

Spotted Salamander Egg Masses Exhibit a Unique Internal
Microbiome in Relation to Their Environment

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Abstract

Microbial studies have led to various ecological and medicinal discoveries. Amphibian eggs, like those of *Ambystoma maculatum*, exhibit natural resistance to bacterial invasion that may be microbiome related. The purpose of this research was to sequence the external and internal microbiome of *A. maculatum* egg masses and compare with the environmental microbiome. It was hypothesized that the external and internal microbiome would bear more similarity to each other than the water. A significant difference was observed between the microbiome of the inside surface of the egg mass and the water but not the outside surface of the egg and the water, suggesting that the internal environment consists of unique microbes possibly acquired through vertical transmission or interactions with other organisms.

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Amphibian Egg Masses

Of terrestrial vertebrates, amphibians are some of the most unique, as the amphibian life cycle plays out in two distinctive phases: aquatic and terrestrial. The aquatic phase includes eggs, tadpoles, and metamorphs, while the terrestrial phase includes subadults and mature adults ready to breed (Prest et al., 2018). For many species of amphibians, adults return to aquatic sites for courtship, egg deposition, and fertilization (Altig & McDiarmid, 2007). The larvae that hatch begin with an aquatic life, remaining in this environment until sufficient growth and development has occurred for them to metamorphosize and move onto land. Due to the variable nature of the appearance of individual eggs, which leads to inconsistent interpretation, an approach focusing on clutch morphology is often used to describe amphibian eggs that are being studied. The amphibian developmental stage for frogs and toads uses the 1960 Gosner Staging System, while the spotted salamander is staged using the 1969 R. Harrison Staging System (Virginia Herpetological Society, 2021).

Structurally, eggs contain an ovum, vitelline membrane, and surrounding jelly layers (Altig & McDiarmid, 2007). These jelly layers vary in toughness, density, thickness, and many other traits, but collectively serve a variety of purposes. Egg jelly plays an important role in the attaching of eggs to each other and nearby structures, allowing the formation of egg masses, and also ensuring that the correct sperm enters for fertilization (Pintar, 2017). Jelly layers also play an essential role in the protection of eggs from environmental threats, which can include predation, contaminants, environmental stressors, and pathogenic microbes. Egg jelly can often be separated into distinctive zones, with most eggs consisting of 3-6 layers depending on the

analytical technique that is used to define each zone (Altig & McDiarmid, 2007). Aquatic eggs are often spherical when submerged but are unable to maintain this shape out of the water, whereas terrestrial eggs have increased membrane strength and turgidity to maintain their shape outside of water. The egg jelly of some species of salamanders, such as *Ambystoma maculatum*, can contain unique glycoprotein crystals, which cause white-colored jellies rather than the usual clear jellies (Altig & McDiarmid, 2007). While the functions of these crystals are unknown, they are suspected to offer concealment from predators or reflect light to benefit the developing embryos. Some species of salamander eggs also contain symbiotic algae within the inner layers of jelly (Altig & McDiarmid, 2007).

Although extensive research has been completed on early amphibian development, much less is understood about amphibian eggs that remain in their natural habitat (Altig & McDiarmid, 2007). Many questions remain about the specific processes by which these eggs are maintained, including how the composition of the jelly layers contributes to the protection of these developing embryos. Further research exploring these avenues has the potential to uncover discoveries that can inform and improve amphibian conservation efforts.

Ambystoma maculatum

Ambystoma maculatum, commonly referred to as the yellow-spotted salamander (Figure 1), is a species that occupies large swaths of forested wetlands throughout eastern North America (D'Errico et al., 2020). This species is known to prefer deciduous forests with abundant rivers and bodies of water. Adequate breeding sites, which include freshwater ponds, temporary ponds, and wetlands, are relatively isolated from one another, leading to population differentiation on a small spatial scale (D'Errico et al., 2020; Zamudio & Wieczorek, 2007). Females lay their eggs underwater, which take 4-7 weeks to hatch based on water temperature and exposure. Aquatic

larvae emerge from the eggs and remain in the water for 2-4 months before undergoing metamorphosis to enter their juvenile land stage (D'Errico et al., 2020).



Figure 1. A spotted salamander (*Ambystoma maculatum*). From “Microbiome Associations Between Spotted Salamander (*Ambystoma maculatum*) Egg Masses and the Environment,” by Leonard, E., Baker, C., Becker, M., Wilson, G., & Harris, K. (2021, August). [Poster Session]. Liberty University Research Symposium, Lynchburg, VA, United States. Reprinted with permission.

A. maculatum is well understood to have a symbiotic relationship with *Oophila amblystomatis*, a species of green algae that colonizes egg masses and the tissues of the embryo (Baxter et al., 2015; Burns et al., 2020). Symbiosis between the two species offers a variety of potential benefits, including increased oxygen provision to the embryo and removal of nitrogenous products like ammonia (Baxter et al., 2015). *O. amblystomatis* produces oxygen photosynthetically but consumes oxygen at night, leading to daily fluctuations in oxygen partial pressure within the egg, so developing embryos experience periodic hypoxia. Embryos nearest to the center of egg masses experience the lowest oxygen levels at night, which can lead to slowed development compared to embryos on the surface (Valls & Mills, 2007). Despite these hypoxic cycles, increased algal density has been correlated with increased embryonic development in *A. maculatum* (Baxter et al., 2015).

One of the most distinctive traits of *A. maculatum* is the exhibition of egg mass polymorphism. Eggs primarily contain either clear or white jelly, although an intermediate phenotype with grey jelly can occur, albeit rarely. Females produce a single morphology in their lifetime, as this variance is due to genetic differences in a single gene (Pintar & Resetarits, 2017). This gene influences the concentration of hydrophobic protein crystals in the outer layers of jelly, where higher concentrations lead to increased opacity in white and intermediate morphs. In clear morphs, this crystal is replaced with a water-soluble protein instead, which is normally found in the inner layers of all egg masses regardless of polymorphism (Ruth et al., 1993).

White egg masses appear in greater abundance in low nutrient environments and are subject to significantly less predation, but it is unknown why the phenotype for clear masses is maintained, as there is no difference in algal density or larval survival between the two morphologies (Pintar & Resetarits., 2017; D'Errico et al., 2020). Both morphs produce larvae of the same size with the same hatching success rate in various light and pH conditions, although white egg masses produce significantly more embryos (Pintar & Resetarits, 2017). The high survival rate of both morphs suggests that while these polymorphisms have been around for thousands of years, neither morph serves as a strong selecting force favoring specific environments.

Historically, a sizeable amount of research has been performed on *A. maculatum* and its eggs. Many of these studies have focused heavily on the symbiosis between *A. maculatum* eggs and *O. amblystomatis*, and some research has been done to explore the differences caused by polymorphic variation in egg jelly. While reviews have found significant variation between the cutaneous microbiome of adult, juvenile, and larval *A. maculatum*, no research has been done on the microbiome found on egg masses (Stevens, 2021). Exploration into this topic could lead to

helpful insight into the mechanism that allow these eggs to develop safely in a hostile heterotrophic environment, further advancing amphibian conservation efforts.

