

Batrachochytrium dendrobatidis
survey in Ncandu Nature Reserve and
fungal ecology

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Abstract

Amphibians across the globe have been experiencing rapid declines, especially in areas with a high amphibian population density. It was assumed that this decline was caused by habitat destruction and climate change. A closer investigation led to the discovery of a parasitic fungus called *Batrachochytrium dendrobatidis* (*Bd*), or batrachochytrids as they are collectively known, which is now considered to be the leading cause of amphibian morbidity and mortality. This parasitic fungus causes a disease known as chytridiomycosis, which compromises the osmoregulation and respiratory processes of amphibians. The effect of chytridiomycosis on amphibians' fitness is significant as adults tend to become lethargic as vital metabolic processes that promote optimal organ functions are compromised. Therefore, increasing the risk of predation depending on the degree of infection. This study focused on the amphibian species *Amietia delalandii*, the common river frog, in the Ncandu Nature Reserve (NNR). The purpose of this study was to determine the presence of *Bd* in the NNR and, if detected, to determine the distribution and prevalence of *Bd*. Prevalence was determined by utilizing two isolation approaches on amphibian samples collected at various sites. The purpose of using two different approaches was to determine the isolation success of each approach respectively.

Bd was found to be present at the NNR, although some perennial streams and pools tested negative for the presence of *Bd*. Eleven sampling sites were screened for *Bd*, with nine being found positive. Therefore, it is clear that the NNR is in a pre-enzootic state, but further monitoring needs to be conducted as it was found that some intermittent pools are positive and are within close proximity to negative sites. Two separate surveys were conducted to determine the seasonal influence on *Bd* prevalence and found that *Bd* proliferates more during the summer period having an overall prevalence of 26%. In contrast, the survey conducted in the winter had a 17% overall prevalence. The two sites with the highest prevalence are Steep Glen Kloof and Steep Glen River; these sites are perennial and flow into the lower-lying valley, therefore being a potential cause for the distribution of *Bd* to downstream localities.

Isolation of *Bd* was done using two approaches: the liquid media approach and the agar approach. Although considerable contamination occurred for both approaches, the agar approach proved to be more successful as contamination of samples could be seen while in the field and could be successfully removed in most instances. The isolation success of the two approaches yielded questionable results as the liquid media approach had an isolation success of 0%, whereas the agar approach had a 72.2% isolation success, making the agar approach more effective. Two *in vitro* experiments were conducted to determine the effect of a competitor/contaminant on the fitness of *Bd*. It was discovered that *Bd* could be significantly

inhibited by a competitor that can secrete inhibitory substances. Therefore, the lack of isolation success for the liquid media approach was caused by the inhibition of *Bd* by a fungal competitor, complicated by the use of liquid media for attempted isolation of *Bd* that facilitates the spread of inhibitory substances.

Keywords: *Batrachochytrium dendrobatidis*, isolation success, contamination, prevalence, batrachochytrids, Ncandu Nature Reserve, fungal ecology.

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Chapter 1: General Introduction and Literature Overview

1.1 Introduction

Amphibian density and populations are rapidly decreasing around the world, of which about 500 species have suffered rapid declines in populations, sometimes leading to the extinction of species (Skerratt *et al.*, 2007). Various anthropogenic threats, such as pollution of habitats and urbanization contribute to this decline, resulting in habitat loss. However, one threat, in particular, a parasitic chytrid fungus known as *Batrachochytrium dendrobatidis* (*Bd*) (Phillips & Puschendorf, 2013), has caused rapid declines in amphibian species on a global scale (Kriger & Hero, 2007; Penner *et al.*, 2013; Scheele *et al.*, 2019; Skerratt *et al.*, 2007; Wilber *et al.*, 2017). *Batrachochytrids* in this thesis refers to *B. dendrobatidis* (*Bd*) as a simplified replacement for *B. dendrobatidis*. *Batrachochytrids* cause a disease known as chytridiomycosis, which compromises the host's osmoregulation and respiration, leading to death (Berger *et al.*, 1998; Wilber *et al.*, 2017). Transmission occurs during the early larval stages of amphibian tadpoles and up to later life cycles of adult amphibians. Adult amphibians can contract *Bd* for the first time during activities with an infected individual, such as mating and migrating to an infected water source (Briggs *et al.*, 2010). Infections usually become diagnosable when tadpoles reach Gosner stage 33 when the first indentations on the foot paddle become evident (Waddle *et al.*, 2019). *Batrachochytrids* feed on the keratin of amphibians, commonly found in the mouth parts of tadpoles, as well as the skin of post-metamorphic amphibians. *Batrachochytrids* primarily infect the rostradonts and keratodonts of tadpoles and spread to other parts of the body as the tadpole progresses through the developmental stages to an adult frog. Once an infected tadpole reaches the end of metamorphoses, *Bd* has the potential to spread to the skin of the adult frog, thus compromising osmoregulation as well as the respiration of the frog. Extensive infections that significantly prevent a frog's ability to perform these bodily functions can result in the frog becoming lethargic. When a frog becomes lethargic, it is less able to escape predation or acquire adequate nourishment to survive.

This study aims to assess the prevalence of *Bd* in the Ncandu Nature Reserve near Newcastle, South Africa. The area is of conservation concern as it contains various species of amphibians, and whether or not *Bd* is present in the protected area is unknown, nor do we know if it poses any threat to the amphibian populations. The reserve is the northernmost part

of the Drakensberg mountain range, which is known to be a *Bd* hotspot in South Africa (Griffiths *et al.*, 2018; Smith *et al.*, 2007). Therefore, disease surveillance of the area can be considered a conservation priority.

1.2 Discovery of Batrachochytrids (*Bd*)

The rapid decline of montane river frogs in Queensland, Australia, and Central America identified a need for research and monitoring as to the possible reasons leading to the decline in amphibian diversity (Skerratt *et al.*, 2007). The studies conducted led to the identification of *Bd* in infected frogs (Berger *et al.*, 1998). Years later, genome sequencing of the global *Bd* isolates revealed that the fungus originated from Asia and subsequently spread through trade (Kolby *et al.*, 2014; O'Hanlon *et al.*, 2018; Schloegel *et al.*, 2012; Weldon *et al.*, 2007).

Batrachochytrids have been found in Africa, but further investigations are required to determine the distribution throughout the continent. Only a few African countries have reported *Bd* infections, namely Nigeria, Cameroon, the DRC, Gabon, and Morocco (Doherty-Bone *et al.*, 2013; Doherty-Bone & Gvoždík, 2017; Penner *et al.*, 2013). This leaves more than 85% of Africa to be screened as there is no distribution data for West Africa whatsoever. Therefore, it is important to establish data points of *Bd* lineages as significant gaps need to be filled. *Bd* has caused severe declines in amphibian populations, especially those that live at higher elevations, such as mountains (Doherty-Bone *et al.*, 2019). Batrachochytrids have been present in Southern Africa for approximately 90+ years (Gower *et al.*, 2012), with the first case of *Bd* occurring in 1937 (Weldon *et al.*, 2004). Although *Bd* was found in South Africa, it was of minimal concern as it had a prevalence of 2.7% and exhibited no significant differences between regions, seasons, species, or time period (Weldon *et al.*, 2004). Even though the presence of *Bd* was known in many regions, little had been done to determine the threat *Bd* poses to native species, particularly those that are Red Listed.

For this reason, (Tarrant *et al.*, 2013) investigated *Bd* in threatened species and found that their distribution overlapped with the predicted occurrence of *Bd* and that the average prevalence was higher than the previous recorded national average of 14.8%. However, there is weak evidence that *Bd* in South Africa is associated with declining amphibian populations. Although *Bd* has been present in South Africa for some time, the divergence of lineages is a more recent event, which may have been initiated by environmental pressures (O'Hanlon *et*

al., 2018; Soto-Azat *et al.*, 2010). A more significant threat than widespread occurrence and high infection rates in the African landscape may be that existing lineages recombine to form more virulent strains.

1.3 Lineages and Species

One lineage was thought to be the cause of chytridiomycosis but was later found to be a wide range of lineages (Fisher *et al.*, 2018). It has become evident that *Bd* has spread to all stretches of the earth except for Antarctica, but as animals adapt to a changing environment so do batrachochytrids (Stevenson *et al.*, 2013; Sun *et al.*, 2016), this resulted in various lineages across the globe. Previously the only accepted lineages were *BdGPL* (global), *BdCAPE* (African), *BdCH* (European), and *BdBRAZIL* (Brazilian). However, studies led to the discovery of a new hyper diverse lineage in amphibians native to the Korean peninsula (*BdASIA-1*) (O'Hanlon *et al.*, 2018). *BdCH* was believed to be enzootic in Switzerland but is now grouped along with the *BdASIA-1* lineage (O'Hanlon *et al.*, 2018). Another lineage *BdASIA-2*, found in Korea correlates closely to that of *BdBRAZIL*, this hybrid lineage was found in the North American bullfrog which most likely found its way to Korea through the amphibian trade. The inability to infer the direction of the intercontinental spread between the isolates of the lineage led to *BdASIA-2/BdBRAZIL* (O'Hanlon *et al.*, 2018). It was concluded that the main lineages are *BdGPL*, *BdCAPE*, *BdASIA-1* (which includes the single *BdCH* isolate), and *BdASIA-2/BdBRAZIL* (O'Hanlon *et al.*, 2018). Isolates that were cultured in the wild Korean peninsula suggest that the Korean peninsula is a global center of *Bd* diversity with an ancestral association with *Bd* in East Asia (O'Hanlon *et al.*, 2018). Using Bayesian based haplotype clustering found that *BdASIA-1* shares more diversity with the global population of *Bd* than any other known lineage, leading to the assumption that chytrid originated from Asia or the Korean peninsula (O'Hanlon *et al.*, 2018).

The discovery of a new batrachochytrid species in Europe and South-East Asia sparked the interest of scientists not only because of its novelty but because it only infects salamanders and, like *Bd* resulted in population declines (Fisher *et al.*, 2018; Laking *et al.*, 2017; Martel *et al.*, 2013). This new species was named *B. salamandrivorans* (*Bsal*), which forms a clade with *Bd* and is grouped in the order Rhizophydiules (Laking *et al.*, 2017; Spitzen-van der Sluijs *et al.*, 2016). *Bsal* is believed to be more robust than the other *Bd* lineages as it can tolerate temperatures as low as -5°C, but growth is optimal at temperatures between 10-15°C (Laking *et al.*, 2017). Lineages found in South Africa include *BdCAPE* and *BdGPL* (Farrer *et*

al., 2011), as well as a hybrid lineage assumed to be the result of the recombination of *BdCAPE* and *BdGPL*. Despite batrachochytrids being present in South Africa for more than 90 years, no mass die-offs have been reported. This supports the statement that endemic *Bd* does not cause nearly as many fatalities as would recently introduced *Bd* (Antwis & Weldon, 2017; Farrer *et al.*, 2011). Although *BdGPL* is more prevalent across South Africa, it mainly dominates the western part of South Africa; *BdGPL* is found to be significantly more lethal than that of *BdCAPE*, which dominates the eastern highlands of the Eastern Cape (Ghosh *et al.*, 2021; O’Hanlon *et al.*, 2018). Batrachochytrids *BdCAPE* and *BdGPL* are the two lineages expected to occur predominately in the Drakensberg mountain range, which the Ncandu Nature Reserve is the northernmost part of (Tarrent *et al.*, 2013; Ferrar *et al.*, 2011). There has been no data collection concerning infected areas in Ncandu Nature Reserve. For this reason, more data is required to accurately provide a distribution map of batrachochytrids in the reserve.

1.4 Ecosystem dynamics and interactions

Amphibians are an essential part of an ecosystem, participating in important ecological processes that promote ecosystem health. They also play a pivotal role as predators by preying on pests as well as serving as prey to other organisms. Amphibians not only keep pests such as flies and termites at bay but also serve as a biological control agent for mosquito larvae and mosquitoes, which is an essential factor when faced with species that transmit dengue and malaria affecting human health. Tadpoles predate and compete against mosquito larvae resulting in biological control of disease transmission through mosquito vectors (Bowatte *et al.*, 2013; Mokany, 2007; Mokany & Shine, 2003; Murugan *et al.*, 2015; Raghavendra *et al.*, 2008).

Batrachochytrids are waterborne and, therefore, primarily affect amphibians that come in contact with water. Therefore, almost all amphibians are at risk of infection as their natural habitat is located around a body of water. Amphibians, especially montane and stream-dwelling, become infected through activities that take place in water, such as the deposition and fertilization of their eggs (Gründler *et al.*, 2012). Infection commonly starts at the larval stages of tadpoles; for this reason, tadpoles are constantly in contact with water and are thus at risk of infection. Therefore, it could be concluded that amphibians located at permanent ponds and water sources have a higher risk of susceptibility than that of amphibians in terrestrial biomes and wetlands (Gower *et al.*, 2012; Gründler *et al.*, 2012; Kriger & Hero,

2007; Lips *et al.*, 2006; Lips *et al.*, 2008; Lips *et al.*, 2003). Therefore, all surveys regarding *Bd* should focus on aquatic amphibians in areas with a high population density.

