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Microbial dynamics and core microbiome of red-backed salamanders (*Plethodon cinereus*)

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Microbial Dynamics and Core Microbiome of Red-backed Salamanders (*Plethodon cinereus*)

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A thesis submitted to the Graduate Faculty of

JAMES MADISON UNIVERSITY

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Dedication:

I would like to dedicate my thesis to my parents, Howard and Kathy, my sister, Sarah, my great uncle and aunt, John and Estelle Ursu, and cousin John Ursu. Without their support and interest I would never have been successful. I would also like to dedicate this work to all salamanders everywhere.

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Abstract:

Beneficial cutaneous bacteria on amphibians can protect against the lethal fungal disease chytridiomycosis, which has decimated many amphibian species. The stability of these bacterial communities likely influences health outcomes, and is investigated here for the first time. We describe the diversity of bacteria on red-backed salamanders (*Plethodon cinereus*) in the wild, and the stability of these communities over time in captivity using culture-independent Illumina sequencing. In the field, there was no correlation between the diversity of salamanders' microbial communities and the diversity of their substrates' microbial communities. Salamanders were brought into the laboratory to test for the effect of an environmental reservoir (soil) in maintaining diversity and stability and were sampled every 7 days ending at day 28. In the laboratory, the alpha diversity of salamanders in the 'no bacterial reservoir' treatment decreased, whereas it remained constant in the 'bacterial reservoir treatment'. The treatment groups diverged from each other, yielding significant differences in beta-diversity. Eight OTUs defined a core community, i.e., present on >90% of salamanders through time, and a majority of these taxa such as Pseudomonadaceae, are known to secrete antifungal metabolites. Alpha diversity decreased in the treatment lacking a soil reservoir, one core OTU from the phylum Verrucomicrobia dominated the community. This result suggests that the non-core community on the salamanders regulate the core community and that the non-core community is dependent on the soil reservoir. Bacterial community structure in both treatments changed when their salamander hosts were brought into the laboratory. Diversity was more constant in the bacterial reservoir treatment. Defensive function of salamanders' cutaneous microbiota may depend on the diversity and stability of the core community.

Chapter I: Literature Review

Introduction

Emerging infectious diseases are a major threat to biodiversity, particularly in the class Amphibia. The fungal disease chytridiomycosis has been documented causing amphibian extinctions and declines globally (Berger et al. 1998, Crawford et al 2010, Lips et al. 2006, Rachowicz et al. 2006, Skerratt et al. 2007) and is considered the largest disease threat to biodiversity (Kilpatrick et al. 2010). One defense possessed by amphibians to protect against chytridiomycosis is a beneficial cutaneous bacterial community (Becker and Harris, 2010); numerous cutaneous bacterial species have been documented to inhibit *Batrachochytrium dendrobatidis* (*Bd*), the causal agent of chytridiomycosis (Harris et al. 2006, Lauer et al 2007, Woodhams et al. 2007).

Key antifungal microbes have been identified on amphibians' skin that inhibit *Bd* (Harris et al. 2006, Lauer et al. 2007, Lauer et al. 2008, Woodhams et al. 2007); however, their relative abundance and stability within their community is unknown. Stability is defined as resistance to a disturbance (Tilman, 1999). If bacterial communities are variable and unstable, it could greatly affect their ability to protect against pathogens. I investigated the constancy and stability of communities across time after a perturbation of captivity and the absence of a bacterial reservoir.

The ability of a host to maintain a stable microbiome may be important in disease prevention. The prevalence of chytridiomycosis has a seasonal pattern such that it is more prevalent in cooler months (Andre et al. 2008, Berger et al. 2004, Kriger and Hero 2007, Savage et al. 2011, Pullen et al. 2010). If the stability of amphibian bacterial communities is influenced by seasonality, and if the stability of bacterial communities is correlated with bacterial defenses against chytridiomycosis, then seasonal disease outbreaks may be

caused by a less defensive community. It is important to establish baseline knowledge of the stability of amphibians' microbiomes as well as their responses to perturbations. Therefore, I investigated the structure and stability of cutaneous bacterial communities of the red-backed salamander (*Plethodon cinereus*) in regards to several major perturbations: moving individuals from the field to the laboratory and housing individuals with and without natural soil.

Amphibians

Among vertebrates, amphibian biodiversity is most at risk with 41% of all species considered threatened and 20% either classified as critically endangered or endangered (Hoffmann et al. 2010). As of 2004, 427 species were on the brink of extinction and 9 to 122 amphibian species had gone extinct (Stuart et al. 2004). Wake and Vredenburg (2008) suggest that we may be in the midst of the sixth mass extinction as the current calculated extinction rate of amphibians is 211 times the historical extinction rate of amphibians (McCallum, 2007). Collins and Storfer (2003) outlined six hypotheses for amphibian decline that may work together synergistically. They include: alien species, over exploitation, land use change, global change such as UV radiation and climate change, pollutants such as pesticides, and emerging infectious diseases. Among those factors habitat loss, climate change, and disease are thought to be the major factors leading to amphibian extinctions (Wake and Vredenburg, 2008). While it is clear how to solve some environmental problems, such as land protection to stop habitat loss, there are no proven strategies to mitigate the effects of chytridiomycosis among amphibians. One current strategy to prevent the spread of chytridiomycosis is disinfecting nets and boots

between study sites (Phillott et al. 2010). Another strategy is to drain ponds to create unfavorable habitats for the pathogen (Woodhams et al. 2011); however, the effectiveness of this strategy has not been proven and would be difficult to implement on a large scale. Additionally, artificially selecting amphibians for disease tolerance has been suggested (Venesky 2012); however this is untested and would require massive resources in money and people hours, and may take decades to accomplish. One promising mitigation strategy that has been tested is bioaugmenting the cutaneous bacterial communities of imperiled amphibians to achieve protection from disease (Harris et al. 2009a,b, Woodhams et al. 2011, Bletz et al. 2013). This approach has been successful in laboratory experiments (Becker et al. 2009, Becker and Harris 2010, Harris et al. 2009b) and one field trial (Vredenburg et al. 2011). Understanding the dynamics of bacterial protection against chytridiomycosis has important implications for disease mitigation in that it can determine the time and frequency with which bioaugmentation should be implemented.

Amphibians are important in maintaining healthy ecosystems. They act as important consumers as well as prey in habitats across the globe. For example, the decline in stream dwelling amphibians may have lasting effects in neotropical ecosystems by altering algal community structure, primary production, organic matter dynamics, energy transfers between habitats as well as effects on other consumers (Whiles et al. 2004). Salamanders contribute greatly to ecosystems by being predators and thus controlling insect populations, transferring energy between aquatic and terrestrial landscapes during migrations, contributing to soil dynamics through burrowing, and acting as a quantifiable metric for ecosystem health (Davic and Welsh, 2004). Therefore,

the ecological role of amphibian species within communities and ecosystems is vital in maintaining ecosystem function, and amphibian declines can yield negative consequences.

Chytridiomycosis

Chytridiomycosis is a fungal disease that infects a wide range of amphibians and is considered the largest disease threat to biodiversity (Kilpatrick et al. 2010). Skerratt et al. (2007) found strong evidence that chytridiomycosis is a main cause of decline and extinction in anurans found in pristine conditions. The causal agent of chytridiomycosis, *Batrachochytrium dendrobatidis* (*Bd*), was first described in 1999 (Longcore et al. 1999). *Bd* is a chytridiomycete fungus that belongs to the chytridiales (Longcore et al. 1999); it has a motile zoospore life stage as well as a stationary sporangia stage that develops in the epidermis of amphibians. *Bd*'s maximum growth rate occurs between 17-25°C and between a pH of 6-7; above its temperature optimum, *Bd* has a 50% mortality rate at 30°C (Piotrowski et al. 2004).

The evolutionary origin and original location of *Bd* are disputed, and indeed multiple hypotheses has been advanced. Weldon et al. (2004) hypothesized that the pathogen originates from Africa and has a co- evolutionary history with the frog, *Silurana* (*Xenopus*) *leavis*. Through examining museum specimens the earliest occurrence of *Bd* in Africa was in 1938 and for 23 years no other cases were observed outside of Africa (Weldon et al. 2004). In this hypothesis, *Bd*'s spread across the globe is attributed to the exportation of *S. leavis*, which was used for human pregnancy tests starting in 1934 (Weldon et al. 2004). However, Goka (2009) suggests that *Bd* was found

on museum specimens in Japan in 1902 on giant Japanese salamanders (*Andrius japonicas*). Based on a genetic analysis, they suggest that the strain is endemic to Japan and as co-evolved with giant Japanese salamanders. Since 1902 is the earliest that *Bd* has been detected, Fisher et al. (2009) suggests that Japan or Asia could be the original source of *Bd*. Both hypotheses support the novel pathogen hypothesis that suggests the pathogen had been newly introduced into geographic locations (Rachowicz et al. 2005, Skerratt et al. 2007). In addition, strains of *Bd* have been recognized that are derived from different geographical regions, and one strain is considered hypervirulent and has been recognized as the strain found globally (Farrer et al. 2011). Another hypothesis, the competing endemic pathogen hypothesis, suggests that *Bd* has been widespread and that either the pathogen or host changed, thus altering the relationship between the two organisms (Rachowicz et al. 2004, Skerratt et al. 2007); however it is currently less favored. Rachowicz et al. (2004) supported the novel pathogen hypothesis due to the wave-like expansion of *Bds*' geographic range, which is a pattern that suggests the introduction of a novel pathogen to naïve host species. However, anthropogenic factors such as climate change and pollution may alter the relationship between host and pathogen and therefore support the endemic pathogen. It is possible that *Bd* is novel to new environments and then anthropogenic stressors such as climate change are making the pathogen's environment more optimal for growth. More surveys and research, particularly on *Bd*'s genetics, are needed to fully understand the origin and spread of *Bd*.

Species that are susceptible to chytridiomycosis, but do not exhibit morbidity or mortality, are potential disease reservoirs. Captive bullfrogs (*Lithobates catesbeiana*) have been experimentally shown to act as a vector of chytridiomycosis (Daszak et al. 2004)

and infected bullfrogs have been found globally in commercial farms where they are raised for food (Fisher et al. 2009, Schloegel et al. 2010). In addition, Woodhams et al. (2008b) found captive northern leopard frogs (*Lithobates pipiens*) potentially acting as a reservoir. The Pacific chorus frog, *Pseudacris regilla*, has been determined to be a *Bd* reservoir in the Sierra Nevada Mountains and is thought to spread the pathogen to naïve populations of *Rana muscosa* (Reeder et al. 2012). Not only do tolerant amphibians act as disease reservoirs and vectors, the occurrence of alternative taxa as hosts is also under investigation.

Alternative hosts are important because they affect the transmission of *Bd*. *Bd* has been found on lizards and snakes in Central America using molecular methods; however, no proof of pathogenesis or growth and reproduction on reptiles was observed (Killburn et al. 2011). It is possible that *Bd* was detected just due to its occurrence in the ecosystem, such as hitchhiking, rather than living on reptiles as an alternative host. Additionally, *Bd* was detected on the feet of geese (Garmyn et al. 2012), and growth was observed in assays containing scales from geese feet. Positive chemotaxis of *Bd* toward geese feet scales was also observed, but it is still unclear if live *Bd* can be transferred by waterfowl. *Bd* has also been reported to kill the nematode *Caenorhabditis elegans* (Shapard et al. 2011); however, the means of mortality and the ability for the pathogen to reproduce were not determined. Recently, McMahon et al. (2013) found *Bd* living and reproducing within the gut of crayfish where it caused morbidity and mortality. Indeed, they also found that the occurrence of *Bd* within a habitat was best explained by the occurrence of crayfish rather than other *Bd* reservoirs such as bullfrogs. The interaction between *Bd* and crayfish needs further investigation, particularly since crayfish biodiversity is also greatly

threatened (Taylor et al. 2007). The understanding of alternative *Bd* hosts is vital in understanding disease transmission and maintenance in a habitat and ultimately in mitigating disease.

Seasonality and Chytridiomycosis

The prevalence of *Bd* in the field has been shown to be greatest in cooler temperatures and is therefore influenced by seasonality. Berger et al. (2004) surveyed 30 species of frogs in Australia and found that *Bd* prevalence and mortality due to chytridiomycosis was more common in the winter. In addition, a study in central Virginia showed that disease prevalence was greatest in the spring and occurred in thirteen amphibian species (Pullen et al. 2010). A five year study of *Lithobates yavapaiensis* in Arizona found infection occurring more frequently in winter months (Savage et al. 2011). A field study that sampled *Litoria wilcoxii* over a 21 month period found highest infection rates in early spring, with a prevalence of 58.3 %; a prevalence of 0% was observed twice, once in the summer and once in early autumn (Kriger and Hero, 2007). *Bd* prevalence increased starting in the late fall and continued into the winter when temperatures favored the pathogen (Kriger and Hero, 2007). Therefore, previous studies have demonstrated that *Bd* prevalence is influenced by seasonality.

