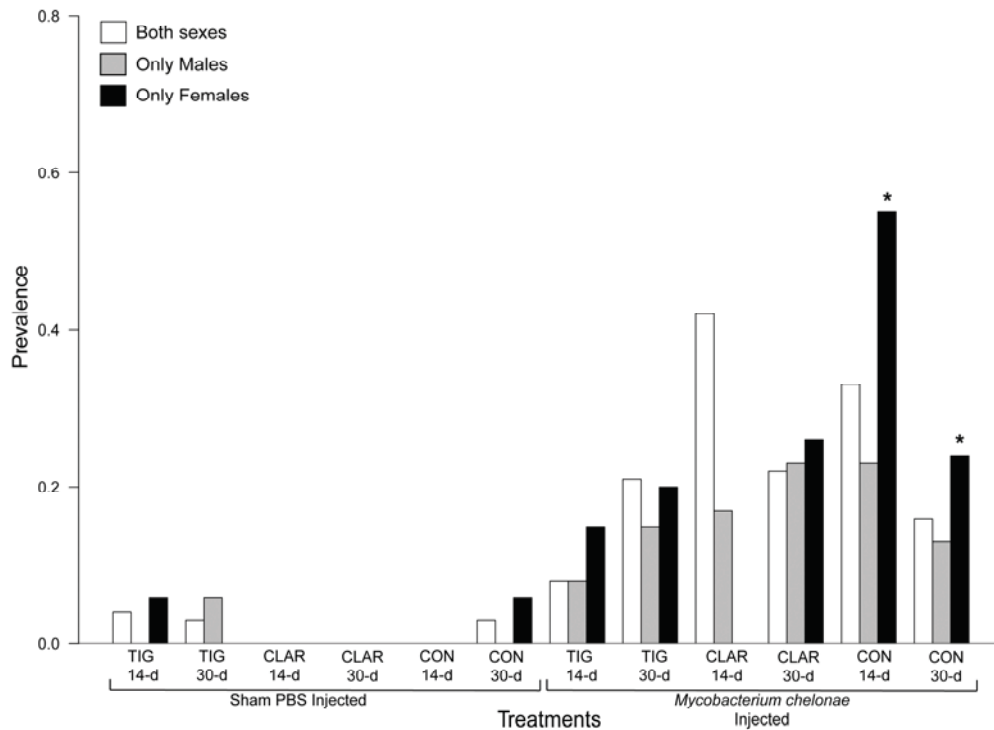


Figure 5-4. Barplot displaying prevalence of acid fast positive bacilli (AFB) for both sexes (white bars), males only (grey bars), and females only (black bars) for both sham-injected and *M. chelonae*-injected zebrafish treated with 14-d or 30-d tigecycline (1 $\mu\text{g/g}$), clarithromycin (4 $\mu\text{g/g}$), or control feeds. A significantly higher prevalence is observed (*) for 14-d control females compared to 14-d tigecycline and clarithromycin treated females. Significantly higher AFB prevalence is also observed for 30-d control females compared to 30-d clarithromycin females (*).

Treatment Efficacy - AFB Prevalence

The prevalence of acid-fast bacilli (Supplemental Table 1). varied between experimental groups and sexes (Figure 5-4). The prevalence of internally-located acid-fast bacilli was also compared between experimental groups. For females, there was a significant difference in the prevalence of AFB ($p=0.0536$). Tigecycline and clarithromycin treated groups for 14-d, resulted in significantly lower prevalence compared to the 14-d untreated group (*). AFB in the 14-d clarithromycin treated group was also significantly lower than 30-d untreated females (+) (Figure 5-4).

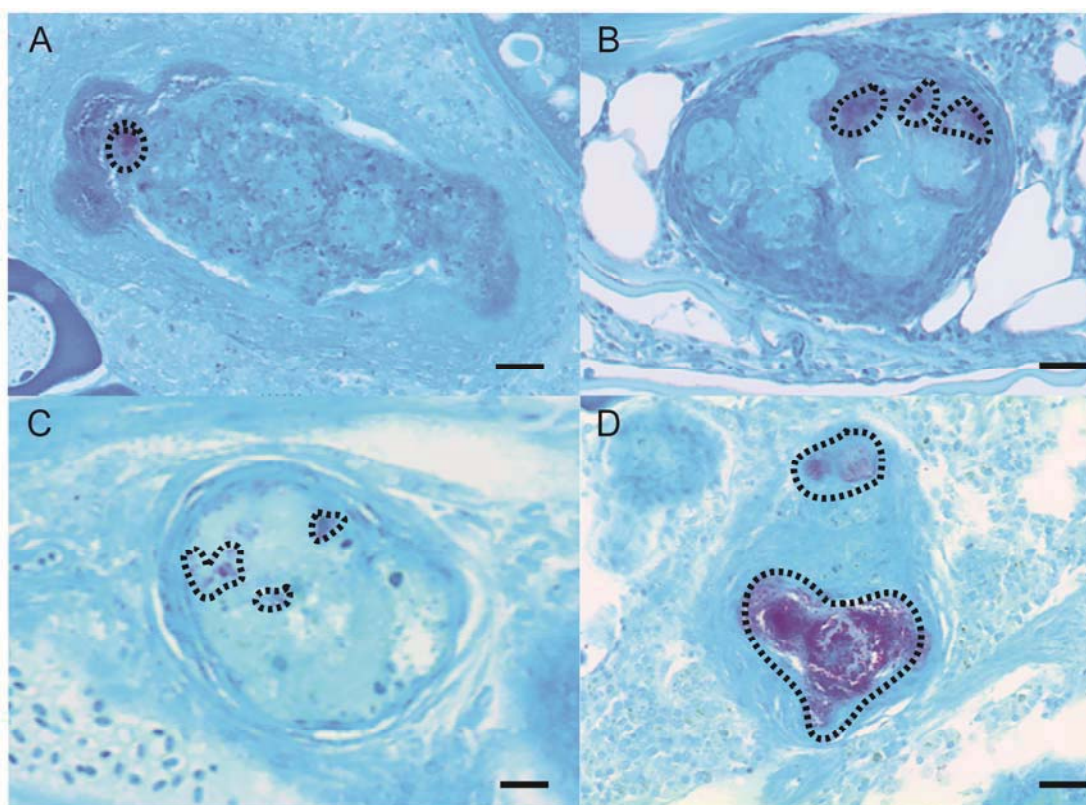


Figure 5-5. Representative images of acid fast stained granulomas with acid fast positive bacilli (enclosed in dashed line trace) for (A) 14-d clarithromycin treatment located in the ovaries, (B) 30-d clarithromycin treatment located ventral to the liver in a male fish , (C) 14-d tigecycline treatment located in the ovaries, and (D) 30-d control treatment located in the ovaries.

Treatment Efficacy - Granuloma Descriptive

Through visual observations of acid-fast positive granulomas there was a general trend in the amount of AFB located within positive granulomas of untreated and treated groups. This is most clear in representative images of AFB positive granulomas from tigecycline, clarithromycin and untreated fish (Figure 5-5). Untreated *M. chelonae* injected fish had granulomas containing visually more acid-fast bacilli relative to tigecycline and clarithromycin treated fish.

5.5 Discussion

Treatment Tolerance

In the tolerance study there were no differences in behavioral observations, morbidity or mortality between zebrafish experimental groups. This suggests that the tigecycline and clarithromycin treatments were tolerated well by the zebrafish and that there is potential for higher dosages to be further considered. To our knowledge there are no previous reports of treatment of zebrafish with these two particular drugs; however, fish have been treated with antibiotics for mycobacterial infections at much higher dosages than those used in this study (Bernut, Le Moigne, Lesne, Lutfalla, Herrmann & Kremer 2014; Strike et al. 2016). The dosages we used were in the ranges used for the treatment of human mycobacterial infections (Muralidharan et al. 2005; Kim et al. 2011) which suggests this might be safe for other vertebrates. It should be noted that these dosages were targeted and may not equal the exact amount of antibiotic available to the fish as oral delivery is subject to loss of drug during digestion (discussed in Chang et al., 2015). There were no observed detrimental effects on zebrafish growth as there was no significant difference in growth post-treatment or 11 weeks post-treatment that differed significantly from untreated groups. Regarding effects on fecundity, there was no breeding cessation; however, treatment with tigecycline did significantly increase

average embryo count and increase 24-hour percent morality compared to other treatment groups. Altogether, all experimental groups were able to reproduce following treatment allowing for the potential for treated fish population to be re-derived from embryos in the case of an outbreak in a zebrafish facility. Although this difference did not result in severely low embryo counts or percent mortality, impacts of tigecycline treatment on fecundity may be more severe over a longer treatment period or higher dosage.

Treatment Efficacy

Observation of H&E stained fish revealed that granulomas were predominately located within the reproductive tissues and the swim bladder which is consistent with observations from other studies using the intraperitoneal injection mode of infection (Watral et al. 2007).

The highest prevalence of granulomas was observed in untreated *M. chelonae*-injected groups followed by *M. chelonae*-injected fish treated with clarithromycin, *M. chelonae*-injected fish treated with tigecycline, and lastly sham injected fish. No difference between experimental groups was observed when sexes were pooled; however, when groups of single sex were analysed separately a significant difference is observed between sexes for the 14-d tigecycline treatment. This finding indicated that further analyses should look at differences between sexes. Further analyses using acid fast staining techniques to identify AFB in granulomas was necessary in order to differentiate naturally occurring granulomas [e.g. in the case of egg degeneration (Kent et al., 2004)].

The locations of acid fast positive granulomas were similar to that observed for the H&E stained granulomas. None of the sham injected fish were observed to have any acid fast positive granulomas, which was expected as these fish were not exposed to *M. chelonae*. Generally the prevalence of acid fast positive granulomas was lower than H&E identified granulomas with the

exception of two female fish where acid fast staining allowed for the detection of a positive granuloma in the ovaries where previous H&E stained fish could not be differentiated from the appearance of a degenerating egg. This difference provides further support of using acid fast staining to correctly diagnose a mycobacterial infection (Noga, 2010). When groups of pooled sexes were analysed there was no significant differences between experimental groups. Further analyses of single sex groups indicate that for males, 14-d clarithromycin treatment resulted in a higher prevalence of acid fast positive granulomas than 14-d tigecycline, 30-d tigecycline or 30-d clarithromycin treatments. Treatments with either 14-d tigecycline or 30-d clarithromycin were less effective for female fish compared to males as females in these groups had a higher prevalence of acid fast positive granulomas for these treatments.

Importantly, we also evaluated the intensity of acid fast positive granulomas in order to better understand the severity of infections following different antibiotic treatments. There was a significant difference in acid fast positive granuloma intensity between the different experimental groups with the untreated groups having the highest intensity, followed by the 14-d clarithromycin treated group, then the 30-d tigecycline treated group, then the 30-d clarithromycin treated group, and the lowest intensity was observed for the 14-d tigecycline treated group. This trend is also observed for females. Males had a similar trend as well, however the 14- and 30-d tigecycline and the 30-d clarithromycin treated groups shared an intensity of zero. Increased infection severity in untreated fish compared to tigecycline and clarithromycin treated fish was also observed visually in the amount of AFB seen within granulomas. It appears as though the antibiotic treatments help to reduce the amount of observed acid fast bacteria present within granulomas; however, there are still some remaining bacilli that have not been eradicated. Thus, while tigecycline and clarithromycin treatments may not have resulted in a

reduction in the prevalence of acid fast positive granulomas, these treatments were effective at decreasing infection severity.

Conclusions and Recommendations

This study used a conservative dosage of tigecycline and clarithromycin based on *in vitro* minimum inhibitory concentration (MIC) values for the strain of *M. chelonae* used in this study and commonly found in zebrafish. Because there were only minimal effects on fecundity and no morbidity or mortality, alternative doses are likely to be tolerated by zebrafish. Also, because a complete eradication of AFB and granulomas was not observed following treatment, higher dosages and/or longer treatment durations should be investigated to determine if the effect on infection correlates positively with these factors. If a higher dose or longer treatment duration is observed to be effective eliminating the infection, additional effects on growth and fecundity should be examined as we did observe some effect on embryo count and 24-hour mortality following treatment with tigecycline. Further differentiation of these two treatments effectiveness could then be evaluated following these additional studies. Additionally, evaluation of a combined tigecycline/clarithromycin treatment should be examined. Future studies should also investigate the effect of these treatments on zebrafish infected with more severe *M. marinum* or *M. haemophilum* infections in order to evaluate if these treatments are effective at decreasing the severity of these infections.

We found that tigecycline and clarithromycin treatments of 14- and 30-d are effective at decreasing the severity of infections but not eliminating infections. Generally, we do not advocate for treatment of whole colonies of zebrafish, and treatment should only be reserved for preserving valuable fish lines. As latency and reinfection could occur, treated fish should be used to re-derive valuable lines using surface disinfected embryos (Whipps et al., 2012). We

recommend treated fish not be placed back on a main system as they may still be an infection risk factor for other fish. Also, treatment alone in order to control an outbreak is not enough, system disinfection is also required or else reinfection can occur from mycobacteria in surface biofilms (Whipps et al. 2012). Disease prevention and detection should still be primary mechanisms for controlling mycobacteriosis in zebrafish populations.

5.6 Acknowledgements

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5.7 References

Adekambi, T. & Drancourt, M. (2004) Dissection of phylogenetic relationships among 19 rapidly growing *Mycobacterium* species by 16S rRNA, hsp65, sodA, recA and rpoB gene sequencing. International Journal of Systematic and Evolutionary Microbiology, **54**: 2095-2105.

Aragon, T., Fay, M. & Wollschlaeger, D. (2012) Epitools: R Package for Epidemiologic Data and Graphics. R Package version 0.5-7. <https://cran.r-project.org/web/packages/epitools/epitools.pdf>

Astrofsky, K.M., Schrenzel, M.D., Bullis, R.A., Smolowitz, R.M. & Fox, J.G. (2000) Diagnosis and management of atypical *Mycobacterium* spp. infections in established laboratory zebrafish (*Brachydanio rerio*) facilities. Comparative Medicine, **50**: 666-672

Bernut, A., Le Moigne, V., Lesne, T., Lutfalla, G., Herrmann, J.L. & Kremer, L. (2014) *In vivo* assessment of drug efficacy against *Mycobacterium abscessus* using the embryonic zebrafish test system. Antimicrobial Agents Chemotherapy, **58**: 4054-4063.

Chang, C.T., Colicino, E.G., Dipaola, E.J., Al-Hasnawi, H.J. & Whipps, C.M. (2015) Evaluating the effectiveness of common disinfectants at preventing the propagation of *Mycobacterium* spp. isolated from zebrafish. Comparative Biochemistry and Physiology Part C, **178**: 45-50.

Chang, C.T. & Whipps, C.M. (2015) Activity of Antibiotics against *Mycobacterium* Species Commonly Found in Laboratory Zebrafish. Journal of Aquatic Animal Health, **27**: 88-95.

Dahm, R. & Geisler, R. (2006) Learning from small fry: the zebrafish as a genetic model organism for aquaculture fish species. Marine Biotechnology, **8**: 329-345.

Falkinham, J.O., 3rd (2009) Surrounded by mycobacteria: nontuberculous mycobacteria in the human environment. Journal of Applied Microbiology, **107**: 356-367.

Falkinham, J.O., 3rd, Norton, C.D. & Lechevallier, M.W. (2001) Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other Mycobacteria in drinking water distribution systems. Applied Environmental Microbiology, **67**: 1225-1231.

Feitsma, H. & Cuppen, E. (2008) Zebrafish as a cancer model. Molecular Cancer Research, **6**: 685-694.

Fox, J. & Weisberg, S. (2011) An (Kent P. T.) Companion to Applied Regression. In. Sage, Thousand Oaks, CA, USA.

Gaind, N. (2016) This week in science: Trendwatch. Nature, **536**: 131.

Gerhard, G.S. (2003) Comparative aspects of zebrafish (*Danio rerio*) as a model for aging research. Experimental Gerontology, **38**: 1333-1341.

Griffith, D.E., Aksamit, T., Brown-Elliott, B.A., Catanzaro, A., Dalet, C., Gordin, F., Holland, S.M., Horsburgh, R., Huitt, G., Iademarco, M.F., Iseman, M., Olivier, K., Ruoss, S., Von Reyn, C.F., Wallace Jr, R.J. & Winthrop, K. (2007) An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculosis mycobacterial diseases. American Journal of Respiratory and Critical Care Medicine, **175**: 367-416.

Kent, M.L., Feist, S.W., Harper, C., Hoogstraten-Miller, S., Mac Law, J., Sanchez-Morgado, J.M., Tanguay, R.L., Sanders, G.E., Spitsbergen, J.M. & Whipps, C.M. (2009) Recommendations for control of pathogens and infectious diseases in fish research facilities. Comparative Biochemistry and Physiology Part C, **149**: 240-248.

Kent, M.L., Spitsbergen, J.M., Matthews, J.M., Fournie, J.W., Murray, K.N. & Westerfield, M. (2012) Diseases of Zebrafish in research facilities. In. Zebrafish International Resource Center, Bulf Breeze, Fl. Available: https://zebrafish.org/wiki/health/disease_manual/start (November 2016)

Kent, M.L., Whipps, C.M., Matthews, J.L., Florio, D., Watral, V., Bishop-Stewart, J.K., Poort, M. & Bermudez, L. (2004) Mycobacteriosis in zebrafish (*Danio rerio*) research facilities.

Comparative Biochemistry and Physiology Part C, **138**: 383-390.

Kim, E.Y., Chi, S.Y., Oh, I.J., Kim, K.S., Kim, Y.I., Lim, S.C., Kim, Y.C. & Kwon, Y.S. (2011) Treatment outcome of combination therapy including clarithromycin for *Mycobacterium avium* complex pulmonary disease. The Korean Journal of Internal Medicine, **26**: 54-59.

Morales, M., With Code Developed by the R Development Core Team & With General Advice from the R-Help Listserv Community and Especially Duncan Murdoch (2012) sciplot: Scientific Graphing Functions for Factorial Designs. R package version 1.1-0 ed. <https://cran.r-project.org/web/packages/sciplot/sciplot.pdf>

Muralidharan, G., Micalizzi, M., Speth, J., Raible, D. & Troy, S. (2005) Pharmacokinetics of tigecycline after single and multiple doses in healthy subjects. Antimicrobial Agents and Chemotherapy, **49**: 220-229.

Noga, E. 2010. Fish disease: diagnosis and treatment, 2nd edition. Wiley-Blackwell Scientific, Ames, Iowa.

Phillips, J.B. & Westerfield, M. (2014) Zebrafish models in translational research: tipping the scales toward advancements in human health. Disease Models and Mechanisms, **7**: 739-743.

Pohlert, T. (2015) PMCMR: Calculate Pairwise Multiple Comparisons of Mean Rank Sums. R Package version 1.1. <https://cran.r-project.org/web/packages/PMCMR/vignettes/PMCMR.pdf>

R Core Team (2013) R: A language and environment for statistical computing. In. R Foundation for Statistical Computing, Vienna, Austria.

