DETERMINING HOW ENVIRONMENTAL CHANGES IMPACT GROWTH OF *BATRACHOCHYTRIUM DENDROBATIDIS* USING A NOVEL *IN VITRO* SYSTEM

By

Amanda D. Layden, B.S.
East Stroudsburg University of Pennsylvania

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of Master of Science in Biology
to the office of Graduate and Extended Studies of
East Stroudsburg University of Pennsylvania

May 8, 2020

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ABSTRACT

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology to the Office of Graduate and Extended Studies of East Stroudsburg University of Pennsylvania.

Student's Name: Amanda D. Layden, B.S.

Title: Determining how Environmental Changes Impact Growth of *Batrachochytrium dendrobatidis* Using a Novel *In Vitro* System

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Thesis Chair: Joshua Loomis, Ph.D.

Thesis Member: William Loffredo, Ph.D.

Thesis Member: Emily Rollinson, Ph.D.

Abstract

Chytridiomycosis, caused by the etiologic agent *Batrachochytrium dendrobatidis*, affects the keratinocytes of the amphibian epithelium. While there have been several studies done on *B. dendrobatidis* both *in vivo* and *in vitro*, there is still little known about what environmental factors influence the growth of this fungus. To better understand such factors, a novel, high-throughput *in vitro* system was developed that utilized tissue culture plates as a submerged in vitro substrate. After analyzing *B. dendrobatidis's* life cycle in this new system, studies were conducted to determine the impact of pH, phosphate and nitrate concentration, and protein concentration on its growth. Results showed that *B. dendrobatidis* completed its life cycle in submerged tissue culture wells and that growth rates were sensitive to concentrations of protein and environmental pH. Results suggest that *B. dendrobatidis* can regulate its growth kinetics depending on access to environmental nutrient sources.

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Chapter I
Introduction

Chytridiomycosis is an emerging infectious wildlife disease that affects the keratinized epidermal cells of the amphibian epithelium. The etiologic agent that causes this disease is *Batrachochytrium dendrobatidis,* a spherical-shaped fungus that is found in a variety of water sources and moist soil environments. *B. dendrobatidis* taxonomically falls in the Phylum Chytridiomycota, Class Chytridiomycetes, and Order Rhizophydiales (Figure 1). Chytridiomycota (chytrids) is the only phylum of true Fungi that reproduces with posteriorly uniflagellate, motile spores (zoospores)[48,97.]. The order Rhizophydiales was formed on the basis of molecular monophyly and zoospore ultrastructure, in which three new families and two new genera were delineated[60]. Of these three families, the Rhizophydiaceae includes a species known as *Rhizophydium globosum*, which has been included in numerous chytrid inventories[35,55,59,60,72,104]. *R. globosum* is sparsely described as having a spherical sporangium with 2-4 discharge papillae and occurs as a parasite on *Closterium* and other algal hosts[16,60]. Although *B. dendrobatidis* has not been officially assigned a taxonomic family, there are other chytrids in the order Rhizophydiales that act as a parasite on other organisms.

B. dendrobatidis is the only known parasitic chytrid fungus of vertebrates. It has been implicated as the main factor in severe amphibian population declines and has been confirmed on every major continent except Antarctica (where amphibian fauna are not present)[32] (Figure 2). *B. dendrobatidis* infects over 350 amphibian species and has been implicated in driving the decline of over 200 of them[32,103]. Some of these affected species have been categorized as critically endangered (CR), endangered (EN) and in some cases extinct (EX) on the IUCN Red List as a result of this emerging disease[4,5,7,13,37,62,63,73,86,89,100,110,111] (Table 1).

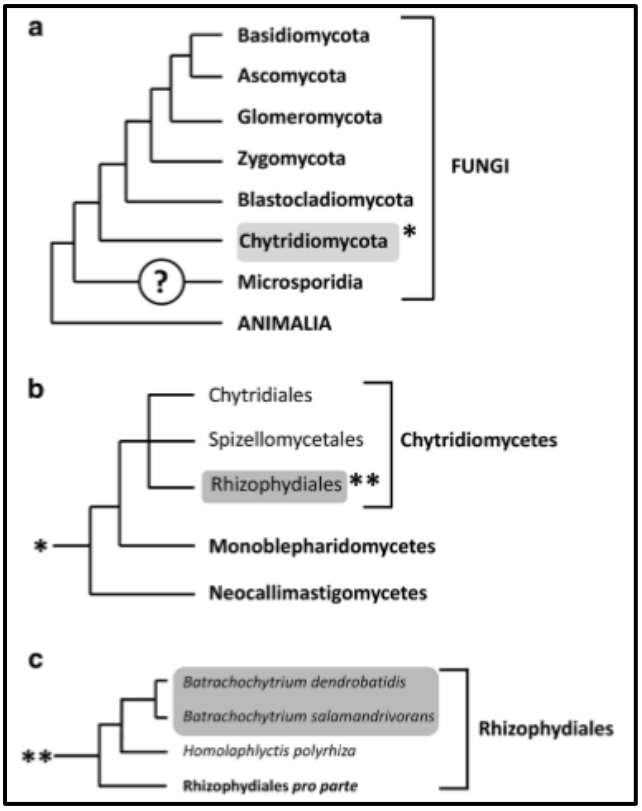


Figure 1. Cladogram indicating taxonomy of *B. dendrobatidis* showing that it falls in the a) kingdom Fungi, b) phylum Chytridiomycota and c) order Rhizophydiales. (Adapted from Van Rooij et al. 2015: the topology is derived from Martel et al. 2013, Longcore et al. 1999 and Hibbett et al. 2007)[45,66,69,113]

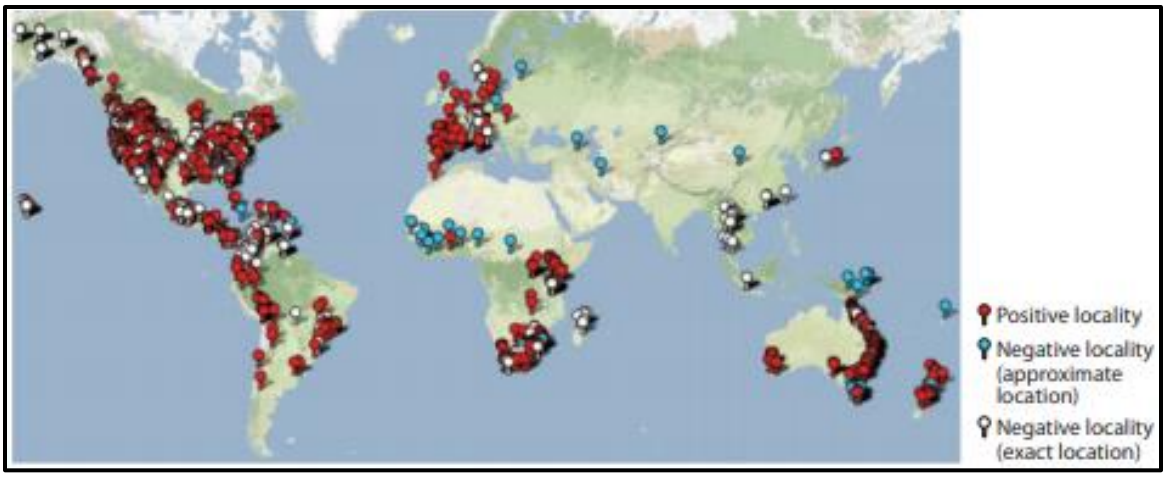


Figure 2. Worldwide distribution of *B. dendrobatidis*. (Adapted from Fisher et al. 2009)[32]

Table 1. Examples of affected wild amphibian species due to chytridiomycosis[4]

Species	**Common Name**	**Habitat**	**Mechanism**	**Conservation Status (IUCN red list)**	**Reference**
Rheobatrachus vitellinus	Eungella gastric-brooding frog	Australia	Pathogenic	Extinct (EX) between 1985-1986	Retallick, et al. 2004
Atelopus zeteki	Panamanian golden frog	Panama	Pathogenic	Still listed as Critically Endangered (CR); but most likely extinct (EX)	Gewin, 2008
Taudactylus acutirostris	Sharp-snouted day frog	Australia	Pathogenic	Extinct: between 1993-1994	Schloegel et al. 2005
Anaxyrus boreas	Western toad	USA	Pathogenic	Near threatened (NT)	Muths et al. 2003
Atelopus chiriquiensis	Chiriqui harlequin frog	Costa Rica	Pathogenic	Critically endangered (CR)	Berger et al. 1998; Lips, 1999
Euproctus playtcephalus	Sardinian brook salamander	Italy	Pathogenic	Endangered (EN)	Bovero et al. 2008

Gastrotheca cornuta	Horned marsupial frog	Costa Rica, Panama, Ecuador, Columbia	Pathogenic	Endangered (EN)	Lips et al. 2006
Leiopelma archeyi	Archey's frog	New Zealand	Pathogenic	Critically Endangered (CR)	Bell et al. 2004
Rana muscosa	Mountain yellow-legged frog	USA; California	Pathogenic	Endangered (EN)	US Fish and Wildlife Service, 1999; Rachowisz et al. 2006
Eleutherodactylus jasperia	Golden coqui	Puerto Rico	Pest and disease transmission	Critically Endangered (CR)	US Fish and Wildlife Service, 2013

The evidence implicating *B. dendrobatidis* in the amphibian declines is compelling. Firstly, chytrid fungus can be pathogenic to amphibians in both the field and the laboratory[7,79]. A study done by Berger et al. utilized experimental transmission of cutaneous chytridiomycosis on captive-bred sibling frogs (*Mixophyes fasciolatus*)[7]. The sample was taken from a dead frog of the same species that had naturally acquired the infection[7]. In the results, it was noted that chytrid sporangia were seen during histological examination of the captive-bred sibling *M. fasciolatus* frogs[7]. Furthermore, they concluded that chytrids are associated with a transmissible fatal disease of anurans in the field and in the laboratory[7].

Secondly, there is genetic evidence suggesting the emergence of a hypervirulent strain of chytrid fungus that shows genetic signal consistent with range expansion[29,49,79]. A study done by Farrer et al. collected samples of *B. dendrobatidis* isolates from locations on every continent except Antarctica and found that there was a much greater

diversity of *B. dendrobatidis* than was previously recognized[29]. They also noted that multiple lineages were being vectored between continents by the trade of amphibians[29]. One of those lineages, (BdGPL=global panzootic lineage) had been characterized with hypervirulence, suggesting that the emergence and spread of chytridiomycosis is largely due to the globalization of the recently emerged recombinant lineage[29,31]. Ultimately, the researchers concluded that the global trade in amphibians is resulting in contact and cross-transmission of *B. dendrobatidis* among previously naïve host species which resulted in intercontinental pathogen spread and an increase in recombinant genotypes generated[29].

Lastly, amphibian population declines appear to have followed a broad wave-like pattern consistent with the spread of a novel pathogen[63,64,79]. A study done by Lips et al. discussed analyses supporting a classical pattern of disease spread across naïve populations (at odds with the CLEH (climate-linked epidemic hypothesis) proposed by Pounds et al., 2006) and how their analyses cast doubt on CLEH[64,81]. In their results, they found evidence of directional spread of *B. dendrobatidis* along most of the principal cordilleras of Lower Central America and the Andean region, supporting the hypothesis that this is an exotic pathogen that was introduced into South America in the late 1970s-early 1980s and has caused multiple amphibian declines in the past 30 years[20,58,63,64,68,93]. One of these declines (the 1987 amphibian decline at Monteverde Cloud Forest Reserve in Costa Rica) is widely assumed to have been caused by an outbreak of *B. dendrobatidis*; however, direct evidence does not exist[64]. Prevalence of *B. dendrobatidis* was noted in 2003, indicating that the pathogen is now endemic to that area[64]. The researchers examined museum specimens for evidence of *B. dendrobatidis* prior to 1986

and found that most of the specimens showed histological evidence of *B. dendrobatidis* infection[64]. Ultimately, their analyses supported a hypothesis that *B. dendrobatidis* is an introduced pathogen that spreads from its point of origin in a pattern typical of emerging infectious diseases[64].

What is a Wildlife Disease?

A wildlife disease can be defined as a pathological condition occurring in a susceptible population in nature. Emerging infectious diseases (EIDs) of free-living wild animals can be classified into three major groups on the basis of key epizootiological criteria. The first group involves EIDs associated with "spill-over" from domestic animals to wildlife populations living in proximity. The second group involves EIDs related directly to human intervention, via host or parasite translocations. The final group of EIDs is related with no overt human or domestic animal involvement[24]. These diseases have two major biological implications: first, many wildlife species are reservoirs of pathogens that threaten domestic animal and human health, and second, wildlife EIDs pose a substantial threat to the conservation of global biodiversity[24].

The USGS National Wildlife Health Center (NWHC) works to safeguard our nation's wildlife from diseases by studying their causes and by developing strategies to prevent and manage them[75]. Aside from chytridiomycosis, other wildlife diseases exist and have not only caused devastating declines in wildlife populations globally but have also caused issues in human populations. An example of a wildlife disease that has caused issues in human populations is Lyme disease. Lyme disease is spread by the

blacklegged tick (*Ixodes scapularis*) and the CDC estimates reports of approximately 30,000 confirmed cases each year[25]. There are many wildlife diseases aside from Chytridiomycosis that cause harm to populations found in nature; however, the top three are Chytridiomycosis, White-Nose Syndrome, and Snake Fungal Disease (SFD). White-Nose Syndrome affects all life stages of hibernating bats, and mortality at newly-affected hibernacula can be very high, resulting in substantial and rapid decreases in bat abundance[33,82]. Millions of North American bats have died from this disease, and population declines for heavily impacted species could result in regional extirpation of some previously common species such as the little brown bat (*Myotis lucifugus*) and northern long-eared bat (*M. septentrionalis*)[17,28,33,34,82,90,106]. Snake Fungal Disease (SFD) has been confirmed in numerous species of snakes and is caused by the fungus *Ophidiomyces ophiodiicola*[107]. As of August 2017, this fungus has been detected in at least 23 states and one Canadian province; however, researchers suspect that SFD may be more widely distributed due to limitations in monitoring snake populations[107]. Studying disease ecology in wildlife can be challenging but understanding wildlife epidemiology is important for the benefit of human health, animal welfare, productivity in agricultural systems, and global biodiversity[24,26,70,122].