Microbiome

Microbes can be found almost everywhere on the globe. Microbiome is a term used in reference to the microorganisms that colonize specific areas, such as a stagnant pool of water, the outside surface of a tree, or the digestive tract of a human (Fahad, 2021). Microbiomes can be located anywhere, including the external surfaces of various objects and the internal environments of living creatures. A wide variety of research has shown that microbiome symbiosis plays a variety of important roles in the health and wellness of all species of animals (Kueneman et al., 2013). Microbiome has been established as an important factor in the prevention of pathogenic uptake. Residual microbes can prevent successful colonization by parasites and opportunistic pathogens, although their protective ability can be compromised by the release of metabolites that affect symbiont abundances, virulence factors that cause host inflammatory response, and the release of symbionts by pathogens themselves (Hernandez-Gomez et al., 2019). Significant steps have been made to expand understanding about microbiomes of a variety of species, but over 90% of vertebrate studies have explored microbiomes on mammals only, with significantly less research on other vertebrates (McKnight et al., 2020). Amphibians are one such group where research has begun, but much remains to be uncovered about their external and internal microbiomes at varying stages in their life cycles, especially considering the dramatic shifts that the microbiome undergoes as amphibians transition from aquatic to terrestrial life. External microbiomes are of special interest because of the unique nature of amphibian skin and its susceptibility to affect the wellbeing of the entire animal.

Amphibian skin bacteria can be acquired and maintained via three methods: horizontal transfer from host-host interactions, environmental transfer, and vertical transfer (parent to offspring) (Hughey, 2017). Of these types, environmental transfer and vertical transfer are of the most interest for early development of the microbiome.

While environmental exposure occurs constantly in any animal's life, there are two points in development where the skin microbiome experiences strong disturbance events: when larvae hatch from their eggs and are first exposed to their environment, and when aquatic tadpoles metamorphosize into their semi-terrestrial juvenile stage and are newly exposed to terrestrial microbes (Prest et al., 2018). This is supported by discoveries about how the amphibian microbiome during early-life plays an essential role in the development of an effective immune response later in an amphibian's life. Disruptors during the tadpole phase of the amphibian lifecycle led to reduced bacterial diversity in adults frogs, ultimately leading to increased susceptibility to infection and parasitic invasion (Knutie et al., 2017). These results suggest that early life is a window of opportunity in which the amphibian microbiome is most vulnerable to defining changes, and this could be especially true of development in the egg. Egg microbiomes are an essential factor in the health of organisms, as they offer an opportunity for vertical transmission of symbionts between parent and offspring but face increased risk of fouling and disease due to the abundance of microorganism found in their environments (Nyholm, 2020). Environment plays an important role in the development of the egg microbiome; in *Anaxyrus boreas*, commonly known as boreal toads, it was discovered that eggs had the highest percentage of aquatic sediment-derived bacteria relative to other aquatic life stages, suggesting the environment is also a heavy influencer on egg microbiome, especially for eggs that rest in the sediment before hatching (Prest et al., 2018).

However, significant research has found that vertical transmission plays a larger role in egg and embryo microbiome than environment. In terrestrial salamanders, more bacterial similarity has been observed between females and their eggs than between eggs and the soil within their nests (Banning et al., 2008). This suggests that egg microbiome is not simply a product of the environment, but experiences lasting impact from adult amphibians. This observation was collaborated by a study performed on *Hyalinobatrachium colymbiphyllum*, a species of glassfrog. Both antimicrobial skin peptides and mutualistic microbiota can be transferred from adult to embryo, which can help create a protective environment during embryonic development (Walke et al., 2011). Glassfrog eggs were found to maintain a diverse microbiome despite differing environments and shared more bacterial similarity to father *H. colymbiphyllum* regardless of whether the males played an active role in the caretaking of the eggs, suggesting that a certain level of bacterial maintenance was attained without continual exposure to the males (Hughey et al, 2017).

Further research has demonstrated that microbiomes can contribute to antimicrobial activity. In the salamander *Hemidactylium scutatum*, approximately 27% of adult females had a skin bacterium that resists *Mariannaea sp.*, a fungal pathogen; in communal nests containing at least one female with this bacterium, embryos were more likely to survive a visible *Mariannaea sp.* infection (Banning et al., 2008). Achieving a better understanding of the microbes present on the surfaces of eggs and where they originated from has the potential to reveal beneficial information that could be used by conservationists to help combat diseases and infections in affected amphibian populations.

While a substantial amount of research has explored amphibian eggs and their microbiome as a whole, no specific research has been found describing the microbiome found on

the eggs of *A. maculatum*. Additionally, no studies have specifically isolated the microbiome on the inside of an egg mass to compare with the microbiome present on the outside of the egg mass. Considering all of the research that supports the idea that microbiome is an important part of the ability of eggs to combat pathogens and prevent bacterial or fungal invasion, it is a worthwhile endeavor to explore these unknown microbiomes so that more discoveries can be made to advance the fields of ecology and conservation.

Project Focus

Historically, the study of microbiomes has uncovered a variety of discoveries that have improved the fields of ecology, conservation, and medicine. Microbiomes are intricate and complex but play a significant role in health and wellness; there is increased acknowledgement that understanding microbiomes is an important factor in establishing conservation strategies (McKnight et al., 2020). Amphibian conservation is of special interest in the scientific community considering the notable declines in amphibian populations that have been observed across the world in the last 20 years, related to habitat loss, invasive species, disease, and chemical contamination (Baxter et al. 2015). One of the most prominent causes of this decline is *Batrachochytrium dendrobatidis* (*Bd*), a fungal pathogen that affects amphibian skin and has decimated amphibian populations around the world (Kueneman et al., 2013). Some bacteria found on the skin of reptiles confers resistance to *Bd* (Hughey et al., 2017). Studies have demonstrated that introducing probiotic bacteria that is effective against dangerous pathogens to adult amphibians, such as Sierra Nevada yellow-legged frogs (*Rana sierrae*), is not conducive to long-term adoption into the microbiota, but can be administered therapeutically as short-term treatments for infection (Woodhams et al., 2019). It is possible that the microbiome is more susceptible to addition of microbes by researchers at earlier stages in life, which could offer

long-term resistance to infection rather than short-term treatment. Considering how varied an amphibian's microbiome is throughout the various stages of its life, it is also possible that there could be unique microbes that are effective against pathogens that are only maintained in early life. Since significant research has been done to explore amphibian skin microbiome but not nearly as much has been done on amphibian eggs, more studies on amphibian egg microbiomes could uncover new microbes with antibacterial or antifungal applications or could enlighten researchers on how to successfully introduce beneficial microbes to at-risk amphibians so that they remain part of the amphibian's microbiome well into their adult life. By exploring the internal and external microbiomes of amphibian egg masses, which are not well understood, insight could be made into new avenues for conservation focused on the prevention and fighting of disease in the amphibian community.

Previous Research

This current study was inspired by previous research performed by this lab that explored the protective features of amphibian eggs. The first study investigated the protective features of wood frog (*Lithobates sylvaticus*) eggs by placing a single egg in motility agar and observing for 72 hours to see if a zone of inhibition was formed, indicating the secretion of antimicrobial compounds from jelly layers of the egg (Figure 2). This study revealed that no observable zones of inhibition were formed. These findings suggested that wood frog eggs had a defensive response to heterotrophic aquatic microorganisms rather than an offensive response. It is hypothesized that this defensive response is specific to a unique microbe on the eggs or chemical substances in the egg jelly (Figure 3).