R Studio (2012) R Studio: Integrated development environment for R. In. R Studio, Boston, MA, USA.

Sciarra, J.B., Tyler, A.T. & Kolb, A. (2014) A gelatin-based diet for oral dosing juvenile to adult zebrafish (*Danio rerio*). Laboratory Animal Science Professional, **June**: 32-35.

Strike, T.B., Feltrer, Y., Flach, E., Macgregor, S.K. & Guillaume, S. (2016) Investigation and management of an outbreak of multispecies mycobacteriosis in Australian lungfish (*Neoceratodus fosteri*) including the use of triple antibiotic treatment. Journal of Fish Diseases. doi:10.1111/jfd.12535.

Truong, L., Harper, S.L. & Tanguay, R.L. (2011) Evaluation of embryotoxicity using the zebrafish model. Methods in Molecular Biology, **691**: 271-279.

Watrall, V. & Kent, M.L. (2007) Pathogenesis of *Mycobacterium* spp. in zebrafish (*Danio rerio*) from research facilities. Comparative Biochemistry and Physiology Part C, **145**: 55-60.

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., Lieggi, C. & Wagner, R. (2012) Mycobacteriosis in Zebrafish Colonies. Institute for Laboratory Animal Research, **53**: 95-105.

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.

Wong, K., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Roy, S., Goodspeed, J., Suci, C., Tan, J.L., Grimes, C., Chung, A., Rosenberg, M., Gaikwad, S., Denmark, A., Jackson, A., Kadri, F., Chung, K.M., Stewart, A., Gilder, T., Beeson, E., Zapolsky, I., Wu, N.D., Cachat, J. & Kalueff, A.V. (2010) Analyzing habituation responses to novelty in zebrafish (*Danio rerio*). Behavioural Brain Research, **208**: 450-457.

Wu, T.S., Chiu, C.H., Yang, C.H., Leu, H.S., Huang, C.T., Chen, Y.C., Wu, T.L., Chang, P.Y., Su, L.H., Kuo, A.J., Chia, J.H., Lu, C.C. & Lai, H.C. (2012) Fish tank granuloma caused by *Mycobacterium marinum*. Plos One, **7**: e4

Chapter 6

Source or Sink: Examining the Role of Biofilms in Transmission of *Mycobacterium* spp. in Laboratory Zebrafish

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6.1 Abstract

The zebrafish (*Danio rerio*) is a popular aquatic animal model used in many research fields. Zebrafish health is a primary research concern because it is prudent to conduct research on disease-free fish or fish with known disease status. Mycobacteriosis is a common bacterial disease in wild and captive fishes, including zebrafish. Despite its prevalence, the dynamics of transmission and potential sources of mycobacterial infections in zebrafish are only partially understood. One suspected natural infection source are surface biofilms on tanks and other system components. This study investigates the role that tank biofilms play in mycobacteriosis in laboratory zebrafish by evaluating the establishment of biofilms from bacteria shed from fish, and conversely the acquisition of infections in fish from surface biofilms. We found that zebrafish infected with *Mycobacterium chelonae* shed bacteria through feces and bacteria are transmitted to tank biofilms from one to 16 weeks post-infection. We also found that zebrafish acquire *M. chelonae* infections as soon as 2 weeks when introduced to tanks with establish *M. chelonae* biofilms. The results from this study highlight the role that tank biofilms play as both a reservoir and source of mycobacterial infections in zebrafish. Results support the inclusion of biofilm surveillance and prevention as part of a disease control program in zebrafish research facilities.

6.2 Introduction

The zebrafish (*Danio rerio*) is a popular aquatic model organism that has been recognized as a “rising star” among aquatic animal models of human disease (Gaird 2016). With increased popularity and investment from the research community, increased value is placed on the maintenance of health stocks of both mutant and wild-type lines. The health of laboratory zebrafish is a primary concern, as it is prudent to maintain and conduct research on healthy organisms. Ideally, research would be conducted on animals free of background infections, and if this is not possible, the disease status of the research subjects should be reported. The impacts of such infections is sometimes obvious when resulting in mortality or declines in reproductive fitness, but equally concerning are subclinical infections that can be a source of uncontrolled experimental variance (Kent 2012). Due to these concerns, measures to prevent and monitor disease in laboratory zebrafish should be taken.

One common infection in laboratory zebrafish is mycobacteriosis. Mycobacteriosis affects approximately 40% of zebrafish facilities submitting cases to the Zebrafish International Resource Center’s (ZIRC) Diagnostic Services (Zebrafish International Resource Center (ZIRC) Health Services 2017). There is no single etiological agent of mycobacteriosis, as it is caused by several species and strains of *Mycobacterium* (Astrofsky, Schrenzel et al. 2000, Kent, Whipps et al. 2004, Whipps, Matthews et al. 2008). The severity of mycobacterial infections in zebrafish is variable and species specific, ranging from high mortality with *Mycobacterium marinum* and *Mycobacterium haemophilum* to few or no mortality with *Mycobacterium abscesses* and *Mycobacterium chelonae* (Watrall and Kent 2007, Whipps, Butler et al. 2007, Whipps, Dougan et al. 2007, Whipps 2012). Similarly, morbidity is also variable and includes non-pathognomic signs including: skin lesions, emaciation, raised scales, swollen abdomen, and behavior that is

irregular or lethargic (Astrofsky, Schrenzel et al. 2000, Kent 2012). Internal signs include granulomas, particularly on hematopoietic organs (Astrofsky, Schrenzel et al. 2000, Kent, Whipps et al. 2004). Subclinical infections lack any observable signs and may go unnoticed (Kent, Whipps et al. 2004, Whipps 2012).

Control and management measures for mycobacteriosis in zebrafish facilities highlight the importance of prevention, as established infections are difficult to eliminate (Whipps 2012, Mason, Snell et al. 2016). Current preventative measures include quarantine of imported fish and those that appear morbid, disinfection of embryos and fomites, ultraviolet sterilization of water, and sentinel programs (Kent, Feist et al. 2009, Whipps 2012). While these measures have, and continue, to effectively prevent the establishment of mycobacterial infections in zebrafish facilities more insight is needed into additional factors contributing this disease. One factor that has been speculated to be involved in the mycobacterial disease cycle in zebrafish facilities is surface biofilms.

Non-tuberculosis mycobacteria (NTM) normally inhabit a variety of habitats that are shared with both humans and animals including water distribution systems (Falkinham, Norton et al. 2001). Piscine mycobacteria species are able to persist in surface biofilms that form in aquaria (Beran, Matlova et al. 2006, Whipps, Matthews et al. 2008). The hydrophobic and oligotrophic characters of mycobacteria allow for survival in environments with low levels of dissolved carbon while readily adhering to surfaces (Falkinham, Norton et al. 2001, Falkinham 2009). Thus, mycobacteria are well adapted to survive in “clean” water systems such as aquaria (Whipps 2012). Many different species of mycobacteria can be found in biofilms (Schulze-Robbecke, Janning et al. 1992), with several species isolated specifically from aquaria (Beran, Matlova et al. 2006, Whipps, Dougan et al. 2007, Whipps 2012). In zebrafish facilities, there

have been reports of detection of the same species of mycobacteria in both biofilms and fish, but many more species are only found in the biofilms (Whipps, Dougan et al. 2007, Whipps, Matthews et al. 2008). In addition to bacterial communities within aquatic biofilms, protozoans are an important component of biofilms (Arndt, Schmidt-Denter et al. 2003). Protozoans rapidly colonize aquatic biofilms, occurring in high abundances (Arndt, Schmidt-Denter et al. 2003). These biofilm-dwelling protozoans may support bacterial survival and virulence as the protozoan/bacteria interaction allows for survival, replication, and distribution of pathogenic bacteria (Barker and Brown 1994).

Genetic comparisons between *Mycobacterium* isolates identify identical strains in both fish and biofilms of *M. chelonae* in zebrafish (Whipps, Matthews et al. 2008) and *M. marinum* in pompano *Trachinotus carolinus* (Yanong, Pouder et al. 2010). These results demonstrate a link between fish and biofilms, but not the direction of transmission; i.e., whether the fish are the source for biofilms, biofilms the source for the fish, or that both are possible.

Biofilms can be found on all surfaces in the aquatic environment, and it is thought that these biofilms and detritus at the bottom of tanks are the source of mycobacterial infections in zebrafish (Whipps, Lieggi et al. 2012). In a study where zebrafish found in the sump tank were examined, they were found to be infected with mycobacteria (Whipps, Lieggi et al. 2012). The only source of food for these fish was the surface biofilms and tank detritus. This is consistent with what is known about the feeding habits of zebrafish, as they feed in the water column and on tank surfaces and substrates (Spence, Gerlach et al. 2008) as generalist consumers (Lawrence 2007). Sentinel zebrafish residing in system sumps have also been observed to hunt for benthic organism and slow zooplankton along the biofilm scaffold (Whipps, Lieggi et al. 2012). Larvae (8-10 mm SL) are very active as hunters and grazers, which may result in the incidental

consumption of mycobacteria (Whipps 2012). A previous study found that zebrafish in larger tanks had a higher prevalence of *M. chelonae* infections compared to fish in smaller tanks (Murray, Bauer et al. 2011). This difference in prevalence was attributed to the different tank cleaning methods used for removing biofilms for the two tank sizes. The zebrafish in the larger tanks remained in the tanks during cleaning compared to zebrafish in the smaller tanks, which were swapped into new tanks before tanks were cleaned. The fish in the larger tanks were likely feeding on the tank debris that were suspended in the water column during the cleaning while the fish in smaller tanks were not exposed to this. Additionally, the oral route of infection has been demonstrated as a natural mode of mycobacterial transmission in zebrafish (Harriff, Bermudez et al. 2007). Conversely, support for biofilms as the sink for mycobacteria includes the natural shedding of bacteria from infected fish (Noga 2010, Whipps 2012) which contributes to biofilms. However, mycobacterial shedding and subsequent uptake into tank biofilms from zebrafish has not yet been studied.

The goal of this current study is to investigate the route of transmission of mycobacteria between zebrafish and biofilms and elucidation of the role of biofilms as source or sink in these infections. We first investigate the transmission of mycobacteria from zebrafish with established *M. chelonae* infections (both through experimental IP injection infection and also experimental natural oral infection) to feces and tank biofilms over a 16 week period. We then investigate the transmission of *M. chelonae* GFP mutant from established tank biofilms to *casper* (*nacre* *-/-* *roy* *-/-*) zebrafish over a 16 week period using bi-weekly live fluorescent imaging of zebrafish. We hypothesize that biofilms play both roles and simultaneously are a source of infection at the same time as being a sink for mycobacteria shed from infected zebrafish. Results from this study will

contribute towards our understanding of zebrafish mycobacteriosis and contribute to disease control and management improving the overall health of laboratory zebrafish.

6.3 Methods

Fish

All fish used in this study were bred and maintained in the zebrafish facility at the SUNY-ESF Center for Integrated Teaching and Research in Aquatic Science. Adult AB wild type zebrafish (n=144; 72 male and 72 female; age = 6 months) and *casper* (*nacre*^{w2/w2}; *roy*^{a9/a9}) zebrafish (n=30; 15 male and 15 female; age = 6 months) lines, originally obtained from the SARL at Oregon State University (Corvallis, OR) and bred for two generations at SUNY-ESF, were utilized in this study. Animals were housed at a density of 6-10 fish/liter in 1.8 L tanks on a timed, flow-through housing system (Aquaneering, San Diego, CA). The housing system included ultraviolet disinfection of dechlorinated (carbon filter) municipal tap water as the source, which was maintained at pH 7.6, conductivity of 600-700 us/cm², a temperature of 28.5°C, and ammonia levels ranging from 0-0.25 ppm. The zebrafish facility maintained a 14:10 light:dark photoperiod. Fish were fed a commercial feed for zebrafish (Gemma, Skretting) twice daily on weekdays and once daily on weekends during periods where they were not being fed a treatment gelatin feed. Prior to the experiment, routine cleaning of all equipment (e.g. tanks, lids, baffles, nets, and tubing) consisted of bi-weekly washing and scrubbing in warm water with a new soft sponge and bleaching in 1000 ppm chlorine bleach for 30 minutes, followed by rinsing three times in dechlorinated water and drying. All tanks, lids, and baffles were also autoclaved using a program specified by the tank manufacturer that reaches a temperature of 105-110°C for 15 minutes. During experiments this tank cleaning procedure occurred when the placement of

fish in a new tank is mentioned in the methods below. All animal work was approved by the SUNY-ESF Institutional Animal Care and Use Committee, protocol #151001.

Transmission to Biofilms

Experimental Infections

Prior to the set-up of experimental groups, mycobacterial infections or sham injections were established in the fish.

IP Injection: *Mycobacterium chelonae* injected wild type zebrafish (N=36 fish) were injected intraperitoneally (IP) with 5.0×10^4 cfu/fish of *Mycobacterium chelonae* (H1E2) mutant expressing green fluorescence protein (GFP) provided by M.L. Kent, and sham injected fish (N=36) injected with sterile saline, following the method described in Watral et al. (2007). The concentration of the bacterial inoculum was determined using a nephelometer (Sensititre) and confirmed with colony counts following culture on Middlebrook 7H10 agar plates. After IP injection, fish from each injection type (*M. chelonae* or sham) were randomly divided equally into three new tanks (12 fish/tank) and maintained on the flow-through system for 1 week prior to the beginning of the experimental sampling. During this week period fish were fed a commercial feed (Gemma, Skretting) twice daily on weekdays and once daily on weekends.

Oral Ingestion Infection: *Mycobacterium chelonae* ingestion wild type zebrafish (N=36) were experimentally infected through the ingestion of a gelatin feed containing *Mycobacterium chelonae* (H1E2) mutant expressing green fluoresce protein (GFP) through five consecutive daily feeds containing 1.0×10^4 cfu/feed/fish to reach a total ingestion of 5.0×10^4 cfu/fish. Gelatin feed (Gelly Belly, Florida Aqua Farms) was prepared as described in a previous study (Sciarra, Tyler et al. 2014). Once gelatin feed was set overnight at 4°C, a flame-sterilized metal spreader was used to spread a known concentration of *M. chelonae* culture diluted in sterile water to make

a final volume of 1 ml. The inoculated gelatin plate was then dried for approximately 24h at 28.5°C in an incubator. Following this a flame sterilized razor blade was used to mince the gelatin and aliquots for each treatment tank were measured out by mass and stored at -20 °C until feeding. At feeding, aliquots were emptied into treatment tanks. Sham ingestion wild type zebrafish (N=36) were fed a gelatin feed containing sterile saline for five consecutive daily feeds. After ingestion infection, fish from each ingestion type (*M. chelonae* or sham) were randomly divided equally into three new tanks (12 fish/tank) and maintained on the flow-through system for 1 week prior to the beginning of the experimental sampling. During this week period fish were fed a commercial feed (Gemma, Skretting) twice daily on weekdays and once daily on weekends. Tanks were siphoned daily following feeding during this period.

Sample Collection and Processing

Sample collection started on the second week post-infection. Sampling was repeated weekly for 16-weeks total. For each sampling event, zebrafish from each treatment tank were transferred to a new tank and incubated overnight in the new tank on the bench top (static water). The old tank was also kept on the bench top. Following overnight incubation, a biofilm swab sample was taken for each old tank as previously described (Whipps, Dougan et al. 2007) and stored in 1% cetyl pyridinium chloride (CPC) (Kent and Kubica, 1985) in a sterile 2.0 ml screwcap tube. Collection of feces/detritus from the overnight incubation tank was also collected using a sterile disposable pipette and stored in 1% (CPC) in a sterile 2.0 ml screwcap tube. Biofilm and fecal samples were stored in the 1% CPC tubes for 2 hours at room temperature prior to sample processing. Following sample collection, fish were returned to their old tank and placed back on the zebrafish system racks unless otherwise noted below.

Processing of the biofilm swab samples consisted of swab samples stored in 2.0 ml screw cap tubes containing 200 μ l 1% CPC being vortexed vigorously for 10 seconds. Next, flame-sterilized forceps were used to remove the swab, which was disposed of in 95% EtOH. To the remaining solution in the screw cap tube, 900 μ l of autoclaved dH₂O was added, followed by vortexing. The vortexed tubes were then centrifuged for 10 minutes at 8000 x g in order to form a pellet. The supernatant was removed from the screw cap tubes, and 100 μ l of autoclaved dH₂O was added, followed by vortexing. Next, 10 μ l of this solution was streaked onto a Middlebrook (MC) 7H10 agar plate, which was sealed with Parafilm and incubated at 28°C for 5-7 days. Following incubation, resulting colonies were observed under fluorescence in order to identify those colonies expressing GFP. The number of tank biofilm swabs resulting in the growth of GFP positive colonies was recorded. In the case that two or more out of three replicate tanks for each treatment were recorded as positive, then fish incubated overnight for sample collection were placed back on system racks in the new tank instead of returning to their old tank so that future observation of transmission of bacteria to tank biofilms could be detected.

Processing of the fecal/detritus samples consisted of fecal samples stored in 1.5 ml tubes containing 200 μ l 1% CPC being homogenized using a sterile plastic tissue pestle. Next, 900 μ l of autoclaved dH₂O was added, followed by vortexing. The vortexed tubes were pelleted and subsequently cultured on MB agar plates as the biofilm samples were described previously. The number of tank fecal samples resulting in the growth of GFP positive colonies was recorded.

End Analyses

After the 16th week of sampling all fish were euthanized by submersion in 0.3 g/L MS222 buffered to a pH of 7.5 for a minimum of 10 minutes following the loss of opercular movement. Once euthanized, fish were dissected to create an approximately 5 mm by 5 mm window on the

lateral side of so that internal organs could be observed under FITC fluorescence for signs of GFP expression. Images were taken when GFP expression was observed. Liver and spleen samples were also taken and stored for 1 hour in 1% CPC followed by the same homogenization, pellet, and culture procedure described above. Following imaging, euthanized fish were fixed in Davidson's solution for 48h and then dehydrated to 70% EtOH for histology. Each fish was sectioned and stained with hematoxylin and eosin (H&E) and Kinyoun's acid fast stain. Following staining, slides were examined for granulomas in H&E sections. For acid fast-stained sections, the presence of granulomas containing acid fast bacilli (AFB) was recorded. The location of granulomas containing AFB was recorded, and the location of AFB not contained in granulomas was also recorded. AFB not within granulomas were considered to be internally located if they were located inside the epithelial boundary (e.g. not in the gut lumen or on the outside of the skin/scales). The prevalence of granulomas, granulomas containing AFB, and free AFB was calculated.

Statistics

There was detection of bacteria in sham infection fish (both IP injection and oral feed) and these groups were excluded from further analyses and figures. For each set of three replicate tanks for each infection type (*M. chelonae* IP injection, *M. chelonae* gelatin feed), a Chi-squared analysis with a Monte Carlo simulation method using 20,000 replications was used to compare replicate tanks in order to determine whether data from replicate tanks can be pooled for further comparisons. No significant differences between replicate groups were identified, and replicate data for each infection type group was pooled. Fisher's exact test for count data was used to compare prevalence between infection types using the "stats" package (R Core Team 2013). Comparisons of prevalence were made between diagnostic methods (*in vivo* GFP expression,

liver/spleen culture, and histology) as well as between sexes. The results were organized into bar plots using the “sciplot” package (Morales, with code developed by the R Development Core Team et al. 2012).