Experiments in laboratory studies have found that amphibian susceptibility to *Bd* is dependent on temperature and therefore corresponds with greater disease prevalence and intensity in cooler seasons. In laboratory experiments with great barred frogs (*Mixophyes fasciolatus*), 100% mortality occurred at 17° and 23°C, whereas only 50% mortality occurred at 27°C, which showed that mortality rates increase in lower

temperatures that were less optimum for the amphibian (Berger et al. 2004). Andre et al. (2008) experimentally showed that *Rana muscosa* mortality was greater at 17° C than at 22° C. Garner et al. (2011) determined that when *Bufo bufo* were exposed to an unnatural hibernation regime that was favorable to *B. dendrobatidis*' optimal temperature, an increased prevalence of chytridiomycosis occurred. Therefore, *Bd* infection is influenced by temperature; indeed *Bd*'s physiology has also been shown to be influenced by temperature.

Bd modifies aspects of its life history in response to changes in temperature. Woodhams et al. (2008a) found that life history trade-offs in *Bd* occurred such that fitness differences were minimized as *Bd* was cultured in less than optimal temperatures. Fitness was still lower at less optimal temperatures, however, not as much as expected if tradeoffs did not occur. When culture temperature was decreased, growth slowed. However, fecundity increased and zoospores remained infectious for longer (Woodhams et al. 2008a); survivorship still decreased after 30°C. Whereas disease outbreaks have been associated with *Bd*'s optimal temperature, it is unknown if temperature fluctuations or any disturbances affect amphibians' microbial communities, which can affect amphibian defenses.

Amphibians also experience tradeoffs at different temperatures that make them more or less likely to be infected with chytridiomycosis. For example, antimicrobial peptides (AMPs) from tiger salamanders have been shown to be less effective against *Bd* at lower temperatures (Sheafor et al. 2008) when *Bd* performs best. In addition the gene expression for AMPs was down-regulated in frogs in cooler temperatures (Ribas et al. 2009). Other functions of amphibian immunology are also affected by temperature, and

amphibians experience a lag of immunity that makes them more susceptible to disease after experiencing temperatures that are below their optimum temperature (Raffel et al. 2006). These examples show that *Bd*-amphibian interactions are context dependent. To further complicate the system, the interactions between symbiotic bacteria and amphibians are also context dependent and mutualisms may not occur under all conditions (Daskin and Alford 2012). To successfully mitigate amphibian disease an in-depth understanding of the interactions associated with *Bd*, amphibians and symbiotic bacteria are vital. This knowledge of the context-dependent interactions present within this disease system will allow conservationists to implement mitigation strategies under the appropriate circumstances to improve the chances for success.

Amphibian Immunity

Immunity is crucial in maintaining health and is divided into two categories: innate and adaptive. Innate immune responses are non-specific and are the first line of defense. They include non-specific leukocytes and antimicrobial peptides. Adaptive immunity, also known as acquired immunity, is specific and recognizes previously encountered pathogens.

The evidence of adaptive immune response of amphibians to *B. dendrobatidis* is limited and contested. Rosenblum et al. (2009) found that genes associated with adaptive immunity were either not affected or were down regulated in infected *Silurana (Xenopus) tropicalis* three days post inoculation and after morbidity occurred within the experiment. Additional studies with *Rana muscosa* have also found no evidence of an adaptive immune response (Rosenblum et al. 2012). Indeed, *Bd* may be evading the amphibian

immune system by producing a chemical compound or suite of chemical compounds that kill T cells (Rollins-Smith, personal communication). On the contrary, Ramsey et al. (2010) found elevated levels of pathogen-specific IgM and IgY serum antibodies and pathogen-specific IgM and IgY and IgX antibodies in mucous secretions of *S. leavis* after exposure to *B. dendrobatidis*. Additionally, Savage and Zumudio (2011) demonstrated in laboratory experiments that immunogenetic variation associated with major histocompatibility complex (MHC) genotypes affected survivorship in *Lithobates yavapaiensis*. MHC genes are important in acquired immunity, as MHC genes code for cell-surface glycoproteins that regulate adaptive response. Although there are only a handful of studies on adaptive immunity with respect to chytridiomycosis, it appears an adaptive response may occur, however at a slow rate that is ineffective. Further research is needed in a wider variety of species to draw broader conclusions.

A widely studied form of amphibian innate immunity against chytridiomycosis is the secretion of antimicrobial peptides. Antimicrobial peptides (AMPs) are a complex mixture of defensive molecules secreted from dermal granular glands into the skin's mucous layer (Rollins-Smith 2009). AMPs are thought to disrupt the cell membrane of both the infectious zoospores and the zoosporangia of *Bd*. Receptors on the dermis of amphibians' skins may initiate synthesis of antimicrobial peptides; granular cells may also possess receptor cells (Rollins-Smith 2009). Woodhams et al. (2012a) found that AMPs are likely suppressed when amphibians are infected with *Bd*. Antimicrobial peptides in combination with bacterially produced anti-fungal metabolites have also been shown to synergistically inhibit *Bd*; therefore, a lower abundance of both AMPs and bacterial produced metabolites are required to protect against disease (Myers et al. 2012).

AMPs may also play an important role in maintaining the compositions of bacterial communities and need to be fully considered when studying amphibians' microbial communities.

Bacterial Community

Amphibians possess a cutaneous bacterial community that has been shown to inhibit *B. dendrobatidis* (Harris et al. 2006). Lauer et al. (2008) found using culturable methods that four-toed salamanders (*Hemidactylium scutatum*) possessed 48 different species of bacteria within 14 genera, 16 families and 4 phyla on their skins. When comparing bacterial community fingerprints via denaturing gradient gel electrophoresis (DGGE), which is culture independent technique, four-toed salamander within two populations shared approximately 25% of their community members. Using culture-based techniques the bacterial communities on four-toed salamanders shared 50% of their community between populations and with red-backed salamander. The greater similarity of the cultured microbiota likely reflects a culture bias, in that species that are easily cultured, even if rare on the salamanders, are represented in each sample.

Intensely studied anti-fungal bacterial species cultured from amphibians' skins have been shown to produce anti-fungal metabolites. For instance, the bacteria *Lysobacter gummosus* produces the metabolite 2,4-diacetylphloroglucinol (Brucker et al. 2008a), and *Janinobacterium lividum* produces the metabolites indole-3-carboxaldehyde and violacein (Brucker et al 2008b). Importantly, these metabolites have been found on the skin of red-backed salamanders at high enough quantities to inhibit the growth of *Bd* (Brucker et al. 2008b).

Little is known on how amphibians acquire their microbial community. Three forms of microbial transmission may occur: vertical, horizontal, and environmental. In vertical transmission communities are passed from the parent to the offspring. Walke et al. (2011) showed that male frogs of the species *Hyalinobatrachium colymbiphyllum* pass their antimicrobial peptides and cultivable microbes to their egg masses. Vertical transmission has also been shown in other phyla such as hydra and pea aphids (Baumann 2005, Fraune et al. 2010). Horizontal transmission occurs when two or more individuals come in contact. This may most likely occur in social organisms and during mating. For example, the pea aphid, *Acyrtosiphon pisum*, transmits its symbiotic microbe, *Buchnera aphidicola*, sexually (Moran and Dunbar 2006). Organisms may also obtain their microbial symbionts through their environment. For example the squid *Euprymna scolopes* obtains its bioluminescent microbe, *Vibrio fischeri*, through seawater (Nyholm et al. 2000). Understanding how different species of amphibians acquire their skin bacterial communities will allow for smarter disease mitigation practices using bacterial probiotics.

Amphibians have a wide array of life histories that would expose them to different environmental pressures. These environmental pressures may influence microbial community structure and as a result may influence protection against disease. For example, amphibians that are totally aquatic would be exposed to different environmental factors than arboreal amphibians. Understanding the different pressures that select for amphibians microbiomes with varying life histories will be important when determining which probiotics to use. For example, an aquatic bacterium that has a

symbiotic relationship with an aquatic frog may not be a good probiotic candidate for an arboreal frog; the bacterium would most likely not persist on and protect its new host.

Interestingly, closely related amphibians with similar environmental pressures possessed statistically different structural bacterial communities. McKenzie et al. (2011) showed that amphibians whose larvae live in the same ponds have different bacterial communities; community structures were specific for each species rather than specific for each pond. Amphibian species-specific bacterial communities may mean that some form of selection for bacterial communities within amphibian species potentially based on different skin physiologies, mucous types, antimicrobial peptides mixture, or nutrients in the mucous layer is occurring.

Research on human cutaneous bacterial communities has identified different selective forces that lead to the structure of skin bacterial communities. Selective forces include the environment of the integument, the host demographic variables such as age, transmission of the microbes, behavioral characteristics of the host and host genetics (Rosenthal et al. 2011). It is likely that amphibian communities are influenced by similar stimuli.

Antimicrobial peptides (AMPs) may play a crucial role in regulating the community of bacteria on amphibians' skin by selecting which bacterial species can colonize and persist (Walke et al. 2011). AMPs selecting for bacterial communities has been documented on embryos of *Hydra* (Fraune et al. 2010). Therefore, particular antimicrobial peptides may be selecting for specific microbial communities. If AMPs are important in regulating bacterial community structure and they are suppressed in amphibians with chytridiomycosis (Woodhams et al 2012a), then the structure of

microbial communities may be abnormal and deter from their protective properties. It is also possible that bacterial communities that are relatively unaffected by AMPs due to suppression are placed in a state of bacteria competition that produces more anti-bacterial metabolites than normal, and thus inhibit *Bd*, thus following models on how to construct beneficial microbiomes, as discussed in Scheuring and Yu (2012). Further research on AMP and bacterial interactions is necessary to understand disease protection and bacterial community structure.

Evidence of a change in bacterial community structure after *B. dendrobatidis* arrives has been documented. Woodhams et al. (2007) and Lam et al. (2010) found evidence of such change in wild populations of yellow-legged frogs (*Rana muscosa*). Populations that were less affected by chytridiomycosis had a greater proportion of individuals with antifungal bacterial species than populations that were more afflicted by the disease. This pattern suggests that frogs without protection died, whereas frogs with protection survived and indicates selective pressure for anti-fungal species is strong. In addition, Flechas et al. (2012) surveyed the culturable bacteria from three species of *Atelopus*, one of which has tested positive for *Bd*. Interestingly they found the species that tested positive for *Bd* had the bacteria with the strongest anti-*Bd* activity. These results suggest that a protective community is selected for and the bacterial communities on amphibians are heritable.

Previous Research on amphibian skin microbes

My research incorporated the latest DNA sequencing technology, which has allowed me to have a more detailed understanding on the structure of amphibian bacterial communities. Past experiments on amphibian cutaneous bacterial diversity have primarily used culturing methods to examine diversity and anti-fungal properties (Harris et al. 2006, Lauer et al. 2007, Lauer et al. 2008). Molecular techniques such as Denaturing Gradient Gel Electrophoresis (DGGE) have also been incorporated to determine bacterial community fingerprints (Lauer et al. 2007, Lauer et al 2008) and give a broader range of diversity since up to 99.8% of bacteria are difficult to culture (Streit and Schmitz, 2004). However, when using DGGE, abundance of microbes and species in low quantities are not recognized. In past studies salamanders in the Harris Lab have been housed in containers with a water source, but with no substrate or a minimal substrate such as autoclaved filter paper. I present the effect of captivity and housing on salamanders on bacterial community stability and diversity.

Chapter II: Microbial dynamics and the effect of environmental reservoirs on red-backed Salamanders (*Plethodon cinereus*)

Introduction

Host associated bacterial communities affect health in many species including humans (Fierer et al. 2012), corals (Rosenberg et al. 2007), insects (Dillon et al. 2005) and amphibians (Harris et al. 2009a,b). The cutaneous microbial community of amphibians provides a defensive function against pathogens such as the fungus *Batrachochytrium dendrobatidis* (*Bd*) (Woodhams et al. 2007, Becker and Harris, 2010). *Bd* causes the fungal disease chytridiomycosis and has caused amphibian extinctions and declines globally (Berger et al. 1998, Crawford et al 2010, Lips et al. 2006, Rachowicz et al. 2006, Skerratt et al. 2007). Previous studies have not examined the dynamics of amphibians' defensive bacterial communities. In order to more fully understand the association of microbiota and health, it is necessary to more fully characterize the microbial community. We have experimentally examined the stability and diversity of red-backed salamander (*Plethodon cinereus*) microbiomes.

The stability of the microbiome may affect the health of the host. A fluctuating community structure may result in a fluctuating defensive function, whereas a stable microbiota may provide more continual protection from pathogens. Most surveys of amphibians' cutaneous bacteria have only encompassed one time point (Lauer et al. 2007, Lauer et al. 2008, Woodhams et al. 2007); therefore, it is unclear if these bacterial communities are stable. Stability of amphibians' cutaneous microbiota may depend on bacterial reservoirs such as soil for re-colonization (Belden and Harris 2007) since soil harbors diverse (Lauber et al. 2009) and abundant microbial communities (Whitman et al.

1998). Understanding the dynamics of amphibian bacterial communities will also help determine if a core microbiome exists and if so, whether members of the core are culturable for possible use as probiotics in disease mitigation.