A similar factor between many emerging wildlife diseases is that the global trade of wildlife provides disease transmission mechanisms for these pathogens[54]. Outbreaks resulting from wildlife trade have caused hundreds of billions of dollars of economic damage globally[54]. For instance, white-nose syndrome is hypothesized to have been introduced to North America from Europe or Asia[33,82]. Since there is no bat migration occurring between North America and Europe, it is very likely that this fungus was

introduced to North America from global movement of humans, animals, and trade[120]. Similarly, examination of historical fungal isolates has demonstrated that *O. ophiodiicola* was present in captive snakes in the eastern USA since at least 1986[67,102]. Furthermore, no wild snake isolates are known prior to 2008, indicating that introduction by spillover of *O. ophiodiicola* from captive to wild snake populations represents a plausible explanation for the sudden emergence of SFD[67]. In regard to Chytridiomycosis, the global trade of a specific species of anuran has enabled *B. dendrobatidis* to be transmitted throughout the world.

Origin and Dissemination

Discovering the origin of an infectious disease is critical for determining how to prevent and treat it. To date, the origin of *B. dendrobatidis* is something still argued by herpetologists, mycologists, and epidemiologists around the world. The earliest case of chytridiomycosis was recorded in 1938 from an African clawed frog (*Xenopus laevis*) in southern Africa[119] (Figure 3). Chytridiomycosis was a stable, endemic infection in southern Africa for 23 years before any positive specimens were found outside of Africa[119]. Some emerging infectious diseases arise when pathogens that have been localized to a single host or small geographic region go beyond previous boundaries and according to research; it is highly likely that this is how *B. dendrobatidis* emerged as well[119]. African clawed frogs are considered natural carriers of *B. dendrobatidis* and are not overly susceptible to its disease symptoms. After 23 years of globally trading African clawed frogs for educational and research purposes, the first case of chytridiomycosis

outside of Africa was noted in North America in 1961, specifically in Quebec, Canada[114,119]. After the case in Canada, the earliest cases from other countries follow sequentially over a period of 38 years from 1961 to 1999[119] (Figure 3).

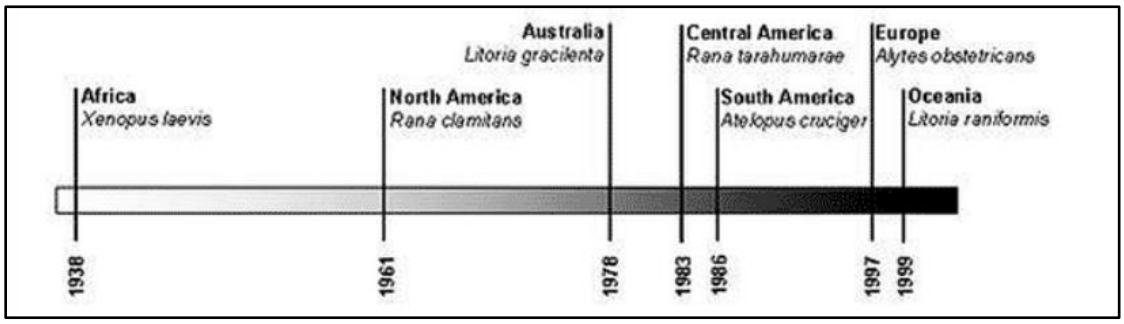


Figure 3. Time bar showing the first occurrence of chytridiomycosis in Africa in 1938, the first occurrence outside of Africa in 1961, (Quebec, Canada, North America) and records outside of Africa following the 23-year gap. (Adapted from Weldon et al. 2004, Quellet 2003, Berger 1999, Speare 2001, Bonaccorso 2003, Rollins-smith 2002, Bosh 2000, Waldman 2001.)[8,11,12,84,92,105,118,119]

Life Cycle

The life cycle of *B. dendrobatidis* begins with a motile zoospore and is approximately 4-5 days. Once the zoospore attaches to a substrate, it morphologically changes into a growing organism called a thallus. Once matured, the thallus body grows into a single zoosporangium (container for zoospores)[6] (Figure 4). The contents of the zoosporangium (also known as the sporangium) cleave into new zoospores which exit the sporangium through one or more discharge papillae (also called discharge tubes)[6].

While sexual reproduction has not been seen in this organism to date, there is another variation in the life cycle known as 'colonial development' resulting from the formation of more than one sporangium from one zoospore[66]. Zoosporangia undergoing colonial development have a septum dividing the contents of the zoosporangium. The life

cycle of this fungus has been found to be the same in culture (*in vitro*) as it is on amphibian skin (*in vivo*)[9,66].

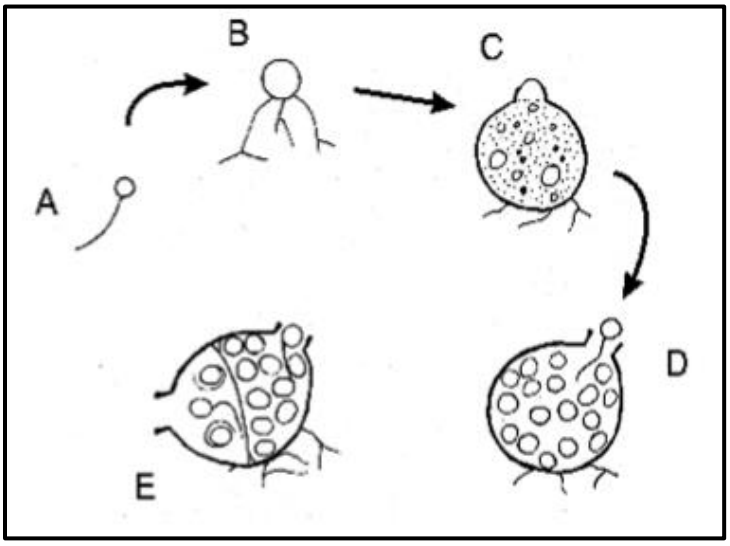


Figure 4. Life cycle of *B. dendrobatidis* in culture: A=zoospore, B=germling, C=mature zoosporangium, D=monocentric zoosporangium, E=colonial zoosporangium with a dividing septum. (Adapted from Berger et al. 2005)[6]

Overview of Morphology

Zoospore and Germling

Zoospores are the waterborne, motile stage of the life cycle. Zoospores of *B. dendrobatidis* are unwalled and mostly spherical shaped but can also be elongate and amoeboid when they are first released from the zoosporangium[6,66]. The zoospores are approximately 3-5 µm in diameter with a posteriorly directed flagellum[66]. Zoospore ultrastructure is used to differentiate orders and genera among the Chytridiomycota. The features of the zoospore of *B. dendrobatidis* that are common to the order Chytridiales are that the nucleus and kinetosome are not associated, ribosomes are aggregated into a core surrounded by endoplasmic reticulum, the microbody partially surrounds the lipid

globules, and the nonflagellated centriole (NFC) is parallel and connected to the kinetosome[65,66]. A key feature of *B. dendrobatidis* is the numerous small lipid droplets with the microbodies that are associated with the edge of the ribosomal mass[66] (Figure 5). Additionally, *B. dendrobatidis* is aneuploid, with copy numbers of the chromosomal regions (contigs) within a single isolate running up to 5[30,94,101,113]. After a period of motility and dispersal, the zoospore encysts, the flagellum is resorbed, and a cell wall forms[6]. Once the zoospore has encysted, fine branching rhizoids grow from one or more areas of the zoospore and it is then known as a germling[6].

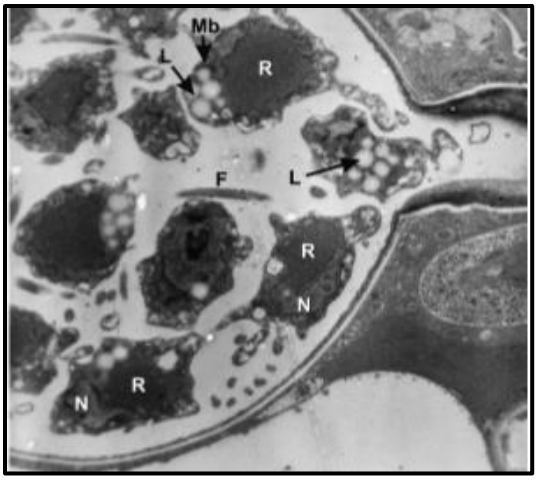


Figure 5. Image showing a formalin-fixed *B. dendrobatidis* zoospore with multiple small lipid droplets (L) taken from the skin of a Cane toad (*Bufo marinus*) (N = nucleus, R = ribosomes, Mb = microbody, L = lipid droplet) (Adapted from Berger et al. 2005)[6]

Developing Zoosporangia

As the germling develops, the thallus grows and the cytoplasm becomes more complex[6]. As this occurs, the thallus becomes multinucleate by mitotic divisions[6]. The contents then cleave and mature into rounded, flagellated zoospores[6]. At this point, the

swollen part of the thallus is now known as a zoosporangium[6]. Simultaneously, one or more discharge papillae (tubes that stick out away from the zoosporangium that aid in zoospore release) form. Some thalli that undergo colonial growth become divided by thin septa and each compartment grows into a separate sporangium with its own discharge tube[6]. These mature zoosporangia contain fully formed flagellated zoospores[6]. Zoospores are released when the plug blocking the discharge tube is dissolved. Once all the zoospores are released, it is considered an empty sporangium. The chitinous walls of the sporangia remain and may eventually collapse. Sometimes, zoospores do not escape and grow within the sporangia[6].

Optimal Growth Environment

Growth and survival of *B. dendrobatidis* is dependent on many environmental factors. Optimal growth of *B. dendrobatidis* is observed between 17 and 25°C and at pH 6-7 *in vitro* (agar and broth culture) which is similar to what is observed in amphibian skin *in vivo* and in the environment[80,113]. *B. dendrobatidis* grows slowly at 10°C and ceases growth at 28°C or higher[50,80,113]. Additionally, *B. dendrobatidis* zoospores are killed within four hours at 37 °C[50,80,113]. Desiccation is poorly tolerated as this species requires wet or moist environments[36,50,113]. It has also been noted that 5% NaCl solutions are lethal to this pathogen[36,50,113]. *In vitro*, *B. dendrobatidis* has been shown to grow on a variety of keratin containing substrates such as autoclaved snakeskin, 1% keratin agar, frog skin agar, feathers and geese paws[36,66,80,113]. *B. dendrobatidis* can also grow on chitinous carapaces of crustaceans[71,113]. Although *B. dendrobatidis* grows well on these

substances, it grows best in tryptone or peptonized milk in both agar and broth *in vitro*[66,113].

The type of growth system used for studying *B. dendrobatidis* ultimately depends on the research questions under investigation. An *in vitro* system would be ideal for studying specific environmental factors on the growth of *B. dendrobatidis* because the variables can be easily manipulated. In contrast, an *in vivo* study involving specific environmental factors would be difficult because not all individual amphibians from the same species are exactly the same in regard to their immune system, skin microbiome, or other host defenses. Studies that require specific pathogen-host interactions can best be observed using an *ex vivo* or *in vivo* approach to obtain specific host defense data.

Transmission and Clinical Signs

In terms of virulence, *B. dendrobatidis* has an extremely broad host range, infecting at least 520 species of anurans (frogs and toads), urodeles (salamanders and newts) and caecilians[39,113]. Transmission among hosts is typically due to infection of motile waterborne zoospores or through direct contact with infected amphibians (ex. during mating)[97,113]. Another factor involving *B. dendrobatidis*'s virulence is that it can survive in water and moist soil for weeks up to several months, which makes it hard for amphibians to not become infected once they have entered an infected water source[51,52,113]. Additionally, *B. dendrobatidis* is able to saprobically grow on sterile bird feathers, arthropod exoskeletons, keratinous paw scales of waterfowl and can survive in the gastrointestinal tract of crayfish[36,51,52,66,71,113]. Being able to grow on many different

substances also increases this pathogen's spreading capability and increases its chances of being transmitted to a new host.

In anuran larvae, clinical signs of chytridiomycosis are generally limited to depigmentation of the mouthparts, low foraging, lethargy, and poor swimming abilities[7,86,113]. Although this does not cause mortality, chytridiomycosis can commonly contribute to reduction in anuran larvae body size[43,113]. In metamorphized amphibians, clinical signs are variable and range from significant skin disorder to sudden death without obvious disease symptoms[113]. The most common signs of chytridiomycosis are excessive shedding of the skin, erythema (redness), or discoloration of the skin[78,113] (Figure 6). In frogs and toads, the skin of the ventral abdomen, especially the pelvic patch, feet and toes, are predilection sites of infection[9,83,113]. In contrast, salamanders are more prone to infection in the pelvic region, fore and hind limbs and the ventral side of the tail[113,114]. Other clinical signs of chytridiomycosis include lethargy, anorexia, abnormal posture, and neurological signs such as loss of righting reflex and flight response[78,113].

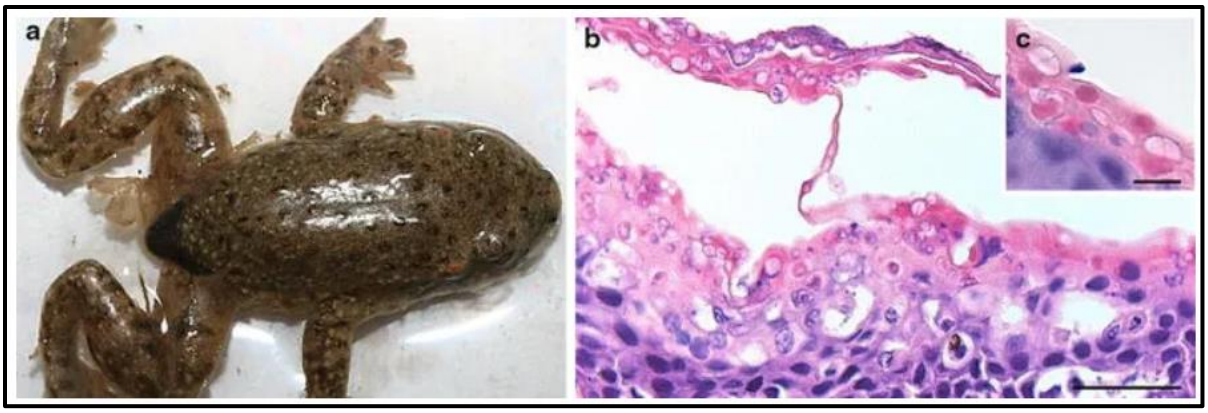


Figure 6. Clinical signs of chytridiomycosis. a) naturally infected moribund common midwife toad (*Alytes obstetricans*) with abduction of the hind legs and loose sloughed skin. b) section through the ventral skin (drink patch) of the same infected toad showing epidermal hyperkeratosis and hyperplasia combined with the presence of numerous zoosporangia. c) detail of intracellular septate zoosporangia. (Adapted from Pessier 2008)[78]

Pathology and Pathogenesis

In metamorphized amphibians, chytridiomycosis caused by *B. dendrobatidis* is diagnosed by the presence of immature chytrid thalli or maturing sporangia found intracellularly in the keratinized layers of the amphibian skin[113]. Infection is associated mainly with a mild to severe irregular thickening of the outermost keratinized layers of the epidermis (hyperkeratosis of the stratum corneum and stratum granulosum)[113]. Infection can also cause erosion of the stratum corneum and increased tissue growth (hyperplasia) of the stratum spinosum, which lies beneath the keratinized superficial skin layers[113]. Dissemination to the deeper layers of the skin or internal organs does not occur[78,113]. Instead, amphibian mortality is caused by *B. dendrobatidis* disrupting normal regulatory function of their skin[96]. Infection in anuran larvae is limited to the keratinized mouthparts[78,113]. It is only when the anuran larvae undergoes metamorphosis that the infection is able to spread to the epithelia of the body, limbs, and tail.

