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Probiotic bioaugmentation of an anti-B*d* bacteria, *Janthinobacterium lividum*, on the amphibian, *Notophthalmus viridescens*: Transmission efficacy and persistence of the probiotic on the host and non-target effects of probiotic addition on ecosystem components

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ABSTRACT

Amphibians worldwide are threatened by the fungal disease chytridiomycosis, caused by the skin pathogen *Batrachochytrium dendrobatidis*. Mutualistic skin bacteria are a critical element in amphibians' defenses against chytridiomycosis. Probiotic bioaugmentation of beneficial, anti-*Bd* bacteria on amphibians is a potential conservation strategies. Outdoor experimental ponds were used to investigate transmission efficacy and persistence of the anti-*Bd* bacteria, *Janthinobacterium lividum*, on the amphibian, *Notophthalmus viridescens*. More specifically, this research investigated whether a short-term individual bath, environmental bioaugmentation, or both are necessary to afford transmission and persistence of *J. lividum* on *N. viridescens*. Additionally, this research investigated the effectiveness of these different probiotic bioaugmentation methods in ameliorating *Bd* infection in *N. viridescens*. Lastly, this research investigated the non-target effects of *J. lividum* on leaf decomposition, periphyton production, and zooplankton. *Bd* introduction into the experimental ponds was successful, and infection of newts occurred as expected; however, morbidity effects associated with *Bd* did not occur, and no probiotic treatment reduce *Bd* prevalence or increase proportional change in *Bd* loads below the levels found in the *Bd* only treatment. Interestingly, the bath+water (combination of individual bath of the amphibian and environmental bioaugmentation) treatment did reduce morbidity and *Bd* prevalence in comparison to the bath only treatment and water only treatment. This was likely associated with the transmission efficacy and persistence of *J. lividum* on the newts being greater in the bath+water treatment. These results suggest that the ideal treatment method to afford probiotic establishment and persistence on the host may be the combination of a probiotic bath and

environmental bioaugmentation. Furthermore, alternations to leaf decomposition, periphyton production, or zooplankton community structure were observed as a result probiotic treatments. Therefore, probiotic conservation strategies may be unlikely to harm other organisms and disrupt ecosystem processes; however, additional studies are required before treatment of natural environments is conducted. Developing an understanding of the transmission and persistence of probiotic bacteria is crucial for determining how to administer them to amphibians effectively and efficiently. Probiotic bioaugmentation is a new conservation frontier that requires continued research in order to develop effective and efficient methods for combating the amphibian fungal disease chytridiomycosis.

INTRODUCTION

Currently amphibian species are more threatened and are declining more rapidly than any vertebrate class (Stuart *et al.* 2004, Hoffman *et al.* 2010). Although several anthropogenic factors including habitat loss and over-exploitation are contributing to global amphibian decline, many population declines and extinctions have occurred in pristine areas and cannot be linked to anthropogenic activities (Skerratt *et al.* 2007). The emerging infectious disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis(Bd)*, is considered the leading cause of these enigmatic amphibian declines in areas undisturbed by human activity (Collins and Storfer 2003, Stuart *et al.* 2004, Collins 2010).

Mutualistic cutaneous bacteria have been found to be a critical element in amphibians' defenses to chytridiomycosis (Woodhams *et al.* 2007, Harris *et al.* 2009, Bletz *et al.* 2013). The use of these beneficial anti-*Bd* bacteria as probiotics for susceptible species of amphibians may prove to be a feasible conservation strategy (Harris *et al.* 2009a, b, Vredenburg *et al.* 2011, Muletz *et al.* 2012). How anti-*Bd* bacteria are transmitted and maintained and how long they persist on the host and in the environment have strong implications for probiotic conservation strategies. The primary objective of this research was to investigate the most effective method or combination of methods to transmit beneficial bacteria to amphibians to allow continued protection from *Bd* infection. In particular, is transmission through a single, short-term probiotic bath of individual amphibians, transmission through environmental inoculation or both necessary to achieve an effective defense against *Bd*? This research will also help develop a better understanding of transmission and persistence of bacteria on amphibians, which is

essential in order to determine how to add beneficial bacteria to amphibians successfully and efficiently. An understanding of persistence of the probiotic bacteria on the amphibians is needed in order to know if additional treatments (probiotic baths or environmental inoculations) are required.

Amphibian decline

Although new amphibian species are still being discovered (AmphibiaWeb- accessed June 2012), the current extinction rate of amphibians is over 200 times that of the historic rate (Wake and Vredenburg 2008). Amphibian declines were first recognized in the late 1980s in Australia and the Neotropics, and amphibians now are confronted with an extinction crisis (Wake and Vredenburg 2008, Fisher 2009). Forty-one percent of amphibians are classified as 'threatened' by the International Union for the Conservation of Nature, and 43% are experiencing decreases in population abundance (Wake and Vredenburg 2008, Hoffmann *et al.* 2010). The Global Amphibian Assessment predicts that at least nine and as many as 122 species may have gone extinct since 1980 (Stuart *et al.* 2004, Mendelson *et al.* 2006). Many declines and extinctions are associated with tropical, upland regions, which is also where high species endemism occurs (Young *et al.* 2001, Alford *et al.* 2001, Lips *et al.* 2005, Wake and Vredenburg 2008, Ryan *et al.* 2008, Cheng *et al.* 2011). In one study it was found that all Neotropical harlequin frog species from elevations greater than 1000 meters experienced population declines and 75% had disappeared (La Marca *et al.* 2005). In lowland areas 58% of harlequin frogs experienced declines and 38% had vanished (La Marca *et al.* 2005). In 1996, 14 stream dwelling frog species in eastern Australian montane forest were experiencing population declines and disappearances (Laurance *et al.* 1996). Three-quarters of the surveyed anuran species in

montane regions in Costa Rica and Panama have experienced reductions in population size (Collins and Storfer 2003). Declines, although greatest in magnitude in the tropics, are not limited to these areas. In the United States *Rana muscosa* populations in the Sierra Nevadas have declined (Briggs *et al.* 2005). *Leiopelma* species, native to the moist forests of New Zealand, also have experienced declines (Bishop *et al.* 2009).

There is no single cause of amphibian declines. Land-use change and habitat destruction, over-exploitation, and exotic species have negatively impacted amphibian populations for centuries (Collins 2010). So the question arises: what changed in the 1980s to cause a striking increase in population decline and the extinction rate of amphibians, particularly in pristine habitats? In the twentieth century, new factors including global climate change, contaminants, and infectious disease have been implicated in the disappearance of amphibian species (Collins and Storfer 2003, Collins 2010). These factors most likely are interconnected and may work synergistically to the detriment of amphibian populations. While declines occur in both disturbed and undisturbed areas, some of the most drastic population crashes and extinctions have been in pristine habitats. The infectious disease chytridiomycosis is suspected to be the major causal agent of the enigmatic declines and extinctions of amphibians in Monteverde and other relatively pristine areas around the world (Cheng *et al.* 2011). Over 200 species are thought to have declined as a result of *Bd* (Kilpatrick *et al.* 2010) and over 350 species have been recorded as infected by this pathogen (Fisher 2009). Chytridiomycosis has been confirmed as the cause of the extinction of the Australian gastric-brooding frogs, *Rheobatrachus spp*., the golden frog, *Atelopus zeteki*, and the sharp-snouted day frog, *Taudactylus acutirostris* (Fisher 2009). Eleven species of Australian frogs rapidly

declined or disappeared in northern Queensland potentially due to *Bd* (McDonald and Alford 1999), and 30 *Atelopus* species in Latin America have disappeared rapidly in pristine areas and are potentially extinct as a result of *Bd* (La Marca *et al.* 2005). The full extent of amphibian declines and extinctions is not known and most likely has been underestimated as many species are data deficient and their population statuses are unknown (Crawford *et al.* 2010).

Chytridiomycosis

Chytridiomycosis was first linked to amphibian declines and disappearances in Australia and Central America by Berger *et al.* (1998). In the next year, Longcore *et al.* (1999) identified and named the new monotypic species, *Batrachochytrium dendrobatidis,* as the causative agent of this devastating disease. *Bd* is a member of the phylum Chytridiomycota and the class Chytridiomycetes. Although members of the Chytridiomycota have been known to parasitize insects, algae, plants and nematodes, *Bd* is the first and only reported chytrid parasite of vertebrates (Berger *et al.* 1998).

Emergence of *Bd*

Two hypotheses have been proposed to explain the emergence of the pandemic disease, chytridiomycosis, in amphibians: (1) the endemic pathogen hypothesis and (2) the novel pathogen hypothesis (Rachowicz *et al.* 2005). It is still debated whether *Bd* was already present in amphibian decline areas and a change in pathogen virulence or environmental conditions that favors *Bd* has arisen and caused massive declines (i.e., the endemic pathogen hypothesis) or if *Bd* has been introduced recently around the world to naïve amphibian populations and subsequently causing amphibian declines (i.e., the

novel pathogen hypothesis) (Rachowicz *et al.* 2005, Kilpatrick *et al.* 2010). Support for the endemic pathogen hypothesis mainly stems from the hypothesis that global warming has caused new environmental conditions that favor *Bd* reproduction and transmission (Rachowicz *et al.* 2005, Pounds *et al.* 2006). *Bd* grows optimally at approximately 23^0 C (Piotrowski *et al.* 2004). As global warming occurs, the temperature of some areas moves into *Bd's* optimal temperature while other areas shift out of this optimum; therefore, a correlation between global warming and *Bd* emergence would be location dependent.

The competing hypothesis, the novel pathogen hypothesis, is better supported and accepted than the endemic pathogen hypothesis. A novel pathogen typically spreads rapidly through naïve populations and causes catastrophic loss of species as seen with *Bd* in Central America (Rachowicz *et al.* 2005). The low genetic variation found among pathogenic *Bd* isolates from locations around the world is characteristic of a recently introduced and spreading pathogen. Fifty-nine isolates of *Bd* from five continents were sampled and were equally closely related (James *et al.* 2009). In addition, the sudden massive mortality and wave-like spread of *Bd* coincides with typical patterns of introduced pathogens. The Global Panzootic Lineage (*Bd*GPL) of *Bd* is responsible for much of the amphibian decline worldwide (Farrer *et al.* 2011). *Bd*GPL is one of four distinct *Bd* strains that have been identified. The others include the Cape Lineage (*Bd*CAPE), which is found in South Africa and the island of Mallorca, the Swiss lineage (*Bd*CH), which is found in Switzerland, and the Asian Lineage, which is found in Japan (Farrer *et al.* 2011). The GPL lineage has been characterized as the hypervirulent strain that has decimated amphibian populations (Farrer *et al.* 2011). It was postulated that recombination between two previously reproductively isolated strains of *Bd* may have

occurred recently bringing about this hypervirulent *Bd*GPL that consequently has resulted in massive amphibian declines. However, with recent genetic analysis of *Bd* strains the picture is becoming more complex (Rosenblum, pers. comm.). *Bd* has been thought to be a recently evolved pathogen, but according to new genome sequencing, the evolutionary divergence of *Bd* from its most recent common ancestor was 72,000 - 129,000 years ago, however, it is not known when the *Bd*GPL emerged. It will be important to reconcile this deeper evolutionary history with its recent spread to determine the true origin of *Bd.* In addition, the novel pathogen hypothesis requires a mechanism of spread between continents and countries. It has been suggested that the amphibian trade of the African clawed frog, *Xenopus laevis,* and the American Bullfrog, *Lithobates catesbeianus,* have spread *Bd* worldwide as they are resistant to chytridiomycosis (Fisher and Garner 2007, Kilpatrick *et al.* 2010). Fisher (2009) explains that the original 'out of Africa' origin of *Bd* is less parsimonious than a North American origin because *Bd* on *L. catesbeianus* exhibits greater genetic diversity. Additionally, recent identification of *Bd* in preserved specimens from 1902 in Japan suggests the potential for an 'out of Asia' origin (Fisher 2009); however, more data are needed for definitive conclusions to be made about the origin of *Bd*. It is possible the true answer is not one or the other hypothesis, but a combination and integration of both hypotheses.

Spread of *Bd* within a given geographic region most likely occurs through frog movement and water transport of zoospores; however, reptile, bird, human, and insect movement have also been suggested as additional sources (Kilpatrick *et al.* 2010, Kilburn *et al.* 2011). Importantly, crayfish have been identified recently as an alternative host for

Bd, which provides an additional vector for spread and persistence of *Bd* in the environment (McMahon *et al.* 2012).

Bd lifecycle and characteristics

Bd is a chytrid fungus that has two known life stages: a sessile growing thallus that produces a single reproductive zoosporangium, which produces motile, flagellated zoospores. During *Bd*'s four to five day asexual lifecycle, a zoospore will attach to the host and develop into a monocentric thallus, which is single zoospore-producing sporangium. The zoospores are released through papillae, or discharge tubes, that project distally allowing the zoospores to re-infect the host or to be released into the environment where they can infect a new individual (Berger *et al.* 2005, Longcore *et al.* 1999, Kilpatrick *et al.* 2010, Piotrowski *et al.* 2004).

Bd infection of amphibians is initiated by waterborne zoospore contact with host tissue or by amphibian-to-amphibian contact, transferring zoospores to a new individual (Berger *et al.* 1999). The mechanism of how the zoospores actually infiltrate amphibian skin has only recently been investigated. Longcore *et al.* (1999) hypothesized that zoospores encyst on the skin surface and insert *Bd'*s nuclear material through a germ tube into the host's epidermal cells; however, evidence for such a mechanism was lacking until recently. Using microscopy, germ tube invasion of the epidermis has been documented for some amphibian species (Van Rooji *et al.* 2012). Additionally, zoospores have been found to secrete enzymes that rapidly disrupt intercellular junctions in amphibian skin, which might allow the germ tube to reach several cell layers deep in the epidermis before piercing an epidermal cell (Brutyn *et al.* 2012). Expanded gene families (i.e. more members present) in the *Bd* genome including the fungalysin metallopeptidase

family and serine-like protease family may be important parts of the infiltration process (Rosenblum *et al.* 2009). Furthermore, the *Bd* genome contains 38 metallopeptidases, 32 serine-type proteases and 99 aspartyl proteases, which is four to ten times more than *Homolaphlyctis polyrhiza,* a non-pathogenic chytrid (Joneson *et al.* 2011). Although the role of these proteases in pathogenicity is unknown, a potential mechanism is that the peptidases allow the zoospore to adhere to and the germ tube to penetrate keratinized tissues. This allows the zoospores to parasitize the cells of the deeper epidermal layers, which is where immature zoosporangia are observed. The zoosporangia then develop at a rate that corresponds with the maturation of the infected amphibian cells. As the epidermal cells move outward and become keratinized the zoosporangia also progress toward maturity (Berger *et al.* 2005). Therefore, the mature, zoospore-releasing zoosporangia reside in the outer stratified keratinized layer, known as the *stratum corneum* (Berger *et al.* 2005). Due to this association with keratinized tissue at maturation, tadpole infection normally is seen in its keratinized mouthparts (Berger *et al.* 2005, Longcore *et al.* 1999). In adults, keratinized tissue exists across the skin surface and therefore *Bd* infects across an amphibian's skin. Infection intensity is typically greater in the ventral region, limbs and feet (North and Alford 2008).

Disease symptoms and cause of death

Bd infection intensity can increase exponentially after exposure to the fungus, and chytridiomycosis and its effects can develop very quickly (Briggs *et al.* 2010). Infected individuals may exhibit hyperkeratosis, irregular hyperplasia, disordered or fused epidermis cell layers, spongiosis and erosions or ulcerations of the skin (Berger *et al.* 2005, Kilpatrick *et al.* 2010). Severity of these symptoms may vary depending on

infection level and susceptibility. Sub-lethally infected individuals may present these symptoms but never succumb to the disease. Severely infected amphibians that are succumbing to disease also tend to be lethargic and discolored, have excessive sloughed skin, exhibit a loss of righting reflex, and have a depressed body position (Berger *et al.* 1999). In order for death to occur it has been suggested that a threshold infection load of 10,000 zoospores must be surpassed (Vredenburg *et al.* 2010). Therefore, it is not until a host is colonized by thousands of zoospores and the resulting zoosporangia that death by *Bd* may occur. The specific cause of death associated with lethal *Bd* infection has been linked to asystolic cardiac arrest due to improper ion and water exchange. Epidermal disruptions caused by *Bd* inhibit electrolyte transport across the ventral skin regions. This causes reductions in plasma electrolyte concentrations and deterioration of cardiac electrical functioning, resulting in cardiac arrest (Voyles *et al.* 2011, Voyles *et al.* 2009).

Susceptibility to chytridiomycosis

Susceptibility appears to be mediated by environment, host life history, and host defenses (Berger *et al.* 1999, Collins 2010). The Panamanian Golden frog, *Atelopus zeteki*, and the Australian gastric brooding frogs, *Rheobatrachus spp*., likely were extirpated by the emergence of *Bd* whereas other species carry the infectious pathogen with little to no manifestation of disease or evidence of population decline (Fisher 2009). Many researchers have documented that amphibian populations at higher elevation and cooler temperature environments are at greater risk for *Bd* infection (Stuart *et al.* 2004, Berger *et al.* 1998, La Marca *et al.* 2005, Young *et al.* 2001, Bosch *et al.* 2001). This follows logically as cooler temperatures are favorable for *Bd* survival and reproduction. Lips *et al.* (2005) concluded that the most threatened amphibian species are those living

at high elevation with an aquatic larval stage and high ecological specialization, where they potentially are restricted to habitats also optimal for *Bd*. Environmental conditions at a landscape level have also been found to strongly influenced host-pathogen dynamics (Woodhams and Alford 2005, Puschendorf *et al.* 2011). More specifically, Puschendorf *et al.* (2011) found two traditionally rainforest dwelling *Litoria* species that were thought to be extinct surviving in a tropical dry forest habitat. It was hypothesized that reduced canopy cover in tropical dry forests allowed frogs to bask, which reduced growth and reproduction of the fungus. Furthermore, in Australia, infection rates were distributed non-randomly between frog breeding habitats, with permanent water-body breeders experiencing higher infection rates (Kriger and Hero 2009).

While the environment and life history influence susceptibility, it is also affected by host-specific biology and genetics. Host factors, such as the cutaneous microbial community (Lauer *et al.* 2008, Lam *et al.* 2010, Harris *et al.* 2006, Harris *et al.* 2009, Harris *et al.* 2009, Becker *et al.* 2009, Brucker *et al.* 2008b), host-produced antimicrobial peptides (Conlon 2011), host adaptive immunity (Ramsey *et al.* 2010) and host MHC genotype (Savage and Zamudio 2011) also mediate amphibian susceptibility to chytridiomycosis. These factors can vary interspecifically and intraspecifically. *Bd* infections span both taxonomic and geographical barriers.

Amphibian defenses

Defenses against disease are critical to survival. Amphibians have been shown to have cutaneous microbial defenses as well as innate and adaptive immune responses protecting them from the pathogenic fungus, *Bd (*Harris *et al*. 2006, Rollins-smith *et al*.

2009; Ramsey *et al.* 2010). In addition, a genetic component of resistance to *Bd* infection has been identified recently (Savage and Zamudio 2012).

Cutaneous defenses

Amphibians, like all organisms, harbor microbes on their skin. These microbial communities vary interspecifically among amphibian species (McKenzie *et al.* 2011), meaning that different amphibian species harbor distinct microbial communities. Numerous bacteria species residing on amphibian skin have been found to inhibit the fungus, *B. dendrobatidis* (Harris *et al.* 2006, Harris *et al.* 2009) as well as other pathogens including *Mariannaea elegans* and *Rhizomucor variabilis* (Lauer *et al.* 2007, 2008). Forty-eight and 28 bacteria, from *Hemidactylium scutatum* and *Plethodon cinereus* respectively, have antifungal activity that can inhibit *M. elegans* and *R. variabilis* have identified (Lauer *et al.* 2007, 2008). Additionally, bacteria genera from both *P. cinereus* and *H. scutatum* have been identified that inhibit *Bd* (Harris *et al.* 2006). This anti-fungal activity is linked to the production of metabolites, such as violacein, 2, 4-diacetylphloroglucinol, or indole-3-carboxaldehyde, by these species of bacteria (Becker *et al.* 2009, Brucker *et al.* 2008b, Brucker *et al.* 2008a). In addition, *Bd* zoospores have been shown to exhibit negative chemotaxis in the presence of two of these metabolites (Lam *et al.* 2011). Therefore, zoospores would tend not to colonize amphibians with protective bacteria. Additional ecological interactions between cutaneous microbes and *Bd* such as space competition, microenvironment alterations, and bacteria secreted antimicrobial compounds may reduce the ability or likelihood of *Bd* infiltrating host tissue (Becker and Harris 2010). Bacterial reduction experiments showed that reduction of the community of skin microbes on *P. cinereus* caused greater morbidity

when exposed to *Bd* (Becker and Harris 2010). Furthermore, probiotic bacterial addition experiments with *Janthinobacterium lividum*, a violacein producer, have shown it can reduce *Bd* infection and amphibian morbidity and mortality (Harris *et al.* 2009a, Harris *et al.* 2009b, Becker *et al.* 2009). Additionally, the persistence of certain amphibian populations in the Sierra Nevadas was correlated to the proportion of individuals possessing anti-fungal bacteria (Lam *et al.* 2010). More specifically, it was suggested that if approximately 80% of individuals within a population possess cutaneous anti-*Bd* bacteria, coexistence with *Bd* can occur due to a mechanism similar to herd immunity (Lam *et al.* 2010).

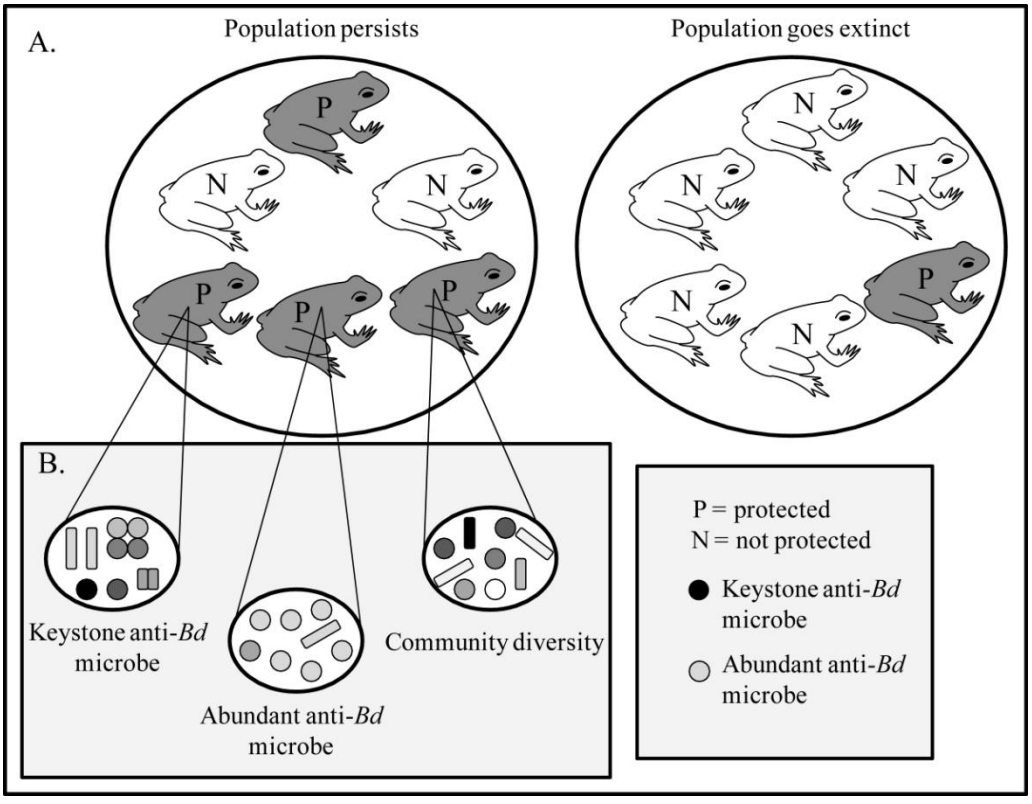


Figure 1. Population and community mechanisms of protection from *Bd*. A) Herd effect in which a population persists with *Bd* because a large proportion of the individuals are protected by beneficial microbes; B) Individuals are protected by one of three possible mechanisms: a keystone anti-*Bd* microbe restructures the cutaneous microbial community into one that is stable and provides increased defensive function, an abundant anti-*Bd* microbe provides a major portion of the defensive function, or a high level of microbial

diversity is associated with defensive function. A goal of probiotic bioaugmentation is to increase the proportion of protected individuals in populations via one of these mechanisms thereby allowing the population to persist with the pathogen. Shading of frogs indicates protection. (from Bletz *et al.* 2013)

Innate immunity

Innate immune activity provides the first line of host-mediated defense against pathogenic microbial invasion (Conlon 2011). The production of antimicrobial peptides (AMP) is considered to be the main component of innate immune response by amphibians (Conlon 2011). The regulation of AMP synthesis and release is not understood completely. However, it is known that AMPs are produced and are released from the dermal granular glands onto the skin surface. AMP synthesis is increased with exposure to pathogenic microbes (Mangoni *et al.* 2001). The secreted AMPs typically are thought to inhibit pathogens by interfering with microbial membranes (Rollins-Smith 2009). *In vivo* experimentation carried out on *Xenopus laevis* showed that *Bd* infection increased after AMPs production was experimentally reduced thus demonstrating that AMPs play a role in reducing zoospore colonization and confirming the importance of these molecules in defense against chytridiomycosis (Ramsey *et al.* 2010). Recent *in vitro* studies have determined that, in the presence of beneficial bacteria, lower concentrations of AMPs are necessary to successfully inhibit *Bd* growth (Myers 2011). This finding suggests there is a synergistic relationship between amphibian-produced AMPs and the metabolites produced by resident anti-*Bd* bacteria.

Adaptive immunity

The existence of an adaptive immune response to *Bd* continues to be debated; however, some evidence for adaptive immunity exists. Rosenblum *et al.* (2009) examined gene expression patterns in amphibian tissue in response to *Bd* infection and detected no evidence suggestive of an immune response in tissue, such as the skin, liver, and spleen. In fact, immune function genes were found to be down regulated in infected *Silurana tropicalis,* suggesting that no immune response is mounted against this fungal infection (Rosenblum *et al.* 2009). Contrary to these findings, antibodies that could bind to *Bd* were found in the cutaneous mucus of *Xenopus laevis,* suggesting that mucosal antibodies may play a role in reducing zoospore colonization of host tissue (Ramsey *et al.* 2011). In addition, immune-suppressed individuals were found to have greater numbers of zoospores and experienced greater sub-lethal effects, which suggests the involvement of leukocytes may help control *Bd* infection levels. However, the suppression of the immune system via X-ray irradiation also removed any microbes and their possible defenses. Immune response to *Bd*, if it occurs at all, is mounted very slowly (Ramsey *et al.* 2010). Furthermore, new evidence suggests that evasion or suppression of the amphibian immune system by *Bd* is occurring (L. Rollins-Smith, pers. comm.)

Recent experimentation has confirmed that another aspect of the adaptive immune system is playing a role. Genetic polymorphisms at MHC loci most likely contribute to amphibian resistance to chytridiomycosis in at least one species of frogs. Mortality risk was reduced for populations that were MHC heterozygotes and for individuals that possessed the MHC allele Q (Savage and Zamudio 2011). This suggests a genetic component of resistance to this fungal disease.

Behavioral mechanisms of defense

Behaviorally mechanisms including behavioral fevers and basking have been documented in amphibians as a means of reducing *Bd* infection. For example, as *Bd* entered populations of the Panamanian golden frog the mean body temperature increased (Richards-Zawacki 2010). More specifically, it was found that post infection the average body temp was 2.4 °C higher than pre-infection levels and 11 % had body temperatures above 28°C, which is known to halt *Bd* growth (Richards-Zawacki 2010). In addition, there was a correlation between body temperature and infection load, such that with increasing temperature zoospore loads decreased (Richards-Zawacki 2010). While *A. zeteki* is possibly extinct in the wild, this correlation suggests that behavioral thermoregulation can be used as a behavioral defense to *Bd* infection and may prevent other species from succumbing to chytridiomycosis.

Applications for conservation

Mitigation of chytridiomycosis requires procedures that can be implemented by conservation biologists. Three types of protocols have been identified to date: measures to limit spread of *Bd*, selection for resistance, and manipulation of cutaneous microbial defenses. Important protocols to limit the spread of *Bd* to new areas are amphibian trade restrictions and the use of appropriate cleaning procedures when leaving infected area, such as cleaning boots and equipment. While these measures are necessary to limit introduction of *Bd* to naïve areas, it cannot help already infected areas. Attempts to manipulate adaptive immune systems (i.e. vaccines) have yet to be successful (Stice and Briggs 2010) but would be ideal for treating animals in survival assurance colonies before repatriation. Additionally, prior exposure has been found to have no effect on

survival or infection intensities, demonstrating that Bd infection do not stimulate a protective adaptive immune response in *Litoria booroolongensis* (Cashins *et al.* 2012).

Selection for tolerance also has been proposed as a possible mechanism to allow extirpated amphibians to be reintroduced (Venesky *et al.* 2012). However, such a technique would involve long-term selection programs and at the present time no research is being conducted in this area. Bioaugmentation of anti-*Bd* cutaneous microbes has the potential to be implemented immediately through probiotic treatments using individual baths (Harris *et al.* 2009, Vredenburg *et al.* 2011) or possibly through environmental bioaugmentation (Muletz *et al.* 2012, Bletz *et al.* 2013). It can provide immediate aid to declining amphibian populations, protect naive amphibian communities and possibly allow the reintroduction of amphibian species that are extinct in the wild. Cutaneous microbial defense is the only mechanism that is not intrinsic to individual amphibians, and can be manipulated and that has been shown to work in a field trial (Vredenburg *et al.* 2011).

In addition, the defenses offered by these mutualistic microbes may keep *Bd* densities low on the skin and provide time for the amphibian to mount an adaptive immune response to the *Bd* infection. Innate and adaptive immunity can serve amphibians that possess them well; however, they offer little hope to susceptible species unless natural selection increases the frequency of individuals with genetically based immune systems that inhibit *Bd*. Extinction of species due to *Bd* shows that natural selection will not be adequate in all cases. A greater understanding of microbial defensive mechanisms and how they are regulated by aspects of the innate and adaptive immune system will lead to a greater ability of researchers and conservation biologists to

manipulate the defensive function of symbiotic microbes, which is imperative to formulating an appropriate disease mitigation plan.