Transmission from Biofilms

Experimental Biofilm Set-Up

An experimental biofilm was created in three 1.8L tanks (Aquaneering). Cleaned and autoclaved tanks were filled with zebrafish system water and inoculated with *Mycobacterium chelonae* (H1E2) mutant expressing green fluoresce protein (GFP) that was diluted to 2.7×10^8 cfu/ml verified by a nephelometer (Sensititre). Following dilution, 3 ml of the diluted bacteria was inoculated into each tank and tank water was stirred using a sterile 25 ml graduated pipette. Tanks were incubated on the benchtop for 4 weeks at room temperature and topped off with zebrafish system water to maintain the water level. Each week, a biofilm swab was taken and processed as previously described. Following two consecutive weeks of isolation of GFP colonies from all three tanks, the tanks were emptied, rinsed, filled with fresh zebrafish system water, and placed on the flow-through zebrafish system. Tanks were then checked weekly to confirm the isolation of GFP colonies from the biofilm of all three tanks throughout the experiment.

Tagging Zebrafish with Individual Markers

Adult AB *casper* (*nacre*^{w2/w2}; *roy*^{a9/a9}) zebrafish (n=30; 15 male and 15 female; age = 6 months) were individually tagged so that fish could be individually tracked over the course of the study. Each of the three tanks received ten *casper* zebrafish (5 male, 5 female) with the following tag designations: male, no tag; male, right-side pink tag; male, right-side orange tag; male, left-side pink tag; male, left-side orange tag; female, no tag; female, right-side pink tag; female,

right-side orange tag; female, left-side pink tag; female, left-side orange tag. Prior to tagging, fish were anaesthetized in 0.15 g/ml MS222 buffered to a pH of 7.5. Following anaesthesia, fish were placed in an autoclaved sponge soaked in the 0.15 g/L MS222 with a slit cut into middle of the sponge to stabilize the fish with its dorsal side exposed. For tagging, a manual injection kit for visible implant elastomer (VIE) tags was used (Northwest Marine technology, Inc., Shaw Island, WA, USA, <http://www.nmt.us>). Elastomer was prepared according to the manufacturer instructions (Northwest Marine Technology, Inc., 2011, 'Manual elastomer injection systems. Instructions for 10:1 visible implant elastomer', http://www.nmt.us/products/vie/manual_vie_instructions.pdf). Tags were injected following the procedure described by (Hohn and Petrie-Hanson 2013) with the syringe needle inserted 2 mm further than the desired tag location, followed by elastomer expression and needle retraction until approximately 1 mm before the injection site. Tags were placed below the dorsal fin base, as (Hohn and Petrie-Hanson 2013) found this site to be optimal for tag retention and visibility. Following tag insertion, fish were put into a recovery tank filled with zebrafish water. Following recovery, fish were placed in system tanks and observed for tag retention and tag-related morbidity for two weeks prior to placement in biofilm tanks.

Bi-Weekly Live-Imaging

Live-imaging occurred immediately prior to placing fish in the biofilm tanks, and bi-weekly thereafter. For live-imaging, fish were anaesthetized in 0.15 g/L MS222. Following anaesthetization, tag location and color were noted and fish were imaged under FITC fluorescence in order to detect GFP expression. If GFP expression was detected, an image was taken and the location of the GFP expression was noted. Following imaging, fish were placed in a recovery tank containing fresh zebrafish system water. Once, recovered, fish were placed back

in their respective biofilm treatment tank. In the case that a fish appeared moribund, it was euthanized in 0.3 g/L MS222 and fixed and stored as previously described.

End Analyses

After the 16th week of sampling all fish were euthanized as above. Once euthanized, four representative fish from each tank (2 male, 2 female) were dissected to create an approximately 5 mm by 5 mm window on the lateral side of so that internal organs could be observed under fluorescence for signs of GFP expression. Images were taken when GFP expression was observed. Liver and spleen samples were also taken and stored in 200 μ l sterile dH₂O followed by the same homogenization, pellet, and culture procedure described above. DNA extraction from the liver/spleen homogenate was performed using the MO BIO Laboratories, Inc. UltraClean® Microbial DNA Isolation Kit following manufacturer protocol. Next, a *M. chelonae*-specific PCR as in (Meritet, Mulrooney et al. 2017) was performed followed by gel electrophoresis on a 1% agarose gel. All euthanized fish were fixed in Davidson's solution for 48h and then transferred to 70% EtOH for storage.

Statistics

A Chi-squared analysis with a Monte Carlo simulation method using 20,000 replications was used to compare replicate tanks in order to determine whether prevalence data from these tanks could be pooled for further comparisons. There was no significant difference between replicate groups and prevalence data was pooled. Fisher's exact test for count data was used to compare prevalence between infection types using the "stats" package (R Core Team 2013). Comparisons of prevalence were made between diagnostic methods (*in vivo* GFP expression, *in vivo* GFP expression with dissection, liver/spleen culture, and liver/spleen PCR) as well as

between sexes. The results were organized into bar plots using the “sciplot” package (Morales, with code developed by the R Development Core Team et al. 2012).

6.4 Results

Transmission to Biofilms

The detection of GFP positive colonies in tanks over time shows variable detection in feces and biofilm samples over time between IP injected and oral feed infection modes (Table 6-1). Detection for the feces and biofilm samples for all *M. chelonae* IP injection replicate tanks occurred as soon as the first week of sampling (Table 6-1) and continued to be consistently detected for the first 9 weeks of sampling in both the feces and biofilm with the exception of week 5. By week 10, detection of GFP positive colonies became more intermittent; however, persisted through the 16 weeks of sampling. For fish infected with *M. chelonae* through oral feed, detection in colonies only occurred in the biofilm of one *M. chelonae* oral feed infection tank for week one. By week three GFP colonies started to be detected intermittently in two or more of the three replicate tanks for the oral feed infection fish. GFP colonies were detected in feces in two or more tanks starting in week two and were detected intermittently through week 16. GFP colonies were not detected in any sham infection tanks with the exception of weeks 7 and 8 where colonies were detected in biofilm samples for oral feed sham infection tanks, for one and two weeks respectively.

We were able to use three different methods in order to evaluate prevalence of *M. chelonae* infections: *in vivo* GFP screening (Figure 6-2A and D), culture of liver and spleen samples, and histology (Figure 6-2G-I). There was no detection of bacteria in sham infection fish.

When comparing the prevalence of *M. chelonae* infections between the two modes of infection (IP injection vs. oral feed), differences depended on the diagnostic method used (Figure 6-1, plus sign). Prevalence determination through dissection and *in vivo* GFP screening (grey bars) resulted in no difference between the two different modes of infection when comparing groups of both sexes, only males, and only females ($p=0.57$). When using culture of liver and spleen samples in order to isolate GFP colonies (black bars), IP injected groups of both sexes and males had a significantly higher prevalence compared to oral feed infected groups of both sexes and males ($p=8.15 \times 10^{-3}$). Histology (white bars) resulted in a significantly higher prevalence in all IP injected compared to all oral feed infected groups ($p<2.2 \times 10^{-16}$).

When comparing the prevalence based on each method within group of the same infection mode and sex (Figure 6-1, asterisk), liver culture resulted in a significantly lower prevalence compared to histology and *in vivo* GFP screening for IP injection infected groups of both sexes ($p=6.02 \times 10^{-8}$), males ($p=8.46 \times 10^{-4}$), and females ($p=5.94 \times 10^{-5}$). For oral feed infection, *in vivo* GFP screening resulted in significantly higher prevalences for groups of all sexes ($p=3.39 \times 10^{-14}$), males ($p=2.57 \times 10^{-9}$), and females ($p=2.57 \times 10^{-9}$).

TABLE 6-1. Transmission of *Mycobacterium chelonae* to feces and tank biofilms from experimentally infected zebrafish over a

period of 16 weeks based on GFP colony formation in culture. The presence (X) or absence (-) of GFP colonies are indicated, with the exception of unavailable biofilm samples (N). When two or more tanks for a given treatment resulted in the formation of GFP colonies fish were placed in new autoclaved tanks (X).

Infection Mode	Tank	Week																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
IP Injection	Sham 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Sham 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Sham 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	<i>M. chelonae</i> 1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	<i>M. chelonae</i> 2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	<i>M. chelonae</i> 3	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	Sham 1	-	-	-	-	-	-	X	X	X	X	X	X	X	X	X	X	X
	Sham 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Sham 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oral Feed	<i>M. chelonae</i> 1	-	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	<i>M. chelonae</i> 2	-	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	<i>M. chelonae</i> 3	-	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

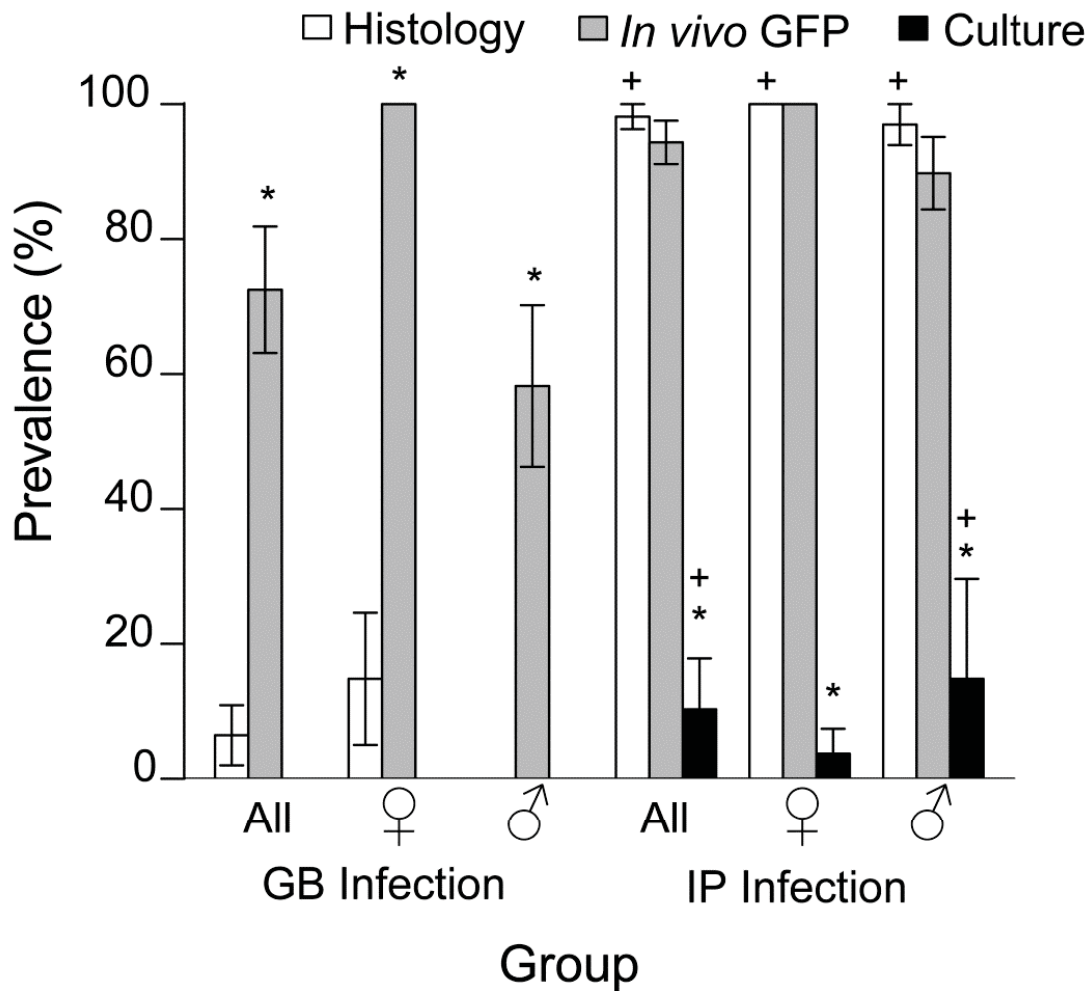


Figure 6-1. Prevalence of *M. chelonae* infections for the two modes of infection [oral Gelly Belly (GB) infection vs. IP injection infection]. Different diagnostic methods include histology (white bars), *in vivo* GFP screening (grey bars, and liver/spleen culture (black bars). Statistically significant differences between diagnostic methods used are indicated (+), as well as those between sexes are also indicated (*)

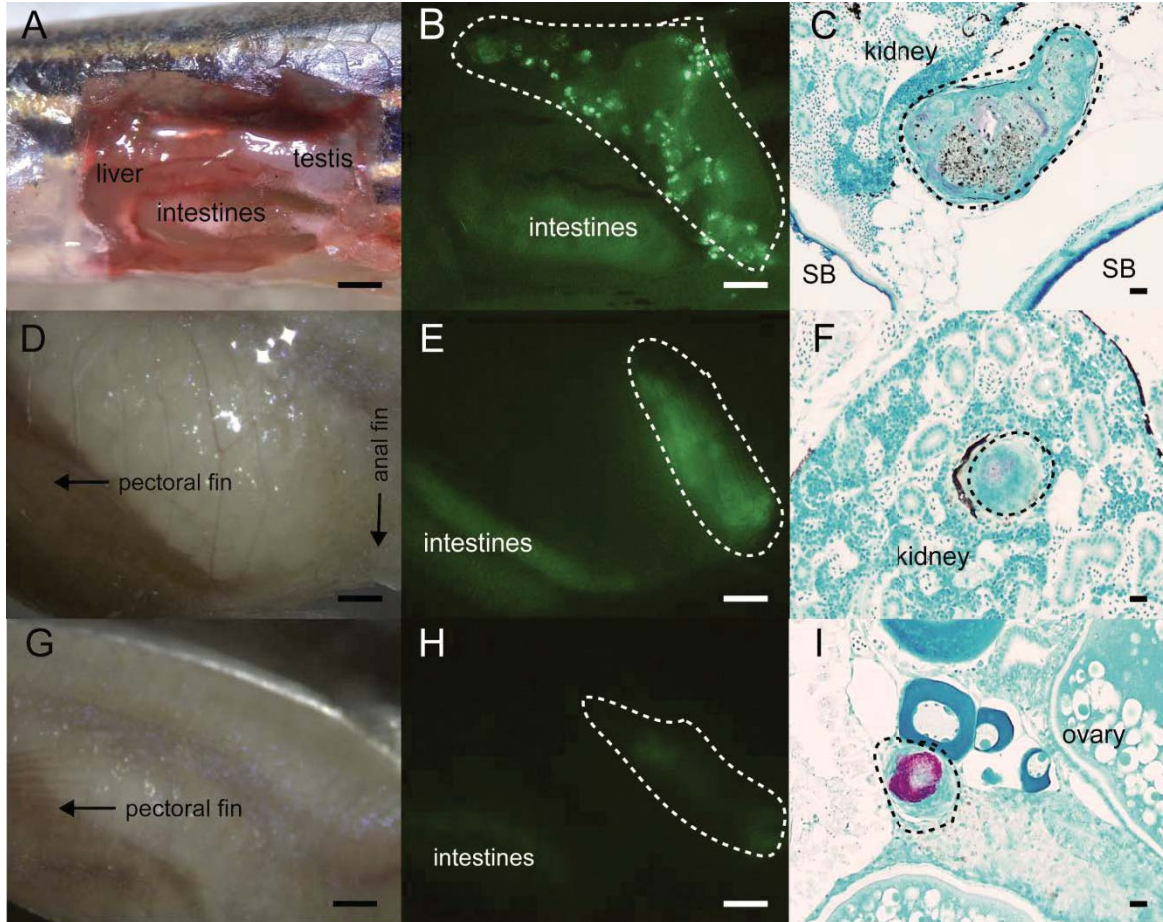


Figure 6-2. Examples of the different diagnostic methods used in this study. Comparative brightfield and FITC screening were used to identify areas of GFP expression (compare A, D, G to B, E, H). For the transmission from fish to biofilms study histology was also used to identify acid-fast bacteria in granulomas (encircled in C, F, I). SB=swim bladder.

Transmission to Fish

The VIE tags were retained for the entirety of the study and we were able to differentiate individual fish from each tank. We also were able to observe GFP expression *in vivo* through the skin/scales of the *casper* mutant fish (Figure 6-2B and C, 6-2E and F). The number of fish with detectable GFP expression increased over the 16 weeks of this study (Table 6-2). When the fish were first placed in the biofilm tanks, no *in vivo* GFP expression was observed. Beginning on week 2, GFP expression was observed in one female fish in Tank 2. At week 6, GFP expression was observed in two female fish in both Tank 2 and Tank 3. The number of fish with GFP expression gradually increased at each sampling time-point, with a higher proportion of females expressing GFP (observed in the ovaries). By the last sampling time point the tank prevalence of GFP expression ranged from 60-100%. Through the dissection of a subset of fish from each tank, additional GFP expression was detected increasing the prevalence (Table 6-2, X) in Tank 2 and 3 to 70%.

Similar to the previous results for the transmission to biofilm, the prevalence of *M. chelonae* infections depended on the method used (Figure 6-3, plus sign). *In vivo* GFP expression and *in vivo* GFP expression post-mortem after subset dissection resulted in a significantly higher prevalence compared to liver and spleen culture or PCR for both sexes ($p=1.48 \times 10^{-4}$), females only ($p=6.90 \times 10^{-4}$). There was no difference in prevalence values for males between diagnostic methods ($p=0.19$). There was also no significant difference ($p>0.05$) between in prevalence between groups when considering each diagnostic method.

TABLE 6-2. Transmission of *Mycobacterium chelonae* to zebrafish from tank biofilms over a period of 16 weeks based on detection of GFP expression in zebrafish during live-imaging. The presence (X) or absence (-) of GFP expression are indicated for each bi-weekly sampling event. The presence of GFP expression only observed following dissection is also indicated (X).