Maintaining functional stability, such as the production of anti-fungal metabolites, may be critical to amphibians in habitats with lethal pathogens such as *Bd*. If an amphibian's protective microbial community is not stable due to perturbations such as skin sloughing or temperature changes when seasons change, then they may be more susceptible to disease. The prevalence and intensity of chytridiomycosis, which is caused by *Bd*, is associated with changing and cooler temperatures (Andre et al. 2008, Berger et al. 2004, Kriger and Hero 2007, Savage et al. 2011, Pullen et al. 2010), that are more optimal for the pathogen (Piotrowski et al. 2004) and less optimal for amphibians' immune systems (Raffel et al. 2006), and this pattern could be caused in part by fluctuations in microbial defensive function. In addition, other anthropogenic disturbances such as climate change, habitat degradation and pollution may also influence the stability of bacterial communities and make amphibians more susceptible to disease.

Advances in DNA sequencing have led to techniques such as high throughput amplicon sequencing that has revolutionized microbial ecology (Hamady and Knight, 2009). In the past, research on the microbial communities on amphibians have been determined using culturing techniques (Harris et al. 2006, Lauer et al. 2007, Lauer et al. 2008, Woodhams et al. 2007) or early community profiling techniques such as Denaturing Gradient Gel Electrophoresis (DGGE) (Lauer et al. 2007, Lauer et al. 2008). These techniques have disadvantages since up to 99.8% of bacteria can be difficult to culture (Streit and Schmitz, 2004), and DGGE does not detect microbes that are present

in low numbers. Recently, McKenzie et al. (2011) performed the first next generation sequencing study on amphibian microbiomes using 454 pyrosequencing to examine the diversity of bacteria on amphibians, which provided a culture independent assay of microbial community structure. Culture independent techniques also allow me to determine the prevalence and abundance of known antifungal culturable bacteria. For instance, the abundance and prevalence of *Janthinobacterium lividum*, a bacterium used in probiotic laboratory and field trials to mitigate *Bd* can be determined. Sampling the microhabitat in addition to host microbiota may provide insight into environmental sources of colonization.

Amphibians' cutaneous microbiota might be strongly influenced by the environment so that different host species in the same environment could share similar microbial communities. However, using 454 pyrosequencing, different species of larval amphibians exposed to the same environment possessed significantly different bacterial community structures (McKenzie et al. 2011). Amphibian species in different ponds maintained species-specific microbial communities, suggesting that community structures were species specific rather than environment specific. Amphibian species-specific bacterial communities suggest that some form of selection for bacterial communities is occurring on amphibians. Amphibian bacterial communities may be species specific but depend on transmission and replenishment from bacterial reservoirs. It is likely that amphibians attain bacteria from soil and water. Soil is an ideal candidate to act as a bacterial reservoir since it contains a high bacterial diversity (Lauber et al. 2009) and a high abundance of microbes (Whitman et al. 1998). For example, one gram is estimated to contain between 10^6 - 10^9 cells (Whitman et al. 1998) and an estimated average of 1017

phylotypes using pyrosequencing; however diversity is expected to be greater (Lauber et al. 2009).

Amphibians are frequently brought into laboratory environments for ecological experiments (Becker and Harris, 2010, Becker et al. 2009, Woodhams et al. 2012b) or into survival assurance colonies, which are critical to many species' survival. Removal from a natural environment is likely to be a major perturbation to amphibians and their microbiota. Typically, the laboratory environment lacks natural bacterial reservoirs, which might strongly affect microbial structure, diversity, and function of the skin microbiota. If the functionality of protective skin microbial communities changes under non-natural conditions, then amphibians may be more susceptible to disease in the laboratory environment.

The first step in understanding the relationship between the microbiome and health is a description of the microbiome in healthy individuals. It would then be possible to understand the effects of perturbations to the microbiome on its function and on the health of the host (Shade and Hendelsman 2012). Additionally, a core microbiome, consisting of species shared between individuals, may be responsible for important functions for their host. For example, it would be beneficial for an amphibian to host a bacterium that produces anti-fungal metabolites that protect against chytridiomycosis and other skin pathogens. Finding a core microbiome on *P. cinereus* may suggest an evolutionary history between these salamanders and the core since it is likely they have evolved a mutualism. In addition members of the core community are likely to persist on amphibians and can potentially be increased in relative abundance by probiotic application (Bletz et al. 2013). I tested the hypotheses that a bacterial reservoir was

required to maintain the stability and diversity of bacterial communities on salamanders through time. Both alpha diversity, the bacterial community diversity per individual salamander, and beta diversity, comparing bacterial communities of salamanders were examined. In addition, I tested the hypothesis that salamanders exposed to a perturbation had a core community. Determining a core microbiome is important for identifying targets to culture and test for disease-resistance or other host-critical function.

Materials and Methods

Experimental design

Red-backed salamanders (*Plethodon cinereus*) were chosen for this study because their bacterial communities have been extensively studied in regards to their capacity to protect against the fungal disease, chytridiomycosis (Lauer et al. 2007, Becker and Harris 2010, Harris et al 2009a,b). Additionally, *P. cinereus* are highly abundant in the Shenandoah Mountain region of Virginia and are tolerant of laboratory conditions. Salamanders were collected from George Washington National Forest in October of 2011 and the soil in this experiment was collected from the same location and at the same time.

After collection, each salamander was immediately rinsed with sterile Provasoli medium (Lauer et al. 2007) three times to remove transient bacteria (McKenzie et al 2011). Salamanders were swabbed 10 times on a randomly chosen left or right side of their ventral surface with a sterile rayon swab (BBL CultureSwab, BD Diagnostics) since an additional swab was collected for another study. The bacterial community of the immediate environment for each salamander was also sampled using the same type of swab. All swabs were stored on ice and then frozen at -80°C until DNA extraction.

Community profiles were obtained from the swabs using Illumina 16S rRNA amplicon sequencing.

Salamanders were transported to the laboratory in 50 mL falcon tubes (BD Diagnostics), and soil was transported in autoclaved plastic containers. Salamanders were randomly assigned to one of two treatments to test the hypothesis that the type of environmental reservoir affects community stability and composition. The first treatment (n=10) consisted of 30 mL of Provasoli media, which will now be referred to as media (Wyngaard and Chinnappa, 1982.) and will be considered the no bacterial reservoir treatment. In previous experiments, red-backed salamanders were housed with media (Becker and Harris 2010, Becker et al. 2009). In the second treatment (n=10), salamanders were housed with 150 grams of soil from the salamanders' natural habitat and will be considered the bacterial reservoir group. The soil was homogenized by hand with sterile gloves and then initial soil samples were taken in triplicate. Salamanders were housed in 17cm x 12cm x 7 cm (L x W x H) containers kept at 17° C on a 12 hour light and 12 hour dark cycle. MEDIA was replaced every 7 days after sampling. Salamanders were swabbed every 7 days until day 28 (Table 1). Soil samples were collected in triplicate on days 0, 14 and 28 from salamanders in the soil treatment. Each salamander was fed 15 fruit flies once a week after sampling took place.

Molecular Techniques

DNA extractions were performed using the MoBio PowerSoil Extraction kit (MO BIO Carlsbad, CA) according to manufacturer's protocols with the following modifications: samples were incubated at 65°C after the addition of reagent C1, the powerbead tubes were vortexed horizontally for 2 minutes (Lauber et al 2008), and the

final addition of reagent C6 was allowed to sit on the filter for 5 minutes before the final elution. PCR reactions contained 11 μL PCR water, 10 μL 5 Prime Master Mix, 1.0 μL each of the forward and reverse primers (10 μM final concentration), 1.0 μL MgCl_2 , and 1.0 μL genomic DNA. PCR primers (F515/R806) were used to target the V4 region of 16S rRNA. This primer pair amplifies the region 533–786 in the *Escherichia coli* strain 83972 sequence (Greengenes accession no. prokMSA_id:470367). The reverse PCR primer contained a 12 base error correcting Golay barcode originally developed by Caporaso et al. (2011). PCR conditions consisted of an initial denaturation step of 94°C for 3 min, followed by 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s; and final extension of 10 min at 72 °C. Each sample was amplified in triplicate and combined. Amplicons were quantified using the Quant-IT Picogreen dsDNA reagent in 1X TE buffer. A composite sample for sequencing was created by combining equimolar ratios of amplicons from the individual samples and cleaned using the MO BIO UltraClean PCR clean up DNA purification kit. The purity and DNA concentration were determined using a NanoDrop spectrophotometer. The samples, along with aliquots of the sequencing primers, were processed on an Illumina HiSeq 2000 at the Biofrontiers Next-Gen Sequencing Facility located at the University of Colorado, Boulder. An aliquot of the DNA was used to test each salamander for the presence of *Bd* using the standard PCR protocol (Annis et al. 2004).

Day of Experiment	Salamanders housed with soil	Salamanders housed with media
0	10 (10)	10 (9)
7	10 (4)	10 (5)
14	10 (4)	10 (6)
21	10 (7)	10 (7)
28	10 (8)	10 (6)

Table 1. Salamander sampling scheme. Number of samples taken for each time point and each treatment. Numbers in parentheses represent the samples that amplified and had enough sequences for analysis.

Sequence analysis

Amplicons were sequenced on 1/3 of an Illumina HiSeq lane at the University of Colorado at Boulder yielding 100bp reads. Quantitative Insights Into Microbial Ecology (QIIME) version 1.5.0 (Caporaso et al. 2010b) was used for all sequence analysis unless otherwise noted. Sequences were filtered for quality and assigned to their respective sample using default settings. The resulting 23.3 million reads were clustered into operational taxonomic units (OTUs) according to the subsampling open reference protocol using the Greengenes October 2012 97% reference sequences (greengenes.secondgenome.com). In total, 83% of the reads hit the reference dataset, and the remaining were clustered into de novo OTUs. OTUs with fewer than 100 reads were filtered out of our analysis (Bokulich et al. 2013), resulting in a total of 21.7 million sequences clustered into 6049 OTUs. Sequences were aligned to the Greengenes references alignment using PyNast (Caporaso et al. 2010b), and a phylogenetic tree, which was used for diversity analyses, was constructed with FastTree according to standard procedures within QIIME. Communities with fewer than 6000 sequences on a

given sample day were removed from the analysis, and this occurred with 40 out of 183 communities. Alpha and beta diversity analyses were conducted on data rarefied to 6300 sequences per sample. The following alpha diversity metrics were determined: richness, Shannon diversity index, PD whole tree and Chao1. PD whole tree provides a quantitative measure of phylogenetic diversity. Phylogenetic diversity is measured by adding the appropriate branch lengths for a subset of taxa (Faith, 1992). Chao1 is a non-parametric measure of OTU richness (Hill et al. 2003). To determine if there were treatment effects on alpha diversity matrices, Linear Mixed Models were performed using the software SPSS. Beta diversity (weighted UniFrac) was calculated within QIIME using weighted metrics. The resulting distance matrices were imported into PRIMER 6 for further analysis. The treatment and temporal effects were statistically analyzed using a permutational multivariate analysis of variance (PERMANOVA (PRIMER 6)) using time as a random effect and treatment as the fixed effect, and were visualized using Principle Coordinate analysis (PCoA); weighted UniFrac was used for this analysis since it takes account of relative abundance in addition of presence of bacterial taxa.

To test for an existence of a core bacterial community on the skin of red-backed salamanders, we used the non-rarified dataset to include all OTUs regardless of abundance bias. The prevalence and proportional abundance of each OTU was determined for the salamanders over time. We defined core OTUs if they were present on 90% or more of the salamander samples taken over the course of the experiment (65 of 100 samples). This cut-off was used in previous studies of core human skin bacteria (Caporaso et al. 2011). The mean relative abundance was determined for 9 OTUs with prevalence greater than 90%, and a heat map was created to visualize changes through

time and treatments. To determine if alpha diversity and the relative abundance of the core OTUs differed between treatments a linear mixed model was used in IBM SPSS Statistics (V. 21).

The most abundant OTU was found in 100% of samples and was in the phylum Verrucomicrobia. A maximum likelihood phylogenetic tree was constructed by placing novel Verrucomicrobia sequences within the Greengenes tree (filtered to sequences with 85% similarity; obtained from http://greengenes.secondgenome.com/downloads/database/12_10) using the EPA algorithm within RAxML (Berger and Stamatakis 2011), including the OTU that corresponds to the highly abundant member of the redback salamander core community (OTU 418941). These sequences were robustly placed within the Verrucomicrobia class Opitutae(Figure 16, appendix).

Results

All environmental samples amplified and had a sufficient number of sequences for analysis. Sixty-five out of 100 salamander samples amplified and had enough sequences for analysis (Table 1). This meant that some salamander samples on some dates could not be used. One negative control (sterile swabs) out of three had amplification and contained sufficient sequences for analysis; beta diversity for this sample was similar to field soil samples and DNA extraction for the control that amplified was done at the same time as the field soil samples; the bacterial community from the negative control was similar to the field soil samples that were extracted at the same time (Fig 8). It was likely that this negative control swab was contaminated with

bacteria from soil. I concluded that the results from the laboratory experiment and field survey were accurate.