Microbial mutualisms

Mutualism is a symbiotic relationship in which both partners benefit, and mutualistic relationships between microbes and metazoans are quite common (McFall-Ngai 1999). While microbial mutualisms can confer a wide variety of functional benefits to host organisms, such as nutrient acquisition in mycorrhizae-plant root systems (Jensen 1982), digestion in all metazoans (Reid *et al.* 2011) and anti-predatory mechanism in the squid *Euprymna scolopes* (McFall-Ngai and Ruby 1991), the ability of beneficial bacteria to provide defense against pathogens for host organisms is particularly relevant to the conducted study. Some species of fungus-farming ants, such as *Atta* species, form symbiotic relationships with a coevolved species in the genus *Pseudonocardia* to inhibit *Escovopsis*, a parasite of ant fungal farms (Currie 2001). *Pseudonocardia* reside on the cuticle of Attine ants and produce antibiotics that target parasite**s** of the *Escovopsis* genus that parasitize the ants' fungal gardens (Cafaro *et al.* 2011). Furthermore, embryos of the lobster, *Homarus americanus*, possess a symbiont that inhibits the crustacean fungal pathogen, *Lagenidium callinectes*, by producing tyrosol, an anti-fungal compound. Specifically relevant to the proposed study is the relationship between resident anti-fungal bacteria and amphibian skin. Resident cutaneous microbes have been found to provide resistance to fungal pathogens such as *Mariannaea elegans* (Lauer *et al.* 2007) and *Bd* (Harris *et al.* 2006, Harris *et al.* 2009 a b).

Transmission

Beneficial microbes that form mutualistic relationships can be transmitted in three different ways in nature including vertically, horizontally, and environmentally. **Vertical transmission** refers to the transfer of microbes from parent to offspring. Harmsen *et al.* (2000) showed that beneficial microbes of the human digestive tract are obtained by newborns during breastfeeding. The Panamanian amphibian species, *Hyalinobatrachium colymbiphyllum,* appears to transmit mutualistic microbiota to deposited embryos, which may protect hatchings from *Bd* (Walke *et al.* 2011). In addition, it appears that female four-toed salamanders, *Hemidactylium scutatum*, transfer their skin bacteria to their embryos (Banning *et al.* 2008). The presence of anti-*Mariannaea* bacteria on *Hemidactylium scutatum* embryos in communal nests was positively correlated with embryo survival (Banning *et al.* 2008). **Horizontal transmission** is the transfer of microbes between individuals of the same life stage and usually of the same species. Horizontal transmission is not well documented in the literature, and in many cases is associated with the transmission of parasitic microbial species. For example, *Salmonella enteritidis* was horizontally transmitted between laying hens (de Vylder *et al.* 2011). The horizontal transmission of beneficial bacteria in amphibians or other species has not been investigated. It is possible; however, that horizontal transmission of beneficial bacteria occurs, especially in social amphibian species or during the mating season where conspecific contact occurs. **Environmental transmission** is the transfer of bacteria to a host from an environmental source and has been documented in multiple species. Kikuchi *et al.* (2007) showed that a *Burkholderia spp.,* a symbiont of *Riptortus clavatus* (broad-headed bug), is acquired environmentally by nymphal insects. In addition, juveniles of

Euprymna scolopes, the bobtail squid, acquires its light-organ symbiont, *Vibrio fischeri*, from the surrounding seawater (McFall-Ngai and Ruby 1991). Terrestrial isopods obtain hepatopancreatic symbionts horizontally from conspecifics and through the environment (Wang *et al.* 2007). Belden and Harris (2007) postulate that amphibians as well as other organisms obtain microbiota from the environment at some point during development. This transfer from the environment may occur continually and may be necessary for persistence of cutaneous microbial communities. Such transfer may be important for reestablishing microbial populations after disturbances such as skin sloughing in amphibians (Meyer *et al.* 2012). Environmental transmission was demonstrated between the salamander *P. cinereus* and *Janthinobacterium lividum,* an anti-*Bd* bacteria species, in laboratory experiments (Muletz *et al.* 2012), suggesting that such transfer occurs in nature.

Probiotic bioaugmentation

Humans are able to take advantage of microbial transmission pathways to bring about a beneficial effect in natural environments. This tactic has been used in agriculture by adding *Azotobacter* or *Azospirillum* species to the soil to promote plant growth (Gentry *et al.* 2004). In addition, inoculation of soils with *P. fluorescens* has been shown to enhance root growth as well as reduce pathogens, such as the potato nematode, *Globodera rostacheinsis* (Cronin *et al.* 1997). Furthermore, Teplitski and Ritchie (2009) proposed the use of bioaugmentation as a biological control for coral disease as beneficial bacteria may assist with disease resistance. In humans, whole-stool implantations have been found to restore appropriate microbial communities in individuals with chronic gut infections, such as *Clostridium difficile* infection (Reid *et al.* 2011, Nood *et al.* 2009).

Environmental bioaugmentation has been successful in aquaculture settings to establish beneficial bacteria in the intestinal tract, which reduces fish mortality. An experiment was performed where rainbow trout were treated with *Pseudomonas fluorescens* (10^5 cells/ml) prior to exposure to the pathogen *Vibrio anguillarum*, and accumulated mortality was reduced by 35% in bacterially treated treatments (Gram *et al.* 1999). Shrimp aquaculture also has benefited from the use of probiotics against luminous *Vibrio* species. In the Philippines, shrimp ponds treated with a probiotic species of *Bacillus* achieved 80-100% survival whereas control ponds had between 0-70 % survival (Moriarty 1998). Bioaugmentation has the potential to be an effective conservation strategy by increasing the amount of anti-*Bd* bacteria on amphibians, thereby protecting amphibians from chytridiomycosis. A bioaugmentation field trial with *Rana muscosa* in the Sierra Nevadas has produced promising results (Vredenburg *et al.* 2011, personal communication). Thirty-nine percent of the treated individuals survived whereas 0% of the untreated controls were found.

In order to implement a probiotic bioaugmentation strategy effectively, an understanding of the persistence of added anti-*Bd* bacteria is necessary. In the field trial conducted by Vredenburg *et al.* (2011), amphibians were immersed in small containers with a concentrated solution containing the anti-*Bd* bacteria, *J. lividum*, for 24 hours and then returned to their natural environment. Treated individuals maintained lower infection intensities in comparison to untreated controls (V. Vredenburg, pers. comm.). While this protocol appears to have been effective, there are several limitations of its feasibility for effective conservation. First, the strategy is labor intensive and requires catching each frog for treatment multiple times. In the *Rana muscosa* system, the frogs

live all year around ponds in the Sierra Nevada Mountains; therefore, it is possible to capture many of them during ice free months. However, in more diverse and complex systems, it may not be possible as large numbers may only congregate for seasonal mating events. In such a system, a direct water or soil probiotic treatment may be more feasible. Second, it is not clear whether probiotic inoculation via a short term (i.e. hours) bath would be long lasting. The anti-*Bd* bacteria used for probiotic treatment may not always be in naturally high abundance in the inhabited pools of the treated individuals. Therefore, the question arises, how long would the anti-*Bd* bacteria persist on the amphibians after probiotic treatment? Would additional, repetitive treatments be necessary? Would an environmental reservoir be needed for continued persistence? Could the augmentation of the aquatic reservoir be a more effective and efficient strategy for probiotic treatment?

In large-scale probiotic bioaugmentation, hand-capturing and bathing amphibians individually in probiotics is not possible in all situations, and environmental treatment may be a more efficient method. The majority of amphibian species that have declined are aquatic breeders (Kriger & Hero 2007); therefore, inoculation of aquatic breeding sites could be a successful strategy. Environmental inoculation of aquacultural ponds has increased survival of farm-raised fish and shellfish species (Moriarty 1998).

One concern with augmentation of the environment with a probiotic is the potential for non-target effects on other organisms and ecosystem processes. The addition of a probiotic may have direct effects on the composition of the existing bacterial community or have direct or cascading effects on higher trophic levels that can in turn affect ecosystem processes. In agricultural settings, studies have shown that probiotic

treatment of soils initially yields changes in the bacterial community but over time this effect is diminished. For example, pathogenic strains of *Fusarium* that cause diseases in crops can be controlled by some non-pathogenic strains of *Fusarium oxysporum*. One study demonstrated that the addition of this species to soil caused the bacterial and fungal community to diverge from control treatments initially, but after 6 months the community structures of treated and control soils were not significantly different (Edel-Hermann *et al.* 2009). In aquaculture the effect of probiotic treatment on bacterial community structure has not investigated; however, such research is necessary (Wang *et al.* 2008).

The effect of a probiotic on higher trophic organisms also needs to be considered. One amphibian anti-*Bd* species, *J. lividum* that has been used in probiotic experiments, produces violacein, and this metabolite can be toxic to nanoflagellates (Matz *et al.* 2004). A reduction in nanoflagellates may affect zooplankton communities because nanoflagellates are important food resources for many zooplankton species (Coveney *et al.* 1977). Nanoflagellates are also bacteria predators and therefore a reduction in their abundance could lead to increased bacterial abundances and an altered community structure. This increase could in turn affect ecosystem processes, such as leaf decomposition, in which bacteria play a significant role. Some bacteria are known to inhibit and even lyse algal cells. For example, *Pseudomonas fluorescens* causes cell lysis and death of *Heterosigma akashixo*, *Alexandrium tamarense*, and *Cochlodinium polykrikoides* perhaps by secreting enzymes (Kim *et al.* 2007). On the other hand, some bacteria can stimulate algae through the production of vitamins and other substances (Cole 1982) and therefore affect primary productivity (Cole 1982). One algal genus, *Chlamydomonas,* was stimulated by *Pseudomonas* and *Flavobacterium* independently

but when in combination these bacteria were inhibitory (Delucca *et al.* 1978). Interestingly, in aquacultural settings it has been suggested that the addition of a probiotic that stimulates microalgae or phytoplankton growth may be beneficial for larviculture as the bivalve and mollusk larvae are dependent of these organisms for growth (Kesarcodi-Watson *et al.* 2008). In the context of amphibian probiotic applications that will be used to treat natural populations, it will be important to consider the probiotic's non-target interactions within the ecosystem and select probiotics that do not have detrimental non-target effects (Bletz *et al.* 2013), which can be elucidated with controlled experiments.

Objectives

As amphibian populations continue to be devastated by *Bd*, it is crucial to develop an effective conservation strategy for combating this amphibian pathogen. Currently bioaugmentation of beneficial microbes appears to be the most feasible conservation option for areas where *Bd* is emerging, for reintroduction of susceptible amphibians, as well as a preventative mechanism for naïve areas. To date, bioaugmentation for amphibians has been largely limited to treating individuals one time with a probiotic bath; however, there are still many unknowns that can limit effectiveness, such as its time intensive. It is essential to investigate and understand how the transmission and persistence of mutualistic bacteria occurs on amphibian skin in order to be able to manipulate these processes to halt the negative effects of *Bd*. With an understanding of transmission we can determine how to augment amphibian cutaneous microbes successfully, and with an understanding of persistence we will know if repetitive treatment is necessary.

My primary objective was to investigate the transmission efficacy and persistence of the anti-*Bd* bacteria, *Janthinobacterium lividum*, on the amphibian, *Notophthalmus viridescens*. More specifically, The experimental design allowed me to investigate whether a short-term individual bath, environmental bioaugmentation, or both are necessary to afford transmission and persistence of *J. lividum* on *N. viridescens*. In addition, this research investigated the effectiveness of these different probiotic bioaugmentation methods to allow continued protection from *Bd* infection in *N. viridescens*. The main hypothesis was that amphibian skin microbiota are maintained through continual replenishment from the environment; therefore, to prevent or reduce *Bd* infection, the existence of an environmental reservoir of the probiotic species, *J. lividum*, is necessary. growth rate, *Bd* loads and *J. lividum* abundance of the newts, as well as *Bd* and *J. lividum* abundance in the aquatic environment were measured. In addition, to determine if probiotic bioaugmentation had any effects on non-target organisms or ecosystem processes, four ecosystem measurements, including zooplankton community composition, primary productivity, and leaf decomposition rate, were taken. Specific hypotheses regarding the main factors of the experiment as well as the ecosystem measurements are provided below:

- T1 (n=5): Newt received probiotic bath, probiotic treatment of aquatic environment present, *Bd* present
- T2 (n=5): Newt received probiotic bath, probiotic treatment of aquatic environment absent, *Bd* present
- T3 (n=5)*:* Newt did not receive probiotic bath, probiotic treatment of aquatic environment present, *Bd* present
- T4 (n=5): Newt did not receive probiotic bath, probiotic treatment of aquatic environment absent, *Bd* present
- T5 (n=5): Newt did not receive probiotic bath, probiotic treatment of aquatic environment absent, *Bd* absent

The following comparisons address the hypotheses of interest:

One-time Individual Treatment

- **$T_2 - T_4$**: Does giving a newt a probiotic bath lead to less weight loss, a lower probability of infection or a lower *Bd* load?
- **$T_2 - T_5$**: Does the effect of a probiotic bath eliminate or greatly reduce weight loss or *Bd* infection such that it is equal to the control?
- **$T_2 - T_3$**: Is a probiotic environmental treatment equal to that of a newt probiotic bath in terms of weight loss, probability of infection or *Bd* loads?

Continuous Treatment/ Environmental Treatment

Environmental Reservoir Only:

- **$T_3 - T_4$**: Does a probiotic environmental treatment lead to less weight loss, a lower probability of infection or a lower *Bd* load? Is environmental transmission adequate to afford protection?
- **$T_3 - T_5$**: Does the effect of a probiotic environmental treatment eliminate or greatly reduce weight loss or *Bd* infection such that it is equal to the control?

Combination Treatment: Individual Bath and Environmental Reservoir

- **$T_1 - T_3$**: When there is a probiotic environmental treatment present, does a newt probiotic bath lead to less weight loss, a lower probability of infection or a lower *Bd* load?
- **$T_1 - T_2$**: When a newt is given a probiotic bath, does a probiotic environmental treatment lead to less weight loss, a lower probability of infection or a lower *Bd* load?
- **$T_1 - T_4$**: Does the combination of a newt probiotic bath and a probiotic environmental treatment lead to less weight loss, a lower probability of infection or a lower *Bd* load?
- **$T_1 - T_5$**: Does the effect of a probiotic bath and environmental treatment greatly reduce weight loss or *Bd* infection such that it is equal to the control?

***Bd* Control Hypothesis**

- **$T_4 - T_5$:** Do newts in a *Bd* positive environment with no added microbial defenses experience greater weight loss, probability of infection or *Bd* loads than in the *Bd* absent control?

Ecosystem Hypotheses

- **T_1-T_5**: Does the addition of a probiotic environmental reservoir and individual newt bath treatment affect the ecosystem variables?
- **T_2-T_5**: Does the individual bath treatment affect the ecosystem variables?
- **T_3-T_5**: Does the addition of a probiotic environmental reservoir affect the ecosystem variables?

METHODS

Experimental design

A replicated, randomized block experiment with five treatments in an array of 25 experimental ponds was performed. Two factors were manipulated in a crossed design: presence and absence of a probiotic bath and presence and absence of the probiotic bioaugmentation of the experimental ponds. These four treatments were in a *Bd* positive environment. An additional treatment without *Bd* or probiotic treatment was used as a control (R. Domangue, pers. comm.). The five treatments were assigned at random to the experimental ponds within each of the five blocks. Each tank housed 2 newts. The 50 newts were assigned at random to 1 of the 5 treatments (Figure 2):

- T1 (n=5): Newt received probiotic bath, probiotic treatment of the aquatic environment present, *Bd* present
- T2 (n=5): Newt received probiotic bath, probiotic treatment of the aquatic environment absent, *Bd* present
- T3 (n=5)*:* Newt did not receive probiotic bath, probiotic treatment of the aquatic environment present, *Bd* present
- T4 (n=5): Newt did not receive probiotic bath, probiotic treatment of the aquatic environment absent, *Bd* present
- T5 (n=5): Newt did not receive probiotic bath, probiotic treatment of the aquatic environment absent, *Bd* absent

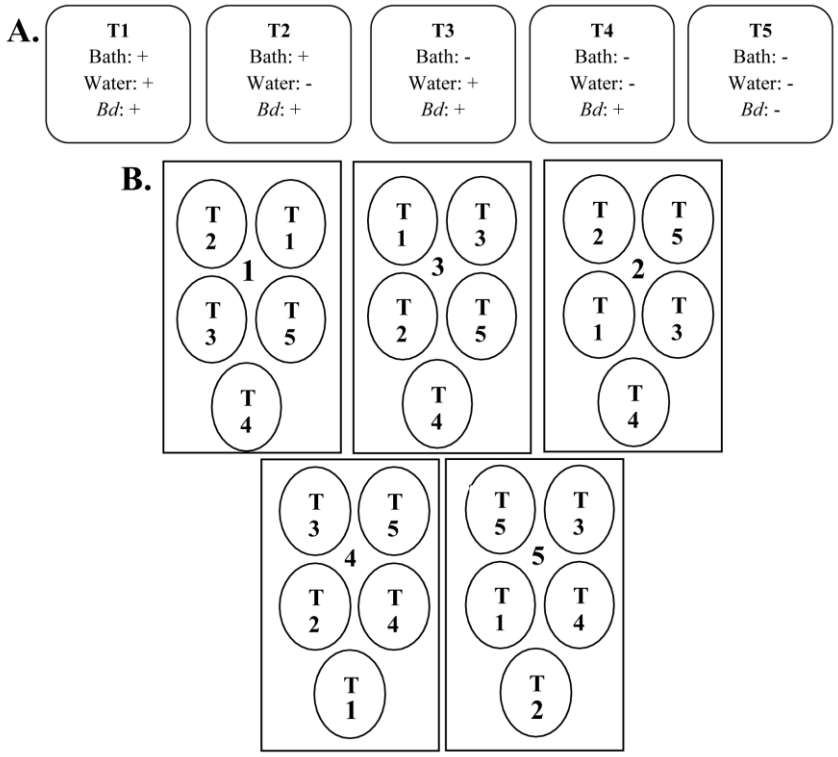


Figure 2: A) Five experimental treatments were used in this experiment. Newt + indicates treatment with probiotic bath, Water + indicates treatment of the experimental ponds with the probiotic, and *Bd* + indicates presence of *Bd*. B) Block design of experimental ponds. Treatments were assigned at random to locations within a block.

Study Species:

Notophthalmus viridescens

Notophthalmus viridescens, the red-spotted newt, is a member of the Salamandridae family. This salamander is common throughout the northeastern United States in wet forested areas and in small bodies of water such as ponds, wetlands, lakes, and slow moving streams, and is abundant in the George Washington National Forest in Virginia (AmphibiaWeb accessed 11 December 2012). *N. viridescens* has four distinct life stages: an aquatic egg, an aquatic larva, a terrestrial eft, and an aquatic or terrestrial adult (AmphibiaWeb accessed 11 December 2012). Red-spotted newts are susceptible to

Bd infection, but it is rarely fatal to this species. Surveys conducted in the GWNF at White Oak Flat Pond, Todd Lake and Mud Pond demonstrated newts can be infected and revealed high prevalence of *Bd* infection during the onset breeding season (Bletz & Harris 2013). Aquatic adults were used as test organisms for this research due to their local abundance, aquatic nature, and their susceptibility to *Bd* infection. Thus, the use of newts was appropriate for testing aquatic bioaugmentation of a probiotic in ameliorating *Bd* infection, and removing newts for experimentation from large populations was not expected to harm those populations (Bakkegard and Pessier 2010, Rothermel *et al.* 2008, Groner and Relyea 2010).

Janthinobacterium lividum

J. lividum is a violacein-producing, pyschrophilic proteobacteria, *J. lividum* has been found on *Plethodon cinereus* (Lauer *et al.* 2007), *Hemidactylium scutatum* (Lauer *et al.* 2008, Harris *et al.* 2009), *Notophthalmus viridescens* (Appendix 1), *Rana muscosa* (Woodhams et al 2007, Lam *et al.* 2010), *Alytes obstrictans* (Woodhams pers. comm.), Ecuadorian frog species (Woodhams pers. comm.), Panamanian frogs (E. Rebollar, pers. comm.), and *Lithobates catesbeianus* (J. Walke, pers. comm.). It has also been found in soil environments in Antarctica (Shivaji *et al.* 1991), in streams in Pennsylvania (Saeger and Hale 1993), and in soil and water environments in Italy and Spain (Pantanella *et al.* 2007). Violacein and indole-3-carboxyaldehyde are secondary metabolites produced by *J. lividum* that have been shown to inhibit *B. dendrobatidis* (Brucker *et al.* 2008b), and *J. lividum* addition to amphibian skin has been shown to reduce mortality (Harris *et al.* 2009a). The strain used in this experiment was isolated originally from *Hemidactylium scutatum*. Several anti-*Bd* bacteria were isolated from *N. viridescens* for possible use;

however, due to their poor performance in preliminary experiments they were not chosen for use in this experiment. *J. lividum* was chosen due to its efficacy in previous studies as an amphibian probiotic (Harris *et al.* 2009, Becker *et al.* 2009, Muletz *et al.* 2012) and in my preliminary experiments (Appendices 2 & 3).

Lithobates sylvaticus

L. sylvaticus (wood frog) is a terrestrial frog with aquatic larvae that develop in ephemeral pools across the eastern United States. Tadpoles are algae grazers, and therefore were used in this experiment as a component of the ecosystem to facilitate nutrient turnover and to control algae levels (Parris *et al.* 2004). It is known to be susceptible to *Bd*, and infection has been fatal at metamorphosis in laboratory experiments (Gahl *et al.* 2011).

Experimental timeline:

This experiment was conducted for 8 weeks, from 8 May 2012 (Day 0) until 3 July 2012 (Day 56). The experimental ponds were filled and ecosystems were developed from 27 March 2012 to 7 May 2012. The following timeline delineates when the steps of experimental pond development and sampling activities occurred (Table 2).

Table 2: Activity timeline for experimental set up and main experiment sampling.

Date	Day	Activity
6 March 2012	-63	*L. sylvaticus* Egg Mass Collection
27 March 2012	-42	Tank Filling Started
10 April 2012	-28	Tank Filling Ended
12 April 2012	-26	Leaf Litter Introduction
16 April 2012	-22	Plankton Collection & Inoculation 1
18 April 2012	-20	Tadpoles Introduction
29 April 2012	-9	Plankton Collection & Inoculation 2 Started Ecosystem Development

30 April 2012	-8	Started Newt Heat Therapy
7 May 2012	-1	Ended Ecosystem Development
8 May 2012	0	Ended Newt Heat Therapy Pre-sampled Newts Probiotic Bath Started
9 May 2012	1	Pre-sampled Experimental Pond Probiotic Inoculation of the Experimental Ponds
10 May 2012	2	Probiotic bath ended Newts Introduction
11 May 2012	3	Ponds Sampled for culture-based (CB) detection *Bd* Introduction and Exposure
17 May 2012	9	Newt and Water Samples 1 (CB Detection)
24 May 2012	16	Newt and Water Samples 2
4 June 2012	27	Newt and Water Samples 3
14 June 2012	37	Newt and Water Samples 4 Periphyton Sample
24 June 2012	47	Newt and Water Samples 5 Periphyton Sample
3 July 2012	56	Newt and Water Sample 6 (CB Detection) Periphyton Sample

Organism collection and treatment:

Plankton collection

Plankton were collected twice from natural ponds for introduction into the experimental ponds. On 16 April 2012, plankton were collected from Mud Pond in the George Washington National Forest using a plankton net (64 µm mesh size). The collected plankton were rinsed with sterile Provasoli medium to remove any transient *Bd* that may have been present. After rinsing, the plankton samples were transported in sterile Provasoli medium (Wyngaard and Chinnappa 1982). Pond water was not used for transport as it may have contained *Bd*. In the laboratory the collected plankton suspension was transferred into a 35 L aquarium. One aliquot of 100 mL was drawn from the aquarium and added to each of 25 unique containers. This procedure was repeated four more times to help ensure an even distribution of planktonic species to each container. Due to the low abundance of plankton in the first collection, a second collection was

completed on 29 April 2012 at White Oak Flat Pond. As previously explained, plankton were rinsed, pooled and transported in sterile Provasoli. In the lab, five 50 ml aliquots were added to 25 unique containers. For both collections, aquatic invertebrates and insect larvae were removed using tweezers. Each inoculum was assigned at random to the experimental ponds and was added on the day of plankton collection. Ostracods, cladocerans, and copepods were present.

Notophthalmus viridescens collection and heat therapy

Fifty-four adult *N. viridescens* were collected from White Oak Flat Pond on 29 April 2012. Newts were collected using a dip net and then placed in a clean plastic container to be sexed. Only males were collected for the experiment to minimize effects on the local populations and to control for any variation that may be due to gender. Each individual newt was rinsed twice in unique sterile tubes of 20 ml of sterile Provasoli to remove transient bacteria. Each newt was swabbed 10 times (1 swab = up and back) on the ventral surface and once on each foot with a sterile MW113 Fine-tip swab (Medical Wire Equipment, Corsham, Wiltshire, England). New nitrile gloves were worn for handling each newt. After swabbing, each newt was housed in an individual plastic container (16.5cm x 10.2cm x 8.9cm) containing 100-200 ml of Provasoli. Each swab was stored in a 1.5 ml sterile Eppendorf tube on ice until transfer to a – 80°C freezer.

The collected newts were housed for 24 hours at room temperature to allow acclimation to the lab. After 24 hours, the newts underwent a heat therapy regime (30°C, 13 light, 11 dark) for 8 days in order to clear any existing *Bd* infection (Chatfield and Richards-Zawacki 2011, Appendix 7). On days 2 and 5 of heat therapy the newts were transferred to new housing containers with 200 ml of sterile Provasoli. On day 2, each

newt was rinsed individually in a unique Falcon tube containing 20 ml of Provasoli to remove zoospores from the skin. On day 8, each newt was swabbed as previously described to assess the presence, if any, of *J. lividum* and *Bd* on the newts prior to the application of the probiotic bath for newts assigned to that treatment and prior to introduction into the experimental ponds. Photographs of each newt's dorsal spot pattern, which are unique, were taken at the termination of heat therapy for individual identification (Gill 1978).

L. sylvaticus collection and rearing

Two *L. sylvaticus* eggs masses were collected from White Oak Flat Pond in the George Washington National Forest on 6 March 2012. Egg masses were transported to the laboratory in Ziploc containers (16.5cm x 10.2cm x 8.9cm). Once in the laboratory, egg masses were transferred immediately to a 35 L aquarium. A sufficient volume of sterile Provasoli was added to the tank to leave only the top of the masses exposed. An air stone was added to the tank to provide adequate aeration. The tank was kept at approximately 18° C and water was changed every 4-5 days until hatching. Upon hatching the tadpoles were transferred to a new 35 L aquarium containing 15-20 liters of Provasoli. An air stone was used to oxygenate the water. The tadpoles were monitored daily, and the water was changed every 3-4 days. Tadpoles were fed Aquatic Tadpole and Newt Pellets (JurassiPet Diet, Madison, GA) every time the water was changed. Tadpoles were held in the lab until their addition to the experiment ponds on 18 April 2012.

Experimental pond development

Twenty-five stock tanks (Rubbermaid Stock Tank, 567 L, 147 cm(L) x 99 cm(W) x 127 cm(H)) were positioned in a randomized block design in an open field on the land

of Dr. Rickie Domangue in Rockingham County, Virginia. In early April experimental ponds were prepared using the ecosystem development parameters explained by Parris and Cornelius (2004) and Morin (1981). Quantities were modified to account for differences in tank size among studies. Each stock tank contained the following components that were added in the following order:

- 420 L of water (added 27 Mar – 10 Apr 2012)
- 0.25 kg of dry leaf litter (added 12 Apr 2012)
- aliquots of 500 ml and 250 ml of plankton suspension (from collection ponds) (added 16 Apr and April 29 2012)
- 14-16 tadpoles (*Lithobates spp*.) (18 Apr 2012)

After these ecosystem components were added to the stock tanks, they were left undisturbed for 10 days to allow ecosystem development (Parris and Cornelius 2004). Tanks were covered with fiberglass window screening lids with weighted edges to prevent predators or other fauna from disrupting or colonizing the experimental ponds and to keep newts from escaping. Bungee cords and nylon cord were used to secure the lids on the tanks. These lids also provided shading of the established experimental ponds. To provide additional shade to the experimental ponds, a shade canopy was created using a wooden frame and greenhouse cloth that blocked 90% of sunlight (Figure 3). The shade cloth was used to prevent water temperatures from surpassing 30°C, which is the lethal temperature for *Bd*. The experiment was conducted from May to July when such pond water temperatures may be reached unless shading is provided.

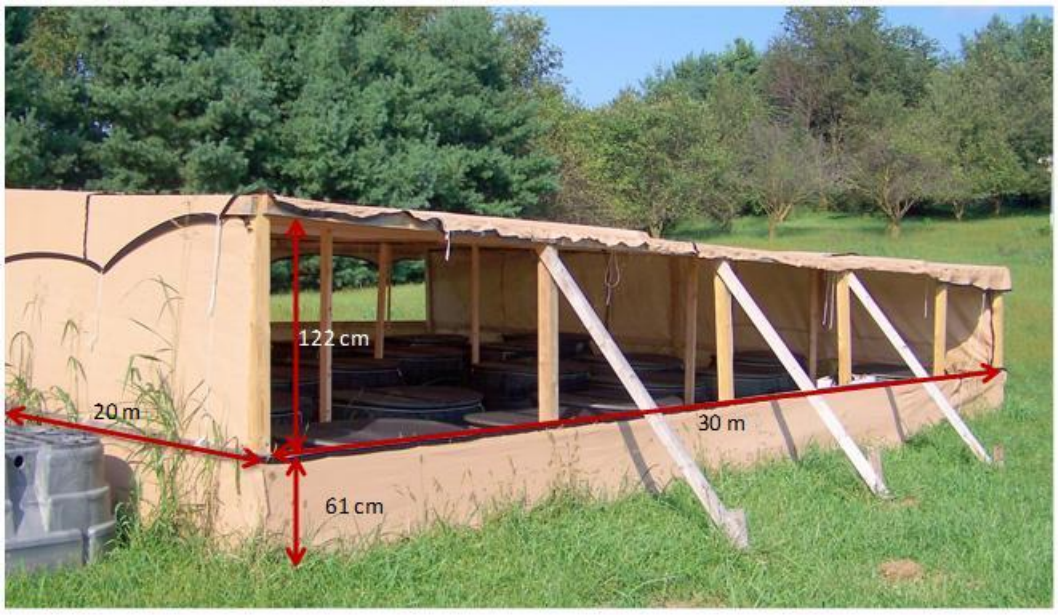


Figure 3: Photograph of shade canopy structure. Shade cloth was secured around the outer perimeter, on the top, and on the east and west sides of the structure.

Selection of bacteria for rifampicin resistance

In order to track the probiotic bacteria added to the experimental ponds using culture-based methods, *J. lividum* was selected for rifampicin resistance, which allowed water samples from the entire aquatic community to be plated on Rif-tryptone plates (1% tryptone, 1 ug/L rifampicin), and only recover rifampicin Resistant (Rif^R) *J. lividum*. To selection for resistance, *J. lividum* was repeatedly cultured on 1% tryptone plates with a rifampicin gradient until growth was seen at the highest rifampicin concentration. At this point, culturing was continued on standard rifampicin-tryptone plates. The resulting Rif^R *J. lividum* was added to experimental ponds and used in probiotic baths.

