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An analysis of prevalence of chytrid fungus in an amphibian assemblage in Middle Tennessee

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Abstract

Chytridiomycosis is an infectious, fungal disease largely seen in amphibians, which is caused by the highly virulent, zoosporic, pathogenic, single-celled fungus *Batrachochytrium dendrobatidis (Bd)*. It is known to cause epidermal hyperplasia, hyperkeratosis, skin ulcerations, and fatalities by asystolic cardiac arrest either from shifts in electrolytes or increased acidity in the blood plasma. Previous research has demonstrated that urban water bodies have a higher prevalence of chytrid fungus than rural water bodies. Researchers have also found that chytrid is more prevalent in open canopy habitats than closed canopy habitats. Furthermore, it is implicated in global population declines and local extinctions in which one-third of extant amphibian species are currently threatened with extinction. This suggests that there is a need for further research into the prevalence of *Bd* and the environmental conditions in which it thrives. I sampled 72 amphibians from four urban and four rural watercourses situated in Davidson and Sumner County in Middle Tennessee. All of the 72 captured amphibians were swabbed for the presence of *Bd*. DNA was extracted using Qiagen DNeasy Blood and Tissue Kits and assayed by PCR in triplicate. Four of out of the 72 sampled amphibians tested positive for the presence of *Bd*. This project provides empirical evidence for the presence of *Bd* in Middle Tennessee, which will aid wildlife and land managers in making adaptive conservation decisions that will better protect amphibians in this region from the foremost threat to amphibian diversity.

Dedication

This thesis is dedicated to two incredibly influential mentors and personal role models, my brother, Jonathan Brocco, and my advisor, Dr. Thomas P. Wilson. My brother inspired my passion for academic involvement and encouraged me to pursue research in the first place alongside being my closest friend. I truly would not be where I am today without him. Dr. Wilson has been a dream to work with. He took me under his wing and taught me more than all the classes I have ever taken at UTC combined. I am forever grateful for his patient, kind guidance. I will never forget the sacrifice, time, and effort he put in to making me a better person and professional.

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Chapter One

I. Introduction

Fungal disease is a global threat to various vertebrate taxa. From mammals to fish and amphibians, populations are experiencing declines from infections like snake fungal disease, white nose syndrome in bats, water molds in fish and amphibians, and chytridiomycosis in amphibians. The causative agents of these diseases are *Ophidiomyces ophiodiicola*, *Pseudogymnoascus destructans*, *Saproglenia sp.*, and *Batrachochytrium dendrobatidis* or *salamandrivorans*, respectively (Hoyt et al. 2017; Martel et al. 2014; Romansic et al. 2009; Tetzlaff, et al. 2015; Voyles et al. 2009). All of these fungal diseases are responsible for extirpations, extinctions, and population declines in vertebrate animals. As such, it is imperative to prevent the spread of and mitigate the impact of these diseases.

Perhaps, the most catastrophic of the aforementioned fungi is *Batrachochytrium dendrobatidis* (*Bd*): a pathogenic, highly virulent, zoosporic, single-celled fungus that is directly responsible for the amphibian fungal disease chytridiomycosis (Voyles et al. 2009). *Bd* is classified in the Phylum Chytridiomycota, Class Chytridiomycetes, and Order Rhyzophidiales (Van Rooij, Martel, Haesebrouck, and Pasmans 2015). Members of the Chytridiomycota, also known as Chytrids, are asexual, unicellular, unwalled spores that swim by undulating a single posterior flagellum (Longcore and Simmons 2012). While *Bd* originally belonged to the Family Chytridiales, it differs morphologically in that its microtubule root runs parallel to the kinetosome, or basal protein structure of the flagellum, into the aggregation of ribosomes, and it is now unclassified at the Family level (Longcore, et al. 1999; Van Rooij et al. 2015). Furthermore, this species of chytrid fungus is differentiated from other members of its genus

indicated by differences in occurrence in Anurans and small subunit-ribosomal DNA sequence, and it is the first of its genus that has been found to inhabit a vertebrate host (Berger, et al. 2005; Longcore et al. 1999).

The life cycle of *Bd* has two stages: a flagellated, mobile, unwalled, aquatic zoospore stage and an encysted thalli stage. Chytrid zoospores range from 3-5 microns in diameter and possess a flagellum that is approximately 19-20 microns in length (Berger et al. 2005). Once these zoospores have located their amphibian host via chemotaxis, they encyst in the epidermis of the host and begin producing the spherical chytrid thallus, which is typically 7-15 μm in diameter (Longcore et al 1999; Van Rooij et al. 2015). The thallus and the zoosporangium, or swollen section of the thallus that contains fully formed zoospores, are responsible for dispersal of zoospores via discharge papillae that protrude out of the zoosporangium (Berger et al. 2005; Longcore et al. 1999) Thalli can exhibit two modes of development. They can cleave and mitotically divide to have multiple sporangia on one thallus with multiple discharge tubes, and this type of development is termed “colonial growth”. The alternative is monocentric growth; wherein, one thallus forms one zoosporangium with one discharge tube (Berger et al. 2005). Monocentric growth is much more common in *Bd* than colonial growth (Berger et al. 2005). Through this life cycle, *Bd* is able to spread like wildfire through a watercourse and infect a large number of amphibians, both adult and larvae. However, the chance of being infected is positively correlated with age because as the individual traverses more of the watercourse, they have a higher chance of picking up zoospores on their epidermis and becoming infected (Thomas P. Wilson, Personal Communication).

Chytridiomycosis is an infectious disease in amphibians caused by *Bd* and is implicated in global population declines, extirpations, and extinctions. *Bd* has been detected in at least 520

species of frogs, salamanders, and caecilians, and approximately 435 species of amphibians have experienced population declines since 1980 (Skerrat et al. 2007; Van Rooij et al. 2015). Factors such as habitat degradation and fragmentation, and exploitation like the pet trade and improper biosecurity practices are significant pressures on amphibian populations as well (Saenz et al. 2015). Nonetheless, it is apparent that chytridiomycosis spread through introduction of exotic species carrying *Bd* spores and humans tracking *Bd* itself through carelessness regarding biosecurity is the most significant factor in the unprecedented global amphibian population declines being observed. This is supported by the presence of the disease during population declines, and the fact that the physiological symptoms largely indicate the fatalities are due to the pathogen. The pathogen is spread in the aforementioned manner and exacerbated by environmental aberrations like climate change, UV radiation, and pollution (Berger et al. 1998; Skerrat et al. 2007). These three factors are worsening on a global scale, so it is increasingly more important to discover where *Bd* is prevalent to mitigate its impact and prevent amphibian die offs in the future.

Bd encysts in the keratinized skin cells of amphibians and is known to be more pathogenic to frogs when compared to other amphibians. However, many caudates have also tested positive. Spread of the disease is confounded and exacerbated by the fact that non-amphibian hosts, even invertebrates, can serve as a vector for zoospores without actually succumbing to infection (McMahon et al. 2013). McMahon et al. (2013) study on crayfish as vectors for *Bd* is of note because it shows that even an invertebrate can transfer zoospores, but non-amphibian vectors are not restricted to just crayfish. Other taxa including lizards, snakes, and fish serve as vectors for *Bd* zoospores. Kilburn, et al. (2011) found *Bd* spores on the skin of 38 *Anolis* lizards out of the 211 they swabbed. Spores were present on three of the eight surveyed

snakes as well. Furthermore, a recent study by Liew et al. (2017) discovered that a non-amphibian host can even be parasitized by *Bd*. Whereas most non-amphibian hosts are typically asymptomatic, the zebrafish in this study displayed fin erosion, cell apoptosis, and muscle degeneration, and the researchers state that these symptoms are a direct result of chytridiomycosis caused by *Bd* (Liew et al. 2017). This reinforces the idea that *Bd* is widespread, detrimental to aquatic and semiaquatic life, and needs to be studied now before the spores become globally ubiquitous.

The disease manifests itself in certain cases as an abnormal increase in the number of epidermal cells, an increase in thickness of the epidermis in some areas, thinning in some areas, and skin ulcerations (Berger et al. 2005; Gray et al. 2015). Research suggests this is because the sporangia infect the keratinized cells of the outer layers, the stratum corneum and stratum granulosum. Interestingly, immature sporangia reside in the more internal layer, the stratum granulosum and move to the stratum corneum only upon maturity, which could potentially be a factor in why *Bd* thrives in such varying conditions and climates because of this buffer to the harsh elements of the environment (Berger et al. 2005). It has been found that the pathogenicity and virulence of *Bd* varies with the strain and the host species (Van Rooij et al. 2015). This is attributed to the fact that *Bd*, which as far as we know from museum specimens has existed and infected vertebrates since 1861, has had sufficient time for coevolution with hosts (Van Rooij et al. 2015). In specialized cases, some species are resistant to their endemic strain of *Bd*, but introduction of an invasive or exotic species may introduce a foreign strain of *Bd* that brings with it a suite of new fungus-host interactions. This foreign strain of *Bd* may cause a large-scale die off in its new host population. The origin of *Bd* is unknown, and more recent studies have

debunked the previously accepted notion that it originated in Africa (Pers. Comm. J. Whitfield Gibbons 2017; Van Rooij et al. 2015).

While the exact mechanism by which *Bd* kills is unknown, it appears that it causes mortality by disrupting the osmoregulation of amphibian skin, which leads to an imbalance of electrolytes and stops the heart (Berger et al 2005; Voyles et al. 2009). This is supported by a study in which afflicted green tree frogs' cardiac electrical activity indicated that they perished from asystolic cardiac arrest either from shifts in electrolytes or increased acidity in the blood plasma (Voyles et al., 2009). Berger et al. (2005) also hypothesized that proteolytic enzymes released by chytrid and absorbed by amphibian skin could play a role in a superficially located disease causing mortality. Furthermore, the time to death from the point of infection and the mortality rate varies based on age class, zoosporic infection load, and temperature (Berger et al. 2005). Based on the severity of the aforementioned symptoms and the global amphibian population declines, it is evident that there is a need for further research into the factors that affect the prevalence of *Bd.*

Amphibian conservation research is paramount to preserving extant biodiversity. Wake and Vredenburg (2008) detail the significance of amphibian research in preventing the sixth mass extinction that many scientists agree humans are driving the planet into via direct and indirect detriments to the environment. Amphibians are currently the only at risk group, which is demonstrated by the fact that one-third of the extant amphibian species are threatened with extinction. However, this does not mean other taxa are not in danger. Amphibians are the first to experience large-scale die offs because of their sensitivity to ambient change. Chytridiomycosis is a significant factor in recent, rapid global amphibian declines. Additionally, amphibians serve

as a bioindicator, which implicates that this research is a significant piece of understanding the sixth great mass extinction (Dodd, 2010).

Urban pools have been compared to rural pools to examine the hydraulic composition, microbiotic community structure, and prevalence of *Bd* in each habitat (Shoffner and Royall, 2008). Pauza and Driessen (2008) found that *Bd* is more prevalent in urban pools than in rural pools. Specifically, the data showed a strong correlation between the presence of gravel roads and the presence of chytrid. Saenz et al. (2015) supported this idea with a comparison of prevalence between urban and rural sites within *Pseudacris crucifer*. These data suggest that urban pools have proportionately more individuals afflicted with chytridiomycosis. Logically, it follows that urban, impacted pools with large amounts of development nearby might have more chytrid than rural, non-impacted pools (Shoffner and Royal, 2008). Prevalence of chytrid in urban versus rural watercourses is one question that will be investigated. Other factors that affect or are affected by *Bd* will be examined.