Batrachochytrids thrive in temperatures between 17-23°C but have also been found at lower temperatures and are often linked to climate and seasons (Hirschfeld *et al.*, 2016; Ruggeri *et al.*, 2018; Voyles *et al.*, 2011). Amphibian species that reproduce seasonally or are restricted to a specific area are at risk of extinction should there be an outbreak of *Bd* (Bielby *et al.*, 2008). Climate change and habitat loss can act as drivers for the spread of *Bd* (Bosch *et al.*, 2007; Bosch *et al.*, 2018). Destruction of natural environments which contain amphibian species that are infected with *Bd* could lead to the migration of individuals from one region to an otherwise pristine area, consequently spreading *Bd* to a new locality (Brem & Lips, 2008; Voyles *et al.*, 2011). Other modes of transmission include behaviours such as territoriality, mating, habitat preferences, and other aggregative behaviours that could lead to the transmission of *Bd* to individuals that are not infected (De Castro & Bolker, 2005).

Another mode of transmission is that of invasive species that come into contact with a healthy amphibian rich area. Invasive amphibian species that migrated or were accidentally introduced to an environment utilize the same pond or water source as the native species. Deposition of fertilized eggs and consequently tadpoles that mature infect the water source as well as the other tadpoles (Miaud *et al.*, 2016). These tadpoles then metamorphose into adults, which are infected with *Bd*. Adult frogs then deposit and fertilize eggs in the infected water source, thus starting the *Bd* lifecycle within a new generation. An example of this is that of the invasive North American bullfrog *Lithobates catesbeianus*, which was hypothesized to have spread *Bd* to Europe (Miaud *et al.*, 2016).

Batrachochytrids are not altitude specific, which means they are not limited by high altitudes. Therefore, all amphibian species are at risk whether they are found at high or low altitude localities (Kriger & Hero, 2008). This poses various questions as to why *Bd* more readily infects amphibians at higher altitudes than lower lying localities. This may be due to natural organisms that outcompete *Bd* to a greater extent in low altitude localities but are limited by altitude, thus giving *Bd* the competitive edge at higher altitudes.

Studies have shown that an introduction of *Bd* to a pre-epizootic locality can have devastating effects on species found in the area. A study done by Brem and Lips (2008) illustrates the

devastation batrachochytrids are capable of when introduced to an otherwise pristine environment located in Panama. A total of 42 amphibian species were present pre-epizootic, all museum specimens that tested negative for *Bd* up to 2002, where several dead amphibians were found by a local naturalist (Brem & Lips, 2008). A study was then conducted in 2003 to assess the cause of these deaths, with the cause of death of amphibians being infected with *Bd*. Amphibian captures were done in 3 trips to determine population density in the area. On the first trip in July, a total of 50 specimens were captured. The second trip in August showed a dramatic decline in amphibians capturing only 11, and on the third trip, a total of 14 specimens (Brem & Lips, 2008). Of the 42 resident species, five commonly found species were not found. In July of the following year, only 13 of the 42 resident species were encountered, which accounts for only 31% of the amphibian diversity pre-epizootic (Brem & Lips, 2008). This study illustrates *Bd*'s devastating effects on a dense population of amphibians. It only takes one infected individual to transmit *Bd* to an entire undisturbed area.

Similarly, in Africa, surveys in Tanzania revealed the extinction in the wild of the Kihansi spray toad (*Nectophrynoides asperginis*). These toads are ovoviviparous and are endemic to a particular spray wetland in the Udzungwa Mountains (Channing *et al.*, 2006). Surveys were conducted from 2001 up to 2003, during which a rapid population decline was observed, of which the actual cause of the extinction was unknown (Channing *et al.*, 2006). Various causes, such as upstream pesticides, safari ants, and *Bd*, were thought to have caused this decline. Collection of the specimens from the population crash in 2003 was examined, and it was found that *Bd* was the cause of this mass population crash that drove the Kihansi spray toad to extinction in the wild (Makange *et al.*, 2014; Weldon *et al.*, 2020). After the population crash in 2003, it took approximately nine months for the remaining amphibians to succumb to *Bd* resulting in the complete extinction of the wild Kihansi spray toad (Weldon *et al.*, 2020).

1.5 Distribution of Batrachochytrids

As stated in section 1.1, *Bd* has been present in South Africa for more than 90 years (Gower *et al.*, 2012). In these years, *Bd* was able to manifest and spread across South Africa. This spread of *Bd* resulted in recombinant lineages. The prevalence of *Bd* is higher in areas with diverse amphibian fauna (Ron, 2005), making South Africa the *Bd* hotspot that it is, as South Africa has approximately 134 described species of amphibians (Du Preez, 2017). Although *Bd* can be found on various continents, they thrive in tropical areas or areas otherwise rich in amphibian density and diversity (Ron, 2005), making parts of the South

African climate and biodiversity ideal for *Bd* to thrive in. This creates concerns in South Africa and other countries with similar environmental conditions. Outbreaks of *Bd* in tropical humid areas can be related to the fact that *Bd* zoospores are dependent on these favourable weather conditions for transmission (Hirschfeld *et al.*, 2016; O'Hanlon *et al.*, 2018; Voyles *et al.*, 2011).

Batrachochytrids can be found in almost all coastal lowlands of the Western, Eastern, and Northern Cape, Swaziland, the majority of KwaZulu-Natal, as well as the entire Drakensberg mountain range, including montane Lesotho (Ghosh *et al.*, 2021; Griffiths *et al.*, 2018). The Drakensberg Mountain range is the ideal habitat as it is comprised of various habitats ranging from grassland habitats to densely forested areas. *Bd* thrives in these habitats since the region receives the highest annual rainfall for South Africa, and high humidity allows for adequate moisture and heat in dry winter months. *Batrachochytrium dendrobatidis* from the Drakensberg is known to occur in *Xenopus laevis*, *Amietia delalandii*, *Strongylopus grayii*, *Amietia hymenopus*, and *Hadromophryne natalensis* (Smith *et al.*, 2007) and is believed to be the cause of a mass mortality event of *Amietia hymenopus* (Weldon & Du Preez, 2004). Globally, approximately 700 species of amphibians have been found positive for *Bd*, with chytridiomycosis being identified as the cause of population declines of over 500 species and the eradication of an estimated 90 species (Ghosh *et al.*, 2021; Scheele *et al.*, 2019).

1.6 Diagnosing Batrachochytrids

Chytrid can be diagnosed through various techniques such as cytological screening, PCR analysis, and histopathology (Boyle *et al.*, 2007; Fisher *et al.*, 2018; Ghosh *et al.*, 2021). Physical investigation of tadpole mouthparts for *Bd* is an excellent method to utilize. This is done on tadpoles as they can be visually screened for batrachochytrids due to their high levels of infections (Fisher *et al.*, 2018). To screen the tadpoles, they must be caught and humanely euthanized (Fisher *et al.*, 2018). Therefore, ethical requirements need to be met. It has been found that the morphological stage of the tadpole correlates with the level of infection; tadpoles that are more developed morphologically exhibit a higher level of infection than that of less developed tadpoles (Boyle *et al.*, 2004; Griffiths *et al.*, 2018; Hanlon *et al.*, 2015; Smith *et al.*, 2007). Therefore, it is evident that there is a correlation between the developmental stages as described by Gosner *et al.* (1960), and the infection level of *Bd*.

Another method is isolating and culturing *Bd* (Fisher *et al.*, 2018). There are various techniques involved in the isolation and culturing process. Over the years these techniques were modified as previous methods involved euthanizing frogs (Fisher *et al.*, 2018). One of

these techniques involved swiping or dragging the leg of an infected frog across a nutrient agar to isolate and cultivate *Bd* (Longcore *et al.*, 1999). Techniques of this nature were frowned upon, which led to alternative techniques which involved euthanizing tadpoles rather than adult frogs (Fisher *et al.*, 2018). Due to their high levels of infection, tadpoles serve as the best method to isolate and culture *Bd* (Fisher *et al.*, 2018). An alternative non-lethal method proposed by Fisher *et al.* (2018) involved clipping the tip of the fourth hind toe and doing non-invasive swabbing of the frog by gently stroking a cotton swab over the hind legs, stomach and the webbing between their toes has been developed.

Polymerase Chain Reaction (PCR) is used to detect the DNA of *Bd*. Samples are derived from swabbing specimens and analysing them using PCR techniques. The process is lengthy, requiring various substances and specific steps. These steps involve obtaining an isolate or a swab used on a specimen. Then the DNA has to be extracted from the sample using a DNA extraction kit such as DNEASY. Once DNA has been extracted from the sample, it is placed into PCR plates along with primers that detect *Bd* during the PCR cycle. Should the sample indicate no fluorescence during the amplification cycle, it can be concluded that the sample is negative (Boyle *et al.*, 2004).

Histopathology used to be readily used, especially in the case of adult and dead frogs, as skin lesions are caused by *Bd* and can be visually observed (Pessier *et al.*, 1999). Tissue samples are subjected to an intensive washing process, which involves steps including dehydration, elucidation, and impregnation of paraffin wax. The wax blocks are then cut into 0.6 µm sections and placed into a warm water bath which allows the wax to stretch. Sections are then placed onto microscope slides and placed into an oven at 36°C for 24 hours. Slides are then stained, followed by permanent fixation with a cover slide. The slide can then be examined for *Bd* using a compound light microscope.

Depigmentation and malformation of tadpole mouthparts are symptoms of oral chytridiomycosis (Smith & Weldon, 2007); by looking at these symptoms, *Bd* can be indirectly identified. Although identification of *Bd* using indirect methods is non-lethal, they could produce errors such as false negatives and false positives (Smith & Weldon, 2007). Indirect methods work on the basis that depigmentation and malformation strongly correlate with the infection of *Bd* (Smith & Weldon, 2007). These errors could significantly alter the outcome of the results as this indirect method does not take into consideration low levels of infection in which depigmentation or malformation has not yet occurred, which could lead to false negatives. False positives can be easily made as not all species react to *Bd* infections the same way and thus would not exhibit the symptoms associated with indirect methods of identifying *Bd* (Smith & Weldon, 2007). The advantages of using indirect methods are simply that it is non-lethal, and tadpoles can be quickly assessed for depigmentation or malformation

and be placed back into the wild (Smith & Weldon, 2007). Therefore, indirect methods represent a trade-off between ethics and experimental accuracy. For this reason, indirect methods are debated as to their scientific as well as ethical principles involved in the accuracy of results.

1.7 Importance of Disease Monitoring

Bd is recognized as the worst wildlife pathogen in recorded history (Scheele *et al.*, 2019). This novel pathogen has caused the mass extinction of amphibian populations across the globe. Therefore, it is of great ecological importance to monitor and investigate *Bd* and its distribution across a natural landscape high in amphibian diversity. Most conservation authorities generally accept that no country has the resources to ensure absolute security of its borders in terms of disease control making them vulnerable to the introduction of diseases (Weldon *et al.*, 2013). Countries that have a high risk of biodiversity loss due to infectious diseases have greater responsibility and as a result, conduct disease monitoring. This allows a country to identify and promptly act on possible emerging diseases in an area (Morner *et al.*, 2002).

Preliminary studies in many countries like Madagascar have identified *Bd* in amphibian populations (Bletz *et al.*, 2015). These countries have confirmed that there was a sudden population decline without environmental factors. Thus numerous studies conducted have shown the presence of *Bd* (Federici *et al.*, 2008). An example of countries that actively monitors infectious diseases is Madagascar (Weldon *et al.*, 2020), and this island paradise has over 500 species of amphibians, with 377 formally described (Perl *et al.*, 2014; Vieites *et al.*, 2009). Surveys should be conducted annually except when traces of *Bd* have been detected; then surveys should be conducted at least twice a year. Monitoring and surveillance of areas rich in amphibian density and species diversity should be prioritized to detect declines of species within a given ecosystem. Regular monitoring and surveillance of potential threats can be minimized allowing governing authorities to implement conservation strategies, resulting in the preservation of amphibian species for a geographical area.

1.8 Mitigation of *Bd*

Amphibian populations have rapidly declined in recent years due to *Bd*, and as a result, there have been various approaches and techniques to minimize and mitigate the effect of *Bd* in amphibian populations. Reduction of host density is an approach that can prevent or decrease

the spread of *Bd*. By reducing the density of susceptible species such as *Xenopus laevis* in a population through culling or translocation can reduce or prevent the excessive spread of *Bd* within the area (Woodhams *et al.*, 2011). A reduction in infected and susceptible individuals could prevent an outbreak of *Bd* within a densely populated amphibian population.