Inoculation of the experimental ponds with *J. lividum*

One day prior to the introduction of newts to the experimental ponds (day 1), tanks in the water treatment (T3) and water + bath treatment (T1) were inoculated with a *J. lividum* suspension as an environmental bioaugmentation treatment. Tanks were

inoculated with a sufficient quantity of bacteria to create a concentration of approximately 1 x 10^6 cells/ml in each pond. Preliminary investigations demonstrated that this concentration would create a stable, persistent population (Appendix 2). *J. lividum* was cultured in 1% tryptone broth at 25° C for 24 hours. After 24 hours, 5 ml of the broth culture was added to 250 ml of 1% tryptone broth containing sterile 3mm micro-beads. This culture was grown at 25^0C on a rotary shaker at 150 rpm until a usable concentration was detected based on OD measurements. Previously, a growth curve was plotted to determine the relationship between optical density (OD) and colony forming units (CFUs) ($y = 3x\ 10^6(e^{28.868x})$). Once an OD reading was reached that was high enough to obtain the target number of bacteria cells, the cells were washed twice via centrifugation (7500 rpm for 10 minutes) in Provasoli to remove any metabolites that might interfere with the bacterial cell persistence and growth (Harris *et al.* 2009, Muletz *et al.* 2012). The collected cells were re-suspended in 15 ml Provasoli and were added to each tank in T1 and T3. Tanks in the remaining treatments received 15 ml of sterile Provasoli. Each tank was stirred 10 with a sterile PVC pipe in a figure eight pattern to distribute the bacteria.

Probiotic bath treatment

To create the probiotic bath solution, *J. lividum* was cultured in 1% tryptone broth at 25° C for 24 hours as previously described (Harris et al. 2009). After 24 hours, 5 ml of the broth culture was added to 250 ml of 1% tryptone broth containing sterile 3-mm diameter microbeads (Kimble Glass Inc., Vineland, NJ). This culture was grown at 25^0C on a rotary shaker at 150 rpm until the OD of the solution indicated that a high enough concentration was available for use. The target number of bacteria cells (~ 4 x 10^9 per

newt) was washed two times via centrifugation (7500 rpm for 10 minutes) to remove any metabolites that might interfere with bacterial cell persistence and establishment (Harris *et al.* 2009). Using 50 ml Falcon tubes, newts in the probiotic bath only treatment (T2) and water and probiotic bath treatment (T1) were bathed in 15 ml of probiotic *J. lividum* solution (4.1 $x10^9$ cells/ml) for 36 hours. To control for the effects of bathing, newts in the remaining treatments received baths of sterile Provasoli. During bath treatment tubes were rotated and aerated every 10-12 hours.

Bd introduction and exposure

Bd was introduced into the ecosystem by placing a plastic container (16.5 cm x 10.2 cm x 8.9 cm) containing 5 *Bd* culture plates attached to the sides of the container (Figure 4). This *Bd* cube was placed in the center of the bottom of each tank (Figure 4). A preliminary trial showed that introduction of *Bd* to the ecosystems with these cubes led to infection of the newts (Appendix 7). A control treatment received a cube with empty plates. Control plates contained no media to prevent bacterial blooms from occurring on initially uncolonized plates. *Bd* culture plates were made by transferring 1 ml of 5 day old liquid *Bd* culture to the surface of tryptone agar plates. The culture plates were incubated for 5 days before attachment to the cube. Plates were assessed for zoospore activity under the microscope before attachment and were found to contain active zoospores in all cases. Plates were assigned at random to each pond, and were

Figure 4: A *Bd* cube used to introduce *Bd* to the experimental ponds.

attached to the cubes with 100% silicone aquarium sealant and rubber bands. Cubes were filled with water and with a piece of bleached tile to keep them at the bottom of each experimental pond. *Bd* cubes were introduced into the experimental ponds on Day 2 of the experiment. The approximate quantity of *Bd* on each plate was determined by harvesting the zoospores from three extra plates and determining the concentration using a hemocytometer. The average was found to be 1.7×10^7 zoospores/plate (range: 1.4×10^7 - 2.0×10^7 zoospores/plate) and therefore approximately 8.4×10^7 zoospores where introduced into each tank upon initial entry into the tanks, which equates to approximately 208 zoospores/ml. The cubes remained in the tanks until the end of the experiment.

Biosafety: containment of *Bd*

Due to the environmental concerns with the potential release of *Bd* to the surrounding environment, precautionary measures were taken. First, water levels were kept 15-25 cm below the brim of the tank to minimize the risk of *Bd* release in the event of heavy rain. Second, water levels were monitored. If the level reached 3 cm or less from the brim of the tank, water was bailed from the tank into a container containing 10% bleach, which kills *Bd* (Walker *et al.* 2007). Third, the shade canopy structure included a shade cloth barrier that extended from the ground to a height of 61 cm and surrounded the tank array (Figure 3). This prevented amphibians or other wildlife from passing through the tank array and contacting *Bd* in the unlikely event it was accidently released from any of the experimental ponds. Fourth, during sampling appropriate equipment sterilization was performed to prevent release of *Bd*. Lastly, the strain of *Bd* that was used was obtained from the eastern USA (Maine). Since *Bd* is already present in Rockingham

County (Bletz and Harris 2013), its accidental release from the experimental ponds would be unlikely to introduce a novel strain of *Bd* to the local environment.

Newt Sampling: Assays of *J. lividum* and *Bd* on newts

Weight measurement

Weight loss is a sub-lethal effect of *Bd* infection (Berger *et al.* 1998), and it was assessed by weighing newts three times (days 0, 37, and 56) to the nearest milligram. Before weighing, each newt was blotted dry with a sterile paper towel to remove excess moisture and then was placed in a tared sterile petri-dish. Weighing was conducted after swabbing since blotting might have reduced the density of skin bacteria or *Bd*.

J. lividum and *Bd* sampling

Newts were swabbed prior to probiotic bath treatment on Day 0 and routinely throughout the experiment on days 9, 16, 27, 37, 47, and 56. During sample collection newts were swabbed in the following treatment order: *Bd* absent, *Bd* only, bath, water and bath+water. Working from the *Bd* absent treatment to the bath+water treatment minimized the possibility of contamination among experimental ponds of different treatments. Newts within each treatment were captured using a hand-held dip net assigned to each treatment. The collection nets were cleaned in 10% bleach and rinsed three times in well water before capturing each newt within a given treatment. New nitrile gloves were worn for handling each newt during swabbing. Before individuals were swabbed, they were rinsed twice in 20 ml of sterile Provasoli (10 inversions) to remove transient bacteria and any pond debris. Newts were identified and swabbed as previously described. Newts were returned immediately to their respective pond after swabbing. Swabs were stored on ice until transfer into a -80 °C freezer in the laboratory.

Water sampling

Sampling to determine the abundance of *J. lividum* and *Bd* in the water was completed on Days 1, 9, 16, 27, 37, and 56. Water samples were collected by dropping a bleached 5.25 cm PVC pipe into the water column (Figure 6, column 1). Caps for the PVC pipe were attached to a piece of nylon monofilament line to allow the cap to be pulled onto the base of the pipe without human contact with the water. This procedure minimized the potential transfer of bacteria from human skin to pond water. The water sample was filtered through 64 um mesh for plankton sampling (see below) and collected into a sterile 1-L bottle respective to each tank. Seven 60 ml aliquots (560 ml) were filtered through 0.45 µm cellulose nitrate filters (Sartorius Stedim, New York) using a 60 ml Luer-Lok syringe (Becton Dickinson Company, Franklin Lakes, NJ). Filters were held in Swinnex® 47 filter holders (Millipore, Billerica, MA) and were attached to the 60 ml syringe via an adaptor (1/4 in. NPTF to F Luer-Lok) and aquarium tubing (Figure 5).

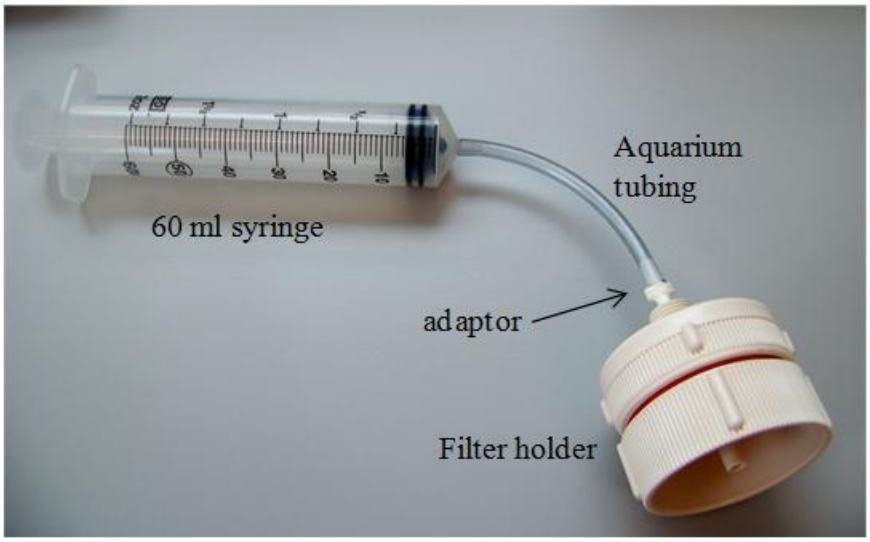


Figure 5: Filtering apparatus used for water sampling for *J. lividum* and *Bd* detection using qPCR-based methods.

Water samples were inverted 5-10 times to ensure mixing before each aliquot was transferred to a syringe for filtering. After filtration, filters were folder with bleached tweezers and placed in sterile 2 ml Eppendorf tubes and stored on ice until transfer to a -80 °C freezer. New sterile syringes were used for each pond sample, and new Swinnex filter holders were used for each treatment. Filter holders, adaptor and tubing were cleaned in 10% bleach solution and rinsed in three consecutive water baths between pond samplings in the same treatment (Walker *et al.* 2007). Control filters were processed to test that the cleaning method was sufficient to clean the filtering apparatus by filtering 120 ml of sterile Provasoli and 120 ml of well water on days 37 and 57 after all tank sampling had been completed. No *J. lividum* or *Bd* was detected in samples after the cleaning process was completed, demonstrating that the cleaning process was sufficient to prevent contamination among water samples. A well water sample was assayed to determine if the well water contained *J. lividum* because it was used for rinsing all sampling instruments after bleaching. A faint band was detected in diagnostic PCR, therefore, it is possible that *J. lividum* was present in the well water. However, the specificity of the traditional PCR primers for *J. lividum* was questionable because multiple bands were obtain when processing preliminary newt samples, thus, it is possible that the primers were amplifying a different, closely related violacein producer.

Water samples taken on days 0, 3, 9, and 56 were used also for culture-based detection of Rif^R *J. lividum* in the laboratory. The remaining water from all other samples was returned to their respective tanks on the day of sampling.

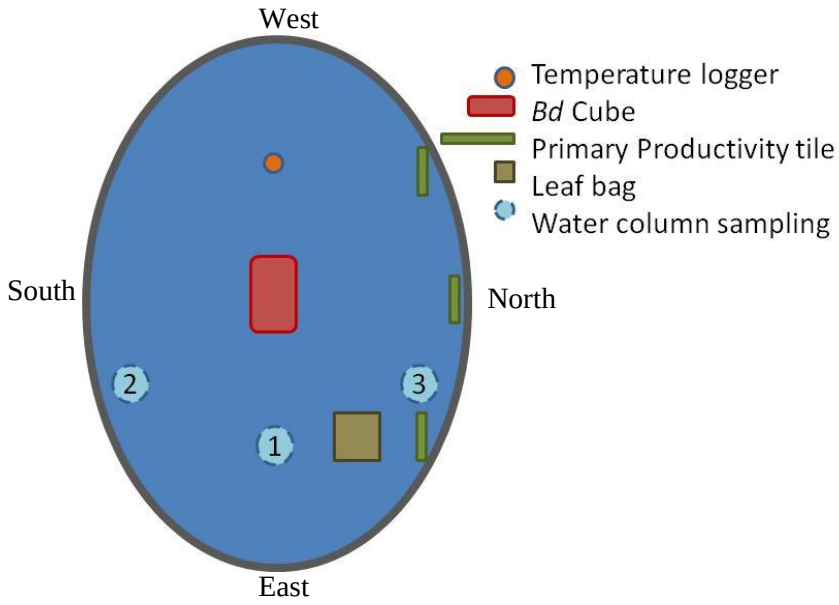


Figure 6: Diagram of experimental pond setup. Water column sample locations are numbered in the order samples were taken.

Cleaning technique under field conditions

For field samples, sampling devices, such as pipes, nets, and filtering devices, were bleached for sterilization and then rinsed in well water. Rinsing was done to minimize transfer of bleach to experimental ponds and to prevent degradation of DNA in filter samples. Rinsing in sterile medium was not practical given the volume that would be required and well water, which was not sterile, was used. There was no bias among treatments in how the sampling devices were bleached and rinsed.

Detection of *J. lividum* and *Bd*

Newts

DNA was extracted from the swabs using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's protocol with minor volume adjustments of Buffer ATL and AL, which were decreased to 200 µl. In addition, 50 µl of

buffer AE was added in the final elution step. These changes were made to maximize use of kit reagents during extraction and because they were found to be adequate in previous trials.

The conventional polymerase chain reaction (PCR) primers and amplification conditions presented in Annis *et al.* (2004) were used to determine if newts were *Bd*-positive prior to experimentation. For the experimental samples, probe-based quantitative PCR (qPCR) was performed in order to quantify the *Bd* zoospore load on each newt using the primers and probe presented in Hyatt *et al.* (2007). KlearKall MasterMix (KBioscience, Herts, England) was used instead of Taqman Universal MasterMix. Due to this change, the qPCR amplification conditions presented in Hyatt *et al.* (2007) were modified to have an activation step of 15 minutes. This step was required due to the nature of the KlearKall taq enzyme. Twenty-five microliter qPCR reactions containing 5 µl of DNA template, 2.3 µl of each primer (stock:10 µM), 0.6 µl of probe (stock:10µM), 12.5 µl of KlearKall PCR Mix (KBioscience, Herts, England) and 5.5 µl PCR-grade H_2O were completed. Standards of the following concentrations were made using the *Bd* 404 strain: 10^5, 10^4 , 10^3, 10^2, 10^1, and 10^0 zoospore equivalents and run along with all qPCR reactions.

To determine the presence of naturally-occurring *J. lividum* on the newts prior to experimentation and the abundance throughout the experiment, extracted DNA from newt swabs was analyzed via probe-based qPCR. An unpublished *J. lividum* quantification protocol designed by V. Vredenburg was used. The primers and probe for *J. lividum* qPCR were developed from the violacein gene. The primers had the following sequences: Forward-3'-ATG CCA CCG ACG GCT AC A-5', Reverse- 3'-ACG GCG

GGA TGG TCA TCA C- 5', and the probe sequence was 5'- 6FAM ACC ATC GTT TGC TGT CCG TTG A MGBNFQ - 3'. Twenty-five microliter (µl) PCR reactions contained 5 µl of DNA template, 0.5 µl of each primer (stock:10 µM), 0.375 µl of probe (stock:10 µM), 12.5 µl of KlearKall PCR Mix (KBioscience, Herts, England) and 6.125 µl PCR-grade H_2O. Amplification reactions were completed with the following conditions: a pre-incubation for 15 minutes at 95°C, followed by 50 cycles of 15 seconds at 95°C for denaturation, 30 seconds at 58°C for annealing, and 30 seconds at 65°C for extension. To create standards of known concentrations, DNA was extracted from a known number of J. *lividum* cells from pure culture using the DNeasy Blood and Tissue Kit. Standards of 10^6-10^0 cell equivalents were amplified along with qPCR reactions to estimate the number of cell equivalents on each newt. Taqman Exogenous Internal positive controls (Invitrogen-TaqMan®) were included following the manufacturer's protocol in one replicate of all newts samples from day 9 to test for PCR inhibition.

All qPCR reactions were completed on a Bio-RAD CFX60 Touch (Bio-RAD, Hercules, CA). For both *J. lividum* and *Bd* quantification, DNA extract samples were run in duplicate and if there was a discrepancy between the duplicates where one indicated a positive sample and the other indicated a negative sample, a third reaction was run.

Water

Culture-based detection

Water samples from days 0, 3, 9 and 56 were used for detecting the Rif^{-R} *J. lividum* added to the experimental ponds. On Day 0, 3, and 9 dilution series of the collected samples were made and 100 µl of each dilution were plated on rif-tryptone plates. On day 56, two 100 µl aliquots were plated directly from the water samples

because the abundance of Rif^{-R} *J. lividum* was expected to be low. Plates were incubated for 48 hours after plating, and colony forming units were counted to determine the concentration of Rif^{-R} *J. lividum* in each tank.

qPCR-based detection

To determine the abundance of *J. lividum* and *Bd* in the aquatic environment in each experimental pond, DNA was extracted from one half of the 0.45 µm filters using a Qiagen DNeasy Blood & Tissue Kit using the manufacturer's protocol. DNA extracts from day 0, 9, 16, and 27 were analyzed via quantitative PCR. The same parameters and primer sets previously described for newt *J. lividum* and *Bd* detection were used.. For both *J. lividum* and *Bd* quantification, duplicate samples were processed, and if there was a discrepancy between the duplicates a third reaction was run. Internal positive controls were also run in one replicate of each samples from day 9 to test for PCR inhibition

Ecosystem monitoring

Four ecosystem measurements, including leaf decomposition rate, periphyton production, and zooplankton community composition were completed to determine if the probiotic bioaugmentation of the water or newts had non-target effects on the pond ecosystem. In addition, ecosystem observations including whether the bottom of the tank was visible and the status of algal growth in the tank walls were recorded every 3-4 days.

Leaf decomposition

To assess leaf decomposition, leaf bags were placed in the experimental ponds for the duration of the experiment. Leaf bags (25 cm x 25 cm) were constructed using black fiberglass window screening. Edges were sealed using a soldering iron. Leaf litter was

collected from the GWNF on 8 April 2012 and dried in a drying oven at 80° C for 24 hours before being placed in leaf bags. Approximately 6-10 g of leaf litter was placed into each bag, and the bags were assigned to tanks at random. On day 0, bags were placed in the northeast corner of each tank (Figure 6). Bags were removed on day 56 and the remaining leaf litter was removed. The leaf litter was collected into foil packets and placed in a drying oven for 72 hours. After drying packages were weighed, final leaf litter weights were determined. The proportion of mass lost per leaf bag was determined. The proportion of mass lost was divided by the length of the experiment, 56 days, to obtain a leaf decomposition rate of each experimental pond.

Periphyton production

Periphyton production was assessed as a measure of primary productivity (APHA 1998). Three bleached ceramic tiles (25.4 cm x 25.4 cm) were placed on the north side of each tank (Figure 6) on day 0. On days 37, 47, and 56 of the experiment one tile was removed and the algal growth was scraped off, dried and weighed. To equalize the scrapping on each tile, the following procedure was followed: using a glass microscope slide (2.54 cm x 7.62 cm), the tile was scraped 10 times from top to bottom, then rotated 90 degrees and process was repeated. Next, the tile was rinsed with a dilute ethanol solution and scraped in the same manner one more time. The obtained algal growth was dried for 48 hours at 80 ˚C in a drying oven. Following drying, algal dry mass per cm^2 was determined.

Zooplankton community composition and structure

Zooplankton communities collected from Mud Pond and White Oak Flat Pond were added to the experimental ponds and monitored throughout the 8-week experiment. Sampling was completed two days prior to the bacterial inoculation of the ponds (day 1) and on days 9, 16, 27, 37, 47, and 56 of the experiment. To sample planktonic communities, water columns were collected at three locations in each tank using a bleached 5.25 cm PVC pipe as previously described for water sampling for *Bd* and *J. lividum* (Figure 6). The collected water was filtered through a nylon mesh filter (64 µm) to collect plankton. Water from the first column was kept for *Bd* and *J. lividum* abundance measurements as described earlier, and water from the second and third column was returned to the tanks. Plankton from the filters were rinsed into collection vials with 95% ethanol to preserve the samples for later identification and enumeration. PVC pipes and filters were cleaned in a ~10% bleach solution followed by three water rinses between each pond sampling, and different pipes were used for each treatment to ensure no cross contamination occurred.

Zooplankton communities on days 1, 16, 37, and 56 were assessed using a dissecting microscope. Using a plankton counting wheel, the total number of cladocerans, copepods (cyclopoids and calanoids), and ostracods were counted. Identifications were verified by Dr. Grace Wyngaard. The concentration of each zooplankton group per liter was determined, and the total abundance of cladocerans, copepods and ostracods was also calculated.

Temperature monitoring

Hobo® (Onset®, Porcasset, MA) temperature loggers were used to monitor the temperature of each experimental pond. Loggers were programmed to measure the temperature every 30 minutes for the duration of the experiment. Each logger was attached to a stainless steel weight with nylon filament to hold it at the base of the ponds. The top of the loggers was suspended approximately 25-30 cm from the base of the ponds in the center on the west side of the ponds (Figure 6).

Statistical analyses

All statistical analyses were performed using SAS statistical software (SAS Institute Inc. Cary, North Carolina). The procedure MIXED was used for continuous dependent variables, and GLIMMIX was used for categorical dependent variables. For all PROC MIXED and PROC GLIMMIX analyses, the experimental treatments were treated as a fixed effect and Block, Block*Treatment (i.e., Tank), and Newt (Block*Treatment) in GLIMMIX only were included as random effects (Rickie Domangue, pers. comm.). In addition, the Kenward-Roger and Satterthwaite method were used to obtain the correct degrees of freedom for all MIXED and GLIMMIX procedures, respectively.

The experimental design had five treatments, in a replicated, randomized block design (Figure 2). Within this design there was a two-way factorial design where two factors were manipulated in a *Bd* positive environment: presence and absence of a probiotic bath (bath treatment) and presence and absence of probiotic inoculation of the experimental ponds (water treatment). These treatments were analyzed with a two-way ANOVA design in PROC MIXED and PROC GLIMMIX to obtain main and interaction

effects. To evaluate *a priori* hypotheses (Table 1), specific treatment comparisons were made using ESTIMATE statements, including comparisons to the *Bd* absent treatment.

Newts

Two newts in the *Bd* only treatment and two newts in the *Bd* absent treatment had *J. lividum* prior to the start of the experiment and were removed from the analysis because they compromised the nature of the *Bd* only treatment, which did not have probiotic treatment.

The effects of treatment on newt proportional weight loss were analyzed using mixed model analyses in PROC MIXED to determine if the bath treatment, the water treatment or the bath+water treatment reduced weight loss experienced by the newts associated with *Bd* infection. The newt weights on day 37 were the dependent variable and the newt weights on day 0 (pre-experiment) was designated as a covariate to adjust for the initial weight of the newts in the model.

The effects of treatment on *Bd* on the newts were analyzed in two ways to test the hypothesis that probiotic treatment would lower the prevalence of infected individuals or *Bd* loads on the newts. Prevalence of newts infected with *Bd* was analyzed using mixed logistical regression analyses in PROC GLIMMIX. A logistical regression approach is appropriate when the response variable is binary (infected or not infected). The *Bd* absent treatment was excluded from this analysis because the predominance of uninfected newts across all experimental ponds in this treatment prevented model convergence. Instead, infection prevalence in the *Bd* absent treatment was compared to the *Bd* only treatment

using a Fisher's exact test with experimental ponds as experimental units (n=5) to determine if *Bd* introduction effectively increased the prevalence of infected individuals.

Bd loads and proportional change in *Bd* loads on the newts were analyzed using mixed model analyses in PROC MIXED. *Bd* loads on the newts were normalized using a log transformation to meet the assumptions of PROC MIXED. To determine if *Bd* introduction had effectively increased the *Bd* loads in the *Bd* positive treatments, *Bd* loads on the newts on day 9 were analyzed as the dependent variable. To investigate the effects of treatment on the *Bd* loads in the newts, proportional change in *Bd* loads was analyzed by treating *Bd* loads on the newts on day 16 as the dependent variable and *Bd* loads on the newts on day 9 as a covariate to adjust for *Bd* loads on the previous sample day in the model. In addition, *Bd* loads on day 27 were analyzed with *Bd* loads on day 9 as a covariate.

Three analyses were performed to investigate *J. lividum* on the newts. To investigate the efficacy of the three probiotic treatments in effectively establishing *J. lividum* on the newts, Prevalence of newts with *J. lividum* on Day 9 was analyzed using mixed logistical regression analyses in PROC GLIMMIX. The effects of treatment on *J. lividum* abundance on the newts were also analyzed in PROC MIXED using mixed model analyses. Average *J. lividum* abundance over days 9, 16, and 27 was used as the dependent variable. Average *J. lividum* abundance on newts was normalized using a log transformation to meet the assumptions of PROC MIXED.

To determine if persistence of the probiotic on the newts over the first 27 days differed among treatments, Fisher's exact tests were used. The number of newts that had *J. lividum* continuously on days 9, 16, and 27 was used as the dependent variable.

Water

To determine the if *Bd* introduction into the tanks was successful and whether treatment affected the abundance of *Bd* in the water, *Bd* abundances on day 9 were analyzed as the dependent variable using mixed model analyses in PROC MIXED. To determine how long the *Bd* environment persisted, *Bd* abundances on day 16 were also analyzed in PROC MIXED. *Bd* abundances on day 27 were not analyzed because the *Bd* in the experiment ponds was absent from most ponds. *Bd* abundances were normalized using a log transformation to meet the assumptions of PROC MIXED.

To determine if *J. lividum* introduction into the tanks was effective at establishing a reservoir of *J. lividum* in the experimental ponds and how long the *J. lividum* reservoir persisted both culture-based and molecular-based abundances were analyzed using mixed model analyses in PROC MIXED. Culture-based *J. lividum* abundances from day 3 were analyzed as the dependent variable to determine if introduction of Rif^{-R} *J. lividum* was effective at creating a environmental reservoir. Molecular-based estimates of abundance on days 9, 16, and 27 and culture-based abundances from day 9 were analyzed separately to determine how long the *J. lividum* reservoir persisted. Data were normalized using log transformation to meet the assumptions of PROC MIXED.

Ecosystem measurements

The effects of the four treatment combinations on three ecosystem variables -- leaf decomposition rate, periphyton production rate, and zooplankton community structure -- were analyzed using PROC MIXED to determine if probiotic bioaugmentation had effects on these aspects of the ecosystem. More specifically, pair-wise comparisons comparing all treatments to the *Bd* absent treatment, which had no *J. lividum* added, were used to assess ecosystem effects using ESTIMATE statements in PROC MIXED. The *Bd* absent treatment was an unmanipulated control and was considered as an ecosystem control. In addition, by comparing the *Bd* only treatment to the *Bd* absent treatment it was possible to investigate the effect of *Bd* on these three ecosystem variables. For analyzing leaf decomposition, the proportion of mass lost per day was the dependent variable. For analyzing periphyton production the average periphyton production rate over time was used as the dependent variable.

Zooplankton data from days 1, 16, 37, and 56 were used for zooplankton community analysis. Ostracods were rarely present in the samples and therefore were not included in analyses related to variance. Zooplankton community structure was analyzed in two ways: 1) using average total abundance per liter as the dependent variable and 2) using the variance ratio derived from the variance ratio method as the dependent variable (Downing *et al.* 2008). The average total abundance was calculated by averaging the total zooplankton abundance per liter across the 4 sampling days. Total abundance was log transformed to meet the assumptions of PROC MIXED. The variance ratio was calculated with the following formula:

$$Ratio = \frac{temporal\ variance\ of\ total\ zooplankton\ abundance}{sum\ of\ the\ variance\ of\ individual\ species\ or\ groups}$$

This ratio has been used to investigate the zooplankton community response to environmental perturbation, such as pH (Klug *et al.* 2000). Therefore, in the context of this experiment it was used to look at whether the addition of the probiotic to the experimental ponds cause the zooplankton community to respond differently than the unmanipulated control experimental ponds. The variance of the total abundance (numerator) is equal to the sum of the individual species variance and their covariances. Therefore, by dividing by the summation of the variances of individual groups, this ratio characterizes the covariation among groups as independent (~ 1), compensatory (<1) or synchronous (>1). If groups vary independently then their covariance is zero and the numerator and the denominator will be equal and thus the variance ratio will be 1. If groups are responding in a compensatory manner, they are negatively correlated, and their covariance will be negative. In this case, the numerator will be less than the denominator, and therefore, the variance ratio will be less than 1. If groups are responding synchronously, they are positively correlated, and their covariance will be positive. In this case the numerator will be greater than the denominator, and the variance ratio will be greater than 1.

Correlations between *Bd* and *J. lividum*

For all correlation analyses, data points containing 0 for both variables were omitted from analysis because we were interested in knowing the responses of one variable to the other.

Correlation between J. lividum in the water and on the newts

To determine if the abundance of *J. lividum* in water predicted whether *J .lividum* was presence on a newt, data from days 9,16, and 27 from the water treatment were analyzed using a logistic regression in PROC LOGISTIC. The bath treatment and the bath+water treatment were omitted from analysis because newts in these treatments had received *J. lividum* bath treatment. For analysis, *J. lividum* abundance in the water was a continuous numerical variable and *J. lividum* on newts was a binary response variable (absence or presence).

Correlation between Bd in the water and on the newts

To determine if the *Bd* abundance in the water was correlated to *Bd* on the newts, abundance data from the *Bd* only treatment on day 9 and 16 and 27 was grouped and analyzed using a nonparametric Spearman's rank correlation test in PROC CORR. A nonparametric correlation was used because the data could not be normalized.

Correlation between Bd and J. lividum abundance in the water

To determine if *Bd* abundance in the water was correlated to *J. lividum* abundance in the water, data from the all four *Bd* positive treatments from day 9, 16, and 27 were pooled. The pooled data were analyzed with a nonparametric Spearman's Rank Correlation Test using PROC CORR. A nonparametric correlation was used because the data could not be normalized.

Correlation between Bd loads and J. lividum abundance on the newts

To determine if *Bd* loads on the newts were correlated with *J. lividum* on the newts, data from the bath+water treatment, the bath treatment, the water treatment and the Bd only treatment from day 9, 16, and 27 were pooled. The pooled data were analyzed with a nonparametric Spearman's Rank Correlation Test using PROC CORR. A

nonparametric correlation was used because the data could not be normalized. In addition, a logistic regression was completed using PROC LOGISTIC to further investigate the relationship between *Bd* and *J. lividum* on the newts. *Bd* was treated as a binary response variable (presence or absence) and *J. lividum* abundance was a continuous predictor variable.

Correlation between Bd loads on the newts and J. lividum abundance in the water

To determine if *Bd* loads on the newts were correlated with *J. lividum* in the water, data from the water treatment and the *Bd* only treatment on day 9, 16, 27 was pooled, and a nonparametric Spearman's Rank Correlation was used for analysis. The bath+water treatment that also had *J. lividum* treatment of the water was omitted due to the newts in this treatment receiving a probiotic bath, which could influence the *Bd* loads on the newts. A nonparametric correlation was used because the data could not be normalized.