Canopy structure may affect the prevalence of chytrid in the area. Beyer et al. (2015) hypothesized that chytrid would be more prevalent in a closed canopy habitat, because high canopy cover causes temperatures to be lower which prevents *Bd* from reaching a critical maximum temperature, which would kill the zoospores. Their findings supported the hypothesis that chytrid was positively correlated with canopy cover. Becker, et al. (2012) also hypothesized that chytrid would be more prevalent in a closed canopy environment, and they also found that *Bd* prevalence increased with canopy density. This makes sense considering the fact that the survival of *Batrachochytrium sp.* is highly dependent on temperature. In a laboratory setting, optimal growth rates for *Bd* occur between 17-25°C, temperatures >28°C will cause growth to cease, and spores will die with exposure to 37°C for longer than 4 hours (Van Rooij et al. 2015).

The provided background information leads me to two hypotheses that will be the primary focus of this investigation into the prevalence of *Bd* in the greater Nashville Area, an understudied region.

This research will elucidate the prevalence of *Bd* in populations of amphibians in the greater Nashville area, specifically Davidson and Sumner County. According to the Tennessee Herpetological Society, Tennessee's amphibian diversity is the 4th highest in the nation (Powers et al. 2008); while, the bordering states of North Carolina, Georgia and Virginia rank 1st, 2nd, and 3rd, respectively (Powers, et al. 2008). Indeed, Tennessee and the Southeastern United states are ideal locations to conduct research on *Bd* due to the immense amphibian diversity. However, there is a lack of data from Middle Tennessee regarding the prevalence of *Bd*. Hence, Middle Tennessee is an ideal location to elucidate the prevalence and distribution of *Bd* in this region.

Hypothesis 1. I hypothesize that potentially impacted urban sites will have a higher prevalence of chytrid than potentially non-impacted rural sites.

Hypothesis 2. I expect to see a positive relationship between canopy structure and the prevalence of chytrid within and across waterbodies.

II. Methods

Ethics Statement

All data was collected under TWRA permit (#3082, Dr. Thomas P. Wilson). Animal use training was completed via the online CITI training program on October 13th and 24th of 2015, and the IACUC board confirmed that the researcher had completed the required training modules. No animals were harmed throughout the duration of this study, and all areas searched were returned to their original state to avoid excessive disturbance of habitat.

Field Methods

This project spanned from mid-summer of 2016 to fall of 2017 with the sampling months including June, July, and August of 2016. Amphibian samples were collected from four rural, potentially non-impacted watercourses (i.e., Bakers Fork and Dry Creek in Davidson County, and Hogan's Branch); and, a potentially non-impacted stretch of Drakes Creek in Sumner County off Capps Gap Road. Furthermore, samples were also collected from four urban, potentially impacted streams including Mansker Creek in Sumner County, a potentially impacted stretch of Drakes Creek in Sumner County off Sandy Valley Road, Pee Dee Creek in Sumner County, and Garrison Branch in Sumner County. A single snake sample was obtained from an urban stream called Madison Creek, which is located on a golf course in Sumner County. There were sampling windows where no animals were found, and additional sites were sampled that were not productive. As such, these sites are not listed because no data was obtained from them. All of the aforementioned stretches of water are located in the Middle Tennessee ecoregion (Region III). See Appendix C for pictures of all of the study sites for a visual reference. See Figure 1 below for a map encompassing all nine sites.

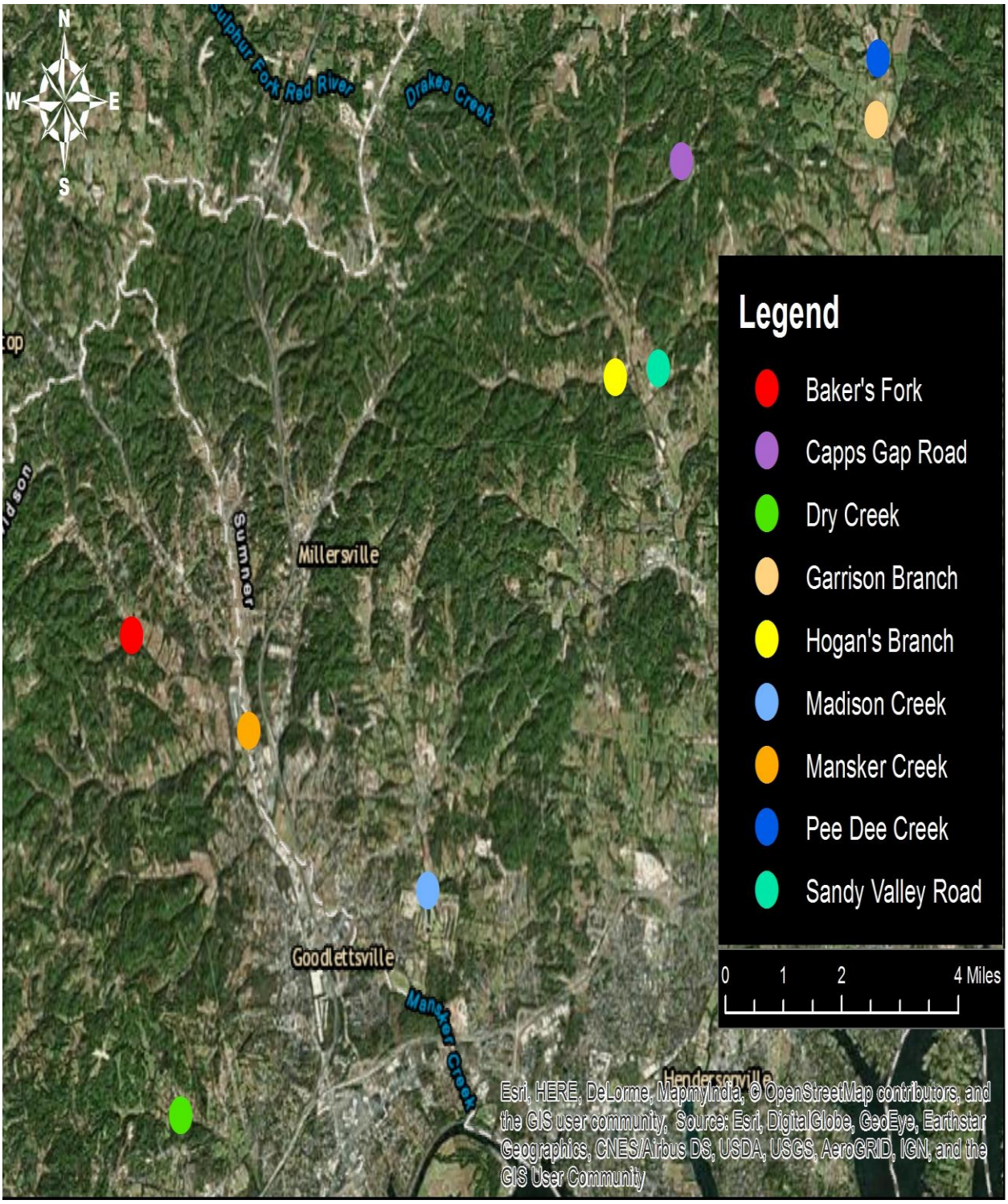

Figure 1: Satellite map illustrating the locations of all nine sites

Study sites were sampled at least three times each, and a total of 72 samples from Plethodontid salamanders, Ranid frogs, a Scincid lizard, and a Colubrid snake were obtained with the latter two serving as vectors for the disease and not potentially infected individuals. Samples were mostly collected from the early morning until noon or early afternoon to evening, as amphibians are largely inactive during the hottest times of the day. Specifically, the species sampled were 36 *Desmognathus fuscus* (Northern Dusky Salamander), 23 *Eurycea cirrigera* (Southern Two-Lined Salamander), 2 *Eurycea longicauda* (Long-Tailed Salamander), 1 *Eurycea lucifuga* (Cave Salamander), 5 *Lithobates clamitans* (Green Frog/Bronze Frog), 2 *Lithobates catesbeianus* (American Bullfrog), 1 *Pseudotriton ruber* (Red Salamander), 1 *Plestiodon fasciatus* (Five-Lined Skink), and 1 *Regina septemvitatta* (Queensnake). Salamanders were classified as larval based on the presence of external gills, as none of the Plethodontids in this data set are paedomorphic. Frogs were classified as larval based on the presence of a tail that is absorbed during metamorphosis. See Appendix A for photographs of all animals organized chronologically by accession number with the scientific name provided.

Amphibians were sampled across a canopy gradient. Biosecurity protocols were followed according to approved animal use protocols and state permit restrictions. Specifically, powder-free nitrile gloves were worn during the handling of animals and were worn and changed frequently during the processing of samples. To thoroughly minimize cross-contamination, animals were temporarily placed in individual plastic bags with a small amount of stream water to decrease handling time. All equipment was disinfected using 70% Ethanol (Hanlon, et al. 2012) before and after contact. All gloves, plastic bags, swabs or similar items were changed between captures and were disposed of according to approved biohazard protocols (Wilson et al. 2015). Other equipment was treated with aqueous chlorine bleach (i.e. 10% by volume; Johnson,

et al. 2003), and the process was replicated independently three times for ten minutes each for a total soak time of 30 minutes. The aqueous chlorine bleach decontamination was conducted before entering and exiting the study area (Wilson et al. 2015)

All captured organisms were measured for snout-vent length, tail length, and head width maximum to the nearest tenth of a millimeter using dial calipers, and weighed using a digital scale. I swabbed the ventral surface of all four limbs, the abdomen, the tail, and the webbing of the rear feet in the case of Ranid frogs for 45 seconds with continuous strokes (Brem et al. 2009). All amphibians were photographed after swabbing. I measured the habitat of each animal using a 1-meter square of PVC pipe in the known location where the animal was found. It was then measured again using the same apparatus in a random location. The sampling quadrat frame was subdivided into 100 equally sized cells, and these cells were then counted to obtain a percent estimate for habitat composition. The same procedure was used in terms of known and unknown locations to measure the how closed the canopy is with a densitometer. For both known and random locations, I measured percent over-story not occupied by canopy (POC). If two or more animals were found under the same cover object, the same canopy and habitat data was used to avoid unnecessary additional disturbance of the habitat. The random location was determined using a random number generator to pick a distance of 1m-30m and a compass bearing of 1º-360º. This distance was walked off using a chain tape at the randomly generated compass bearing. The end of the polyester (Dacron) swabs (Fisherbrand, Cat. #14-959-90; Pittsburgh, Pennsylvania, USA) potentially containing *Bd* DNA were cut off and placed in 1.5-ml microcentrifuge tubes with snap caps with cold 70% Ethanol, labeled for reference, and stored in a -80º C freezer before analysis in the laboratory (Wilson et al. 2015). The end of the swab with no DNA was disposed of according to Brem et al.'s (2009) method.