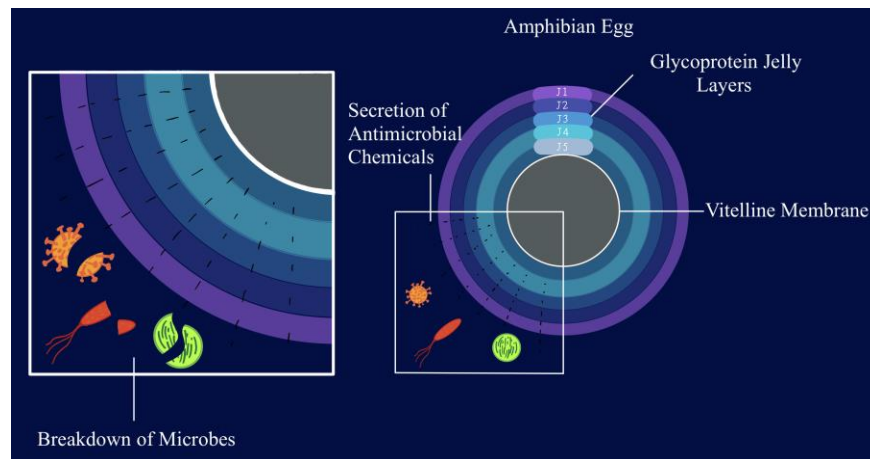


Figure 2. Graphic illustrating glycoprotein layers of an amphibian egg. Amphibian eggs contain 3-6 layers of egg jelly separating the ovum from its environmental. It was suspected that antimicrobial chemicals were released from the egg jelly, resulting in the breakdown of microbes. This was not observed in the study, suggesting a defensive response instead. From “Investigating Antimicrobial Chemicals Released from Amphibian Eggs,” by Sorenson, C., Young, A., Warren, A., Hannah, A., Chapman, C., Piddock, A., Arrasmith, C., Becker, M., Harris, K., & Wilson, G. (2021) [Poster presentation]. Liberty University Research Week, Lynchburg, VA, United States. Reprinted with permission.



Figure 3. A wood frog (*Lithobates sylvaticus*) egg mass, like those used in the zone of inhibition study. The egg jelly is clearly distinguishable from the developing embryos. From “Investigating Antimicrobial Chemicals Released from Amphibian Eggs,” by Sorenson, C., Young, A., Warren, A., Hannah, A., Chapman, C., Piddock, A., Arrasmith, C., Becker, M., Harris, K., & Wilson, G. (2021) [Poster presentation]. Liberty University Research Week, Lynchburg, VA, United States. Reprinted with permission.

Current Project

This study seeks to continue building our understanding of the defensive response of amphibian eggs by isolating and sequencing the microbiome of *A. maculatum* egg masses to compare with the microbes found in their environment. To achieve this, aseptic swabs were taken of the outside surface, inside surface, and aquatic environment of *A. maculatum* egg masses (Figure 4). DNA extractions, PCR, and sequencing was performed on each swab to isolate the 16s gene, commonly used for bacterial identification. QIIME2 was used to analyze the data and perform statistical analysis (Figures 5, 6, 7). The use of these methods allows for increased understanding of the microbiome of amphibian egg masses, which will further inform

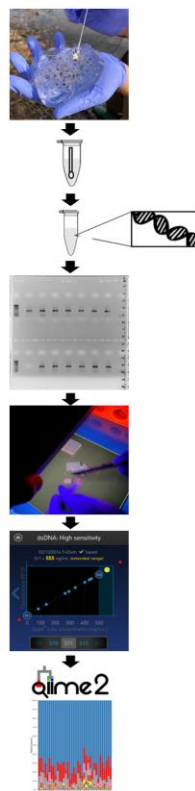


Figure 4. *Experimental methods.* Swabs were collected from the spotted salamander egg masses and water in Sheppe Pond. Microbial DNA was extracted, the 16s gene was amplified, the purity of the samples was confirmed through gel electrophoresis, the bands were extracted from the gel and cleaned, and DNA was sequenced and analyzed using QIIME2.

our understanding of the defensive properties of amphibian eggs that are responsible for protecting them from the abundance of pathogenic microorganisms found in their aquatic environments.

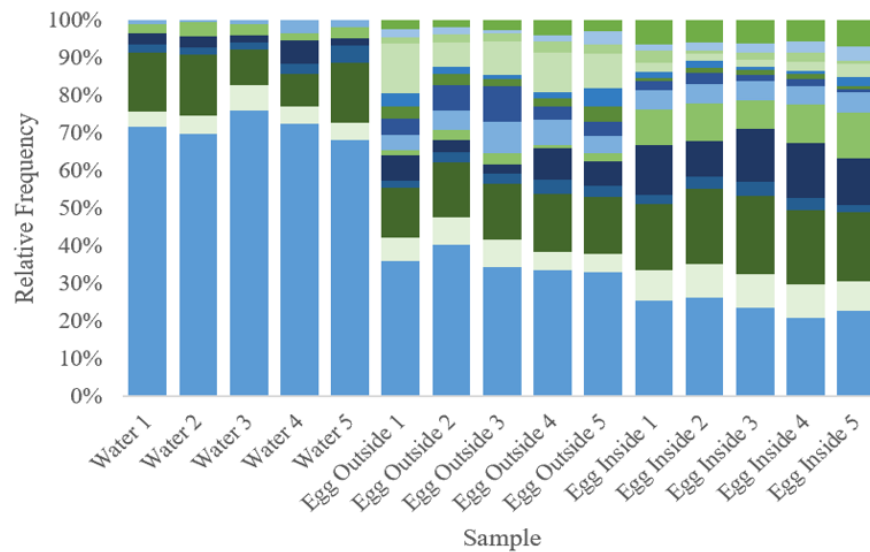


Figure 5. *Taxa Box Plot demonstrating expected taxonomic diversity.* It is expected that the egg surface samples will vary significantly from and demonstrate higher α -diversity than the water samples. While there is expected to be some taxonomic variety between the outside surface and inside surface of the egg masses, significant overlap is also expected in α -diversity.

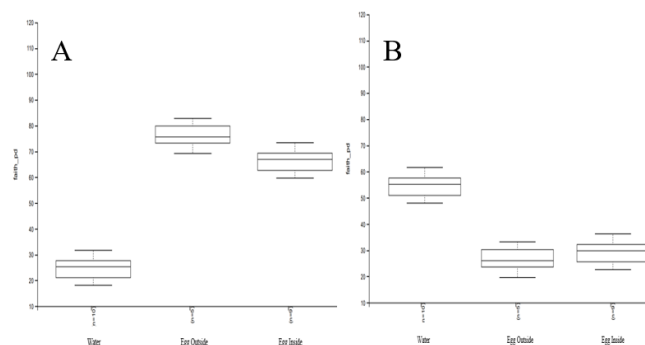


Figure 6. *Alpha Diversity Box Plots representing two possible diversity distributions.* It is expected that the egg mass samples will show significantly more α -diversity than the water sample, and that the outside surface will have slightly more diversity than the inside surface (A). Another possibility is that the water sample has more diversity than the egg samples, with the egg mass samples still showing similar species richness.

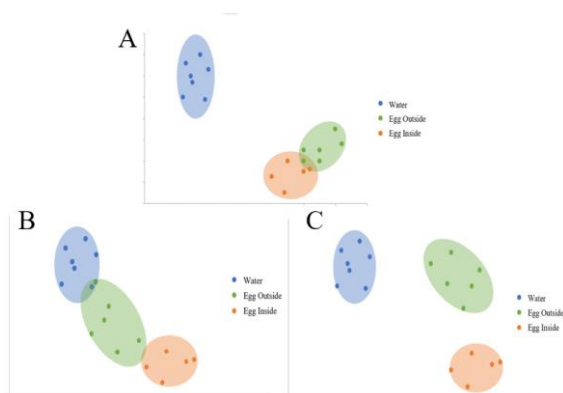


Figure 7. *PCoA plots representing 3 possible results.* It is expected that the microbiome on the outside surface and inside surface of the egg masses will overlap, while the water sample will be diverse with limited overlap with either egg samples (A). Another possibility is that the microbiome on the outside surface of the egg mass will overlap with both the environmental microbiome in the water and the inside surface microbiome in a transitional manner (B). It is also possible that all three sample types have unique microbiomes with limited overlap (C).