RESULTS

Newts

Analysis of morbidity effects from *Bd* across treatments

All newts survived the experiment, with the exception of one newt in the water+ treatment, that likely escaped the experimental pond because its carcass was never found. Pair-wise statistical comparisons were made to investigate specific *a priori* hypotheses (Table 1). To test if *Bd* increased the weight loss experienced by the newts, a comparison between the *Bd* only treatment and the *Bd* absent treatment was made. Weight loss experienced by the newts between day 0 and day 37 did not differ between the *Bd* only and *Bd* absent treatment ($t= 0.13$, $df = 42$, $p = 0.898$, Figure 7).

Although there is no evidence that *Bd* negatively affected proportional growth rate, there was an interaction between the bath and water treatment ($t = 2.05$, $df = 42$, $p= 0.047$, Figure 7). The hypothesis that newts in the bath+water treatment would exhibit less weight loss in comparison to the bath or the water treatments alone was tested to explore the interaction. The bath+water treatment had significantly less weight loss than the water treatment and marginally less than the bath treatments (Bath: $t = 1.83$, $df = 42$, $p = 0.074$; Water: $t = 2.05$, $df = 42$, $p = 0.047$, Figure 7). Therefore, This interaction arose because the combination of bath and water inoculation treatment had less weight loss than either treatment by itself and it surpassed the additive effects of these treatments, leading to a beneficial effect on growth in the bath+water treatment. There was no main effect of the bath+ treatment ($t= 0.91$, $df = 42$, $p=0.366$) or main effect of the water+ treatment ($t= 0.54$, $df = 42$, $p = 0.5916$) in terms of weight loss of the newts.

The interaction between the bath and water treatments in terms of weight loss generated a new hypothesis that probiotic treatment may reduce weight loss regardless of *Bd* infection. To test the hypotheses that bath, water, or bath+water treatment reduced the weight loss experienced by the newts, pair-wise comparisons between probiotic treatments and the pooled *Bd* only and *Bd* absent treatment were made. These treatments were pooled because both did not have probiotic treatment and the proportional weight loss in these treatments was not significantly different. Weight loss experienced by the newts in the bath treatment, the water treatment, and the probiotic bath+water treatment did not differ from the pooled treatments with no probiotic (Bath: $t = -.86$, $df = 42$, $p = 0.394$; Water: $t = -1.15$, $df = 42$, $p = 0.257$; Bath+Water+: $t = 1.25$, $df = 42$, $p = 0.217$ respectively) (Figure 7). Because there was no difference in growth rate between the *Bd* only treatment and the *Bd* absent treatment or the *Bd* only treatment and the probiotic treatments, comparisons between the probiotic treatments and the *Bd* absent control were not investigated.

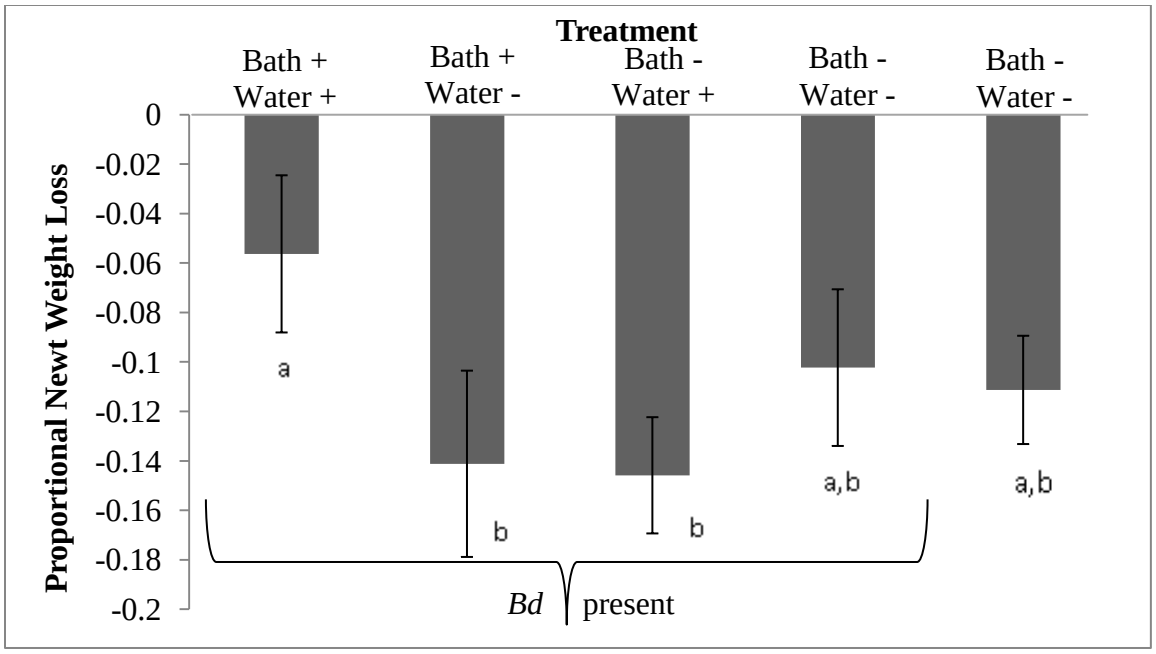


Figure 7. Average proportional weight loss of newts between day 0 and day 37 for each treatment. Bath + indicates the treatment of newts with a *J. lividum* bath and Water + indicates the treatment of the pond with *J. lividum.* Error bars show the standard error of each treatment. Letters represent statistically significant differences.

Prevalence of newts infected with *Bd*

All newts were negative for *Bd* on day 0 before the start of the experiment. All newts in *Bd*-exposed treatments became infected by day 9, and two newts in the *Bd* absent treatment were infected on day 9 (Figure 8). To test whether infection prevalence was greater in the *Bd*-exposed treatment, a comparison of the number of infected individuals in the *Bd* only treatment and the *Bd* absent treatment was made. There was a significantly higher prevalence of *Bd* infection in the *Bd* only treatment than in the *Bd* absent treatment on days 9 and day 16 (Day 9: Fisher's Exact Test, n = 5, p = 0.048; Day 16: Fisher's Exact Test, n = 5, p = 0.008).

On day 9, all newts in the probiotic treatments and in the *Bd* only treatment were infected (Figure 8); however, on day 16 the infection prevalence differed among

treatments. Comparisons were made to test whether probiotic treatment decreased the infection prevalence on day 16. There was no main effect of probiotic bath treatment on prevalence of infection of newts ($t = -0.29$, $df = 36$, $p = 0.7752$), and there was no main effect of water treatment on prevalence of infection of newts ($t = -1.26$, $df = 36$, $p = 0.215$). However, there was a marginally significant interaction between the bath and water treatments ($t = -1.92$, $df = 36$, $p = 0.063$). This interaction was due to the bath+water treatment on day 16 surpassing the additive effects of the bath and water treatments, leading to a greater, synergistic reduction in the prevalence of *Bd* infection of newts. Pair-wise comparisons between the bath and water treatment to the bath+water treatment were investigated to explain this interaction. The prevalence of infection of newts in the bath+ water treatment was significantly lower than the infection prevalence in the bath treatment ($t = -2.09$, $df = 36$, $p = 0.043$, Figure 8), and was marginally lower than the infection prevalence in the water treatment ($t = -1.73$, $df = 33.93$, $p = 0.063$, Figure 8).

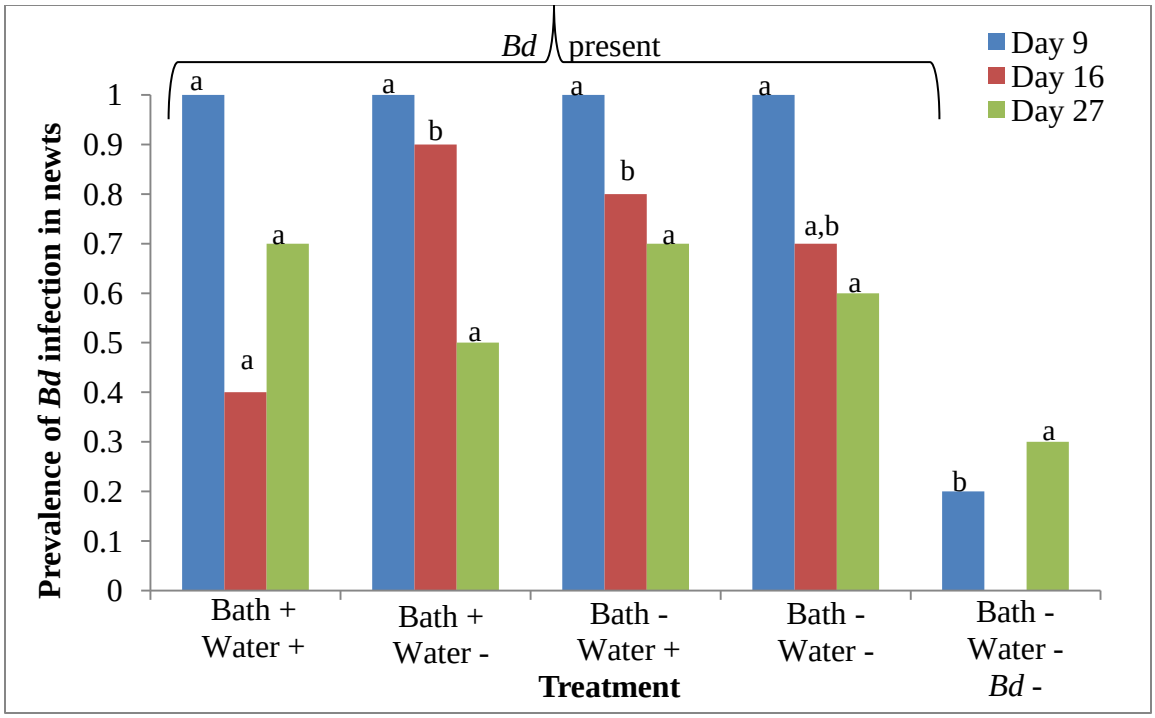


Figure 8. *Bd* infection prevalence on newts over time for each treatment. Colors represent different sample days. Letters represent statistically significant differences within a given sample day.

Additional pair-wise statistical comparisons were analyzed to investigate specific *a priori* hypotheses (Table 1). Although the bath+water treatment reduced infection prevalence more than either the bath or water treatment alone, there was no difference in infection prevalence between the bath+water treatment and the *Bd* only treatment ($t = -1.30$, $df = 26.31$, $p = 0.204$). In addition, the prevalence of *Bd* infection did not differ between the bath treatment and the *Bd* only treatment ($t = 1.06$, $df = 36$, $p = 0.297$) or between the water treatment and the *Bd* only treatment ($t = 0.50$, $df = 35.28$, $p = 0.618$). Because no probiotic treatment reduced infection prevalence below that found in the *Bd* only treatment, it did not make sense to investigate whether probiotic treatments were equal to the *Bd* absent treatment. Thus, treatment comparisons between probiotic treatments and the *Bd* absent treatment were not investigated.

Differences between infection prevalence were lost by the day 27 because the infection prevalence of the bath+water increased to levels equal to the other treatments (Figure 8, Table 3).

Table 3. Mixed logistic regression model statistics for infection prevalence on day 27.

Comparison	**t value**	**df**	**p value**
Main Effect of Water treatment	1.16	35	0.255
Main Effect of Bath treatment	-0.55	35	0.584
Interaction Effect	0.00	35	0.998
Bath+Water to Bath	0.86	35	0.397
Bath+Water to Water	-0.36	35	0.717
Bath+Water to *Bd* only	0.44	35	0.661
Bath to Water	-1.17	35	0.251
Bath to *Bd* only	-0.42	31.17	0.675
Water to *Bd* only	0.78	35	0.439

Bd abundance on newts

As previously mentioned all newts in the *Bd*-exposed treatments were infected with *Bd* on day 9. To assess abundance, pair-wise comparisons were made between the loads of *Bd* zoospores on the newts in *Bd*-exposed treatments and the *Bd* loads on the newts in the *Bd* absent treatment,. All *Bd*-exposed treatments had significantly greater *Bd* loads on the newts on day 9 in comparison to the *Bd* absent control (Bath+: $t = 6.55$, $df = 20$, $p < 0.001$; Water+: t 10.34, $df = 20$, $p < 0.001$; Bath+Water: $t = 8.13$, $df = 20$, $p < 0.001$; *Bd* only: $t = 7.16$, $df = 20$, $p < 0.001$, Figure 9).

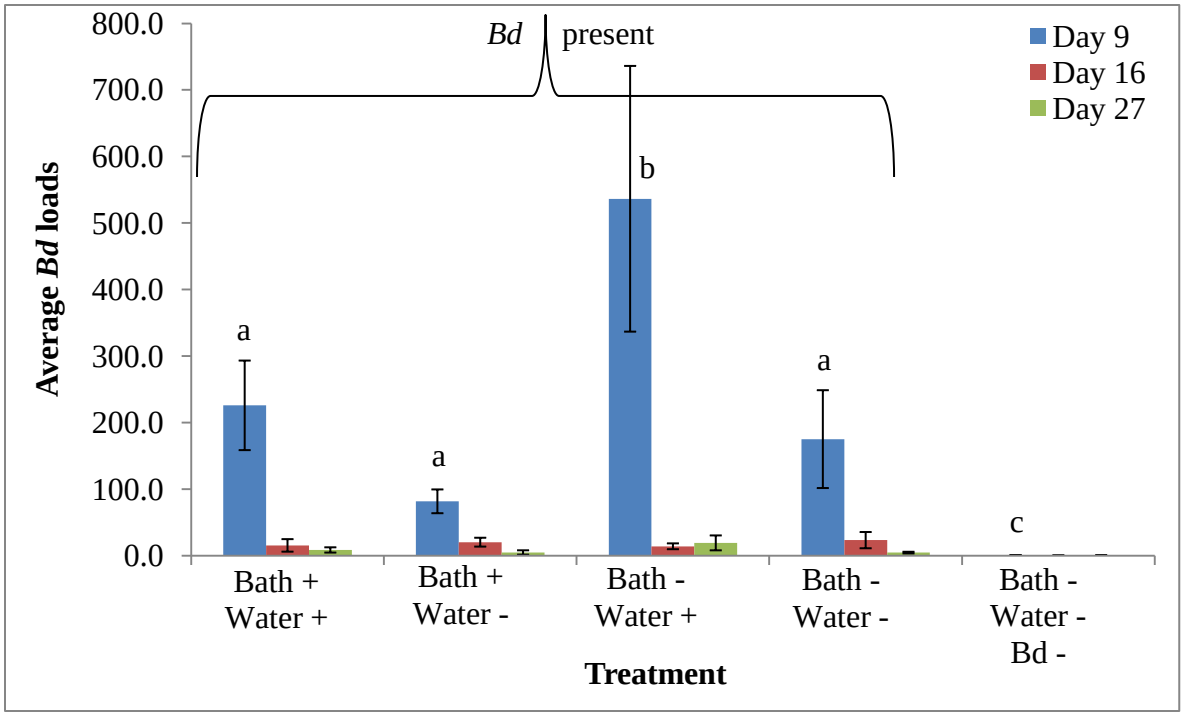


Figure 9. Average *Bd* loads on newts in each treatment on days 9, 16, and 27. Error bars represent standard error. Colors represent different sample days. *Bd* loads on day 9 were different between treatments. Statistically significant differences among treatments on day 9 are indicated by letters. No significant differences were observed on day 16 and 27.

To assess whether probiotic treatments reduced *Bd* loads on newts, proportional change in *Bd* loads was compared among probiotic treatments and the *Bd* only treatment. Proportional change in *Bd* loads differed among treatments. On day 16 there was a main effect of the water treatment ($t = -2.67$, $df = 24.6$, $p = 0.0106$). There was no main effect of the bath treatment ($t = 0.88$, $df = 21.5$, $p = 0.3728$) and no significant interaction in proportional change in *Bd* loads on the day 16 ($t = -0.91$, $df = 20.3$, $p = 0.3728$). Specific pair-wise comparisons were investigated to explain the main effect of water treatment. The bath+water treatment on day 16 had greater proportional loss of *Bd* zoospore loads in comparison to the bath treatment ($t = -2.77$, $df = 20.9$, $p = 0.011$); however, did not differ from the water treatment ($t = -0.01$, $df = 21.9$, $p = 0.9989$). The water treatment also exhibited greater proportional loss of *Bd* zoospore loads than the bath treatment ($t = 2.47$, $df = 25.8$, $p = 0.021$). The greater proportional change in *Bd* loads in the water treatment

than the bath treatment and in the bath+water treatment compared to the bath treatment is driving the main effect of the water inoculation.

Additional pair-wise statistical comparisons were analyzed to investigate specific *a priori* hypotheses (Table 1) with respect to proportional change in *Bd* loads on newts. The comparison of the *Bd* only treatment to the *Bd* absent treatment with respect to proportional change in *Bd* loads on the newts was not investigated. The *Bd* absent treatment was expected to have no *Bd* and because one cannot divide by 0 proportional change could not be calculated. Pair-wise comparisons were used to test whether the bath treatment, the water treatment or the bath+water treatment showed greater proportional loss in *Bd* zoospore loads on day 16 than in the *Bd* only treatment. None of the probiotic treatments had significantly different proportional changes in *Bd* loads than the *Bd* only treatment (Bath: $t = 1.30$, $df = 19.9$, $p = 0.204$; Water: t -1.39, $df = 24.1$, $p = 0.1775$; Bath+Water: $t = -1.53$, $df = 20.1$, $p = 0.142$).

On day 27, there were no significant differences between any treatment comparisons with respect to the proportional change in *Bd* loads on the newts (Table 4).

Table 4. Mixed model statistics for proportional change in *Bd* loads on newts on day 27.

Comparison	**t value**	**df**	**p value**
Main Effect of Water treatment	0.23	24	0.822
Main Effect of Bath treatment	0.76	21.1	0.459
Interaction Effect	0.71	19.8	0.485
Bath+Water to Bath	0.67	20.4	0.5080
Bath+Water to Water	1.01	21.4	0.3242
Bath+Water to *Bd* only	0.74	19.7	0.4696
Bath+Water to *Bd* absent	-0.42	35	0.6765
Bath to Water	0.32	25.1	0.7517
Bath to *Bd* only	0.05	19.4	0.9608
Bath to *Bd* absent	-0.95	32	0.3494
Water to *Bd* only	-0.29	23.6	0.7743
Water to *Bd* absent	-0.90	37.9	0.3762

Transmission efficacy of probiotic treatment

All newts in the three probiotic treatments were negative for *J. lividum* at the beginning of the experiment. To investigate the efficacy of the three probiotic treatment methods in establishing *J. lividum* on the newts, the number of newts that became positive for *J. lividum* on day 9 was compared among treatments. Ninety percent of the newts in the bath+water treatment became positive whereas 60% of the newts in the bath treatment and 40% of the newts in the water treatment became positive on the day 9 (Table 5). The bath+water was marginally more effective at transmitting *J. lividum* to the newts than the water treatment ($t = 1.54$, $df = 25$, $p = 0.062$); however, there was no difference between the bath+water treatment and the bath treatment ($t = 1.25$, $df = 25$, $p = 0.112$). Additionally there was no difference between the bath treatment and the water treatment ($t = 0.36$, $df = 12$, $p = 0.36$). However, 2 newts in the *Bd* only treatment also became positive for *J. lividum* on day 9 (Table 5). It was not possible to distinguish if newts were positive for the inoculated Rif^R *J. lividum* versus other naturally-occurring

strains of *J. lividum* with molecular methods. However, the bath+water treatment had significantly greater transmission than the *Bd* only treatment ($t = 2.42$, $df = 29$, $p = 0.011$), but the bath treatment and water treatment were not (Bath: $t = 1.46$, $df = 15$, $p = 0.08$; Water: $t = 1.12$, $df = 15$, $p = 0.14$).

Table 5. Transmission efficacy of probiotic treatments. Number of newts in each treatment with *J. lividum* on day 9.

Treatment	**# of newts with *J. lividum* on day 9 (n = 10)**
Bath+	6
Water+	4
Bath+Water	9
***Bd* only**	2

J. lividum abundance on the newts

The average *J. lividum* abundance on the newts over the three sample days (9, 16, and 27) was greater in the probiotic treatments than in the *Bd* only and *Bd* absent treatment (Table 6, Figure 10). In addition, there was a main effect of the bath treatment and water treatment (Bath: $t = 2.71$, $df = 40$, $p = 0.01$; Water: $t = 2.98$, $df = 40$, $p = 0.005$).

Table 6. Mixed model statistics for pair-wise comparison of all probiotic treatments to the *Bd* only and *Bd* absent control with respect to average *J. lividum* abundance on the newts.

Treatment comparisons	**df**	**t value**	**p value**
Bath+Water to *Bd* only	40	3.96	0.0003
Bath+Water to *Bd* absent	40	2.46	0.0183
Bath to *Bd* only	40	3.29	0.0021
Bath to *Bd* absent	40	1.80	0.0800
Water to *Bd* only	40	3.40	0.0015
Water to *Bd* absent	40	1.94	0.0598

Furthermore, there was also a significant interaction between the bath and water treatment ($t = -2.02$, $df = 40$, $p = 0.05$). Pair-wise comparisons between the bath and water treatment to the bath+water treatment were investigated to explain this interaction. To determine if the bath+water treatment lead to greater abundances of *J. lividum* on the newts, pair-wise comparisons between the bath+water treatment and individual the bath and water treatments were made. The *J. lividum* abundance on the newts did not differ between the bath+water treatment and the bath treatment ($t = 0.70$, $df = 40$, $p = 0.485$) or between the bath+water treatment and the water treatment ($t = 0.49$, $df = 40$, $p = 0.625$). Therefore, the interaction is driven by the lack of additional *J. lividum* found on the newts in the bath+water treatment.

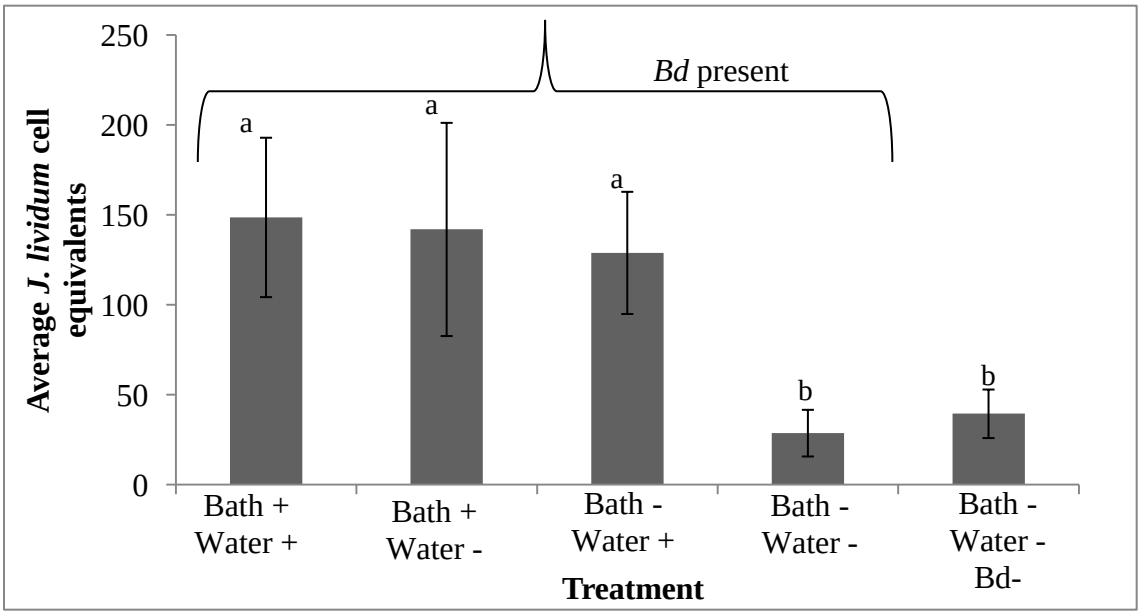


Figure 10. Average *J. lividum* abundance estimates on newts across day, 9, 16 and 27 for each treatment. Error bars indicate the standard error. Letters indicate statistical significant differences among treatments.

Persistence of *J. lividum* on newts

All newts in the probiotic treatments had *J. lividum* on at least one of the three sample days. Persistence was defined as the constancy of the probiotic on the newts. The hypothesis that the presence of water treatment (i.e. an environmental reservoir) would be needed for *J. lividum* to be maintained on the newts was tested by comparing the number of newts that had *J. lividum* on all three sample days among the probiotic treatments. In the bath+water treatment 60% of the newts maintained *J. lividum* across the three sample days (Table 7). In the bath treatment, 2 newts maintained *J. lividum*, and in the water treatment no newts maintained *J. lividum* across all three days. There was greater persistence of *J. lividum* on the newts in the bath+water than in the water treatment (Fisher's exact test (one-tailed), n = 5 p = 0.043). There was no difference in persistence between the bath+water and the bath treatment (Fishers exact test(one-tailed), n = 5, p = 0.152); however, this was likely due to small sample size. Additionally, there was no difference between the persistence of *J. lividum* between the bath treatment and the water treatment (Fisher's exact test, n = 5, p = 0.50).

Table 7. Persistence of *J. lividum* on the newts.

Treatment	**# of newts that maintained *J. lividum* continually (n = 10)**
Bath+	1
Water+	0
Bath+Water	4
***Bd* only**	0

Water

Bd introduction into the experimental ponds

All experimental ponds were negative for *Bd* at the beginning of the experiment (day 0). *Bd* introduction into the experimental ponds was successful. No ponds in the *Bd* absent treatment had *Bd* on day 9. All *Bd*-exposed treatments had *Bd* in the water on day 9 and had significantly greater abundance of *Bd* zoospore equivalents per liter than the *Bd* absent treatment (Figure 11, Table 8).

Table 8. Mixed model statistics for pair-wise comparisons of *Bd* abundance in the experimental ponds of the *Bd*-exposed treatments to the *Bd* absent treatment.

	Day 9			Day 16		
Treatment comparison	**t value**	**df**	**p value**	**t value**	**df**	**p value**
Bath+Water to *Bd* absent	7.82	16	< 0.001	-0.00	16	0.997
Bath to *Bd* absent	8.07	16	< 0.001	-0.24	16	0.817
Water to *Bd* absent	9.52	16	<0.001	1.41	16	0.175
Bd only to *Bd* absent	9.22	16	<0.001	0.10	16	0.921

There was no significant main effect of the water treatment or the bath treatment on day 9 with respect to *Bd* abundance in the water (Water: t = 0.03, df = 16, p = 0.977; Bath: t = -2.02, df = 16, p = 0.060). In addition, there was no interaction (t = -0.38, df = 16, p = 0.706). Additional pair-wise comparisons were not investigated because of the lack of main effects and treatment interactions.

Persistence of *Bd* in experimental ponds

Bd abundance in the water decreased greatly by day 16. *Bd* was detected in 9 of the 20 *Bd*-exposed experimental ponds, and all but two of the tanks had less than 10 *Bd* zoospore equivalents/liter. On day 16 there was no difference in the *Bd* abundance in the water between any of the *Bd*-exposed treatments and the *Bd* absent control (Figure 11,

Table 8; all p's > 0.05). The average *Bd* abundance in the water treatment increased on day 16; however, this was driven by one experimental pond in this treatment having a high *Bd* abundance of 4890 zoospore equivalents per liter (Figure 12, C). *Bd* abundance was not investigated on day 27 because *Bd* remained in only 5 experimental ponds.

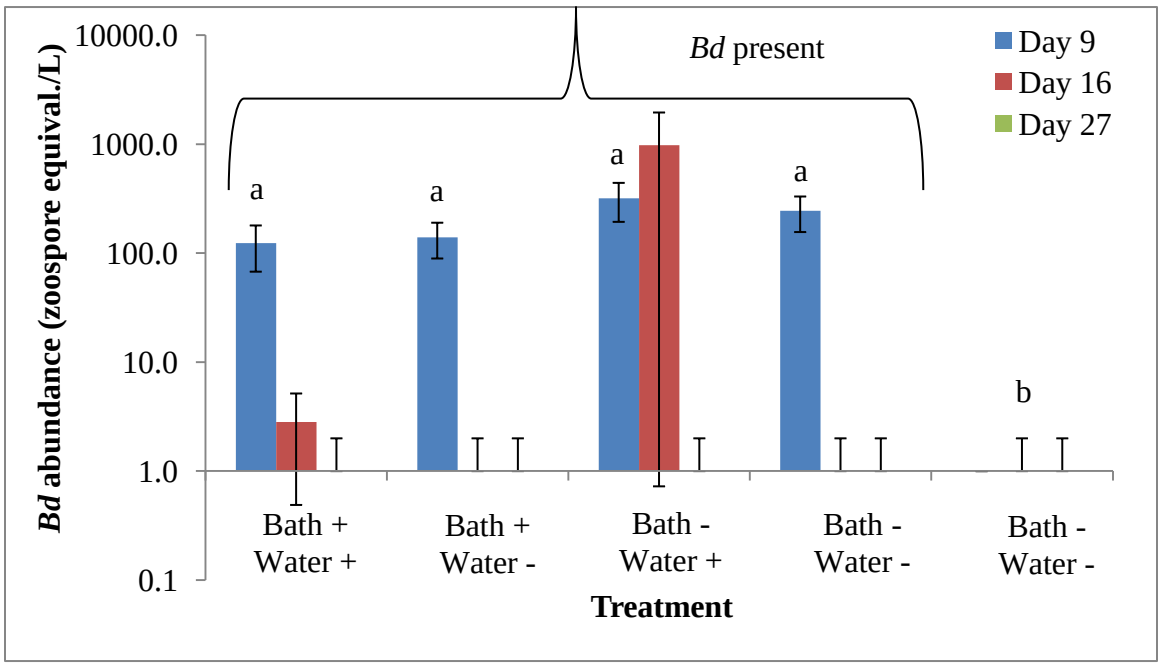


Figure 11. *Bd* abundance per L in the experimental ponds for each treatment on day 9, 6, and 27. *Bd* abundance is presented on a log scale. Letters represent statistically significant differences among treatments on day 9.