With the availability of *B. dendrobatidis*'s full genome, genetic studies have led to an improved understanding of host-pathogen dynamics and the identification of several putative pathogenicity factors with high specificity for skin-related substrates, facilitating colonization or causing host damage[113]. Nevertheless, processes that take place during the whole infection cycle at a molecular and cellular level such as cell signaling, induction of cytoskeletal change and so on are still barely understood and require more attention[113].

Immune Defenses Against *B. dendrobatidis*

Innate and acquired immune components both contribute to the antimicrobial function of amphibian mucus[113]. Firstly, amphibians produce antimicrobial peptides in their dermal glands to act as an innate immune defense mechanism[113]. To date, approximately forty anuran antimicrobial peptides inhibiting *B. dendrobatidis* have been characterized[91,113]. Both purified and natural mixtures of these antimicrobial peptides effectively inhibit *in vitro* (broth and agar) growth of *B. dendrobatidis* zoospores and sporangia[87,91,113,123]. Although these antimicrobial peptides have been found to inhibit growth of *B. dendrobatidis in vitro*, it is unclear how these peptides provide protection against chytridiomycosis *in vivo*[113]. Another innate immune defense mechanism against chytridiomycosis is antifungal metabolites secreted by symbiotic bacteria present on amphibian skin[113]. To date, there have been only 3 inhibitory metabolites identified by the symbiotic bacterial species *Janthinobacterium lividum*, *Lysobacter gummosus, and Pseudomonas fluorescens*[18,113]. These natural metabolites are known as 2,4-DAPG (2,4-diacetylphloroglucinol), indol-3-carboxaldehyde (I_3C) and violacein[18,113]. These

metabolites can inhibit growth of *B. dendrobatidis* both *in vitro a*nd *in vivo*[18,57,74,113]. Myers et al. discovered that these metabolites work synergistically with antimicrobial peptides to inhibit growth of *B. dendrobatidis* at lowered minimal inhibitory concentrations necessary for inhibition by either metabolites or antimicrobial peptides[74,113]. In addition, 2,4-DAPG and I_3C seem to repel *B. dendrobatidis* zoospores[57,113]. A final innate immune defense mechanism with fungicidal potential in amphibian skin mucus is lysozyme; however, this has not been studied in detail[91,113]. Bacterial cells contain two alternating amino acids sugars, N-acetylglucosamine (GlcNAc or NAGA) and N-acetylmuramic acid (MurNAc or NAMA), which are connected by a β-1,4-glycosidic bond[113]. Lysozyme catalyzes bacterial cell lysing of the β-1,4 bonds of peptidoglycan, a polymer of *N*-acetylmuramic acid (GlcNAc) that is found in their cell wall[113]. Since the fungal cell wall consists mainly of chitin, a similar polymer consisting of β-1,4 linked GlcNAc units, it is also a potential target for lysozyme[113].

In contrast, the acquired immune system provides very specific protection against pathogens and involves both cell-mediated and humoral antibody responses. However, many researchers have become confused because of the apparent absence of a robust immune response in susceptible amphibian species[113]. So far, attempts to immunize frogs using subcutaneous or intraperitonial injection of formalin or heat-killed *B. dendrobatidis* failed to elicit an acquired immune response[113]. Only in *X. laevis, B. dendrobatidis* specific IgM, IgX (mammalian IgA-like) and IgY (mammalian IgG-like) antibodies were found in skin mucus after injection with heat-killed zoospores[87,113]. According to Ramsey et al., the mucosal antibodies elicited in *X. laevis* frogs bind with *B. dendrobatidis* zoospores *in vitro* and are suggested to limit colonization of the skin to mild and non-

lethal infections; however, their contribution to actual protection is still undetermined[87,113]. Rollins-Smith et al. observed that as *B. dendrobatidis* infections naturally occur in the skin, it seems likely that introduction of *B. dendrobatidis* antigens directly into the skin may be more effective, but more research needs to be done on this topic[91,113]. Despite this, susceptible amphibians still acquire this disease indicating that this fungus can withstand the host immune defenses.

Attachment and Colonization of Amphibian Skin

B. dendrobatidis infection of amphibian skin begins with the attachment of motile zoospores to the host's skin (Figure 7). It is at this step when *B. dendrobatidis* interacts with the amphibian's mucus barrier (mucosome). The main components of mucus are mucins or mucin glycoproteins[113]. The mucosome may be able to reduce the infection load on the skin during the first 24 hours of exposure, which is critical for colonization and establishing skin infection[112,113]. At this point, the zoospores germinate and adhere to the host surface. To date, the mechanisms and kinetics of adhesion of *B. dendrobatidis* to amphibian skin have only received limited attention[113]. Adhesion has been documented to occur approximately 2-4 hours after exposure to zoospores[112]. After the zoospores have attached, they mature into thick walled cysts on the host epidermis and often cluster in foci of infection[113]. The cysts are anchored into the skin via fine fibrillar projections, rhizoids and some adhesion not yet determined. These fibrillar projections and adhesions are similar to fibrillar adhesins documented for pathogenic fungi affecting human skin (*Trichophyton mentagrophytes*)[113]. Several genes encoding proteins involved in cell

adhesion such as vinculin, fibronectin, and fasciclin have been identified in the *B. dendrobatidis* genome and are expressed more in sporangia than in zoospores[95,113]. *B. dendrobatidis* is also equipped with a chitin binding module (CBM18) that is hypothesized to facilitate survival on its amphibian host[113]. It is suggested that a key role of CBM18 involves pathogenesis and protection against host-derived chitinases[113]. CBM18 also allows *B. dendrobatidis* to attach to non-host chitinous structures (insect or crustacean exoskeletons) allowing vectored-disease spread[1,71,113].

Once the zoospore has encysted, invasion of the epidermis begins. In general, *B. dendrobatidis* develops endobiotically, with sporangia located inside the host cell. This is generally achieved within 24 hours after initial exposure[113]. Colonization is established from the extension of a germ tube (discharge papillae) arising from the zoospore cyst that penetrates the host cell membrane and enables transfer of genetic material (zoospore nucleus and cytoplasm) into the host cell[112,113]. The distal end of the germ tube becomes swollen and gives rise to a new intracellular chytrid thallus[113]. *B. dendrobatidis* continues to use this mechanism to spread to deeper skin layers. Older thalli develop rhizoid-like structures that spread to deeper skin layers[113]. At this point, they form a swelling inside the host cells in the deeper skin layers and give rise to new daughter thalli[113].

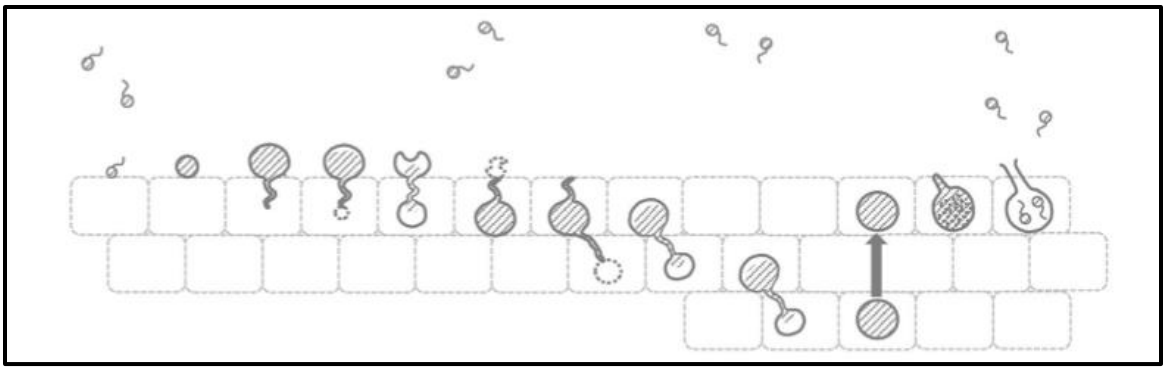


Figure 7. Image showing the infection cycle of *B. dendrobatidis* in a susceptible host. The lifecycle includes invasion mediated by a discharge tube, establishment of intracellular thalli, spreading to the deeper skin layers, and upward migration by the differentiating epidermal cell to finally release zoospores at the surface of the skin (Adapted from Berger at al. 2005, Van Rooij et al. 2012, and Greenspan et al. 2012)[6,40,112]

Environmental factors affecting growth of *B. dendrobatidis*

Changes to the chemical composition of an environmental water source have the potential to drastically alter the growth of microorganisms like *B. dendrobatidis*. For instance, the sudden introduction of nutrients such as nitrogen, phosphorus, and organic waste can trigger massive increases in microbial populations, which can have deleterious effects on the other aquatic life in that water source. Such changes can be caused by sewage infiltration, human pollution, or runoff. According to the USGS, runoff can be defined as the part of the precipitation, snow melt, or irrigation water that appears in uncontrolled water sources[98]. These water sources, surface streams, rivers, drains, or sewers, can be classified according to speed of appearance after rainfall or melting snow as direct runoff or base runoff[98]. Additionally, they can be classified according to source as surface runoff, storm interflow, or groundwater runoff[98]. When rain falls onto the landscape, it doesn't wait to be evaporated by the sun or used as a drinking source by the local wildlife. Instead, it begins to move slowly due to gravity[98]. Some of the rainwater

seeps into the ground to refresh groundwater, but most of it flows down gradient. This is known as surface runoff[98]. As watersheds are urbanized and much of the vegetation is replaced by impervious surfaces, groundwater infiltration is reduced and stormwater runoff increases[98]. Stormwater runoff must be collected by drainage systems and storm sewers that carry the runoff directly to streams.

Stormwater runoff that flows over the land surface can pick up potential pollutants that may include sediments, nutrients (from lawn fertilizers – nitrogen (N) and phosphorus (P)), bacteria (from animal and human waste), pesticides (from lawn/garden chemicals), metals (from rooftops and roadways), and petroleum by-products (from leaking vehicles)[98].

Nitrogen and Phosphorus

Nitrogen (N) and Phosphorus (P) are two important and essential nutrients for healthy soil and aquatic environments. According to the Environmental Protection Agency (EPA), nitrogen is generally used and reused by plants within natural ecosystems, with minimal "leakage" into surface or groundwater, where nitrogen concentrations remain very low[109,117]. However, when nitrogen is applied to the land in amounts greater than can be incorporated into crops or lost in the atmosphere through volatilization or denitrification, concentrations in soil and streams can cause environmental issues[109]. The major sources of excess nitrogen in streams and other agricultural watershed sources are fertilizer and animal waste[109]. Excess nitrate is not toxic to aquatic life, but increased nitrogen may result in overgrowth of microorganisms

like soil bacteria, soil fungi, and algae (known as algal blooms)[108]. This can decrease the dissolved oxygen content of the water, thereby harming or killing fish and other aquatic species[108]. Phosphorus is also an essential nutrient for all life forms, but at high concentrations the most biologically active form of phosphorus, phosphate, can cause water quality problems by also overstimulating the growth of microorganisms (similar to nitrogen). Elevated levels of phosphorus in streams can result from fertilizer used, animal wastes, and wastewater[109]. The EPA states that freshwater streams and ponds fall under one of five categories when looking at nitrate levels (mg/L): <1 mg/L, 1-2 mg/L, 2-6 mg/L, 6-10 mg/L, and 10 mg/L or more[109]. The EPA also states that for phosphate levels, freshwater streams and ponds fall under one of four categories: <0.1 mg/L, 0.1-0.3 mg/L, 0.3-0.5 mg/L, and 0.5 mg/L or more[109]. According to the EPA, the recommended water quality for freshwater ponds and streams consists of <1 mg/L nitrogen and <0.1 mg/L phosphorus[109].

Increased levels of nitrogen and phosphorus can also impact many soil microorganisms. Long-term application of fertilizers can affect the plant-soil-microbe system by changing the composition and structure of plant and soil microbial communities[47]. Increasing the availability of these nutrients can also cause changes in soil pH. This change can affect species richness by causing a decline of plants and soil microbes[47]. These effects can eventually cause issues with some of the nutrient cycles many organisms rely on. Nitrogen cycling in natural ecosystems and traditional agricultural production relies on biological nitrogen fixation primarily by diazotrophic bacteria and sometimes, under specific conditions, free-living bacteria such as cyanobacteria, *Pseudomonas, Asozpirillum,* and *Azobacter*[19,53,77]. Diazotrophic

community structure and diversity have been shown to respond to changes in the nature of nitrogen added and are also especially sensitive to chemical inputs such as pesticides[76,77]. Although there has been a lot of research that focuses on the effects of nitrogen and phosphorus on water chemistry, algae, and bacteria, little has been done to study the effects of these elements on soil fungi and *B. dendrobatidis* in particular.

Study Objectives

Today, we know that *B. dendrobatidis* has a complex interaction with amphibians and that the response of amphibians to this pathogen depends on many ecological, environmental, and genetic factors. While these early studies have shed some light on the pathogenesis of *B. dendrobatidis*, they have provided only a limited understanding of its basic physiological processes. One major limitation is that most experiments with *B. dendrobatidis* have been conducted either using a complex and relatively expensive *ex vivo* system that typically involves the use of isolated frog skin or *in vivo* experiments on amphibians themselves. This study will be the first to utilize a tissue culture system as a novel and cheaper alternative to growing the fungus *ex vivo* or *in vivo* and it will be the first to test the effect of nitrogen and phosphate levels on the growth rate of *B. dendrobatidis*. The objectives were to:

1. Create a new *in vitro* system using tissue culture plates that will attempt to simulate a submerged growth substrate
2. Validate the *in vitro* system using already published data from other *in vitro* and *ex vivo* studies

3. Determine if addition of protein or an excess of nitrogen or phosphorus have an effect on the growth rate of *B. dendrobatidis* using the new *in vitro* system

Chapter II
Materials and Methods

Obtaining *B. dendrobatidis* Strain JEL 423

The original sample of *B. dendrobatidis* was obtained from Dr. Joyce Longcore from the University of Maine Chytrid Laboratory. Isolates of *B. dendrobatidis* were aseptically transferred from 1% tryptone agar plates to 100mL of 1% tryptone broth media. The culture was placed at room temperature (21-23℃) for two weeks and was then stored at 4℃ for prolonged usage.