Materials and Methods

Microbial Swab Collection

In March 2021, *Ambystoma maculatum* egg masses were located anchored to plant matter, like branches and tall grasses, below the surface of Sheppe Pond in Snowden, VA. Upon arrival at the collection site, 15 environment swabs were taken of the water using aseptic technique. Each of the 24 egg masses were double rinsed with autoclaved RO water to remove transient bacteria, one swab was taken of the outside surface of the egg mass, and one swab was taken of the inside surface of the egg mass using aseptic technique. All collected swabs were stored at -20°C until DNA extraction took place.

Total Bacterial DNA Extraction

Total microbial DNA was extracted from the 63 swabs using the Qiagen DNeasy Blood and Tissue kit according to protocol. The extraction product was stored at -20°C until PCR took place.

Polymerase Chain Reaction (PCR)

Following extraction, unique forward primers were added to each sample and PCR was completed according to the Illumina MiSeq protocol to amplify the 16s gene. Each sample was run in singlicate with one negative control per seven samples. The thermocycler protocols were as follows:

1. 94°C for 3 minutes
2. 94°C for 45 seconds
3. 60°C for 1 minutes
4. 72°C for 45 seconds
5. Steps 2-4 were repeated until 40 cycles were completed.
6. 72°C for 5 min

After PCR was completed, if needed, samples were stored overnight at 4°C until gel electrophoresis was completed.

Gel Electrophoresis and Band Extraction

Gel electrophoresis was conducted on all PCR product to confirm DNA presence and check for contamination. PCR product was run through a 1.0% agarose gel containing 24 µL of ethidium bromide for 45 minutes at 200 V. A ChemiDoc Imager was used to visual the bands. DNA bands were then cut out of the gel, and the bands went through an agarose extraction and purification process using the Zymoclean Gel DNA Recovery Kit and protocol.

DNA Quantification and Pooling

In order to standardize the DNA concentration across all samples, DNA was quantified using the ThermoFisher Qubit™ protocol. Differing amounts of each sample were added to two pools so that 25 ng of DNA from each sample was present in both of the final pools. Once

combined, the DNA samples were cleaned using the Qiagen QIAquick PCR Purification Kit. One pool was sent for Illumina sequencing, while the other was reserved as a backup and stored at -20°C.

Data Analysis

The bioinformatics software QIIME2 was used to complete all statistical analysis according to the established protocol (Bolyen et al, 2019). A taxonomic bar plot was created to compare the relative frequency of each phylum, class, and order of bacteria for each of the samples. A Kruskal-Wallis test was used to assess the α -diversity of the three sample types to determine if there was statistical difference ($p < 0.05$). A permutational multivariate anova (PERMANOVA) test was also completed to compare the β -diversity between each sample to look for significant difference ($p < 0.001$) and a PCoA plot was created to visualize the difference.

Results

Taxonomic Bacterial Community Composition

The microbiome of the inside surface of egg mass samples, outside surface of egg mass samples, and environmental water samples showed significant overlap in bacteria present at the phylum, class, and order levels. A total of 59 phyla were identified across all samples: 43 were Bacteria, nine were Eukarya, six were Archaea, and one was unassigned. These phyla represented a total of 150 classes and 376 orders.

Bacterial Community at the Phylum Level

The 16 most abundant bacterial phyla were compared, and it was noted that all 16 were present in all sample types, even if they weren't in every sample (Figure 8). It was also noted that the relative frequency of each phylum varied between sample types (Figure 9). Only four phyla were present in the most abundance in all samples regardless of type: Proteobacteria,

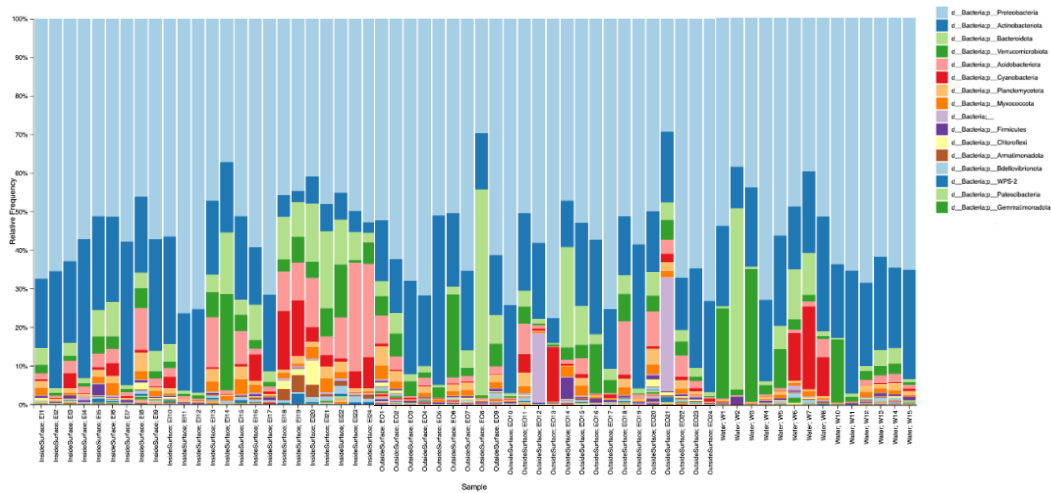


Figure 8. Taxonomic bar plots of bacteria species of each sample type at the phyla level. The 16 most common phyla are labeled and represent $\geq 1\%$ of at least one individual sample.

Actinobacteria, Bacteroidetes, and Verrucomicrobiota (Table 1). Of these, Proteobacteria and Actinobacteria were the most abundant phyla across all sample types, accounting for between 54.9–56.7% and 18.3–22.1%, respectively. A disproportionately high percentage of Acidobacteriota was noted in the inside surface of egg mass sample type, with 6.5% compared to 2.8% in the outside surface of egg mass sample type and 1.0% in the water sample type. It was also noted that the phyla Armatimonadota and Chloroflexi were the ninth and tenth most frequent bacterium in the inside surface samples but were not one of the most abundant for the other two sample types. The water samples also had a unique phylum that was not as abundant in the egg mass samples, which was Bdellovibrionota.

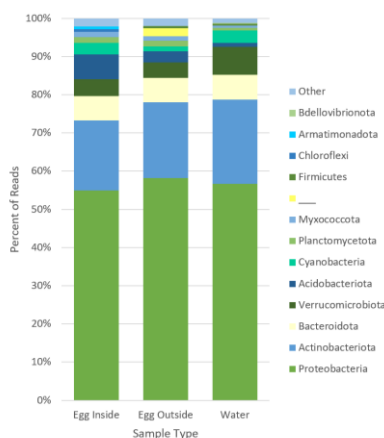


Figure 9. *Percent of reads (community) for each sample type.* The 16 most abundant phyla are shown, with all other phyla grouped together in the “Other” category.

Table 1. *Top 10 most abundant microbial phyla for each sample type.* While the top 10 for each sample is populated by mostly the same phyla, the order of prominence varies between sample type. The group denoted “___” found in the egg mass outside samples refers to an unrecognized phylum that is not present in the Silva database.