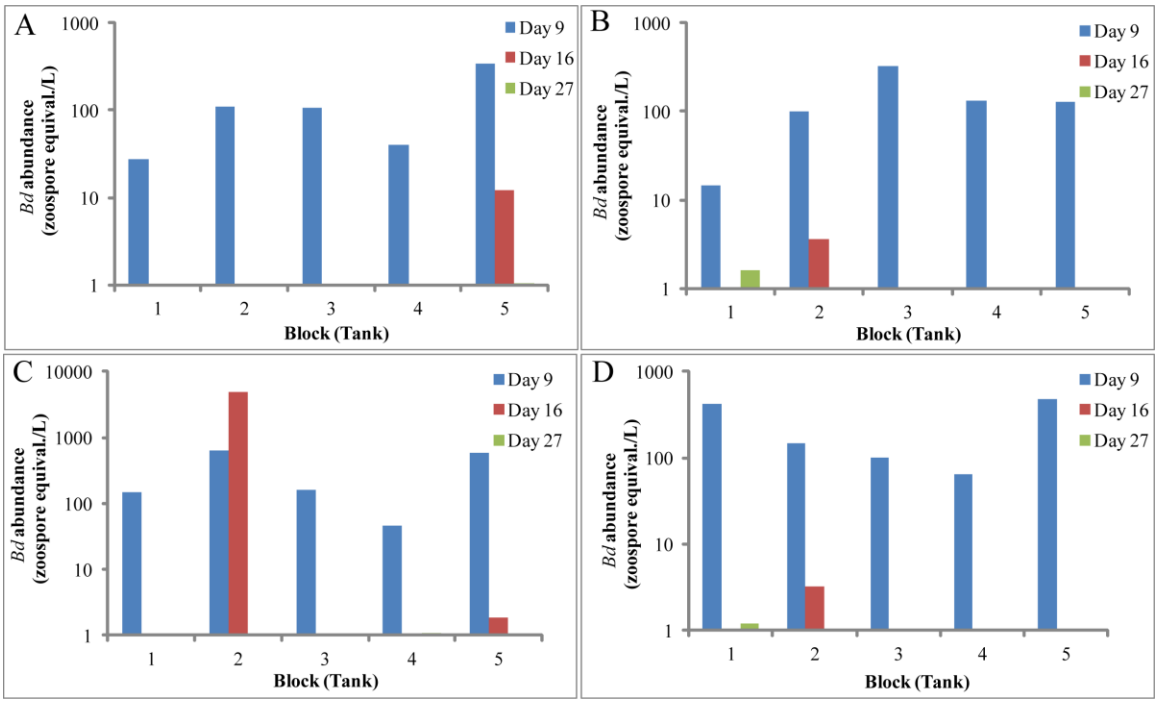


Figure 12. *Bd* abundances in each experimental pond in each treatment. A = Bath+Water treatment, B = Bath treatment, C = Water treatment, D = *Bd* only treatment. *Bd* abundances are presented on a log scale.

<u>*J. lividum* introduction in the experimental ponds</u>

Rif^{-R} *J. lividum* was not detected in the experimental ponds at the beginning of the experiment; however, one pond in the bath+water treatment had naturally-occurring *J. lividum*. *J. lividum* introduction into the experimental ponds was successful (Figure 13). Culture-based sampling showed that on day 3 all ponds in the water treatment and bath+water treatment had Rif^{-R} *J. lividum*; however, the inoculation success in terms of concentration varied between the individual experimental ponds (Figure 14). Nonetheless, there was a significant main effect of the water treatment on day 3 ($t = 23.82$, $df = 20$, $p < 0.001$, Figure 13). The main effect of bath and the interaction between bath and water treatments were not tested since it was not a preplanned comparison. Specific pair-wise comparisons of the water treatment and the bath+water treatment to

the all other treatments, including the bath treatment, *Bd* only treatment, and *Bd* absent treatment show that there was significantly more Rif^{-R} *J. lividum* in the water of the bath+water and water treatments than in the other treatments (Table 9).

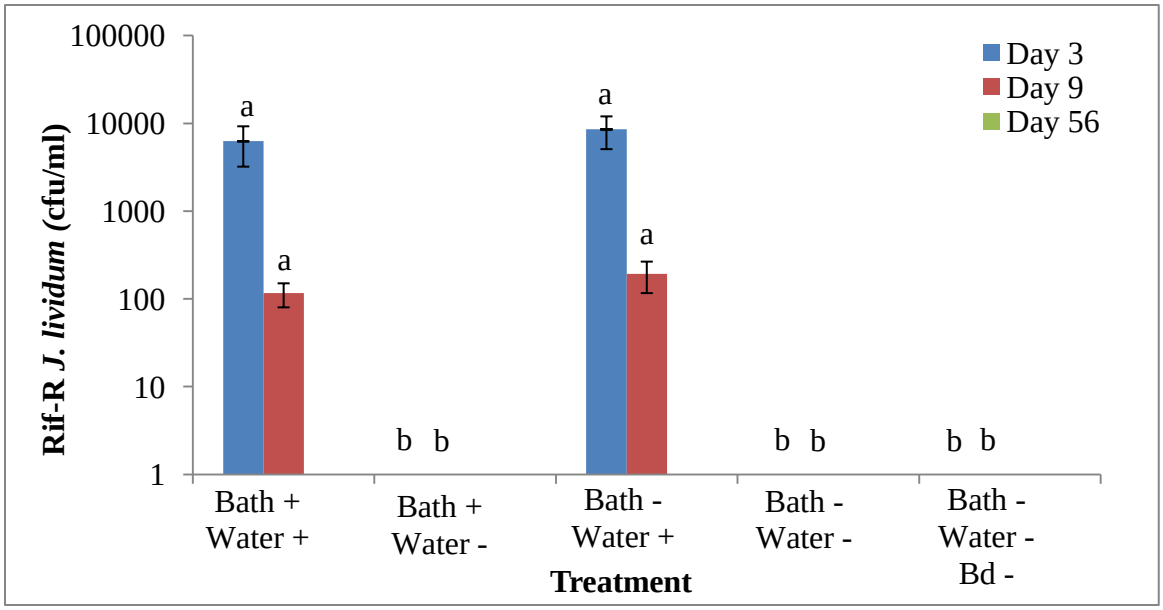


Figure 13. Average Rif^{-R} *J. lividum* in each treatment on Day 3, 9, and 56. *J. lividum* concentration is presented on a log scale. Error bars represent the standard error. Letters represent statistically significant differences within each day.

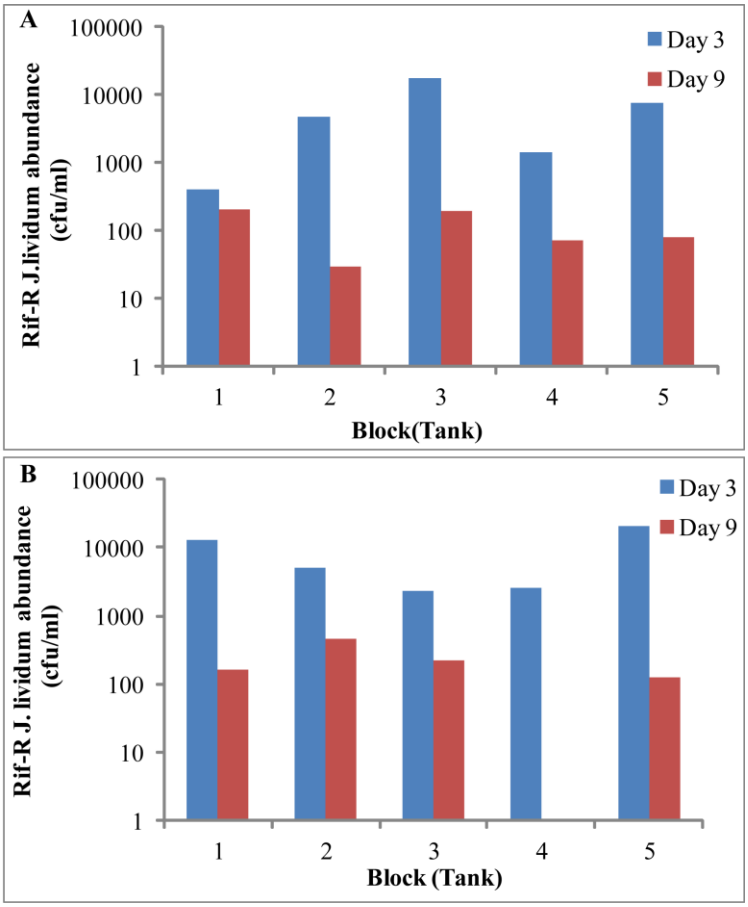


Figure 14. Rif^{-R} *J. lividum* in each experimental pond is the bath+water treatment (A) and the water treatment (B). *J. lividum* abundance is presented on a log scale.

Table 9. Mixed model statistics for pair-wise comparisons of Rif^{-R} *J. lividum* abundance in the water of the bath+water treatment and water treatment to other treatment on day 3 and day 9.

	Day 3			Day 9		
Treatment comparisons	**df**	**t value**	**p value**	**df**	**t value**	**p value**
Bath+Water to *Bd* only	20	15.26	< 0.0001	15.3	6.12	< 0.0001
Bath+Water to *Bd* absent	20	15.26	< 0.0001	15.3	6.12	< 0.0001
Bath+Water to Bath	20	13.90	< 0.0001	15.9	5.70	< 0.0001
Water to *Bd* absent	20	16.44	< 0.0001	15.3	5.78	< 0.0001
Water to *Bd* only	20	16.44	< 0.0001	15.3	5.78	< 0.0001
Water to Bath	20	-15.01	< 0.0001	15.9	-5.38	< 0.0001

Persistence of *J. lividum* in the experimental ponds

Culture-based detection:

J. lividum abundance in the water decreased quickly in the experimental ponds. On day 9, all but one experimental pond contained Rif^R *J. lividum*; however, all concentrations were 456 cfu/ml or lower (Table 10). Despite the lower concentrations, there was a main effect of water treatment on day 9 for the abundance of Rif^R *J. lividum* ($t = 8.63$, $df = 20$, $p < 0.001$), and the water and bath+water treatment contained significantly more Rif^R *J. lividum* than non-water inoculation treatments (Table 9). The main question was whether *J. lividum* persisted and for how long in the treatments where it was introduced into the aquatic environment. The main effect of bath treatment and the interaction between bath and water treatments were not tested since they did not address this question. By day 56, no Rif^R *J. lividum* was detected in any experimental ponds.

Table 10. Concentration of Rif^{-R} *J. lividum* in the bath+water treatment and the water treatment.

Treatment	Tank	Day 3 (cfu/ml)	Day 9 (cfu/ml)
1	2	400	210
1	6	4690	30
1	13	17350	190
1	20	1400	70
1	23	7523	80
3	3	12673	160
3	7	5033	457
3	14	2333	220
3	16	2600	0
3	22	20350	125

qPCR-based detection:

The concentration of *J. lividum* on day 9 as determined by qPCR was higher in the water and bath+water treatments. These results parallel that seen using culturing methods. There was a main effect of water treatment on day 9 and 16 (day 9: $t = 17.86$, $df = 16$, $p < 0.0001$; day 16: $t = 4.46$, $df = 16$, $p = 0.0004$). As previously mentioned, the main effect of bath treatment and the interaction between bath and water treatments were not tested since it was not a pre-planned comparison. Furthermore, the water treatment and the bath+water treatment had significantly greater abundances of *J. lividum* in the water than all other treatments on day 9 and 16 (Table 11). By day 27, there was no water treatment effect, and *J. lividum* was detected in the bath, *Bd* only and *Bd* absent treatments ($t= -1.28$, $df = 16$, $p = 0.220$, Figure 15). It was not possible to determine whether the *J. lividum* was a result of experimental contamination of Rif^{-R} *J. lividum* or a result of naturally-occurring *J. lividum* in the pond ecosystems since only non-culturing methods were used for detection on day 27. Because there was no main effect, specific pair-wise comparisons were not investigated on day 27.

Table 11. Mixed model statistics of pair-wise comparisons of the molecular-based estimates of *J. lividum* abundances in the water of the bath+water and water treatment to all other treatments.

	Day 9			**Day 16**		
Treatment comparisons	**df**	**t value**	**p value**	**df**	**t value**	**p value**
Bath+Water to *Bd* only	16	12.11	< 0.0001	16	2.96	0.0092
Bath+Water to *Bd* absent	16	13.47	< 0.0001	16	2.96	0.0092
Bath+Water to Bath	16	13.47	< 0.0001	16	2.96	0.0092
Water to *Bd* only	16	13.14	< 0.0001	16	3.35	0.0041
Water to *Bd* absent	16	11.78	< 0.0001	16	3.35	0.0041
Water to Bath	16	-13.14	< 0.0001	16	3.35	0.0041

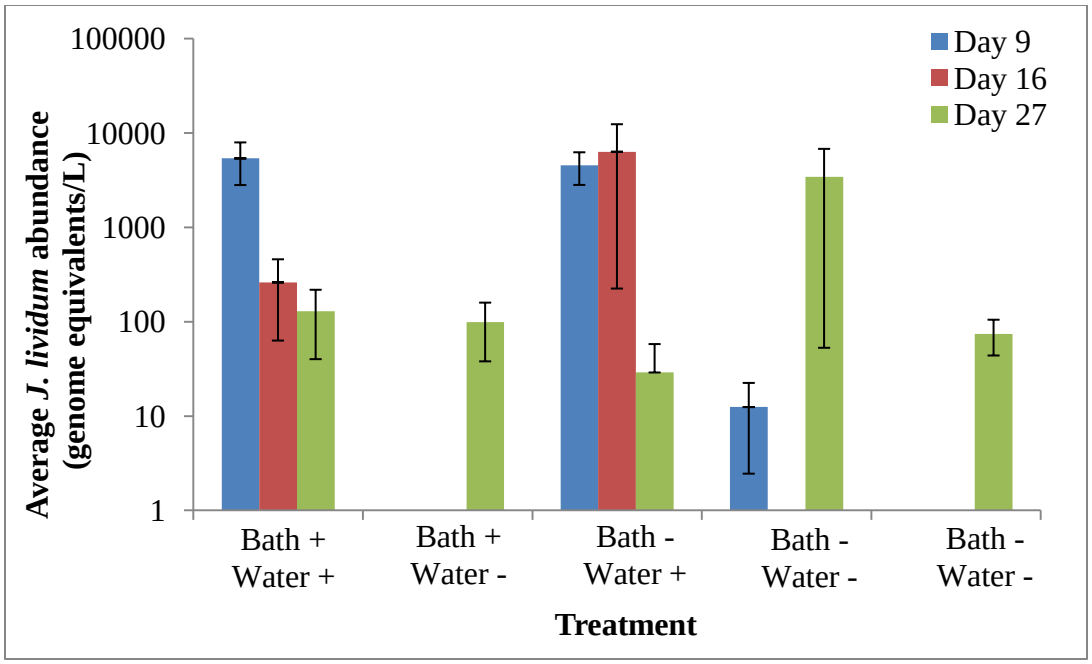


Figure 15. Average *J. lividum* abundance estimates from qPCR-based detection for the experimental ponds of each treatment on day 9, 16, 27. Abundance is presented on a log scale. Error bars represent standard error.

Table 12. Number of experimental ponds in each treatment that had *J. lividum* via qPCR on each sample day.

	# of experimental ponds with *J. lividum* present		
Treatment	**Day 9**	**Day 16**	**Day 27**
Bath+Water	5	4	3
Bath	0	0	2
Water	5	3	1
Bd only	1	0	3
Bd absent	0	0	3

Correlations between *J. lividum* and *Bd*

The abundance of *J. lividum* in the water was not correlated to the presence of *J. lividum* on the newts (logistic regression: n = 38, Wald-Chi-Square = 0.241 p = 0.623). *Bd* abundance in the water was positively correlated with the *Bd* loads on the newts (Spearman rank correlation: r = 0.424, n = 24, p = 0.039, Figure 16); however, it is

possible that this correlation is driven by the data point in the upper right portion of the (Figure 16).

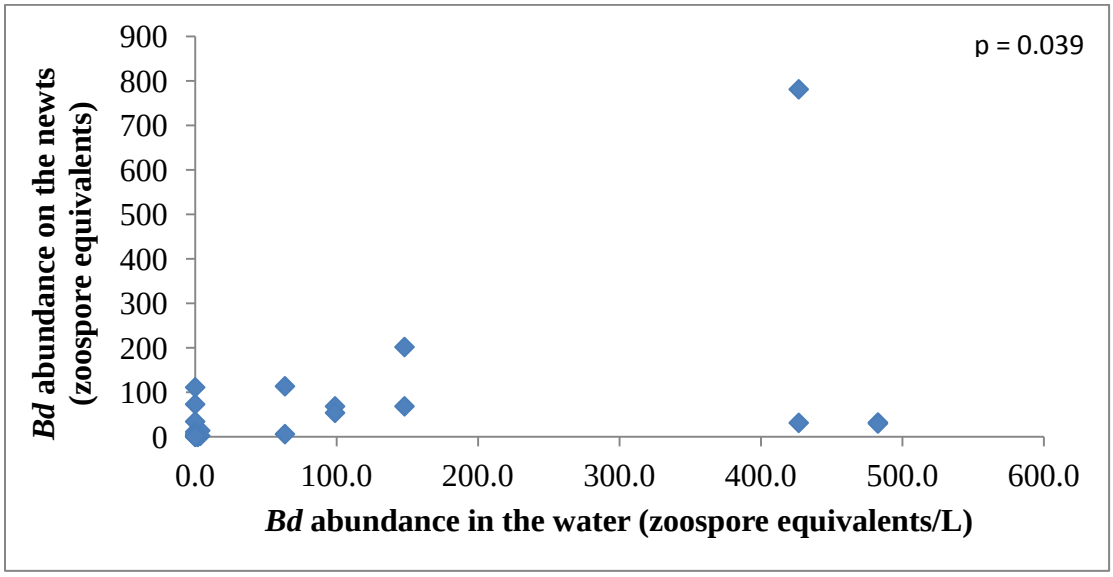


Figure 16. Correlation between *Bd* abundance in the water and *Bd* loads on the newts.

Bd abundance in the water was not correlated with the *J. lividum* abundance in the water (Spearman rank correlation: n = 43, r = -0.019, p = 0.902). *Bd* loads on the newts were negatively correlated to the *J. lividum* on the newts (Spearman rank correlation n = 86, r = -0.395, p = 0.0002, Figure 17). In addition, the logistic regression showed that for every one genome equivalent increase in *J. lividum* abundance the odds of *Bd* infection decreased by 0.998 times. (n=86, Wald Chi-square = 6.27, p = 0.012).

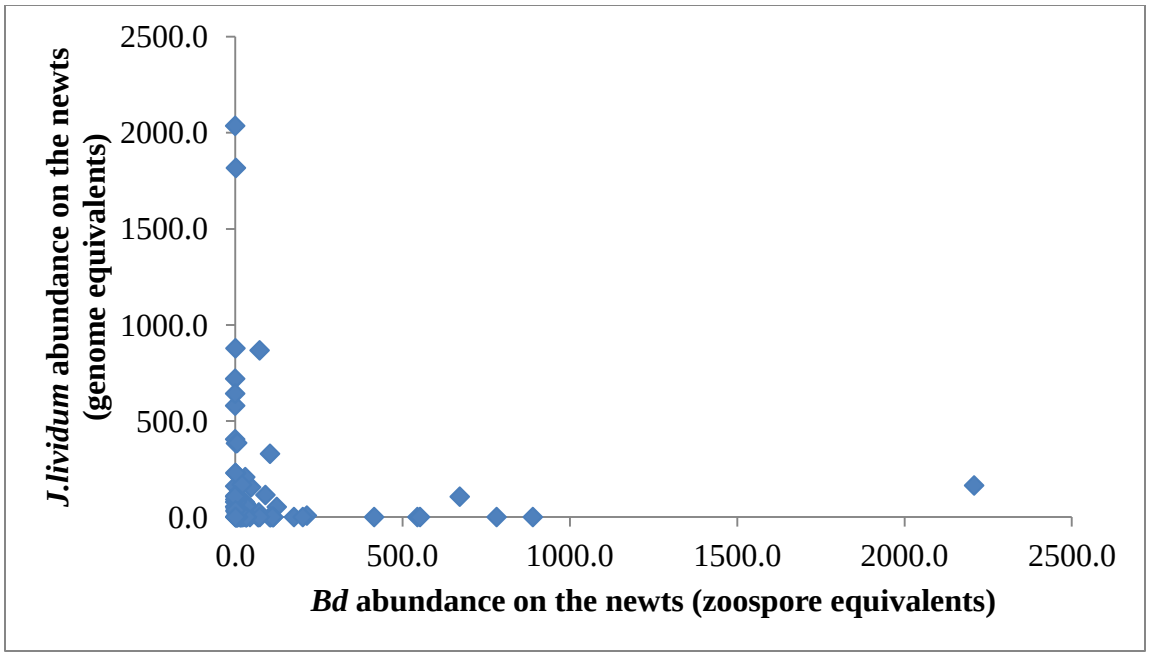


Figure 17. Correlation between *Bd* loads and *J. lividum* abundance on the newts.

Bd loads on the newts were positively correlated with the *J. lividum* abundance in the water (Spearman rank correlation n= 47, r = 0.231, p = 0.118, Figure 18).

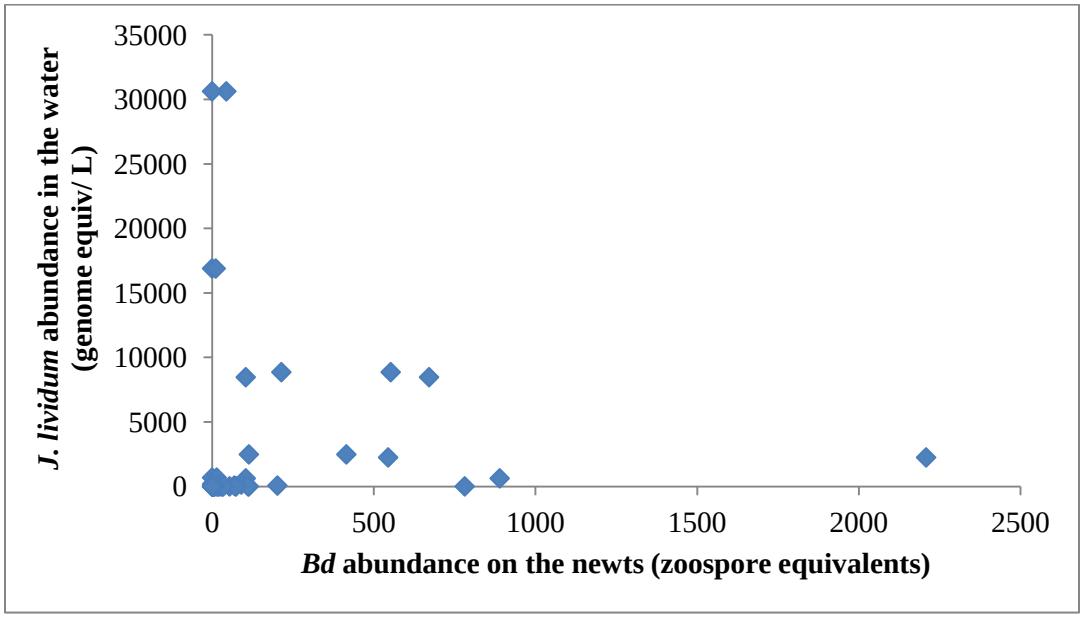


Figure 18. Correlation between *J .lividum* abundance in the water and *Bd* abundance on the newts.

Ecosystem measurements

Leaf Decomposition

Proportional leaf decomposition rate in the experimental ponds varied slightly between treatments (Figure 19); however, no treatments were significantly different than the *Bd* absent treatment, which was the un-manipulated ecosystem control (Table 13).

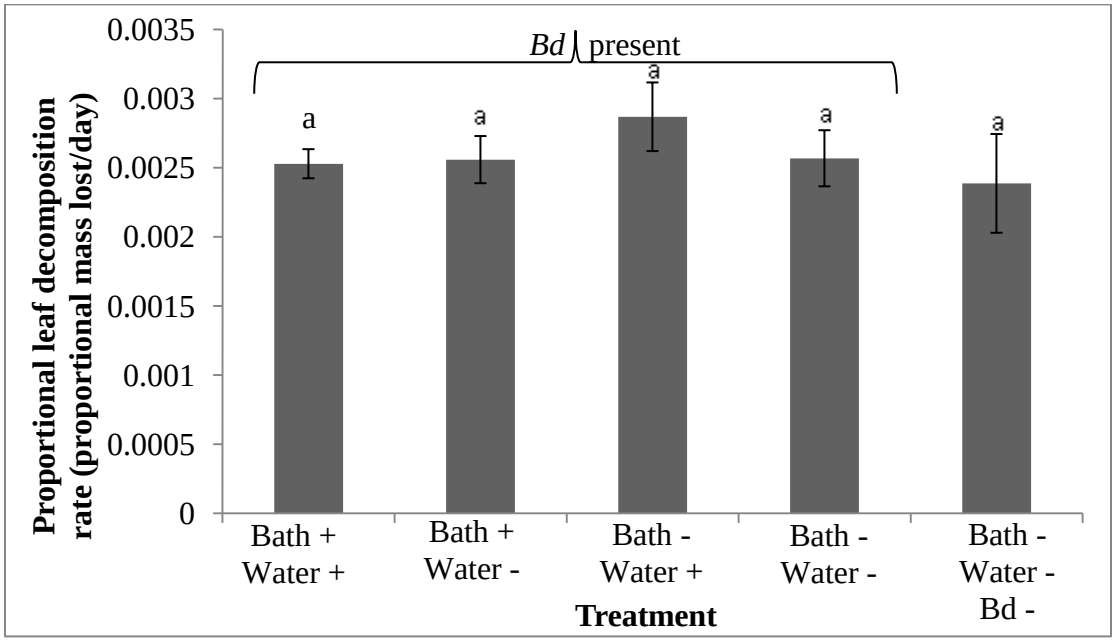


Figure 19. Average leaf decomposition rate for each treatment. Error bars represent one standard error. Letters represent statistically significant differences among treatments.

Table 13. Mixed model statistics of pair-wise comparisons of the proportional leaf decomposition rate of all treatments to the *Bd* absent treatment.

Treatment comparisons	df	t value	p value
Bath+Water to *Bd* absent	16	0.43	0.74
Bath to *Bd* absent	16	1.47	0.381
Water to *Bd* absent	16	0.52	0.693
Bd only to *Bd* absent	16	0.55	0.679

Periphyton Production

Periphyton production was used as a measure of primary productivity for the experimental ponds. Periphyton production rate of any probiotic treatment did not differ from the *Bd* absent treatment (Table 14, Figure 20).

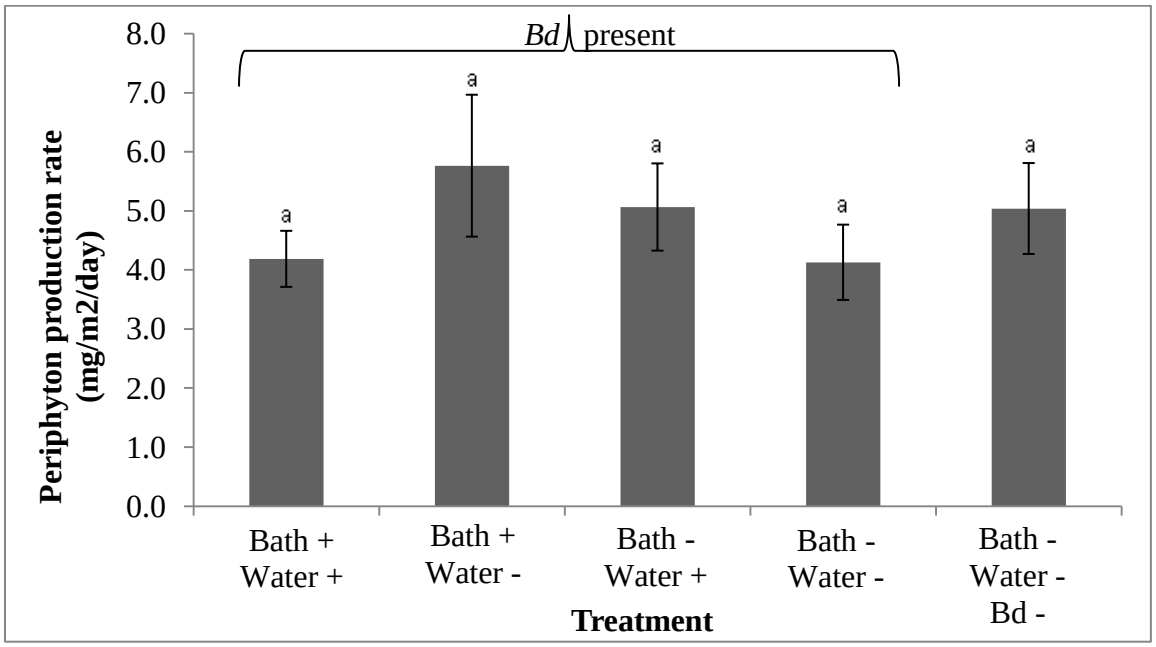


Figure 20. Average periphyton production rate for each treatment. Error bars represent one standard error. Letters represent statistically significant differences among treatments.

Table 14. Mixed model statistics of pair-wise comparisons of the periphyton production rate of all treatments to the *Bd* absent treatment.

Treatment comparisons	df	t value	p value
Bath+Water to *Bd* absent	16	-0.94	0.361
Bath to *Bd* absent	16	0.80	0.435
Water to *Bd* absent	16	0.03	0.978
Bd only to *Bd* absent	16	-1.01	0.330

Zooplankton Community:

Total zooplankton abundance varied across sample day and generally increased throughout the experiment (Figure 21). There were no differences among the average total zooplankton abundance of the manipulated treatments and the *Bd* absent treatment (Table 15). The bath treatment and the *Bd* only treatment spiked on day 16; however, this was driven, in both cases, by one experimental pond having high abundances of cladocerans and copepods respectively (Figure 21).

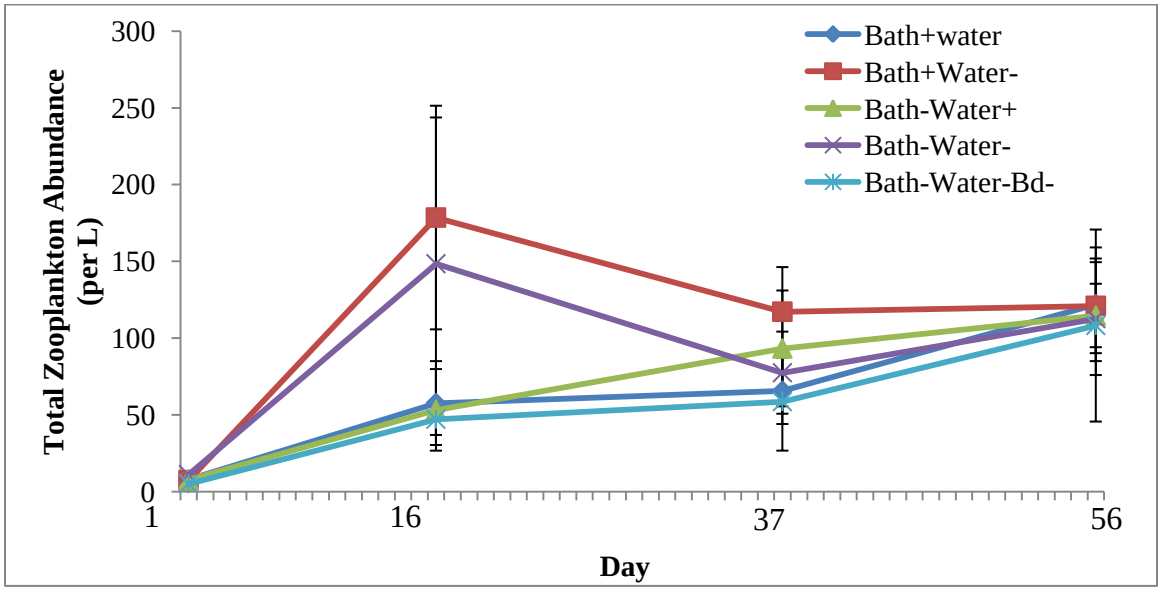


Figure 21. Average total zooplankton abundance per liter throughout the experiment for each treatment.