Habitat Description

All nine sites in Middle Tennessee featured limestone streambeds and were densely shaded with over-story, which is demonstrated by the average POC across all sites of 5.21% ± 13.41%. The area of interest for this project was northeast of Nashville and north of Hendersonville (See Figure 1). These streams are tributaries of the Cumberland River. Baker's Fork (See Figure 6C) is a rural stream that is far from any high-density urban areas or development. The habitat at this location was mostly composed of a limestone streambed lined with medium to large boulders and leafy vegetation along its banks. Mansker Creek (See Figure 7C) is an urban site that is situated adjacent to an industrial complex. As such, it was difficult to find animals at this site, which is likely due to contaminants in the water. The five animals discovered here were found on a cobble, stone, or cement substrate. Dry Creek (See Figure 8C) is a rural stream that was the southwestern-most site in this study. Its substrates were largely composed of cobble, stone, and foliage. Hogan's Branch (See Figure 5C) is a rural stream with a habitat composition of stone, cobble, leafy debris, and aquatic vegetation. The Sandy Valley Road site (See Figure 4C) is a stretch of Drakes' Creek surrounded by housing and roads that runs under a bridge. As such, it experiences runoff from this surrounding development, which probably contributed to difficulty finding animals at this site. Garrison Branch (See Figure 2C) is an urban site that featured cobble, mud, and vegetation as substrates with medium flat boulders serving as cover objects for all the sampled animals at this site. It is located next to housing and a power line repair site. The Capps Gap Road site (See Figure 3C) is a rural stretch of Drake's Creek that is located in a densely forested area. The habitat was largely composed of large tree roots, mud, cobble, leafy debris, and boulders. Finally, Pee Dee Creek (See Figure 1C) is an urban site that was filled with manmade debris. Naturally occurring substrates here included

cobble and small boulders. The only animal discovered at this site was found under a piece of debris.

Laboratory Analysis

Each swab was scraped into the original tube and dried in a speedvac (Labconco, Centrivap DNA Concentrator; Kansas City, Missouri, USA) before the DNA extraction process. DNA was extracted using the animal tissue protocol of a DNA extraction kit (Qiagen, DNeasy Blood and Tissue Kit; Hilden, North Rhine-Westphalia, Germany). Qiagen kits were chosen because they were shown to have the highest efficiency in a comparative study of three leading manufacturers (Bletz, et al. 2015). The pellet was re-suspended in 180 µL of a tissue lysis buffer with 20 µL of proteinase K. Then, the sample was incubated at 56º C for three hours with intermittent vortexing every hour to allow the spore walls to break and release DNA if *Bd* spores were present in the sample. 200 µL of lysis buffer was added and the sample was vortexed. This last step was repeated with 100% Ethanol in place of lysis buffer. The sample was then transferred into the DNEasy minispin column provided in the kit that contains a filter where the DNA present in the sample is suspended while it is washed. The sample was then centrifuged, washed with 500 µL of a wash buffer with 100% Ethanol added, centrifuged again, washed with a different wash buffer with less 100% Ethanol added, and finally eluted twice with the same elution buffer each time. The centrifuge was set to 13,000 rpm and each wash lasted 30 sec; while, each elution lasted 4 minutes. The extracted DNA was quantified using a spectrophotometer (Thermo Scientific, Nanodrop 2000C; Waltham, Massachusetts, USA). A table of quantification readings is provided in Appendix D.

I used a modified Polymerase Chain Reaction (Px2 Thermal Cycler, SN: PX210785 Thermo Electron Corporation, Milford, MA, USA) for analysis according to the methods of

Boyle, Boyle, Olsen, Morgan, and Hyatt (2004) utilizing a controlled reaction containing the chytrid primers 5.8S (5'-AGCCAAGAGATCCGTTGTCAAA-3') and ITS1 (5'-CCTTGATATAATA…TGTGCCATATGTC- 3'). The *Bd* gDNA clone was obtained from the Center for Wildlife Health at the University of Tennessee-Knoxville. Each row of wells contained this standard to serve as a positive control and a negative control with deionized water in place of the plasmid. All samples were run in triplicate using an agarose and TBE gel electrophoresis to separate the DNA fragments with a λ/ hindIII marker. All samples were assayed by PCR independently three times to ensure accurate results (Wilson et al. 2015). Following the methods of Boyle et al. (2004), I programmed the thermocycler to heat the PCR tubes to 50º C for 2 minutes followed by 10 minutes at 95º C. Then, the tubes were put through 50 cycles of 15 seconds at 95º C then 1 minute at 60º C to allow sufficient amplification of very small amounts of *Bd* DNA that may have been present in the sample. This process has been successful in obtaining accurate results in previous studies; which, coupled with the fact that Qiagen kits have the highest efficiency, indicates that accurate results were obtained (Bletz et al. 2015; Chatfield and Richards-Zawacki 2011). All samples that appeared positive from the initial three runs of PCR were then independently assayed three more times to ensure that they were conclusively positive samples.

Statistical Analysis

The Chi-Square Goodness of Fit Test with Yates' Correction for Continuity (Soto-Azat et al. 2013; Wilson et al. 2015) was used to compare the observed prevalence value from this data set with an expected value generated from an average of results of six studies on *Bd* prevalence in Plethodontids and Anurans in southeastern states of the U.S (Byrne, et al. 2008; Chatfield et al. 2009; Chatfield et al. 2012; Davis et al. 2012; Rothermel et al. 2008; Wilson et al. 2015).

Taken together, all of the raw counts of positive animals from the six studies were added together and divided by the total number of samples from the studies. This generated a 7.44% prevalence value due to the fact that 175/2351 anurans and caudates from southeastern states tested positive for the presence of *Bd*. This prevalence value was also used to compare the observed prevalence value of solely the Plethodontids in the data set using the Chi-Square Goodness of Fit Test with Yates Correction. Furthermore, I calculated the Probability of Detection (POD) for detecting at least one *Bd* positive animal in the representative sample using DiGiacomo and Koepsell's (1986) equation: $C = 1 – (1 – p)^n$, where n is the number of samples, and C is the probability of at least one animal testing positive at a hypothetical disease prevalence value (p), which was set to 0.05 for the purpose of the POD calculation in this study (Wilson et al. 2015).

To provide a clearer understanding of the conditions in which *Bd* positive animals were found, basic measures of central tendency like mean, median and mode were calculated for POC values were calculated for each site. Additionally, measures of dispersion such as range standard deviation and variance of POC were calculated for each site. A full listing of POC values for known and random locations can be found in Appendix B. See Table 1 for canopy summaries. No statistical correlations between canopy cover and prevalence can be observed because the number of positive samples is lower than five (Pers. Comm. Thomas P. Wilson).

Chapter Two

I. Results

Out of 72 animals that were swabbed, *Bd* was detected in 2 *D. fuscus* and 2 *E. cirrigera*. This corresponds to an overall prevalence value of 0.0556, or 5.56%. The positive samples were a larval *E. cirrigera* (Accession # 07/12/16 03, lab #19) from Baker's Fork (non-impacted), an

adult *E. cirrigera* (07/15/16 01, lab #23) from Mansker Creek (impacted), a larval *D. fuscus* (07/19/16 03, lab #34) from Hogan's Branch (non-impacted), and an adult *D. fuscus* (08/12/16 04, lab #68) from Capp's Gap Road (non-impacted). The Chi-Square Goodness of Fit Test indicated there was no significant difference between observed and expected *Bd* prevalence (Chi-Square Goodness of Fit Test χ^2=0.364, p=0.5463, df=1, Yates' Correction χ^2=0.140, p=0.7079, df=1; Sokal and Rohlf 1995; Wilson et al. 2015). When restricted to just the 63 Plethodontids, the overall prevalence value was 0.0635, or 6.35%. The Chi-Square Goodness of Fit Test again indicated there was no difference between observed and expected prevalence values (Chi-Square Goodness of Fit Test with Yates Correction χ^2=0.007, p=0.9334, df=1). The POD calculation yielded a C value of 97.5% (DiGiacomo and Koepsell 1986).

Mansker Creek had the highest mean POC value with 13.31% ± 23.89% of the over-story being open. The mean POC at Capps Gap Road was 0% ± 0% open, but this may speak more to the dates of sampling at this site or human error with over-story measurement rather than the actual over-story composition. The other two positive samples came from relatively closed canopy sites. Hogan's Branch yielded a mean POC value of 1.35% ± 1.39% open, and Baker's Fork had a mean POC value of 3.20% ± 12.22%. A full listing of mean, median, mode, range, standard deviation, and variance for known and random POC values of sites where more than one sample was obtained can be found below in Table 1.

Table 1: Mean, median, mode, standard deviation, and variance of POC at seven sites where more than one sample was obtained. Statistics for Madison Creek and Pee Dee Creek are not present in this table because only one sample was obtained, and statistics could not be analyzed.

Site	Mean POC (%)	Median POC (%)	Mode	Range	Standard Deviation of POC(%)	Variance
Baker's Fork (Known)	3.20	0	0	0% - 53.56%	± 12.22	149.35
Baker's Fork (Random)	7.44	6.50	6.76	0.78% - 18.25%	± 5.95	35.36
Mansker Creek (Known)	13.31	0	0	0% - 55.12%	± 23.89	570.76
Mansker Creek (Random)	1.92	0.52	0	0% - 7.28%	± 3.09	9.52
Dry Creek (Known)	10.95	0.78	0	0% - 50.96%	± 21.12	446.19
Dry Creek (Random)	9.82	0.52	0	0% - 42.38%	±17.40	302.92
Hogan's Branch (Known)	1.35	1.30	0	0% - 3.64%	± 1.39	1.94
Hogan's Branch (Random)	4.47	2.34	1.3	0% - 12.74%	± 4.20	17.65
Sandy Valley Road (Known)	12.13	0	0	0% - 36.4%	± 21.02	441.65
Sandy Valley Road (Random)	7.37	0.52	No mode	0.26% - 21.32	± 12.08	146.04
Garrison Branch (Known)	2.86	2.6	No mode	0% - 6.24%	± 2.27	5.16
Garrison Branch (Random)	2.82	2.73	No mode	0% - 5.98%	± 2.37	4.67
Capps Gap Road (Known)	0	0	0	0% - 0%	± 0	0
Capps Gap Road (Random)	2.15	1.04	0	0% - 10.66%	± 3.79	14.37