Top 10 Most Abundant Microbial Phyla (%)						
Egg Mass Inside			Egg Mass Outside		Water	
1	Proteobacteria	54.939	Proteobacteria	58.162	Proteobacteria	56.672
2	Actinobacteriota	18.284	Actinobacteriota	19.907	Actinobacteriota	22.053
3	Acidobacteriota	6.527	Bacteroidota	6.301	Verrucomicrobiota	7.337
4	Bacteroidota	6.438	Verrucomicrobiota	4.158	Bacteroidota	6.490
5	Verrucomicrobiota	4.375	Acidobacteriota	2.831	Cyanobacteria	3.219
6	Cyanobacteria	2.999	___	2.109	Acidobacteriota	1.029
7	Planctomycetota	1.512	Planctomycetota	1.443	Planctomycetota	0.707
8	Myxococcota	1.386	Cyanobacteria	1.314	Myxococcota	0.591
9	Armatimonadota	0.710	Myxococcota	1.158	Firmicutes	0.421
10	Chloroflexi	0.672	Firmicutes	0.645	Bdellovibrionota	0.303

Bacterial Community at the Class Level

Following phyla comparison, the 18 most abundant bacterial classes were compared, and all 18 were present in each sample type (Figure 10). Relative frequency varied more dramatically

at the class level than at the phylum level (Figure 11). The three most common classes were the same between the three sample types: Gammaproteobacteria, Actinobacteria, and Alphaproteobacteria (Table 2). More variations were noted between the inside egg mass sample, most notably being a higher percentage of Acidobacteria, Chlamydiae, Ktedonobacteria, Armatimonadia, WPS-2, and Phycisphaerae than in the outside egg mass samples or the water samples.

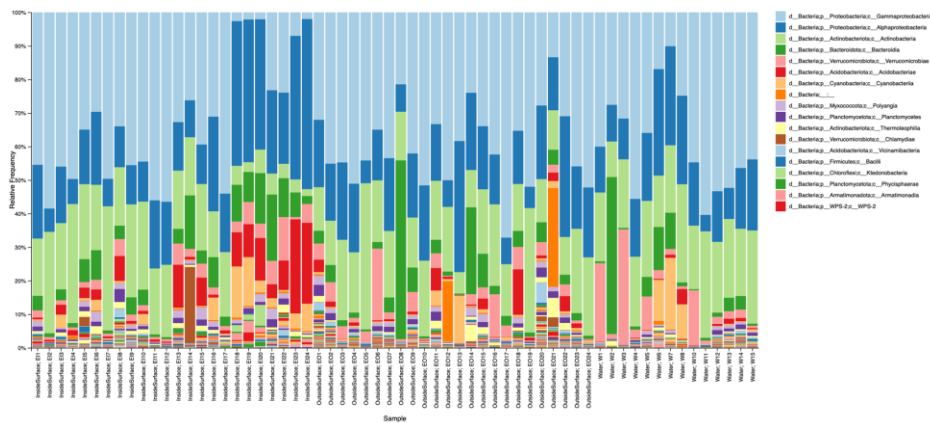


Figure 10. Taxonomic bar plots of bacteria species of each sample type at the class level. The 18 most common classes are labeled and represent $\geq 1\%$ of at least one individual sample.

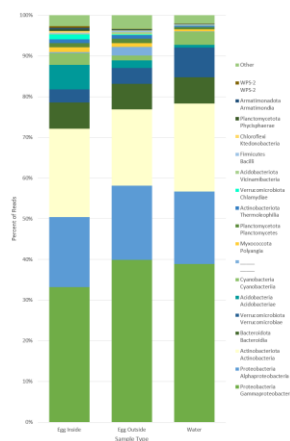


Figure 11. Percent of reads (community) for each sample type. The 18 most abundant classes are shown, with all other classes grouped together in the “Other” category.

Table 2. *Top 15 most abundant microbial class for each sample type.* While the top 15 for each sample is populated by mostly the same classes, the order of prominence varies between sample types. Some classes are unique, however, especially among the egg inside samples, such as Ktedonobacteria and WPS-2.

Top 15 Most Abundant Microbial Classes (%)						
	Egg Inside		Egg Outside		Water	
1	Gammaproteobacteria	33.207	Gammaproteobacteria	39.896	Gammaproteobacteria	38.838
2	Actinobacteria	21.722	Actinobacteria	18.770	Actinobacteria	21.621
3	Alphaproteobacteria	17.223	Alphaproteobacteria	18.247	Alphaproteobacteria	17.813
4	Bacteroidia	6.418	Bacteroidia	6.257	Verrucomicrobiae	7.274
5	Acidobacteriae	6.033	Verrucomicrobiae	3.919	Bacteroidia	6.468
6	Verrucomicrobiae	3.205	—	2.111	Cyanobacteriia	3.203
7	Cyanobacteriia	2.870	Acidobacteriae	1.816	Acidobacteriae	0.699
8	Polyangia	1.168	Cyanobacteriia	1.222	Planctomycetes	0.520
9	Chlamydiae	1.153	Planctomycetota	1.058	Polyangia	0.448
10	Planctomycetes	1.037	Polyangia	0.957	Thermoleophilia	0.318
11	Thermoleophilia	0.907	Thermoleophilia	0.871	Bacilli	0.238
12	Ktedonobacteria	0.458	Vicinamibacteria	0.602	—	0.160
13	Armatimondia	0.399	Bacilli	0.336	Vicinamibacteria	0.153
14	WPS-2	0.372	Phycisphaerae	0.221	Phycisphaerae	0.088
15	Phycisphaerae	0.354	Chlamydiae	0.181	Armatimondia	0.058

Bacterial Community at the Order Level

Analysis was also completed on bacterial orders, allowing a comparison to be made using the 27 most abundant orders (Figure 12). Even more variance in relative frequency of bacterial orders was noted than at the phylum or order level (Figure 13). The inside egg mass had more bacterial diversity, being composed of smaller percentages of a wider range of orders when compared to the outside egg mass and the water samples. The four most common orders were the

same between all three sample types, composed of Micrococcales, Pseudomonadales, Rhizobiales, and Enterobacterales (Table 3). After this, greater bacterial frequency and diversity was noticed between sample types. The inside egg mass samples were composed of a significantly higher portion of Acetobacterales, Acidobacteriales, Chthoniobacterales, Chlamydiales, and Caulobacterales when compared to the egg mass outside and water samples. This divergence at the order level suggests that the bacterial communities between these samples are unique, although there is substantial overlap.

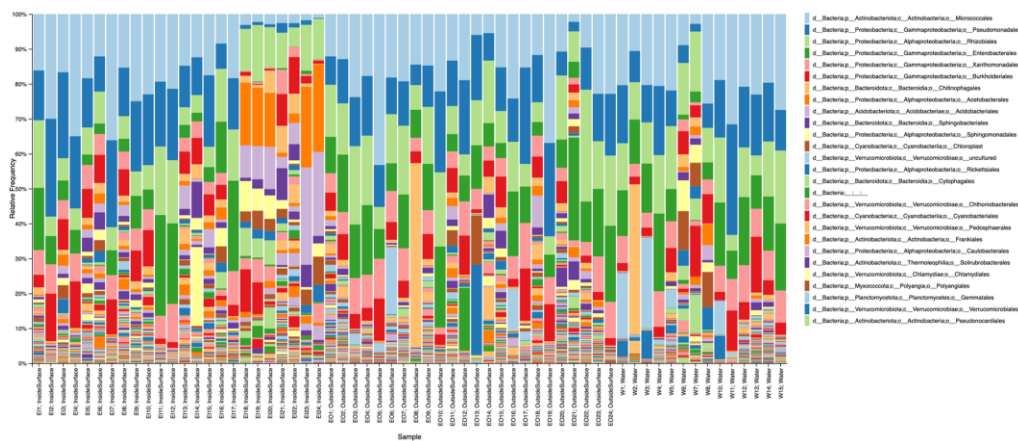


Figure 12. Taxonomic bar plots of bacteria species of each sample type at the order level. The 27 most common orders are labeled and represent $\geq 1\%$ of at least one individual sample.