Treating infected amphibian hosts and habitats is another approach to mitigate the spread of *Bd* in an area. *In vitro*, *Bd* is susceptible to various antibiotics and fungicides, as well as salt and drying out (Johnson *et al.*, 2003; Woodhams *et al.*, 2011). Using antibiotics and fungicides can help reduce pathogenicity and prevent disease outbreaks. When dealing with an artificial pond or area that can be drained of water, consequently eradicating any *Bd* in the water source. Individuals from an infected water source can be removed and treated for infections and reintroduced to the area after they have been cleared. Although this is an effective approach to mitigate the spread of *Bd*, it is also very expensive and only applies to areas that can be drained. Therefore, this approach cannot be used in wild habitats with a constant supply of water, as such measures would be labour intensive and extremely expensive (Griffiths & Pavajeau, 2008; Walker *et al.*, 2008). Biological control using predators that consume *Bd* is an excellent approach to minimize the spread of *Bd* and prevent an outbreak of *Bd* in an ecosystem. "Microcrustacean zooplankton, such as water fleas (Cladocera), copepods (Copepoda), and seed shrimp (Ostracoda), are aquatic grazers and eat the aquatic zoospores of some chytrid fungi" (Schmeller *et al.*, 2014; Hamilton *et al.*, 2012; Kagami *et al.*, 2004). The use of these microcrustaceans could reduce the spread of *Bd* to other regions within the geographical area.

One of the more innovative strategies to mitigate chytridiomycosis is biological control using mycoviruses (Webb *et al.*, 2022; Woodhams *et al.*, 2011). Most mycoviruses are double-stranded RNA (dsRNA), but single-stranded DNA (ssDNA) has also been found (Kotta-Loizou & Coutts, 2017). These are viruses that infect a fungal host, impair the growth of zoosporangia, and inhibit spore production (van Diepeningen *et al.*, 2006). The presence of these viruses would reduce the ability of a pathogenic fungus like *Bd* to cause chytridiomycosis (Webb *et al.*, 2022). If it can be possible to reduce the impact *Bd* has on amphibians, it could lead to amphibians developing immunity toward *Bd*.

1.9 Aim and Objectives

To determine the degree and distribution of batrachochytrid prevalence in the Ncandu Nature Reserve through the utilization of isolation approaches and to determine how seasonal changes influence the severity of *Bd* infection within the Ncandu Nature Reserve.

Objectives include the following:

- To determine the degree of Batrachochytrid prevalence in the species *A. delalandii* at the Ncandu Nature Reserve.
- To determine the distribution of *Bd* in the Ncandu Nature Reserve.
- To determine if seasonal changes affect the degree of prevalence at infected sites.
- To identify the best isolation approach to isolate *Bd*.
- To determine species diversity and distribution of amphibians in the Ncandu Nature Reserve.
- To determine if a competitor's presence influences the fitness of *Bd* (*in vitro*).
- To determine the behaviour of *Bd* in the presence of a competitor using the Tri-Tube Behavioural Chamber (*in vitro*).

1.10 Chapter overview

The introduction and literature overview (Chapter 1) is followed by *Bd* prevalence in the NNR (Chapter 2), which contains all information regarding *Bd* prevalence, distribution, Isolation success, contamination percentage, and amphibian species distribution. Chapter 3 consists of *in vitro* experiments to determine the behaviour *Bd* exhibits in the presence of a competitor. Chapter 4 is the General discussion in which both chapter 2 and 3 are discussed collectively. References for all chapters follow Chapter 4.

Chapter 2: Distribution of Batrachochytrids in Ncandu Nature Reserve

2.1 Introduction

Amphibians provide vital ecological functions, which form part of the more extensive ecological processes that allow an ecosystem to thrive rather than survive. Amphibians are vital to most temperate and tropical ecosystems and are often referred to as indicators of a healthy environment because they are both aquatic and terrestrial. Therefore, an area with a high species density and diversity often indicates that the area or region is in a pristine condition allowing all inhabitants to thrive. For this reason, the conservation of all amphibian species has high ecological relevance.

Amphibian species have suffered rapid declines in populations on a global scale with 43% of species are experiencing declines, and a further 11% are data deficient (IUCN, 2022). Various anthropogenic threats, such as pollution and urbanization, contribute to this decline, resulting in habitat loss. One threat, in particular, is a parasitic chytrid fungus known as *Batrachochytrium dendrobatidis* (*Bd*) (Phillips & Puschendorf, 2013), which has caused rapid declines in amphibian species on a global scale (Kriger & Hero, 2007; Skerratt *et al.*, 2007; Penner *et al.*, 2013). *Bd* causes a disease known as chytridiomycosis, which compromises the host's osmoregulation and respiration, leading to death at later life stages (Berger *et al.*, 1998).

Amphibians serve as excellent bioindicators for ecosystem health because of their unique biology. Amphibians have thin permeable skin that readily absorbs moisture directly from the environment, implying that any dissolved contaminants present in the environment can also be absorbed through the skin of the amphibians (DeGarady & Halbrook, 2006; Dixon *et al.*, 2011).

For this reason, it is vital to assess and document the spread of Batrachochytrids to create a baseline study upon which future surveys can be based. Various localities were surveyed for *Bd*, with the prevalence of each locality being determined. Sampling localities in the area range from lowland ponds and streams to montane river streams. This is done to determine

the prevalence of *Bd* at low and high altitudes. Studies show that all amphibians are susceptible to *Bd* regardless of altitude. It was found that *Bd* infections persist longer throughout summer in upland locations than in lowland areas, implying that montane amphibian populations are more prone to disease outbreaks than lowland species (Kriger & Hero, 2008).

There is still a significant gap regarding the distribution zones of *Bd* in South Africa. South Africa is a biodiversity hotspot as it has various biomes that cater to different species. South Africa also has stringent conservation laws protecting threatened or endangered species, but these laws focus primarily on specific mammals such as rhinos and elephants. Laws regarding the conservation and monitoring of amphibians tend not to grab the interest of officials as amphibians are not the vertebrates that attract tourists to South Africa. As a result, only a fraction of protected areas and national parks have been screened for *Bd*; these include major parks such as the Addo Elephant National Park and the Kruger National Park. These two parks are the largest protected areas in South Africa, yet they have not been screened for *Bd*. Areas surrounding these Parks have been screened, and *Bd* has been positively identified, although more tests need to be run to determine the lineage. Screening shows that *Bd* is present in these localities and should be monitored closely, as the monitoring of amphibians is a lesser concern when considering the health of an ecosystem.

2.2 Preservation of Ncandu Nature Reserve

Kwa-Zulu Natal (KZN) has the highest diversity and density of amphibians than any other province in South Africa. Regions in KZN are subdivided into districts, each of which regulates its conservation approach based on the National Environmental Management Act 107 of 1998, regulated by Ezemvelo KZN Wildlife. Ezemvelo regulates more than 120 protected areas, including the Ncandu Nature Reserve (NNR). Even though there are guidelines on regulating and managing nature reserves, little attention is paid to amphibian conservation within these reserves. Of the 120 known reserves, approximately 9 Nature reserves have been screened of *Bd*. Therefore, it should be of the utmost importance for continuous monitoring within the NNR to assess the extent and prevalence of *Bd* in the area.

Part of the motivation for creating the Ncandu Nature Reserve (NNR) was to protect endangered, rare, endemic species native to the area and to preserve and conserve the area's ecological integrity. The preservation of the ecosystem and all its species is of great importance; however, this is only related to anthropogenic disturbances. The East sub-region

uKhahlamba Region and the Newcastle Magisterial District (EKZNW) protect NNR from various anthropogenic threats such as deforestation and stripping of natural resources from the landscape, but this does not include biological threats such as batrachochytrids. Ncandu Nature Reserve is a diverse, rich landscape that has both montane forests as well as grassland. Due to the nature of the landscape, various frog and toad species have claimed these areas for their habitat. All protected areas in South Africa were not created with amphibians in mind as 85% of South Africa's amphibians are considered to be under protected (SANBI, 2018). Therefore, it is important to monitor amphibians in protected areas for all threats including that of *Bd*.

Two surveys were done to determine the prevalence percentage during the off-season (winter months) and in-season (summer months). The species chosen for the study was *Amietia delalandii*, the common river frog, as its tadpoles can be found throughout the year, with some tadpoles staying in their larval state for up to two years. This species was chosen for its continuous breeding habits, as studies show that breeding increases the prevalence of a site (Conradie *et al.*, 2011). By using this species, a clearer picture can be provided as to the prevalence of *Bd* during the breeding season and outside of the breeding season. Although *A. delalandii* breeds more during winter months, larger tadpoles tend to have a higher degree of infection as they are exposed to *Bd* for a longer period than smaller tadpoles (Smith *et al.*, 2007).

2.3 Methodology

Two processing methods were used to determine the most effective approach to utilize in the field, as contamination is a big issue in the procurement of *Bd* isolates. Isolates provide a baseline of prevalence in the NNR. Future studies at the NNR are essential to provide data points of infected sites and whether or not they are increasing in prevalence or spreading to other sites that were not previously recorded.

2.3.1 Locality and Extent of Ncandu Nature Reserve

Ncandu Nature Reserve (NNR) forms part of the eastern Drakensberg Escarpment and stretches to the Low Berg Escarpment, located approximately 32 km south-west of Newcastle. The protected area of NNR falls within the East sub-region uKhahlamba Region and the Newcastle Magisterial District (EKZNW), 80km from Ladysmith on the north side of the dam

and 19km South of Newcastle on the D210 District Road towards Normandien (Rambarath *et al.*, 2009).

The NNR was proclaimed a protected area in 1925 as the Ncandu Forest Reserve, which later included two separate pieces of state land, which was added in 1989 from the adjoining farms Steep Glen and Buffel's Hoek. With the addition of these two estates, the total size of NNR was expanded to 1858 ha. The protected area extends from 27°51'36" S to 27°58'12" S, and from 29°40'48" E to 29°45'00" E, across an altitude gradient of 1460 m to 1900 m (Figure 1).

The Ncandu Private Forest and Grassland Reserve is under private management but forms part of the NNR. Regarding dominant vegetation types, the NNR includes the Northern KZN Moist Grassland, Southern Mistbelt Forest, and Low Escarpment Moist Grassland (Figure 1).

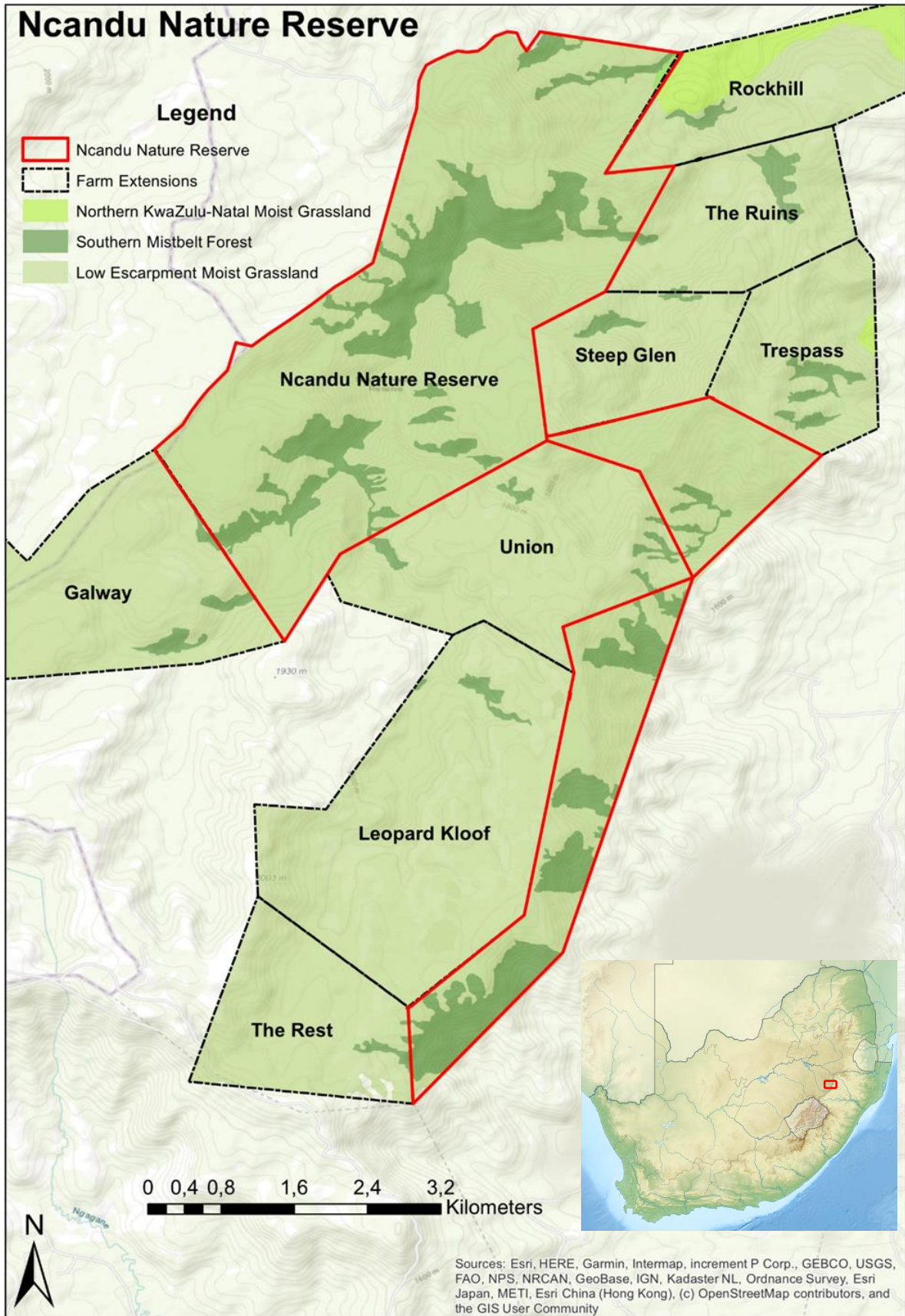


Figure 1 Vegetation map of Ncandu Nature Reserve, indicating the various land borders

2.3.2 Climate and Rainfall

There are no official rainfall figures for the Ncandu Nature Reserve; thus, the data figures presented show the official climate and rainfall for the town of Newcastle, which is approximately 22km from the NNR.