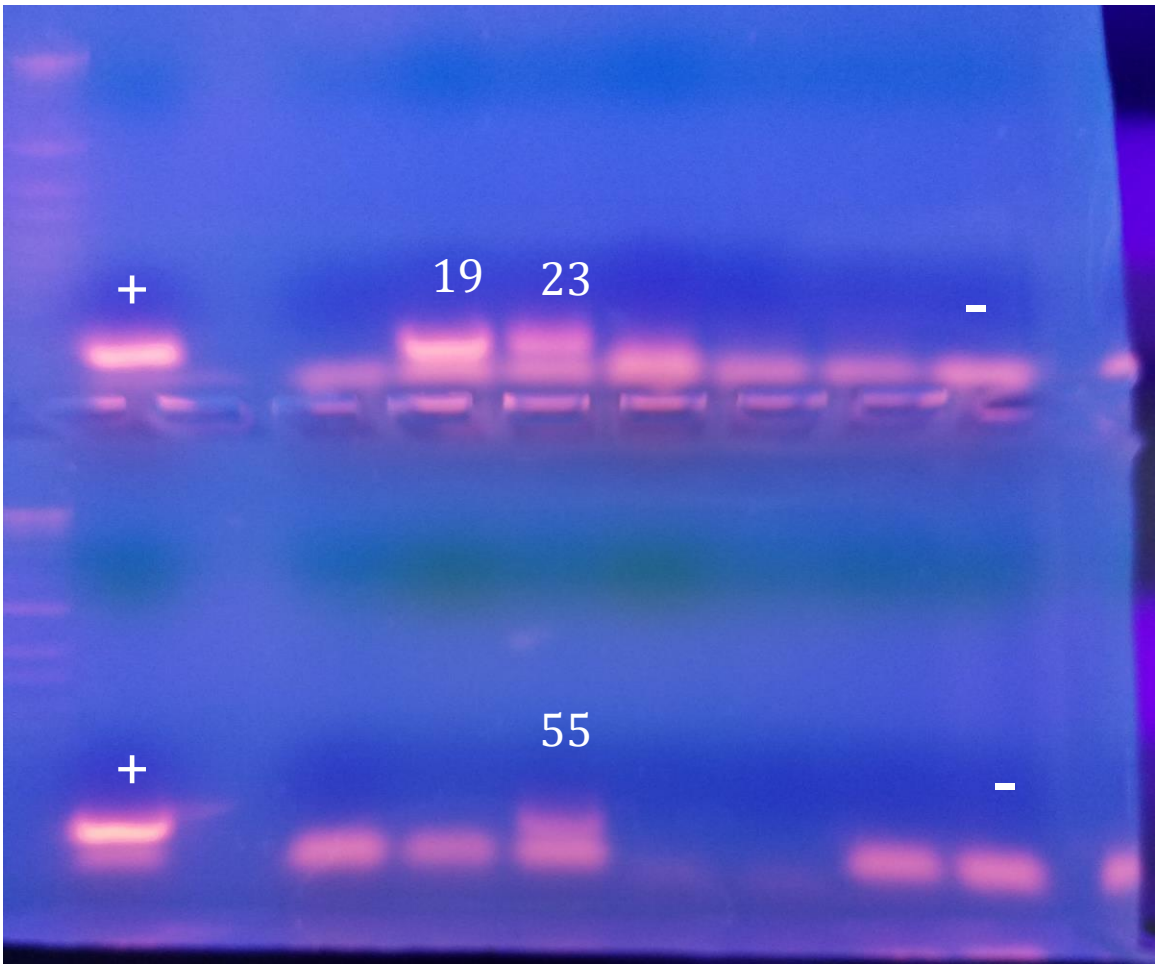

Figure 2: Example gel electrophoresis result showing two confirmed positives with contrast enhanced for increased visibility: Accession # 07/12/16 03, lab #19, which is the first positive on the gel and Accession # 07/15/16 01, lab #23, which is the second positive on the gel. The third suspicious positive (lab #55) on the gel was confirmed to be negative with subsequent runs.

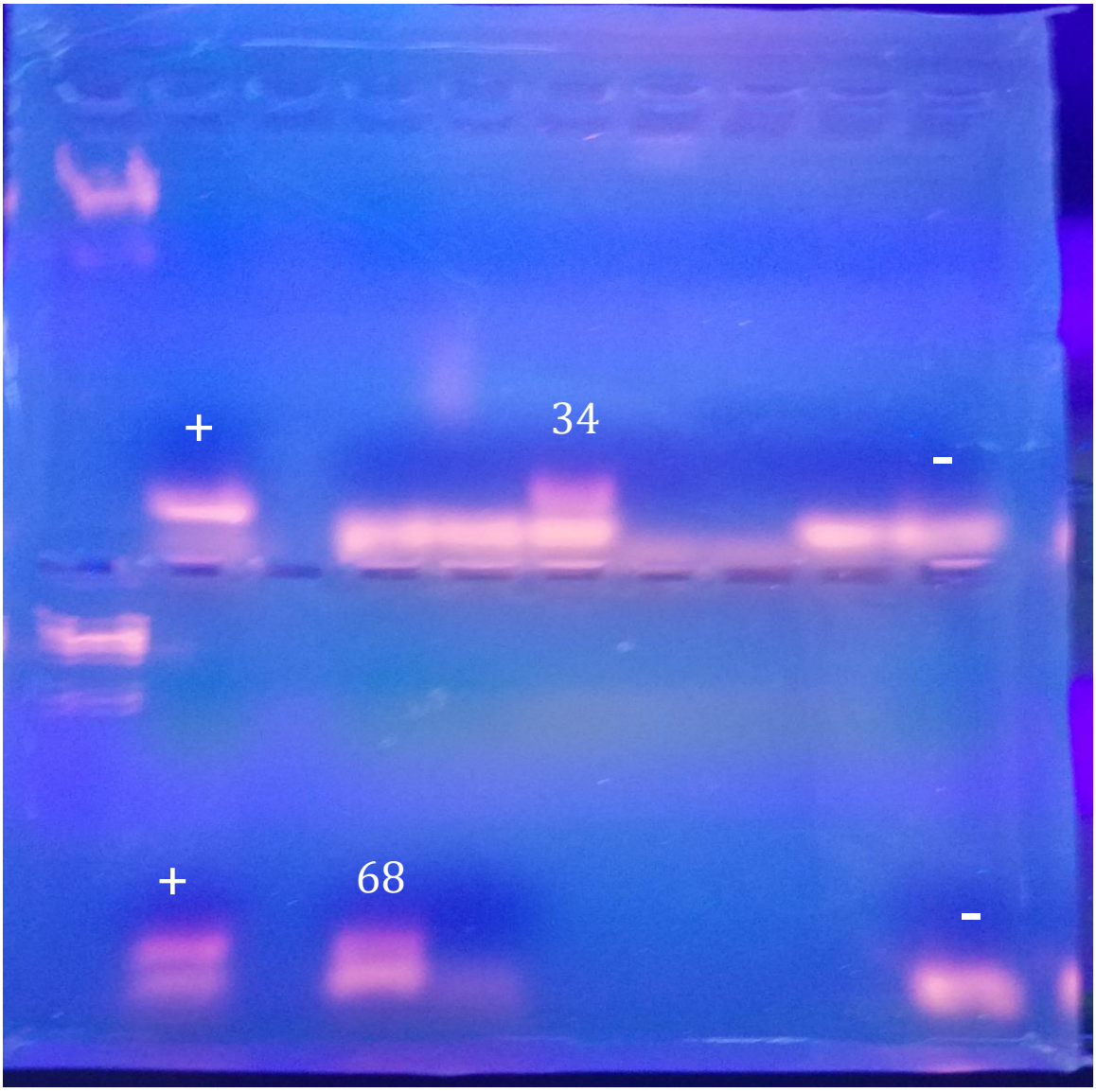

Figure 3: Example gel electrophoresis result showing two confirmed positives: First positive on the gel is Accession # 07/19/16 03, lab #34, second positive is 08/12/16 04, lab #68.

II. Discussion

The objectives of this study were to investigate the prevalence of *Bd* in Middle Tennessee and to see if there was a correlation between canopy cover and *Bd* prevalence and/or urbanization and *Bd* prevalence. As was previously stated, a low number of positives renders a statistical correlation between these two variables and *Bd* prevalence impossible. However, this does not indicate that meaningful results were not obtained from this study. *Bd* was detected in four different streams in Middle Tennessee at an overall prevalence rate of 0.0556, or 5.56%. The p value comparing expected and observed prevalence values was 0.7079, which is greater than 0.05. A p value > 0.05 indicates that there is not a statistically significant difference between the observed value in this data set and the expected value of prevalence in Plethodontids and Anurans in the Southeastern United States. When restricted to just Plethodontids, the p value was still greater than 0.05, but the prevalence increased slightly. The C value of 97.5% from the POD calculation indicates that I was 97.5% likely to detect at least one *Bd* positive animal at a disease prevalence of 5%. The data set certainly fit the POD prediction as I detected at least one positive animal, and the prevalence value was 5.56%. This is a relatively low prevalence value; however, it is in line with what is expected for a largely a Plethodontid data set. Also, the fact that *Bd* was detected in four different streams in Middle Tennessee warrants immediate action to prevent further spread of zoospores.

Byrne et al. (2008) conducted a study on the prevalence of plethodontid salamanders in Horseshoe Bend National Military Park. They found the prevalence of *Bd* within *D. fuscus, E. cirrigera, E. guttolineata, G. porphyriticus, P. glutinosus, and P. ruber* to be 27.63% with 21/76 animals testing positive. Chatfield et al. (2009) investigated the prevalence of *Bd* in 25 species of amphibians with a mixture of pond-breeding, stream-breeding, and fully terrestrial amphibians.

They found that 2.58% (17/659) of total amphibians tested positive for *Bd*, and all of the positive samples came from *N. viridescens*, *Anaxyrus sp.*, *Pseudacris sp.*, or *L. sylvaticus*. Next, fully aquatic salamanders in Florida, Mississippi, and Louisiana were sampled to elucidate *Bd* prevalence in this particularly *Bd*-prone taxonomic group. The researchers found that infection prevalence was 34% with 33/98 samples of *Amphiuma*, *Necturus, and Pseudobranchus* testing positive. Davis et al. (2012) detected *Bd* in 11/219 *A. fowleri* in metropolitan areas of Memphis, TN for a prevalence value of 5.02%. A large-scale study that spanned from 1999-2006 and surveyed anurans and caudates across ten states measured the prevalence of *Bd* throughout 30 sites. The researchers found that 80/1222 animals tested positive for the presence of *Bd*, which corresponds to 6.55% overall prevalence (Rothermel et al. 2008). Their findings are consistent with previous literature in that anurans had a much higher mean prevalence value than caudates. Finally, Wilson et al. (2015) investigated the prevalence of *Bd* of two ranid frogs, *L. catesbeianus* and *L. clamitans* on a former Department of Defense installation in Southeastern Tennessee. They found that the overall prevalence across both species was 16.88% with 13/77 animals testing positive. These are the data that were used to generate the expected value for the Chi-Square Goodness of Fit Test, and this provides a broad summative look at *Bd* prevalence in the Southeastern United States.

Furthermore, it is likely that more animals in these four streams are infected and were not detected. Swabbing as a method for *Bd* detection has some limitations, but it is still the most widely used, conventional method for field studies of *Bd* prevalence. In general, swabbing has the chance to produce false negatives when the swab is taken from an animal with a low infection load (Shin, et al. 2014). Also, results can be inconsistent within a data set when using skin swabs to detect *Bd*. This is likely due to the fact that most of the extracted DNA in a skin

swab comes from the amphibian's epidermal, keratinized epithelial cells. DNA from zoospores and zoosporangia is present in much lower concentrations if it is present at all. Even using histopathology to look for encysted zoosporangia in the stratum corneum can take hours and still fail to detect infection (Shin et al.2014; Hyatt et al. 2007). In addition, an animal that is actually infected can test negative if they have recently shed their skin because the stratum corneum, where mature zoosporangia are found, sloughs off during ecdysis. Furthermore, there is no guarantee that potentially encysted immature zoosporangium have made their way to the stratum corneum because *Bd* begins its life cycle in the deeper stratum granulosum. This makes the method of bathing an animal in a clinical setting over a number of weeks and extracting the DNA from the water that has been run through a filter a more reliable method (Hyatt et al. 2007). However, this requires removing the animal from the wild, which was beyond the scope of this study. Also, swab sampling is the least invasive, most sensible method to conduct the field-sampling portion of this research.