Figure 13. Percent of reads (community) for each sample type. The 27 most abundant orders are shown, with all other orders grouped together in the “Other” category.

Table 3. *Top 20 most abundant microbial orders for each sample type.* While the top 20 for each sample is populated by mostly the same order, the proportion of each order varies between sample types. Some orders are unique, however, especially among the egg inside samples, such as Caulobacteriales and Chlamydiales.

Top 20 Most Abundant Microbial Orders (%)						
	Egg Inside		Egg Outside		Water	
1	Micrococcales	14.849	Micrococcales	17.248	Micrococcales	19.471
2	Pseudomonadales	12.660	Pseudomonadales	13.956	Pseudomonadales	13.725
3	Rhizobiales	10.189	Rhizobiales	13.936	Rhizobiales	13.705
4	Enterobacteriales	6.893	Enterobacteriales	11.658	Enterobacteriales	12.231
5	Acetobacteriales	5.085	Xanthomonadales	6.940	Xanthomonadales	6.742
6	Acidobacteriales	5.030	Burkholderiales	5.765	Burkholderiales	5.580
7	Xanthomonadales	5.000	Chitinophagales	3.320	uncultured Verrucomicrobiae	4.522
8	Burkholderiales	3.821	_____	2.111	Chitinophagales	3.942
9	Sphingobacteriales	2.986	uncultured Verrucomicrobiae	1.714	Chloroplast	2.091
10	Sphingomonadales	2.168	Rickettsiales	1.613	Verrucomicrobiales	1.748
11	Chitinophagales	2.116	Sphingobacteriales	1.218	Sphingomonadales	1.424
12	Chloroplast	1.803	Chloroplast	1.197	Pseudonocardiales	1.118
13	Chthoniobacteriales	1.657	Acidobacteriales	1.094	Cyanobacteriales	1.111
14	Rickettsiales	1.475	Pedosphaerales	0.946	Rickettsiales	1.073
15	Cytophagales	1.414	Sphingomonadales	0.718	Cytophagales	1.001
16	Chlamydiales	1.152	Solirubrobacteriales	0.681	Acetobacteriales	0.829
17	Caulobacteriales	1.049	Cytophagales	0.673	Sphingobacteriales	0.783
18	Cyanobacteriales	1.048	Chthoniobacteriales	0.640	Acidobacteriales	0.548
19	Frankiales	0.921	Gemmatales	0.589	Pedosphaerales	0.398
20	Pedosphaerales	0.820	Frankiales	0.492	Chthoniobacteriales	0.354

Alpha Diversity of Microbial Assemblages

To access the α -diversity between the three sample types, a Kruskal-Wallis test was performed and accessed with a p-value of 0.05. Several noteworthy results were observed in this comparison of the α -diversity of the different sample types, providing insight into the species richness. Most notably, there was a statistically significant difference between the α -diversity of the inside surface of the egg mass and the water samples, with a p-value of 0.011023 (Figure 14).

The inside surface and outside surface of the egg mass, and the outside surface of the egg mass and the water were not significantly different, with p-values of 0.409493 and 0.084546, respectively.

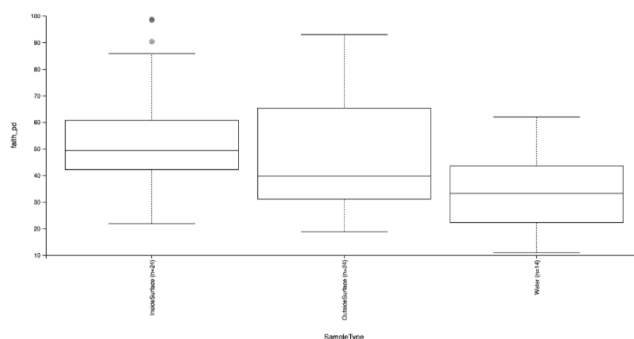


Figure 14. *Box-and-Whisker Plot comparing α -diversity between egg masses samples and environment samples.* Statistical significance was defined by $p \leq 0.05$. Significant difference was noted between the egg mass inside samples and water samples ($p = 0.011023$). There was not significant difference between the egg mass outside and egg mass inside ($p = 0.409493$) or the egg mass outside and water ($p = 0.084546$).

Beta Diversity of Bacterial Composition

To assess beta-diversity, a PERMANOVA test was run based on Jaccard analysis, which does not account for taxonomical abundance. An analysis of beta-diversity found similar results as the α -diversity assessment. The difference between the inside surface of the egg mass and the water sample was statistically significant, with a p-value of 0.001 (Figure 15), as was the comparison between the inside surface and outside surface, with a p-value of 0.002. The comparison between the outside surface and water did not find a statistically significant difference in beta diversity, with a p-value of 0.082. A PCoA plot (Figure 16) was used to visualize the comparison between beta diversity, and while there was significant overlap between sample types, each type resided in a clear region of the plot, suggesting defined, overlapping communities.

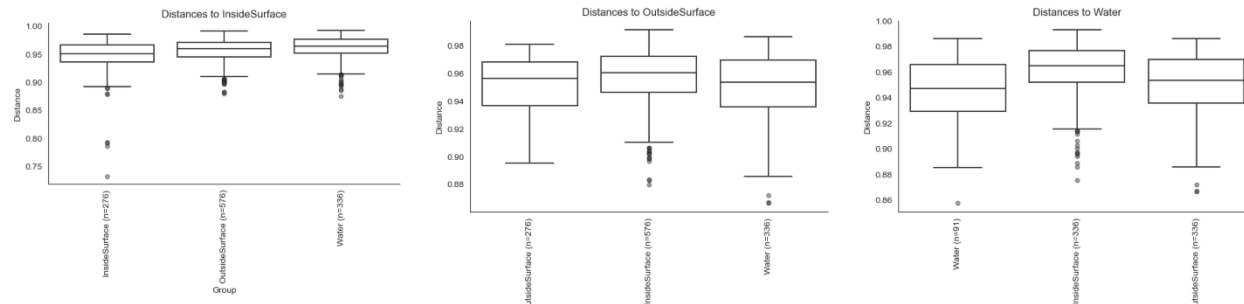


Figure 15. Box-and-Whisker Plot comparing beta diversity between the microbiome composition for each sample type.. The PERMANOVA based on Jaccard analysis/indices showed significant differences in the microbiomes of the inside egg mass in comparison with the outside egg mass surface ($P=0.002$) and water environment ($P=0.001$). However, the outside egg mass surfaces were not significantly different from the surrounding water environment microbiome ($P=0.082$).