Tank	Number	Sex	Week										
			0	2	4	6	8	10	12	14	16		
1	Fish 1	M	-	-	-	-	-	-	-	-	-	-	X
	Fish 2	M	-	-	-	-	-	-	-	-	-	X	X
	Fish 3	M	-	-	-	-	-	-	-	-	X	X	
	Fish 4	M	-	-	-	-	X	X	X	X	X	X	
	Fish 5	M	-	-	-	-	-	-	-	-	X	X	
	Fish 6	F	-	-	-	-	X	X	X	X	X	X	
	Fish 7	F	-	-	-	-	X	X	X	X	X	X	
	Fish 8	F	-	-	-	-	-	-	X	X	X	X	
	Fish 9	F	-	-	-	-	X	X	X	X	X	X	
	Fish 10	F	-	-	-	-	X	X	X	X	X	X	
2	Fish 1	M	-	-	-	-	-	-	-	-	-	-	X
	Fish 2	M	-	-	-	-	-	-	-	-	-	-	-
	Fish 3	M	-	-	-	-	-	-	-	-	-	-	-
	Fish 4	M	-	-	-	-	-	-	-	-	-	-	-
	Fish 5	M	-	-	-	-	-	-	-	-	-	-	X
	Fish 6	F	-	-	-	-	X	X	X	X	X	X	
	Fish 7	F	-	-	-	X	X	X	X	X	X	X	
	Fish 8	F	-	-	-	-	-	X	X	X	X	X	
	Fish 9	F	-	X	X	X	X	X	X	X	X	X	
	Fish 10	F	-	-	-	-	-	X	X	X	X	X	
3	Fish 1	F*	-	-	-	-	X	X	X	X	X	X	
	Fish 2	M	-	-	-	-	-	-	-	-	-	-	-
	Fish 3	M	-	-	-	-	-	-	-	-	-	-	-
	Fish 4	M	-	-	-	-	-	-	-	-	-	-	X
	Fish 5	M	-	-	-	-	-	-	-	-	-	-	X
	Fish 6	F	-	-	-	-	X	X	X	X	X	X	
	Fish 7	F	-	-	-	-	-	X	X	X	X	X	
	Fish 8	F	-	-	-	-	-	-	-	-	-	-	-
	Fish 9	F	-	-	-	X	X	X	X	X	X	X	
	Fish 10	F	-	-	-	X	X	X	X	X	X	X	

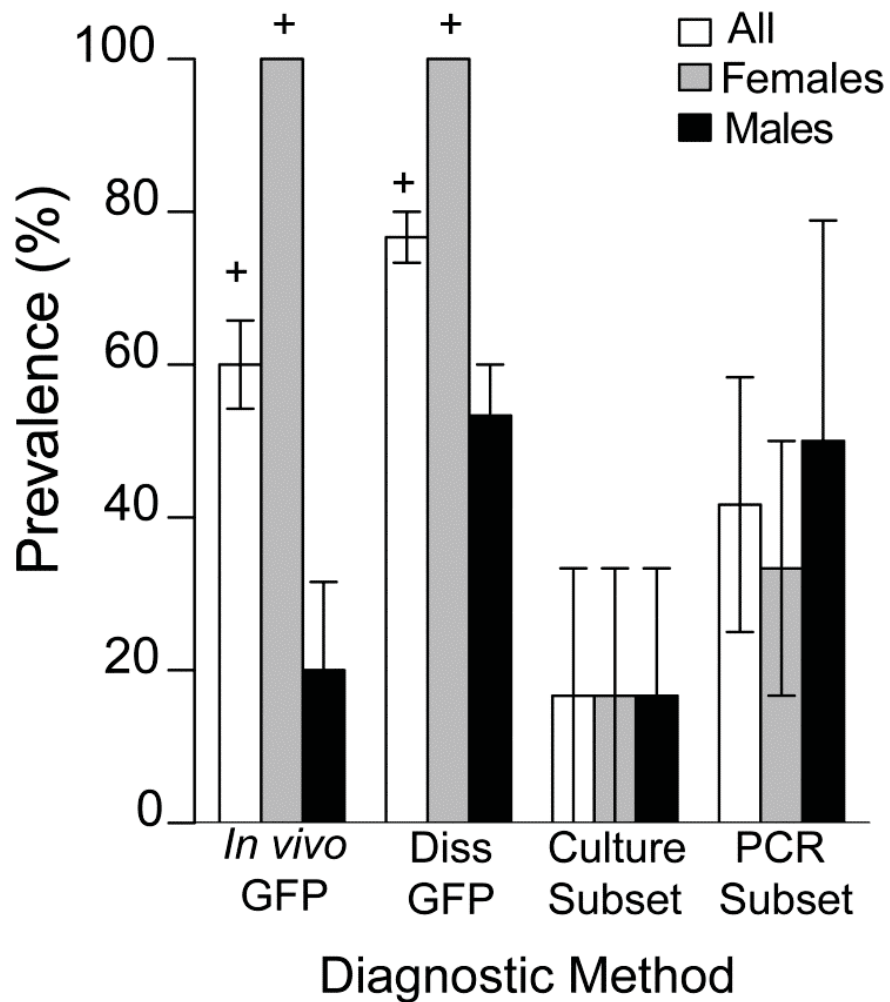


Figure 6-3. Prevalence of *M. chelonae* infections following incubation in *M. chelonae* biofilm tanks based on four diagnostic methods: *in vivo* GFP screening with and without subset dissection, subset liver/spleen culture, and subset liver/spleen PCR. Statistically significant differences between diagnostic methods used are indicated (+).

6.5 Discussion

Measures to prevent and monitor mycobacteriosis in laboratory zebrafish should be taken in order to minimize or eliminate the impact the disease. In this study, transmission of *M. chelonae* between zebrafish and tank surface biofilms was demonstrated. Transmission from infected zebrafish to biofilms occurred within one week post-infection for IP injected fish and for fish exposed orally. Observation of GFP *M. chelonae* in feces occurred in week 1 for IP injected fish, and by week 2 for orally infected fish. Detection of *M. chelonae* in feces and tank biofilms persisted through the end of the study period for both groups; however, as the study progressed, detection became intermittent. This observation of initial consistent transmission of *M. chelonae* to tank biofilms and feces followed by more sporadic transmission is likely due to the amount of bacteria shed by fish during the phases of infection, with a greater amount of shedding following initial infection. There were also two weeks where GFP positive colonies were detected in sham oral infection tanks, which we believe to be due to contamination during sample processing as mycobacteria were never detected in any other sham samples. We expect that the observations from the oral ingestion infection fish simulate what would naturally be observed in zebrafish colonies. It is likely that mycobacteria are shed for an extended period time as we observed shedding and transmission to tank biofilms up to the endpoint of this study (16 weeks). Many studies investigating zebrafish mycobacteriosis end at 8 weeks post-infection, and little is known about infections past these experimental endpoints. It is likely that some natural infections are eventually cleared while some shedding occurs (Harriff, Bermudez et al. 2007); although a stressful event could exasperate the situation and result in an outbreak.

We also observed transmission of *M. chelonae* from tank biofilms to uninfected zebrafish demonstrating that biofilms can be a natural source of infection in zebrafish colonies. Although

we cannot entirely determine whether infection occurred through ingestion from zebrafish feeding on biofilms or through immersion in the case that planktonic mycobacteria detached from biofilms, the originating source of *M. chelonae* in this study was a tank biofilm. This result is significant as biofilms are a common reservoir for *Mycobacterium* spp. in aquatic systems and form readily.

There is still a dearth of clear demonstrations of natural transmission routes of mycobacteria in laboratory zebrafish. Transmission through oral ingestion has historically been suspected as the major source of infection, as major outbreaks in hatchery salmon in the 1950s and 1960s were correlated with unpasteurized fish feed (Ross 1970, Belas, Faloon et al. 1995). In zebrafish, Harriff *et al.* (2007) demonstrated mycobacterial infections through ingestion highlighting the intestinal tract as a route of entry. Infection by oral ingestion has previously been shown to be more infective than by culture alone demonstrated to be a more infective source of infection compared to culture alone. Mosquito larvae infected with *Mycobacterium marinum* were more infective to Japanese medaka (*Oryzias latipes*) compared to immersion challenge at a high dosage (Mutoji 2011). In zebrafish, ingestion of the ciliated protozoan *Paramecium caudatum* containing *M. marinum* and *M. chelonae* resulted in a higher prevalence of infection compared to ingestion of culture alone (Peterson, Ferguson et al. 2013). These results follow previous studies describing an increase in virulence in mycobacteria in tissues (e.g. granulomas) compared to culture (Volkman, Clay et al. 2004). Infection through immersion has also been demonstrated in zebrafish embryos (Davis, Clay et al. 2002). Biofilms as a source of infection in laboratory zebrafish has been suspected (Whipps 2012), but had not yet been experimentally addressed until this study.

The identification of tank biofilms as both a source and reservoir for *M. chelonae* in this study can inform prevention and control of mycobacteriosis in zebrafish colonies. In terms of prevention, biofilm sampling can be used for environmental monitoring in conjunction with testing animals as part of a disease surveillance program (Collymore, Crim et al. 2016); however the intermittent nature of bacterial shedding and detection in the biofilm should be considered when interpreting environmental sampling results. Additionally, regular tank cleaning and removal of biofilms from tanks and other equipment in zebrafish systems should be carried out to minimize potential sources of infection. Removal of biofilms within systems can be labor intensive as pieces of equipment need to be scrubbed and disinfected but has previously been successful in controlling established outbreaks (Whipps 2012). Also, adjustment to tank cleaning measures have previously been demonstrated to be effective for management asymptomatic *M. chelonae* infections in zebrafish stock (Murray, Bauer et al. 2011). These husbandry considerations include tank size, population density, and changes in biofilm cleaning methods (Murray, Bauer et al. 2011). As demonstrated our study, mycobacterial transmission between tank biofilms and zebrafish occur rapidly, emphasizing the need for zebrafish facilities to incorporate these adjustments in husbandry measures. The control of mycobacterial biofilms has already been identified as major challenge in the medical setting, as nosocomial infections due to mycobacterial biofilms have been identified to contaminate water systems and medical equipment reviewed in (Phillips and von Reyn 2001). Also, environmental monitoring including sampling of biofilms, feces, and detritus can be useful for surveillance of *Mycobacterium* spp. pathogenic to zebrafish (Collymore, Crim et al. 2016), as not all infections result in observable signs of disease (Watrall and Kent 2007). Recently, real-time PCR analysis of environmental samples were found to be more sensitive than those of antemortem zebrafish samples for

detecting the presence of mycobacteria in zebrafish facilities (Crim, Lawrence et al. 2017). Sub-clinical infections with *Mycobacterium* spp. such as *M. chelonae* can go unnoticed and be a potential source of non-protocol experimental variation. Biofilm monitoring can assist in detecting *Mycobacterium* spp. in systems before morbid fish are observed and allow for identification and control of potential risk factors. It is also important to note that several species of *Mycobacterium* are ubiquitous in water systems and have not been observed to be pathogenic to zebrafish.

In terms of observable morbidity, the fish in this study showed a range of signs of morbidity and different diagnostic methods allowed for a higher degree of detection of *M. chelonae* infections. First, there were no observed mortalities due to any of the experimental *M. chelonae* infections in this study. This result is not surprising as *M. chelonae* has previously been reported to result in more chronic, low-level infections showing few clinical signs of infection (Watrall and Kent 2007). *In vivo* screening for GFP expression resulted in no difference in prevalence between IP injected fish and orally infected fish; however, IP injected fish resulted in a higher prevalence detected through both liver/spleen culture and histology. This is likely due to IP infections being more severe, as this is not a natural mode of infection and allowing bacteria to surpass natural barriers (e.g., epithelium of the intestinal tract). The difference in prevalence between diagnostic methods follows with oral ingestion infection resulting in fewer detected infections in the liver sample or through the observed histological sections. Results from the fish infected from tank biofilms were similar, as *in vivo* screening resulted in the highest prevalence value compared to liver/spleen culture and PCR when looking at groups of both sexes or only females, this difference did not occur when only comparing males. This difference between males and females could be related to the ovaries as an important location of granuloma

formation. All the female fish infected through tank biofilms had granulomas observed in their ovaries, while males were observed to have GFP expression in their hematopoietic organs, which would influence detection of bacteria in liver/spleen samples.

This study was the first usage of the *in vivo* GFP whole-fish screening used by our group. Differences in detection were observed between this new method compared to histological and liver/spleen culture and PCR methods already established for studies investigating mycobacterial infections in zebrafish. In terms of the sensitivity, the ability to correctly detect positives the *in vivo* GFP screening had a high sensitivity compared to histology. This method was much less specific than histology, because detection was higher in *in vivo* GFP screening fish than histology, and we have no secondary method of diagnostic validation. This could be attributed to additional potential sources of auto-fluorescence within the zebrafish (e.g. gall bladder and degenerating eggs) that may result in false positives, requiring further analyses of bacterial smears or culture. It is important to note that the evaluation of histological sections may not be optimal for detecting a true negative, as only select midline sections were observed and not the entire fish. Future comparison of this new *in vivo* GFP screening method for detecting mycobacterial infections should be carried out and compared with real-time qPCR detection values to gain a better understanding of the specificity of this method. Importantly, it should be emphasized that his method was useful for the purpose of demonstrating the transmission of *M. chelonae* between tank biofilms and zebrafish, which was confirmed by all diagnostic methods (GFP observation, PCR, and histology).

In this study, we have demonstrated that *M. chelonae* can be shed in feces and transmitted to tank biofilms from infected zebrafish, and that zebrafish can naturally acquire *M. chelonae* infections from tank biofilms. Additionally, the demonstration of this mode of

mycobacterial transmission emphasizes other important disease control measures that reduce risk of biofilms forming in zebrafish facilities (e.g. quarantine and “eggs only” policies for fish from an outside facility, quarantine of moribund fish, regular tank cleaning). This transmission of *M. chelonae* between biofilms and zebrafish is also likely true to additional species of *Mycobacterium* as well as other fishes. This study has implications for the control and management of mycobacteriosis in laboratory zebrafish, as well as other fishes.

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6.7 References

Arndt, H., K. Schmidt-Denter, B. Auer and M. Weitere (2003). "Protozoans and biofilms." Fossil and Recent Biofilms: 161-179.

Astrofsky, K. M., M. D. Schrenzel, R. A. Bullis, R. M. Smolowitz and J. G. Fox (2000).

"Diagnosis and management of atypical *Mycobacterium* spp. infections in established laboratory zebrafish (*Brachydanio rerio*) facilities." Comparative Medicine **50**(6): 666-672.

Barker, J. and M. R. Brown (1994). "Trojan horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment." Microbiology **140 (Pt 6)**: 1253-1259.

Belas, R., R. Faloon and A. Hannaford (1995). "Potential applications of molecular biology to the study of fish mycobacteriosis." Ann Rev Fish Dis **5**(133-173).

Beran, V., L. Matlova, L. Dvorska, P. Svastova and I. Pavlik (2006). "Distribution of mycobacteria in clinically healthy ornamental fish and their aquarium environment." Journal of Fish Diseases **29**: 383-393.

Collymore, C., M. J. Crim and C. Lieggi (2016). "Recommendations for Health Monitoring and Reporting for Zebrafish Research Facilities." Zebrafish **13**: S138-S148.

Crim, M., C. Lawrence, R. Livingston, A. Rakitin, S. Hurley and L. Riley (2017). "Comparison of Antemortem and Environmental Samples for Zebrafish Health Monitoring and Quarantine." Journal of the American Association for Laboratory Animal Science **56**(4): 412-424.

Davis, J. M., H. Clay, J. L. Lewis, N. Ghori, P. Herbomel and L. Ramakrishnan (2002). "Real-time visualization of *Mycobacterium*-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos." Immunity **17**(6): 693-702.

Falkinham, J. O. (2009). "Surrounded by mycobacteria: nontuberculous mycobacteria in the human environment." Journal of Applied Microbiology **107**(2): 356-367.

Falkinham, J. O., 3rd, C. D. Norton and M. W. LeChevallier (2001). "Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other Mycobacteria in drinking water distribution systems." Appl Environ Microbiol **67**(3): 1225-1231.

Gaind, N. (2016). "US grants for zebrafish studies on the rise." Nature News & Comment.

Harriff, M. J., L. E. Bermudez and M. L. Kent (2007). "Experimental exposure of zebrafish, *Danio rerio* (Hamilton), to *Mycobacterium marinum* and *Mycobacterium peregrinum* reveals the gastrointestinal tract as the primary route of infection: a potential model for environmental mycobacterial infection." Journal of Fish Diseases **30**(10): 587-600.

Hohn, C. and L. Petrie-Hanson (2013). "Evaluation of visible implant elastomer tags in zebrafish (*Danio rerio*)." Biol Open **2**(12): 1397-1401.

Kent, M. L., S. W. Feist, C. Harper, S. Hoogstraten-Miller, J. Mac Law, J. M. Sanchez-Morgado, R. L. Tanguay, G. E. Sanders, J. M. Spitsbergen and C. M. Whipps (2009). "Recommendations for control of pathogens and infectious diseases in fish research facilities." Comparative Biochemistry and Physiology C-Toxicology & Pharmacology **149**(2): 240-248.

Kent, M. L., Varga, Z. (2012). "Use of Zebrafish in Research and Importance of Health and Husbandry." ILAR **53**(2): 89-94.

Kent, M. L., C. M. Whipps, J. L. Matthews, D. Florio, V. Watral, J. K. Bishop-Stewart, M. Poort and L. Bermudez (2004). "Mycobacteriosis in zebrafish (*Danio rerio*) research facilities." Comparative Biochemistry and Physiology Part C Toxicology and Pharmacology **138**(3): 383-390.

Kent, M. L. S., J. M., Matthews, J. M., Fournie, J. W., Murray, K. N., Westerfield, M. (2012). "Diseases of Zebrafish in Research Facilities." Retrieved July 10, 2014, from <http://zebrafish.org/zirc/health/diseaseManual.php>.

Kent P. T., Kubica, G. P. (1985). Public Health Microbacteriology A Guide for a Level III Laboratory: 146-157.

Lawrence, C. (2007). "The husbandry of zebrafish (*Danio rerio*): A review." Aquaculture **269**(1-4): 1-20.

Mason, T., K. Snell, E. Mittge, E. Melancon, R. Montgomery, M. McFadden, J. Camoriano, M. L. Kent, C. M. Whipps and J. Peirce (2016). "Strategies to Mitigate a *Mycobacterium marinum* Outbreak in a Zebrafish Research Facility." Zebrafish **13**: S77-S87.

Meritet, D. M., D. M. Mulrooney, M. L. Kent and C. V. Lohr (2017). "Development of Quantitative Real-Time PCR Assays for Postmortem Detection of *Mycobacterium* spp. Common in Zebrafish (*Danio rerio*) Research Colonies." Journal of the American Association for Laboratory Animal Science **56**(2): 1-11.

Morales, M., with code developed by the R Development Core Team and with general advice from the R-help listserv community and especially Duncan Murdoch (2012). sciplot: Scientific Graphing Functions for Factorial Designs.

Murray, K. N., J. Bauer, A. Tallen, J. L. Matthews, M. Westerfield and Z. M. Varga (2011). "Characterization and management of asymptomatic *Mycobacterium* infections at the Zebrafish International Resource Center." Journal of the American Association of Laboratory Animal Science **50**(5): 675-679.

Mutoji, K. N. (2011). Investigation into Mechanisms of Mycobacterial Transmission Between Fish. Doctor of Philosophy, University of Louisiana at Lafayette.

Noga, E. J. (2010). Fish Disease: Diagnosis and Treatment. Iowa, USA, Wiley-Blackwell.

Peterson, T. S., J. A. Ferguson, V. G. Watral, K. N. Mutoji, D. G. Ennis and M. L. Kent (2013). "*Paramecium caudatum* enhances transmission and infectivity of *Mycobacterium marinum* and *M. chelonae* in zebrafish *Danio rerio*." Diseases of Aquatic Organisms **106**(3): 229-239.