Cryo-preserving *B. dendrobatidis* Isolates

Isolates of *B. dendrobatidis* were cryo-preserved following the procedure by Boyle et al. 2003[14]. Freezing media was composed of 10% dimethyl sulfoxide (DMSO) and 10% Fetal Bovine Serum (FBS) in 1% tryptone broth. The culture used contained actively released zoospores and sporangia that were grown in 100mL of 1% tryptone media for 2 weeks at room temperature (21-23℃). Two milliliters of the actively growing culture was added to 13 mL of fresh 1% tryptone broth and spun in a centrifuge

at 1700 RPM for 10 minutes. In a Biosafety cabinet, the supernatant was discarded, and the sporangia pellet was resuspended in 1mL 10% DMSO+10% FBS in 1% tryptone broth and transferred to a 1mL cryotube. This was repeated to make 6 cryotubes. All 6 cryotubes were placed in a -80℃ freezer for long-term storage.

Thawing of Cryo-preserved *B. dendrobatidis* Isolates

Each time a cryotube was thawed, it was removed from the -80℃ freezer and placed at 37℃ for 1-2 minutes. Once thawed, the entire contents of the tube were put into 100 mL of fresh 1% tryptone broth. The newly inoculated culture was placed at room temperature (21-23℃) for 2 weeks without shaking to allow for growth.

Novel *in vitro* growth of *B. dendrobatidis*

One milliliter of inoculated culture was aseptically spread onto a 1% tryptone agar plate and placed at room temperature (21-23℃) for 8 days. On day 8, the agar plate was flooded with 5 mL 1% tryptone broth to lift zoospores. The zoospore suspension was then diluted 1:10 in fresh 1% tryptone. Cell density of the 1:10 dilution suspension was then determined using a hemocytometer and the following equation:

Total number of cells/number of 1 mm^2 squares counted x 10,000/mL x dilution factor

After determining cell density, the 1:10 dilution was further diluted in order to achieve a final cell density of 165,000-330,000 zoospores/3mL of media (3mL of media was used in each well). The diluted zoospore suspension was then aseptically transferred

into the wells of sterile, 12-well cell culture plates and allowed to incubate at room temperature for a total of 12 days. Every 3 days, cell density was determined by scraping the cells off of the wells using a rubber policeman and measuring absorbance of the suspension at 495 nm. Wells were always scraped in triplicate in order to achieve more accurate data.

Crystal Violet Staining of *B. dendrobatidis* Isolates

B. dendrobatidis isolates were aseptically stained with crystal violet in the novel *in vitro* system to determine if rhizoid structures were present. One milliliter of culture was transferred into multiple wells in the 12-well culture plate and placed at room temperature (21-23°C) overnight to give the fungus time to adhere to the plastic wells. After 24 hours, the culture was pipetted out of the wells and a 0.5% crystal violet (in 1% formaldehyde) stain was placed into each well for approximately 1-2 minutes. Each stained well was then washed with distilled water to discard any residual stain. Once washed, the plate was observed under an inverted phase-contrast microscope using the 40x objective lens (400x total magnification) to determine presence of rhizoid structures.

Effect of pH on Growth of *B. dendrobatidis*

B. dendrobatidis was grown on 1% tryptone agar plates and zoospores were harvested after 8 days of growth as described above. Zoospores were diluted to a density of 165,000-330,000 cell/3 mL using 1% tryptone media that had been adjusted to various pHs (5-9) using HCl and NaOH. Diluted cell suspensions were applied to cell culture 12-

well plates and cell growth was monitored every 3 days for a total of 12 days. Similar to the previous experiment, growth was measured by absorbance at 495 nm using approximately 2-3mL of the media harvested from each well. Each measurement was obtained from harvesting wells in triplicate and each experiment was repeated three times.

Effect of Keratin on Growth of *B. dendrobatidis*

Two different experiments were conducted in order to determine the effect of keratin on the attachment and growth of *B. dendrobatidis*. In the first experiment, *B. dendrobatidis* was grown on two 1% tryptone agar plates and zoospores were harvested after 8 days of growth as described above. After the first plate was harvested, zoospores were diluted to a density of 165,000-330,000 cells/3 mL using 1% tryptone media and those cells were added to normal cell culture wells or wells that had been pre-coated with a 1% keratin solution for 1 hour. Similarly, zoospores were harvested from a second 1% tryptone agar plate and were diluted to a density of 165,000-330,000 cells/3 mL using 1% tryptone + 1% keratin media and those cells were added to normal cell culture wells. Cell growth was monitored every 3 days for a total of 12 days. Similar to the previous experiment, growth was measured by absorbance at 495 nm using approximately 2-3mL of the media harvested from each well. Each measurement was obtained from harvesting wells in triplicate and each experiment was repeated three times.

In the second experiment, *B. dendrobatidis* was grown on two 1% tryptone agar plates and zoospores were harvested after 8 days as described above. Zoospores were diluted to a density of 165,000-330,000 cells/3 mL using 1% tryptone and 1% tryptone +

1% keratin media and those cells were added to broth tubes. Cell growth was again measured every 3 days for a total of 12 days using spectroscopy. Each measurement was obtained from harvesting broth tubes in triplicate and each experiment was repeated three times.

Effect of Nitrate on Growth of *B. dendrobatidis*

B. dendrobatidis was grown on 1% tryptone agar plates and zoospores were harvested after 8 days of growth as described above. Zoospores were diluted to a density of 165,000-330,000 cell/3 mL using 1% tryptone media that had been adjusted to various concentrations of NO_3^- [0 mg/L (1% tryptone), 5 mg/L, 10 mg/L, and 25mg/L] using solid $NaNO_3$. Diluted cell suspensions were applied to cell culture 12-well plates and cell growth was monitored every 3 days for a total of 12 days. Similar to the previous experience, growth was measured by absorbance at 495 nm using approximately 2-3mL of the media harvested from each well. Each measurement was obtained from harvesting wells in triplicate and each experiment was repeated three times.

Effect of Phosphate on Growth of *B. dendrobatidis*

B. dendrobatidis was grown on 1% tryptone agar plates and zoospores were harvested after 8 days of growth as described above. Zoospores were diluted to a density of 165,000-330,000 cell/3 mL using 1% tryptone media that had been adjusted to various concentrations of PO_4^{-3} [0 mg/L (1% tryptone), 0.05 mg/L, 0.2 mg/L, 0.4 mg/L, and 1mg/L] using solid Na_2HPO_4. Diluted cell suspensions were applied to cell culture 12-

well plates and cell growth was monitored every 3 days for a total of 12 days. Similar to the previous experience, growth was measured by absorbance at 495 nm using approximately 2-3mL of the media harvested from each well. Each measurement was obtained from harvesting wells in triplicate and each experiment was repeated three times.

Statistical Analysis

Statistical analysis was conducted using the statistical computing program R[46,88,121]. A linear model (LM) for each environmental factor (keratin in the *in vitro* system, keratin in *in vitro* broth tubes, pH, phosphate concentration, nitrate concentration) was used to test for the effect between each level of that environmental factor on the absorbance of the sample at day 12. The R code used for the analyses can be seen in Appendix B. For each linear model, all triplicate runs for each environmental factor (Appendix A) was utilized. For this analysis, the level of significance was set to $\alpha = 0.05$.

Chapter III
Results

Creation of a Novel *In Vitro* System

In order to determine if *B. dendrobatidis* can attach to and grow within submerged cell culture wells, zoospores were applied to the wells and growth was monitored for eight days. As shown by microscopy, zoospores successfully attached to the wells and transformed into germlings within the first 2 days (Figure 8A and B). From days 3-5. the newly-formed germlings transformed into zoosporangia and the zoosporangia produced new zoospores (Figure 8C-E). Zoospores continued to be produced and reattach over the next several days (Figure 8F-H). In all, these data suggest that *B. dendrobatidis* is able to complete its life cycle when grown in this submerged *in vitro* system.

To further determine this organism's success in completing its life cycle in this submerged *in vitro* system, specific structures and stages of the life cycle were identified using microscopy. On day 5, newly produced zoospores were observed in the tissue culture wells (Figure 9A). From days 3-8 when zoosporangia were maturing, both types of zoosporangia were observed (Figure 9B). The left arrow shows a developing ‘

monocentric' zoosporangium and the right arrow shows a developing 'colonial' zoosporangium. The colonial zoosporangium contained a septum, which divided the thallus body into two compartments for new zoospores. At days 3-5, rhizoid structures were formed by germlings and maturing zoosporangia (Figure 9C). At any time from days 4-8, zoospores were released from the mature zoosporangia and all that was left was a clear, empty zoosporangia with one (or multiple) discharge tube(s) from one side of the zoosporangia's chitinous wall (Figure 9D).

Different volumes of culture were tested to determine if inoculum size would make any difference in growth rate. Data was collected and quantified every 3 days for 6 days during *B. dendrobatidis*'s log growth phase in culture. Data was quantified by using hemocytometer cell counting (Figure 10A) and by measuring light absorbance of the culture at 495 nm (Figure 10B). Ultimately, volume did not make any significant differences in absorbance. Also, microscopic cell-counting and spectrophotometry produced very similar results. Since spectrometry allows for faster, more high-throughput acquisition of data, it was used for all further experiments.

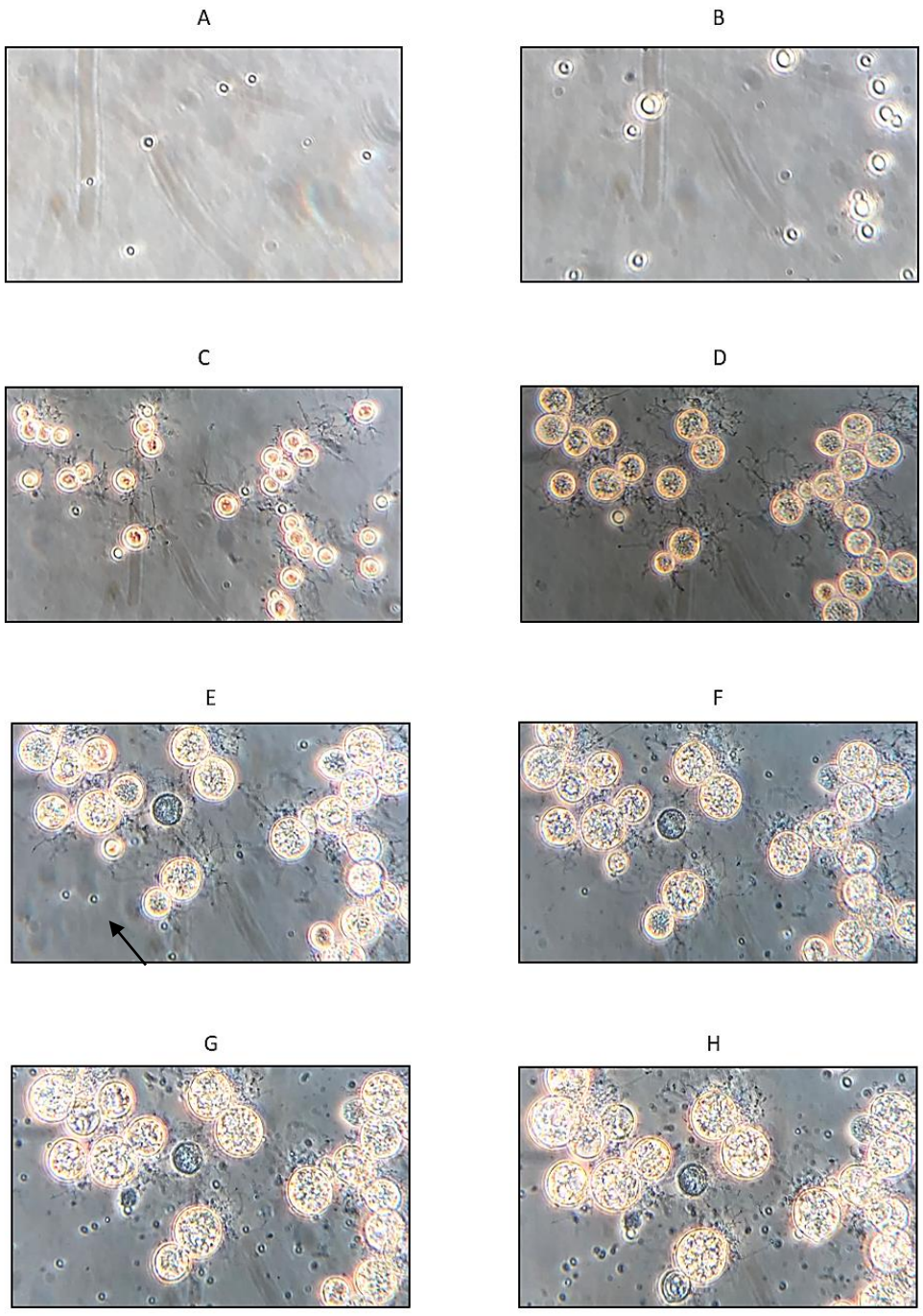


Figure 8. Life cycle of *B. dendrobatidis* as shown from A-H. A= day 1: motile zoospores. B= day 2: germlings. C= day 3: developing zoosporangia/germlings. D-H= days 4-8: developed zoosporangia with note of newly produced zoospores at day 5 shown by black line arrow (Photo Credit to Amanda Layden)