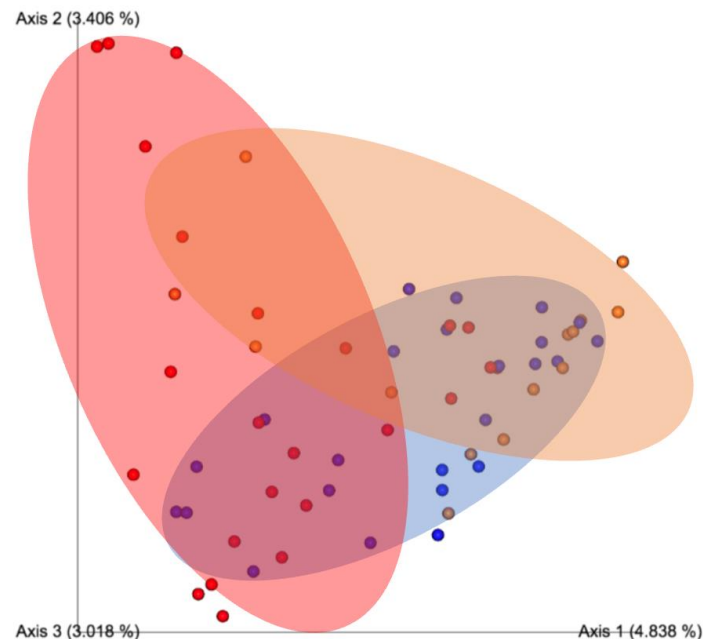


Figure 16. PCoA plot comparing microbiomes of sample types using the Jaccard analysis. Red represents the inside of the egg mass, blue represents the outside of the egg mass, and orange represents the water. While there is clear overlap among all three sample types, suggesting similarity in beta diversity, each sample type resides in a clear region of the plot, suggesting defined communities with some overlap.

Discussion

The purpose of this study was to explore the microbiome of the inside surface and outside surface of *Ambystoma maculatum* egg masses and to compare their diversities with each other

and the surrounding environment. Of the relationships that were considered, only the comparison between the inside surface of the egg mass and the water were found to be significantly different. This supported the hypothesis in part, as it had been predicted that both the inside and outside would differ significantly from the water rather than only the inside.

Bacterial Species

As observed in Figures 8 & 9, and in Table 1, the most abundant bacteria phylum across all sample types was Proteobacteria. Currently, Proteobacteria are the largest phylum within the bacterial domain, and most are Gram negative with heavy lipopolysaccharide presence in the outer membrane (Rizzatti et al., 2017). Proteobacteria are incredibly diverse and can be found in a variety of environments. Some Proteobacteria have already been shown as defensive against amphibian pathogens. A betaproteobacterium known as *Janthinobacterium lividum* produces violacein, an antifungal metabolite that is effective at combating *Bd* (Becker et al., 2009). Due to its abundance across a variety of environments and species, as well as its domination in the bacterial domain, it is not surprising that it would occur with the largest abundance across both egg mass and water samples.

The second most abundant bacteria phylum that was observed in all samples was Actinobacteria. Most Actinobacteria are free-living species found in both terrestrial and aquatic ecosystems (Barka et al., 2015). In these ecosystems, Actinobacteria are crucial for their role in recycling biomaterials, contributing to the refreshing of nutrients. Actinobacteria are also known for their production of bioactive compounds and antibiotics, which can inhibit pathogens (Barka et al., 2015). Given the abundance of this phylum in aquatic environments and the essential role it plays in the recycling of nutrients to maintain a healthy ecosystem, it is not surprising that

Actinobacteria would be found in such abundance and consistency across both egg mass samples and water samples.

Bacteroidota was the third most abundant bacteria phylum on the outside surface of the egg masses, and the fourth most abundant on the inside surface of the egg masses and in the water. Recognized as a marine species, Bacteroidetes are also found in other aquatic habitats but can also be terrestrial (Fernandez-Gomez et al., 2013). Most abundant during and following algal blooms, this phylum has been observed as preferring to grow attached to particles, surfaces, and algal cells, and is recognized as important processors of organic matter. Given its affinity for attaching to particles, it is not surprising that Bacteroidetes would compose a greater relative portion of the outside surface of the egg masses, as it would likely seek to anchor itself to the egg mass. The abundance of this phylum in the water is likely due to algal cells present in the stagnant pool, providing another anchoring point for the bacteria.

The fourth most abundant bacteria phylum, Verrucomicrobiota, was the last of the four phyla found in every sample, regardless of sample type. It was the third, fourth, and fifth most abundant phylum in the water, outside surface, and inside surface samples, respectively. Verrucomicrobiota is recognized as a phylum predominately found in soil, although it can be found in almost all aquatic environments, albeit in low levels (Freitas et al., 2012). Interestingly, this phylum occurred with relatively more abundance in the water samples than in the egg mass samples. As a primarily terrestrial microbe, it would have been expected to see higher levels of it on the eggs, suggesting vertical transfer from the terrestrial microbiome of the adult salamanders. Instead, a higher concentration of Verrucomicrobiota is found in the water, suggesting that the phylum is able to thrive in the stagnant water better than on the surface of the egg masses.

Among the most abundant bacterial phyla that were observed, several were found in higher abundance in the inside surface samples than in the other samples. Acidobacteriota is an incredibly abundant and diverse phylum, with an especially impressive range of soil bacteria, where a vast majority of the bacteria composing this phylum thrive. Despite their abundance, however, not much is understood about the Actinobacteria phylum because of difficulty in lab cultivation (Kielak et al., 2016). This lack of understanding is especially prominent in the few bacteria within the phylum found in aquatic environments, as very little exploration of their traits has occurred (Kristensen et al., 2021). Acidobacteriota was third most abundant phylum for the inside surface of the egg mass samples and was only the fifth and sixth most abundant for the outside surface and water samples, respectively. It is intriguing that a phylum so abundant in the soil would be so prominent on the inside of the egg mass, but not nearly as prominent on the outside surface or in the environment. It is possible that this phylum is transferred vertically from parent to offspring, explaining the higher rates in the isolated environment inside of the egg, and does not survive as well in the aquatic environment, explaining the decrease in prominence on the outside surface, and then in the water itself.

Two additional phyla found in higher abundance in the inside surface samples were Armatimonadota and Chloroflexi. Armatimonadota is a phylum composed of both terrestrial and aquatic bacteria in a variety of environments, including thermophilic environments such as geothermal soil and hot springs (Tamaki et al., 2011). Only three strains of this phylum have been cultured and all three are motile Gram-negative bacteria that produce pinkish pigments and can degrade polysaccharides. The phylum Chloroflexi is abundant in both freshwater and marine environments, composing up to 26% of the community in specific instances, and yet has remained mostly uncultivated and uncharacterized (Mehrshad et al, 2018). Chloroflexi is

suspected to have started as a soil/sediment phylum that has shifted to aquatic life, with higher diversity in freshwater than in brackish or marine habitats. It is believed that Chloroflexi plays an important role in demineralizing nitrogenous organic matter (Mehrshad et al., 2018). The Armatimonadota and Chloroflexi phyla were the ninth and tenth most abundant phyla in the inside surface samples, and were not in the top ten most abundant phyla for either the outside surface or water samples, although all three sample types did contain samples with these phyla. Since neither phyla is well understood, future research exploring the unique attributes of these bacteria could provide fascinating insight into some of the differences between the isolated environment contained inside of the egg mass and the external environment in which the eggs reside.

Just as the inside surface of the egg mass samples had a few unique phyla contained within the ten most abundant phyla, so too did the water samples. In the water samples, the phylum Bdellovibrionota was the tenth most abundant, but did not appear in the ten most abundant for either egg mass sample type. Bdellovibrionota is a phylum of Gram-negative predators often found in marine environments (Qing-Mei et al., 2021). Bdellovibrionota can live a prey-dependent or prey-independent lifestyle depending on environment and has been shown to be useful as an agent against bacterial pathogens. The increased abundance of Bdellovibrionota in the water samples compared to the egg mass samples could be symptomatic of the predatory nature of this phylum, as it likely thrives best as a motile hunting organism rather than colonizing a surface and hunting in that more limited environment.