Phillips, M. S. and C. F. von Reyn (2001). "Nosocomial infections due to nontuberculous mycobacteria." Clinical Infectious Diseases **33**(8): 1363-1374.

R Core Team (2013). R: A language and environment for statistical computing. . Vienna, Austria, R Foundation for Statistical Computing.

Ross, A. J. (1970). Mycobacteriosis among Pacific salmonid fishes. A Symposium on Diseases of Fishes and Shellfishes. S. F. Sniesko. Washington, D.C., American Fisheries Society: 279-283.

Schulze-Robbeke, R., B. Janning and R. Fischeder (1992). "Occurrence of Mycobacteria in Biofilm Samples." Tubercle and Lung Disease **73**(3): 141-144.

Sciarra, J. B., A. T. Tyler and A. Kolb (2014). A gelatin-based diet for oral dosing juvenile to adult zebrafish (*Danio rerio*). Laboratory Animal Science Professional. **June**: 32-35.

Spence, R., G. Gerlach, C. Lawrence and C. Smith (2008). "The behaviour and ecology of the zebrafish, *Danio rerio*." Biological Reviews **83**(1): 13-34.

Volkman, H. E., H. Clay, D. Beery, J. C. Chang, D. R. Sherman and L. Ramakrishnan (2004). "Tuberculous granuloma formation is enhanced by a *Mycobacterium* virulence determinant." PLoS Biol 2(11): e367.

Watrall, V. and M. L. Kent (2007). "Pathogenesis of *Mycobacterium* spp. in zebrafish (*Danio rerio*) from research facilities." Comparative Biochemistry and Physiology Part C Toxicology and Pharmacology **145**(1): 55-60.

Whipps, C. M., W. R. Butler, F. Pourahmad, V. G. Watrall and M. L. Kent (2007). "Molecular systematics support the revival of *Mycobacterium salmoniphilum* (ex Ross 1960) sp. nov., nom.

rev., a species closely related to *Mycobacterium chelonae*." International Journal of Systematic Evolution and Microbiology **57**(Pt 11): 2525-2531.

Whipps, C. M., S. T. Dougan and M. L. Kent (2007). "*Mycobacterium haemophilum* infections of zebrafish (*Danio rerio*) in research facilities." FEMS Microbiol Lett **270**(1): 21-26.

Whipps, C. M., C. Lieggi and R. Wagner (2012). "Mycobacteriosis in Zebrafish Colonies." Ilar Journal **53**(2): 95-105.

Whipps, C. M., J. L. Matthews and M. L. Kent (2008). "Distribution and genetic characterization of *Mycobacterium chelonae* in laboratory zebrafish *Danio rerio*." Diseases of Aquatic Organisms **82**(1): 45-54.

Yanong, R. P., D. B. Poudel and J. O. Falkinham, 3rd (2010). "Association of mycobacteria in recirculating aquaculture systems and mycobacterial disease in fish." Journal of Aquatic Animal Health **22**(4): 219-223.

Zebrafish International Resource Center (ZIRC) Health Services. (2017). "Summary of Submissions to the ZIRC Diagnostic Service 2006-2016." Retrieved October 4, 2017, from https://zebrafish.org/wiki/_media/health/submission/submissions_summary_2006-2016.pdf.

Chapter 7

Investigating transmission of *Mycobacterium* spp. in laboratory zebrafish through live feeds.

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7.1 Abstract

The zebrafish (*Danio rerio*) is a popular vertebrate model organism used in a wide range of research fields. Mycobacteriosis, caused by *Mycobacterium* species is particularly concerning because it is a common disease and is associated with chronic infections and zebrafish mortality and introduces experimental variability which may impact research results. Food sources have been highlighted as a risk factor as live feeds for zebrafish are common and include paramecia (*Paramecium caudatum*), brine shrimp (*Artemia franciscana*), and rotifers (*Branchionus* spp.). Although nutritionally beneficial, live feeds pose a biosecurity risk. In this study we investigate transmission of *Mycobacterium chelonae* and *Mycobacterium marinum* through consumption of live food vectors. We show that mycobacterial infections are transmitted through all three feed vectors. This observation emphasizes the need for live feed vectors to be considered as a source of biosecurity risk. This study is of importance to other beyond the zebrafish community, including those of additional aquatic models.

7.2 Introduction

Currently, mycobacteriosis are an important infectious disease in zebrafish research occurring in approximately 40% of facilities (Kent, Spitsbergen et al. 2012). Disease management recommendations highlight the importance of prevention, as eradication, although effective, is intensive and requires re-derivation of fish strains and complete facility disinfection (Whipps, Lieggi et al. 2012). Despite mycobacteriosis being commonly reported in zebrafish, much remains to be understood regarding the modes of transmission.

The natural route of transmission of mycobacteria in zebrafish is thought to be predominantly through ingestion. This is due to inference from transmission in salmonids, where outbreaks in the 1950s and 1960s were correlated to unpasteurized fish feed (Ross 1970, Belas, Faloon et al. 1995). Mutoji (2011) showed significantly increased infection of *Mycobacterium marinum* in Japanese medaka by infection through feeding mosquito larvae with *M. marinum* in the gastrointestinal tract than feeding cultured mycobacteria alone. Transmission through ingestion has been demonstrated in zebrafish and the intestinal tract was identified as a route of mycobacterial entry (Harriff, Bermudez et al. 2007). Ingestion as a source of natural mycobacterial transmission in zebrafish highlights feed as a potential risk factor.

Live feeds for zebrafish are both common and recommended and include the ciliated protozoan *Paramecium caudatum*, brine shrimp *Artemia franciscana* as well as rotifers *Branchionus* spp. Despite nutritional benefits gained from the usage of these live feeds, live fish feeds have previously been shown to be risk factors for pathogen transmission. In aquaculture, brine shrimp and rotifers are common feeds for larval fish and crustaceans. However, bacteria present in these feeds have been shown to be transmitted to larvae and negatively affect survival and growth of fishes (Gatesoupe 1989). *Artemia* feeds have been shown to be contaminated with *Mycobacterium* spp. (Beran, Matlova et al. 2006), highlighting the risk to laboratory fish culture.

Additionally when *Mycobacterium* spp. are transmitted through a live food vector experimentally, an increase in bacterial virulence has been observed. Mosquito larvae infected with *Mycobacterium* spp. were more infective to Japanese medaka (*Oryzias latipes*) than cultured mycobacteria (Mutoji 2011) and *Mycobacterium* spp. transmitted to zebrafish through the ciliated protozoan *Paramecium caudatum* resulted in a higher prevalence of infections than bacteria transmitted from culture plates alone (Peterson, Ferguson et al. 2013). *Mycobacterium* spp. infected *Acanthamoeba castellanii* were also more infective to zebrafish (Harriff, Bermudez et al. 2007) relative to control fed.

Free-living aquatic protozoans (e.g., *Acanthamoeba* and *Dictyostelium*) have evolved as phagocytic cells, actively preying on bacteria within the air water interface of biofilms (Barker and Brown 1994). These aquatic protozoans inhabit the same environmental niches as mycobacteria, and they digest mycobacteria in food vacuoles. However in some instances, mycobacteria persist. This mycobacterial persistence within free-living aquatic protozoan host has been examined in the amoebae host where an endosymbiotic relationship between the bacteria and the amoebae has been hypothesized (Winiecka-Krusnell and Linder 2001). More than 25 species of *Mycobacterium* shown to have some form of endosymbiotic relationship with free-living amoebae (Steinert, Birkness et al. 1998). Many bacterial traits co-evolved with amoebae in order to resist predation and digestion, including: bacterial encapsulation, toxin secretion, blockage of phagolysosomal fusion, intracellular replication within host (Cosson and Soldati 2008).

Amoebae are thought to act as surrogates for macrophages causing *Mycobacterium* spp. to upregulate virulence genes in the intracellular environment (Harriff, Bermudez et al. 2007). Similar mechanisms may be responsible for the increase in virulence observed in paramecium

and mosquito larvae (Mutoji 2011; Peterson, Ferguson et al. 2013). More work investigating the virulence of *Mycobacterium* spp. when transmitted through a live feed vector is required as live feeds used in zebrafish culture pose a risk factor for enhanced mycobacterial transmission.

The goal of this study is to investigate the virulence of *Mycobacterium* species that commonly infect laboratory zebrafish when they are transmitted through different live food vectors. First an experimental model for the transmission of *Mycobacterium* species to the zebrafish host through live feed vectors was developed, a more natural exposure route than the commonly used intraperitoneal (IP) injection or bath treatment. Next, this experimental model was used to compare differences between the virulence of *Mycobacterium chelonae* (H1E2) and *Mycobacterium marinum* (OSU214) when transmitted to the zebrafish host through three different feed vectors: paramecia (*Paramecium caudatum*), brine shrimp (*Artemia franciscana*), rotifers (*Branchionus plicatilis*), and through ingestion without a vector. Insight from this study can be utilized by the zebrafish research community in order to make recommendations regarding pathogen risk factors associated with the usage of live feeds, directly impacting zebrafish health and welfare.

7.3 Methods

Fish

The fish used for this study were bred and maintained in the zebrafish facility at the SUNY-ESF Center for Integrated Teaching and Research in Aquatic Science. Adult *casper* (*nacre*^{w2/w2}; *roy*^{a9/a9}) zebrafish (n=396; 198 male and 198 female; age = 6 months) lines, originally obtained from the SARL at Oregon State University (Corvallis, OR) and bred for two generations at SUNY-ESF, were utilized in this study. Animals were housed at a density of 6-10 fish/liter in 1.8 L tanks on a timed, flow-through housing system (Aquaneering, San Diego, CA).

The housing system included ultraviolet disinfection of dechlorinated (carbon filter) municipal tap water maintained to pH 7.6, a conductivity of 600-700 us/cm², and a temperature of 28.5°C, and ammonia levels ranging from 0-0.25 ppm. The zebrafish facility maintained a 14:10 light:dark photoperiod. Fish were fed a commercial feed for zebrafish (Gemma, Skretting) twice daily on weekdays and once daily on weekends during periods where they were not being fed a treatment feed. Prior to the experiment, routine cleaning of all equipment (e.g. tanks, lids, baffles, nets, and tubing) consisted of bi-weekly washing and scrubbing in warm water with a new soft sponge and bleaching in 1000 ppm chlorine bleach for 30 minutes, followed by rinsing three times in dechlorinated water and drying. All tanks, lids, and baffles were also autoclaved using a program specified by the tank manufacturer that reaches a temperature of 105-110°C for 15 minutes. During experiments this tank cleaning procedure occurred when the placement of fish in a new tank is mentioned in the methods below. All animal work was approved by the SUNY-ESF Institutional Animal Care and Use Committee, protocol #151001.

Fish were randomly divided into 33, 1.8L tanks (n=12, 6 male and 6 female). Three tanks per treatment were designated: sham artemia, *M. chelonae* artemia, *M. marinum* artemia, sham rotifers, *M. chelonae* rotifers, *M. marinum* rotifers, sham paramecium, *M. chelonae* paramecium, *M. marinum* paramecium, *M. chelonae* no vector, *M. marinum* no vector.

Bacterial culture and growth media

For this study *Mycobacterium chelonae* (H1E2) mutant expressing green fluorescence protein (GFP) provided by M.L. Kent and *Mycobacterium marinum* (OSU214) were used. The *M. chelonae* was maintained at 28-30°C on solid-phase Middlebrook 7H10 agar supplemented with oleic albumin dextrose catalase (OADC) for 5 days. *Mycobacterium marinum* was cultured

at 28-30°C on solid-phase Middlebrook 7H10 agar supplemented with OADC and 60 µM hemin for 10-14 days.

Vector Culture and Dosage Determination

Artemia

For artemia experiments, Grade A Brine Shrimp Eggs were used (Brine Shrimp Direct). Manufacturer instructions were used for preparing artemia hatching water with a salinity of 25 parts per thousand and a pH of 8. Following preparation, the hatching water was autoclaved and cooled to room temperature. Prior to hatching, artemia cysts were disinfected and decapsulated by bleaching. For bleaching, cysts were placed in a mesh bottom container, nested within a glass beaker. The glass beaker was maintained in an ice bath. Cysts were bleached in a 1:1 ratio of Clorox® to hatching water under constant aeration. Bleaching was done until cysts were decapsulated and orange in color. Following bleaching, cysts were rinsed three times with hatching water. Next, rinsed cysts were placed in hatching cones containing hatching water (Aquaneering). Hatching cones were maintained on a bench top with constant overhead lighting at room temperature (25°C).

Initial bacterial incubations were conducted to gain insight into the uptake of *Mycobacterium* spp. by vectors. For initial bacterial incubations with artemia, artemia nauplii were collected and concentrated in a mesh-bottom filter at 24 h post hatching. Concentrated artemia were distributed into sterile petri dishes and incubated at a 3:1 ratio with *M. chelonae* diluted in autoclaved dH₂O to a concentration of 2.7×10^8 cfu/ml using a nephelometer where 1 ml of diluted *M. chelonae* per 3 ml of concentrated artemia (Sensititre). Incubations were done for 4 time-points: 10 minutes, 30 minutes, 1 hour, and 24 hours. Control incubations with no bacteria were also

conducted. Following incubation periods, artemia were filtered and rinsed using a mesh-bottom conical tube and resuspended in autoclaved hatching water.

For imaging, artemia were immobilized using carbonated water (grocery store) and imaged using a Leica inverted compound microscope under both brightfield and fluorescence.

Representative images of artemia from each incubation time-point were taken.

For qPCR, triplicate 200 μ l samples from each time-point were taken from the concentrated and rinsed artemia. An additional 200 μ l sample was taken and set aside so that the number of organisms in the sample could be counted. Artemia were homogenized using a sterile plastic tissue pestle. Homogenized samples were then used for DNA extraction using the MO BIO Laboratories, Inc. UltraClean[®] Microbial DNA Isolation Kit following manufacturer protocol. Following DNA extraction, a real-time qPCR was performed with technical triplicates for each triplicate sample using primer, probes, and protocol previously published (Meritet, Mulrooney et al. 2017). C_t values of serial dilutions of either *M. chelonae* or *M. marinum* were used to create a standard curve and calculate the colony forming units for each sample (Excel 2010, Microsoft).

Paramecium

Starter cultures of paramecia were obtained from Carolina Biological. Paramecia were cultured following previously described directions (ZFIN, 2014, <https://wiki.zfin.org/display/prot/Paramecium+Recipes+for+Large+and+Small+Facilities>). Initial bacterial incubations with paramecia were performed similarly to those previously described above for artemia. With the amendment of paramecia being immobilized for imaging using 0.3 g/L MS222 dropped into rotifer suspension using a sterile plastic pipette until movement of paramecia ceased, imaging was conducted similarly to artemia as well. The qPCR assay was also performed the same as those previously described for the artemia.

Rotifers

A starter culture of 1 million live marine L-type rotifers was obtained from Reed Mariculture Inc. along with a Compact Culture System (CCS) for benchtop-scale culture of the rotifers. Rotifers were cultured following directions from the manufacturer with a 20% daily harvest and feeding of RGComplete (Reed Mariculture, http://apbreed.com/product_compact_culture_system.php). Initial bacterial incubations were performed as previously described for the paramecium, with the amendment of rotifers being evaluated only by qPCR at the 24-h time-point.

Vector Feed Preparation

From the previous qPCR assays, it was determined that 24 hour incubation was optimal for highest bacterial dose. Vectors (artemia, paramecium, rotifers, and bacteria only) were incubated with either *M. chelonae*, *M. marinum*, or no bacteria for 24 hours as described above. Following incubations, vectors were filtered and rinse. Triplicate 200 μ l samples were taken while the remaining concentrated vectors were stored at 4°C. From these samples a DNA extraction followed by qPCR assay was performed as described above to verify the amount of vector required to reach the desired infective dose. This inoculant dose was calculated to determine the amount of concentrated vector needed based on each fish receiving 1.0×10^4 cfu per feed each day for five days (resulting in a total dose of...) at a daily feeding amount of 5% body weight based on an average fish size of 0.4 g.

Gelatin feed (Gelly Belly, Florida Aqua Farms) was prepared as previously described (Sciarra, Tyler et al. 2014) with 10 g of Gelly Belly being dissolved in 20 ml (minus volume of inoculant) of clam juice (grocery store brand) warmed to approximately 50°C. Once the gelatin mix was mixed thoroughly using a flame sterilized metal spatula. The inoculant was then added

to the warmed mixture to and mixed well. Following mixing the entire mixture was spread on a sterile plastic petri dish and allowed to set at 4°C. After the gelatin mix had set, it was minced using a flame-sterilized razor blade and aliquoted into the mass of feed needed for one tank's (N=12) daily feed. Aliquots were stored at -20°C until feeding time. Treatment tanks were fed the treatment feeds for five consecutive days. Aliquoted treatment feeds were briefly thawed from storage in the freezer and then emptied into treatment tanks. Following 30 min of feeding tanks were observed for any excess food, none was noted. Following the 5 treatment feeds, fish were returned to regular feeding and care as listed in the details above.

Live Imaging of *M. chelonae* infected zebrafish

For *M. chelonae* treatments (*M. chelonae* broth, *M. chelonae* artemia, *M. chelonae* paramecium, and *M. chelonae* rotifers) live imaging of fish was done at 4 weeks post-infection. For live-imaging, fish were anaesthetized in 0.15 g/L MS222 and imaged under FITC fluorescence in order to detect GFP expression. If GFP expression was detected, an image was taken and the location of the GFP expression was noted. Following imaging, fish were placed in a recovery tank containing fresh zebrafish system water. Once, recovered, fish were placed in new clean 1.8L tanks and returned to the zebrafish system.

End Analyses

After 8 weeks week of sampling all fish were euthanized by submersion in 0.3 g/L MS222 buffered to a pH of 7.5 for a minimum of 10 minutes following the cessation of opercular movement. For *M. chelonae* treatments (*M. chelonae* broth, *M. chelonae* artemia, *M. chelonae* paramecium, and *M. chelonae* rotifers) euthanized fish were imaged under both brightfield and fluorescence. In the case that no GFP expression was initially detected, a 5 mm by 5 mm window was made on the lateral side of the fish so that internal organs could be observed under FITC

fluorescence for signs of GFP expression. Images were taken when GFP expression was observed with corresponding brightfield images. Samples of any GFP expressing tissue along with liver, and spleen were also taken and stored in 200 µl sterile dH₂O. These samples were further processed with homogenization using a sterile plastic tissue pestle. Next, 900 µl of autoclaved dH₂O was added, followed by vortexing. The vortexed tubes were pelleted and subsequently cultured on MB agar plates. Resulting growth with GFP positive colonies was recorded. Finally, real-time qPCR of the homogenate was conducted using *M. chelonae* specific primers as previously described (Meritet, Mulrooney et al. 2017). Finally, euthanized fish were fixed in Davidson's solution for 48h and then transferred to 70% EtOH for storage.