It is a perfect paradise for amphibian species in the area as it has an average annual rainfall of 895 mm, which provides more than adequate water for species in the environment. Rainfall varies in altitude, with the lower grasslands receiving approximately 680mm to the montane forests receiving up to 1900mm annually (WWC, 2022). The area is also prone to flash floods, with some areas experiencing over 200mm of rainfall within 24 hours (Figure 2).

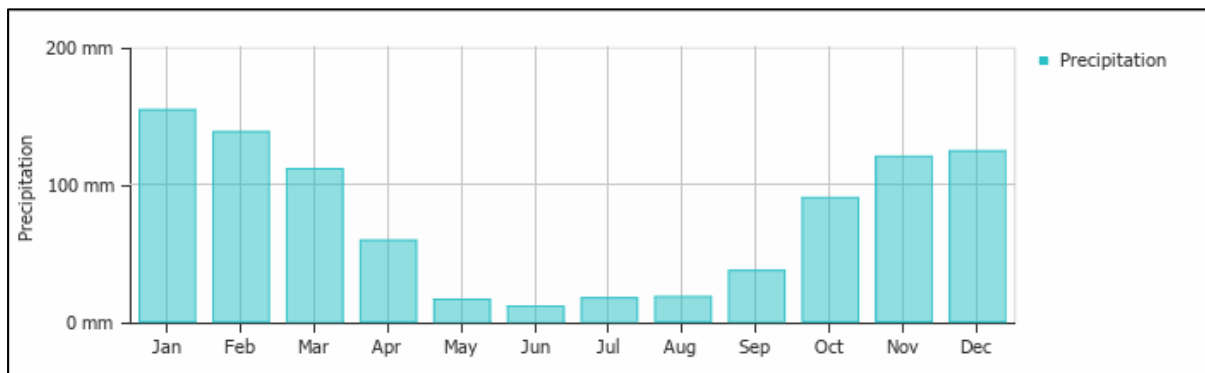


Figure 2 Average monthly precipitation in Newcastle, South Africa for the past 12 years (WWC, 2022).

The Climate of NNR is primarily cold and stable, with a mean temperature of 20°C; this temperature fluctuates in response to altitude. The summer periods, spanning from September to the end of March, average on temperatures between 10°C and 29°C. Winter periods, spanning from April to the end of August, average on temperatures between 1°C and 24°C (Figure 3).

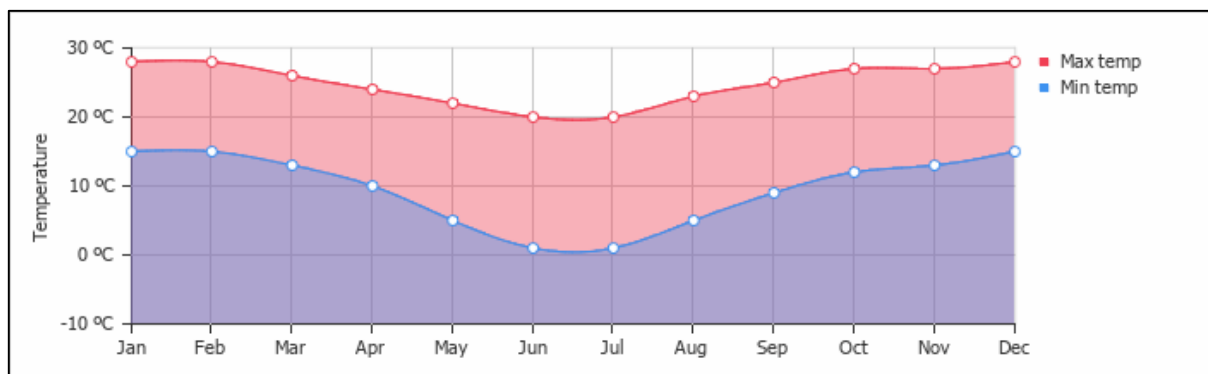


Figure 3 Average Minimum and maximum temperatures in Newcastle, South Africa for the past 12 years (WWC, 2022).

Humidity is relatively constant throughout the year at 60%, dipping slightly below 50% during the peak winter months. Humidity plays a significant role as *Bd* thrives in areas with higher humidity (Figure 4). The higher the humidity in a locality, the more moisture there is in the air. The humidity would also prevent water bodies from drying out.

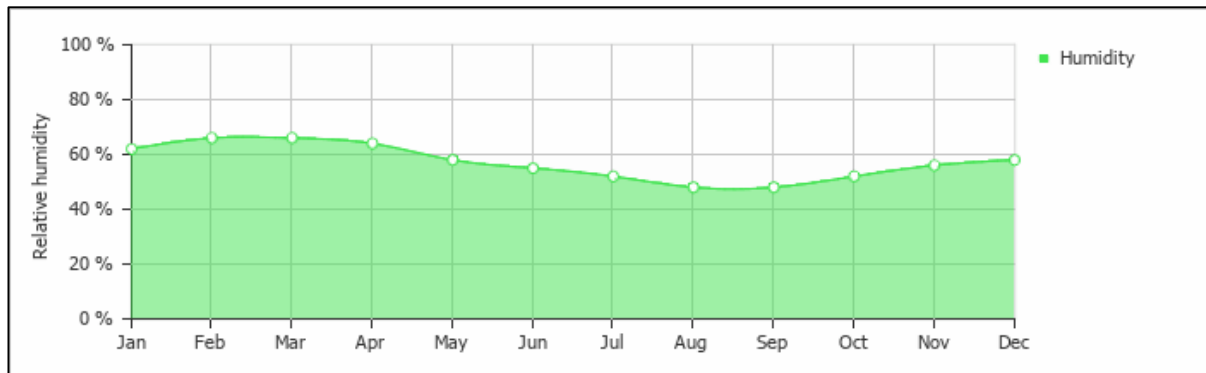


Figure 4 Average relative humidity in Newcastle, South Africa (WWC, 2022).

2.3.3 Sampling Locality

A total of 11 sites were screened for batrachochytrids. These sites included areas in the Ncandu Nature Reserve as well as some of the bordering forest and grassland reserves that have been merged with the Ncandu Nature Reserve. Site selection was made by selecting perennial water bodies, primarily undisturbed by human and cattle activity. Sites were also selected based on the species of frogs that occur at the body of water, as it was necessary for *A. delalandii* to be present as it is the focal species. Marshes or intermittent bodies of water were also included based on the amphibian activity at the site areas, with a higher population density or species diversity being of greater concern as more species in the area are at risk. It was important to establish distribution throughout the entire reserve to ascertain the spread and prevalence of *Bd* in both highland and lowland areas.

Eight of the eleven sites are highland montane areas, and three are lowland valley areas (Figure 5). The lowland areas include Marsh, Eikenhof Dam, River Bend Stream, and the highland areas include Protea Outlook, Steep Glen Kloof, Steep Glen River, Union, Mushroom Rock, Dampad, Leopard Kloof, and Bloekombos. These areas are considered pristine as no agricultural activity readily occurs in these areas. Therefore, it was vital to determine whether *Bd* was present at any of these sites.

2.4 Construction of Geographical Charts

The map of Ncandu Nature Reserve and the amphibian distribution maps were created using ArcMap Ver. 10.7.2. To determine the geographical position of the NNR, a topographic reference map of the world featuring parks and landmarks was used as a reference map. Since farms were added to the reserve, and the reserve's geographical range is now larger than what is indicated on publicly available maps, a more updated map from Rambarath *et al.* (2009) was spatially fitted and georeferenced according to the contour of the NNR as shown on the reference map. In addition, a map of South African vegetation was retrieved from BGIS <http://bgis.sanbi.org/vegmap> and clipped to the newly georeferenced map, which contained the NNR and the farm expansions that make up the reserve. The geographical scope of NNR is shown in red on publicly available maps. Furthermore, the additional farms for the reserve are denoted by a dashed black line.

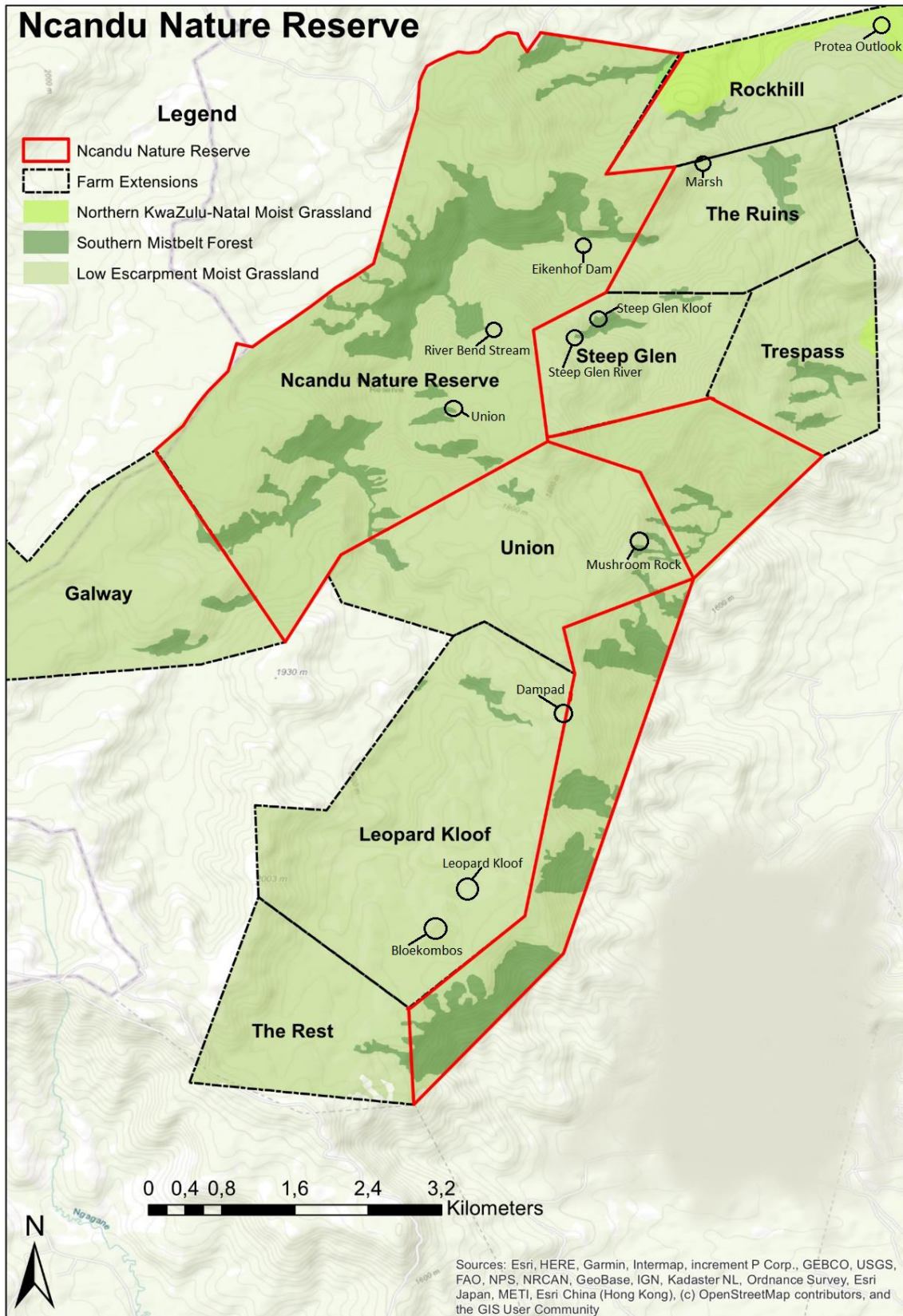


Figure 5 Sampling sites screened for Bd in the Ncandu Nature Reserve.

2.5 Project Authorization

The collection permit was issued in pursuance of the provisions of the Nature Conservation Ordinance No 15 of 1974, Chapter 7, and the Regulations framed thereunder. The permit allows for collecting twenty adult frogs per species to obtain toe clips and complete collections (tadpoles) per site within the Ncandu Nature Reserve. For sampling to occur, obtaining a permit to sample from Ezemvelo and ethical clearance from the AnimCare Committee was required. After the permits were obtained, an application for ethics was lodged with the AnimCare council. Sufficient motivation was provided, and the application was successful, permitting the collection of samples needed for this study.

2.6 Project sampling methodology

2.6.1 Preparation of lab materials needed for sample collection

For the first survey, the liquid media method was used as described by Fisher *et al.* (2018). We prepared antibacterial 1% tryptone agar plates, as well as 1% tryptone broth in 1ml eppendorf tubes for use in attempted isolation and culture of *Bd*.