Having a reservoir strongly affected cutaneous microbial diversity on salamander skins. Shannon diversity index was significantly lower in the no bacterial reservoir treatment ($F_{(1, 30.587)}=128.734$; $P<0.001$). Day of the experiment was also a major factor affecting diversity ($F_{(4, 21.103)} = 25.038$; $P < 0.001$), and there was a significant interaction between treatment and day ($F_{(4, 21.103)}=12.3$; $P<0.001$; Fig. 1). The diversity stayed relatively constant in the bacterial reservoir treatment, whereas it declined rapidly from a high diversity on day 0 to a lowest diversity on day 21 in the no bacterial reservoir treatment, leading to the significant interaction between treatment and day.

Supplementary alpha diversity measures are presented in Figures 10, 11 and 12 in the appendix. Additionally, a table presenting the richness of salamanders' bacterial communities is presented in Table 3 in the appendix.

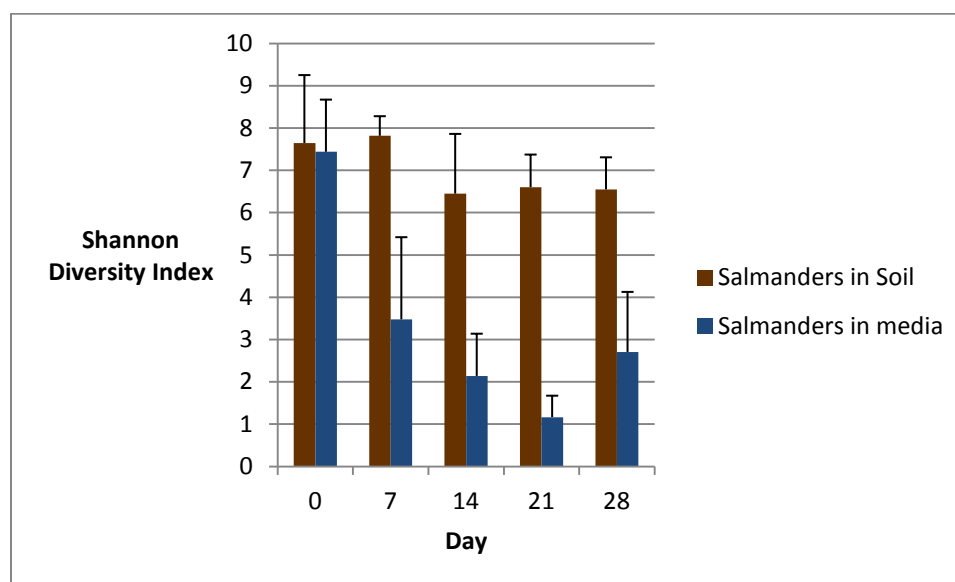


Figure 1. Shannon Diversity Index (alpha diversity) for salamanders in soil (bacterial reservoir) and in media (no bacterial reservoir) over time. Salamanders housed without a bacterial reservoir had lower Shannon diversity indices than salamanders with reservoirs. Error bars indicates standard deviations.

There was no correlation between alpha diversity of salamanders' microbes on day 0 and that of the microbial community of their immediate environment on day 0 ($r=0.14792$; $P=0.5256$; Fig. 2). There was no correlation between the alpha diversity of salamanders' cutaneous microbial communities on day 0 and day 28 for salamanders in the no bacterial reservoir treatment ($r=-0.118$; $P=0.802$; Fig. 3), and for salamanders in the bacterial reservoir treatment ($r= -0.218$; $P = 0.972$; Fig. 4).

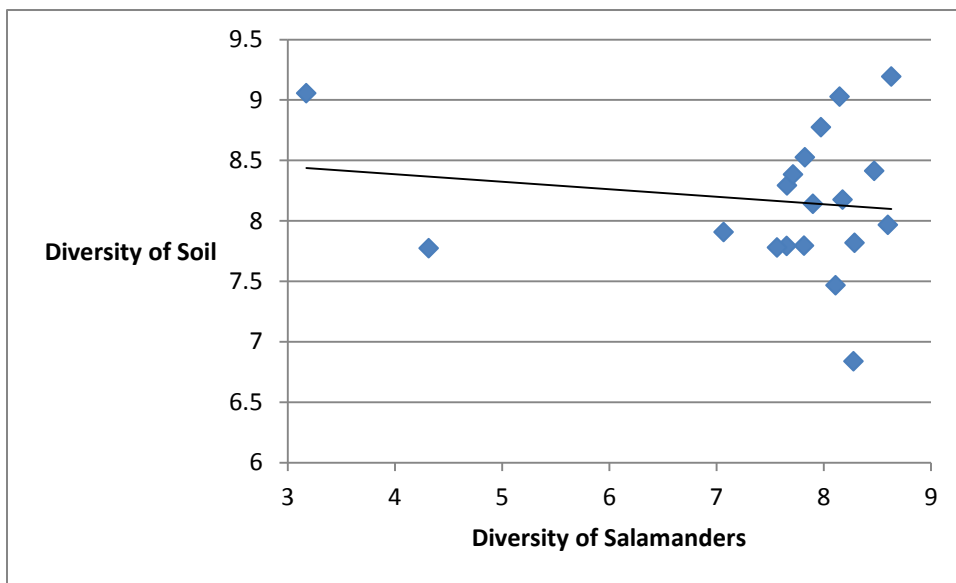


Figure 2. Correlation of Shannon diversity of salamanders in the field compared to their respective environment. This demonstrated that the diversity of bacterial communities on salamanders is not dependent on their immediate habitat.

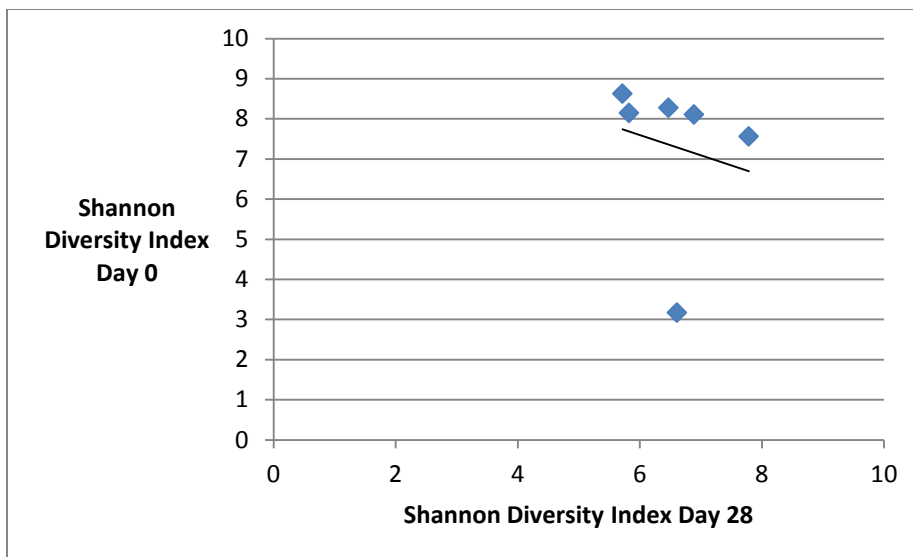


Figure 3. Correlation between Shannon diversity indexes of salamanders housed in soil (with a bacterial reservoir) on day 0 and day 28. This indicates that the initial diversity of salamander's bacterial communities in the field did not predict the bacterial diversity at the end of the experiment.

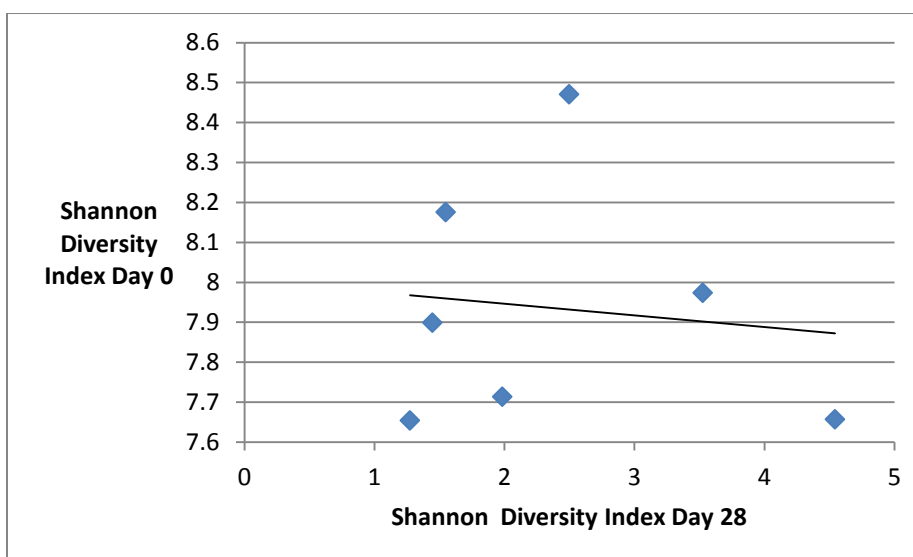


Figure 4. Correlation between Shannon diversity indexes of salamanders housed media (without a bacterial reservoir) on day 0 and day 28. This indicates that the initial diversity of salamander's bacterial communities in the field did not predict the bacterial diversity at the end of the experiment.

Having access to a bacterial reservoir lead to a different microbial community structure on salamanders compared to salamanders that had no access to a bacterial reservoir. A permutational multivariate analysis of variance (PERMANOVA) was performed on Weighted Unifrac Beta diversity of salamanders (Fig. 5); this indicated that the bacterial communities in the treatments were significantly different (Pseudo- $F_{(1)}=7.852$; $P=0.031$), the day of the experiment was significant (Pseudo- $F_{(4)}=7.702$; $P=0.001$) and there was an interaction between treatment and day of experiment (Pseudo- $F_{(4)}=2.867$; $P=0.001$). The interaction arose because the difference between the treatments increased over time. The bacterial communities on salamanders without a bacterial reservoir had fewer OTUs and were dominated by Verrucomicrobia.

The difference in community structures between microbial communities in laboratory soil, field soil and in media was significantly different (Pseudo- $F_{(2)}=77.7$; $P<0.001$). Bacterial community structures in the soil differed between day 0 and days 14 and 28. Field soils had greater variation than initial lab soil and both were different than water on day 28 (Fig 6). The initial community structure of the laboratory soil changed over the course of the experiment (Pseudo- $F_{(2)}=14.092$; $P=0.001$; Fig 7). All data points are presented in Figure 8 in the appendix. Importantly, as the communities on salamanders housed with or without a bacterial reservoir diverged, they became more similar to their respective substrates.

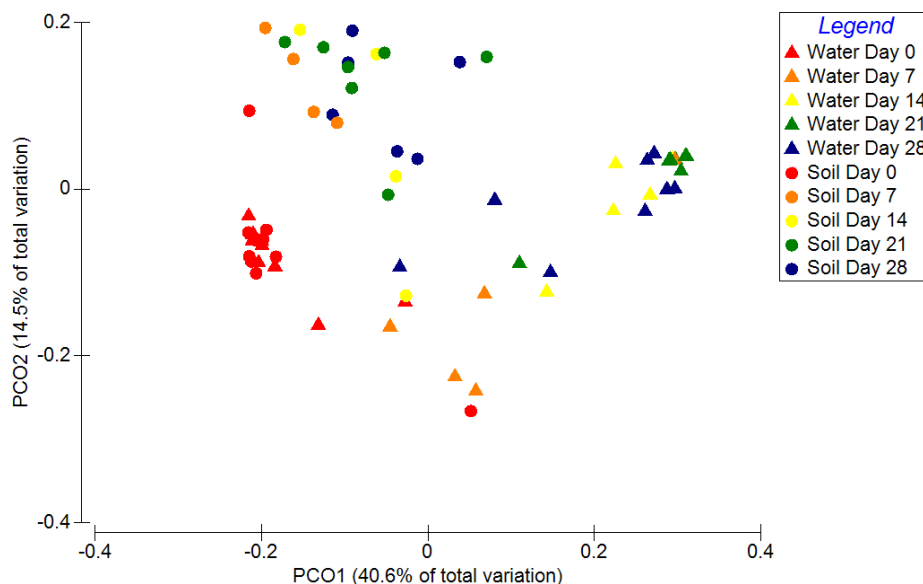


Figure 5. Principal coordinates plot of salamanders in each treatment (media (Water) and Soil) through time. Each point represents a bacterial community from one red-backed salamander. Salamanders housed with soil (bacterial reservoir) are denoted by circles and salamanders housed with Provasoli media are denoted by triangles. Color indicates the day of sampling.