Table 15. Mixed model statistics of pair-wise comparisons of the average total abundance of zooplankton of all treatments to the *Bd* absent treatment.

Treatment comparisons	**df**	**t value**	**p value**
Bath+Water to *Bd* absent	20	0.45	0.660
Bath to *Bd* absent	20	1.51	0.147
Water to *Bd* absent	20	0.58	0.565
Bd only to *Bd* absent	20	0.25	0.808

The variance ratio, which was used to assess the stability of the zooplankton communities, also did not differ between the manipulated treatments and the *Bd* absent treatment (Table 16,17). In addition, the variance of cladocerans and variance of copepods were compared between manipulated treatments and the *Bd* absent control, and no differences were detected (Table 18, Figure 22).

Table 16. Mean and variance of the variance ratio for each treatment.

Treatment	**Mean**	**Variance**
Bath+Water	0.872	0.026
Bath	0.481	0.131
Water	0.790	0.144
Bd only	0.843	0.047
Bd absent	0.758	0.149

Table 17. Mixed model statistics of pair-wise comparisons of the variance ratio of zooplankton communities of all treatments to the *Bd* absent treatment.

Treatment comparisons	**df**	**t value**	**p value**
Bath+Water to *Bd* absent	16	0.60	0.558
Bath to *Bd* absent	16	-1.45	0.168
Water to *Bd* absent	16	0.17	0.868
Bd only to *Bd* absent	16	0.44	0.665

Table 18. Mixed model statistics for the cladoceran variance and copepod variance of all treatments in comparison to the *Bd* absent treatment.

	Cladoceran Variance			**Copepod Variance**		
Treatment comparisons	**df**	**t value**	**p value**	**df**	**t value**	**p value**
Bath+Water to *Bd* absent	16	-0.27	0.793	16	-0.62	0.541
Bath to *Bd* absent	16	0.14	0.889	16	0.39	0.699
Water to *Bd* absent	16	-0.29	0.775	16	0.50	0.627
Bd only to *Bd* absent	16	-0.61	0.551	16	1.23	0.236

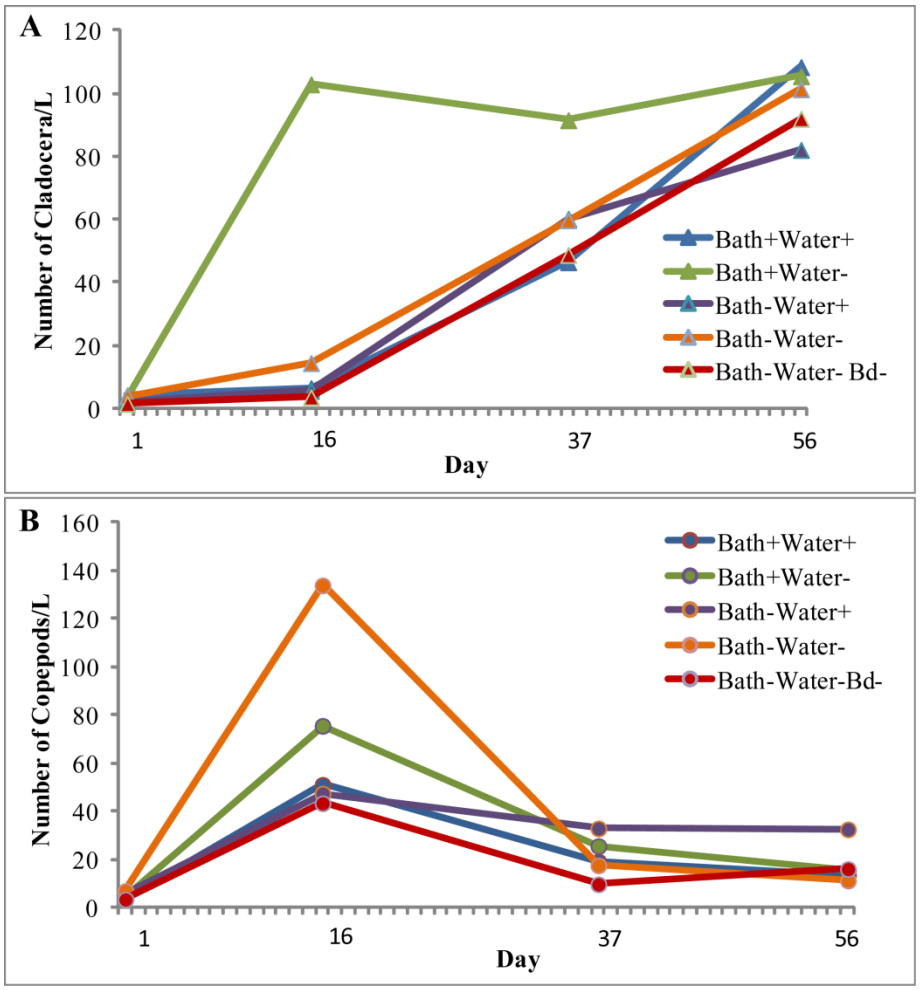


Figure 22. Cladoceran (A) and Copepod (B) abundances over time for each treatment.

DISCUSSION

The goal of this research was to find a means to effectively protect amphibians from the lethal fungal disease, chytridiomycosis. The specific objectives of this experiment were to determine the most effective method to transmit probiotic bacteria to the red-spotted newt for protection from *Bd* and to investigate whether probiotic treatment causes non-target ecosystem effects. I begin by summarizing the results of this study and then explore the major findings. First, the efficacy of probiotic treatment in establishing and maintaining *J. lividum* on the newts is discussed. Second, the persistence of *J. lividum* in the experimental ponds is discussed, and third the effectiveness of the probiotic treatments with respect to *Bd* is discussed. Finally, the implications of this experiment for probiotic conservation strategies and future directions for research are noted.

Bd introduction into the experimental ponds was successful, and infection of newts occurred as expected based on preliminary trials and results from previous studies in the literature (Appendix 4, Parris and Cornelius 2004). Additionally, *Bd* on the newts was positively correlated with *Bd* in the water. By day 16, *Bd* was absent from most ponds and was in low abundance on newts. Introduction of *J. lividum* into the ponds was also successful and persisted for at least 9 days. Probiotic transmission efficacy to the newts varied depending on treatment. The bath+water treatment had greater efficacy of establishing *J. lividum* on newts and resulted in greater persistence of *J. lividum* on the newts in comparison to the individual bath and water treatments. Weight loss associated with *Bd* infection did not occur, and the three probiotic treatments did not reduce *Bd* prevalence or lead to greater proportional change in *Bd* loads compared to the levels

found in the *Bd* only treatment. However, the bath+water treatment did reduce morbidity and *Bd* prevalence in comparison to the bath and water treatment. The proportional change in *Bd* loads showed a slightly different result, with both the water treatment and the bath+water treatment having greater proportional reduction in *Bd* loads than the bath treatment. *Bd* abundance on the newts was negatively correlated to both *J. lividum* abundance on the newts and *J. lividum* abundance in the water, which suggests *J. lividum* may be inhibiting *Bd*, although the experimental results do not suggest a protective effect. In addition, no ecosystem effects were seen in leaf decomposition, periphyton production, or zooplankton community structure as a result of probiotic treatments.

Transmission efficacy of probiotic treatment

Efficacy of treatment was defined as how well the probiotic bacteria transmitted to and established on the newts, and persistence was defined as the constancy of the probiotic bacteria on an individual throughout all sample days. Efficacy and persistence of the probiotic bacteria on the newt over time varied between the treatment methods. The results suggest that the combination of individual baths and environmental bioaugmentation is the most effective at establishing and maintaining a probiotic on the amphibian, because the bath+water treatment had the highest treatment efficacy and probiotic persistence on the host. However, the bath+water was only significantly greater than the water treatment, not when compared to the bath treatment suggesting that the treatment of the host with the probiotic bath is driving this difference. However, there was not significantly greater efficacy in the bath treatment in comparison to the water treatment; therefore, the bath treatment in itself is not adequate. With further

experimentation with increased sample size the effects of these treatment methods can be further elucidated.

The interpretation of these results becomes more complex because there were individuals in the controls that also obtained *J. lividum* during the experiment. Rif^{-R} *J. lividum* was used in the experiment to differentiate between naturally-occurring *J. lividum* and experimental *J. lividum*, but due to time constraints all newt sampling was qPCR-based. Culture-based detection was only used for determining *J. lividum* abundance in the water. The occurrence of *J. lividum* on newts in the non-manipulated treatments was likely a result of naturally-occurring *J. lividum* on the newts that was not detected before the start of the experiment or naturally-occurring *J. lividum* in the experimental ponds. When sampling the newts, only their ventral region and each foot were swabbed; therefore, *J. lividum* could have been residing on non-sampled areas of the newts, such as the dorsal region. In addition, *J. lividum* was found to survive the digestive track of *P. cinereus,* serving as a potential reservoir of beneficial bacteria that could recolonize the host after defecation (Wiggins *et al.* 2011). The same process could be occurring on newts and suggests a means of *J. lividum* presence on newts in control ponds. Culture-based water sampling confirms that there was no Rif^{-R} *J. lividum* in the experimental ponds in these treatments on any of the culture-based sampling days; therefore *J. lividum* on the newts in non-probiotic treatments is unlikely to be experimental contamination.

The experimental ponds had leaf litter, zooplankton communities, and tadpoles added to them to create aquatic ecosystems, and the addition of these components each could have introduced *J. lividum* into the experimental ponds. *J. lividum* has been found

in soils in Antarctica and Alaska (Shivaji *et al.* 1991, Schloss *et al.* 2010), streams in Pennsylvania (Saeger and Hale 1993) and soil and water environments in Italy and Spain (Pantanella *et al.* 2007). Additionally, recent research has found *J. lividum* in stream water in the lowland tropical rainforests (E. Rebollar, pers. comm.) and in the soils in the Appalachian mountains in western Virginia (A. Loudon, pers. comm.). Therefore, it is likely to be found in the leaf litter and soil in the area where leaf collection was made, which could have resulted in it being in the experimental ponds. Furthermore, the addition of wood frog tadpoles to the experimental ponds is another potential source of *J. lividum* introduction. The cutaneous microbiota of wood frogs has not been investigated, but the microbiota of other related and sympatric species has been (Lauer *et al.* 2007a,b). Species including *N. viridescens, Lithobates catesbeianus, Hemidactylium scutatum,* and *P. cinereus* have all been found to have *J. lividum* (Appendix 1, Lauer *et al.* 2007a, 2007b, J. Walke, pers. comm.) The wood frogs were collected as eggs and hatched in the laboratory; therefore, the presence of *J. lividum* would have to be a result of it being on the egg masses and then transferring to the tadpoles as they developed. If *J. lividum* was present on the tadpoles it could have been transmitted from tadpole to newt via horizontal transmission or pseudo-environmental transmission. Zooplankton also have associated microbiota and therefore the addition of collected zooplankton communities from natural ponds could have introduced *J. lividum* to the experimental ponds. Interestingly, *J. lividum* was not detected in the bath, *Bd* only, or *Bd* absent treatment by qPCR or culture based sampling of the pond water on the first two sample days. This does not exclude the possibility of *J. lividum* being at the bottom of the experimental ponds in the leaf litter or on the tadpoles because the substrate and wood frogs were not sampled before entry into

or during the experiment to determine if *J. lividum* was present. In the future, substrate sampling of the leaf litter and pre-sampling the wood frogs would be logical additions to the experimental procedures.

Transmission efficacy from the environmental treatment alone was low, and this may be explained by inadequate inoculation dosage and low probiotic persistence in the water, an existing stable microbial community on the host preventing establishment of a new species, or environmental transmission itself being a limited force in shaping microbial communities. Only two newts in the water treatment obtained *J. lividum* within the first nine days of the experiment, suggesting that environmental transmission did not occur at a high rate. This could be due to the inoculation dosage of the aquatic environment not being sufficiently high to allow environmental transmission. In a recent study conducted with environmental transmission between soil and *P. cinereus*, the soil was inoculated with 2.9×10^7 *J. lividum* cells/g of soil, which is roughly equivalent to 2.9×10^7 cells/ml, and it was effectively transmitted to all exposed salamanders (Muletz *et al.* 2012). This concentration is greater than the target concentration of the present study (1×10^6 cells/ml), suggesting that increasing the target concentration may afford transmission. In addition, in the Muletz *et al.* (2012) study, *J. lividum* persisted at greater concentrations than in the present study, suggesting that higher persistence of the bacteria in the environment may also afford transmission. In the aquaculture literature, it was suggested that a concentration between 1×10^4- 1×10^6 cells/ml in the water may be ideal sufficient for the probiotic bacteria to improve survival of the species being farmed; however, probiotic strain and the fish, mollusk or bivalve species being treated likely influence the idea inoculation conditions (Vine *et al.* 2006). A study conducted with blue

crab larvae used a probiotic concentration of 1 x 10^6 cells/ml, and it effectively increased crab larvae production (Nagomi and Maeda 1992). Additionally, a study conducted with bivalve larvae found that a concentration of 1 x 10^5 cells/ml was optimal for the enhancement of oyster larvae growth (Douillet and Langdon 1994). However, some studies with shrimp larviculture used lower concentrations of approximately 1 x 10^3 cells/ml and it was sufficient to improve survival and increase weight gain (Garriques and Arevalo 1995, Zherdmant *et al.* 1997). These results suggest that various concentrations can be effective and that effective concentrations can differ between hosts; therefore, it will be important to identify the appropriate concentration or concentrations that are effective for amphibian species. It is possible that in order for there to be transmission to the newts the concentration needed to be higher than that achieved in the ponds in the conducted study. Preliminary experiments showed transmission of *J. lividum* to newts through environmental inoculation of aquariums (Appendix 3). It is possible that the concentration of *J. lividum* in the aquariums established and persisted at higher concentrations whereas in the main study in experimental ponds the concentration of the probiotic bacteria in the water declined rapidly after introduction minimizing transmission to the newts. Additionally, The bath treatment in this experiment was at a concentration of 10^9 cells/ml and it was successful at establishing *J. lividum* on 60% of the newts. Future experiments can explore the use of this concentration for environmental bioaugmentation. Introducing this high of a concentration into the environment could be more effective for probiotic transmission to amphibians, but it may not be ideal for other aspects of the ecosystem (see non-target effects section).

The lack of transmission to the newts in the water treatment also may be due to the presence of an already existing stable and protective microbial community on the newts that prevented the successful establishment of a new bacterial species. If other microbes are already occupying potential niches on the host and perhaps producing inhibitory compounds as a byproduct of microbial competition among existing community members it may be difficult for *J. lividum* to get a competitive advantage and establish in the existing community on the amphibian. *J. lividum* successfully established on newts in some experimental ponds and on newts in preliminary experiments (Appendix 3), suggesting that *J. lividum* can survive host-produced defenses and colonize newts. However, the conditions on newts and their microbial community likely vary between individuals and over time making transmission a function of each individual at a given time. In future experiments it will be important to monitor the microbial community as a whole through next generation sequencing methods to determine the microbial community structures that allow and do not allow probiotic transmission. Furthermore, in other laboratory experiments with bath (Harris *et al.* 2009, Vredenburg *et al.* 2011) and environmental bioaugmentation (Muletz *et al.* 2012) amphibian hosts have been treated with peroxide or antibiotics to reduce the existing microbial community and open a niche for the probiotic species being added. Therefore, successful transmission and establishment of the probiotic bacteria could be a function of this treatment reducing microbial competition. In the present study, this was not done because it would be more feasible to not pre-treat the amphibians for conservation strategies. A pretreatment protocol is only possible with individual capture, and it minimizes the potential for synergies of the added probiotic bacteria with other resident microbial community

members. Being a strong competitor that can survive host defenses and compete with other microbes in order to establish within an existing resident microbial community will be an important characteristic of an effective probiotic bacterium (Bletz *et al.* 2013).

The low occurrence of transmission in the water treatment may also be due to environmental transmission being a relatively minor force shaping cutaneous microbial communities on newts, meaning that the microbial community of newts is independent of that of the aquatic environment. In this experiment this explanation is supported by the absence of a correlation between the abundance of *J. lividum* in the water and the presence of *J. lividum* on the newts. In addition, preliminary evidence of the microbial communities of red-spotted newts suggested that there was little overlap between the newt's microbial community and the community of its environment over time (M. Becker, pers. comm.). In another species, recent evidence demonstrated that the existence of an environmental reservoir facilitated maintenance of microbial diversity on *P. cinereus,* suggesting that transmission from the environment may be important (A. Loudon, pers. comm). These different results demonstrate that the role of environmental transmission for the establishment and maintenance of amphibians' microbial communities is in need of further investigation and likely varies among amphibian species. While environmental transmission may be essential for initial establishment of an amphibians' microbial community (Belden and Harris 2007), it may play less of a role after establishment in some species or only play a role during periods of disturbance, such as skin shedding (Meyer *et al.* 2012, Bletz *et al.* 2013). This stresses the importance of choosing bacteria for use as probiotics that are effective at establishing on larval amphibians. It is at this point in development that the resident microbial community is

developing in terms of structure and composition and the host immunity system is maturing, therefore; probiotic bioaugmentation of the cutaneous community may occur more readily (Bletz *et al.* 2013).

It is possible that with optimization of inoculation concentration and selection of probiotic bacteria that are strong competitors and are able to persist in the aquatic environment more effectively, environmental transmission through aquatic inoculation can be an effective means to transfer probiotics to amphibian hosts and allow persistence.

This research suggests that water inoculation may be needed in addition to bath treatment in order to maintain the probiotic bacteria on the host amphibian. In the bath+water treatment *J. lividum* was maintained on the newts more than in the water treatment and there was a trend toward it being more than the bath treatment. The lack of a significant difference between the bath+water and the bath treatment is likely due to low sample size. In addition, *J. lividum* on the newts in the bath treatment was not maintained more than in the water treatment. Thus, the treatment of water with the probiotic in addition to bath treatment appears to be crucial for persistence of the probiotic bacteria on the host. It is interesting that the water treatment was not effective at establishing *J. lividum* on the newts but there was a trend of it being important in terms of maintaining *J. lividum* on the host in the bath+water treatment. Taken together the results of the transmission efficacy and persistence of the probiotic suggest that the bath is needed to establish the probiotic bacteria on the host and that environmental inoculation is important for persistence of the probiotic on the host. Additionally, this suggests that lower bacterial concentrations can facilitate persistence of the probiotic even if they do not afford transmission and establishment on the host.

Persistence of *J. lividum* in the water

J. lividum persistence in the experimental ponds was low. By day 9 abundances were approximately 10^2 cells/ml and lower. The observed low persistence of the Rif^{-R} *J. lividum* in the experiment may have been due to sampling method, grazing by other organisms, competition from other bacteria, non-optimal water conditions or ineffective inoculation concentration. As previously mentioned, the sampling technique for the water targeted the water column and did not effectively sample the bottom leaf litter. It is possible that the added *J. lividum* settled to the bottom of the experimental ponds in the leaf litter and therefore, was not detected accurately by the water sampling. In the future, sampling of the substrate will be an important addition to experiments.

Bacterivorous organisms, such as protozoa and heterotrophic nanoflagellates, in the water or sediment also could have reduced the abundance of *J. lividum* in the experimental ponds. Bacterial grazers have been found to mediate bacterial production and community structure and diversity in aquatic environments (Hahn and Hofle 2006, Berdjeb *et al.* 2011), and some predators selectively feed on certain species or morphotypes (Pernthaler 2005). In one microcosm study, the presence of grazers shifted the size structure of the microbial community and also lead to an increase in the relative abundance of the initially rarer bacterial phylotypes (Sime-Ngando and Ram 2005). Some bacterivorous protists graze selectively on medium-sized bacterial cells (Hahn and Hofle 2006). Bacteria of 0.4-1.6 µm and 1.6-2.4 µm are classified as "graze-vulnerable" and "graze-suppressed" respectively, and *J. lividum* is typically between around 1.5-2.3 µm (Nakamura *et al.* 2002, Matz *et al.* 2004); therefore, it falls within this two groups. *J. lividum's* size possibly made it more prone to predation by protozoan. In addition, some

zooplankton including *Daphnia galeata* and *D. rosea* and even tadpoles can feed on bacteria (Peterson *et al.* 1978, Kupferburg 1997) providing other avenues of *J. lividum* reduction. While it is possible that grazing led to the reduction in *J. lividum* abundance in the experimental ponds, it is important to note that this may not occur because it produces the metabolites, violacein and I3C (Brucker *et al.* 2008). Violacein has been found to be cytotoxic to some nanoflagellates bacterivorous predators (Matz *et al.* 2004) so grazing may be avoided due to its production.

Effective environmental inoculation and persistence of a probiotic is likely associated with the bacterial community composition in the aquatic environment. It is possible that *J. lividum* was outcompeted by other bacterial species in the aquatic environment. Some species in the experimental ponds may have had the ability to more effectively acquire resources or may produce metabolites that are inhibitory to *J. lividum*.

Bacterial survival also can be influenced directly by water conditions, such as temperature, pH or other abiotic factors. Temperature was monitored in the experimental ponds, and throughout the experiment temperatures did not go above 30°C in any pond (data not shown). The optimal temperature for *Janthinobacterium spp*. is 25°C and the maximum is 32°C (Bergey 1994); therefore, the temperature in the experimental ponds should have been adequate for *J. lividum*. However, different *Janthinobacterium* species likely vary in their optimal temperatures and *J. lividum* is typically considered a psychrophile meaning it prefers cooler temperatures. Therefore, it is possible that temperature played a role in persistence of *J. lividum*. *J. lividum* is also known to be sensitive to pH (Bergey 1994, Shivaji *et al.* 1991). The strain of *J. lividum* used in this study did not survive well in soils with a pH of 5 and below (Muletz, 2011). pH was not

monitored in this experiment; so, it is possible that the pH of the aquatic environment was sub-optimal for *J. lividum.*

In the present study, experimental ponds were inoculated with *J. lividum* at 1 x 10^6 cells/ml and it did not persist at this concentration; in fact, it declined rapidly within the first 9 days. It is possible that a greater inoculation concentration is needed for persistence of the probiotic to occur. For example, in a laboratory-based amphibian transmission experiment, soil inoculated with 2.9 x 10^7 cells/g dropped to 1 x 10^5 cells/g by the 8th day and then persisted for 41 days at a steadily declining concentration (Muletz, 2011). Perhaps by increasing the inoculation dosage to 10^7 or 10^8 persistence of *J. lividum* in the experimental ponds could be achieved. It will be important to determine at what environmental concentration transmission to the amphibian host is achieved, and work to get the probiotic bacteria to establish and persist in the environment at that appropriate concentration.

The observed low persistence of *J. lividum* in the experimental ponds is possibly the reason why transmission efficacy of *J. lividum* on the newts in the water treatment was low. In addition, the low persistence of *J. lividum* in the water may explain why there was no correlation between *Bd* and *J. lividum* in the water. It is also possible that *J. lividum* and *Bd* were occupying different locations in the experimental ponds. For example, *J. lividum* may have inhabited the leaf litter whereas *Bd* occupied the water column as *Bd* has a motile zoospore stage.

Improving the persistence of probiotics introduced via environmental bioaugmentation will involve optimizing inoculation dosage. In addition, it will be

important to develop an understanding the abiotic factors which the probiotic candidates can and cannot tolerate. For environmental probiotic bioaugmentation strategies the ideal probiotic will be a product of not only the intended amphibian host but also the abiotic factors of application area.

The probiotic's effectiveness against *Bd*

Growth rate

Bd did not have a significant negative effect on mortality or on the growth rate of newts, and therefore it was not possible to determine if the probiotic treatments reduced this aspect of morbidity associated with *Bd*. None of newts had *Bd* loads that were near or surpassed the lethality threshold of ten thousand zoospores that has been proposed in the literature (Vredenburg *et al.* 2010), and therefore it is not surprising that no mortality was seen in this experiment. In fact, *Bd* loads on all but one newt were below 10^3 zoospore equivalents on day 9 and by day 16 all were below 10^2 zoospore equivalents. This also helps explain why the sublethal effect of weight loss was not present (Berger *et al.* 1998).

Red-spotted newts population declines have not been documented across its range in the eastern US, but *Bd* has been detected in low prevalence (Rothermel *et al.* 2008, Bakkegard and Pessier 2010, Groner and Relyea 2010, Raffel *et al.* 2010, Pullen *et al.* 2010, Hossack *et al.* 2010, Gratwicke *et al.* 2011). Therefore, it is likely that this species has adequate defensive mechanisms, such as adaptive immunity, innate immunity or microbial defenses. This species' adaptive and innate immunity have not been investigated thoroughly; however, newts were found to possess anti-*Bd* bacteria on their skin (Appendix 1); therefore, it is a possibility that the existing microbial community of

the newts, perhaps in concert with the newt's antimicrobial peptide secretions, allowed clearance or reduction of *Bd* regardless of probiotic treatment.

In this experiment, a local strain of *Bd* from Maine (*Bd* JEL404) was used as a biosafety precaution, and it is possible that this strain has reduced virulence in comparison to the hypervirulent lineage that is causing amphibian declines globally. *Bd* JEL404 has not been used in the previous laboratory experiments. Instead, a strain from Panama (JEL 310), which is known to be virulent, has been used. Interestingly, a study with green frogs (*Lithobates clamitans*) and wood frogs (*Lithobates sylvaticus*) tested the effects of two *Bd* strains (JEL423 (Panama) and JEL404 (Maine)) on survival and found that these species were affected differently by the two *Bd* strains (Gahl *et al.* 2011b). While wood frogs experienced mortality regardless of strain, green frogs experienced mortality when exposed to the Panamanian strain but not the Maine strain (Gahl *et al.* 2011b). In light of this evidence, it is very possible that the use of the less virulent Maine strain of *Bd* explains the lack of morbidity effects in newts in the experiment. The genomics of different *Bd* strains and what genes are associated with its virulence are still under investigation (Rosenblum *et al.* 2009, Joneson *et al.* 2011). Partial sequencing of 39 *Bd* strains from around the world showed little genetic difference among the strains (James *et al.* 2009); however, as more sequences become available, the conclusions regarding phylogeny of *Bd* will likely become more complex. Thus, it is possible that strain 404 is not part of the virulent *Bd*GPL lineage.

Interestingly in this study, the bath+water treatment has a beneficial effect on growth rate in comparison to the individual bath and water treatments. This is likely associated with the inoculation efficacy and persistence of *J. lividum* in the bath+water

treatment being better than the individual bath and water treatments. The reduced weight loss associated with the bath+water treatment cannot be associated with amelioration of the effects *Bd*; therefore, it suggests that the probiotic may have a benefit to the host unrelated to clearance of *Bd* infection. It is possible that *J. lividum* provided defense against other pathogens, but additionally, benefits independent of defense against pathogens are not uncommon. In aquacultural settings, the addition of probiotics to larvicultural ponds is beneficial in terms of disease resistance and in terms of increasing growth rate by facilitating microalgae growth on which the mollusk and bivalve larvae feed (Kesarcodi-Watson *et al.* 2008). The main food resources for newts in the experimental ponds were zooplankton and potentially tadpoles. There were no changes in the zooplankton abundance and community structure associated with probiotic treatment. However, it is possible that the newts were feeding on organisms in the leaf litter, such as ostracods, that were stimulated by the probiotic bacteria, providing an increased food resource for the newts. The sampling method targeted the water column not the benthos; therefore this cannot be known for certain.

Many probiotic bacteria that improve growth rate are associated with improvements of the intestinal microbial community structure (Musa *et al.* 2009). *J. lividum* has been found in the gut of *P. cinereus,* and therefore it is possible that *J. lividum* could colonize the intestinal tract of newts. The benefits of *J. lividum* for digestion are not known. It is possible that it could have a direct benefit or act as a keystone probiotic (Bletz *et al.* 2013) and cause a shift in the intestinal microbial community that has an improved nutrient acquisition function. Improved digestive activity through the synthesis of vitamins or improvements in enzyme activity by

probiotics have been documented in aquacultural studies (Ninawe and Selvin 2009). The addition of *Bacillus spp*. to fish intestines increased feed conversion ratio, protein efficiency ratio, and growth (Bairagi *et al.* 2004). Furthermore, probiotics in some situations can be stabilizers of intestinal pH and lead to increased absorption of some nutrients (Mountzouris *et al.* 2007). In chickens, pigs, sheep, goats, cattle and equine, probiotics improved feed intake, feed conversion rate, and weight gain (Musa *et al.* 2009). In addition, in agriculture certain probiotic bacteria increase the growth of crops by solubilizing phosphorus in the soil for plants to use (Islam and Hossain 2012). Similarly, newts may have benefited from improved nutrient acquisition.

Bd on the newts

There was greater prevalence of infection and average *Bd* loads in all *Bd* treatments than in the *Bd* absent treatment, which demonstrates that a *Bd* environment was created and infection of the newts occurred. However, no probiotic treatment in itself reduced the *Bd* prevalence or *Bd* loads to levels below that found in the *Bd* only treatment. Interestingly, there was a negative correlation between *J. lividum* and *Bd* on the newts, suggesting that *J. lividum* may be reducing *Bd*. Although cause and effect cannot be known, we have *in vitro* evidence that *J. lividum* can inhibit *Bd*, whereas there is evidence that *Bd* cannot inhibit *J. lividum* (data not shown). Despite this in vitro evidence, it is possible that *J. lividum* is a less effective competitor and *Bd* is a more effective competitor in vivo. *Bd* has been shown to show different gene expression patterns when grown in different substrates (Rosenblum *et al.* 2012), therefore, it is likely to act differently on an amphibian host. However, the same is true for *J. lividum*; it has been shown to effectively reduce *Bd* infection in vivo (Harris *et al.* 2009, Muletz *et al.*

2012). It is interesting that this correlation exists considering the lack of treatment effects. This result is likely due to the efficacy of the probiotic treatments in establishing *J. lividum* on the newts; not all *J. lividum* treatment newts were *J. lividum* positive. The existence of this correlation between *Bd* and *J. lividum* suggests that with further optimization of treatment methods in establishing *J. lividum* on the host, *J. lividum* treatment could effectively reduce *Bd* infection in amphibians.

The pattern seen with the growth rate response was paralleled in the prevalence of *Bd* infection in the newts. No probiotic treatment reduced *Bd* infection prevalence of newts more than that seen in the *Bd* only treatment. The bath+water treatment reduced infection prevalence in comparison to the individual bath treatment and water treatment on day 16, suggesting that the combination treatment may be the most effective treatment method. Despite this beneficial reduction in infection prevalence, by day 27 this effect was gone and equal numbers were infected among treatments. The *Bd* loads on this day were lower overall, but there was no difference between the *Bd* loads on the newts among treatment.

No treatment was effective at increasing the proportional change in *Bd* loads in comparison to the *Bd* only treatment. However, the proportional change in *Bd* loads of the bath+water treatment and the water treatment were greater than that of the bath treatment, suggesting that these treatments were more effective at reducing *Bd* loads. The effect observed in the water treatment was driven by this treatment have higher average *Bd* loads on day 9, which was in part driven by one newt in this treatment having a high *Bd* load of 2×10^3 zoospores.