Preparation of the antibacterial 1% tryptone agar was done a week before fieldwork to ensure the effectiveness of the nutrient agar and that of the antibacterial component (Figure 6).

1. Exactly 1L of distilled water was poured into an autoclavable 1L Scott bottle, 1L is enough for the preparation of 40 plates. This process was done twice.
2. 10g of tryptone was then weighed out and added to the distilled water.
3. 10g of No2 bacterial agar was then weighed and added to the distilled water.
4. The mixture was shaken well to ensure that all the substances have been dissolved.
5. The Scott bottle was then placed into the autoclave for a 20 min sterilization at 121 °C.
6. Once the autoclave was done the mixture was removed with caution and placed aside to cool down.
7. After enough time has passed for the mixture to have cooled down enough to be placed against the cheek without burning, it was then taken back to the scale, making sure that the area it was placed in is sterile.
8. 200mg of ampicillin and 350mg of streptomycin were weighed out, added to the lukewarm mixture and mixed well to ensure that all the antibiotics have dissolved into the solution.

9. Ager plates, as well as the solution, were sterilized with 70% EtOH to ensure that no contaminants are carried into the sterile laminar flow.
10. Once fully sterilized the solution was carefully decanted equally into the 20 agar plates and left to solidify.
11. Once the agar has fully solidified it was placed in a sterile bag and sealed to avoid contamination and placed in a refrigerator at approximately 14°C.

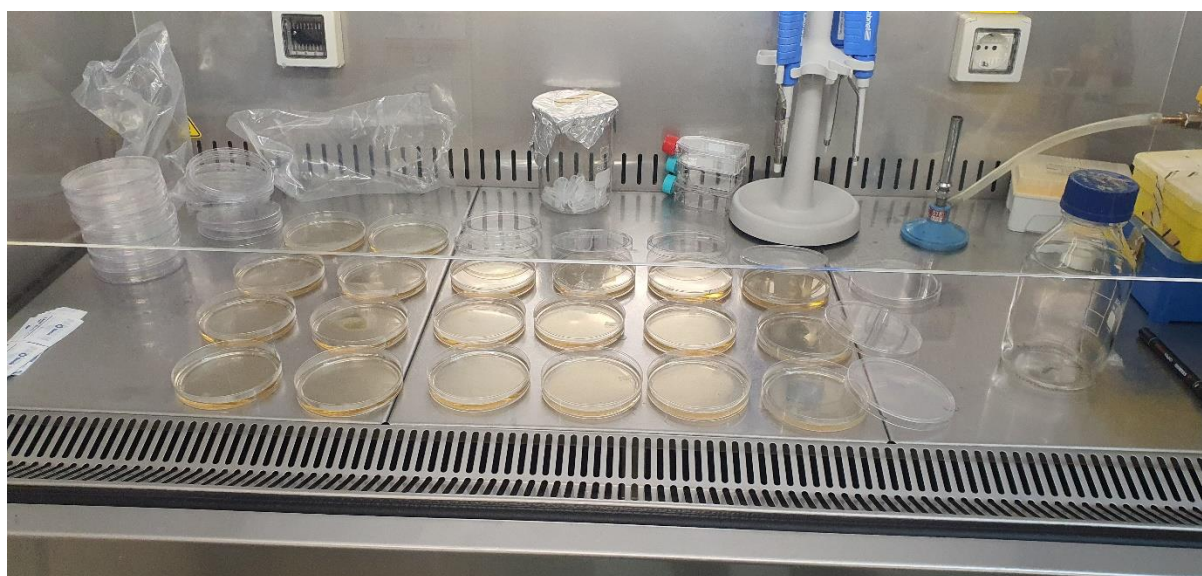


Figure 6 Agar preparation done inside a Laminar Flow Cabinet

Preparation of the antibacterial 1% tryptone broth was prepared a week before fieldwork to ensure the effectiveness of the nutrient-rich broth as well as the antibacterial component.

1. Exactly 1L of distilled water was poured into an autoclavable 1L Scott bottle.
2. 10g of tryptone was then weighed out and added to the distilled water.
3. The mixture was then shaken well to ensure that all the substances have been dissolved.
4. The Scott bottle was then placed into the autoclave for a 20 min sterilization at 121°C.
5. Once the autoclave cycle finished the mixture was removed with caution and placed aside to cool down.

6. After enough time has passed and the mixture was cool enough to be placed against the cheek without burning, it was then taken back to the scale making sure that the area it was placed in is sterile.
7. 200mg of ampicillin and 350mg of streptomycin were weighed out and added to the lukewarm mixture and mixed well to ensure that all the antibiotics have dissolved into the solution.
8. The solution was then sterilized before being placed into the laminar flow where it was transferred to 2ml eppendorf tubes.
9. Exactly 1ml of the solution was transferred to the eppendorf tubes via a pipet.
10. After the solution has been transferred to approximately 200 Eppendorf tubes they were sealed and refrigerated until fieldwork.
11. Excess solution was sealed and refrigerated for later use.

2.6.2 Field Sampling

During field sampling, a total of 10 *Amietia delalandii* tadpoles were sampled per site with the use of dip nets. Strong sweeping movements were used while moving through the water to catch tadpoles (Figure 7). After each sweep, the net was examined, and the largest tadpoles were removed within the Gosner stage 33 and up. Once enough tadpoles have been sampled, they were placed into plastic bags and then into plastic containers for protection with sufficient air for the tadpoles to survive. Containers were then marked according to the site they were sampled from. Toe clips were also taken from adult frogs that were found using a surgical scissor, which is sterilized before use by dipping in 70% EtOH and then flamed; the sterilization process was repeated before being used. The frog was then secured with the belly of the frog pointing upward and the legs grasped between the pinkie and index finger, stretching the legs outward to avoid any sudden movements. Using the surgical scissor, the tip of the longest toe was excised, embedded in an agar plate with antibiotics, and then marked. The foot was topically treated with disinfectant ointment (Betadine). The frog was then released back into the environment, and the instruments were thoroughly cleaned before being used again to prevent cross-contamination.



Figure 7 Active collection of tadpole samples using dip nets.

2.6.3 Field sample processing

After all the samples of the day have arrived at the base camp, processing of the samples needs to take place to avoid contamination. Animals were transported alive to basecamp to prevent any form of decay; tadpoles were then euthanized using MS-222 (tricaine methane sulphate), which were placed into designated containers. Five tadpoles at a time were subjected to euthanasia. Once the tadpoles were euthanized, the excision of mouthparts took place.

After putting on gloves and sterilizing your hands with 70% EtOH the first step was to take a tadpole and place it on its left side using forceps that remain in the container with the MS-222. The second set of forceps and the surgical scissors were then dipped in 70% EtOH and flamed to prevent contamination. Using the non-dominant hand, the tadpole was picked up using forceps, gripping the tadpole just below the mouth. By adding slight pressure to the forceps, the mouthparts can be pushed outward, allowing one to excise the mouthparts more effectively. Once the mouthparts have been excised, the tadpole was placed into a biohazard bin.

Mouth parts were then carefully placed on a sterile petri dish and cut into four equal segments. These segments were then placed onto an antibacterial agar plate and dragged through the agar using a zig-zag movement with the aid of a sterilized 27 gauge needle (Figure 8). Once the individual segment has been thoroughly dragged through the agar, it was placed into the eppendorf tubes containing the antibiotic 1% tryptone broth and was clearly marked. All equipment used was then sterilized before starting with the next specimen. The same method was utilized for the processing of toe clips.

After all the tadpoles have been processed, the eppendorf tubes were refrigerated to delay the growth of possible contaminants. After processing, all equipment was thoroughly cleaned, used agar plates were disposed of as biohazard material, and the surface was cleaned with 70% EtOH.



Figure 8 Field Sample Processing: (Left) Capturing of field data after processing took place; (Right) Mechanical cleaning of tissue samples during the isolation process of Bd.

2.6.4 Culturing of *Bd* isolates

The eppendorf tubes containing the samples were placed into the incubator set at 20°C for three days. The container with the eppendorf tubes was thoroughly sterilized and placed into the laminar flow along with sterile 24 well plates and antibacterial 1% tryptone broth. The content of each eppendorf tube was then transferred to its corresponding well and topped up

with fresh tryptone broth to promote growth. Each well was clearly marked to identify the specimen. Each 24 well plate was then sealed using parafilm (Parafilm M®), which allows for gaseous exchange, allowing the specimens to receive oxygen. After all specimens have been transferred to 24 well plates; they were placed into the incubator to facilitate growth.

Daily observation was required of all the wells to ensure no contamination spreads from 1 well to another and to see if any *Bd* zoospores were present in any of the specimens (Figure 9). Should the contamination problem arise, it was necessary to save the specimen by removing the contaminant within the laminar flow. In the case that the specimen itself was contaminated, the immediate termination of the well using 70% EtOH was required to prevent any possible cross-contamination to adjoining wells.

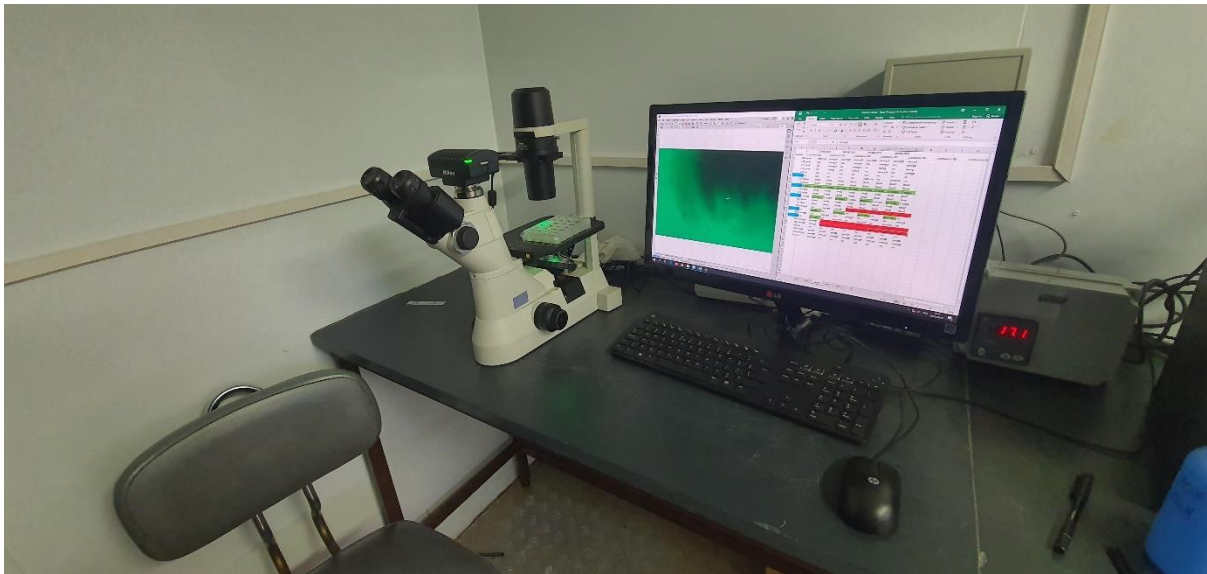


Figure 9 Inverted Light Microscope used for screening of specimens using the NS Elements software to identify positive samples.

Once *Bd* was identified in a well, it was then labelled positive and has to be observed more frequently as the specimen must be transferred once large quantities of active zoospores can be observed (Figure 10). When adequate growth has taken place the specimen, along with the zoospores, were carefully extracted and placed onto an antibacterial 1% tryptone agar plate. Once the specimen was placed onto the agar plate it was equally spread to allow a larger growth area, which also allowed for the removal of contaminants should a section become contaminated. The agar plate was left in the laminar flow until the liquid media evaporated, leaving behind the zoospores. The plate was then sealed with parafilm and placed

upside down in the incubator, and left to grow. Daily observations were done to see if any growth was taking place. Once sufficient growth has taken place, and no contamination was found, the section of isolated *Bd* was carefully removed, placed into a culture flask with 1% tryptone broth, and then placed in the incubator for five days, followed by refrigerated storage of the isolate.

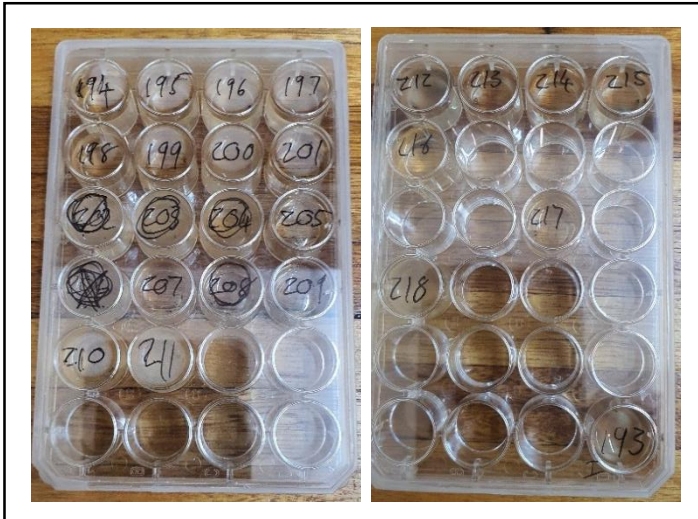


Figure 10 24-well Plates containing active growing *Bd* isolates.