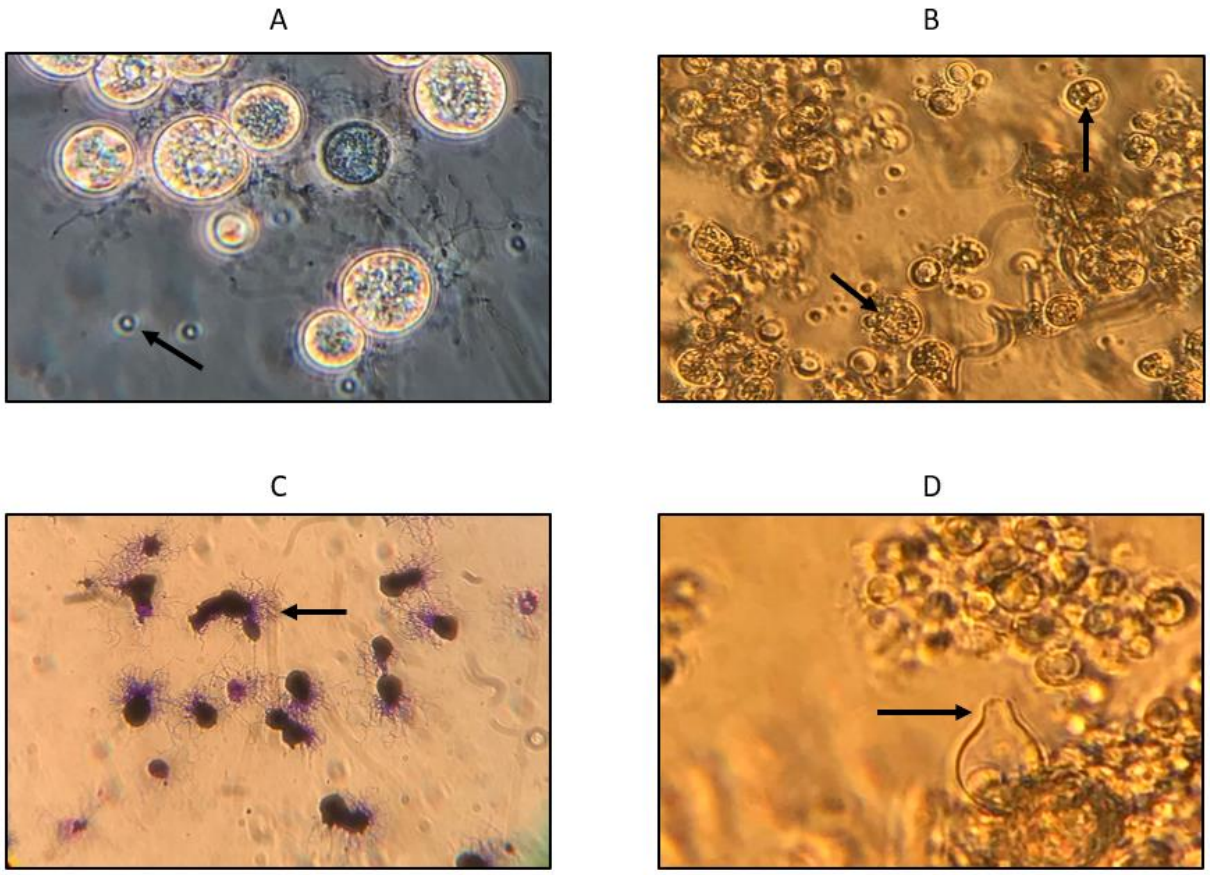
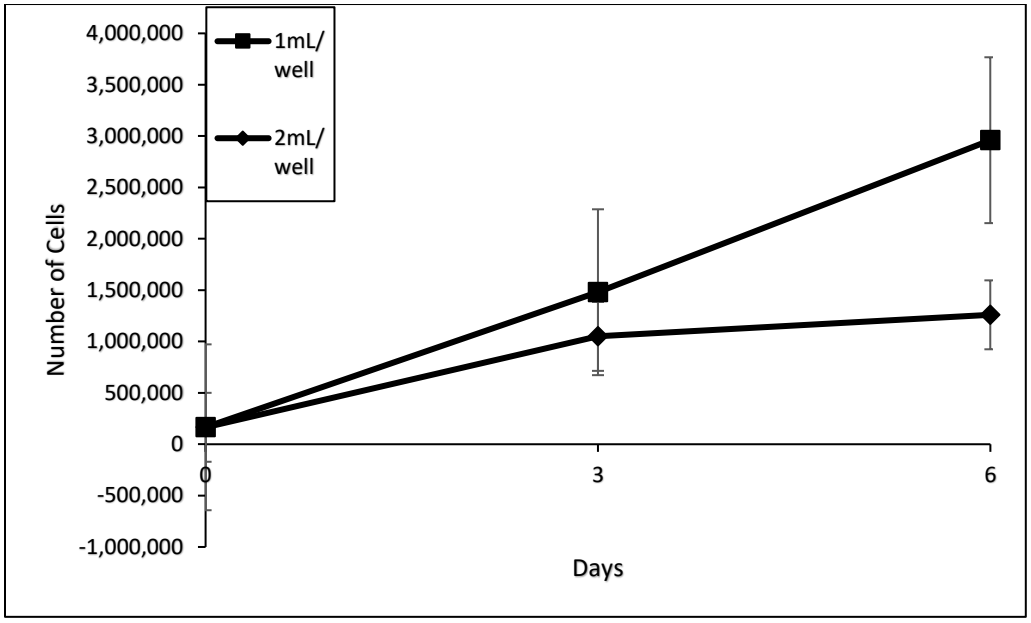


Figure 9. Structures and stages of the life cycle of *B. dendrobatidis* as shown from A-D. A= zoomed in view of Day 5 from life cycle in tissue culture plates *in vitro* to show newly produced zoospore. B= left arrow shows a developing monocentric zoosporangium and right arrow shows a mature colonial zoosporangium with a septum dividing the thallus body into two compartments. C= germlings stained with crystal violet to show rhizoid structures noted by arrow. D= a clear, empty zoosporangium with a single discharge papillae (tube) noted by arrow (Photo Credit to Amanda Layden)

A



B

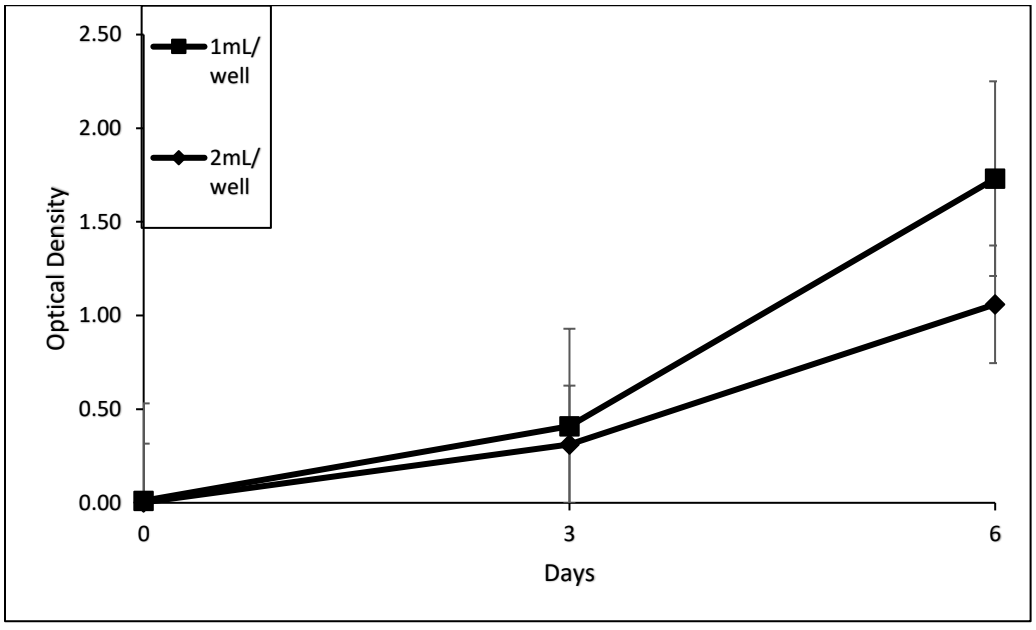


Figure 10. Growth of *B. dendrobatidis* using this novel in vitro system. A= growth quantified using cell counts on a hemocytometer, B= growth quantified using absorbance (495 nm) values from a spectrophotometer

Effects of pH on the Growth of *B. dendrobatidis*

Different pH values were tested to validate whether *B. dendrobatidis* would grow similarly in this novel *in vitro* system when compared to other *in vitro* models (1% tryptone broth and agar), *in vivo* models (host amphibians), and the natural environment [80,113]. Five pH values were chosen based on previously published data about this organism's optimal growth environment [80,113]. Growth of *B. dendrobatidis* in this novel *in vitro* system was observed in pH values ranging from approximately 5-9 (Figure 11). There was a significant difference among pH treatments in growth of *B. dendrobatidis* (LM; df=4,10; F=29.23; P=0) (Table 2). Overall, *B. dendrobatidis* grew well in pH values of 6 and 7 in this system, similarly to what it favors in the environment and in other *in vitro* systems.

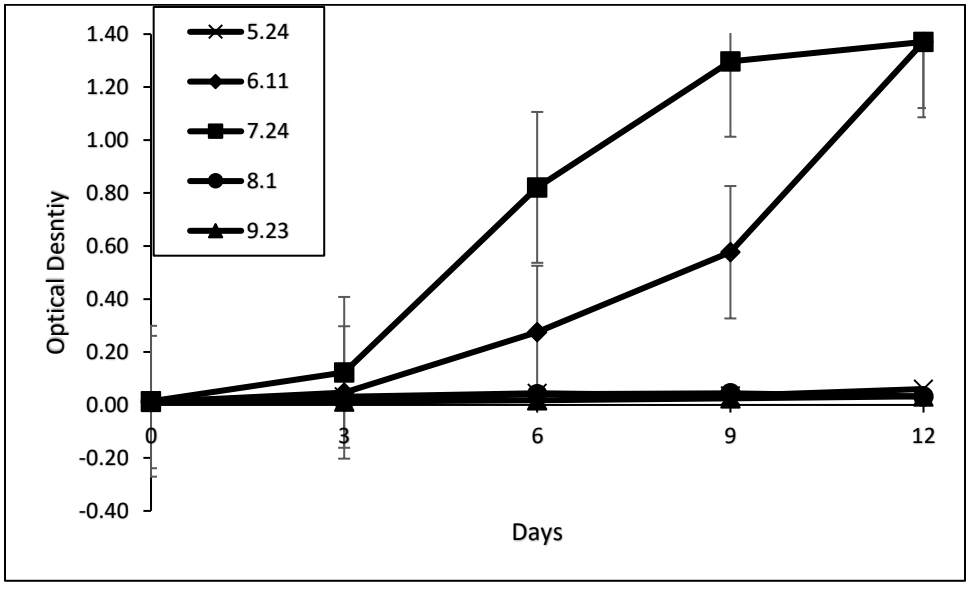


Figure 11. Effect of pH values on growth of *B. dendrobatidis* in the novel *in vitro* system

Table 2. pH Analysis of Variance

	Df	Sum Sq	Mean Sq	F value	Pr (>F)	
ID	4	3.9771	0.99427	29.23	1.702e-05	***
Residuals	10	0.3402	0.03402			

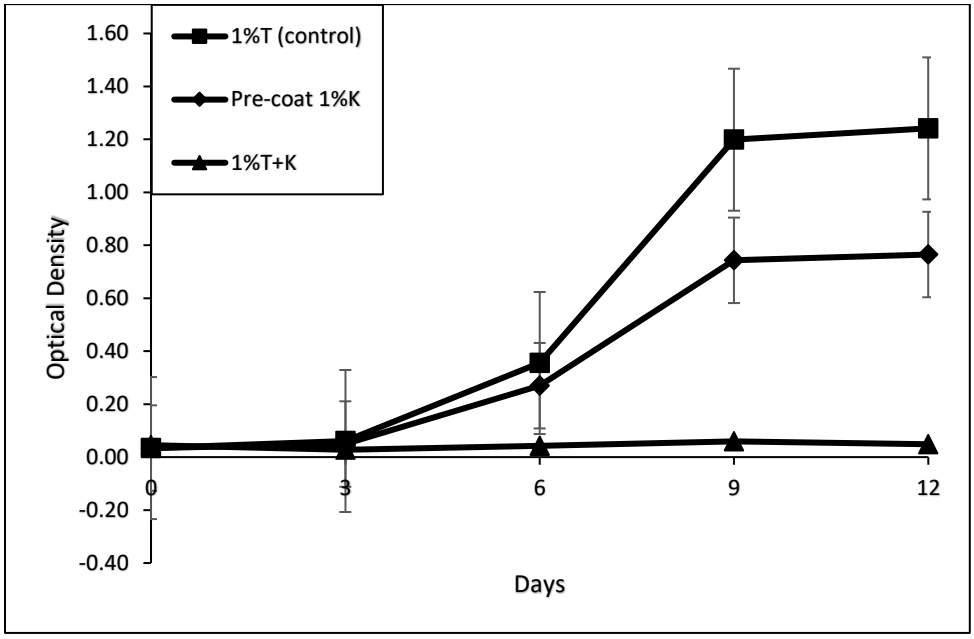
Effects of Keratin on the Growth of *B. dendrobatidis*

Previous *in vitro* studies with *B. dendrobatidis* suggest that its growth may be impacted by increased concentrations of tryptone[80]. Since tryptone is a stable product of protein digestion, other proteins were tested to determine if they have any effects on the growth of *B. dendrobatidis*. Addition of keratin to the 1% tryptone media and as a pre-coat on the tissue culture wells was tested to determine whether higher levels of protein affect the growth of *B. dendrobatidis* in our system (Figure 12A).