For *M. marinum* treatments (*M. marinum* broth, *M. marinum* artemia, *M. marinum* paramecium, and *M. marinum* rotifers) euthanized fish were imaged under both brightfield followed by a 5 mm by 5 mm window was made on the lateral side of the fish so that internal organs could be further observed for signs of granulomas. Images were taken for all fish. Samples of any granulomas, liver, and spleen were also taken and stored in 200 µl sterile dH₂O. These samples were further processed as described for the *M. chelonae* treatment fish with the amendment of *M. marinum* specific primers (Meritet, Mulrooney et al. 2017) being used for qPCR. Finally, euthanized fish were fixed in Davidson's solution for 48h and transferred to 70% EtOH for storage. This same process was repeated for control (no bacteria) artemia, paramecium, and rotifer treatment fish with *Mycobacterium* species-specific primers (Meritet, Mulrooney et al. 2017) being used for qPCR.

End Analyses Vector

The quantity of *Mycobacterium* spp. that were taken up by each vector was compared following the collection of real-time qPCR data using R 3.1.0 (R Core Team, 2013) and R Studio

(R Studio, 2012). Prior to statistical analyses the resulting quantity of bacteria following qPCR was calculated by both sample (50 µl DNA extraction product) and also by organism based on counts of organisms in each sample. For each treatment descriptive statistics were obtained using the “psych” package (Revelle, 2014). Data were also checked for normality equal variances using the “stats” package (R Core Team, 2013) and “car” package (Fox and Weisberg, 2011) respectively. Since all data sets were found to have non-normal distributions ($p < 0.05$) and unequal variances ($p < 0.05$) the non-parametric Kruskal-Wallis rank sum test was used to compare percent survival values using the “stats” package (R Core Team, 2013). In the case of a significant result, indicating differences between disinfection treatments, post-hoc tests for pairwise multiple comparisons of the ranked data were performed using the “PMCMR” package (Pohlert, 2015). Data were then visualized as a clustered bar graph using the “sciplot” package (Morales et al., 2012).

End Analyses Fish

For *M. chelonae* and sham infections, analyses of end prevalence values based on three diagnostic techniques (*in vivo* screen for GFP, dissection followed by *in vivo* screen for GFP, and real-time qPCR of liver/spleen samples) was carried out. Prior to statistical comparisons between treatment groups, replicate tanks for the same treatment were compared to see if results could be pooled using a Chi-squared analysis with a Monte Carlo simulation method using 20,000 replications. There was no significant difference between replicate groups thus prevalence data was pooled for each treatment for further analyses. To compare the prevalence values obtained by each diagnostic method between treatments, Fisher’s exact test for count data was used to compare prevalence between infection types using the “stats” package (R Core Team 2013). This comparison was carried out separately between groups of both sexes, males only, and females

only. It should be noted that prevalence via qPCR was not available for sham infection treatments, so sham treatments were excluded from comparisons of this diagnostic method. Prevalence values and statistical results were organized into bar plots using the “sciplot” package (Morales, with code developed by the R Development Core Team et al. 2012).

For *M. marinum* treatment groups, where only qPCR prevalence data was available, treatment groups were compared. Similar to the analyses described above, replicate tank data was compared in order to see if replicate data could be pooled for each treatment group. There was statistical difference between replicate groups, so data was pooled for further analyses. To compare treatments, an ANOVA test was performed using the “stats” package (R Core Team 2013). Tukey post-hoc analyses was conducted using the “agricolae” package (CITE). Prevalence values and statistical results were organized into bar plots using the “sciplot” package (Morales, with code developed by the R Development Core Team et al. 2012).

7.4 Results

Vector Analyses

Both *M. chelonae* and *M. marinum* were taken up by all vectors, and when evaluated, the GFP expression was observed in both artemia and paramecia (Figure 7-1). In the artemia, the *M. chelonae* was observed to be collected in the organism’s gut. In the paramecia, the *M. chelonae* was observed to be taken up by the organism’s vacuoles. Based on real-time qPCR analysis, the amount of both *M. chelonae* and *M. marinum* taken up by each vector was quantified as follows. For *M. chelonae*, there were more bacteria detected in DNA extracts from paramecia, as compared to artemia, at both the 30 minute and 1 hour incubation time point [$F(5)=21.55$, $p=1.3 \times 10^{-5}$] (Figure 7-2, asterisk). However, when corrected for the quantity of bacteria per organism,

there was no difference between vectors [F(5)=0.972, p=0.475]. When comparing incubation time points for each vector individually, artemia had the significantly highest quantity of *M. chelonae* at the 10-minute incubation point (Figure 7-2, plus sign) compared to longer incubations. Similarly, paramecium had the lowest quantity at the 24-hour incubation time point [F(5)=21.55, p=1.3 x 10⁻⁵]. For *M. marinum*, the amount of *M. marinum* quantified from the DNA extraction did not differ significantly between vector organisms at the 24-hour incubation time-point [F(5)=0.972, p=0.475].

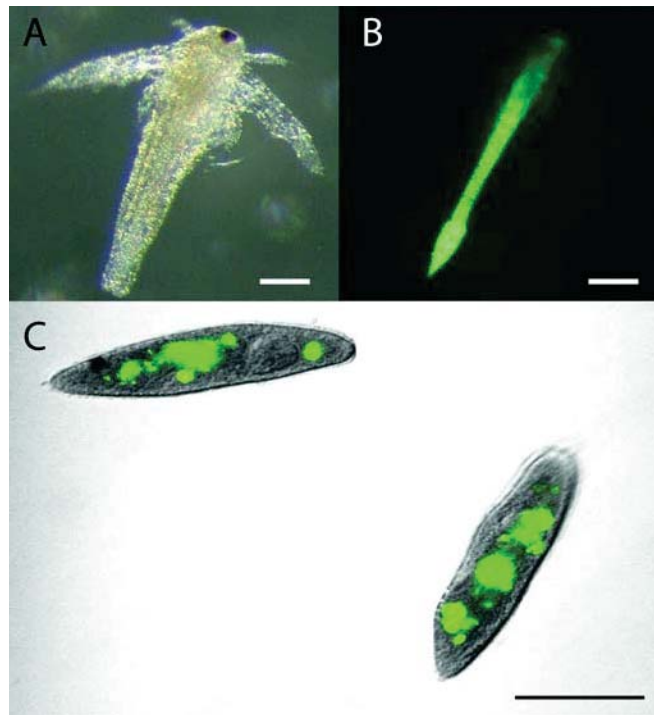


Figure 7-1. Expression of *M. chelonae* GFP mutant in zebrafish feed vectors. (A) Artemia is shown in bright field and under (B) FITC fluorescence where GFP is observed in the gut of the artemia. (C) GFP expression is observed in the paramecium vacuoles. Scale = 100 μ m.

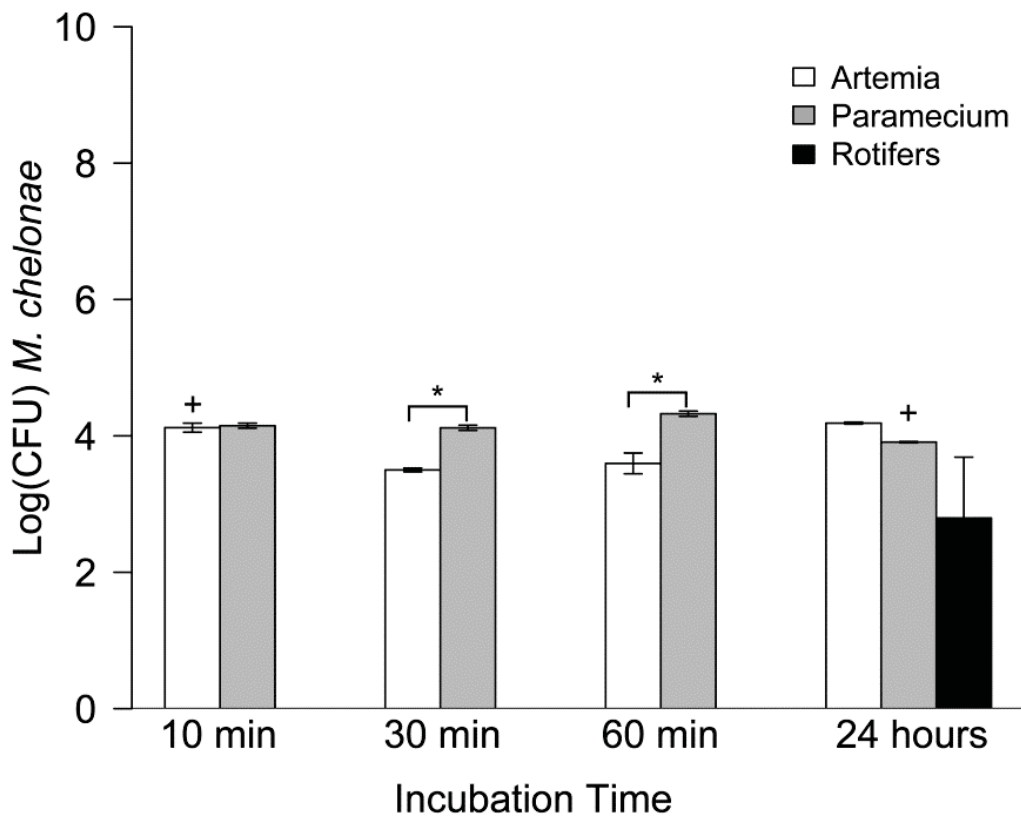


Figure 7-2. Quantity of *M. chelonae* in live feed vectors based on real-time qPCR analyses. Artemia (white bars), paramecium (grey bars), and rotifers (black bars) are shown. Significant differences between vectors at the same time-point is shown (*) and significant differences between time-points for the same vector is shown (+).

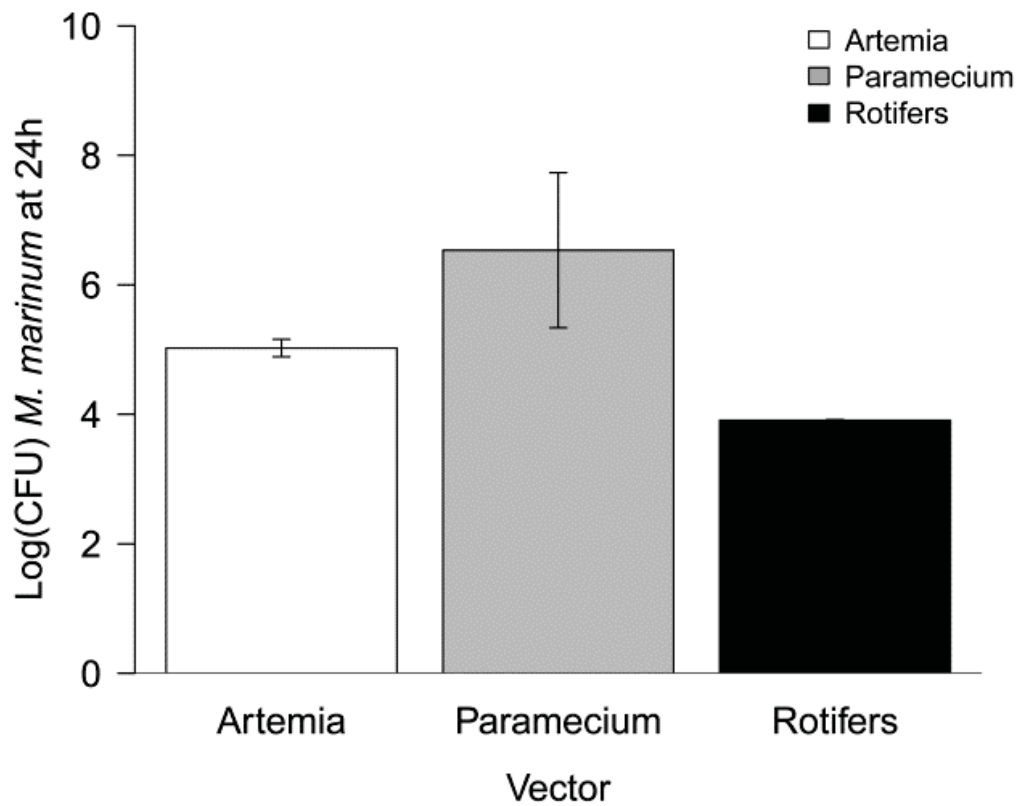


Figure 7-3. Quantity of *M. marinum* in live feed vectors based on real-time qPCR analyses following 24 hours of incubation. Artemia (white bars), paramecium (grey bars), and rotifers (black bars) are shown. No significant differences in bacterial uptake were observed.

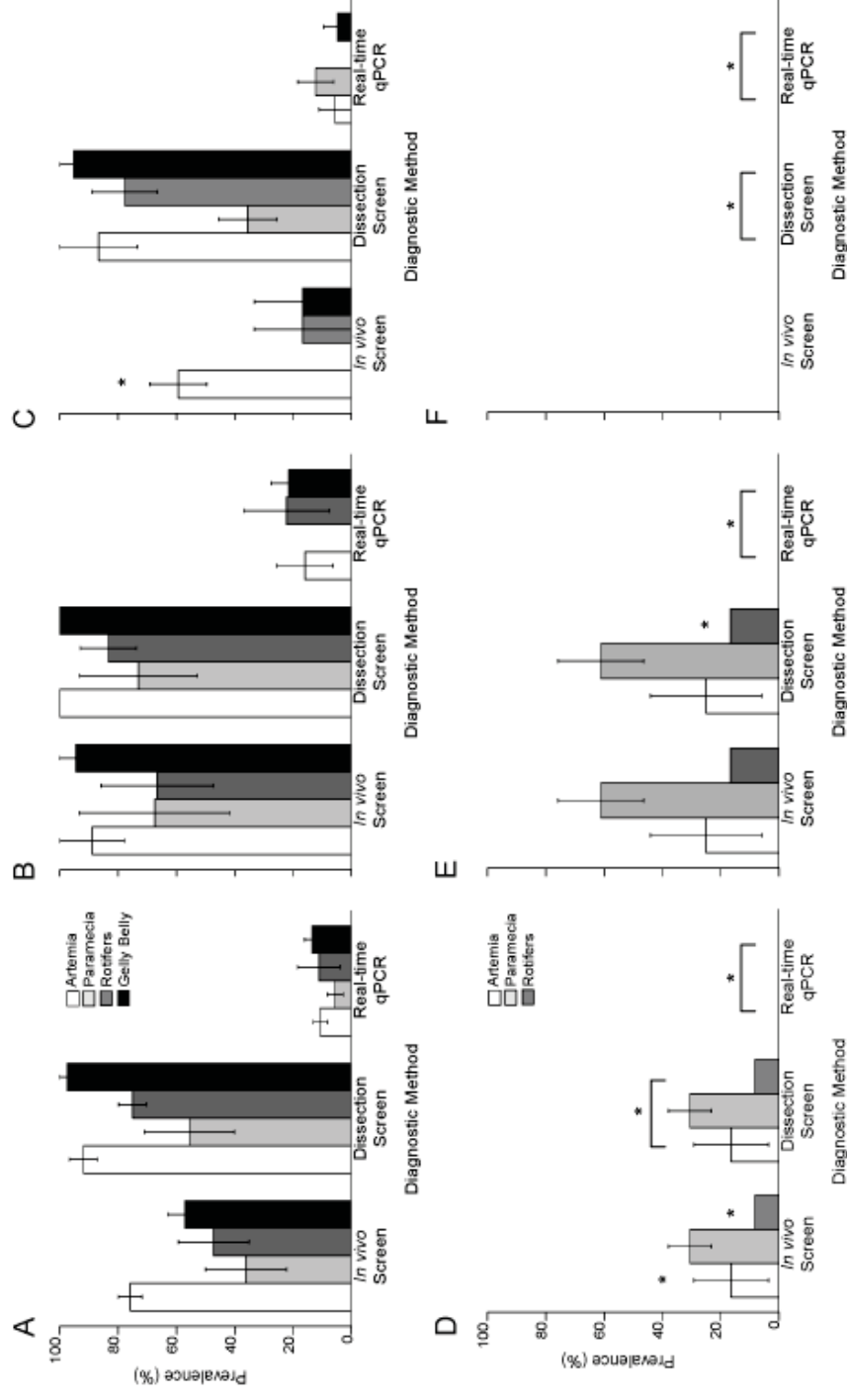


Figure 7-4. End prevalence of infection in zebrafish for *M. chelonae* (A-C) and sham infection (D-F) treatments are shown for each diagnostic technique. (A, D) Treatment groups consisting of both sexes (B, E) only males and (C, F) only females are shown with statistically significant differences in prevalence shown for each diagnostic technique (asterisk).

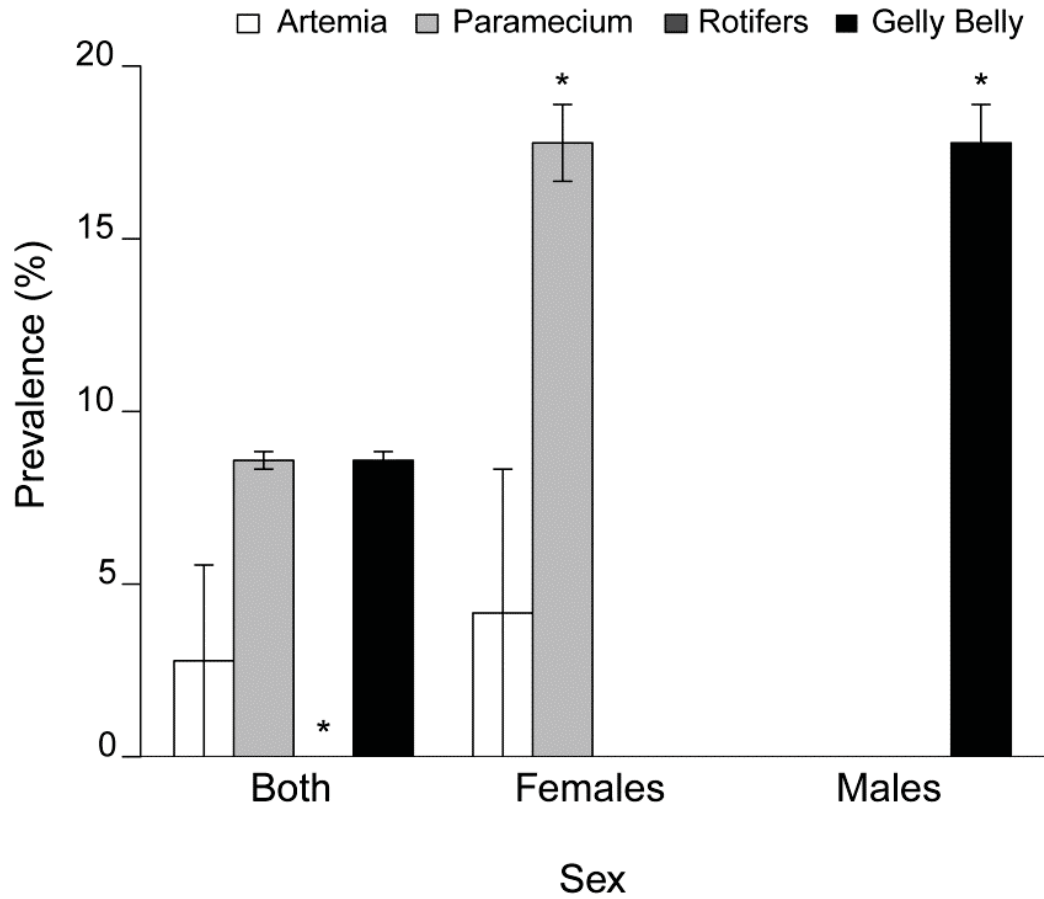


Figure 7-5. End real-time qPCR prevalence values for the detection of *M. marinum* in liver and spleen samples are shown for each treatment group. Differences between treatment groups consisting of both sexes only males and only females are shown (asterisk). Note sham controls were all negative and are not shown.