At the class level, a higher abundance of Acidobacteriae, Chlamydiae, Ktedonobacteria, Armatimonadia, WPS-2, and Phycisphaerae were observed in the inside egg mass samples when compared to both the outside egg mass samples and the water samples. Ktedonobacteria is a

class known for terrestrial microbes, often found in forests and sediments, and WPS-2, also known as *Eremiobacterota*, is an acid-tolerant soil microbe; the presence of both classes could be indicative of vertical transfer from terrestrial adults (Yabe et al., 2017; Ji et al., 2021). Notably, genomic study of the *Ktedonobacteria* has found a variety of novel and diverse bioactive compounds with unknown functions, which may possess antimicrobial or antifungal qualities that could be contributing to the protection of these amphibian eggs against heterotrophic aquatic microorganisms (Zheng et al., 2019).

At the order level, a higher portion of *Acetobacterales*, *Acidobacteriales*, *Chthoniobacterales*, *Chlamydiales*, and *Caulobacterales* were observed in the inside egg mass samples than in the outside surface samples. *Chlamydiales* is common in association with fish and arthropod hosts, and *Caulobacterales* can often be found in nutrient-rich waters and soils, such as brackish ponds, and relies on surface adhesion (Pillonel et al., 2015; Chepkwony et al., 2019). These two orders consist of bacteria that demonstrate a preference for adhesion or host interaction, which could explain why these orders are found in higher frequency in the egg samples than in the water. *Chthoniobacterales* is a soil microbe with a known endosymbiotic relationship with species of nematodes (Janssen & Hedlund, 2011). This particular order is transmitted maternally in nematode species and could easily have been acquired by the egg masses in a similar vertical transmission.

Alpha and Beta Diversity

The Kruskal-Wallis test, which assesses for α -diversity, found that the inside surface of the egg mass was significantly different from the water samples but not from the outside surface of the egg mass, with p-values of 0.011023 and 0.409493, respectively. This result was expected, as the internal environment created within the egg mass is protected from the outside

environment, which could prevent microbes found in the water from colonizing the internal surface of the egg masses. The outside surface of the egg mass bore more similarity to the inside with a substantially higher p-value, indicating more overlap in the species richness of both egg mass sample types. This is likely due to inherit uniformities in the microbiomes of the eggs from when they are laid, which could be explained by retained microbes from the parental microbiome, a phenomenon which has been observed in terrestrial salamanders and glassfrogs (Banning et al., 2008; Hughey et al, 2017). The outside surface of the egg mass did not differ significantly from the water samples, with a p-value of 0.084546. This is likely due to the fact that the outside surface of *A. maculatum* egg masses remain in constant exposure to their water environment, allowing opportunity for microbes found within the water to colonize the external surface of the egg mass. However, it was noted that the p-value of 0.084546 was relatively close to 0.05 than the p-value representing the difference between the inside and outside surfaces, which was 0.409493. This suggests that while the difference between the water sample and outside surface sample was not significant, the α -diversity of the outside surface of the egg mass was more similar to that of the inside surface of the egg mass than the water in which the egg masses are found. This supports the idea that while the environment has some influence on the α -diversity of the outside surface of the egg masses, the egg masses are still able to maintain a vertically acquired microbiome that is unique in its species richness.

Similar results were observed in the PERMANOVA test for beta-diversity. As with the α -diversity test, the inside surface of the egg mass was significantly different from the water environment (p-value = 0.001) and the outside surface of the egg mass (p-value = 0.002). This indicated that the inside surface of the egg mass varied significantly from the water and the outside of the egg mass not only in the number of bacteria (α -diversity), but also in the types of

bacteria (beta-diversity). Once again, the outside surface of the egg mass did not have a statistically significant difference when compared to the beta-diversity of the water samples, with a p-value of 0.082. This indicated that the beta-diversity of the outside surface bears more similarity to that of the water than to the inside surface, suggesting that the external microbiome on the surface of the egg mass shared more similarity with its environment than with the inside of the egg mass, which is protected from the environment. The unique microbiome of the internal surface was likely acquired vertically or from interactions with other organisms (Banning et al., 2008; Hughey et al, 2017). The PCoA plot (Figure 16) provides a visualization of the beta diversity, demonstrating that while there is significant overlap in the microbes composing each community, aspects of these communities are still distinct.

Limitations

A number of limitations were associated with this study. First, samples were only collected from a single pond, so while the results are assumed to be indicative of the population, this has not been confirmed by using other sample collecting sites. Second, all samples were collected at a single point in time. While many observations can be derived from a snapshot of the microbiome found on the surfaces of *A. maculatum* egg masses, any progressive microbial changes that occur as the egg masses remain in the water would not have been recorded. As this study was not a longitudinal study, further research would be needed to examine changes throughout larval development.

Future Research

Further research would be incredibly beneficial to expand upon the conclusions of this study. Future studies that expanded the sample pool and examined the microbiome of more egg masses from a variety of collection sites would confirm that the results of this study were

indicative of the entire population. An additional study could also collect microbial swabs at different times to see if the outside surface microbiome continues to shift and become more like that of its environment over time. Longitudinal studies examining the change of egg microbiome over time have occurred for reptile eggs, such as *Sceloporus virgatus*, the striped plateau lizard, which showed that vertically transferred microbes were retained at high levels 25 days after they were laid, but while amphibian egg studies often include eggs at varying stages of development, an intentional longitudinal study comparing the same eggs at two points in time has not been performed (Bunker et al., 2021; Prest et al., 2018).

Future research could also expand on the discoveries of this study to further explore the features of amphibian eggs that protect them from potential pathogens in their environments. Further studies could explore the specific microbes that were observed on both the external and internal surfaces of the egg masses to see which specific microbes might be contributing to defensive responses. Another avenue of study could be the chemical analysis of the egg jelly to see if defensive substances can be identified as components of the jelly. Additional research can also investigate the eggs of other amphibian species to see if antimicrobial characteristics are observed in the microbiome. Some research has been done on investigating the adult microbiome of *Ambystoma maculatum* and *Ambystoma mexicanum*, but more can be discovered, especially in regards to the egg microbiome (Stevens, 2021; Demircan et al., 2018).

Conclusion

Microbiomes are of increasing interest to the scientific community, as their study has led to various discoveries in the fields of ecology, conservation, and medicine. Amphibian egg masses, which reside in a hostile environment filled with potential pathogens, exhibit a natural resistance to the invasion of that bacteria that may be attributed to the unique features of the

microbiome of the egg mass. Through the completion of molecular techniques and bioinformatic analysis, bacterial DNA was successfully sequenced and analyzed to provide insight into the microbial differences between the internal surface of egg masses, external surface of egg masses, and the water in which they reside. The results of this study indicate that the internal microbiome of the egg mass is significantly different from the water in which it resides, but that the external microbiome is not significantly different from either the internal microbiome or the microbiome found in the water. This indicates that the internal environment is unique, hosting different microbes than the water around it, which were likely acquired from parental exposure and maintained in the protection provided by its internal location. The outside surface, exposed to the aquatic microbes abundant in the water, maintains some of its unique microbiome that it shares with the internal surface, but also acquired some of its microbiome from the water that surrounds it. Additional research should explore these microbial differences to verify whether the unique microbiome maintained by the internal surface of the egg mass contributes to the defensive features of the egg mass that prevent bacterial invasion.

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