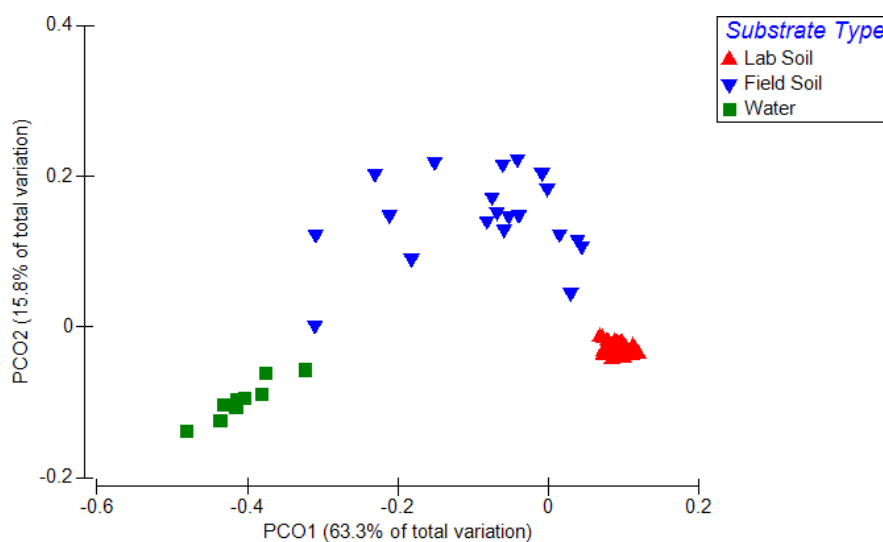


Figure 6: Principal coordinates plot of the salamanders' environment in the field and in captivity. Each point represents a bacterial community from the environment of one red-backed salamander. Soil in the lab is denoted as red triangles, soil in the field is represented by blue upside down triangles and water from day 28 is represented by green squares.

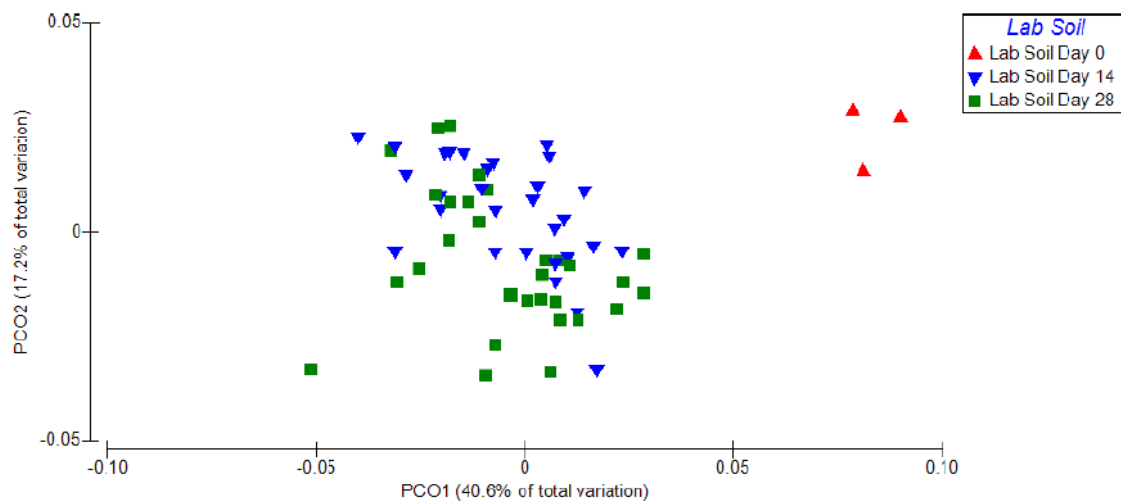


Figure 7. Principal coordinates plot of laboratory soil through time. Each point represents a bacterial community from the environment of one red-backed salamander. Day zero is represented by red triangles, day 14 is represented by blue upside down triangles and day 28 is represented by green squares.

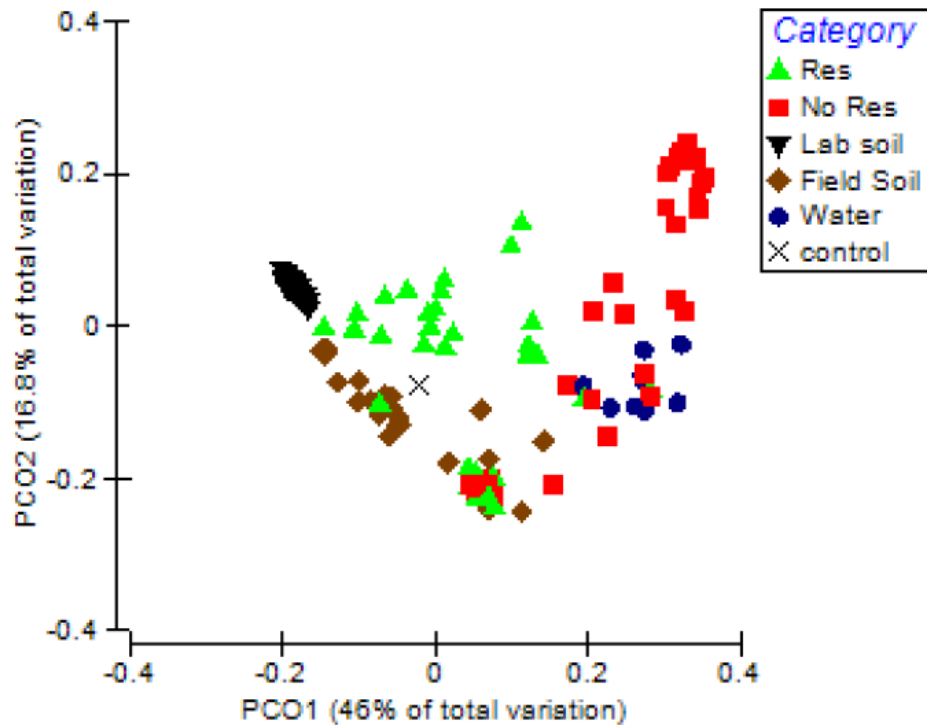


Figure 8. Principal coordinates plot of all samples. Each point represents a bacterial community from the environment of one red-backed salamander. Green triangles represent salamanders housed with a bacterial reservoir. Red squares represent salamanders housed without a bacterial reservoir. Black upside down triangles represent laboratory soil and brown diamonds represent field soil. Blue circles represent water. The negative control that amplified is represented as an X.

A core community consisted of 8 OTUs that were found on greater than 90% of salamanders in the field and over all time points in the experiment in both treatments (Table 2). The relative abundance of the core community increased ($F_{(1,26,182)} = 40.982$; $P < 0.001$) more in the no bacterial reservoir treatment over time than in the bacterial reservoir treatment, leading to an interaction between treatment and day ($F_{(4, 21,771)} = 6.856$; $P < 0.01$). In the soil treatment, the core community comprised a relatively small

fraction of the overall community. However, the core community comprised as much as 93.5% of the core community on day 21 of the experiment in the no bacterial reservoir treatment (Fig. 9). One OTU in the phylum Verrucomicrobia became the most abundant, and it greatly increased in the no bacterial reservoir treatment, comprising as much as 92.5% of the entire community. A phylogenetic tree that includes Verrucomicrobia is presented in Figure 16 in the appendix. Day of experiment also greatly affected the relative abundance of the core community ($F_{(4,21.771)}=15.926$ $P<0.001$). There was a negative correlation of alpha diversity and the abundance of the Verrucomicrobia OTU ($r=-0.84358$; $P<0.0001$; appendix, Fig. 10), and a negative correlation between the abundance of the core and the alpha diversity ($r=-0.88324$; $P<0.0001$; appendix Fig. 11). The antifungal bacterium, *Janthinobacterium lividum*, which has been found on *P. cinereus* in previous studies, was not in the core as defined; however, it was found on 94 percent of the salamanders that were sampled in the field (day 0). There was no relationship between *J. lividum*'s abundance and alpha diversity ($r= -0.00310$; $P=0.9803$; appendix, Fig. 12); its prevalence was not different between treatments throughout experiment (Fisher's exact test, $P>.05$). In addition, five out of the eight core OTUs were Pseudomonadaceae, which are easily and commonly cultured from red-backed salamanders.

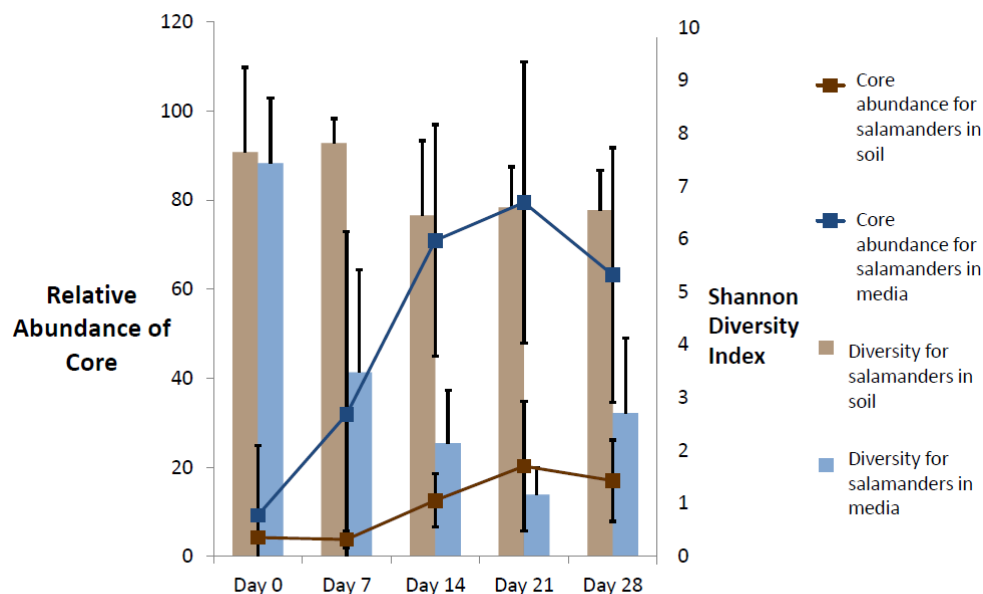


Figure 9. The relative abundance of all 8 core OTUs and the alpha diversity (Shannon Diversity Index) of all salamanders through the course of the experiment. As alpha diversity decreased the abundance of the core OTUs increases.

Consensus Lineage	Soil Day 0	Water Day 0	Soil Day 7	Water Day 7	Soil Day 14	Water Day 14	Soil Day 21	Water Day 21	Soil Day 28	Water Day 28
Pseudomonadaceae; g_ s_	2.115	6.690	0.399	11.382	0.449	3.521	3.718	1.470	3.723	4.601
Opitutae	0.182	0.089	2.912	18.017	8.799	65.677	24.748	77.559	12.221	56.660
Pseudomonadaceae; g_ s_	0.038	0.127	0.005	0.244	0.007	0.078	0.075	0.024	0.062	0.088
Pseudomonadaceae; g_Pseudomonas; s_	0.126	0.351	0.024	0.668	0.022	0.179	0.206	0.073	0.185	0.310
Comamonadaceae; g_ s_	0.091	0.076	0.006	0.006	0.007	0.003	0.028	0.002	0.006	0.007
Pseudomonadaceae; g_ s_	1.563	1.495	0.038	0.734	0.076	0.984	0.715	0.118	0.011	0.605
Staphylococcaceae; g_ s_	0.102	0.296	0.408	0.691	3.197	0.437	0.762	0.204	0.622	0.698
Pseudomonadaceae; g_ s_	0.025	0.076	0.005	0.124	0.007	0.032	0.041	0.016	0.032	0.048
Oxalobacteraceae; g_Janthinobacterium ; s_lividum	0.441	0.777	0.216	1.985	1.325	0.593	0.815	0.371	0.162	0.154

Table 2. Heat map of the core OTUs and the *Janthinobacterium lividum* OTU for each treatment through time. The color scaled heat map represents the relative abundance of the core OTUs found within all salamanders through all time points. Red represents the most abundant and green represents the least abundant. An OTU from the class Opitutae (phylum Verrucomicrobia), was the most abundant bacterium and its abundance was greatest in salamanders without a bacterial reservoir. Five out of the eight core OTUs were Pseudomonadaceae, which are easily and commonly cultured from red-backed salamanders.

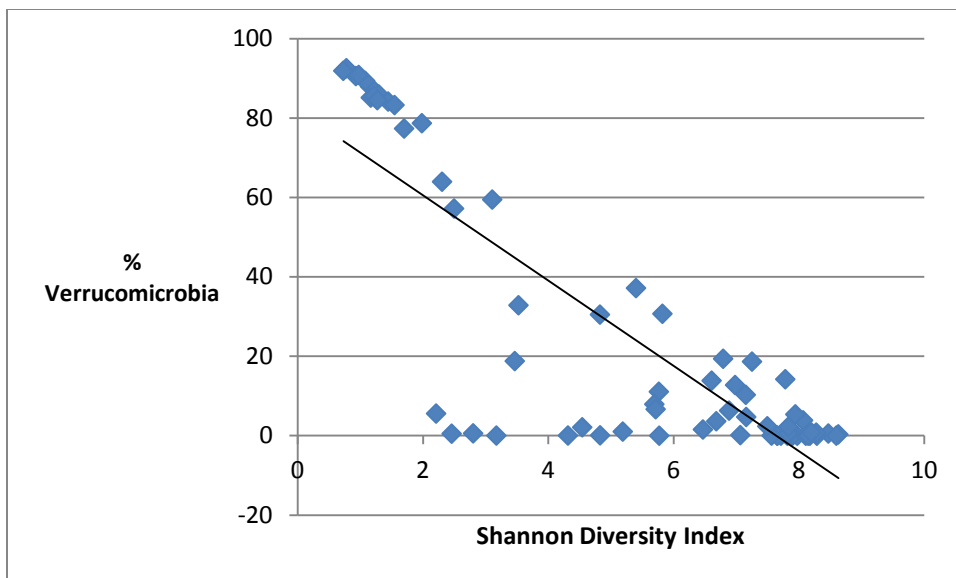


Figure 10. Correlation between relative abundance of Verrucomicrobia and Shannon diversity Index of all red-backed salamanders in this study. This demonstrated that as diversity decreases the relative abundance of the Verrucomicrobia increases.