End Prevalence Analyses

The feeding all three invertebrates to zebrafish, as well as the Gelly Belly diet, resulted in transmission of *M. chelonae*. Overall, there was no significant difference in resulting prevalence in fish fed the different invertebrate vectors based on real-time qPCR testing. When comparing treatment groups consisting of both sexes (Figure 7-4 A and D), differences were observed based on *in vivo* screening for GFP between treatment and sham groups. Rotifer and artemia sham infection prevalence was significantly less than all *M. chelonae* treatment groups ($p=0.003$). Similarly based on dissection followed by *in vivo* screening for GFP, all sham infection treatments had a significantly lower prevalence than *M. chelonae* treatments ($p=3.56 \times 10^{-7}$). No significant difference was observed between any of the treatments based on qPCR prevalence results. When comparing treatment groups consisting of males only (Figure 7-4 B and E) differences were observed based on *in vivo* screening for GFP between treatment and sham groups. The artemia *M. chelonae* treatment resulted in a significantly higher prevalence than the paramecium *M. chelonae* treatment and also the sham treatments ($p=0.00014$). Based on dissection followed by *in vivo* screening for GFP, all sham infection treatments had a significantly lower prevalence than *M. chelonae* treatments ($p=0.249 \times 10^{-7}$). No significant difference was observed between any of the treatments based on qPCR prevalence results. For groups consisting of only females (Figure 7-4 C and F) no significant differences were observed based on *in vivo* screening for GFP between treatment and sham groups. Based on dissection followed by *in vivo* screening for GFP, all sham infection treatments had a significantly lower prevalence than *M. chelonae* treatments ($p=0.024$). No significant difference was observed between any of the treatments based on qPCR prevalence results.

Feeding all three invertebrates to zebrafish, as well as the Gelly Belly diet, resulted in transmission of *M. marinum* to fish except when rotifers were used as vectors. Overall, as no transmission was detected via real-time qPCR analysis for rotifers, all other invertebrates and Gelly Belly feed resulted in significantly higher prevalence following transmission relative to sham feeds. End prevalence values for *M. marinum* treatments are shown for real-time qPCR of liver and spleen samples (Figure 7-5). When comparing treatment groups consisting of both sexes, rotifer prevalence was significantly lower than paramecium and Gelly Belly treatments [F(3)= 9.461, p=0.005]. For females only, paramecium was significantly higher than other treatments [F(3)=15.27, p=0.00113]. Finally, for males only the Gelly Belly prevalence was significantly higher than the other treatments [F(3)=256, p=2.77 x 10⁸].

7.5 Discussion

All vectors readily took up the mycobacteria, which was not surprising as the uptake of bacteria in live feed vectors has been demonstrated for other pathogens (Rombaut, Dhert et al. 1999, Sahul Hameed and Balasubramanian 2000) and in previous studies looking at *Mycobacterium* spp. (Harriff, Bermudez et al. 2007, Peterson, Ferguson et al. 2013). No significant difference was observed between vectors in terms of the quantity of bacteria taken up. This study incubated feed vectors with a high dose of mycobacterial inoculant, and differential uptake of bacteria between vectors may occur with a lower dose of inoculant. We also only looked at uptake over a maximum period of 24 hours, and continued bacterial uptake or elimination cannot be ruled out. In terms of management insight, it is important to note that artemia do not take up bacteria until feeding by mouth begins at approximately 24 hours post-hatch. Thus, the use of younger artemia may help prevent vector transmission of pathogens.

The observed virulence of mycobacterial infections through different methods resulted in variation in the prevalence of *Mycobacterium* spp. infections depending on the end point (e.g. *in vivo* screening for GFP vs. liver and spleen qPCR). We also observed some GFP expression in sham female fish that could be due to auto-fluorescence in the ovary due to degenerating eggs. However when smears were prepared from these tissues, no GFP rods were observed, and could be confidently categorized as uninfected. This study was the second usage of the *in vivo* GFP whole-fish screening used by our group. Similar to our previous study (unpublished), we found that the *in vivo* GFP screening was high in sensitivity, but low in specificity when compared to a gold standard like qPCR. This low specificity can be attributed to additional potential sources of auto-fluorescence within the zebrafish (e.g. gall bladder and degenerating eggs) that may cause false positives. These can be examined further for the presence of GFP rods, to verify the presence or absence of bacteria. Despite the low specificity of the *in vivo* GFP screening method, it is important to emphasize that we detected transmission of mycobacteria from live feeds to zebrafish for all vectors examined using our most specific test, PCR.

Following end-analyses of *M. chelonae* treated fish, no significant differences were observed for any of the diagnostic methods when considering groups composed of both sexes or females. When only considering males, *in vivo* screening for GFP resulted in a significantly higher prevalence in artemia *M. chelonae* treated fish. End real-time qPCR analyses of *M. marinum* treated fish resulted in rotifer *M. marinum* treated fish having the no prevalence for groups of both sexes, Gelly Belly *M. marinum* treatment resulting in the highest prevalence among males, and paramecium *M. marinum* treatment resulting in the highest prevalence among females. Thus no clear trend in virulence between vector feed treatment was observed.

Previous studies (Harriff, Bermudez et al. 2007, Peterson, Ferguson et al. 2013) have evaluated the influence of feed vectors separately, and this is the first study that compared virulence following treatment of multiple live-feed types. However, previous studies have found transmission through a live feed vector resulted in an increased virulence compared to treatment with culture (no live feed). We did not observe this increased virulence that could be due to several possible reasons. First with regard to dose, adult zebrafish in a previous study (Peterson, Ferguson et al. 2013) were fed an experimental dose of *M. chelonae* and *M. marinum* for 14 days compared the 5 days of feeding used in this study. A longer treatment period or higher dose may result in more severe infections and greater difference between feed vectors. Second in Peterson et al. (2013), end point analyses consisted of histological analyses, thus future histological analyses will help to further inform mycobacterial prevalence and infection severity in these fish. Third, we used exclusively adult *casper* (*nacre*^{w2/w2}; *roy*^{a9/a9}) zebrafish. Differences may occur between wild-type and mutant fish, as well as zebrafish at different developmental time-points.

Although no differences were observed between vector feeds used in this study, we did observe mycobacterial transmission through live feed vectors commonly used in zebrafish facilities. This observation emphasized the need for live feed vectors to be considered as a source of biosecurity risk. Future studies should be conducted to increase our understanding of the role live feeds play in mycobacterial transmission within zebrafish facilities, as live feeds have been shown to be a beneficial nutrition for zebrafish culture. These future studies, in addition to this one, will help not only zebrafish research facilities, but also those of additional aquatic models (i.e., Japanese medaka, swordtails, killifish, and Mexican cavefish).

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7.7 References

- Barker, J. and M. R. Brown (1994). "Trojan horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment." Microbiology **140** (Pt 6): 1253-1259.
- Belas, R., R. Faloon and A. Hannaford (1995). "Potential applications of molecular biology to the study of fish mycobacteriosis." Annual Review of Fish Diseases **5**(133-173).
- Beran, V., L. Matlova, L. Dvorska, P. Svastova and I. Pavlik (2006). "Distribution of mycobacteria in clinically healthy ornamental fish and their aquarium environment." Journal of Fish Diseases **29**(7): 383-393.
- Cosson, P. and T. Soldati (2008). "Eat, kill or die: when amoeba meets bacteria." Current Opinion in Microbiology **11**(3): 271-276.

Gatesoupe, F. J. (1989). Further advances in the nutritional and antibacterial treatments of rotifers as food for turbot larvae, *Scophthalmus maximus L.* Bredene, Belgium, European Aquaculture Society.

Harriff, M. J., L. E. Bermudez and M. L. Kent (2007). "Experimental exposure of zebrafish, *Danio rerio* (Hamilton), to *Mycobacterium marinum* and *Mycobacterium peregrinum* reveals the gastrointestinal tract as the primary route of infection: a potential model for environmental mycobacterial infection." Journal of Fish Diseases **30**(10): 587-600.

Kent, M. L., J. M. Spitsbergen, J. M. Matthews, J. W. Fournie, K. N. Murray and M. Westerfield. (2012). "Diseases of Zebrafish in research facilities." Retrieved 10/25/16, 2016.

Meritet, D. M., D. M. Mulrooney, M. L. Kent and C. V. Lohr (2017). "Development of Quantitative Real-Time PCR Assays for Postmortem Detection of *Mycobacterium* spp. Common in Zebrafish (*Danio rerio*) Research Colonies." Journal of the American Association of Laboratory Animal Science **56**(2): 131-141.

Morales, M., with code developed by the R Development Core Team and with general advice from the R-help listserv community and especially Duncan Murdoch (2012). sciplot: Scientific Graphing Functions for Factorial Designs.

Mutoji, K. N. (2011). Investigation into Mechanisms of Mycobacterial Transmission Between Fish. Doctor of Philosophy, University of Louisiana at Lafayette.

Peterson, T. S., J. A. Ferguson, V. G. Watral, K. N. Mutoji, D. G. Ennis and M. L. Kent (2013). "*Paramecium caudatum* enhances transmission and infectivity of *Mycobacterium marinum* and *M. chelonae* in zebrafish *Danio rerio*." Diseases of Aquatic Organisms **106**(3): 229-239.

R Core Team (2013). R: A language and environment for statistical computing. . Vienna, Austria, R Foundation for Statistical Computing.

Rombaut, G., P. Dhert, J. Vandenberghe, L. Verschuere, P. Sorgeloos and W. Verstraete (1999). "Selection of bacteria enhancing the growth rate of axenically hatched rotifers (*Branchionus plicatilis*)." Aquaculture **176**(3-4): 195-207.

Ross, A. J. (1970). Mycobacteriosis among Pacific salmonid fishes. A Symposium on Diseases of Fishes and Shellfishes. S. F. Sniesko. Washington, D.C., American Fisheries Society: 279-283.

Sahul Hameed, A. S. and G. Balasubramanian (2000). "Antibiotic resistance in bacteria isolated from *Artemia* nauplii and efficacy of formaldehyde to control bacterial load." Aquaculture **183**(195-205).

Sciarra, J. B., A. T. Tyler and A. Kolb (2014). "A gelatin-based diet for oral dosing juvenile to adult zebrafish (*Danio rerio*)." Laboratory Animal Science Professional **June**: 32-35.

Steinert, M., K. Birkness, E. White, B. Fields and F. Quinn (1998). "*Mycobacterium avium* bacilli grow saprozoically in coculture with *Acanthamoeba polyphaga* and survive within cyst walls." Applied and Environmental Microbiology **64**(6): 2256-2261.

Whipps, C. M., C. Lieggi and R. Wagner (2012). "Mycobacteriosis in Zebrafish Colonies." ILAR Journal **53**(2): 95-105.

Winiecka-Krusnell, J. and E. Linder (2001). "Bacterial infections of free-living amoebae." Research in Microbiology **152**(7): 613-619.

Chapter 8: Conclusions

8.1 Overview

The general goal of this thesis was to investigate mycobacteriosis in laboratory zebrafish in order to increase our understanding of this disease with the intention of influencing control and management practices. As mycobacteriosis is the second most common pathogen affecting zebrafish, disease control is a priority within the zebrafish research community. This thesis has several aims that contribute towards improved disease management measures.

First I aimed to investigate disease prevention through embryo disinfection. I evaluated the effectiveness of disinfectants commonly used for embryo disinfection in the aquaculture setting, but with unknown effectiveness against the species of *Mycobacterium* found in zebrafish. Once I identified povidone-iodine as an effective disinfectant against *Mycobacterium* spp. *in vitro*, I evaluated the treatments effect on zebrafish embryo health by conducting a subsequent *in vivo* study.

Second, I aimed to examine the efficacy of antibiotic treatment against mycobacteriosis in zebrafish, as antibiotics are the established treatment in mycobacterial infections in other animals. I first was able to identify tigecycline and clarithromycin as key drug candidates as they were effective against preventing mycobacterial growth *in vitro*. I was then able to test both the tolerance and efficacy of these treatments in adult zebrafish, where treatments were well tolerated and resulted in a decreased severity in established mycobacterial infections.

Finally, I aimed to examine natural modes of transmission of mycobacteria in zebrafish. I first examined transmission between tank biofilms and zebrafish and demonstrated the role mycobacterial biofilms play as both a reservoir for and source of *Mycobacterium* spp. in zebrafish tanks. Finally, I examined the role that live feeds play as vectors for mycobacterial

transmission to zebrafish. I demonstrated that common zebrafish feeds are able to transmit *Mycobacterium* spp. to zebrafish. Altogether, these studies contribute to our current knowledge of mycobacterial infections in laboratory zebrafish and help to inform disease management. These results are also of use to other fish species as well.

8.2 Additional Considerations

Through these studies, three underlying considerations were identified and merit additional discussion. While these considerations were not directly tested in this thesis, they were noted, and along with results from recent literature contributions, should be considered in future research that investigates mycobacteriosis in laboratory zebrafish.

The first consideration is the importance of the reporting of standard conditions and reagent preparation. This was first identified in this thesis through the research aim investigating disinfection. As has previously been identified, many factors influence the effectiveness of disinfectants including: temperature, time of contact, pH, concentration as well as the presence of organic matter (Mainous and Smith, 2005). In the context of a zebrafish facility, environmental conditions (e.g., temperature, lighting) may impact the integrity of chemicals. Current long-term storage practices for disinfectants may not be appropriate and alternative measures are necessary (e.g., refrigeration). We identified that the preparation of working solutions of disinfectants influences the effectiveness of treatment, as working solutions of povidone iodine had the iodine concentration diminish over 24 hours. We found that some users of iodine disinfectants did not find their treatments as effective at preventing *Mycobacterium* spp. growth (Mason et al., 2016); however, details regarding the source, storage, and preparation of the working solutions were not reported and could have caused this discrepancy. In general within the zebrafish community, the importance of standardization and reporting in zebrafish studies in order to conduct repeatable

and comparable studies (Collymore, Crim et al. 2016, Goodwin, Karp et al. 2016, Watts, Lawrence et al. 2016) cannot be overemphasized. It is prudent that zebrafish research includes the reporting of the health status of fish so that implications of background infections can be taken into account.

The second consideration is differences between mycobacterial infection prevalence and severity between male and female zebrafish. I had decided to conduct additional analyses comparing sexes, as sex as a biological variable (SABV) is a recent concern highlighted by the National Institutes of Health (NIH) (Tannenbaum, Schwarz et al. 2016). I also saw SABV as potentially informative for disease management in zebrafish. This initiative turned out to be important as during the *in vivo* antibiotic study (Chapter 5), I observed differences in mycobacterial prevalence and severity between male and female fish. I also subsequently observed similar differences in the biofilm transmission study (Chapter 6) and the vector transmission project (Chapter 7). In all of these studies I observed that experimental mycobacterial infections were both more prevalent and severe in female zebrafish, with the majority of granulomas being observed in the ovaries. Mycobacteria infections have been previously discussed to target the ovaries (Kent, Whipps et al. 2004, Whipps, Matthews et al. 2008) and mycobacteriosis is commonly found in zebrafish with widespread chronic inflammation. Previous studies discuss the association between mycobacterial infections and Egg Associated Inflammation and Fibroplasia (EAIF), a common condition in laboratory zebrafish (Kent, Whipps et al. 2004). As EAIF occurs commonly with no pathogen present, it is thought that EAIF provides a macrophage-rich environment within the ovaries for mycobacterial infections to be established (Kent, Whipps et al. 2004, Kent, Watral et al. 2016). These results support further investigation of SABV in zebrafish mycobacteriosis. Understanding SABV in

mycobacteriosis will benefit those interested in improving disease control and management within zebrafish but it will also help inform studies studying mycobacterial disease using the zebrafish model for biomedical research.

The final consideration, is the choice of diagnostic end-point for determining mycobacterial prevalence. In our studies we looked at a variety of end-points including histology, *in vivo* GFP screening before and after dissection, liver and spleen culture, and liver and spleen real-time qPCR. These different end points resulted in variable prevalence values. This difference was more pronounced in fish infected through a more natural mode of infection compared to IP injected fish. For example, in the biofilm transmission study (Chapter 6), *in vivo* GFP screening resulted in a higher prevalence compared to histology for oral feed infected fish. Whereas this difference between diagnostic methods was not observed in IP injection fish. Both modes of infection still resulted in a lower prevalence through liver culture. Previous studies have found a high agreement in prevalence between culture and histology for diagnosing mycobacterial infections in zebrafish (Whipps, Matthews et al. 2008). Depending on the severity of mycobacterial infections, agreement between diagnostic methods may vary. More severe infections would result in a greater probability of detecting mycobacteria in the haematopoietic organs or through midline histological sections. Less severe infections, particularly those isolated in the ovaries, may not be detected by histology or haematopoietic organ culture/PCR, but may be observed through *in vivo* GFP screening. Additionally, the timing sampling in relation to the infection course may impact detection. The progression of granuloma pathology and growth of mycobacteria within the zebrafish is both time and dose dependent, as higher doses and/or longer incubations result in more severe infections with a more detectable mycobacteria (Swaim, Connolly et al. 2006). Also, little is known about the natural mycobacterial infection clearance in

zebrafish, which has been previously attributed to low bacterial counts in experimentally infected fish (Swaim, Connolly et al. 2006). More research is needed to compare diagnostic methods across modes of infection and timing of experimental end-points to better understand detection of mycobacteriosis in zebrafish.

For Chapters 6 and 7, we started using the *in vivo* GFP screening method in order to detect mycobacterial prevalence *in vivo*. While we experienced issues with the specificity of this method, it was still useful for the purpose of our studies tracking transmission of mycobacteria infections to zebrafish as we were able to easily detect infections in zebrafish without needing to euthanize the fish or utilize more expensive or time consuming diagnostic methods. This *in vivo* GFP method work well for initially detecting transmission of mycobacteria; however, more specific methods (e.g., real-time qPCR) may be more appropriate in other situations where a more specific diagnosis is required. Further evaluation of this *in vivo* GFP method should be done to improve the specificity of this method.

The need for further understanding of mycobacterial infections within laboratory zebrafish research facilities contributed to the broad nature of the studies presented in this thesis. Results from these studies have contributed to what we understand about the prevention, treatment, and transmission of mycobacteriosis in zebrafish. Protocols for embryo disinfection and antibiotic treatment of zebrafish were developed, as well as, natural disease transmission models to further this understanding. Results from these studies help to inform the zebrafish community, emphasizing the importance of proper husbandry techniques, particularly disease control and management measures as a means to prevent or control mycobacteriosis in these facilities. While it may not be possible to entirely eliminate mycobacteria from laboratory

zebrafish facilities, results from this dissertation demonstrate measures can be taken to control and minimize infection risks.