Overall, *B. dendrobatidis* favored 1% tryptone media for growth. The 1% keratin pre-coat slightly decreased growth and the 1% tryptone + keratin media showed little to no growth of *B. dendrobatidis*. There was a significant difference among keratin novel, *in vitro* system treatments in growth of *B. dendrobatidis* (LM; df=2,6; F=22.608; P=0.001) (Table 3). Additionally, keratin added to 1% tryptone broth tubes (Figure 12B) and 1% tryptone agar plates (Figure 12C) showed similar inhibitory effects. There was a significant difference among keratin *in vitro* broth tube treatments in growth of *B. dendrobatidis* (LM; df=1,4; F=63.141; P=0.001) (Table 4). A second, unrelated protein (bovine serum albumin) was also added as a supplement to 1% tryptone agar to determine whether protein concentration in general is impacting fungal growth (Figure 13D). Similar to what was seen for keratin, the addition of bovine serum albumin showed little

to no growth of *B. dendrobatidis* when compared to the standard 1% tryptone broth and agar. BSA was also tested in the novel *in vitro* system one time (data not shown) but results for this were inconclusive and needs further investigation. These preliminary results might suggest that increased concentrations of protein may indeed inhibit growth of *B. dendrobatidis*, but they are inconclusive and further studies will need to be performed to verify the effect or lack of effect of protein concentration on growth of *B. dendrobatidis.*

A


1%T (control)
Pre-coat 1%K
1%T+K
Optical Density
1.60
1.40
1.20
1.00
0.80
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0.40
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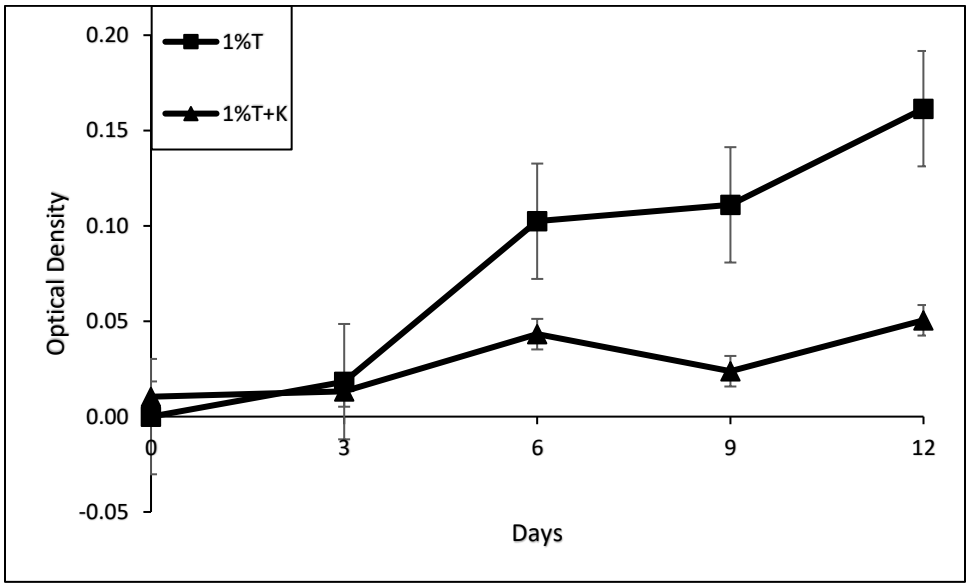
0.00
-0.20
-0.40
0
3
6
9
12
Days

B


1%T
1%T+K
Optical Density
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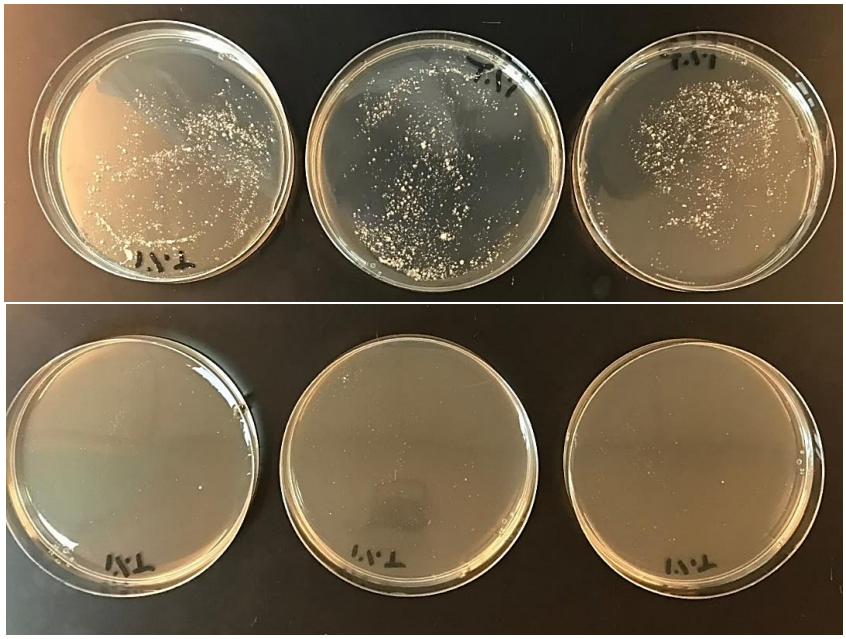
3
6
9
12
Days

C



D

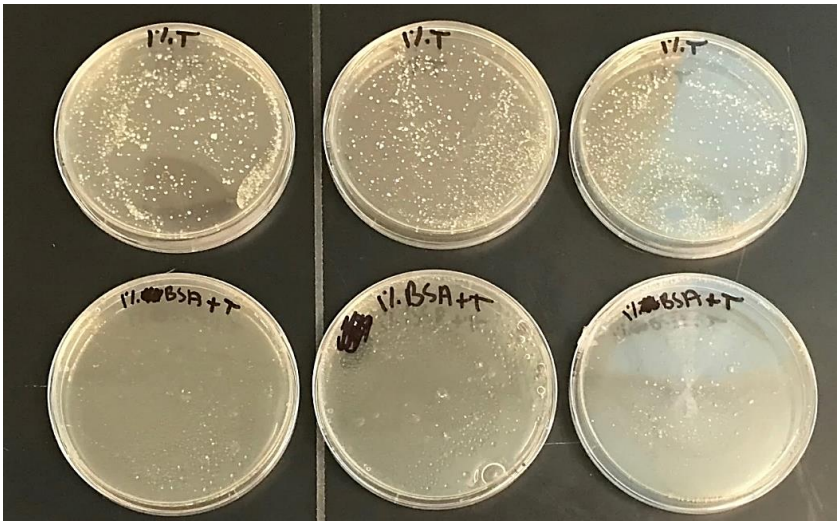


Figure 12. Effect of protein on growth of *B. dendrobatidis*. A= quantitative data showing effect of keratin on growth on *B. dendrobatidis* in novel *in vitro* system. B= quantitative data showing effect of keratin on growth on *B. dendrobatidis* in broth tubes. C= image showing effect of keratin on growth on *B. dendrobatidis* when added to 1% tryptone agar. 1% tryptone agar is shown on the top row and 1% tryptone + keratin agar is shown on the bottom row. D= image showing effect of bovine serum albumin on growth of *B. dendrobatidis* when added to 1% tryptone agar. (Photo Credit to Amanda Layden)

Table 3. Keratin Broth Tubes Analysis of Variance

	Df	Sum Sq	Mean Sq	F value	Pr (>F)	
ID	1	0.0184704	.0184704	63.141	0.001358	**
Residuals	4	0.0011701	.00002925			

Table 4. Keratin Analysis of Variance

	Df	Sum Sq	Mean Sq	F value	Pr (>F)	
ID	2	3.9274	1.96370	22.608	0.001608	**
Residuals	6	00.5212	0.08686			

Effect of Phosphate on the Growth of *B. dendrobatidis*

Different concentrations of phosphate were tested to see their effect on the growth of *B. dendrobatidis*. Concentrations tested were 0 mg/L (1% tryptone), 0.05 mg/L, 0.2 mg/L, 0.4 mg/L, and 1.0 mg/L (Figure 13). Similar growth patterns were observed with all concentrations; however, at day 6, the 1.0 mg/L concentration showed a steeper spike in growth when compared to the other concentrations. Growth of the 1.0 mg/L concentration remained steady between days 6-9 until day 12 when there was a second spike in growth observed. There was no significant difference among phosphate concentration treatments in growth of *B. dendrobatidis* (LM; df=4,10; F=2.0192; P=0.1) (Table 5). Overall, data showed that higher concentrations ($\geq$1 mg/L) of phosphate led to increased growth of *B. dendrobatidis* when compared to the traditional 1% tryptone broth.

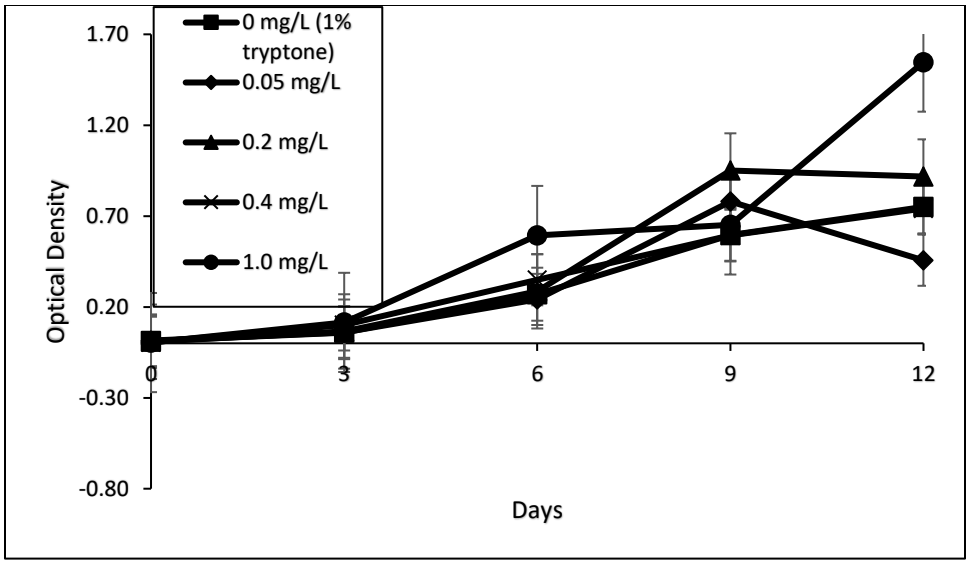


Figure 13. Effect of phosphate concentration growth of *B. dendrobatidis*

Table 5. Phosphate Analysis of Variance

	Df	Sum Sq	Mean Sq	F value	Pr (>F)	
ID	4	1.9829	0.49572	2.0192	0.1676	
Residuals	10	2.4551	0.24551			

Effect of Nitrate on the Growth of *B. dendrobatidis*

Different amounts of nitrate were tested to see their effect on the growth of *B. dendrobatidis*. Concentrations tested were 0 mg/L (1% tryptone), 5 mg/L, 10 mg/L, and 25 mg/L (Figure 14). Similar growth patterns were observed between all concentrations; however, at day 6 the 25 mg/L concentration showed a steeper spike in growth when compared to the other concentrations. After day 9, it was noted that the optical density of the 25 mg/L concentration decreased. Similarly, by day 12, all concentrations tested had decreased from the day 9 observations. There was no significant difference among nitrate concentration treatments in growth of *B. dendrobatidis* (LM; df=3,8; F=0.0805; P=0.1)

(Table 6). Overall, data showed that higher concentrations (≥ 25 mg/L) of nitrate may cause an initial increase of growth during log phase, and then lead to a decrease over time.

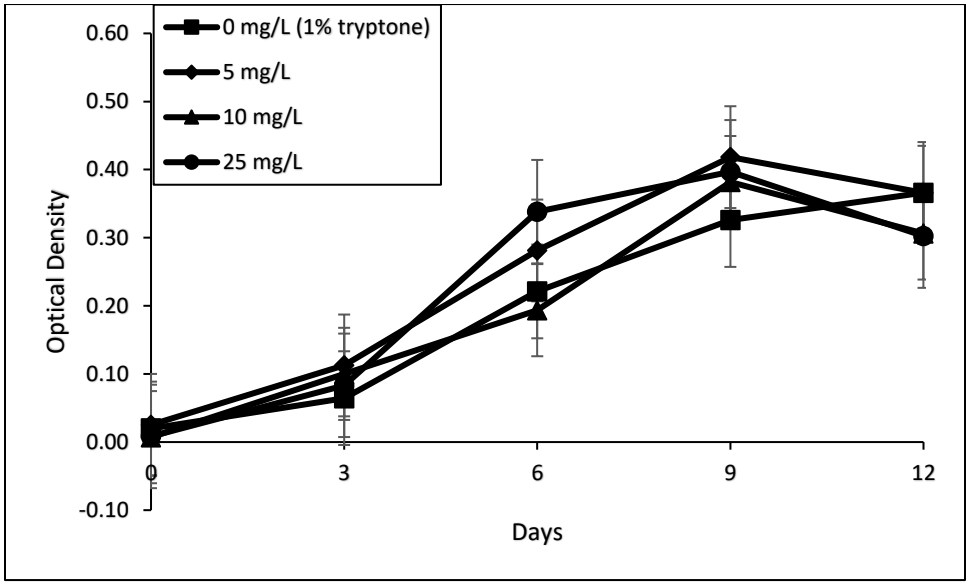


Figure 14. Effect of nitrate concentration on growth of *B. dendrobatidis*

Table 6. Nitrate Analysis of Variance

	Df	Sum Sq	Mean Sq	F value	Pr (>F)	
ID	3	0.01142	0.003808	0.0805	0.9688	
Residuals	8	0.37847	0.047309			

Chapter IV
Discussion

Chytridiomycosis is an emerging infectious wildlife disease that is continuing to cause massive declines in amphibian populations on a global scale. As mentioned, *B. dendrobatidis* infects over 350 amphibian species and has been implicated in driving the decline of over 200 of these species[32,103]. *B. dendrobatidis* induced chytridiomycosis was first described 20 years ago and several studies have documented *B. dendrobatidis* growth and development at morphological and ultrastructural levels[6,7,40,112]. Understanding what environmental factors affect the growth of *B. dendrobatidis* is important in figuring out how to treat and prevent this disease. Aside from this, having the ability to utilize a novel, high-throughput *in vitro* system would enable researchers to study these factors more efficiently and in more detail by being able to look more closely at what factors effect this pathogen's life cycle. This is the first study utilizing tissue culture plates as a novel, submerged *in vitro* growth system to test different environmental factors on the growth of this emerging environmental pathogen.

Creation of a Novel *in vitro* System

B. dendrobatidis has been studied for decades; however, there is still much that is not known about its basic biology. To date, *in vivo* experimentation is still widely utilized in *B. dendrobatidis* research in order to understand host-pathogen interactions[116]. Others have turned to various types of ex vivo systems that involve inoculated amphibian skin explants. A study done by Verbrugghe et al. discussed pathogen-host interactions using primary amphibian keratinocytes, followed by internalization of *B. dendrobatidis* in these host cells[116]. They also developed an invasion model using *X. laevis* kidney epithelial cell line A6 mimicking the complete *B. dendrobatidis* colonization cycle in vitro[116]. That said, although *in vivo* research has tremendous value for understanding disease processes, the availability of a cost-effective *in vitro* system could provide a first line tool to gain insight into host-pathogen interactions and understanding the pathogen itself, which will reduce the number of animals used in infection experiments[99,116].

Understanding what factors can affect a pathogen's life cycle is important in understanding how it's able to cause disease. Infectious diseases are commonly studied *in vitro* by assessing the interaction of a pathogen with host cells[116]; however, this study showed that *B. dendrobatidis* is capable of completing its life cycle in a submerged, *in vitro* environment without the use of host cells. Since this pathogen has a stage of its life cycle where it is not attached to amphibian skin, understanding its growth behavior outside of host cells is extremely important. *In vitro* studies offer the advantage of being simplistic and easy to perform and repeat when studying a pathogen's behavior in a specific environment or answering unknown questions regarding a pathogen. Also, it is relatively simple to determine if there are any environmental factors (temperature, pH,

salinity, etc), biotic triggers, or even purified host defenses that affect its life cycle or cell structure. As mentioned earlier, the amphibian host has both innate and acquired immune components that contribute to fighting chytridiomycosis infection[113]. To date, approximately 40 anuran antimicrobial peptides inhibiting *B. dendrobatidis* have been discovered and both purified and natural mixtures of these antimicrobial peptides have effectively inhibited *in vitro* broth growth of *B. dendrobatidis*[113]. Although these antimicrobial peptides have shown results in *in vitro* broth studies, this method does not allow one to determine how these antimicrobial peptides are inhibiting *B. dendrobatidis*'s life cycle or at what stage the life cycle is being affected. Additionally, having a novel, submerged *in vitro* assay similar to how this pathogen would grow *in vivo* on amphibian skin would be time efficient, cost efficient, and require no animal test subjects or field studies that could lead to low prevalence data and potential bias. This study showed that not only did *B. dendrobatidis* attach and grow successfully in this novel, submerged *in vitro* system, it proved that this system can be successfully utilized to test environmental factors, other aspects of *B. dendrobatidis*'s life cycle, genetic factors that control its life cycle, attachment proteins, antifungal drugs, water quality parameters, and a variety of other factors on the growth of this pathogen.