2.6.5 Weeding

Weeding is the method used to remove contaminated sections from a specimen through sterile techniques (Figure 11). Before weeding can take place the contaminated container needs to be thoroughly cleaned with 70% EtOH along with a scalpel, lighter, and a beaker of 70% EtOH. All equipment and isolates were then placed into the sterile laminar flow while using disposable gloves. Once inside, the parafilm was carefully removed and placed onto tissue paper soaked in 70% EtOH. The lid was then carefully lifted and cleaned using 70% EtOH and left to evaporate. The Scalpel was then dipped in the 70% EtOH and flamed before carefully cutting out the contaminated section in the agar. Once the contaminant has been removed, it was placed with the contaminant side down onto a tissue paper soaked in 70% EtOH and enclosed within. The lid was then placed back onto the agar plate and sealed with parafilm. The plate was then placed upside-down in the incubator once again and carefully observed should there be traces of the contaminant left. Instruments were thoroughly cleaned, and the laminar flow was wiped down and set for a 20 minute UV cycle to kill any trace of contaminants.



Figure 11 Weeding process in which contamination is removed.

2.6.6 Agar Isolation Methodology

2.6.6.1 Pre-fieldwork preparation

For the second survey, a different isolation approach was utilized. Pre-fieldwork preparation was similar to that of survey one. A 1L antibacterial 1% tryptone agar was made to prepare 80 agar plates to be used for cleaning specimens for preparation refer to 3.1.1. An additional 1L antibacterial 1% tryptone agar was prepared and equally poured into ten 24 well plates. These plates were then sealed and stored upside-down in the refrigerator for fieldwork. The purpose of the 24 well plates is to fix the specimens after they have been thoroughly dragged through the agar plates. The 24 well plate design is for easy access weeding (section 3.1.5) should a specific specimen become contaminated.

2.6.6.2 Fieldwork methodology: Agar approach

Field sampling was done the same as in survey one, with the only difference being that specimens were placed in the 24 well plates filled with agar instead of eppendorf tubes. Once each plate was filled, it was sealed and clearly marked according to the locality of the specimen. Since daily contamination can be observed visually, it was possible to weed out the contaminated specimen in the field allowing for a higher probability of isolation success.

2.6.6.3 Laboratory methodology: Agar approach

Each 24 well plate was placed under a light microscope to see if any *Bd* zoospores are present. Each well was thoroughly observed every day for 12 days to conclusively determine whether specimens were infected with *Bd*. Should a well show active zoospores, it was frequently observed for any traces of contamination, or if it has shown sufficient zoospore growth, it was then transferred to a 1% tryptone agar plate. The method used for extraction was the same method used for weeding (section 3.1.5). Once the specimen was extracted from the well, it was streaked across the agar plate to spread as many zoospores across the surface as possible to allow for accelerated growth. The agar plate was then sealed with parafilm and placed upside-down in the incubator, and observed daily. Once a visual mound of *Bd* has developed (Figure 12), it was carefully cut out, placed in a culture flask along with 1% tryptone broth, and stored in the incubator for five days, followed by refrigerated storage of the isolate.

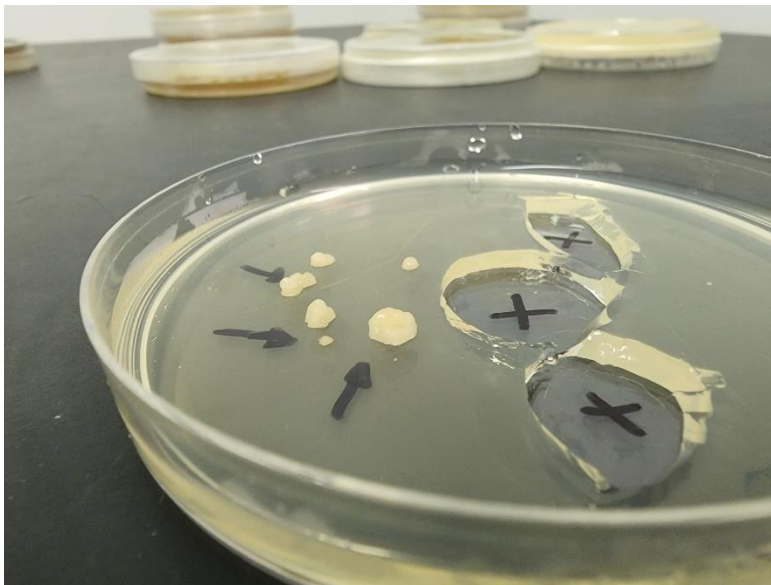


Figure 12 Culture plate indicating sections of agar that have been removed due to contamination. Arrows indicate active growing *Bd* colonies.

2.7 Results

2.7.1 Prevalence and distribution data

The first sampling survey of Ncandu Nature Reserve was in April of 2021 at the onset of the cold season, marking the end of the breeding season for most Amphibian species. Although the target species of the study was *Amietia delalandii*, sites with the African clawed frog *Xenopus laevis* present were also processed for *Bd*. It was found that six of the eight sites showed traces of *Bd*, with Steep Glen River and Union having the highest prevalence of 40%, while the sites Bloekombos and Mushroom Rock tested negative for *Bd* (Figure 13).

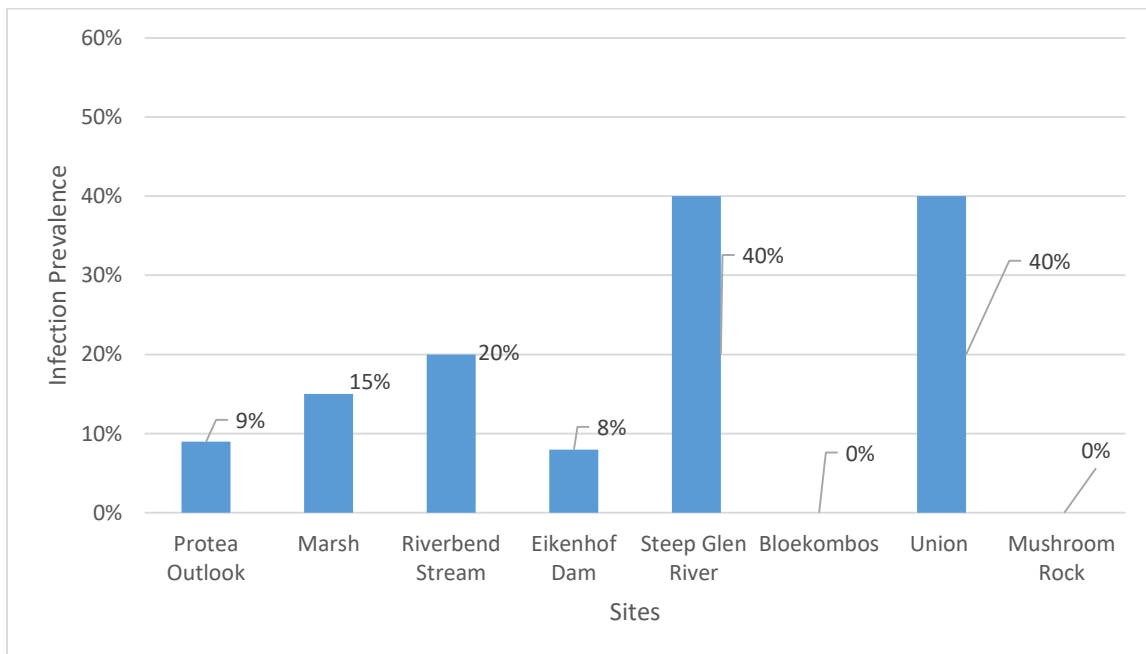


Figure 13 Batrachochytrid prevalence for the winter season for survey 1.

The amphibian species *Xenopus laevis* was found at the Eikenhof Dam along with *Amietia delalandii*. Although our target species, *A. delalandii* had a prevalence of 8%, this was not the case for *X. laevis*. Only one tadpole of *A. delalandii* was found positive at the site, while none of the five adult *A. delalandii* were positive. In the case of *X. laevis*, an overall prevalence of 57% was found, in which 4 of the 7 adult *X. laevis* tested positive for *Bd* (Figure 14).

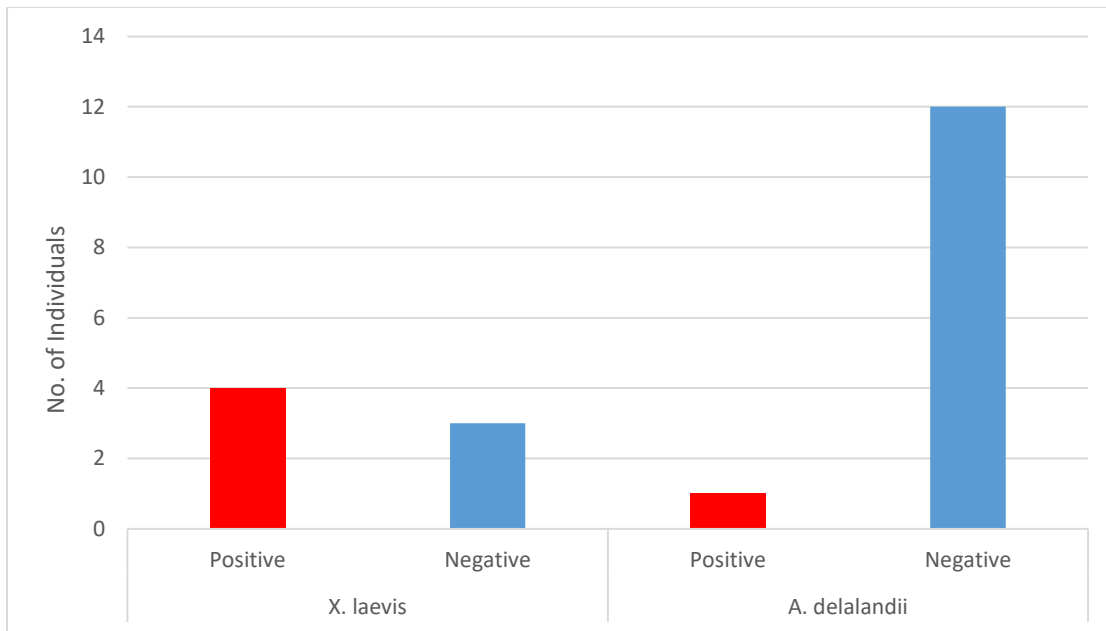


Figure 14 Batrachochytrid prevalence between the species *Xenopus laevis* and *Amietia delalandii*.

The second survey was conducted in October of 2021, in which a total of six sites were screened for *Bd*. Of the six sites, four were found to be positive for *Bd*, with the Steep Glen River and Steep Glen Kloof having the highest prevalence. Steep Glen River had a prevalence of 70%, Steep Glen Kloof had a prevalence of 60%, Leopard Kloof had a prevalence of 15%, Dampad had a prevalence of 10%, while Bloekombos and Mushroom Rock both tested negative for *Bd* (Figure 15).

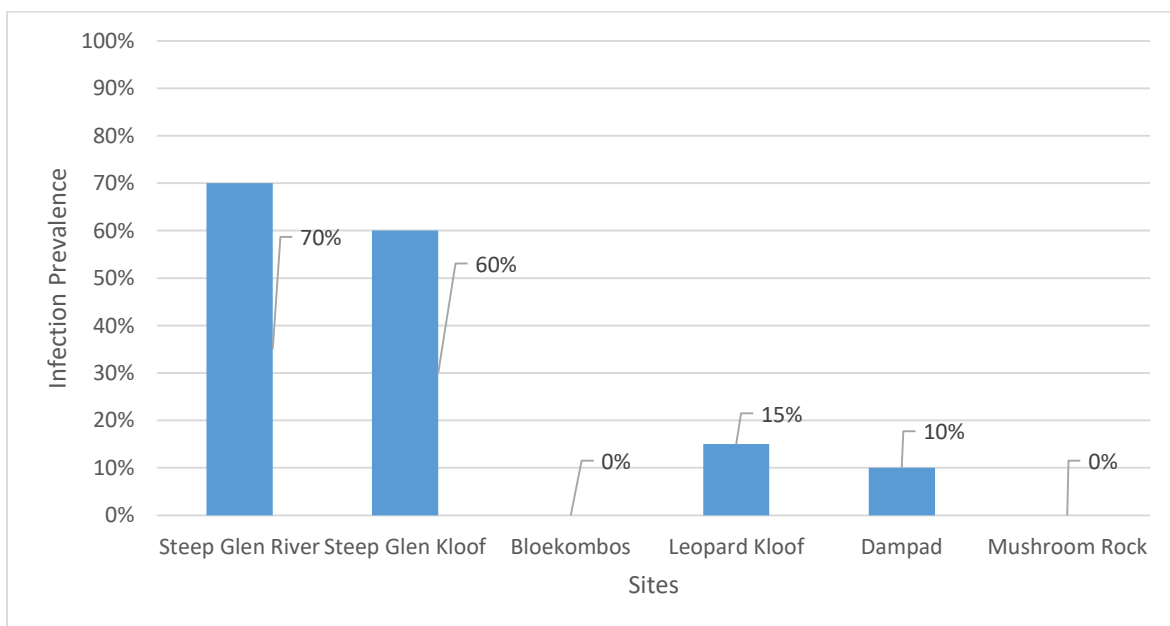


Figure 15 Batrachochytrid prevalence for the summer season for Survey 2.