8.3 References

Collymore, C., M. J. Crim and C. Lieggi (2016). "Recommendations for Health Monitoring and Reporting for Zebrafish Research Facilities." Zebrafish **13 Suppl 1**: S138-148.

Goodwin, N., N. A. Karp, S. Blackledge, B. Clark, R. Keeble, C. Kovacs, K. N. Murray, M. Price, P. Thompson and J. Bussell (2016). "Standardized Welfare Terms for the Zebrafish Community." Zebrafish **13 Suppl 1**: S164-168.

Kent, M. L., V. G. Watral, N. S. Kirchoff, S. T. Spagnoli and T. J. Sharpton (2016). "Effects of Subclinical *Mycobacterium chelonae* Infections on Fecundity and Embryo Survival in Zebrafish." Zebrafish **13 Suppl 1**: S88-95.

Kent, M. L., C. M. Whipps, J. L. Matthews, D. Florio, V. Watral, J. K. Bishop-Stewart, M. Poort and L. Bermudez (2004). "Mycobacteriosis in zebrafish (*Danio rerio*) research facilities." Comparative Biochemistry and Physiology C-Toxicology & Pharmacology **138**(3): 383-390.

Swaim, L. E., L. E. Connolly, H. E. Volkman, O. Humbert, D. E. Born and L. Ramakrishnan (2006). "*Mycobacterium marinum* infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity." Infection and Immunity **74**(11): 6108-6117.

Tannenbaum, C., J. M. Schwarz, J. A. Clayton, G. J. de Vries and C. Sullivan (2016).

"Evaluating sex as a biological variable in preclinical research: the devil in the details." Biology of Sex Differences **7**: 13.

Watts, S. A., C. Lawrence, M. Powell and L. R. D'Abramo (2016). "The Vital Relationship Between Nutrition and Health in Zebrafish." Zebrafish **13**(S1): S72-S76.

Whipps, C. M., J. L. Matthews and M. L. Kent (2008). "Distribution and genetic characterization of *Mycobacterium chelonae* in laboratory zebrafish *Danio rerio*." Diseases of Aquatic Organisms **82**(1): 45-54.

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PUBLICATIONS

Refereed Journal Articles

Rand, T.G., **Chang, C.T.**, McMullin, D.R., and J.D. Miller. (2017) Inflammation-associated gene expression in RAW 264.7 macrophages induced by toxins from fungi common on damp building materials. *Toxicology In Vitro* 43: 16-20.

Chang, C.T., Doerr, K.M., and Whipps, C.M. (2017) Antibiotic treatment of zebrafish mycobacteriosis: tolerance and efficacy of treatment with tigecycline and clarithromycin. *Journal of Fish Diseases*. DOI: 10.1111/jfd.12619

Chang, C.T., Amack, J.D., and Whipps, C.M. (2016) Zebrafish embryo disinfection with povidone iodine: evaluating an alternative to chlorine bleach. *Zebrafish* 13(S1): S96-S101.

Chang, C.T., Colicino, E.G., DiPaola, E.J., Al-Hasnawi, H. J., and Whipps, C.M. (2015) Evaluating the effectiveness of common disinfectants at preventing the propagation of *Mycobacterium* spp. isolated from zebrafish. *Comparative Biochemistry and Physiology – Part C: Toxicology and Pharmacology* 178: 45-50.

Chang, C. T., and Whipps, C.M. (2015) Activity of antibiotics against *Mycobacterium* spp. commonly found in laboratory zebrafish. *Journal of Aquatic Animal Health* 27 (2): 88-95.

Chang, C.T., and Franz-Odenaal, T.A. (2014) Perturbing the developing skull: Using laser ablation to investigate the robustness of the infraorbital bones in the zebrafish (*Danio rerio*). *BMC Developmental Biology* 14(44): 1-13.

Chang, C., and Franz-Odenaal, T.A. (2014) The zebrafish infraorbital bones: a descriptive study. *Zebrafish* 11(1): 50-56.

AWARDS & HONORS

2017 Spotlight on Student Research First Place Presentation, SUNY-ESF (\$100 USD)
2017 Best Student Paper, American Fisheries Society Fish Health Section (\$200)
2017 Best Student Poster, American Fisheries Society Fish Health Section (\$200)
2017 Snieszko Student Travel Award, American Fisheries Society (\$674 USD)
2017 Alumni Association Memorial Scholarship, SUNY-ESF (\$1,200 USD)
2017 Graduate Student Travel Grant, SUNY-ESF (\$500 USD)
2017 Tuition Scholarship, SUNY-ESF
2016 Postgraduate Scholarship – Doctoral Program, NSERC (\$42,000 CAD)
2016 Alumni Association Memorial Scholarship, SUNY-ESF (\$1,200 USD)
2016 Graduate Student Travel Grant, SUNY-ESF (\$500 USD)
2016 Tuition Scholarship, SUNY-ESF
2015 Fish Health Section Best Student Paper, American Fisheries Society (\$100 USD)
2015 Snieszko Student Travel Award, American Fisheries Society (\$565 USD)
2015 Graduate Student Association Travel Grant, SUNY-ESF (\$250 USD)
2015 Spotlight on Student Research First Place Presentation, SUNY-ESF (\$150 USD)
2015 3-Minute Thesis Competition Bracket Winner, Syracuse University
2015 Tuition Scholarship, SUNY-ESF
2014 Tuition Scholarship, SUNY-ESF
2012-2013 Faculty of Graduate Studies and Research Award, Saint Mary's University
2011-2013 Faculty of Graduate Studies and Research Fellow, Saint Mary's University
2011 Ontario Graduate Scholarship, Government of Ontario (declined)

CONFERENCE ACTIVITY

April 25, 2017. SUNY-ESF Spotlight on Student Research, Syracuse, NY. Can strain typing help us understand *Mycobacterium marinum* outbreaks at zebrafish research facilities? **Chang, C.T.**, Clemons, B.M., Whipps, C.M [Poster]

April 3, 2017. American Fisheries Society Fish Health Section / Great Lakes Fishery Consortium Fish Health Committee Joint Meeting, Lansing, MI. Tolerance and efficacy of tigecycline and clarithromycin for the treatment of mycobacteriosis in laboratory zebrafish. **Chang, C.T.**, Doerr, K.M., Whipps, C.M [Oral]

April 2, 2017. American Fisheries Society Fish Health Section / Great Lakes Fishery Consortium Fish Health Committee Joint Meeting, Lansing, MI. Can strain typing help us understand *Mycobacterium marinum* outbreaks at zebrafish research facilities? **Chang, C.T.**, Clemons, B.M., Whipps, C.M [Poster]

February 21, 2017. Aquaculture America, San Antonio, TX. Tolerance and efficacy of antibiotic treatments of zebrafish mycobacteriosis. **Chang, C.T.**, Doerr, K.M., Whipps, C.M [Oral]

December 9, 2016. Mid-Atlantic Regional Zebrafish Meeting, National Institutes of Health, Bethesda, MD. Preventing mycobacteriosis in zebrafish colonies through embryo disinfection. **Chang, C.T.**, Whipps, C.M. [Poster]

April 19, 2016. SUNY-ESF Spotlight on Student Research, Syracuse, NY. Antibiotic treatment of zebrafish mycobacteriosis: efficacy of treatment with tigecycline and clarithromycin. **Chang, C.T.**, Doerr, K.M., Whipps, C.M. [Poster]

April 19, 2016. SUNY-ESF Spotlight on Student Research, Syracuse, NY. Investigating tolerance, growth, and fecundity of laboratory zebrafish (*Danio rerio*) clarithromycin and tigecycline antibiotics. **Doerr, K.M.**, **Chang, C.T.**, Whipps, C.M. [Poster]

April 19, 2016. SUNY-ESF Spotlight on Student Research, Syracuse, NY. Investigating transmission of *Mycobacterium* spp. from experimentally infected zebrafish (*Danio rerio*) to tank biofilms. **Adler, A.**, **Chang, C.T.**, Whipps, C.M. [Poster]

February 23-26, 2016. Aquaculture America, Las Vegas, NV. Zebrafish embryo disinfection with povidone iodine: evaluating an alternative to chlorine bleach. **Chang, C.T.**, Amack, J.D., Whipps, C.M. [Oral]

July 13-15, 2015. American Fisheries Society Fish Health Section Annual Meeting, Ithaca, NY. Toxicity of iodine to zebrafish embryos, an alternative to chlorine disinfection for preventing mycobacterial spread. **Chang, C.T.**, Amack, J.D., Whipps, C.M. [Oral]

April 15-16, 2015. SUNY-ESF Spotlight on Student Research, Syracuse, NY. Investigating the effectiveness of disinfectant treatments for inhibiting the growth of *Mycobacterium* spp. isolated from laboratory zebrafish (*Danio rerio*). **Chang, C.T.**, Colicino, E.G., DiPaola, E.J., Chen, T., Al-Hasnawi, H.J., Whipps, C.M. [Poster]

April 15-16, 2015. SUNY-ESF Spotlight on Student Research, Syracuse, NY. Strain typing *Mycobacterium marinum* from outbreaks at zebrafish research facilities. **Clemons, B.M.**, **Chang, C.T.**, Whipps, C.M. [Poster]

December 13-18, 2014. The 7th Aquatic Animal Models of Human Disease Conference, Austin, TX.) Investigating the effectiveness of disinfectant treatments for inhibiting the growth of *Mycobacterium* spp. isolated from laboratory zebrafish (*Danio rerio*). **Chang, C.T.**, Whipps, C.M. [Poster]

August 31-September 5, 2014. Seventh International Symposium on Aquatic Animal Health, Portland, OR. Activity of antibiotics against *Mycobacterium* spp. commonly found in zebrafish. **Chang, C.T.**, Whipps, C.M. [Oral]

May 25-29, 2014. Genomes to Biomes, Montreal, QC. Skull development in zebrafish: tracking skeletogenic condensations. **Chang, C.**, Jabalee, J., Franz-Odendaal, T.A. [Poster]

April 15-16, 2014. SUNY-ESF Spotlight on Student Research, Syracuse, NY. Fishy infections: investigating mycobacteriosis in laboratory zebrafish. **Chang, C.T.**, Whipps, C.M. [Poster]

April 15-16, 2014. SUNY-ESF Spotlight on Student Research, Syracuse, NY. Analysis of disinfectants on *Mycobacterium* spp. Colicino, E.G., DiPaolo, E.J., **Chang, C.T.**, Whipps, C.M. [Poster]

April 15-16, 2014. SUNY-ESF Spotlight on Student Research, Syracuse, NY. The effect of bleach and hydrogen peroxide on *Mycobacterium* species. DiPaolo, E.J., Colicino, E.G., **Chang, C.T.**, Whipps, C.M. [Poster]

July 9-13, 2013. The 8th European Zebrafish Meeting, Barcelona, Spain. Testing developmental resilience through targeted laser ablation of zebrafish infraorbital bones. **Chang, C.**, Franz-Odendaal, T.A. [Poster]

May 13-17, 2013. Canadian Society of Zoologists Annual Meeting, Guelph, ON. Perturbing the developing skull: Using laser ablation to investigate the robustness of the infraorbital bones. **Chang, C.**, Franz-Odendaal, T.A. [Oral]

January 3-7, 2013. Society of Integrative and Comparative Biology Annual Meeting, San Francisco, CA. Condensations to mineralizations: the development of the zebrafish (*Danio rerio*) infraorbital bones. **Chang, C.**, Franz-Odendaal, T.A. [Poster]

May 7-11, 2012. Canadian Society of Zoologists Annual Meeting, Sackville, NS. Investigating the developmental morphogenesis of the zebrafish (*Danio rerio*) infraorbital bones. **Chang, C.**, Franz-Odendaal, T.A. [Poster]

TEACHING EXPERIENCE

State University of New York College of Environmental Science and Forestry

Microbial Pathogenesis & Host Defenses Seminar, Co-Instructor (Spring 2016)

Research Problems in Biotechnology, Co-Supervisor (Spring 2014-2017, Fall 2016)

Internship in Biotechnology, Co-Supervisor (Summer 2015, Fall 2016)

Research Apprenticeships in Biotechnology, Co-Supervisor (Fall 2015)

Saint Mary's University

Human Organ Systems, Teaching Assistant (Fall 2012, Fall 2013)

Animal Tissues, Teaching Assistant (Spring 2013)

Molecular and Cell Biology, Teaching Assistant (Fall 2011, Fall 2012)

Organismal and Ecological Biology, Teaching Assistant (Spring 2012)

Animal Development, Teaching Assistant (Fall 2011)

RESEARCH EXPERIENCE

Research Project Assistant: Whipps Fish and Wildlife Disease Lab, Department of Environment and Forest Biology, SUNY-ESF – State University of New York College of Environmental Science and Forestry, Jan 2014-present

- Investigating mycobacteriosis in laboratory zebrafish (*Danio rerio*) using both *in vitro* and *in vivo* modelling, molecular diagnostics, bacterial culture, and histological techniques.

Research Technician: Department of Biology, Saint Mary's University, Fall 2013, (research advisor: Dr. Thomas Rand).

- *In vitro* study of murine macrophage cytokine expression following exposure to fungal metabolite isolates using ELISA analysis.

Masters Researcher: Department of Biology, Mount Saint Vincent University, 2011-2013, (research advisor: Dr. Tamara Franz-Odenaal)

- *In vivo* analyses of the growth and development of the zebrafish craniofacial bones and sensory system using laser ablation and histological analyses.

Undergraduate Honors Research: Department of Biology, University of Waterloo, 2009-2010, (research advisor: Dr. John C. Semple).

- Cladistical analysis of morphological characters of the goldenrod genus *Solidago* L.

SERVICE TO PROFESSION

Manuscript Reviewer, *Journal of Fish Diseases* (1)

Manuscript Reviewer, *Journal of Aquatic Animal Health* (1)

Graduate Student Orientation Volunteer, SUNY-ESF, Spring 2017

Graduate Student Member, SUNY-ESF Academic Governance Research Committee, Fall 2016 - Spring 2017

Treasurer, SUNY-ESF Chapter of the American Fisheries Society, 2016 - 2017.

Graduate Student Orientation Volunteer, SUNY-ESF, Fall 2016

Graduate Student Committee, SUNY-ESF Department of Environment and Forest Biology Faculty Search, Spring 2015 & 2016

Judge, SUNY-ESF Spotlight on Student Research, 2015

Session Instructor, SUNY-ESF Smart Scholars Biotechnology Week, 2014

Volunteer, 7th Aquatic Animal Models of Human Disease Conference, 2014

COMMUNITY INVOLVEMENT/OUTREACH

Mentor, Global Stem Alliance 1000 Girls 1000 Futures Program, 2015-2016

Volunteer, NSERC Women in Science and Engineering Girls Get WISE Camp, 2012

Session Organizer, NSERC Women in Science and Engineering WISE Day, 2012

Volunteer, NSERC Women in Science and Engineering School Visits, 2011-2012

Mentor, Techsploration Goes to School, 2011-2012

PROFESSIONAL MEMBERSHIPS

American Fisheries Society, Fish Health Section

Zebrafish Husbandry Association

New York Academy of Sciences

Appendix

Table A-1.

IP Injection	Experimental Group	Replicate	N		H&E Granuloma			AF Granuloma			Interior AFB				
			N _{Total}	N _{Male}	N _{Total}	N _{Male}	N _{Female}	N _{Total}	N _{Male}	N _{Female}	N _{Total}	N _{Male}	N _{Female}		
Sham Saline	Tigecycline 14-d	1	12	4	8	1	1	0	0	0	0	0	0	0	
	Tigecycline 14-d	2	12	3	9	0	0	0	0	0	0	1	1	0	
	Tigecycline 14-d	Pooled	24	7	17	1	1	0	0	0	0	1	1	0	
	Untreated 14-d	1	15	6	9	0	0	0	0	0	0	0	0	0	
	Untreated 14-d	2	9	3	6	0	0	0	0	0	0	0	0	0	
	Untreated 14-d	Pooled	24	9	15	0	0	0	0	0	0	0	0	0	
	Clarithromycin 14-d	1	13	4	9	0	0	0	0	0	0	0	0	0	
	Clarithromycin 14-d	2	12	4	8	0	0	0	0	0	0	0	0	0	
	Clarithromycin 14-d	Pooled	25	8	17	0	0	0	0	0	0	0	0	0	
	Tigecycline 30-d	1	18	9	9	0	0	0	0	0	0	0	0	0	
	Tigecycline 30-d	2	17	7	10	0	0	0	0	0	0	0	0	0	
	Tigecycline 30-d	Pooled	35	16	19	0	0	0	0	0	0	0	0	0	
	Untreated 30-d	1	16	8	8	0	0	0	0	0	0	0	0	0	
	Untreated 30-d	2	15	6	9	1	0	1	0	0	0	1	0	1	
	Untreated 30-d	Pooled	31	14	17	1	0	1	0	0	0	1	0	1	
	Clarithromycin 30-d	1	15	8	7	0	0	0	0	0	0	0	0	0	
	Clarithromycin 30-d	2	18	8	10	0	0	0	0	0	0	0	0	0	
	Clarithromycin 30-d	Pooled	33	16	17	0	0	0	0	0	0	0	0	0	
	<i>M. chelonae</i>	Tigecycline 14-d	1	11	4	7	3	0	3	0	3	0	3	2	0
		Tigecycline 14-d	2	14	8	6	3	0	3	4	0	4	1	1	0
Tigecycline 14-d		Pooled	25	12	13	6	0	6	7	0	7	3	1	2	
Untreated 14-d		1	12	7	5	5	2	3	3	1	2	2	1	1	
Untreated 14-d		2	12	6	6	6	3	3	3	2	1	7	2	5	
Untreated 14-d		Pooled	24	13	11	11	5	6	6	3	3	9	3	6	
Clarithromycin 14-d		1	12	7	5	4	3	1	3	3	0	2	2	0	
Clarithromycin 14-d		2	12	5	7	2	0	2	2	0	2	0	0	0	
Clarithromycin 14-d		Pooled	24	12	12	6	3	3	5	3	2	2	2	0	
Tigecycline 30-d		1	16	7	9	6	2	2	2	0	2	4	2	2	
Tigecycline 30-d		2	12	6	6	5	1	3	2	0	2	1	0	1	
Tigecycline 30-d		Pooled	28	13	15	8	3	5	4	0	4	5	2	3	
Untreated 30-d		1	17	7	10	10	3	7	5	0	5	3	0	3	
Untreated 30-d		2	15	8	7	7	2	5	6	3	3	3	2	1	
Untreated 30-d		Pooled	32	15	17	17	5	12	11	3	8	6	2	4	
Clarithromycin 30-d		1	17	7	10	3	2	1	3	0	3	5	3	2	
Clarithromycin 30-d		2	15	6	9	3	1	3	2	0	2	3	0	3	
Clarithromycin 30-d		Pooled	32	13	19	7	3	4	5	0	5	8	3	5	