Effects of pH, Nitrate and Phosphate on the Growth of *B. dendrobatidis*

Understanding associations between *B. dendrobatidis* infection dynamics and environmental factors is important for mitigating adverse effects of the chytrid on amphibian populations[56]. The data in this study showed that *B. dendrobatidis* favored an

environmental pH of roughly 6-7. A study done by Karvemo et al. discussed that pond pH was strongly positively associated with *B. dendrobatidis* infection prevalence, particularly when pH was higher than 6.5[56]. This is consistent with observations of increases in *B. dendrobatidis* growth rates with increases in pH in previous experimental and field studies[10,22,56,80]. Environmental pH is influenced by abiotic (ex. acid-neutralizing capacity) and biotic (ex. organic carbon, aquatic plant community) characteristics of a system[22,42,44,61]. A lower pH can inhibit microbial metabolism[21,22] and changes in pH are related to the acid-neutralizing capacity in a system. This is strongly tied to the amount of organic carbon present[22,44,61] which in turn, is an important nutrient for aquatic fungi[22,38]. Although it is not clear as to why *B. dendrobatidis* does not grow well in a lower pH, Chestnut et al. suggested that it may be due to reductions in metabolic rates of the fungus and organic carbon substrates, which are important nutrients for aquatic fungi in low pH environments[22,56]. Aside from pH playing a major role in metabolic rates of fungi, it's possible that *B. dendrobatidis* may favor an environmental pH of roughly 6-7 because that may be the external pH of amphibian skin. However, further investigation on this topic is needed to confirm or deny this hypothesis.

As mentioned above, changes to the chemical composition of the environmental water source has the potential to drastically alter the growth of microorganisms like *B. dendrobatidis*. Nitrogen (N) and phosphorus (P) are two important and essential nutrients for healthy soil and aquatic environments. In addition, nitrogen and phosphorus are two of the many additives found in traditional fertilizers used for lawn care and plant food and fertilizers used to enhance agricultural productivity. Fertilizers influence both the aboveground biomass and the belowground microbial biomass[124]. Soil microbial

communities consist mainly of bacteria, fungi, and archaea and play critical roles in ecosystem function and regulate key processes such as carbon and nitrogen cycles[3,15,124]. Determining whether nitrogen and phosphorus fertilization impacts a microbial community is difficult because the soil microbe communities in various ecosystems are different and thus their responses to similar fertilizations might also be different[124].

A well-known outcome of an increase of nitrogen and phosphorus in aquatic environments is known as an algal bloom[27]. Algae that undergo these algal blooms are classified as microalgae, which includes dinoflagellates and bacillariophyta (diatoms)[27]. In the past several decades, a growing number of studies concerning the environmental factors of algal bloom outbreaks and decline have been explored[125]. According to Zhang et al., excessive exogenous nitrogen and phosphorus, high temperature, and adequate light intensity have been identified as major abiotic triggers of algal blooms[125]. Although algal blooms are seen only in aquatic environments, there are other microorganisms (bacteria, fungi, archaea) that coexist in these ecosystems and may also be affected by these environmental factors. As mentioned, *B. dendrobatidis* is found in a variety of water sources and moist soil environments. Similar to algae, *B. dendrobatidis*'s growth is known to be affected by abiotic triggers such as temperature fluctuations[80]. That said, with chytridiomycosis infection and the use of fertilizers in agriculture increasing over the last few decades, understanding if a similar event occurs with *B. dendrobatidis* is important to determine if these abiotic factors cause changes in the growth of this pathogen in the environment.

Overall, the data in this study showed that the lower concentrations of nitrate and phosphate added to 1% tryptone growth media did not have any effect on the growth of *B. dendrobatidis*. The higher concentrations tested ($\geq$ 25 mg/L NO_3 and $\geq$ 1.0 mg/L PO_4) indicated slight increased growth of *B. dendrobatidis*, but not enough to make a statistical significance. According to the EPA, naturally occurring amounts of nitrogen and phosphorus vary substantially between water sources[109]. Appropriate reference levels for normal water quality range from 0.12 to 2.2 mg/L total nitrogen and 0.01 to 0.075 mg/L total phosphorus[109]; however, nuisance algal growths are not uncommon in rivers and streams below the low reference level (0.1 mg/L) for phosphorus. Additionally, the EPA noted that excess nitrate is not toxic to aquatic life, but increased nitrogen may result in overgrowth of algae, which can decrease the dissolved oxygen content of the water, thereby harming or killing fish and other aquatic species[108,109]. Furthermore, this indicates that nitrate and phosphate may not cause a significant impact on the growth of this pathogen, but further exploration of this hypothesis is needed. To do so, a wider range of concentrations of nitrate and phosphate should be tested on the growth of this pathogen to determine if these environmental factors have an effect on the growth of this pathogen.

Effect of Keratin on the Growth of *B. dendrobatidis*

As mentioned, chytridiomycosis is an emerging infectious wildlife disease that affects the keratinized epidermal cells of the amphibian epithelium, which is an extremely important organ in amphibians. In infected amphibians, *B. dendrobatidis* is found in the cells of the epidermis and pathological abnormalities include a thickening of

the outer layer of the skin[7,96]. Cutaneous fungal infections in other vertebrates are not usually lethal, but amphibian skin is unique because it is physiologically active, tightly regulating the exchange of respiratory gases, water, and electrolytes[96]. That said, the physiological importance of the skin makes amphibians particularly vulnerable to skin infections[96]. Since this is a cutaneous infection of amphibians, a major theory regarding *B. dendrobatidis* is that it utilizes keratin as a nutrient source[23]. This is a major topic of discussion because this pathogen infects the keratinocytes of the stratum corneum and can only infect the keratinized mouthparts of tadpoles[23].

This study showed that keratin being added to 1% tryptone broth tubes *in vitro,* 1% tryptone agar *in vitro,* and to 1% tryptone in the novel, submerged *in vitro* system had a statistically-significant negative impact on the growth of *B. dendrobatidis*. Ultimately, adding keratin to the 1% tryptone media resulted in a decrease in growth of *B. dendrobatidis* when compared to the standard 1% tryptone media. It's possible that keratin might be impacting the growth of *B. dendrobatidis* by altering cell signaling, cell-to-cell communication, or some other unknown mechanism. It is also possible that the free form of keratin is not a viable nutrient source as compared to keratinized skin cells.

Despite the increasing scientific attention to chytridiomycosis, mechanisms that influence host characteristics and *B. dendrobatidis* population densities still remain poorly understood[115]. Quorum sensing (QS) is a mechanism of cell-to-cell communication that allows unicellular organisms to determine their population density in order to regulate their population behavior, including growth[2,115]. A study done by Verbrugge et al. showed that *B. dendrobatidis* is capable of controlling its cell

populations, in which individual cells communicate with each other by secreting tryptophol in order to assess the population density and to coordinate their growth response[115]. When a certain density is achieved, they start producing tryptophol with an autostimulatory mode of action, and when tryptophol reaches high concentrations in the exponential/stationary phase of growth, this results in growth reduction[115]. According to Verbrugge et al., it could be suggested that nutrient limitation occurs during these growth phases, leading to growth decreases[115]. That said, when keratin was added to 1% tryptone media, there were no indications of a log growth phase. This suggests the possibility that keratin may not be a necessary nutrient for *B. dendrobatidis*. It's also possible that *B. dendrobatidis* did not recognize the added keratin, due to it not being expressed within or on cells of the amphibian nonprofessional immune cells (keratinocytes, fibroblasts)[41]. Similarly, it could also be possible that an increase of protein concentration could potentially be causing *B. dendrobatidis* to halt its growth, cause cell death, or act as a signal for this fungus to switch from growth to invasion mechanisms.

B. dendrobatidis is capable of growing on a variety of growth media *in vitro* such as 1% keratin agar, frog skin agar, feathers, geese paws, and chitinous carapaces of crustaceans[36,66,71,80,112,113]. Although *B. dendrobatidis* grows well on these substances, it grows best in tryptone or peptonized milk in both agar and broth *in vitro*[66,113]. Tryptone and peptonized milk are both digests of casein, a protein readily found in mammalian milk. A study done by Piotrowski et al. discussed that *B. dendrobatidis* does not require sugars other than those that were added to the 1% tryptone and that high percentages of sugar or tryptone (greater than 2%) hinder growth[80]. Similarly, throughout our study, it

was observed that 1% tryptone media with the addition of 1% keratin (a roughly 2% protein-rich growth media) hindered growth of this pathogen.

Conclusions

The overall increase in chytridiomycosis over the last few decades has had a severe impact in amphibian populations globally. *In vivo* studies on chytridiomycosis are valuable to obtain pathogen-host interactions; however, *in vitro* studies provide a faster, inexpensive, high-throughput way to test multiple environmental factors at once that could potentially be impacting growth of *B. dendrobatidis*. The results discussed in this paper and others suggest that *B. dendrobatidis* may be impacted by abiotic factors such as temperature, environmental pH, and increased protein concentrations. Other microorganisms, such as microalgae, are affected by similar abiotic factors and it is important to understand whether these abiotic factors also cause an impact on this pathogen as well.

Future Studies

As this was the first study utilizing tissue culture plates as a novel, submerged *in vitro* assay, there is ample opportunity to continue using this assay to test similar and new environmental factors that could potentially impact the growth of *B. dendrobatidis*. Although nitrogen, phosphorus, and pH are important components of water quality parameters, there are others that play a significant role as well. Future studies could look at some of these other important water quality parameters such as ammonium, dissolved

oxygen, alkalinity, and water hardness. The most interesting discovery of these results was that keratin concentration seemed to have a negative effect on the growth of *B. dendrobatidis*. Future studies could utilize this new-found information and determine at what stage the life cycle is being altered, or test if there are other amphibian surface proteins or generic proteins that show a similar result.

With the availability of this system and the results of this study, it is important to continue researching what environmental factors, other aspects of *B. dendrobatidis*'s life cycle, genetic factors that control its life cycle, attachment proteins, antifungal drugs, water quality parameters, and a variety of other factors that have an effect on the growth of this fungus. Additionally, it is also important to continue observing a wider range of concentrations of nitrogen, phosphorus, and proteins to determine if any other concentrations outside of what was observed in this study show a similar or adverse effect on the growth of this pathogen.

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Appendix A: Raw Data

Table 7. Environmental Factors Raw Data

Variable	Run	ID	Days	Absorbance
pH	1	5.24	0	0.0067
pH	1	5.24	3	0.0141
pH	1	5.24	6	0.0521
pH	1	5.24	9	0.0210
pH	1	5.24	12	0.0417
pH	2	5.24	0	0.0156
pH	2	5.24	3	0.0427
pH	2	5.24	6	0.0546
pH	2	5.24	9	0.0416
pH	2	5.24	12	0.0748
pH	3	5.24	0	0.0254
pH	3	5.24	3	0.0344
pH	3	5.24	6	0.0273
pH	3	5.24	9	0.0424
pH	3	5.24	12	0.0639
pH	1	6.11	0	0.0120
pH	1	6.11	3	0.0107
pH	1	6.11	6	0.0755
pH	1	6.11	9	0.2253
pH	1	6.11	12	0.0530
pH	2	6.11	0	0.0095
pH	2	6.11	3	0.0145
pH	2	6.11	6	0.0804
pH	2	6.11	9	0.7199
pH	2	6.11	12	0.4832
pH	3	6.11	0	0.0116
pH	3	6.11	3	0.1165
pH	3	6.11	6	0.6705
pH	3	6.11	9	0.7845
pH	3	6.11	12	0.5769
pH	1	7.24	0	0.0138
pH	1	7.24	3	0.1025
pH	1	7.24	6	0.4597
pH	1	7.24	9	1.2923
pH	1	7.24	12	1.2273
pH	2	7.24	0	0.0099
pH	2	7.24	3	0.0699

pH	2	7.24	6	0.2803
pH	2	7.24	9	0.7826
pH	2	7.24	12	1.1688
pH	3	7.24	0	0.0182
pH	3	7.24	3	0.1968
pH	3	7.24	6	1.7243
pH	3	7.24	9	1.8168
pH	3	7.24	12	1.7162
pH	1	8.10	0	0.0162
pH	1	8.10	3	0.0221
pH	1	8.10	6	0.0099
pH	1	8.10	9	0.0262
pH	1	8.10	12	0.0265
pH	2	8.10	0	0.0178
pH	2	8.10	3	0.0240
pH	2	8.10	6	0.0590
pH	2	8.10	9	0.0337
pH	2	8.10	12	0.0163
pH	3	8.10	0	0.0083
pH	3	8.10	3	0.0196
pH	3	8.10	6	0.0520
pH	3	8.10	9	0.0719
pH	3	8.10	12	0.0525
pH	1	9.23	0	0.0089
pH	1	9.23	3	0.0249
pH	1	9.23	6	0.0111
pH	1	9.23	9	0.0363
pH	1	9.23	12	0.0000
pH	2	9.23	0	0.0163
pH	2	9.23	3	0.0088
pH	2	9.23	6	0.0050
pH	2	9.23	9	0.0390
pH	2	9.23	12	0.0645
pH	3	9.23	0	0.0083
pH	3	9.23	3	0.0043
pH	3	9.23	6	0.0352
pH	3	9.23	9	0.0000
pH	3	9.23	12	0.0335
KeratinBroth	1	T	0	0.0000
KeratinBroth	1	T	3	0.0106
KeratinBroth	1	T	6	0.0949
KeratinBroth	1	T	9	0.1360