Bd has been detected previously in both *E. cirrigera* and *D. fuscus*, which are the two species that tested positive in this study (Byrne et al. 2008). So, it is unsurprising that these two species tested positive, but the life stage of the positive animals in this study is surprising. *Bd* is known to occur in larval frogs and salamanders; however, infection of larvae is more rare than in adults (Blaustein, et al. 2005; Parris and Beaudoin 2004). Interestingly, two of the four positive animals, one from each species, were larvae. This may indicate that infection rates are actually higher *in situ* than were observed in the study due to the fact animals could have perished before they were able to be sampled. Larvae in this study were often found under the same large cover object as adults, and it could be that the adult made a groove between the cover object and the cobble. Larvae then could have followed these trails under the cover object in the hot summer

months to avoid desiccation. It is likely that the larvae could pick up zoospores from the shallow pool of water in these tunnels made by conspecific adults. This notion is supported by the fact that Parris and Beaudoin (2004) found that high intraspecific density of amphibians has a strong reductive influence on metamorphic body mass when *Bd* was present.

It is important to note that the findings of this study echo the findings of other studies on canopy cover and chytrid prevalence in that the known locations of all of the positive animals in this study had very low POC values, which was one of the hypotheses stated at the beginning of the study. Beyer et al. (2015) and Becker et al. (2012) both concluded that there is a positive correlation between the density of the over-story and *Bd* prevalence. These studies support this observation, but no statistical correlational test was conducted due to low sample sizes (<5). Researchers suggest the reason for this association is that dense over-story serves as a buffer to temperature increase from the sun. This allows zoospores to survive because their optimal growth occurs at 17ºC, and spores cannot live for longer than four hours at a temperature of 37ºC (Van Rooij et al. 2015). From analysis of POC measurements across at all sites, it is apparent that all of the sites in this study had relatively high canopy density, so it is of particular concern that Becker et al. (2012) state that amphibians in temperate zones with high canopy density have increased risk of infection. Temperature has a strong effect on amphibian thermoregulation because they are ectotherms. Abnormal thermoregulation can compromise an amphibian's immune response due to the fact that they are dependent on ambient temperature for thermoregulation. Thereby, a weakened immune response can increase susceptibility to *Bd* and other pathogens (Becker et al. 2012). This idea can have some seemingly strange implications for disease management and prevention. It may suggest that systematic removal of over-story above streambeds of inoculated areas could increase temperature past the C_T max of the fungus (37ºC)

and reduce zoosporic loads to potentially save populations from declines. However, this could have an adverse effect on myriad other organisms within the habitat or the amphibians themselves. So further research into this type of disease mitigation needs to be conducted on a small scale before attempting to use this method as a solution. One limitation to this inference that must be stated is that the dates of sampling are mid-late summer, so animals could have been seeking areas with high canopy density to avoid desiccation from the sun and not due to the presence or absence of *Bd*.

Three of the positive animals came from rural sites, and one positive came from an urban site. Although no statistical correlation could be made, this result is the inverse of the stated hypothesis for this study. The subject of prevalence of *Bd* in urban versus rural streams is a controversial debate among ecologists and epidemiologists. The results of two studies suggest that urbanization increases *Bd* prevalence because of higher traffic bringing spores into an area (Pauza and Driessen 2008; Saenz et al. 2015). However, a competing theory has emerged that suggests there is no association between urbanization and the presence of *Bd* (Pullen, et al. 2010). Pullen et al. (2010) postulate that *Bd* is associated with seasonality with peak chytrid season occurring in May. Seasonality in *Bd* prevalence is supported by the results Geiger et al. (2017), although the peak chytrid season was August-December in this study. Based on these data, the sampling window, June to August is less than optimal because it did not fall in either of those aforementioned sampling windows. This reason alone could be a contributing factor for the prevalence of *Bd*. If true, that would validate a higher need for further research in Middle Tennessee with more ideal sampling dates beginning in the spring. Pullen et al. (2010) also hypothesize that rural environments experience unique contaminants like herbicides, pesticides, and excess nitrogen that can compromise amphibian immune systems and make them more

susceptible to *Bd*. These findings parallel the current study's findings based on the fact that three out of the four positive samples came from rural sites.

Considering the bigger picture of the ramifications of the global spread of *Bd*, immediate development of new strategies to reduce the prevalence of and prevent naïve areas from being exposed to this disastrous fungus is imperative. There are various management plans that currently exist that have some traction but have not demonstrated the ability to lower *Bd* prevalence on a large scale or with long lasting results. The first step that must be taken is a continued, integrative global effort to measure the prevalence of *Bd* worldwide (Phillips et al. 2010). This is where much of the significance of the current study is derived from in that this data functions to provide some information about where *Bd* is present in Middle Tennessee, an understudied area with significant amphibian diversity. Baseline data about the current distribution is paramount to taking the first step in saving global amphibian biodiversity from complete destruction. A recent study by (Geiger et al. 2017) explored the efficacy of treating wild caught tadpoles of the midwife toad, *Alytes obstetricans,* with an antifungal solution (General Tonic) then releasing that treated cohort of tadpoles back into the wild. This strategy yielded mixed results. The researchers did observe a temporary reduction in prevalence, but the prevalence reduction caused by the antifungal treatment only lasted for a year. Itrocanazole is another antifungal that has been demonstrated to be effective at killing zoospores in clinical trials (Jones et al. 2012).

Another measure that can be used immediately upon introduction of *Bd* to a naïve population is quarantine. Isolating known *Bd* positive areas from other naïve surrounding areas can be useful to prevent the zoospores from spreading into these surrounding areas and stopping a massive outbreak. Previously established antifungal agents can be used to treat the infected

amphibians *in situ* and stop an outbreak in its tracks, but this must be combined with quarantine to be effective (Jones et al. 2012; Phillips et al. 2012). This method is relatively inexpensive and effective, but it does require a massive effort in terms of manpower and is contingent upon knowing that an introduction of *Bd* into a naïve area has occurred almost as soon as it happens. This is not the case with most *Bd* infections as it has been spreading and infecting amphibian populations since at least 1861 (Van Rooij et al. 2015). Approximately 32.5% of amphibians are listed as vulnerable, endangered, or critically endangered, and 43% of species are experiencing declines (Wake and Vredenburg 2008). The last ditch effort for saving the most critically imperiled of these animals is captive breeding programs. These programs are labor intensive and expensive and require removal of endangered species from the wild but they may be the only hope for some species that will go extinct in the near future without intervention. Captive breeding programs would be used in concert with habitat restoration projects to eventually repatriate these animals into their natural habitats once the habitat has been made suitable (Phillips et al. 2012).

These *in situ* treatment measures appear to be somewhat effective and are being tested in the field (Phillips et al. 2012), but another method that outwardly appears as a more sustainable long-term solution to the problem is promoting natural resistance to *Bd* within amphibians. The former methods function more like triage in preventing disaster and should be used in this way; however, species-specific susceptibility may be the key to significantly reducing the prevalence of and eventually eradicating chytridiomycosis. It is understood that certain amphibian species are resilient to the symptoms of *Bd* infection, while others quickly succumb to infection. The cause of this resilience appears to be some combination of co-evolution, which was previously discussed, antimicrobial peptides produced by the granular glands on amphibian skin, or

microbial assemblages on the skin of the animals that may outcompete *Bd* in some animals (Rollins-Smith and Conlon 2005; Walke et al. 2015). A better understanding of the latter two factors could be the best way to eventually encourage intrinsic resistance to *Bd* and prevent declines in areas where populations are already infected. Presently, *Bd* occurs on all continents that amphibians can be found, so finding ways to prevent declines in already infected areas is one of the most important courses of action (Wake and Vredenburg 2008). It has been shown that the aforementioned antimicrobial peptides can inhibit growth of *Bd*, and microbial assemblages of frogs change when exposed to *Bd* in clinical trials (Rollins-Smith and Conlon 2005; Walke et al. 2015). The problem herein becomes converting this information into a useful tool to combat *Bd*. Microbial research with *Bd* may play a big role if bacteria that produce antibiotic compounds found on resistant species thought to inhibit *Bd* could be translocated to susceptible species and function in a similar way (Harris et al. 2006) Similarly to habitat restoration projects, this microbial research could be coupled with captive breeding programs to encourage the development of intrinsic resistance within species that are currently susceptible. Specifically, more research needs to be conducted on what species harbor the bacteria responsible for inhibition of *Bd*, outside of *P. cinereus* and *H. scutatum*, and what species with similar life histories could benefit from these microbial translocations as more of a natural transition (Harris et al. 2006; Walke et al. 2015).

All of these ideas are in the preliminary stages and require more research and funding to become viable solutions. However, there are immediate steps that are inexpensive and can be immediately implemented, and these are more relevant to the current study. Awareness about *Bd* is extremely lacking in the general community. Scientists are largely knowledgeable about *Bd* and its affects, but the general population needs to be educated about this epidemic. Following

established biosecurity practices is simple and inexpensive, yet community outreach is so lacking that people are largely unaware that *Bd* exists. Encouraging people to spray their boots with a cheap fungicide could be very effective in terms of preventing the spread of *Bd*. This could be accomplished in several ways. For example, brief pamphlets describing proper biosecurity protocols could be distributed that briefly outline the destructive nature of *Bd* and what can be done to stop it. These protocols and pamphlets exist within the Partners in Amphibian and Reptile Conservation (PARC) organization, but distribution of this type of literature is severely lacking. Furthermore, signs could be posted at trailheads of known *Bd* positive areas to encourage people to spray their boots and gear with fungicide to prevent spreading it to naïve areas and populations.

In closing, this study should contribute to the scientific community's wealth of knowledge about the characteristics of *Bd*, specifically where it is found, what conditions it thrives in, and in what taxonomic groups it is most prevalent in. It can be impactful in that it documents the presence of *Bd* in four streams that have never been surveyed for *Bd*. This is all done in an effort to aid wildlife and land managers in making decisions that will protect and conserve amphibians in this region from the foremost threat to amphibian diversity and overall health. Because of the statistical and ecological similarities to three other projects on *Bd* that have been conducted or are ongoing at UTC, the data can later be compiled into one article that will be submitted for publication to a scientific, peer-reviewed journal and will be presented as poster and/or podium presentations at various conferences. Taken together, this project is part of a larger whole that has the potential to be impactful for both the UTC Honors College and the Department of Biology, Geology and Environmental Science because this project showcases

amphibians as key bioindicators and provides a mechanism to better understand the sixth mass extinction.

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Appendix A: Photograph Documentation of each Sample with Accession # Provided

Accession # is in the format of date MM/DD/YY 01=1st sample collected that day. No animals sampled showed outward signs of infection.