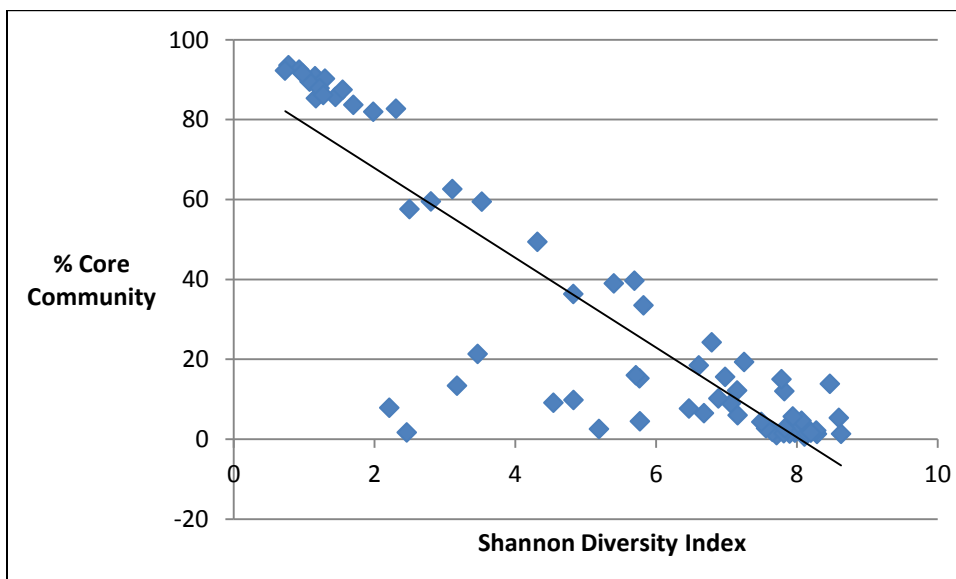


Figure 11. Correlation between relative abundance of the core and Shannon diversity Index of all red-backed salamanders in this study. This demonstrated that as diversity decreases the relative abundance of the core OTUs increases.

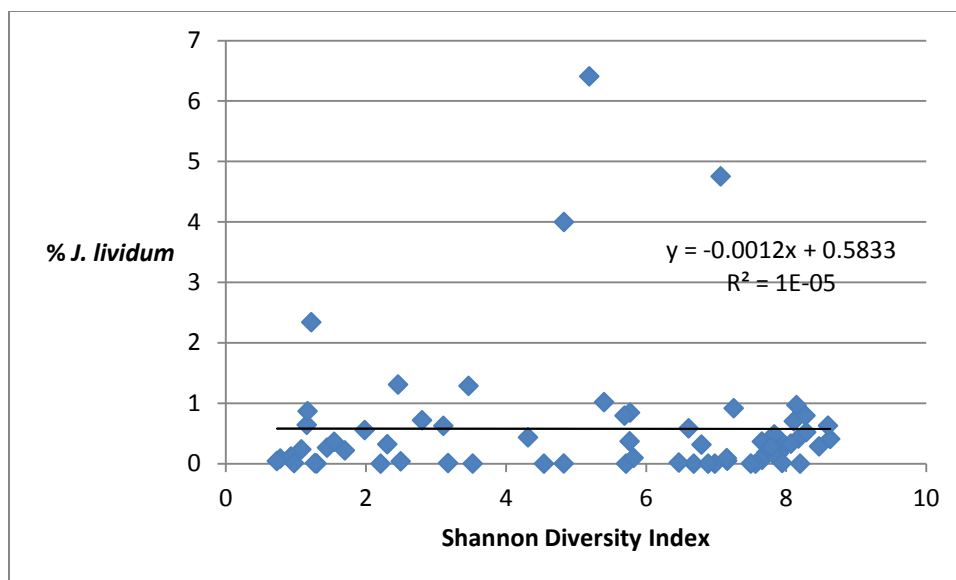


Figure 12. Correlation between relative abundance of *J. lividum* and Shannon diversity Index of all salamanders. This demonstrated that there is no relationship with the diversity of bacterial communities on red-backed salamanders and the relative abundance of *J. lividum*.

Discussion

Host microbiomes perform a number of important functions for their hosts, such as disease resistance (Becker and Harris 2010, Harris et al. 2009, Rosenberg et al. 2007, Dillon et al. 2005). It is likely that the stability and diversity of host microbiomes are related to the consistency and quality of bacterially provided functions. In both the bacterial reservoir and no bacterial reservoir treatments stability was not evident as communities changed rapidly from their initial state in the field. In human microbiome studies, there has also been lack of stability with these microbiomes changing through time (Caporaso et al. 2011). From the perspective of the host, it is more important to have functional stability, in which the function of the microbiome remains constant, than community taxonomic stability. Microbiomes with different community structures may share similar genes due to horizontal gene transfer (HGT) and hence share a specific,

important, function for the host. Analysis of the transcriptomes of amphibians' bacterial communities will be important to determine if different assemblages of microbial communities produce similar genes. The relationship between structural stability and functional stability is unknown in many microbial communities. However, in the human gut, microbiomes were not stable, but shared genes were found across individuals (Turnbaugh et al. 2009); this indicates that these bacterial communities are likely to be functionally stable despite lacking structural stability.

Amphibians' cutaneous communities may have limited stability through time due to seasonal temperature variation or host-related disturbances, such as skin sloughing (Meyer et al. 2012). An important question for amphibians threatened by chytridiomycosis is whether the defensive function of the microbial community remains relatively constant even if community structure varies after a perturbation. For example, constancy of microbial disease function such as the production of anti-fungal metabolites is likely to be important in resistance to chytridiomycosis, but this has yet to be determined for amphibians. In sponges, stressors such as such as climate change, habitat destruction and pesticides have led to changes in microbial community structure (Fan et al. 2013, Webster et al. 2008) that affect the constancy of disease function. If this occurs in amphibians, it could lead to loss of structural and possibly functional stability in amphibians and lead to disease outbreaks.

The alpha diversity of the microbiomes of salamanders without a bacterial reservoir was significantly lower than salamanders with a soil bacterial reservoir. The importance of bacterial diversity in resistance to the fungal disease, chytridiomycosis, has not been determined for amphibians. Humans are less attractive to malaria mosquitoes if their

microbiomes are diverse (Verhulst et al. 2012). Additionally, locusts with diverse gut microbiomes were less susceptible to disease (Dillon et al. 2005). Greater diversity may lead to functional redundancy leading to a constancy of function. Functional redundancy is present in some microbiomes, but absent in others. For example, some environmental microbiomes in soil or ponds lack functional redundancy (Finlay et al. 1997, Allison and Martiny 2008); however, the host associated microbiomes of the human gut has functionally redundancy in term of genes that aid in digestion (Ley et al. 2006). I suggest that host associated microbiomes may be more likely to have functional redundancy since the microbiome and the host are acting together as a selective unit (Rosenberg and Zibler-Rosenberg, 2011). Functionally redundant communities are likely to impart a higher fitness to hosts than non-functionally redundant communities since an important function would still occur after a disturbance. Studies determining the role of bacterial diversity and functional redundancy on amphibian disease susceptibility are needed.

There was no relationship between alpha diversity of salamanders' microbiomes and the microbiomes of the salamanders' immediate habitat at capture in the field. Therefore, the bacterial diversity of the salamanders' habitat had no effect on the diversity of the salamanders' skin bacteria. This suggests that there is independence between salamander microbiomes and substrate microbiomes. Another study found no correlation of *J. lividum* abundance between the skin of salamanders and soil (Muletz et al. 2012). These results suggest that amphibians are dictating the species and abundance of bacteria that are present in their skin microbiota. Host selection and regulation of specific bacterium may be due to differences in skin physiologies, mucous types, antimicrobial peptides mixtures, or nutrients in the mucous layer.

I found a core microbiome that consisted of eight species level OTU's that were present on 90% of all salamanders through time and in both experimental treatments. Core microbiomes have been defined in various ways such as joint membership on hosts in a population at one time and persistence on individuals through time (Shade and Hendelsman 2012, Turnbaugh et al. 2009). Research on core microbiomes has just begun; however, it is likely that species-level core microbiomes on hosts are absent in some systems (e.g., humans), while present in others (e.g., sponges). For example, a core microbiome at finer taxonomic levels, e.g., species, is probably not present in humans (Fierer et al. 2012). This conclusion is supported by the marked variability of bacteria communities through time on human hosts with few taxa present across time points (Caporaso et al. 2011, Costello et al. 2009). On the contrary, a small core microbiome was found among 32 species of marine sponges across a large geographic range; only 0.1% of all the OTUs present among sponges were among the core, when defining an OTU as 97% sequence similarity (Schmitt et al. 2012). Although only a small portion of OTUs were deemed to be within the core microbiome of marine sponges, it is important to note that the comparison was between 32 species throughout a large geographical range, whereas the research on human core microbiomes has been on a limited population with one species. Thus, we can expect considerable variation in the existence of a core community among invertebrate and vertebrate species.

Determining a core microbiome for amphibians' skin has important disease mitigation implications. Augmenting naturally occurring antifungal amphibian bacteria is a promising disease mitigation strategy (Bletz et al. 2013, Vredenburg et al. 2011). For example, a successful bioaugmentation field trial was performed in the Sierra Nevada

Mountains with *Rana muscosa*: all survivors after one year were in the bioaugmentation treatment. It is important to choose a bacterial species for bioaugmentation that inhibits *Bd* in laboratory bioassays and that is prevalent throughout healthy populations of amphibians. A high prevalence of a specific bacterium may indicate an evolutionary relationship between the host and symbiont and therefore be a good candidate for bioaugmentation. The goal of bioaugmentation is to make the prevalent anti-*Bd* species numerically dominant on as many host individuals as possible with the aim of increasing its ability to suppress *Bd*.

The high throughput sequencing performed in this study allowed us to determine the relevance of commonly cultured bacteria to the entire bacterial community. Our results show that five out of the eight species level OTUs that made up the core are Pseudomonadaceae, which are readily cultured from amphibian skin and have been found to be antifungal (Lauer et al 2007, 2008. Brucker et al. 2008a). The anti-fungal bacterium, *J. lividum*, that has been used in past bioaugmentation experiments (Harris et al. 2009, Becker et al. 2009, 2011, Muletz et al. 2012) was not in the core, but was present on 87 % of salamanders at all sample points and in both experimental treatments and on 94% of salamanders in the field. These results indicate that bacterial groups that are readily cultured from amphibians are part of the core and are common members of the community.

The relative abundance of the core microbiome and alpha diversity were negatively correlated. When communities became less diverse the abundance of the core increased and in some cases composed as much as 93.5% of the community. Of course when alpha diversity decreases dramatically this means that a few OTUs have become

dominant; however, we have found that the OTUs that became dominant are members of the core microbiome. A novel, dominant core OTU from the phylum Verrucomicrobia and class Opitutae comprised as much as 92.5 % of the bacterial community in the no reservoir treatment. Verrucomicrobia is commonly found within intestines (van Passel et al. 2011b) and soil (Bergmann et al. 2011, van Passel et al. 2011a). This phylum has been under represented due to PCR bias (Bergmann et al. 2011), which may have affected detection in earlier studies of amphibians systems. Some Verrucomicrobia within intestines are known mucus degraders; however, this OTU's niche on amphibian skins is unknown. Interestingly, Verrucomicrobia in the human gut became dominant after a disturbance due to antibiotic therapy (Dubourg et al. 2013), and this group became dominant in this study after the major disturbance of captivity and lack of a soil bacterial reservoir. It is unknown if the increase of Opitutae on salamanders without a bacterial reservoir was a direct environmental effect on Opitutae growth and reproduction, an environmental effect on the salamander that led to an increase in Opitutae, or an environmental effect on the other bacteria, including the transient bacteria, that led to increased Opitutae abundance. In future studies, it will be important to investigate if the increase in the abundance of the core and certain taxa such as Opitutae in the absence of a bacterial reservoir is because the core is most prevalent before a perturbation and therefore it is likely that one of its members will increase. It is also possible that Opitutae or other core members are more likely than transient species to be opportunistic, which can be an adaptive response of the bacterial species to host stress (Kinney et al. 1999, 2000), or it is possible that the increase in abundance is an adaptive response by the host. The host may produce different secretions to change their microbiome for an adaptive

reason that make the microbiome more beneficial as stress to the host increases. An adaptive response may suggest an evolutionary relationship between the host and the core microbiome, including Opitutae. Further research is necessary to determine the function and importance of the core and Opitutae.