KeratinBroth	1	T	12	0.1479
KeratinBroth	1	TK	0	0.0313
KeratinBroth	1	TK	3	0.0396
KeratinBroth	1	TK	6	0.0480
KeratinBroth	1	TK	9	0.0087
KeratinBroth	1	TK	12	0.0720
KeratinBroth	2	T	0	0.0000
KeratinBroth	2	T	3	0.0288
KeratinBroth	2	T	6	0.1081
KeratinBroth	2	T	9	0.1068
KeratinBroth	2	T	12	0.1596
KeratinBroth	2	TK	0	0.0000
KeratinBroth	2	TK	3	0.0000
KeratinBroth	2	TK	6	0.0423
KeratinBroth	2	TK	9	0.0337
KeratinBroth	2	TK	12	0.0347
KeratinBroth	3	T	0	0.0000
KeratinBroth	3	T	3	0.0156
KeratinBroth	3	T	6	0.1043
KeratinBroth	3	T	9	0.0903
KeratinBroth	3	T	12	0.1769
KeratinBroth	3	TK	0	0.0000
KeratinBroth	3	TK	3	0.0000
KeratinBroth	3	TK	6	0.0394
KeratinBroth	3	TK	9	0.0290
KeratinBroth	3	TK	12	0.0448
Keratin	1	T	0	0.0179
Keratin	1	T	3	0.0385
Keratin	1	T	6	0.2992
Keratin	1	T	9	1.6870
Keratin	1	T	12	1.9600
Keratin	1	PK	0	0.0179
Keratin	1	PK	3	0.0330
Keratin	1	PK	6	0.1815
Keratin	1	PK	9	0.9960
Keratin	1	PK	12	1.4600
Keratin	1	TK	0	0.0428
Keratin	1	TK	3	0.0376
Keratin	1	TK	6	0.0479
Keratin	1	TK	9	0.0641
Keratin	1	TK	12	0.0525
Keratin	2	T	0	0.0368

Keratin	2	T	3	0.0572
Keratin	2	T	6	0.4578
Keratin	2	T	9	1.5590
Keratin	2	T	12	1.2971
Keratin	2	PK	0	0.0368
Keratin	2	PK	3	0.0560
Keratin	2	PK	6	0.2325
Keratin	2	PK	9	0.4575
Keratin	2	PK	12	0.8586
Keratin	2	TK	0	0.0316
Keratin	2	TK	3	0.0368
Keratin	2	TK	6	0.0291
Keratin	2	TK	9	0.0585
Keratin	2	TK	12	0.0395
Keratin	3	T	0	0.0480
Keratin	3	T	3	0.0871
Keratin	3	T	6	0.6643
Keratin	3	T	9	1.5490
Keratin	3	T	12	1.7090
Keratin	3	PK	0	0.0480
Keratin	3	PK	3	0.0594
Keratin	3	PK	6	0.3953
Keratin	3	PK	9	0.7762
Keratin	3	PK	12	0.7419
Keratin	3	TK	0	0.0597
Keratin	3	TK	3	0.0087
Keratin	3	TK	6	0.0521
Keratin	3	TK	9	0.0558
Keratin	3	TK	12	0.0548
PO4	1	0	0	0.0106
PO4	1	0	3	0.0208
PO4	1	0	6	0.1425
PO4	1	0	9	0.4015
PO4	1	0	12	0.4976
PO4	2	0	0	0.0000
PO4	2	0	3	0.0784
PO4	2	0	6	0.3273
PO4	2	0	9	0.5628
PO4	2	0	12	0.7029
PO4	3	0	0	0.0290
PO4	3	0	3	0.0765
PO4	3	0	6	0.3407

PO4	3	0	9	0.8284
PO4	3	0	12	1.0512
PO4	1	0.05	0	0.0077
PO4	1	0.05	3	0.0311
PO4	1	0.05	6	0.0904
PO4	1	0.05	9	0.3719
PO4	1	0.05	12	0.2279
PO4	2	0.05	0	0.0132
PO4	2	0.05	3	0.0940
PO4	2	0.05	6	0.3483
PO4	2	0.05	9	0.5199
PO4	2	0.05	12	0.5275
PO4	3	0.05	0	0.0247
PO4	3	0.05	3	0.0579
PO4	3	0.05	6	0.2857
PO4	3	0.05	9	1.4483
PO4	3	0.05	12	0.6164
PO4	1	0.2	0	0.0087
PO4	1	0.2	3	0.0492
PO4	1	0.2	6	0.2021
PO4	1	0.2	9	0.4472
PO4	1	0.2	12	0.3831
PO4	2	0.2	0	0.0118
PO4	2	0.2	3	0.1216
PO4	2	0.2	6	0.4792
PO4	2	0.2	9	1.1607
PO4	2	0.2	12	1.2352
PO4	3	0.2	0	0.0050
PO4	3	0.2	3	0.0249
PO4	3	0.2	6	0.1776
PO4	3	0.2	9	1.2437
PO4	3	0.2	12	1.1340
PO4	1	0.4	0	0.0151
PO4	1	0.4	3	0.0692
PO4	1	0.4	6	0.2298
PO4	1	0.4	9	0.3847
PO4	1	0.4	12	0.3172
PO4	2	0.4	0	0.0003
PO4	2	0.4	3	0.1799
PO4	2	0.4	6	0.5249
PO4	2	0.4	9	0.7961
PO4	2	0.4	12	1.0033

PO4	3	0.4	0	0.0025
PO4	3	0.4	3	0.0531
PO4	3	0.4	6	0.2930
PO4	3	0.4	9	0.6028
PO4	3	0.4	12	0.9047
PO4	1	1.0	0	0.0044
PO4	1	1.0	3	0.0476
PO4	1	1.0	6	0.1686
PO4	1	1.0	9	0.3981
PO4	1	1.0	12	0.6620
PO4	2	1.0	0	0.0044
PO4	2	1.0	3	0.1808
PO4	2	1.0	6	0.6067
PO4	2	1.0	9	0.8047
PO4	2	1.0	12	2.3970
PO4	3	1.0	0	0.0037
PO4	3	1.0	3	0.1169
PO4	3	1.0	6	1.0044
PO4	3	1.0	9	0.7505
PO4	3	1.0	12	1.5823
NO3	1	0	0	0.0267
NO3	1	0	3	0.1437
NO3	1	0	6	0.3668
NO3	1	0	9	0.3887
NO3	1	0	12	0.3663
NO3	2	0	0	0.0179
NO3	2	0	3	0.0393
NO3	2	0	6	0.1037
NO3	2	0	9	0.3531
NO3	2	0	12	0.3287
NO3	3	0	0	0.0151
NO3	3	0	3	0.0105
NO3	3	0	6	0.1930
NO3	3	0	9	0.2362
NO3	3	0	12	0.4034
NO3	1	5	0	0.0362
NO3	1	5	3	0.1095
NO3	1	5	6	0.4006
NO3	1	5	9	0.3211
NO3	1	5	12	0.2287
NO3	2	5	0	0.0042
NO3	2	5	3	0.0418

NO3	2	5	6	0.1939
NO3	2	5	9	0.6997
NO3	2	5	12	0.5815
NO3	3	5	0	0.0355
NO3	3	5	3	0.1862
NO3	3	5	6	0.2494
NO3	3	5	9	0.2342
NO3	3	5	12	0.2870
NO3	1	10	0	0.0114
NO3	1	10	3	0.0570
NO3	1	10	6	0.0873
NO3	1	10	9	0.1581
NO3	1	10	12	0.0683
NO3	2	10	0	0.0059
NO3	2	10	3	0.0866
NO3	2	10	6	0.2345
NO3	2	10	9	0.6871
NO3	2	10	12	0.6909
NO3	3	10	0	0.0043
NO3	3	10	3	0.1568
NO3	3	10	6	0.2591
NO3	3	10	9	0.3000
NO3	3	10	12	0.1594
NO3	1	25	0	0.0071
NO3	1	25	3	0.0609
NO3	1	25	6	0.3150
NO3	1	25	9	0.1733
NO3	1	25	12	0.2084
NO3	2	25	0	0.0000
NO3	2	25	3	0.0506
NO3	2	25	6	0.2654
NO3	2	25	9	0.7975
NO3	2	25	12	0.5293
NO3	3	25	0	0.0177
NO3	3	25	3	0.1384
NO3	3	25	6	0.4344
NO3	3	25	9	0.2196
NO3	3	25	12	0.1694

Appendix B: R Code

The R function utilized in the analyses:

```{r}
install.packages("tidyverse")
library("tidyverse")
library(readxl)

#KeratinBrothLM

Kbroth<- read_excel("KeratinBrothData.xlsx")
> View(Kbroth)
> Kbroth$Days<- as.factor(Kbroth$Days)
> Kbroth$ID<- as.factor(Kbroth$ID)
> Kbroth$Run<- as.factor(Kbroth$Run)
> str(Kbroth)
Classes ‘tbl_df’, ‘tbl’ and 'data.frame':	30 obs. of  4 variables:
$ Run : Factor w/ 3 levels "1","2","3": 1 1 1 1 1 1 1 1 1 1 ...
$ ID  : Factor w/ 2 levels "T","TK": 1 1 1 1 1 2 2 2 2 2 ...
$ Days: Factor w/ 5 levels "0","3","6","9",..: 1 2 3 4 5 1 2 3 4 5 ...
$ ABS : num  0 0.0106 0.0949 0.136 0.1479 ...
> Kbrothfin<- Kbroth %>% filter(Days=="12")
> Kbrothfin$Days <- factor(Kbrothfin$Days)
> View(Kbrothfin)
> Kbrothfinaov<- lm(ABS ~ ID, data=Kbrothfin)
> anova(Kbrothfinaov)
```

Analysis of Variance Table

Response: ABS

Df Sum Sq Mean Sq F value Pr(>F)

ID 1 0.0184704 0.0184704 63.141 0.001358 **

Residuals 4 0.0011701 0.0002925

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

#KeratininvitroLM

Kvitro<- read_excel("KeratinData.xlsx")

> View(Kvitro)

> Kvitro$Run<- as.factor(Kvitro$Run)

> Kvitro$Days<- as.factor(Kvitro$Days)

> Kvitro$ID<- as.factor(Kvitro$ID)

> str(Kvitro)

Classes ‘tbl_df’, ‘tbl’ and 'data.frame': 45 obs. of 4 variables:

$ Run : Factor w/ 3 levels "1","2","3": 1 1 1 1 1 1 1 1 1 1 ...

$ ID : Factor w/ 3 levels "PK","T","TK": 2 2 2 2 2 1 1 1 1 1 ...

$ Days: Factor w/ 5 levels "0","3","6","9",..: 1 2 3 4 5 1 2 3 4 5 ...

$ ABS : num 0.0179 0.0385 0.2992 1.687 1.96 ...

> Kvitrofin<- Kvitro %>% filter(Days=="12")

> Kvitrofin$Days <- factor(Kvitrofin$Days)

> View(Kvitrofin)

> Kvitrofinaov<- lm(ABS ~ ID, data=Kvitrofin)

> anova(Kvitrofinaov)

Analysis of Variance Table

Response: ABS

Df Sum Sq Mean Sq F value Pr(>F)

ID 2 3.9274 1.96370 22.608 0.001608 **

Residuals 6 0.5212 0.08686

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

#pHLM

pH <- read_excel("pHData.xlsx")

> pH$Run<- as.factor(pH$Run)

> pH$Days<- as.factor(pH$Days)

> pH$ID<- as.factor(pH$ID)

> str(pH)

Classes ‘tbl_df’, ‘tbl’ and 'data.frame': 75 obs. of 4 variables:

$ Run : Factor w/ 3 levels "1","2","3": 1 1 1 1 1 2 2 2 2 2 ...

$ ID : Factor w/ 5 levels "5.24","6.11",..: 1 1 1 1 1 1 1 1 1 1 ...

$ Days: Factor w/ 5 levels "0","3","6","9",..: 1 2 3 4 5 1 2 3 4 5 ...

$ ABS : num 0.0067 0.0141 0.0521 0.021 0.0417 0.0156 0.0427 0.0546 0.0416 0.0748 ...

> pHfin <- pH %>% filter(Days=="12")

> pHfin$Days <- factor(pHfin$Days)

> View(pHfin)

> pHfinaov <- lm(ABS ~ ID, data=pHfin)

> anova(pHfinaov)

Analysis of Variance Table

Response: ABS

Df Sum Sq Mean Sq F value Pr(>F)

ID 4 3.9771 0.99427 29.23 1.702e-05 ***

Residuals 10 0.3402 0.03402

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

#PO4LM

PO4 <- read_excel("PO4Data.xlsx")

> View(PO4)

> PO4$Run<- as.factor(PO4$Run)

> PO4$ID<- as.factor(PO4$ID)

> PO4$Days<- as.factor(PO4$Days)

> str(PO4)

Classes ‘tbl_df’, ‘tbl’ and 'data.frame': 75 obs. of 4 variables:

$ Run : Factor w/ 3 levels "1","2","3": 1 1 1 1 1 2 2 2 2 2 ...

$ ID : Factor w/ 5 levels "0","0.05","0.2",..: 1 1 1 1 1 1 1 1 1 1 ...

$ Days: Factor w/ 5 levels "0","3","6","9",..: 1 2 3 4 5 1 2 3 4 5 ...

$ ABS : num 0.0106 0.0208 0.1425 0.4015 0.4976 ...

> PO4fin <- PO4 %>% filter(Days=="12")

> PO4fin$Days <- factor(PO4fin$Days)

> view(PO4fin)

> PO4finaov <- lm(ABS ~ ID, data=PO4fin)

> anova(PO4finaov)

Analysis of Variance Table

Response: ABS

Df Sum Sq Mean Sq F value Pr(>F)

ID 4 1.9829 0.49572 2.0192 0.1676

Residuals 10 2.4551 0.24551

#NO3

```
NO3 <- read_excel("NO3data.xlsx")
> View(NO3)
> NO3$Run<- as.factor(NO3$Run)
> NO3$ID<- as.factor(NO3$ID)
> NO3$Days<- as.factor(NO3$Days)
> str(NO3)
Classes ‘tbl_df’, ‘tbl’ and 'data.frame': 60 obs. of 4 variables:
$ Run : Factor w/ 3 levels "1","2","3": 1 1 1 1 1 2 2 2 2 2 ...
$ ID : Factor w/ 4 levels "0","5","10","25": 1 1 1 1 1 1 1 1 1 1 ...
$ Days: Factor w/ 5 levels "0","3","6","9",..: 1 2 3 4 5 1 2 3 4 5 ...
$ ABS : num 0.0267 0.1437 0.3668 0.3887 0.3663 ...
> NO3fin<- NO3 %>% filter(Days=="12")
> NO3fin$Days <- factor(NO3fin$Days)
> View(NO3fin)
> NO3finaov<- lm(ABS ~ ID, data=NO3fin)
> anova(NO3finaov)
```

Analysis of Variance Table

Response: ABS

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
ID	3	0.01142	0.003808	0.0805	0.9688
Residuals	8	0.37847	0.047309		