Survey 1 occurred during the start of the winter season, while survey 2 occurred during the beginning of the summer season. Survey 1 had an overall prevalence of 17%, while survey 2 had an overall prevalence of 26% (Figure 16). From the results, it is evident that climate plays a role in the prevalence of *Bd* but not in the overall distribution.

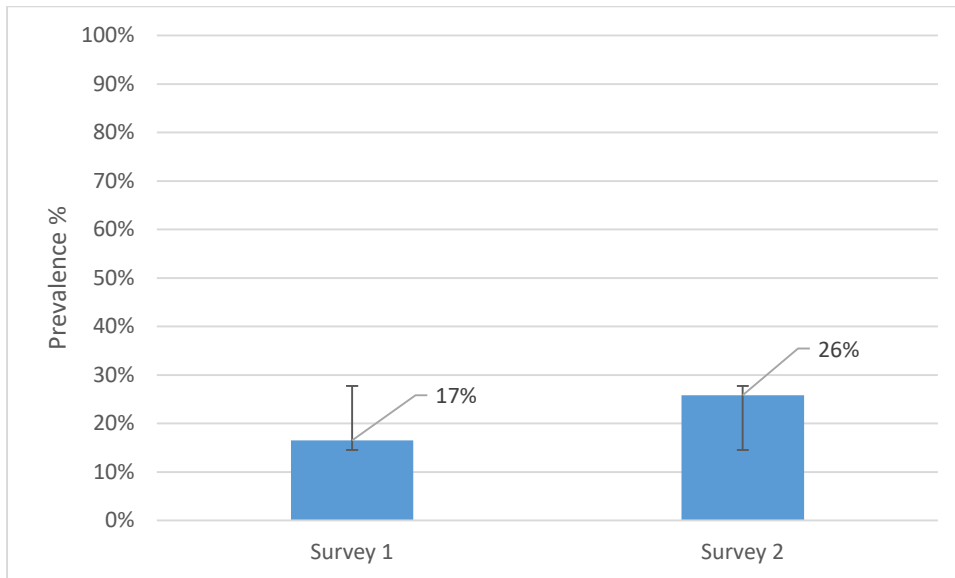


Figure 16 Overall *Bd* prevalence of survey 1 and 2 with standard deviation error bars.

Steep Glen River exhibited the highest prevalence of all sites. Survey 1, which took place during the winter, indicated a 40% prevalence for the site, while survey 2, which occurred during the summer, indicated a 70% prevalence for the site. From the results, it can be concluded that *Bd* prevalence rose by 30% in the summer (Figure 17), thus indicating that *Bd* thrives at optimum temperatures between 18°C - 23°C in summer compared to the lower temperatures experienced in the winter 1°C - 14°C.

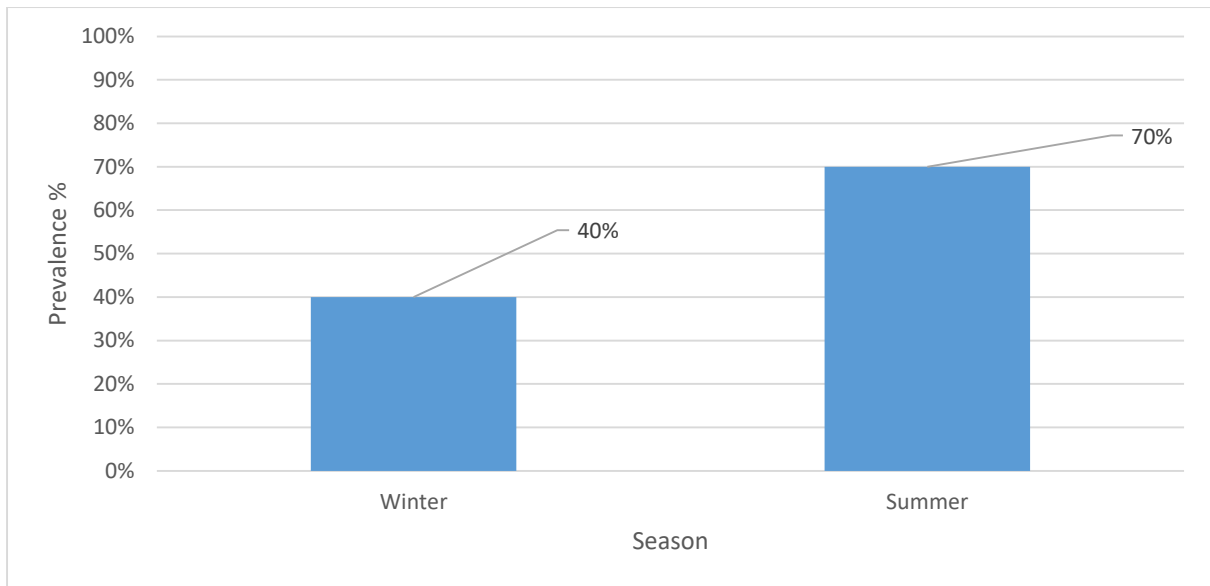


Figure 17 Seasonal change of *Bd* prevalence for the site Steep Glen River.

Batrachochytrid prevalence varies across the NNR, but it is evident that *Bd* has a wide distribution, as seen in (Figure 18). Of the 11 sites, three sites have a moderate *Bd* prevalence (20%-60%), six sites with low *Bd* prevalence (<20%), and two sites with no *Bd* prevalence (0%). By looking at the distribution and prevalence, it can be concluded that *Bd* is widespread but has a relatively low prevalence among the majority of sites.

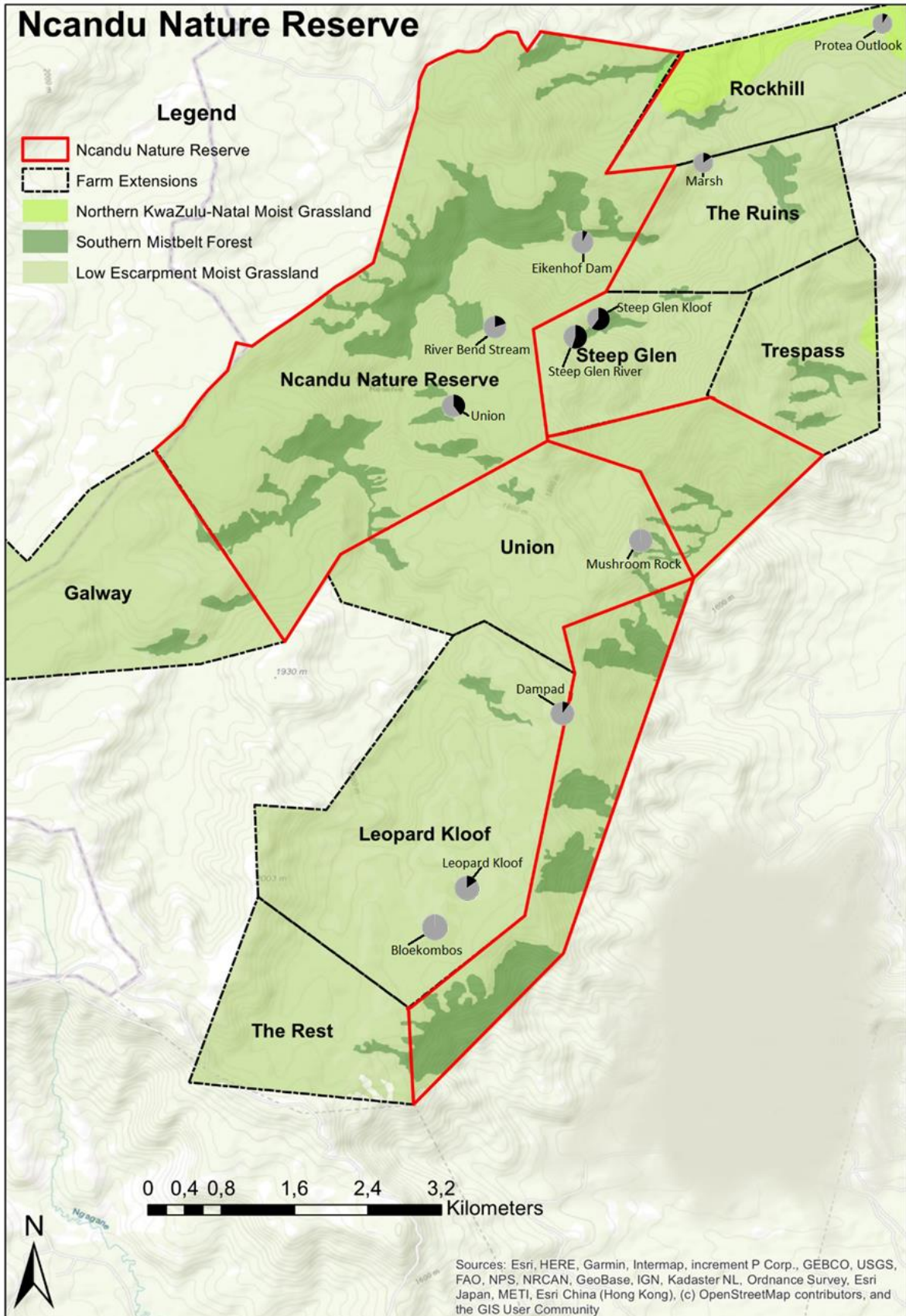


Figure 18 Distribution of Bd in Ncandu Nature Reserve: Positive individuals are represented by the black accent, and Negative individuals are represented by the grey accent.

In Survey 1, the liquid media approach was utilized, and samples were incubated for 14 days upon return to the lab. Isolation resulted in significant contamination of all sampling areas. The site River Main had the lowest contamination of 70%, whereas all Union and Mushroom Rock isolates were completely contaminated by day 14 of incubation (Figure 19).

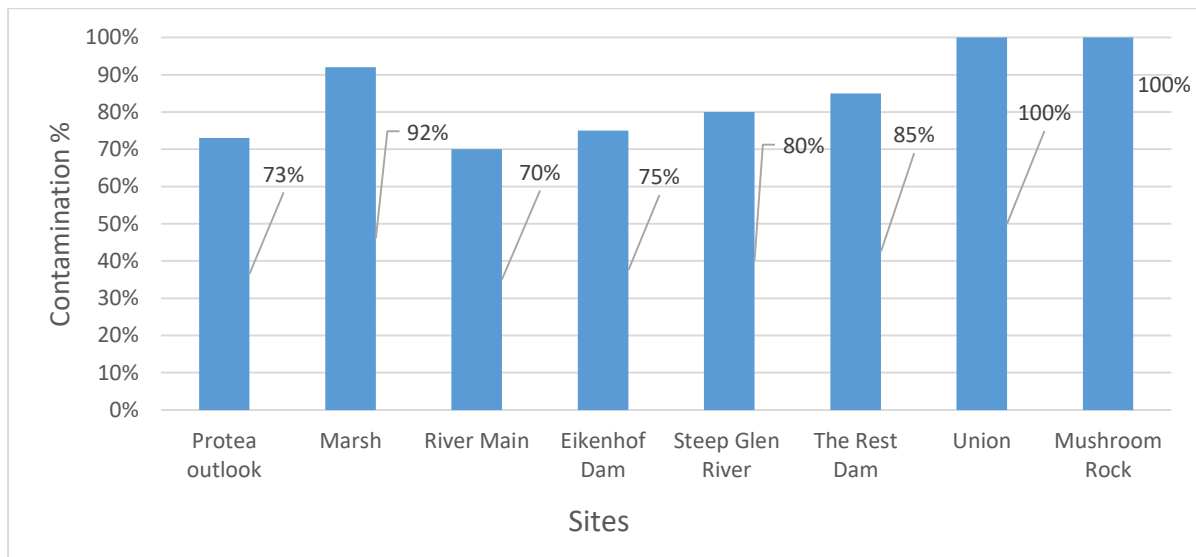


Figure 19 Total Sample Contamination for Survey 1.

In survey 2, we made use of agar plates with antibiotics to isolate *Bd* with subsequent incubation for 14 days. Steep Glen River had the highest level of contamination of 69%, with Mushroom Rock having the lowest level of contamination at 30%. Steep Glen Kloof had a contamination value of 63%, Bloekombos had a contamination value of 63%, Leopard Kloof had a contamination value of 56%, and Dampad had a contamination value of 64% (Figure 20).