The core OTUs accounted for a small proportion of the microbiomes on salamanders in the field and salamanders in the laboratory housed with a bacterial reservoir, whereas the core OTUs accounted for a large proportion of the bacterial communities for salamanders without a bacterial reservoir. This result may suggest that a diverse bacterial reservoir, such as soil, supports the presence of a large number of transient bacterial species that suppress the abundance of the core microbes due to competitive interactions. If competition is no longer occurring due to the absence of a diverse bacterial reservoir, then core bacteria can become numerically dominant. An alternative explanation is that some bacteria need to be regularly seeded on an amphibian to be common and therefore they become less common without a reservoir. If the initial stress from capture and placement into captivity caused a decrease in diversity due to the new conditions favoring a few bacterial species, it is likely that the communities would remain in low diversity in the no bacteria reservoir treatment since immigration is being prevented. It would be necessary in the future to have a sterile soil control to assure that changes in diversity are due to the presence or absence of bacteria, rather than the presence and absence of soil. However, Figure 8, which shows all data points, demonstrates that the bacterial communities of the salamanders are more similar to the bacterial communities of their respective substrate; therefore suggesting the bacteria in the soil was of importance rather than the presence or absence of soil itself. Determining the consequences of amphibians'

microbiomes with low diversity and the factors that cause low diversity will be important in laboratory experiments, captive rearing programs and disease mitigation protocols.

Five of the eight core OTUs are in the family Pseudomonadaceae, which are within gamma Proteobacteria. The family comprises of five genera: *Pseudomonas*, *Azotobacter*, *Azomonas*, *Azorhizophilus* and *Cellvibrio*. They all share certain physiological properties such as aerobic and chemoorganotrophic metabolism, and the inability to undergo photosynthesis and fermentation. In addition, they are able to use a wide range of organic substrates. One of the core OTUs within the family Pseudomonadaceae is in the genus *Pseudomonas*. This genus is known for its rapid growth, genetic elasticity and metabolic versatility; due to these traits they are found in all environments. As of the eighth edition of Bergey's manual, there were a total of 235 described species of *Pseudomonas* (Dworkin et al. 2006). One of the core OTUs is in the family Staphylococcaceae, which is a Firmicutes and comprises of Gram positive bacteria with cocci cell morphology. The most studied genus within the family is *Staphylococcus* and is commonly cultured from human skin (Dworkin et al. 2006). Another OTU in the core is in the family Comamonadaceae, which is a beta proteobacteria. Comamonadaceae is considered phylogenetically coherent, but physiologically diverse (Dworkin et al. 2006). Interestingly, Comamonadaceae is in the same order, Burkholderiales, as *Janthinobacterium lividum*. The last core OTU is a novel Opitutae, which has been discussed above. Out of the eight core OTUs, 7 were Gram negative (five Pseudomonadaceae, one Comamonadaceae and one Opitutae).

The beta diversity of the laboratory soil did change throughout the experiment. It is unknown if the change was due to the salamander shedding bacteria and introducing nutrients, or due to the soil's captivity. A control that just involves soil without salamanders would be necessary to determine if the salamanders affected the soil. It is likely, however, that salamanders could shift the soil bacterial community since the salamanders are introducing nutrients and gut bacteria into the system.

The hologenome theory suggests that a host and its microsymbionts act together as a selective unit (Rosenberg and Zibler-Rosenberg 2011). This concept has been applied to corals; however, it has never been applied to amphibians. This theory would suggest that amphibians share an evolutionary past with their bacteria, and their bacteria provide a beneficial function. For example, a mutualism could occur between an amphibian and an anti-fungal bacterium; in this scenario the amphibian provides the bacterium with a substrate and nutrients, and the amphibian is provided with protection from fungal pathogens. The fact that important known anti-fungal bacteria such as *J. lividum* and Pseudomonadaceae are highly prevalent on red-backed salamanders suggests that they are providing an adaptive function for the host and are members of the salamanders' holobiont, and therefore, the salamanders and bacteria are acting together as a selective unit.

My results show that captivity and animal maintenance affect skin microbiomes, which has implications for amphibian microbial ecology experiments that are conducted in the laboratory (Becker and Harris 2010, Becker et al. 2009, Harris et al. 2009). I have found that the microbiome changed upon entering captivity and that the diversity of the bacterial communities is likely dependent on the availability of a bacterial reservoir in

soil. In many of the experiments on the role of bacteria protecting amphibians against *Bd*, the amphibians have been housed in containers with sterile artificial pond water (Becker et al. 2009, Becker and Harris, 2010, Becker et al. 2011, Harris et al. 2009), which are conditions similar to our no bacterial reservoir treatment. We suggest that laboratory conditions make amphibians more susceptible to disease since they quickly attain atypical microbial community structures with a low diversity. This may mean that past laboratory studies do not reflect results that would be obtained in the field if microbiomes are less defensive when amphibians are housed in captivity without bacterial reservoirs. In addition, these results may be important in captive rearing programs where animals are raised in pristine conditions with the intentions of release into the wild. For instance, captive rearing programs are occurring in the USA for hellbenders (*Cryptobranchus alleganiensis*), boreal toads (*Anaxyrus boreas boreas*), mountain yellow-legged frog (*Rana muscosa*) and chiricahua leopard frogs (*Lithobates chiricahuensis*) (Bodinof et al. 2012, Muths et al. 2001, Fellers et al. 2008, Soorae, 2011, respectively). These amphibians are likely to have depauperate and atypical microbiomes since they have no natural bacterial reservoir. It is important to determine how captivity affects the microbiomes of animals in repatriation programs in order to establish natural microbiotas prior to release. In addition, investigating if bacterial communities attain a similar structure and function after repatriation as wild animals will be important in assisting repatriation programs and retaining atypical communities may be ameliorated through the use of probiotics. If the microbiomes of captive animals are dissimilar to wild animals and if they lack key anti-fungal bacteria, then it is possible these animals will be more susceptible to disease when first reintroduced into the wild. To restore atypical and

depauperate bacterial communities, it may be possible to establish the typical climax communities for amphibians using bacteria found on wild, healthy, animals. Indeed probiotic therapy is a promising disease mitigation strategy for future efforts (Bletz et al. 2013). It will be important to determine the structure of microbiomes for each species of amphibians that are candidates for bacterial bioaugmentation since microbiome variations occur between amphibian species (McKenzie et al. 2011). Further knowledge of the microbial dynamics and the role that known culturable bacteria play within microbial communities will benefit probiotic strategies and amphibian conservation efforts.

Chapter III: Justification of research and future directions

I seek to develop mitigation strategies against the fungal disease chytridiomycosis, which has caused global amphibian declines. One strategy to mitigating disease is enhancing the hosts' defenses against the pathogen. Efforts have been made to study the defensive responses that amphibians have against chytridiomycosis; they are: adaptive immunity, innate immunity, and beneficial symbiotic bacteria. Research has demonstrated that amphibians have little to no adaptive immune response against the disease (Rosenblum et al. 2009, 2011); indeed the fungus may evade the adaptive immune system (Rollins-Smith, personal communication). Innate immunity, such as the production of antimicrobial peptides (AMPs) has been shown to be important in disease resistance (Rollins-Smith 2009), but is also less active and efficient under amphibians' suboptimal environmental conditions (Sheafor et al. 2006). Furthermore, both AMPs and the adaptive immune system cannot be easily modified to achieve greater disease resistance. Lastly, beneficial symbiotic bacteria on the skin of amphibians are important in disease resistance and when added to amphibians provide protection from disease (Becker and Harris 2009).

Studying bacterial communities has greatly advanced with the advent of culture independent techniques that allow for bacteria that are not easily cultured to be detected. In recent years the importance of bacteria in amphibian health has become an important focus of research, and it has been proposed to manipulate the bacterial communities of amphibians to enhance disease protection (Bletz et al. 2013); however, little is known about the entire bacterial communities on amphibians. My thesis incorporates culture

independent techniques that allow me to study the complete bacteria community regardless of culture bias. I determined a baseline bacterial community structure on red-backed salamanders in the wild, then experimentally determined that the communities were structurally not stable and that the diversity of those bacterial communities was dependent on the availability of a bacteria reservoir. In this chapter, I discuss future research directions to answer questions that arise from my research.

To advance my thesis research further it will be important to determine if amphibians with a lower skin bacterial diversity have an increase in disease susceptibility. This could be done by repeating this experiment to create high and low diversity communities and then exposing salamanders to *Batrachochytrium dendrobatidis* (*Bd*). Infection prevalence and intensity could then be determined using quantitative polymerase chain reaction (qPCR; Boyle et al. 2004). In addition, past *Bd* studies have found that growth rate is a reliable indicator of morbidity (Becker et al. 2009, Becker and Harris 2010).

Determining the proportion of bacteria that are culturable compared to the entire bacterial community will be important in amphibian probiotic mitigation strategies. This can be determined by combining culture independent techniques such as Illumina sequencing with culture based surveys. This is important because in some microbial systems as many as 99.8% of the bacteria species are difficult to culture (Streit and Schmitz, 2004), and bacteria that are used as probiotics for amphibians will need to be easily cultured. In previous culture surveys, morphotypes of bacteria were separated and a representative of each morphotype would then be put into pure culture and sequenced for identification based on the 16s RNA gene. However, this protocol may skew the

actual diversity of culturable bacteria because different species of bacteria may have the same morphotype and appear similar to the researcher. In future surveys of culturable bacteria on amphibians it will be important to culture and identify all colonies, regardless if they look similar. This will allow for the total diversity of cultured bacteria to be established. It is likely that a small proportion of the bacteria on amphibians are easily cultured since there was an average of about 1000 OTUs on each wild salamander in my thesis research.

It will also be important to determine the function of the bacterial communities on amphibians to determine the main purpose of the community and determine if there is functional redundancy. This can be done in a number of ways, such as acquiring transcriptomes, metabolite profiles or functional assays from skin secretions.

Transcriptomes from the skins of amphibians can be determined by making messenger (mRNA) into complementary DNA (cDNA), which is then sequenced. This would allow me to determine which genes are being transcribed; it can be assumed that the genes detected with this method would ultimately be translated. This would allow us to determine which genes for defensive compounds are being transcribed and if there are other genes for major functions occurring on the skin of amphibians such as mucus degradation. One problem with this technique is that all mRNA would be sequenced including mRNA from the host; however techniques allow for eukaryotic genes to be sorted out. The technique is also expensive and technically difficult.

Another method to examine bacterial community function is to observe the metabolites found on amphibians' skins that function in defense. This technique uses high pressure liquid chromatography-mass spectrometry (HPLC-MS) to determine a

profile of small organic compounds that are soluble in methanol and is done in the laboratory of Dr. Kevin Minbiole (Villanova). Each chemical can be identified if it has been detected in previous studies, or identified as an unknown compound that chemist can then identify. This technique of acquiring metabolite profiles would be useful in determining if particular metabolites are associated with particular bacteria communities. In addition, coupling this technique with a *Bd* exposure experiment would establish if certain metabolite profiles are associated with disease resistance. The shortcomings of acquiring metabolite profiles are that they exclude large groups of chemicals. For instance, this technique does not allow for the identification of any proteins that could be made by bacteria. It is possible that the main antifungal compound produced by a particular bacterium is a protein rather than a metabolite and therefore would not be observed. It is also possible that the metabolites observed with this technique would not be bacterially derived since amphibians could also produce compounds detected with this method; however, all of the metabolites that have been studied from the skin of amphibians to date have been bacterially produced (Brucker et al. 2009ab).

Testing the secretions from amphibians' skin against *Bd*, as mentioned in Bletz et al. (2013), will determine the total inhibitory properties of all the chemicals found on the skin of amphibians. However, there is no way to separate chemicals that are host or bacterially derived. Furthermore, the extent to which the chemicals are host or bacterially derived is not known. However, amphibians within the same population probably produce similar antifungal products, such as antimicrobial peptides. Therefore, any differences in inhibition from the sample may be explained by the hosts' bacterial communities, which can then be correlated to the bacterial community structure. This

assay, coined the total chemical assay, is currently being developed by me and the laboratory of Dr. Kevin Minbiole (Villanova University), and may have an important role in determining how successful a particular probiotic is without having to do clinical trials with *Bd*. In this case, the experimental design would have two groups: control and probiotic. Total chemicals from each group would be collected and then used in a bioassay with *Bd* to determine if the probiotic treatment has greater inhibitory properties.

Using HPLC-MS, transcriptomes, and the total chemical assay to examine the function of microbial communities may also be used to determine if amphibians' bacterial communities are functionally redundant when disturbed. For instance, if low diversity bacterial communities, have the same defensive function as the high diversity bacterial communities, then it could be concluded that the bacterial communities are functionally redundant. This would establish if there were different bacterial species present that shared the same functional role. This may be important to amphibians that are in conditions with constant disturbances such as seasonality that may affect bacterial communities' structures. It is possible that two different species of bacteria that have different optimal temperatures both possess a gene for an important anti-fungal metabolite and therefore the host is protected regardless of the temperature. This gene sharing could be explained by horizontal gene transfer (HGT), where important genes are passed amongst the bacterial community. It will also be important to determine the role of the core microbiome since it was shared between the low and high diversity communities in my thesis. If bacterial communities on amphibians maintain their function regardless of perturbations, it may be due to the core, as it was present on salamanders regardless of treatment.