Bd did not cause morbidity in the newts and probiotic treatments did not lead to amelioration of morbidity effects from *Bd*, most likely because newts are resistant to *Bd* due to existing immune or microbial defenses or because the *Bd* JEL404 strain is not highly virulent. However, it is intriguing that bath+water had a beneficial effect in terms of weight loss and *Bd* prevalence. This is likely caused by the efficacy and probiotic persistence of this treatment being greater than the other probiotic treatments. This evidence suggests that both the bath and water treatment are necessary for probiotic conservation strategies.

Non-target effects of probiotics on the aquatic ecosystem

One concern with probiotic bioaugmentation of the environment is the potential for non-target effects on other organisms and ecosystem processes. This study provides preliminary evidence that the effects of the probiotic, *J. lividum*, do not extend beyond the host organism. Probiotic addition had no effects on leaf decomposition, periphyton production and zooplankton community structure and dynamics.

Because *J. lividum* is an anti-fungal bacteria, it is plausible that it negatively affects other fungi in the aquatic ecosystem thereby resulting is alterations in leaf decomposition since fungi are a key decomposers (Wong *et al.* 1998). However, probiotic addition at a concentration of 400-2 x 10^4 cells/ml did not alter leaf decomposition rates suggesting that the probiotic bacteria did not negatively affect fungal decomposers in the ecosystem.

Probiotic addition had no effect on periphyton production. In aquaculture, some probiotic species are used with the intention of facilitating phytoplankton growth and

production because this is a major food resource for bivalve and mollusk larvae (Kesarcodi-Watson *et al.* 2008). Therefore, it was thought that addition of the probiotic bacteria to the experimental ponds may lead to alternations in the periphyton production. For example, the addition of *Pseudomonas sp*. 002 strain to algal cultures of *Asterionella glacialis* stimulated growth via the bacterial production of a glycoprotein that acted as a growth factor for the algae (Riquelme *et al.* 1988). Additionally, *Flavobacterium sp*. DM-10 promotes the growth of the marine diatom, *Chaetoceros gracilis* (Suminto and Hirayama 1997). In another study, *Flavobacterium sp*. 5N-3 was found to inhibit *Gymnodinium mikmitoi,* the algae associated with red tide (Fukami *et al.* 1997). Nonetheless, in this experiment addition of *J. lividum* did not affect periphyton production.

Probiotic addition had no effect on total zooplankton abundance or the variance in abundance over time of the cladocerans or the copepods. In addition, there was no effect of probiotic addition on the variance ratio, which characterized the population dynamics between the groups of zooplankton. All treatments exhibited compensatory patterns between cladocerans and copepods, meaning that these groups were negatively correlated. Violacein, which is an anti-fungal metabolite produced by *J. lividum*, has been found to be mildly cytotoxic to nanoflagellates (Matz et al 2004). Therefore, it was thought that it could be toxic to organisms at higher trophic levels, such as zooplankton, or that by affecting the nanoflagellate community, there could be indirect consequences on higher trophic levels through food web dynamics. Nevertheless, there was no effect of the probiotic bacteria addition on the zooplankton community structure and abundance.

This result of non-target effects related to the probiotic addition may be due to the symbiotic relationship between amphibians and *J. lividum* where it may only exhibit measurable inhibitory effects, such as violacein or I3C secretion, when it is on amphibian skin. When not on an amphibian, *J. lividum* might not produce defensive metabolites and therefore it will not negatively affect other species in the ecosystem. Differential inhibitory effects while on the amphibian could be a result of amphibian-microbe interactions or microbe-microbe interactions (Bletz *et al.* 2013). Synergy between amphibian-produced antimicrobial peptides (AMPs) and symbionts has been documented in vitro between *Rana muscosa* AMPs and 2,4-diacetylphloroglucinol (DAPG), a metabolite produced by *Pseudomonas spp*. (Myers *et al.* 2012). Additionally, preliminary evidence has shown that pair-wise combinations of certain bacteria from *P. cinereus* when cultured together synergistically inhibit *Bd* (Holland 2013). When a probiotic bacteria is added to the skin of an amphibian its effectiveness is a product of any or all of these interactions. Interestingly, there was no correlation between *J. lividum* and *Bd* in the water, suggesting that *J. lividum* was not killing *Bd* in the water. If *J. lividum* had inhibitory effects in the water, it likely would be negatively correlated with *Bd* abundance in the water; therefore, it is possible that the lack of a correlation was driven by *J. lividum* not producing inhibitory metabolites when off its host.

The absence of non-target effects should be taken with caution because the inoculation dosage in this experiment was not sufficient to lead to a beneficial effect on newts in the water only treatment, therefore, it may be necessary to increase the inoculation dosage to get effective reduction in *Bd* loads or reduction in weight loss associated with *Bd* infection. In this experiment, the intention was to inoculate the ponds

with 1 x 10^6 cell/ml; however, this concentration was not maintained in any tank and the concentration was variable between the inoculated experimental ponds. Optimization of inoculation methods, by altering inoculation concentration, could improve the success of environment treatment. It will be important to monitor ecosystem effects under different inoculation conditions to test whether changes in the dosage and persistence of the probiotic cause non-target ecosystem effects. In addition, difference amphibian species and amphibian communities will have different ideal probiotics or probiotic mixtures, and therefore the ecosystem effects of each probiotic may vary and must be tested under controlled settings before initiating field-based trials in nature.

Implications for probiotic conservation strategies and future work

Understanding transmission and persistence of probiotics on amphibian hosts, and persistence of probiotics in the environment is essential in order to determine how to add beneficial bacteria to amphibians effectively and efficiently. This research suggests that transmission of bacteria to amphibians may be concentration dependent. It is apparent that if the amphibian receives a concentrated bath solution, transmission to the host is increased. This is perhaps because the probiotic bacteria is given a competitive advantage when in such a high density. Additionally, this study suggests that the ideal treatment method to afford establishment and persistence of the probiotic bacteria on the amphibian host is the combination of the probiotic bath and inoculation of the aquatic environment and this combination treatment also has a beneficial effect on growth rate. However, this study had several limitations, including the fact that no probiotic treatment was in itself effective at reducing *Bd* infection below that in the *Bd* only treatment, and that *Bd* had minimal effect on the amphibian host, likely due to the use of a non-susceptible species.

Therefore, additional studies are needed to further explore what treatment method is most effective and efficient for providing protection from the lethal fungus. The use of more susceptible species in probiotic experiments may lead to more definitive results. Perhaps the use of *L. sylvaticus* or *L. clamitans*, which have experienced mortality as a result of *Bd* infection, would be possible experimental species for future studies (Searle *et al.* 2010, Gahl *et al.* 2011).

The microbial community structure of different amphibian hosts may vary in terms of stability, and probiotic establishment is likely a function of community stability. If a microbial community is less stable it may be easier to establish the probiotic bacteria species because niches frequently are being opened; however, it also may make the community more prone to loss of species, including the probiotic bacteria, due to stochastic events. Monitoring the microbial community composition and structure over time and in the context of probiotic addition will reveal the response, stability and dynamics of the microbial community on amphibians. Such surveys and experiments will provide insight into the relationship among the stability of amphibian microbial communities, the modes of probiotic action and the development of probiotic conservation strategies.

Further research is necessary to investigate whether probiotic application through environmental bioaugmentation in itself is sufficient to afford protection from *Bd*, as it is the only method that does not require individual capture of amphibians. Selecting the optimal probiotic species or species mixtures for the amphibian host in the context of the intended environmental conditions in addition to determining the dosage needed to afford transmission to amphibian hosts and to achieve persistence in the environment will be

essential for effective conservation strategies. It is important to note that lasting persistence in the environment may not be essential; the probiotic bacteria needs to persist long enough for transmission to occur. If the dynamics of the probiotic bacteria on the host are independent of that of the environment then environmental persistence may not be needed. On the other hand, if a probiotic bacteria species was lost from an amphibian due to a stochastic event, then persistence in the environment could afford a mechanism of reestablishment. Furthermore, the microbial community composition of an aquatic environment varies seasonally due to abiotic factors (Kritzberg *et al.* 2006) and therefore the ability of a probiotic bacteria to establish and persist in an environment may be a function of seasonal community dynamics. One likely avenue of environmental bioaugmentation strategies for amphibian conservation is the inoculation of ponds where amphibians congregate to breed; therefore, the seasonal conditions at the onset of breeding will be important to consider. Experimentation with probiotic persistence in collected pond water in laboratory experiments or in developed mesocosms in field-based experiments can be performed to further investigate probiotic persistence under different environmental conditions.

Ideal probiotic bacterial species for bioaugmentation must be effective competitors against other bacteria on the host and in the environment, relatively invulnerable to grazing, and suited for the environmental conditions of the intended application area in the context of the season of application. Of course, selecting probiotics that are from amphibians in the local area will increase the likelihood of those species persisting in the environmental conditions of the application area (Bletz *et al.* 2013). Additionally, continued experimentation with different probiotic dosages, such as

a target concentration of 1×10^7 and 1×10^8 cells/ml will be important to determine if increased concentrations can afford transmission and create a persistent population. It is possible that the persistence of a probiotic bacteria is also a function of the bacterial species; therefore, it will be important to perform trials will multiple probiotic bacteria candidates and possibly probiotic bacteria mixtures and determine which candidates are ideal for environmental bioaugmentation. Additionally, probiotic application methods will need to be optimized for different hosts and amphibian communities from different habitats and geographic areas so experimentation with phylogenetically diverse hosts and in different habitats is critical.

Importantly this study suggests that probiotic addition has no ecosystem effects, therefore, probiotic conservation strategies are unlikely to harm other organisms and disrupt ecosystem processes. Continued testing of ecosystem effects will be essential as different probiotic species and inoculation concentrations are explored. In addition, more aspects of the ecosystem should be tested including benthic organisms, phytoplankton, and the aquatic bacterial community to determine the effects of probiotic addition on a wider variety of ecosystems parameters.

Probiotic bioaugmentation is a new conservation frontier that requires continued research in order to develop effective and efficient methods for combating the amphibian fungal disease, chytridiomycosis. In doing so we will gain an understanding of symbiosis between microbes and amphibians, microbial community dynamics, and microbe interactions with other aspects of the ecosystem. The interplay of basic ecological study and applied method development will be critical to the success of amphibian disease mitigation programs.

APPENDICES

Appendix 1: Identification of Anti-*Bd* bacteria on *N. viridescens*

Introduction:

The aim of this preliminary investigation was to isolate and identify a usable probiotic bacterium from *Notophthalmus viridescens*, the study species for the main experiment. *N. viridescens* is an aquatic organism that resides in pond ecosystems; therefore, the microbial species and strains may differ from those previously identified and used in probiotic experiments with the terrestrial salamander, *Plethodon cinereus*. An effective probiotic must inhibit *Bd*, colonize and persist on newts (Appendices 3 & 5), and colonize and persist in an aquatic environment (Appendix 2) (Bletz *et al.* 2013). Isolates were tested to see if they meet these criteria.

Methods:

Field Collection and Microbial Swabbing

In order to identify candidate anti-*Bd* bacteria that reside on *N. viridescens* two different collections were completed. Twenty individuals (5 females, 18 males) were collected at Gauley Ridge Pond (GRP) in the George Washington National Forest, Virginia on 26 October 2011, and 21 individuals were collected at Todd Lake in the George Washington National Forest on 27 February 2012. To prevent cross contamination each captured newt was handled with a new set of nitrile gloves. Individuals collected at GRP were processed in the field and promptly released whereas the individuals collected at Todd Lake were taken into the laboratory, processed and kept for use in other preliminary investigations.

To sample the resident cutaneous bacteria, each newt was rinsed twice in different sterile 50 ml Falcon tubes containing 20 ml sterile Provasoli medium prior to swabbing. This procedure removes transient bacteria and pond associated materials (Culp *et al.* 2007; Lauer *et al.* 2007). Using a FineTip MW113 swab (Medical Wire & Equipment, Corsham, Wiltshire, England) moistened with Provasoli, each newt was swabbed 10 times on the ventral and lateral surfaces (1 swab = up and back) and each foot was swabbed once. The swabs were streaked directly onto a 1% tryptone plate and wrapped with parafilm. The plates then were incubated at 25^0 C.

Isolation of Bacteria

Gauley Ridge Sampling:

The goal of this collection was to isolate resident symbiotic bacteria from newts in order to find *Bd*-inhibitory isolates that naturally occur on newts. After the bacterial cultures from the individual newts were incubated at 25^0 C for 72-96 hours, each morphologically distinct colony was labeled on the petri dish. A representative single colony of each distinct type from each newt was isolated with a sterile toothpick and streaked onto fresh 1% tryptone agar plates until pure cultures were obtained. Original mass culture plates were checked daily for new distinct isolates for one to two weeks to ensure that all distinct species including slow growing strains had been isolated. Each isolate was characterized by color, shape, texture, form, and surface appearance (Table 1). Approximately 200 bacterial isolates were obtained from the 23 newts sampled at GRP. Morphologically similar isolates grouped together and were considered to be the same operational taxonomic unit (OTU). Forty-two OTUs were isolated from two or more newts, and 30 OTUs were isolated from one newt. If an OTU was isolated from three or more newts it was said to be ‘commonly present on *N. viridescens',* and it was

maintained for future use. Thirty-three groups were maintained for challenge assays and given a Challenge Assay Group (CAG) number. Isolates from these groups were maintained on 1% tryptone plates until challenge assays with *Bd* were completed.

Todd Lake Sampling:

The aim of this collection and sample was to isolate a newt strain of *Janthinobacterium lividum.* Isolates from the Todd Lake newts were not isolated and maintained unless they were purple, which is a characteristic of *J. lividum*.

Challenge Assays

Thirty-three isolates from GRP and five purple isolates from Todd Lake were examined for their inhibition activity against *Bd* using agar-plate challenge assays (Harris *et al.* 2006). For the challenge assays, zoospores were harvested from *Bd* stock plates. One milliliter aliquots of the zoospore-suspension from stock plates were pipetted onto fresh 1% tryptone plates. The liquid was spread equally across the surface of the plate and allowed to dry. As soon as the plates dried two bacteria isolates (one control non-inhibitory, one test strain) were streaked on either side of the plate with a sterile toothpick. Plates were covered, sealed, and incubated upright for 48-96 hours at 25^0C. After incubation, bacterial isolates were scored as either inhibitory (clear zone of inhibition developed between the bacterial streak and *Bd* culture) or not inhibitory (no zone of inhibition developed). Inhibition zones were also measured in order to compare inhibitory isolates. Each bacteria isolated was tested at least two times. All challenge assay plate preparations were performed in the laminar flow hood.

Storage of Bacteria Isolates

After challenge assays had been completed two samples of each of the isolates

that were inhibitory to *Bd* were prepared and stored in the -80^{0}C freezer to maintain them for future use as a potential probiotic. To do this, bacteria was collected from a pure culture on an agar plate using a sterile toothpick and placed in a centrifuge tube with 1 ml of TYSE + glycerol (25%) stock. After 60 minutes at room temperature, the tubes were transferred to the -80^{0}C freezer.

Identification of Bacteria

Bacterial isolates from Gauley Ridge newts that were found to be inhibitory towards *Bd* were candidates for use as a probiotic and were identified by 16s rRNA gene sequencing methods. DNA was extracted from pure cultures using the MoBio UltraClean Microbial DNA Isolation Kit (Carlsbad, CA, USA) following the manufacturer's protocol. After extraction, the 16S rRNA gene was amplified using 16S rRNA specific primers, 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'GGTTACCTTGTTACGACTT-3') (Lane 1991). Twenty-five µl PCR reactions were run containing, 1µl (10µM) of each primer, 2.5 µl of 10x Buffer A, 1µl (10µM) of dNTPs, 0.2 µl of Taq polymerase, 0.5 µl of DNA, and 18.8 µl of water. The thermo-cycling program parameters for sequencing reactions were: 94^{0}C for 4 min., 35 cycles of 94^{0}C for 30 sec., 53^{0}C for l min., 72^{0}C for 2 min., followed by a final 10 min. at 72^{0}C (Lauer *et al.* 2007). All PCR products were electrophoresed in a 1% agarose gel and stained with Gel Red to ensure that amplification of the desired product had occurred. All PCR reactions contained a negative control. PCR products were sent to Agencourt Bioscience Corporation (Beverly, MD) for DNA sequencing. An attempt to align the forward and reverse sequences was made in Sequencher. However, it was not possible to align most obtained sequences due to poor quality chromatograms and short sequencing

reads. The poor quality reads may have been due to inadequate sample volume or contamination. Regions of clean reads were selected from the sequences and entered into a NCBI GenBANK database search in attempt to identify the bacteria. (http://www.ncbi.nlm. nih.gov) (Lam *et al.* 2010). Identification at genus level was possible for some isolates.

Isolates suspected to be *J. lividum* from the Todd Lake newts were verified via PCR with species-specific primers, ViolF (5'-TACCACGAATTGCTGTGCCAGTTG-3') and ViolR (5'-ACACGCTCCAGGTATACGTCTTCA-3') (Becker *et al.* 2009). The thermo-cycling program parameters for *J. lividum* were: 94^0C for 4 min., 30-35 cycles of 94^0C for 1 min, 58^0C for l min., 72^0C for 1.5 min., followed by a final 10 min at 72^0C. PCR products of isolates were run on gels with a positive control of known *J. lividum* to confirm their identity.

Results

For the thirty-three bacterial groups challenged against *Bd*, 11 (30%) showed inhibition in at least two agar plate challenge assays. CAG 10 had an inhibition zone of 9.5 mm (Table 1), which was the largest recorded zone. CAG 10 was selected for preliminary experimentation as a possible probiotic for the main experiment in experimental ponds. CAG13, which was one of the most prevalent bacterial strains (Table 2), had the second largest zone, measuring 9.0 mm (Table 1). CAG 4, 11, 12, 16, 18, 20, 25, and 31 were also inhibitory, having zones ranging from 3.5-8 mm (Table 1). CAG 1 consistently appeared to have inhibited all *Bd* and bacteria growth on the challenge plate, and no precise zone could ever be measured. Because *Bd* activity and

control bacteria growth were consistently eliminated, it likely is a very strong inhibitor of *Bd*. In the future, a 96 well-plate challenge assay should be completed using the crude extract of this bacterial strain to confirm its inhibitory nature. 96-well challenge assays involving using the cell-free supernatant, which can be serially diluted to test inhibition of various concentrations of this isolates metabolites.

Five newt swabs from the Todd Lake collection had purple colonies that were isolated and tested to confirm whether they were *J. lividum*. Four of the 5 purple isolated colonies were confirmed to be *J. lividum* via PCR and gel electrophoresis. The four

CAG	Inhibitory?	Average inhibition zone (mm)	Genus ID	Percent match to GenBANK isolate

confirmed isolates were tested for *Bd* inhibition and exhibited small but visible inhibition zones, characteristic of *J. lividum*. One of these strains (13A) was selected for use in preliminary probiotic bath and environmental transmission experiments.

Due to low sequencing quality, inhibitory isolates could not be identified to the species level. Some sequencing reads were clean enough to get genus level identification (Table 1); however, 16s rRNA gene sequencing should be conducted again to confirm the identification of these isolates.

Table 1: Inhibitory isolates from N .viridescens and zone measurements. Control strains are labeled. Genus ID is provided for isolates that had usable sequencing reads.

1*	yes	Not measurable	none	
4*	yes	4	*Serratia sp.*	93
10*	yes	9.5	*Pseudomonas sp.*	91
11*	yes	6.5	*Pseudomonas sp.*	98
12*	yes	7	none	
13*	yes	9	none	
16*	yes	5	none	
18*	yes	5.5	none	
20*	yes	8	*Pseudomonas sp.*	98
21*	no	Control	*Sphingomonas sp.*	98
25*	yes	5	none	
31*	yes	3.5	none	
33*	no	Control	*Sphingomonas sp.*	93

Table 2: Morphological characteristics of challenge assay bacterial groups. Color, shape, texture and surface character are given. The number of newts possessing each isolate is provided in the second column.

CAG	# of newts	Color	Shape	Texture	Surface
1	11	white	round	mucousy	Wet
2	5	yellowy-orange	round	smooth	Wet
3	5	clearish white	round	smooth	Wet
4	7	milk- white	round	smooth	Wet
5	6	white-opaque	round	rough	Dry
6	7	creamsicle color	round	smooth	Wet
7	8	white-clearish	round	smooth	Dry
8	10	light yellow, creamy	round	smooth	Wet
9	7	solid yellow-orange	round	smooth	Wet
10	4	white, greenish hue	irregular	non-smooth	Wet
11	4	white, greenish hue	round	smooth	wet
12	5	milky white	round	smooth	Wet
13	8	gloppy white	Dome, spreader	smooth	Wet

14	10	opaque white	round	smooth	Wet
15	3	orange	round	smooth	Wet
16	6	white- greenish hue	round	smooth	Wet
17	4	white	round	smooth	Wet
18	3	off-white	filamentous	curly	Dry
19	5	yellow-white	round	smooth	Wet
20	3	hazy white	round-oval	smooth	Wet
21	3	yellow	round	smooth	Wet
22	3	clear, white	round	smooth	Dry
23	4	white-off-white	irregular edge	smooth	Wet
24	3	light yellow	round	smooth	Wet
25	4	white	irregular	smooth	Dry
26	4	yellowy-orange	round	smooth	Wet
27	4	opaque white	irregular	smooth	Wet
28	3	yellowy-white	round	smooth	Wet
29	4	off white	round	smooth	Wet
30	4	white, clearer center	round, distinct edge	smooth	Dry
31	5	distinct white	round, defined edge	smooth	Wet

Discussion

The resident bacterial community of *N. viridescens* contains bacteria that can inhibit the pathogen, *Bd*. Inhibitory isolates found on *N. viridescens* included

Pseudomonas species and *J. lividum*, which have been cultured from numerous other amphibian species (Lauer *et al.* 2007, 2008, McKenzie et al 2012).

Anti-*Bd* bacteria isolated from *N. viridescens* were not selected as the probiotic bacteria for the main experiment. For the proposed experiment in experimental ponds there were three main requirements for the selected bacteria including inhibition of *Bd*, persistence in an aquatic environment, and presence on or ability to colonize *N. viridescens*. Both CAG 10, a top inhibitory isolate, and *J. lividum* from *N. viridescens* were inhibitory and present on *N. viridescens*; however, they did not persist in the aquatic inoculation experiments (see Appendix 2). Experimentation with *J. lividum* originally isolated from *Hemidactylium scutatum* was conducted in conjunction with the newt isolates since this isolate has been a successful probiotic in experiments with *Plethodon cinereus* and *Rana muscosa* (Harris *et al.* 2009, Becker *et al.* 2009, Vredenburg *et al.* 2011, Muletz *et al.* 2012). Because it performed well in the aquatic inoculation trials (Appendix 2) and was able to be transmitted successfully to newts in the preliminary transmission trial (Appendix 3) it was selected for use in the main experiment. While the original goal was to use a bacterial species isolated from newts, by using *J. lividum* from *H. scutatum* I tested whether bacteria isolated from a different amphibian species from the same local area can be an effective probiotic. This choice evaluated the possibility that a broad spectrum probiotic can be effective in inhibiting *Bd.*

Appendix 2: Determination of probiotic dosage for water in aquariums and stock tanks

Introduction

The main experiment required probiotic inoculation of experimental ponds in order to create a sustained probiotic reservoir. The objective was to find a dosage that was large enough to create a sustained reservoir but small enough so that it would not have significant detrimental or adverse effects on the health and functioning of the pond ecosystem.

Methods

Aquarium Experiments

Laboratory experiments using glass aquariums (capacity 37.85 L) and outdoor experiments using stock tanks (440 L) were performed to determine an adequate inoculation dosage. Three preliminary water inoculation experiments were carried out in aquariums. These experiments tested different probiotic candidates at varying concentrations. For all experiments bacteria were selected for rifampicin resistance to allow tracking of bacteria using culture based methods.

In the first experiment, three aquariums containing approximately 20 L of sterile Provasoli were inoculated with one of the following bacterial suspensions, $1x10^4$ cells/ml, $1x10^5$ cells/ml, $1x10^6$ cells/ml, to determine which dosage created the most persistent population. These concentrations were based on those used in aquacultural probiotic experiments (Moriarty 1998, Boutin *et al.* 2011). Two bacterial isolates, including *Janthinobacterium lividum* Rif^{-R} isolated from *Hemidactylium scutatum* and Rif^{-R} isolate 10 from *N. viridescens*, were grown for 24 hours in 20 ml of tryptone broth. Next, for

each strain, five ml of the broth culture was added to a unique flask containing 250 mL of tryptone broth with microbeads (3-mm) and grown on an incubated shaker (100rpm; 25^0C). Once an adequate concentration of cells was reached (determined by previously collected growth curve data), bacteria cells were washed twice via centrifugation (7500 rpm for 10 minutes) to remove any metabolites that may interfere with the bacterial cell persistence and growth. The collected cells were re-suspended in Provasoli (Harris *et al.* 2009). The Provasoli-bacteria suspension containing the appropriate number of cells was added to each aquarium, and the water was stirred. Water samples were taken every day for the first 3 days, including the day of inoculation and then every 3 days for 26 days. Bacteria concentrations were determined by culture-based colony forming unit (CFU) methods. Using a 10 mL pipette, two water column samples were taken from each tank and pooled together. Serial dilutions were made in PBS (phosphate-buffered saline, pH 7.4) and 100 µl was plated on 1% tryptone-Rif agar plates. After 48 hours of incubation (25°C), the colonies were counted to determine the concentration present in the aquaria.

The second inoculation experiment was conducted with a newly isolated strain of *J. lividum* from *N. viridescens*. The bacteria were prepared as previously described. Three aquariums (~ 20L) were inoculated with enough *J. lividum* to create a concentration of 10^6 cells/ml. After inoculation, water samples were taken following the same regime as stated previously; however, this experiment was terminated at 15 days. Sampling was completed as described in the first aquarium experiment.

The third inoculation experiment conducted in aquariums was performed in tandem with the environmental transmission experiment (Appendix 3). Four tanks were inoculated with *J. lividum* isolated from *H. scutatum* to create a concentration of 10^6

cells/ml. In addition, four control tanks were set up and were inoculated with an equivalent volume of sterile Provasoli. These control were used mainly for the environmental transmission experiment (Appendix X), but also used to confirmed that there was not experimental contamination during sampling. *J. lividum* cultures were prepared as previously described and sampling took place on day 0, 1, 2,4,10, and 15. Sampling of the control tanks occurred only on day 0 and 1 and 15. Sampling was completed as described in the first aquarium experiment.

Stock Tank Experiments

The results of the aquarium dosage experiments were used to suggest an adequate dosage to be used in the much larger stock tanks. The 1 x 10^6 cells/ml dosage was tested in experimental stock tanks (~567 L) to determine if it was an adequate inoculation dosage for persistence of the probiotic.

Three tanks containing approximately 440 liters of water were inoculated with the enough bacteria to create a 10^6 cell/ml concentration. *J. lividum* from *N. viridescens* was prepared as previously described and added to three stock tanks. Water samples were taken on day 0 (day of inoculation), days 3, 6, and 10 to monitor the persistence of *J. lividum*. To sample the water in the cattle tanks, a 2.54 cm diameter PVC pipe was used to collect a column of water. The column was transferred to a sterile 1-L sample bottle and brought back to the lab for serial dilution and plating as previously described. Concentrations were determined though culture-based colony counting methods.

Results

In the first aquarium trials with *J. lividum*, inoculation at all three concentrations lead to establishment of *J. lividum*; however, persistence varied. Initial bacterial concentrations were 4.06 x 10^3 cell/ml, 3.6 x 10^4 cells/ml, and 2.69 x 10^5 cells/ml for the targeted inoculation concentrations of 10^4, 10^5, and 10^6 cells/ml respectively (Table 1). All three inoculations showed a decline in concentration over the first two days. The tanks inoculated with10^5 and 10^6 cells/ml showed an increase in concentration on day 3 (Figure 1). This increase was not seen in the 10^4 inoculation trial; however, colonies were not countable on day 1 and 2 due to the plated bacteria spreading because of excess moisture and there is no replication in this experiment. After cay 3, the 10^5 and 10^4 inoculation trial concentrations declined to between 10-100 cells/ml, while the 10^6 inoculation declined slightly but stabilized at approximately 10^4 (Figure 1). This suggested that 10^6 would be an adequate inoculation dosage to create a persistent population. The results of this experiment were used for the 2 additional aquarium experiments as well as the cattle tank inoculation experiment.

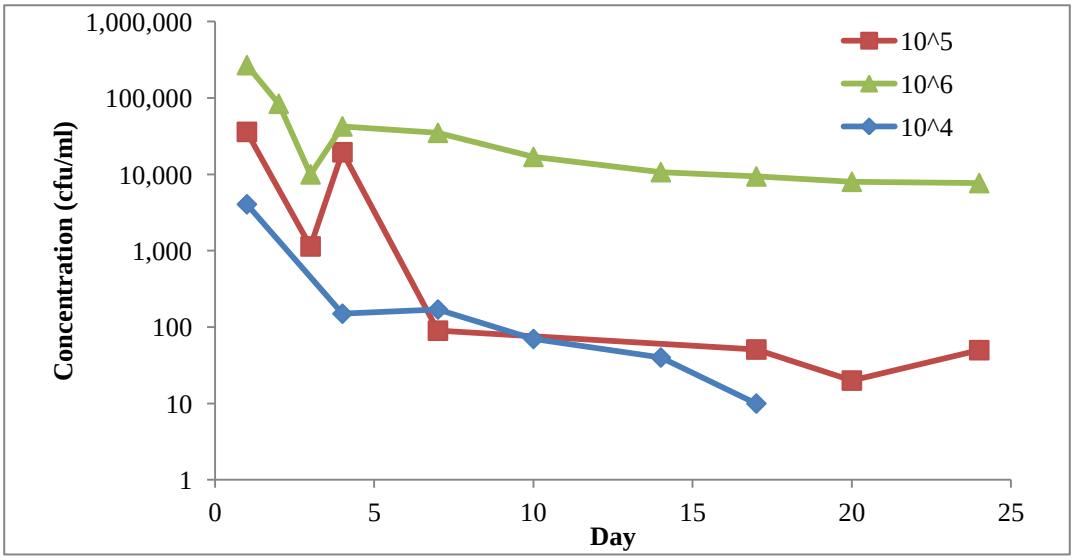


Figure 1: Persistence of *J. lividum H.s.* strain in Aquarium Trial 1 over time. The concentration of *J. lividum* is presented on a log scale.

In the aquarium trial with CAG 10, inoculation lead to establishment in the water; however the established populations were in lower concentrations than expected by the inoculation concentration of 1 x 10^4, 1 x 10^5, and 1 x 10^6 cells/ml (Figure 2). All three inoculations decreased gradually over time to levels between 10 and 100 cells/ml (Figure 2).