A (Just Before Release) B

06/20/2016 01 06/20/16 02

Figure 1A: A is an adult Eurycea cirrigera. B is a larval L. clamitans. 06/21/16 01 and 06/21/16 03 both L. clamitans. Picture files were corrupted. C is an adult E. cirrigera

06/21/16 02 C

A

06/28/16 01 *Regina septemvittata*

B

07/05/16 01 *Desmognathus fuscus*

C

07/05/16 02 *D. fuscus*

D

07/05/16 03 *D. fuscus*

Figure 2A: Photos B,C,D are all D. fuscus. Photo A is an adult R.septemvittata.

07/05/16 04 *E. cirrigera* A

07/05/16 05 *E. cirrigera* B

07/05/16 06 *E. cirrigera* C

07/11/16 01 *E. cirrigera* D

Figure 3A: Pictures A-D are all E. cirrigera.

07/11/16 02 *E. cirrigera* A

07/11/16 03 *E. cirrigera* B

07/11/16 04 *D. fuscus* C

07/12/16 01 *D. fuscus* D

Figure 4A: Pictures A and B are E. cirrigera. Pictures A and D are both D. fuscus.

07/12/16 02 *D. fuscus* A 07/12/16 03 *D. fuscus* B

07/12/16 04 *E. cirrigera* C 07/12/16 05 *D. fuscus* D

Figure 5A: Pictures A,B, and D are all immature D. fuscus. Picture C is a larval E. cirrigera.

07/12/16 06 *D. fuscus* A 07/14/16 01 *E. cirrigera* B

07/14/16 02 *E. cirrigera* C 07/18/16 01 *D. fuscus* D

Figure 6A: Photos B and C are both E. cirrigera, Pictures A and D are both larval D. fuscus.

07/18/16 02 *D. fuscus* A 07/18/16 03 *D. fuscus* B

07/18/16 04 *D. fuscus* C 07/18/16 05 *E. cirrigera* D

Figure 7A: Photos A-C are all larval D. fuscus, and photo D is a larval E. cirrigera.

07/18/16 06 *D. fuscus* A

07/18/16 07 *D. fuscus* B

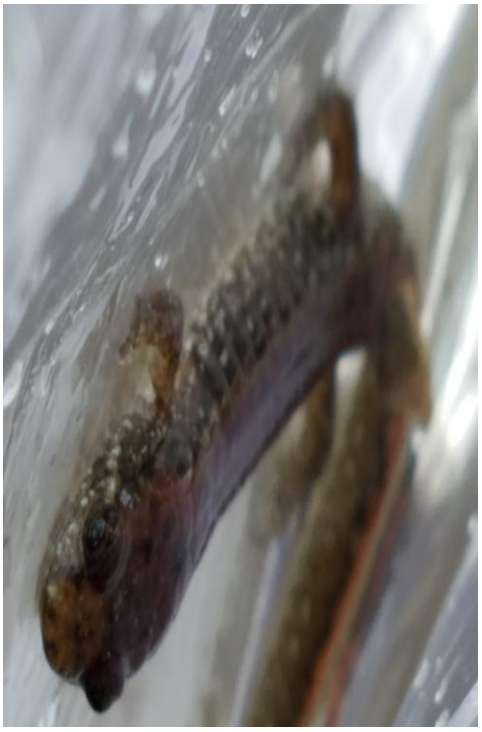
07/19/16 01 *Lithobates clamitans* C

07/19/16 02 *D. fuscus* D

Figure 8A: Pictures A,B, and D are all mature D. fuscus. C is an adult L. clamitans.

07/19/16 03 *D. fuscus* A

07/19/16 04 *D. fuscus* B



07/19/16 05 *D. fuscus* C

07/19/16 06 *D. fuscus* D

Figure 9A: Photographs A-D are all D. fuscus.

07/20/16 01 *D. fuscus* A 07/20/16 02 *D.fuscus* B

07/21/16 01 *D. fuscus* C 07/21/16 02 *D. fuscus* D

Figure 10A: Photos A-D are all adult D. fuscus.

07/21/16 03 *D. fuscus* A 07/21/16 04 *D. fuscus* B

07/21/16 05 *D. fuscus* C 07/21/16 06 *D. fuscus* D

Figure 11A: Photos A-D are all adult D. fuscus

07/21/16 07 *L. clamitans* A 07/21/16 08 *D. fuscus* B

07/26/16 01 *E. cirrigera* C 07/26/16 02 *Plestiodon fasciatus* D

Figure 12A: A is an adult L. clamitans, B is an adult D. fuscus. C is an adult E. cirrigera. D is an adult P. fasciatus.

07/26/16 03 *E. cirrigera* A 07/27/16 01 *E. cirrigera* B

C D

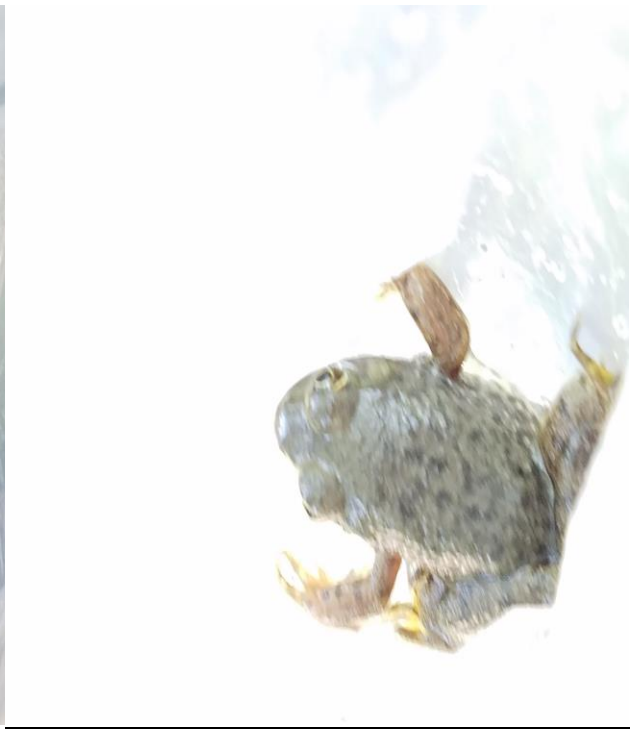
Figure 13A: A and B are adult E. cirrigera. C is an adult E. lucifuga, and D is an adult E. longicauda

07/27/16 02 *E. lucifuga* 08/01/16 01 *E. longicauda* (Herringbone Pattern)

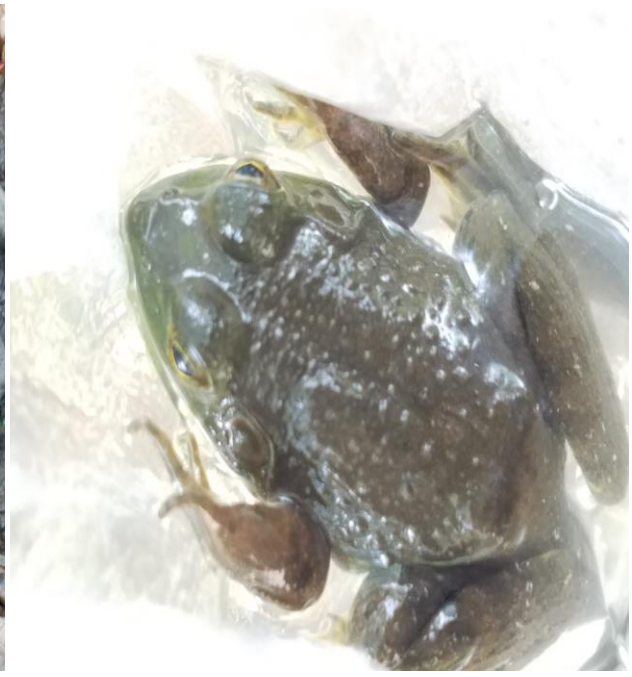
08/02/16 01 *D. fuscus* A 08/02/16 02 *D. fuscus* B

08/02/16 03 *D. fuscus* C 08/02/16 04 *D. fuscus* D

Figure 14A: A-D are all adult D. fuscus



08/03/16 01 *D. fuscus* A 08/03/16 02 *Lithobates clamitans* B



08/03/16 03 *Pseudotriton ruber* C 08/09/16 01 *Lithobates catesbeianus* D

Figure 15A: A is an adult D. fuscus. B is an adult L. clamitans. C is an adult P. ruber. D is an adult L. catesbeianus.

f

08/09/16 02 *Eurycea longicauda* A 08/10/16 01 *Lithobates clamitans* B

08/10/16 02 *D. fuscus* C 08/12/16 01 *D. fuscus* D

Figure 16A: A is an adult E. longicauda. B is an adult L. clamitans. C and are adult D. fuscus.

08/12/16 02 *D. fuscus* A

08/12/16 03 *D. fuscus* B

08/12/16 04 *D. fuscus* C

08/15/16 01 *D. fuscus* D

Figure 17A: A-D are all adult D. fuscus.

08/15/16 02 *E. cirrigera* A

08/15/16 03 *D. fuscus* B

08/15/16 04 *D. fuscus* C

Figure 18A: A is an adult E. cirrigera. B and C are adult D. fuscus.

Appendix B: Canopy and Habitat Measurements Organized by Accession Number and Location

Table 1B: Canopy coverage reported as Percent Overstory not Occupied by Canopy (POC) and habitat measurements from the location the animal was found and a random location. These measurements are from Baker's Fork, a rural site.

Accession #	Genus species	Known POC Given as a %	Known Habitat	Random POC Given as a %	Random Habitat
06/26/16 01	*E. cirrigera*	1.82	5% Boulder, 95% Stone	3.12	80% cobble, 20% stone
06/20/16 02	*L. clamitans*	53.56	45 Dry stone, 55% Wet stone	11.18	40% cobble 30% dry stone, 30% wet stone
07/12/16 01	*E. cirrigera*	0.26	80% cobble, 20% stone	0.78	80% cobble, 20% stone
07/12/16 02	*D. fuscus*	0.26	80% cobble, 20% stone	0.78	80% cobble, 20% stone
07/12/16 03	*E. cirrigera*	2.34	100% cobble	6.50	90% wet stone, 10% cobble
07/12/16 04	*E. cirrigera*	2.34	100% cobble	6.50	90% wet stone, 10% cobble
07/12/16 05	*D. fuscus*	0	40% cobble, 40% stone, 20% small rocks	18.25	20% green foliage, 80% mossy wet stone
07/12/16 06	*D. fuscus*	0	40% cobble, 40% stone, 20% small rocks	18.25	20% green foliage, 80% mossy wet stone
07/18/16 01	*E. cirrigera*	0	80% large boulders, 20% cobble	6.76	50% cobble 50% wet stone
07/18/16 02	*D. fuscus*	0	80% large boulders, 20% cobble	6.76	50% cobble 50% wet stone
07/18/16 03	*E. cirrigera*	0	100% medium boulder	0.52	100% stone
07/18/16 04	*E. cirrigera*	0	50% muddy cobble, 50% medium boulder	15.08	20% reeds, 80% stone
07/18/16 05	*E. cirrigera*	0	50% muddy cobble, 50%	15.08	20% reeds, 80% stone

			medium boulder		
07/18/16 06	*D. fuscus*	0.26	40% medium boulder, 10% foliage, 50% stone	1.3	70% muddy stone, 30% cobble
07/18/16 07	*D. fuscus*	0	50% cobble, 50% medium boulders	4.16	60% wet stone covered in mollusks, 20% cobble, 20% small boulders
08/15/16 01	*D. fuscus*	0	50% grassy vegetated overhang, 20% cobble, 10% small stone, 20% muddy stone	6.24	82% submerged stone, 18% leafy debris
08/15/16 02	*E. cirrigera*	0	55% cobble with leafy debris, 45% medium boulders	1.04	100% muddy submerged stone
08/15/16 03	*D. fuscus*	0	48% med mossy boulders, 22% leafy debris, 30% mossy muddy stone	12.22	30% aquatic grass, 40% dry stone, 30% leafy debris
08/15/16 04	*D. fuscus*	0	62% cobble, 18% medium boulders, 20% leafy debris	6.76	82% muddy stone, 18% leafy debris

Table 2B: Habitat and canopy data for Mansker Creek, an urban stream.