In sum, I established a baseline bacterial community structure on red-backed salamanders in the wild, then experimentally determined that the communities were structurally not stable and that the diversity of those bacteria communities was dependent on the availability of a bacteria reservoir. In this chapter, I discussed the future directions to further my research and ultimately benefit the conservation of amphibians, who are greatly threatened by disease and other anthropogenic disturbances such as habitat loss and climate change at this time.

Chapter IV: Appendix

Phylogenetic Diversity (PD) Whole tree rarefaction provides a quantitative measure of phylogenetic diversity. Phylogenetic diversity is measured by adding the appropriate branch lengths for a subset of taxa (Faith, 1992). PD Whole tree rarefaction decreased over time on salamanders in MEDIA but remained constant on salamanders in soil (Fig. 5). There was a significant different in treatment ($F_{(1, 26.058)} = 42.275$; $P < 0.0001$), day of experiment ($F_{(4, 26.558)} = 15.673$; $P < 0.0001$), and interaction ($F_{(4, 20.558)} = 4.205$; $P = 0.012$).

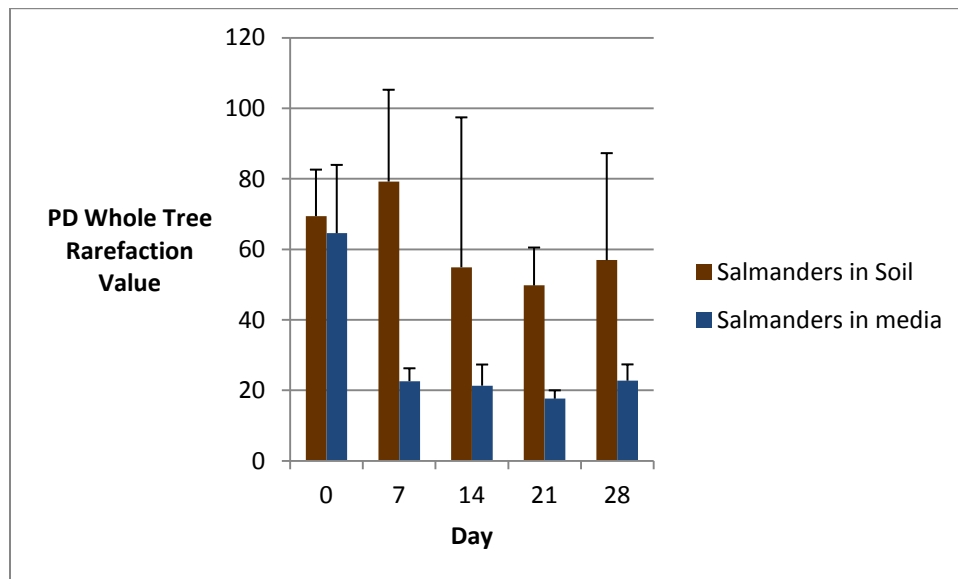


Figure 13. PD Whole Tree for salamanders in soil (bacterial reservoir) and in media (no bacterial reservoir) over time. Salamanders housed without a bacterial reservoir had lower Shannon diversity indexes than salamanders with reservoirs. Standard deviations are included.

Chao1 is a non-parametric measure of OTU richness (Hill et al. 2003). Chao 1 value declined in the media treatment, but remained similar in the soil treatment (Fig. 6). There was a significant different in treatment ($F_{(1, 26.228)} = 35.156$; $P < 0.0001$), day of experiment ($F_{(4, 20.676)} = 14.742$; $P < 0.0001$), and interaction ($F_{(4, 20.676)} = 3.015$; $P = 0.042$).

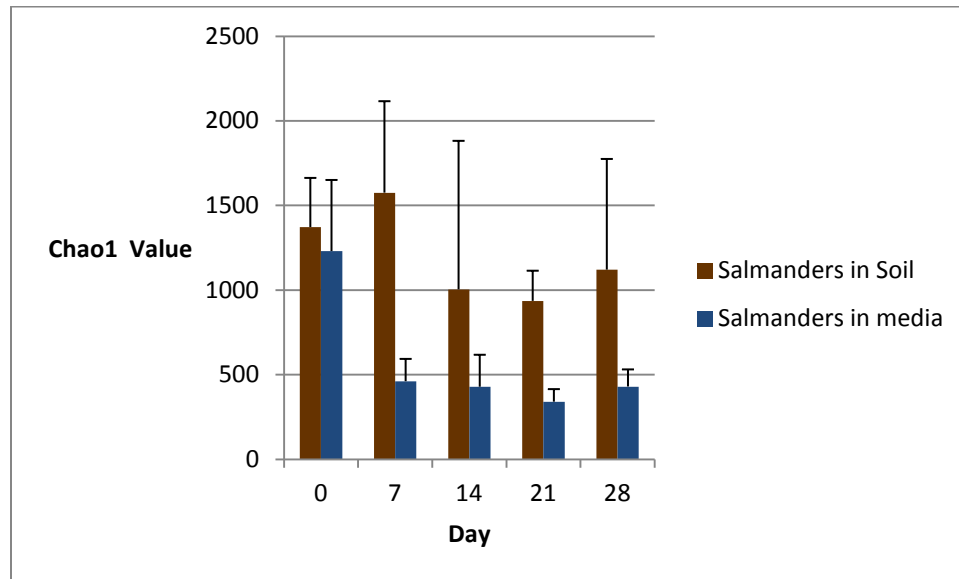


Figure 14. Chao1 for salamanders in soil (bacterial reservoir) and in media (no bacterial reservoir) through time. Salamanders housed without a bacterial reservoir had lower Shannon diversity indexes than salamanders with reservoirs. Standard deviations are included.

OTU richness is the number of different OTUs found on each salamander. Salamanders housed without a bacterial reservoir had a lower OTU richness than salamanders housed with a bacterial reservoir (Fig 7, Table 3). There was a significant difference in treatment ($F_{(1, 30.587)}=128.734$; $P<0.0001$), day of experiment ($F_{(4, 21.103)}=25.038$; $P<0.0001$), and interaction ($F_{(4, 21.103)}=12.300$; $P=0.012$).

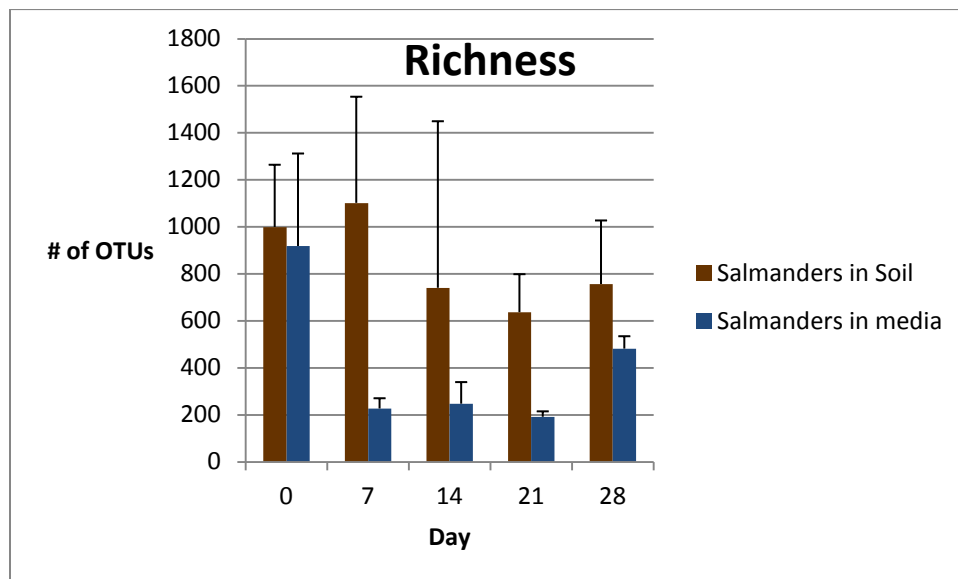


Figure 15. Richness for salamanders in soil (bacterial reservoir) and in media (no bacterial reservoir) through time. Salamanders housed without a bacterial reservoir had lower Shannon diversity indexes than salamanders with reservoirs. Standard deviations are included.

The most abundant OTU found on salamanders was within the Verrucomicrobia class Opitutae and is novel. Four novel Verrucomicrobia OTUs were found in this study; however New Opitutae 164589 was most abundant and was present on all salamanders. Figure16 shows where all of the Verrucomicrobia class Opitutae OTUs place within a tree of Verrucomicrobia.

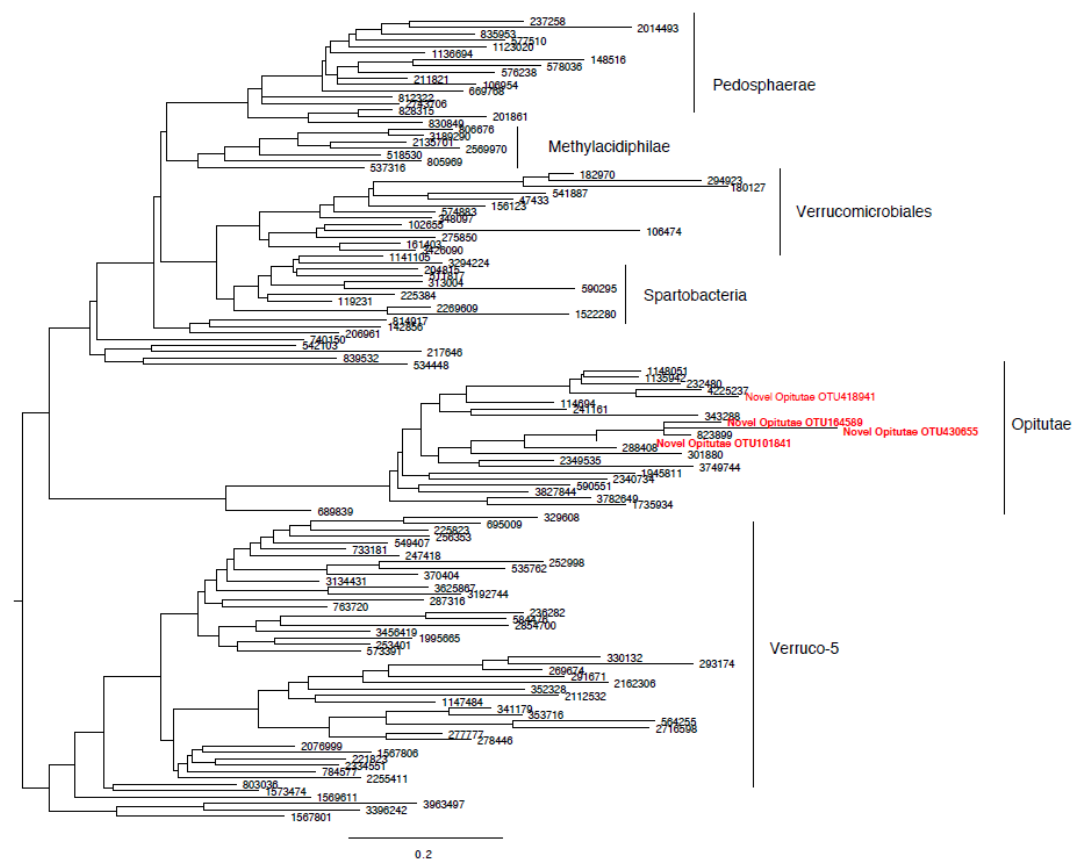


Figure 16. Tree was constructed by placing novel Verrucomicrobia sequences within the Greengenes tree (filtered to sequences with 85% similarity; obtained from http://greengenes.secondgenome.com/downloads/database/12_10) using the Evolutionary Placement Algorithm within RAxML, including the OTU that corresponds to the highly abundant member of the redback salamander core community (OTU 418941). These sequences are robustly placed within the Verrucomicrobia class Opitutae. Tree is labeled with Greengenes prokMSA ids.

Day	Salamanders with Soil	Salamanders with media
0	998 ± 265	918 ± 394
7	1102 ± 452	226 ± 44
14	740 ± 709	247 ± 92
21	636 ± 191	191 ± 23
28	756 ± 271	481 ± 53

Table 3. Richness of OTUs for salamanders in soil (bacterial reservoir) and in media (no bacterial reservoir) over time. Salamanders housed without a bacterial reservoir had lower Shannon diversity indexes than salamanders with reservoirs. Standard deviations are included.

Janthinobacterium lividum, a known antifungal symbiote of red-backed salamanders (Brucker et al. 2008b) was found on 18/19 wild salamanders (day 0). *J. lividum*'s presence on salamanders throughout the experiment is presented in Table 4.

Day	Salamanders with Soil	Salamanders with media
0	90% n=10	100% n=9
7	75% n=4	100% n=5
14	100% n=4	100% n=6
21	71% n=7	86% n=7
28	67% n=6	88% n=8

Table 4. Prevalence of *Janthinobacterium lividum* on salamanders through time. There were no statistical differences between salamanders housed with soil (bacterial reservoir) versus salamanders housed with media (no bacterial reservoir).

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