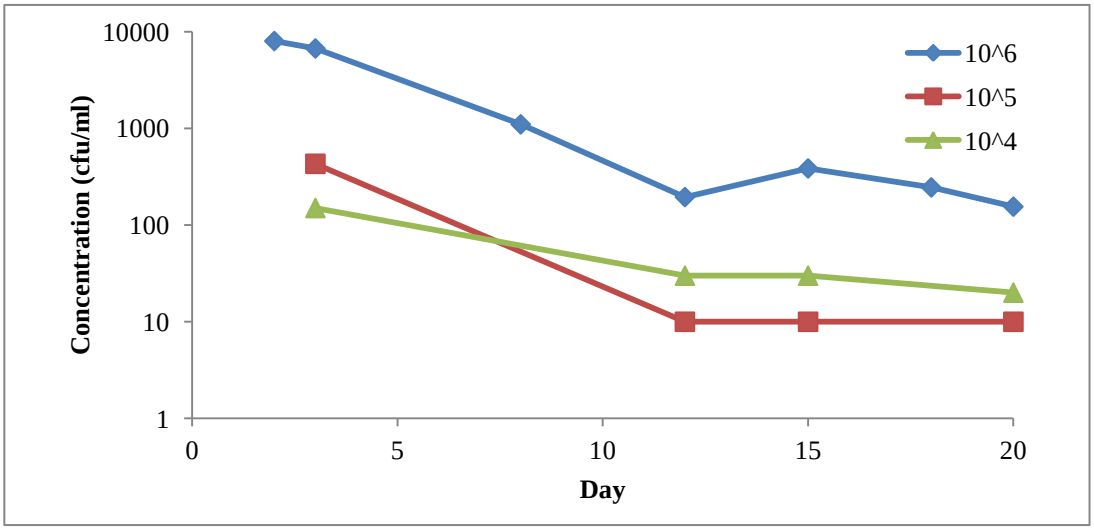


Figure 2: Persistence of *N. viridescens* Isolate 10 over time in aquarium trial. The concentration of *J. lividum* is presented on a log scale.

In the second experiment with *J. lividum* isolated from *N. viridescens*, only 2 of the 4 aquariums had cell concentrations close to the intended inoculation dosage at the first sampling. In two aquariums, while there was evidence of the presence of the added *J. lividum*, less than 10 cells were detected at the initial sampling (Table 1). In the other two aquariums initial concentrations were 1.86x10^5 and 2.42 x 10^5cells/ml. The *J. lividum* concentrations in both tanks decreased over time. In aquarium 2, it dropped to 25 cfu/ml by day 15 and aquarium 4 decreases to 1000 cfu/ml (Table 1).

In the third trial, the *J. lividum (H. scutatum* strain) inoculation established, *J. lividum* in the water but did not achieved stable populations (Table 2). By day 15,

concentrations in tank 1 and 2 had dropped to 200 and 300 cfu/ml respectively. Tank 3 maintained a slightly greater concentration of $2.8x10^3$cfu/ml (Table 2); however this was much lower than the expected inoculation dosage of 1×10^6.

Table 1: *J. lividum* concentrations over time in aquarium trial 2. Concentrations are given in CFUs/ml.

Replicate	Expected	Day 0	Day 1	Day 3	Day 15
Tank 1	1×10^6	1	0	0	0
Tank 2	1×10^6	2.42×10^5	0	45	25
Tank 3	1×10^6	3	0	0	0
Tank 4	1×10^6	1.86×10^5	3.2×10^4	TMTC	1000

Table 2: *J. lividum* concentrations over time in aquarium trial 3. Concentrations are given in CFUs/ml.

Replicate	Expected	Day 0	Day 1	Day 3	Day 15
Tank 1	1×10^6	1.6×10^4	3.3×10^3	3.2×10^4	200
Tank 2	1×10^6	1.7×10^4	4.6×10^3	1.5×10^5	300
Tank 3	1×10^6	3.7×10^4	3.7×10^3	6.3×10^4	2.8×10^3

Discussion

J. lividum from *H. scutatum* was the only bacterium to achieve stable populations in the aquarium inoculation trials when the aquarium was inoculated with 10^6 cells/ml, which suggested that this would be an adequate dosage to create a persistent population in the main experiment. This concentration was used for the main experiment. While the proposed experiment aimed to use a bacterium isolated from *N. viridescens* the two bacteria strains from *N. viridescens* (Isolate 10 and *J. lividum N. viridescens*) did not persist at consistent levels. If more time had been available, additional bacteria strains isolated from *N. viridescens* could have been tested at a range of inoculation concentrations.

Appendix 3: Environmental transmission of *J. lividum* to *N. viridescens*

Introduction

The aim of this preliminary experiment was to determine if the chosen beneficial bacterium, *Janthinobacterium lividum*, can be transmitted through an aquatic reservoir to *Notophthalmus viridescens*. Environmental transmission experiments have been completed using the terrestrial salamander, *Plethodon cinereus*, in a soil environment (Muletz *et al.* 2012); however, no experiments to date have used aquatic environments. To test whether transmission can occur in an aquatic environment (experiment 1 & 2) and determine if *J. lividum* originally isolated from *H. scutatum* can be transmitted to *N. viridescens* (experiment 2) preliminary experiments were completed.

Methods

Newt collection

N. viridescens for the transmission experiments were obtained from field sites located in the George Washington National Forest (White Oat Flat and Todd Lake). New nitrile gloves were worn for collection of each newt. Each newt was sexed and only males were kept. Collected *N. viridescens* were placed in sterile containers for transportation with 25 ml of Provasoli. Because newts have unique dorsal spot patterns photographs were taken for future identification of individuals (Gill 1978). While housed in the lab, prior to the start of experimentation, newts were given pellet food (JurrasDiet).

Water inoculation with bacteria

To create a bacterial reservoir, aquariums containing 20 L of well water (R. Domangue residence) were inoculated with the enough bacteria to create a concentration of 10^6 cells/ml. To do so, *J. lividum* was cultured in 1% tryptone broth at 25^0C for 24 hours. After 24 hours, 5 ml of the broth culture was added to 250 ml of 1% tryptone broth containing sterile 3 mm micro-beads. This culture was grown at 25^0C on a rotary shaker at 150 rpm until a usable cell concentration, as determined by optical density, was reached. The appropriate amount needed for water inoculation was washed twice via centrifugation as explained in Appendix 2 and suspended in fresh sterile Provasoli. The bacterial suspension was added to each aquarium and gently stirred.

Transmission Experiment

To determine whether environmental transmission occurs, two experiments were completed. In experiment 1, *J. lividum* isolated from *N. viridescens* was used and in experiment 2, *J. lividum* isolated from *H. scutatum* was used due to its success in water inoculation and persistence trials (Appendix 2).

Experiment 1:

N. viridescens were assigned at random to one of two treatments: treatment 1 (n=4) had a bacteria reservoir (bacteria +) and treatment 2 (n=3) was a control, with no added bacteria (bacteria –). After the water was inoculated with 1 x 10^6 *J. lividum* cells/ml (bacteria+) or sterile Provasoli (bacteria-), tanks were undisturbed for 24 hours. After 24 hours, 2 newts were added at random to each aquarium and monitored for two weeks. Newts were swabbed, prior to tank entry, and on days 6 and 14 of the experiment as described in Appendix 1. Swabs were frozen (-80^0C) until further processing was completed. DNA from the swabs was extracted with the Qiagen QIAamp DNA Micro Kit (Germantown MD, USA) according to the manufacturer's protocol except the volumes of

Buffer ATL and AL were decreased to 200 µl. Diagnostic PCR was performed to assess the presence or absence of *J. lividum* on the newts (Harris *et al.* 2009). Positive and negative controls were run in all PCR reactions.

Experiment 2:

Three aquaria were inoculated with 1 x 10^6 *J. lividum* cells/ml (bacteria+) and aquarium were undisturbed for 24 hours. After 24 hours, 2 newts were added at random to each aquarium and monitored for 1 week. Newts were swabbed, prior to tank entry, and on day 7 of the experiment as explained in Appendix 1. Swabs were frozen (-80^0C) until further processing was completed. Extraction and PCR were preformed as explained above.

Results:

Experiment 1

All newts tested negative for *J. lividum* before the experiment began, except one newt for one newt in control aquarium 5. After 6 days, 3 of the 8 newts in bacteria + aquarium tested positive for *J. lividum*. After 14 days, 3 of the 8 newts were positive; however, only one of these positive newts was positive on day 6 (Table 1). The newts in the control bacteria – treatment were all negative except for one newt in aquarium 5 (Table 1). This newt also tested positive for *J. lividum* before the experiment began; therefore, this result was not due to contamination but because the newt already possessed *J. lividum*. Throughout the experiment 5 of the 8 newts were positive for *J. lividum*. The low rate of transmission of the *J. lividum* isolated from *N. viridescens* lead to the decision to run an additional transmission experiment with the *J. lividum* isolated from *H. scutatum* that had been used in previous transmission experiments in soil (Muletz *et al.* 2012).

Table 1: *J. lividum (N. viridescens* strain) presence on newts throughout the environmental transmission experiment. + indicates the presence of *J.liv*, - indicates the absence of *J.liv* and NT indicates not tested.

Aquarium	Environmental Reservoir	Initial	Day 6	Day 14
1	+	-	-	-
		-	-	-
2	-	-	NT	-
		-	NT	-
3	+	-	-	+
		-	-	+
4	+	-	-	-
		-	+	-
5	-	+	NT	+
		-	NT	-
6	-	-	NT	-
		-	NT	-
7	+	-	+	-
		-	+	+

Experiment 2

All newts tested negative for *J. lividum* before the experiment began, except one newt in aquarium 1. All newts tested positive for *J. lividum* on day 7 of the experiment (Table 2).

Table 2: *J. lividum (H. scutatum* strain) presence on newts in 2nd environmental transmission experiment. + indicates the presence of *J.liv*, - indicates the absence of *J.liv*.

Aquarium	Environmental Reservoir	Initial	Day 7
1	+	+	+
		-	+
2	+	-	+
		-	+
3	+	-	+
		-	+

Discussion

In the first experiment, transmission *J. lividum* to the newts was low and maintenance of *J. lividum* on the newts was inconsistent. This is most likely due to the inoculation of the reservoir and persistence of the bacteria in the water being variable. Inoculation was not successful in aquariums 1 and 4 (Appendix 2), and therefore it is not surprising that the newts in these aquariums? did not acquired *J. lividum*. Aquariums 3 and 7 both had successful inoculations, although concentrations declined over time. Both newts in aquarium 7 and one newt in aquarium 3 acquired *J. lividum*. The fact that even a short period of persistence of environmental sources of *J. lividum* allowed transmission has positive implications for environmental treatment probiotic conservation strategies. This finding suggests that a probiotic may not need to persist for long periods of time to allow for transmission, which will minimize potential non-target effects caused by the addition of a probiotic. *J. lividum (H. scutatum* strain) was effectively transmitted to at least five of six newts, and therefore it was chosen as the optimal candidate of the tested isolates for the main experiment in experimental ponds.

Appendix 4: Batrachochytrium dendrobatidis exposure methods

Introduction

There are multiple ways of introducing *Bd* into the experimental ponds, including tank inoculation and individual newt exposure. Tank inoculation is a more accurate representation of environmental conditions but does not ensure that individuals will become infected. Therefore, preliminary experimentation was conducted to determine if tank inoculation was a successful method of infecting newts.

Methods

Experimental pond ecosystems were created in three stock tanks. First, approximately 440 liters of water were added to each tank. Next, leaf litter (25g) and plankton suspension (500 ml) were added. After leaving the tanks undisturbed for one week, two newts were added to each tank. Newts were collected from White Oak Flat, George Washington National Forest, on 5 May 2012. Newts were swabbed prior to entry to confirm they were negative for *Bd* at the start of the experiment.

Bd Introduction

To introduce *Bd* to the environment, 6 *Bd* stock plates that had been incubated for 5 days were attached to a water-filled container using aquarium sealant. These '*Bd* cubes' were placed on the bottom of each stock tank. The newts were swabbed on day 3, 6, 14 and 23. DNA was extracted from the swabs using the Qiagen Qiaamp Micro Kit swab extraction protocol with the exception of using 200 ul buffer ATL and AL as recommended in the lab protocol book. Diagnostic PCR was performed to determine if the newt were infected with *Bd* following the protocol and primer sets in Annis *et al.* (2004).

Results

On day 3 one newt tested positive for *Bd*; however, by day 6 all newts were positive for *Bd*. The infection status of all newts remained positive through day 14; however, by day 23 only 50 % were infected (Table 1).

Table 1: Infection status of newts over time.

	Newt ID	**Day 3**	**Day 6**	**Day 14**	**Day 23**
Tank 1	8	-	Faint +	Faint +	-
	9	-	+	+	+
Tank 2	1	-	Faint +	+	-
	10	Faint +	+	+	-
Tank 3	4	-	+	+	+
	7	-	+	Faint +	+

Discussion:

The experiment demonstrated that tank introduction of *Bd* was an effective method to establish *Bd* on newts. This method was used in the main experiment to introduce *Bd* into the experimental ponds.

Appendix 5: Probiotic inoculation of individual newts experiment

Introduction

Individual probiotic treatment is meant to augment the microbial community of the amphibian's skin to contain greater amounts of the chosen probiotic species, which for this experiment was *J. lividum*. In previous experiments (Becker and Harris 2010) reduction of the cutaneous microbiota through antibiotic treatment has been performed to provide open niches for the probiotic bacteria. However, the main experiment was designed to be representative of a feasible disease mitigation strategy in the field. The use of antibiotic treatments prior to probiotic application in large-scale field situations is not feasible. To determine if probiotic bath treatment without prior antibiotic treatment can successfully augment the microbial community to contain the probiotic species, probiotic bath trials were performed.

Methods

To create the probiotic solution, the probiotic bacterial species was cultured on a shaker for 24 hours in a flask containing 1% tryptone broth. After approximately 8 hours, to remove any metabolites, the appropriate number of cells were washed in Provasoli and centrifuged two times and then re-suspended in Provasoli (Harris *et al.* 2009). Five newts were bathed in 15 ml of probiotic *J. lividum* (*N. viridescens* strain) solution (conc. 4.3 $x10^9$ cells/ml) in 50 ml Falcon tubes for 2 hours. Every 30 minutes tubes were rotated. After 24 hours, newts received a second bath under the same conditions as the first bath for two hours. Newts were kept in sterile plastic containers(16.5 cm x 10.2 cm x 8.9 cm) with 100 ml of sterile Provasoli between baths. Twenty-four hours after the second bath, newts were rinsed 2 times in 20 ml of Provasoli to remove transient bacteria and swabbed

as described in Appendix 1. DNA extraction and *J. lividum* PCR was completed as previously described.

One newt was also bathed in a concentrated bacterial solution (9.2×10^{10}) of *J. lividum* from *H. scutatum* for 24 hours. Only one newt was used because there was only one remaining from initial collection for preliminary experiments. The Falcon tube was aerated and rotated every 10-12 hours. Twenty-four hours after the newt was bathed, it was rinsed and swabbed as described in Appendix 1.

Results

The first probiotic bath trial using two 2 hours baths, resulted in 3 of the 5 newts becoming positive for *J. lividum*; however, one of these individuals also tested positive for *J. lividum* prior to treatment. In the second trial with the extended bath, *J. lividum* was successfully transferred to the 1 newt in the trial.

Discussion

Two of the 5 newts were effectively inoculated with *J. lividum* in the first trial. Two of the newts that did not have *J. lividum* at the end of the experiment were shedding during the bath. The effect of shedding on skin bacteria has only recently been investigated (Meyer *et al.* 2012). It is plausible that such a disruption could affect the ability of the probiotic to establish itself on the newt. For the main experiment, it was decided that an extended bath for 36 hours would be used and no antibiotic treatment would be performed because the two, two-hour baths were not sufficient in this preliminary experiment. Additionally, in previous probiotic experiments, longer bath exposure times have been successful (Vredenburg *et al.* 2011).

Appendix 6: Occurrence of *Batrachochytrium dendrobatidis* in populations of *Notophthalmus viridescens* in northwestern Virginia

Introduction

Batrachochytrium dendrobatidis (Bd) (Longcore *et al.* 1999), the causative agent of the amphibian disease chytridiomycosis, has caused global amphibian population declines and extinctions (Berger *et al.* 1998, Lips *et al.* 2005, Fisher *et al.* 2009). Little is known about the occurrence of *Bd* in northwestern Virginia, USA and continued efforts to sample for *Bd* are needed to provide a more complete understanding of its distribution and what species are infected (Gratwicke *et al.* 2011). Data on the presence, prevalence, and abundance of *Bd* on host amphibian populations in this region will provide baseline data for *Bd* in local populations to form a basis for continued monitoring of the pathogen. In addition, if amphibians are surviving in this region despite *Bd* infection, it may suggest they possess adequate defenses, such as microbial defenses, that could be investigated to help conserve susceptible amphibians (Harris *et al.* 2006, Vredenburg *et al.* 2011).

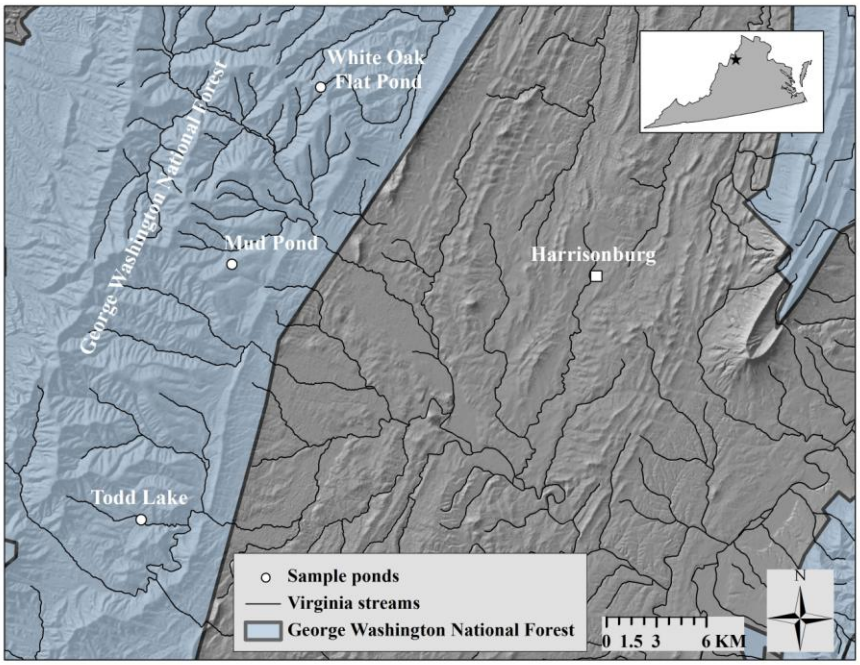
Notophthalmus viridescens, the red-spotted newt, is found throughout the northeastern United States and is abundant in the George Washington National Forest (GWNF) in northwestern Virginia. Declines in newt populations have not been reported in any areas of its range, and given that this species is not cryptic and is present in ponds for several months each year, it is likely that major declines would have been detected (Hunsinger and Lannoo 2005). Therefore, this species is either not infected by *Bd* because *Bd* is not found locally, individuals are able to persist despite infection because they have adequate defensive mechanisms, such as innate immunity or microbial defenses (Harris *et al.* 2006, Rollins-Smith 2009) or limited surveys assessing population

trends of this species have been conducted to accurately document population dynamics. Few studies have investigated *Bd* infection in *N. viridescens*, but they suggest that newts can be infected. One study in Alabama found two dead newts that were confirmed positive for *Bd*, and it was suggested that these individuals may have died as a result of chytridiomycosis (Bakkegard and Pessier 2010). In western Pennsylvania, a survey detected *Bd* infection in newts at six locations with varying prevalence (Groner and Relyea 2010), and in central Pennsylvania, infected individuals were found at 12 of 16 ponds (Raffel *et al.* 2010). Rothermel *et al.* (2008) surveyed locations throughout the southeastern USA and found newts infected with *Bd* in eastern North Carolina, northern Mississippi and southeastern Virginia. Only two newts were sampled in Virginia, and both were positive for *Bd*, but showed no signs of disease. An additional study conducted in central Virginia sampled seven newts, all of which were negative for *Bd* (Pullen *et al.* 2010). Two other studies in Virginia, one in the central Appalachians in Maryland and Virginia (Hossack *et al.* 2010) and one in Warren Country, Virginia (Gratwicke *et al.* 2011), surveyed different amphibian hosts, and found low infection prevalence across the sampled populations. Few studies have surveyed amphibian populations in Virginia and no studies to our knowledge have been completed in the GWNF in northwestern Virginia. We surveyed *Bd* infection status in *N. viridescens* populations in northwestern Virginia to determine if *Bd* was present and to assay the prevalence of infection.

Methods

Three populations of *N. viridescens* in the GWNF, including populations at Todd Lake, White Oak Flat Pond, and Mud Pond (Fig. 1), were surveyed between 27 February and 1 May 2012. Todd Lake, a medium-sized lake located at an elevation of 579 m, was surveyed on 27 Feb 2012. White Oak Flat Pond, a pond located on top of Shenandoah

Mountain at an elevation of 1034 m, was surveyed on 5 March 2012 and 1 May 2012. Mud Pond, a natural pond located at an elevation of 864 m, was surveyed on 27 March 2012. Other amphibian species, including *Lithobates sylvaticus, Pseudacris crucifer* and



Ambystoma maculatum, were seen at the sampled locations.

Figure 1: Topographic map of sampling locations for *Batrachochytrium dendrobatidis* on *Notophthalmus viridescens* in the George Washington National Forest in northwestern Virginia, USA.

During each survey, adult newts were captured using a dip net, and the presence of *Bd* was assessed using established methods that involve swabbing the skin and traditional PCR (Annis *et al.* 2004, Hyatt *et al.* 2007). Unique gloves were worn for capture and swabbing of each animal to ensure that *Bd* was not transferred between individuals. Nets and boots were not cleaned between individual captures but were cleaned in 10% bleach solution in between sample locations. Each newt was rinsed two

times in 20 ml of sterile Provasoli medium (Wyngaard and Chinnappa 1982) before swabbing and then swabbed 10 times (1 swab = up and back) on the ventral body surface and one time on each foot using a Fine Tip MW 100 swab (Medical Wire and Equipment, Corsham, UK). Swabs were stored on ice until transfer to a -80 C freezer. Captured newts were visually assessed for signs of chytridiomycosis, such as lethargy, hemorrhagic lesions, reddening of the ventral region and skin sloughing (Berger *et al.* 2005). Newts from Mud Pond were returned to the pond immediately following swabbing. Newts from the remaining surveys were collected for laboratory experiments (data not shown).

DNA was extracted from the swabs using Qiagen QiaAMP DNA micro kit (Qiagen, Valencia, CA) following the manufacturer's protocol for DNA extraction from swabs with minor modifications. The volume of buffers ATL and AL were reduced to 200 µl. Conventional PCR was performed to detect infection. Twenty-five µl PCR reactions were completed containing the following: 2 µl of DNA extract, 2.5 µl 10X Buffer A (Fisher Scientific, Pittsburgh, PA), 0.7 µl of 25 mM MgCl, 0.5 µl dNTPs, 2.5 µl of each primer (10 µM), 0.2 µl Taq DNA polymerase (Fisher Scientific), and 14.1 µl of PCR-grade water. Primers *Bd*1a (5'-CAG TGT GCC ATA TGT CAC G-3') and *Bd*2a (5'-CAT GGT TCA TAT CTG TCC AG-3'), as described by Annis *et al.* (2004), were used. Thermocycler parameters were as follows: 94°C for 4 min, followed by 29 cycles of 94°C for 30 sec, 58°C for 1 min, and 72°C for 2 min, and a final step of 72 °C for 10 min. Positive and negative controls were completed during DNA extraction and PCR. All negative controls were negative for *Bd,* and all positive controls were positive for *Bd.*

Results

Newts were infected with *Bd,* but infection prevalence varied between location and time of survey. At the first survey at White Oak Flat Pond, 1 of 10 sampled newts

was *Bd*-positive, whereas at the second survey, 33 of 39 newts were *Bd*-positive, showing an increased infection prevalence (Fisher's Exact Test, $p = 0.037$) (Table 1). Twenty of 22 individuals sampled at Todd Lake were *Bd*-positive (Table 1). At Mud Pond, 3 of 10 sampled newts were *Bd*- positive (Table 1). The February Todd Lake sample and the May White Oak Flat Pond sample revealed variation in band intensity, suggesting variable levels of infection. It was not possible to estimate variation for the March White Oak Flat Pond and March Mud Pond samples because of the low number of positive individuals. No newts showed prominent signs of chytridiomycosis.

Table 1: Prevalence of *Batrachochytrium dendrobatidis* (*Bd)* infection at three sampled locations in the George Washington National Forest, Virginia, USA.

Location (date)	Number sampled (*Bd*-positive)	Prevalence
White Oak Flat Pond (5 March 2012)	10 (1)	10%
White Oak Flat Pond (1 May 2012)	39 (33)	84%
Todd Lake (27 Feb 2012)	22 (20)	90 %
Mud Pond (27 March 2012)	10 (3)	30 %

Discussion

Bd detection in these ponds expands the known distribution of *Bd* to northwestern Virginia. Differences in *Bd* prevalence between sampling times and locations suggest that *Bd* may be responding to differences in environmental conditions, such as temperature or habitat composition, changes in transmission frequency or variation in host susceptibility. Increases in the number of amphibians entering ponds, or the frequency of contact between individuals in ponds may increase *Bd* transmission (Lips *et al.* 2006). *Bd* prevalence increased during the time when *P. triseriata* congregated to breed in ponds in Arizona (Hyman and Collins 2012). Furthermore, newts have an elaborate courtship ritual involving periods of sustained amplexus; therefore, the onset of courtship is an

additional explanation for increased *Bd* prevalence at White Oak Flat that bears further consideration. Continued and repeated monitoring of these sites in northwest Virginia is an avenue of future research that can further investigate the possibility of temporal variation in *Bd* prevalence within these newt populations. This study in tandem with other studies document *Bd* infection across the newts' geographical range with no signs of chytridiomycosis or striking population declines. This result suggests that newts may have a defensive mechanism, such as antimicrobial peptides or cutaneous antifungal symbionts (Harris *et al.* 2006, Rollins-Smith 2009). The potential defenses of newts against *Bd* warrant further investigation.

Appendix 7: Heat therapy duration experiments

The main experiment required newts to be negative for *Bd* at the beginning of the experiment. Initially I expected to find a population that did not have *Bd*; however, all sampled locations were found to have newts with *Bd* (Appendix 6). Because of this it was necessary to develop a way to remove the fungal infection. Heat therapy has been shown to be effective at eliminating infection because *Bd* cannot survive at temperatures above 28°C (Piotrowski *et al.* 2004). This also avoids the use of fungicide chemicals, such as itraconazole, which can be harmful to amphibians. It was necessary to determine the appropriate amount of time for heat therapy to eliminate *Bd* infection and therefore experimentation with infected newts was performed.

Methods

Infected newts collected from Todd Lake were put into heat therapy for different periods of time in order to determine the adequate length necessary to eliminate *Bd*. All heat therapy trials were completed in Percival incubators with a 12 hour light/dark cycle at 30°C. Newts were housed in medium-sized Ziploc containers with 200 ml of sterile Provasoli medium. On 5 March 2012, six newts were put into heat therapy and swabbed four days and nine days after entry into heat therapy (Trial 1). On 8 March six additional newts were placed into heat therapy and swabbed 6 days after entry (Trial 2). On 9 March, the remaining eight newts were placed in heat therapy and swabbed 9 days after entry (Trial 3). All swabbing was conducted as explained in Appendix 1. DNA was extracted from the swabs as explained in Appendix 4. For all trials, newt housing containers and media was not changed during heat therapy. They were changed after the last swabbing of the experiment.

Results

In trial 1, all newts were still positive for *Bd* 9 March but by 14 March all but one were negative for *Bd* (Table 1). This newt remained in heat therapy until 24 March and was still infected. In trial 2, all newts were negative by 14 March, and in trial 3 all newts were negative by 18 March 2012. On 24 March all newts in trial 1 and 2 were swabbed and all were still negative except for the one newt that maintained its infection. There was no mortality of newts during these trials.

Table 1: Heat therapy infection data for three trials. X indicates no swabbing occurred. Sample sizes are in parentheses.

	Swab Date			
Start Date	**9 March**	**14 March**	**18 March**	**24 March**
5 March (6)	6/6	1/6	X	1/6
8 March (6)	X	0/6	X	0/6
9 March (8)	X	X	0/8	X

Discussion

The heat therapy trials revealed that at least 6 days of heat therapy were required to remove infection with the exception of the one newt in trial one that remained positive throughout the experiment. Elimination of infection may be hastened by changing the newts' housing container and Provasoli to remove zoospores that could re-infect the host.

Appendix 8: Wood frog capture and laboratory care

Wood frog sampling

L. sylvaticus tadpoles were added to the ecosystem because they are a typical part of pond ecosystems and they facilitate nutrient turnover by grazing on algae. Tadpoles began to metamorphose in late May and were collected at night every 2-3 days. Individuals were collected when at least one of their forearms has emerged, which is the definition of anuran metamorphosis.

Wood Frog Capture

Experimental ponds were visited every other night when metamorph emergence was greatest and three nights a week once metamorph emergence lessened. Each tank was visited twice to maximize the chance of collecting all metamorphic individuals. Metamorphosis was defined as the emergence of at least one forelimb. Head lamps and spotlights were used to locate metamorphs, and hand held dip nets were used for capture. Each metamorph was transferred to a small plastic container containing a small volume of water from their respective tank. Containers had 3-5 holes in the lids to provide oxygen. Metamorphs found dead were removed from the tanks and placed in individual plastic containers with no water. Collection nets were cleaned in 10% bleach and rinsed in three consecutive water baths between tanks. Unique nets were used for each treatment. After collection was completed, wood frogs were transported back to the laboratory and housed in the vivarium prep room.

Weight measurements and swabbing

In the laboratory, metamorphs were weighed to the nearest milligram and swabbed for *Bd* to test for treatment effects when they reached Gosner stage 45 (Gosner

1960). Each metamorph was swabbed on its ventral surface, legs, and feet using sterile FineTip MW113 swabs. Swabs were stored in 1.5 ml Eppendorf tubes in a -80 °C freezer until processing. DNA was extracted from the swabs using the same protocol used for newt swabs. Conventional PCR was used to determine the presence of *Bd* on the wood frogs. The PCR parameters and primer sets described in Annis *et al.* 2004. Wood frogs were maintained in the laboratory to assess mortality or morbidity effects as a function of *Bd* infection.

Wood Frog Laboratory Care

Metamorphs were kept in the laboratory in order to assess morbidity or mortality effects as a function of treatment. Metamorphs were feed and put in 15 ml of new sterile Provasoli weekly. Pin-head crickets (Grubco Inc.), fruit flies (The Fruit Fly Company), and aquatic tadpole pellets (JurassiDiet) were used as food sources throughout the period of wood frog housing in the laboratory. On 7 July 2012, the environmental chamber housing the metamorphs failed causing mass mortality of the wood frogs and therefore morbidity and mortality could no longer be assessed accurately.

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