Accession #	Genus Species	Known POC (%)	Known Habitat	Random POC (%)	Random Habitat
06/21/16 01	*L. clamitans*	0	30% foliage, 70% cement	0	100% dry cobble
06/21/16 02	*E. cirrigera*	11.44	80% med boulder, 20% cobble	1.82	100% cobble
06/21/16 03	*E. cirrigera*	0	20% small boulder, 80% stone	0.52	100% stone
07/15/16 01	*E. cirrigera*	0	70% small boulder, 30% cobble	0	10% intact log, 90 wet cobble
07/15/16 02	*E. cirrigera*	55.12	100% cobble	7.28	40% foliage, 60% wet cobble

Table 3B: Habitat and canopy data from a rural stream called Dry Creek.

Accession #	Genus species	Known POC (%)	Known Habitat	Random POC (%)	Random Habitat
07/05/16 01	*D. fuscus*	0	10% small foliage, 60% dry stone, 30% small cobble	0	20% dry stone, 50% wet stone, 30% small cobble
07/05/16 02	*D. fuscus*	0	10% wet cobble, 30% foilage, 10% small stone, 50% dry stone	0	10% leaves, 90% muddy stone
07/05/16 03	*D. fuscus*	0	30% cobble, 70% wet stone	1.04	100% wet stone
07/05/16 04	*E. cirrigera*	1.56	40% cobble, 50% medium boulder, 10% wet stone	3	50% dry stone, 40% wet stone, 10% foilage
07/05/16 05	*E. cirrigera*	3	100% dry cobble	0	100% wet stone
07/05/16 06	*E. cirrigera*	3	100% dry cobble	0	100% wet stone
07/11/16 01	*E. cirrigera*	50.96	70% cobble, 30% stone	42.38	100% grass
07/11/16 02	*E. cirrigera*	50.96	70% cobble, 30% stone	42.38	100% grass
07/11/16 03	*E. cirrigera*	0	100% cobble	9.36	20% medium boulder, 30% crab grass, 50% cobble
07/11/16 04	*E. cirrigera*	0	100% cobble	0	50% tree roots, 50% dead foilage

Table 4B: Habitat and canopy metrics from Hogan's Branch, a rural site.

Accession #	Genus species	Known POC (%)	Known Habitat	Random POC (%)	Random Habitat
07/19/16 01	*L. clamitans*	0	20% stone, 30% vegetation, 30% cobble, 20% small stones	1.3	90% cobble, 10% log
07/19/16 02	*D. fuscus*	3	60% cobble, 40% stones	10	90% muddy stone, 10% cobble
07/19/16 03	*D. fuscus*	1.3	50% small stone, 20% cobble, 20% stone, 10% foilage	10.14	70% wet stone, 30% cobble
07/19/16 04	*D. fuscus*	1.3	50% small stone, 20% cobble, 20% stone, 10% foilage	10.14	70% wet stone, 30% cobble
07/19/16 05	*D. fuscus*	1.3	50% small stone, 20% cobble, 20% stone, 10% foilage	10.14	70% wet stone, 30% cobble
07/19/16 06	*D. fuscus*	3.64	70% vegetation, 20% small stone, 10% cobble	4.16	50% submerged cobble, 50% submerged stone
07/21/16 01	*D. fuscus*	0	20% small boulders, 20% vegetation, 10% cobble, 50% wet stone	12.74	60% submerged cobble, 40% wet stone
07/21/16 02	*D. fuscus*	0	80% large boulders, 20% cobble	1.82	90% cobble, 10% wet stone
07/21/16 03	*D. fuscus*	0	90% muddy stone, 10% cobble	0	20 foilage, 50 stone, 30 med boulder
07/21/16 04	*D. fuscus*	0	80% small stones, 20% muddy cobble	0.78	60 muddy wet stone, 40 mossy dry stone
07/21/16 05	*D. fuscus*	2.08	35% leafy vegetation, 45% med flat mossy boulder, 20% mud	2.34	80% small boulder, 20% muddy cobble

07/21/16 06	*D. fuscus*	2.08	35% leafy vegetation, 45% medium flat mossy boulder, 20% mud	2.34	80% small boulder, 20% muddy cobble
07/21/16 07	*L. clamitans*	0.78	40% leafy vegetation, 60% mossy boulder	1.82	20% leaves, 80% mossy stone
07/21/16 08	*D. fuscus*	2.86	60% foilage, 40% medium flat mossy boulder	1.30	70% small boulders, 10% debris, 20% mossy muddy stone
08/02/16 01	*D. fuscus*	3.38	83% medium flat boulder, 17% cobble	3.38	63% cobble, 37% submerged stone
08/02/16 02	*D. fuscus*	1.30	13% submerged mossy stone, 60% small flat boulder, 27% cobble	1.30	92% submerged stone, 8% cobble
08/02/16 03	*D. fuscus*	0	100% flat large mossy boulder	0.78	13% moss, 25% mud, 20% cobble, 22% green vegetation, 20% leafy debris
08/02/16 04	*D. fuscus*	0	82% medium flat mossy boulders	6.76	60% debris, 20% vegetation, 20% mixed mud and cobble
08/10/16 01	*L. clamitans*	3.90	65% debris, 22% muddy cobble, 13% small stones	8.06	100% submerged cobble
08/10/16 02	*D. fuscus*	0	54% leafy debris and sticks, 12% muddy cobble, 34% medium boulders	0	40% cobble, 55% stone, 5% debris

Table 5B: Canopy and habitat information from a potentially impacted stretch of Drakes Creek off of Sandy Valley Road.

Accession #	Genus species	Known POC (%)	Known Habitat	Random POC (%)	Random Habitat
07/20/16 01	*D. fuscus*	36.4	30% small boulders, 70% cobble	0.26	80% vegetation, 20% bare soil
07/20/16 02	*D. fuscus*	0	60% dead foilage, 20% mud, 20% small stone	0.52	10% mud, 90% wet stone
08/09/16 02	*E. longicauda*	0	82% med boulder, 18% cobble	21.32	62% tree roots with interspersed leafy debris, 38 %cobble

Table 6B: Habitat and canopy metrics for Garrison Branch

Accession #	Genus species	Known POC (%)	Known Habitat	Random POC (%)	Random Habitat
07/27/16 01	*E. cirrigera*	1.3	63% medium flat boulders, 37% muddy cobble	0	85% muddy flat stone, 15% leafy debris
07/27/16 02	*E. lucifuga*	1.82	80% medium flat boulders, 20% cobble	4.94	95% muddy stone, 5% cobble
08/01/16 01	*E. longicauda*	0	45% med flat boulder	2.34	93% submerged stone, 7% cobble
08/03/16 01	*D. fuscus*	3.38	7% green vegetation, 33% cobble, 60% medium boulder	3.12	85% submerged med boulders, 15% cobble
08/03/16 02	*L. catesbeianus*	6.24	45% cobble, 55% medium flat boulders	5.98	100% wet stone
08/03/16 03	*P. ruber*	4.42	85% cobble, 15% small boulder	0.52	100% submerged cobble

Table 7B: Canopy and habitat information from a potentially non-impacted stretch of Drakes Creek off of Capps Gap Road.

Accession #	Genus species	Known POC (%)	Known Habitat	Random POC (%)	Random Habitat
07/26/16 01	*E. cirrigera*	0	40% med boulders, 60% cobble	10.66	80% green vegetation, 20% cobble
07/26/16 02	*Plestiodon fasciatus*	0	70% flat large boulder, 30% cobble	0	40% leafy debris, 50% dirt, 10% vegetation
07/26/16 03	*E. cirrigera*	0	30% tree roots, 40% cobble, 30% small boulders	0.78	30% vegetation, 20% mud, 50% cobble
08/12/16 01	*D. fuscus*	0	82% small stones, 15% mud and cobble, 3% leafy debris	1.3	42% submerged cobble, 58% muddy stone
08/12/16 02	*D. fuscus*	0	82 small stones, 15% mud and cobble, 3% leafy debris	1.3	42% submerged cobble, 58% muddy stone
08/12/16 03	*D. fuscus*	0	15% leafy debris, 25% small boulders, 60% muddy stone	0	62% cobble, 38% leafy debris
08/12/16 04	*D. fuscus*	0	82% medium boulders, 18% mud and cobble	1.04	20% leafy debris, 60% med stones, 20% dirt and cobble

Table 8B: Habitat and canopy data for Pee Dee Creek, an urban stream.

Accession #	Genus species	Known POC (%)	Known Habitat	Random POC (%)	Random Habitat
08/09/16 01	*L. catesbeianus*	41.6	22% debris, 60% cobble, 18% small boulder	6.76	60% leafy debris, 20% small boulder, 20% cobble

Table 9B: Habitat and canopy data for the only snake sample (Regina septemvittata) in the data set. It was obtained from Madison Creek, which is an urban stream on a golf course.

Accession #	Genus species	Known POC (%)	Known Habitat	Random POC (%)	Random Habitat
06/28/16 01	*Regina septemvittata*	16.12	30% dry cobble, 70% wet cobble	26.00	100% wet cobble

Appendix C: Pictures of all nine study sites to provide a visual reference point.

Figure 1C: A picture of Pee Dee Creek, an urban site.

Figure 2C: Picture of Garrison Branch, an urban site

Figure 3C: A picture of Capps Gap Road, a rural site.

Figure 4C: A picture of Sandy Valley Road, an urban site.

Figure 5C: A picture of Hogan's Branch, a rural site.

Figure 6C: A picture of Baker's Fork, a rural site.

Figure 7C: A picture of Mansker Creek, an urban site.

Figure 8C: A picture of Dry Creek, a rural site.

Figure 9C: Picture of Madison Creek, an urban site.

Appendix D: Quantification readings from spectrophotometer for both elutions of each sample.

Table 1D: Concentration, A 260, A 280, 260/280 ratio, and 260/230 ratio for each sample.

Accession #, Elution #	Concentration (ng/μL)	A 260	A 280	260/280 ratio	260/230 ratio
06/20/16 01, E1	4.5	0.090	0.036	2.49	0.25
06/20/16 01, E2	2.5	0.049	0.013	3.71	0.67
06/20/16 02, E2	2.7	0.055	0.020	2.76	0.27
06/20/16 02, E2	2.2	0.043	0.009	4.96	0.73
06/21/16 01, E1	3.8	0.076	0.024	3.12	0.18
06/21/16 01, E2	2.6	0.051	0.027	1.88	0.27
06/21/16 02, E1	1.8	0.037	0.017	2.14	0.39
06/21/16 02, E2	2.6	0.051	0.029	1.80	0.88
06/21/16 03, E1	1.9	0.027	0.009	3.00	0.47
06/21/16 03, E2	2.5	0.051	0.036	1.41	1.15
06/28/ 16 01, E1	2.1	0.025	0.009	2.71	0.26
06/28/ 16 01, E2	2.2	0.044	0.006	7.92	0.35
07/05/16 01, E1	2.1	0.043	0.017	2.47	0.53
07/05/16 01, E2	2.2	0.044	0.026	1.66	0.53
07/05/16 02, E1	1.1	0.022	0.009	2.44	0.52
07/05/16 02, E2	2.5	0.050	0.012	4.08	0.90
07/05/16 03, E1	2.5	0.037	0.007	2.58	
07/05/16 03, E2	1.8	0.037	-0.007	-5.23	0.65
07/05/16 04, E1	2.1	0.032	0.022	1.42	0.23
07/05/16 04, E2	189.0	3.780	3.145	1.2	0.76
07/05/16 05,	2.1	0.032	0.022	1.42	0.23

E1					
07/05/16 05, E2	1.7	0.034	0.017	1.97	0.42
07/05/16 06, E1	3.6	0.073	0.032	2.26	0.38
07/05/16 06, E2	3.0	0.060	0.027	2.24	0.59
07/11/16 01, E1	2.2	0.049	0.018	2.41	0.78
07/11/16 01, E2	3.0	0.060	0.014	4.16	0.73
07/11/16 02, E1	3.0	0.059	0.011	5.32	0.24
07/11/16 02, E2	3.0	0.059	0.014	4.21	0.75
07/11/16 03, E1	3.9	0.079	0.020	4.02	0.52
07/11/16 03, E2	1.6	0.033	0.009	3.68	0.48
07/11/16 04, E1	3.3	0.065	0.017	3.92	0.23
07/11/16 04, E2	3.4	0.069	0.018	3.87	0.81
07/12/16 01, E1	4.0	0.079	0.015	5.30	0.27
07/12/16 01, E2	3.5	0.069	0.021	3.22	0.531
07/12/16 02, E1	3.5	0.070	0.012	5.57	0.28
07/12/16 02, E2	3.4	0.068	0.022	3.08	0.66
07/12/16 03, E1	2.6	0.052	0.008	6.21	0.43
07/12/16 03, E2	3.3	0.056	0.015	4.43	0.70
07/12/16 04, E1	2.4	0.040	0.000	109.95	0.27
07/12/16 04, E2	2.4	0.047	0.001	86.18	0.40
07/12/16 05, E1	2.3	0.069	0.018	3.87	0.81
07/12/16 05, E2	3.0	0.060	0.025	2.37	1.88
07/12/16 06, E1	2.2	0.044	0.008	5.36	0.99
07/12/16 06,	2.1	0.043	0.008	5.22	4.32

E2					
07/15/16 01, E1	4.3	0.087	0.023	3.76	0.48
07/15/16 01, E2	3.7	0.074	0.025	2.97	0.62
07/15/16 02, E1	5.1	0.102	0.044	2.34	0.30
07/15/16 02, E2	3.9	0.077	0.029	2.68	0.64
07/18/16 01, E1	2.9	0.059	0.021	2.84	0.20
07/18/16 01, E2	1.9	0.039	0.010	3.81	0.86
07/18/16 02, E1	3.1	0.063	0.017	3.62	0.28
07/18/16 02, E2	2.6	0.052	0.008	6.31	0.52
07/18/16 03, E1	3.0	0.059	0.024	2.48	0.33
07/18/16 03, E2	2.7	0.055	0.015	3.71	0.58
07/18/16 04, E1	1.9	0.037	0.016	2.34	0.25
07/18/16 04, E2	1.9	0.038	0.008	4.46	0.51
07/18/16 05, E1	1.9	0.038	0.007	5.22	0.37
07/18/16 05, E2	2.8	0.056	0.013	4.40	0.13
07/18/16 06, E1	1.7	0.033	0.008	4.10	0.46
07/18/16 06, E2	2.6	0.051	0.013	3.83	0.59
07/18/16 07, E1	6.1	0.123	0.059	2.09	0.42
07/18/16 07, E2	4.4	0.088	0.041	2.12	0.60
07/19/16 01, E1	3.7	0.074	0.031	2.38	0.51
07/19/16 01, E2	4.8	0.095	0.044	2.17	0.75
07/19/16 02, E1	2.2	0.044	0.007	6.5	0.56
07/19/16 02, E2	3.1	0.062	0.026	2.41	0.74
07/19/16 03,	2.9	0.058	0.019	3.04	0.91

E1					
07/19/16 03, E2	3.3	0.067	0.014	4.71	0.81
07/19/16 04, E1	2.3	0.046	0.009	4.81	0.24
07/19/16 04, E2	1.7	0.034	0.019	1.74	0.66
07/19/16 05, E1	7.9	0.158	0.086	1.84	0.46
07/19/16 05, E2	8.6	0.172	0.071	2.41	0.40
07/19/16 06, E1	2.7	0.055	0.008	7.20	0.56
07/19/16 06, E2	2.0	0.040	0.006	6.87	0.39
07/20/16 01, E1	2.3	0.037	0.005	7.56	0.25
07/20/16 01, E2	1.9	0.038	0.015	2.62	0.41
07/20/16 02, E1	3.9	0.078	0.045	1.74	0.43
07/20/16 02, E2	2.8	0.057	0.017	3.35	0.43
07/21/16 01, E1	2.9	0.058	0.019	3.06	0.43
07/21/16 01, E2	2.7	0.054	0.005	10.48	0.46
07/21/16 02, E1	3.3	0.065	0.014	4.66	0.68
07/21/16 02, E2	1.9	0.037	0.004	8.77	0.37
07/21/16 03, E1	3.8	0.075	0.020	3.80	0.26
07/21/16 03, E2	4.1	0.083	0.026	3.17	0.33
07/21/16 04, E1	4.2	0.085	0.033	3.54	0.66
07/21/16 04, E2	1.6	0.033	-0.011	-2.88	2.87
07/21/16 05, E1	47.7	0.955	0.700	1.36	0.69
07/21/16 05, E2	2.1	0.042	0.011	3.77	1.99
07/21/16 06, E1	3.3	0.065	0.021	3.18	0.50
07/21/16 06,	1.7	0.034	0.005	7.47	0.92

E2					
07/21/16 07, E1	4.3	0.086	0.037	2.37	0.51
07/21/16 07, E2	1.2	0.025	0.008	3.23	0.70
07/21/16 08, E1	3.0	0.060	0.021	2.89	1.23
07/21/16 08, E2	3.3	0.066	0.015	4.47	0.31
07/26/16 01, E1	2.3	0.045	0.022	2.07	0.72
07/26/16 01, E2	2.8	0.057	0.014	3.91	0.45
07/26/16 02, E1	3.6	0.072	0.019	3.87	0.46
07/26/16 02, E2	1.4	0.028	0.007	4.11	1.02
07/26/16 03, E1	2.9	0.057	0.019	3.06	0.55
07/26/16 03, E2	2.2	0.045	0.005	8.20	0.61
07/27/16 01, E1	3.9	0.059	0.015	3.91	0.55
07/27/16 01, E2	0.9	0.019	0.015	1.27	0.20
07/27/16 02, E1	4.2	0.084	0.024	3.57	2.19
07/27/16 02, E2	1.2	0.024	0.006	4.28	0.18
08/01/16 01, E1	3.5	0.069	0.021	3.23	1.34
08/01/16 01, E2	3.2	0.064	0.015	4.37	0.90
08/02/16 01, E1	313.9	6.277	4.372	1.44	0.70
08/02/16 01, E2	4.6	0.092	0.031	2.96	0.35
08/02/16 02, E1	4.9	0.098	0.045	2.16	0.36
08/02/16 02, E2	4.7	0.094	0.029	3.25	1.84
08/02/16 03, E1	4.2	0.085	0.040	2.13	0.37
08/02/16 03, E2	1.7	0.034	0.028	1.22	0.59
08/02/16 04,	1.5	0.030	0.009	3.24	0.50

E1					
08/02/14 04, E2	0.9	0.018	0.006	2.92	1.56
08/03/16 01, E1	4.5	0.090	0.032	2.80	0.21
08/03/16 01, E2	2.3	0.046	0.012	3.93	0.38
08/03/16 02, E1	2.8	0.055	0.030	1.87	0.32
08/03/16 02, E2	0.9	0.018	0.008	2.24	0.58
08/03/16 03, E1	4.0	0.080	0.042	1.93	0.36
08/03/16 03, E2	1.0	0.020	0.017	1.12	0.39
08/09/16 01, E1	3.7	0.074	0.024	3.05	0.42
08/09/16 01, E2	2.2	0.045	0.009	4.74	0.70
08/09/16 02, E1	3.8	0.076	0.030	2.55	0.59
08/09/16 02, E2	4.0	0.079	0.025	3.14	0.64
08/10/16 01, E1	6.5	0.131	0.080	1.64	0.38
08/10/16 01, E2	4.2	0.084	0.028	3.06	0.72
08/10/16 02, E1	4.1	0.083	0.029	2.83	0.45
08/10/16 02, E2	3.1	0.062	0.024	2.54	0.73
08/12/16 01, E1	3.5	0.070	0.024	2.90	0.70
08/12/16 01, E2	3.1	0.061	0.013	4.56	0.50
08/12/16 02, E1	7.3	0.146	0.076	1.93	0.27
08/12/16 02, E2	2.7	0.055	0.020	2.79	0.69
08/12/16 03, E1	4.2	0.057	0.010	5.48	0.28
08/12/16 03, E2	2.8	0.084	0.013	6.39	0.35
08/12/16 04, E1	7.7	0.153	0.067	2.28	0.25
08/12/16 04,	4.4	0.088	0.024	3.63	0.32

E2					
08/15/16 01, E1	4.7	0.095	0.034	2.76	0.30
08/15/16 01, E2	4.5	0.091	0.036	2.55	0.39
08/15/16 02, E1	2.5	0.049	0.008	5.93	0.74
08/15/16 02, E2	2.7	0.054	0.010	5.44	0.43
08/15/16 03, E1	3.1	0.062	0.014	4.45	0.38
08/15/16 03, E2	2.4	0.049	0.005	10.59	0.74
08/15/16 04, E1	5.3	0.074	0.031	2.40	0.54
08/15/16 04, E2	3.7	0.105	0.033	3.22	0.34

Vita

Cameron Brocco was born in Winter Park, Florida and graduated from Pope John Paul II High School. He has one older brother. He continued his education as a Preprofessional Biology major with a Chemistry minor at UTC and will graduate in December of 2017. Cameron joined the Innovations in Honors Program in the fall of 2014 and joined Team Salamander in the fall of 2014. He plans begin Chiropractic School in the winter of 2018. This thesis was completed as part of the Innovations in Honors Program, a subdivision of the UTC Honors College.