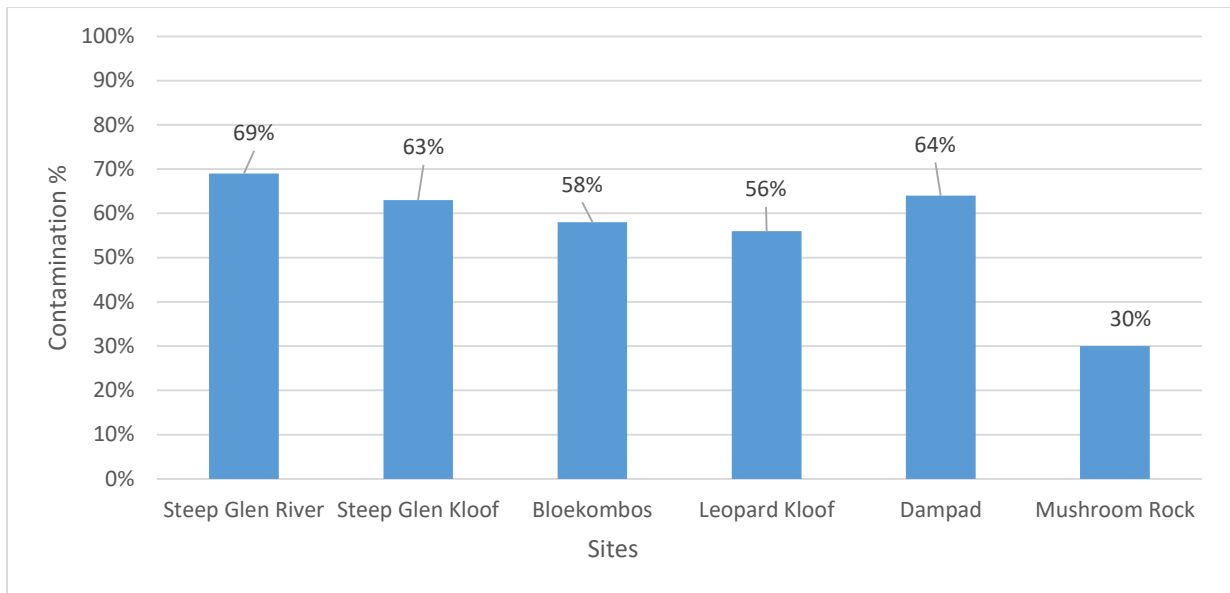


Figure 20 Total Sample Contamination for Survey 2.

The average contamination of survey 1 using the liquid media approach was 84.4%, and the average contamination of survey 2 using the Agar method was 56.66% (Figure 21). The agar approach had 27.74% less overall contamination than that of the liquid media, thus indicating the agar approach is more successful in the control of contamination.

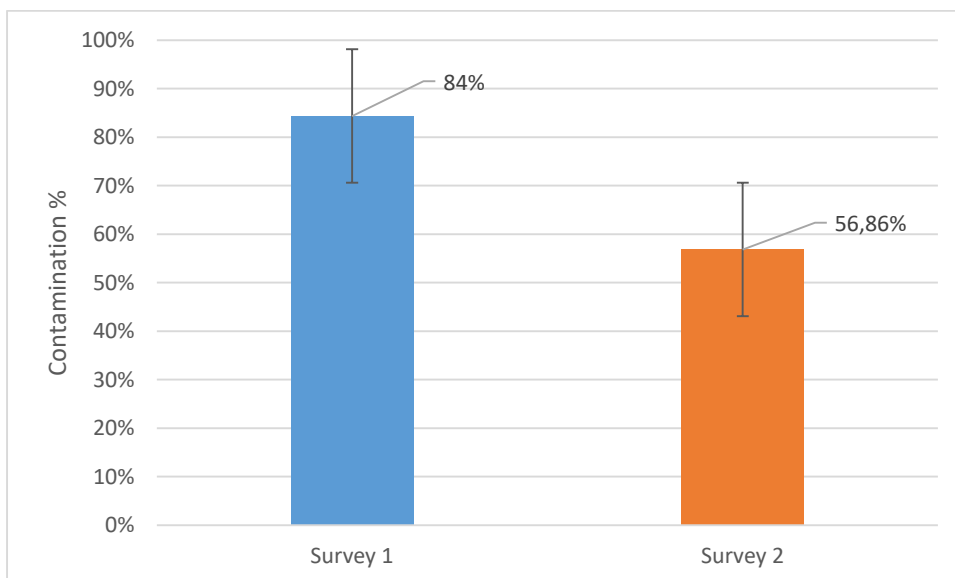


Figure 21 Contamination: (Survey 1) Liquid Media Vs. (Survey 2) Agar.

The next figure (Figure 22) illustrates the negative and positive *Bd* samples, as well as the number of successful isolates derived from positive samples. Survey 1 had a total sample size

of 103, of which 18 were positive, but no successful isolates were derived from the samples. Survey 2 had a total sample size of 216, of which 18 were positive, with 13 of the 18 positives being successfully isolated (Figure 22).

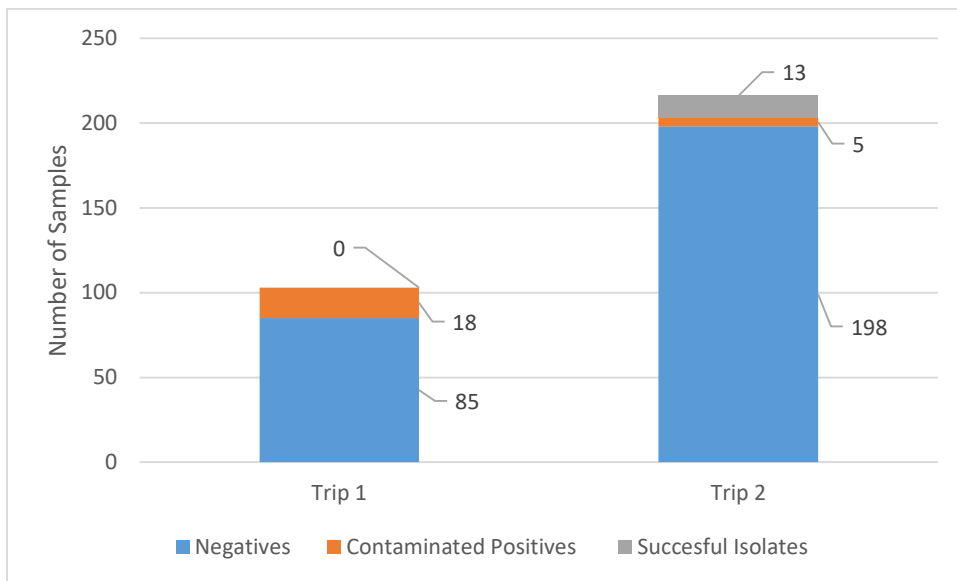


Figure 22 Overall Lab Results for Survey 1 and Survey 2 showing the overall success of *Bd* isolation.

Table 1 Simplified summary of core results compared to sample size

| Site | Species | Overall Prevalence | Seasonal Variation | Topography | Isolation Approach used with resulting Contamination | Sample Size |
|------------------|---------------------------|--------------------|--------------------|-----------------------------|--|-------------|
| Protea Outlook | <i>Amietia delalandii</i> | 9% | N/A | Perennial Montane Stream | Broth 73% | 9 |
| Marsh | <i>Amietia delalandii</i> | 15% | N/A | Perennial Valley Stream | Broth 92% | 15 |
| Riverbend Stream | <i>Amietia delalandii</i> | 20% | N/A | Perennial Valley Stream | Broth 70% | 10 |
| Eikenhof Dam | <i>Amietia delalandii</i> | 8% | N/A | Intermittent Valley Dam | Broth 75% | 13 |
| Steep Glen River | <i>Amietia delalandii</i> | 55% | 30% | Perennial Valley Stream | Broth 80% Agar 69% | 20 |
| Steep Glen Kloof | <i>Amietia delalandii</i> | 60% | N/A | Perennial Valley Stream | Agar 63% | 10 |
| Bloekombos | <i>Amietia delalandii</i> | 0% | 0% | Perennial Montane Dam | Broth 100% Agar 58% | 26 |
| Leopard Kloof | <i>Amietia delalandii</i> | 15% | N/A | Intermittent Montane Stream | Agar 56% | 13 |
| Dampad | <i>Amietia delalandii</i> | 10% | N/A | Intermittent Montane Stream | Agar 64% | 10 |
| Union | <i>Amietia delalandii</i> | 40% | N/A | Perennial Valley Stream | Broth 100% | 10 |
| Mushroom Rock | <i>Amietia delalandii</i> | 0% | 0% | Perennial Valley Stream | Broth 100% Agar 30% | 20 |

2.7.2 Amphibian diversity detected at Ncandu Nature Reserve

Due to the geographical niche of the NNR, various habitats formed, such as steep slopes ideally suited for rain frogs such as *Breviceps verrucosus*, montane river streams for *Amietia delalandii*, and lowland valleys and marshes for species such as *Strongylopus grayii*. We identified eleven amphibian species in the NNR (Figure 23). This shows that the NNR is of biological importance as it is evident that it is an important refuge for amphibians within this region and that continued biomonitoring is of the utmost importance.

Considering the distribution of amphibians, it is evident that *A. delalandii* was an appropriate choice for the *Bd* survey as it had the widest distribution of all species, being present at all 11 sites (Table 1). The site Protea Outlook and Eikenhof Dam had the highest amphibian density among all the sites. Marsh, Union, and Mushroom Rock had the lowest amphibian diversity, as only *A. delalandii* was present at those sites.

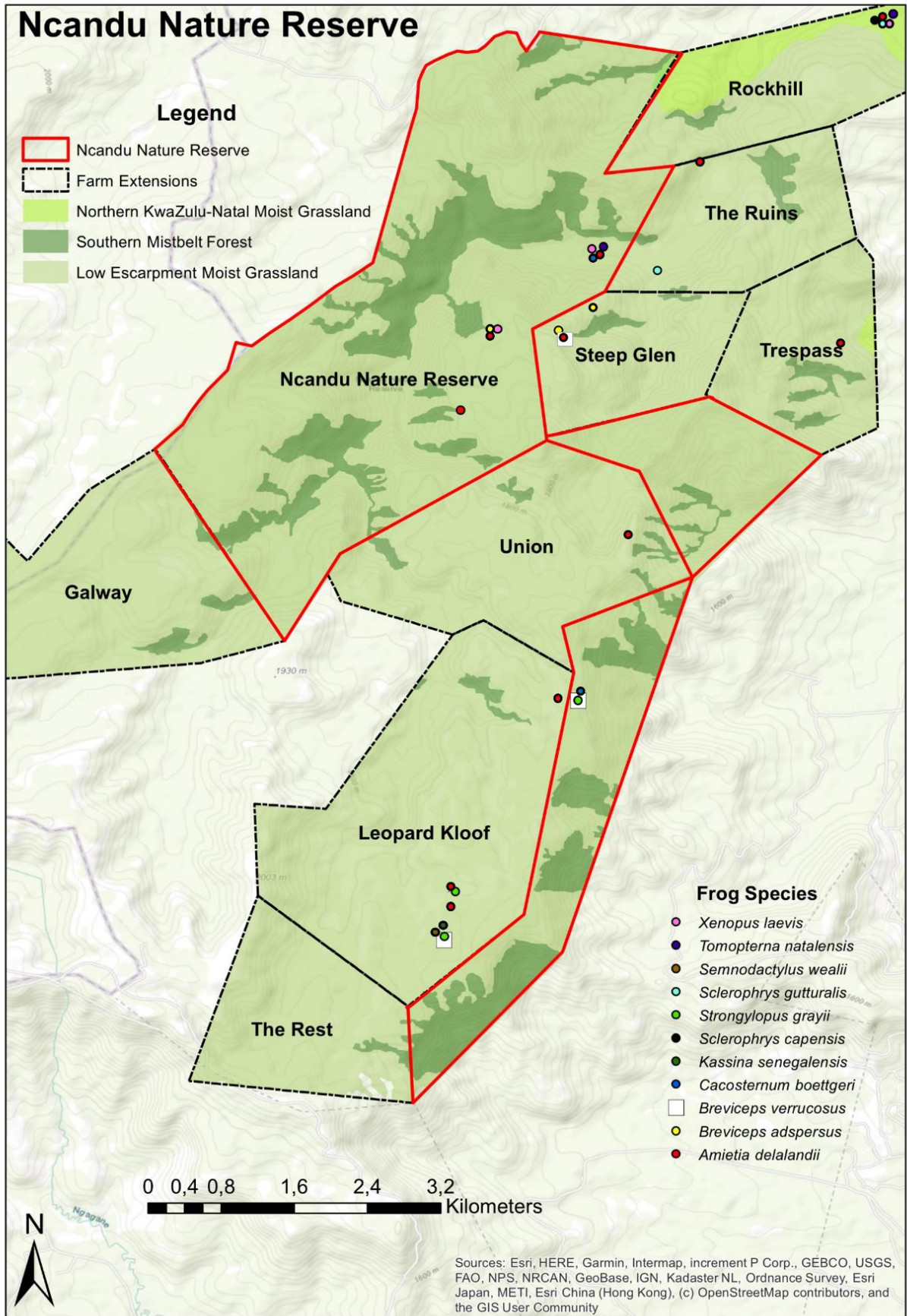


Figure 23 Amphibian species distribution of Ncandu Nature Reserve.

2.8 Discussion

2.8.1 General discussion of the NNR

Amphibians are one of the greatest biological indicators when assessing the health of a specific ecosystem. The reason why amphibians serve as such impressive biological indicators is the fact that their skin is permeable to water. The skin regulates ion transport and respiratory gas diffusion (Enciso *et al.*, 2008; Fellers *et al.*, 2001) and is essential for maintaining homeostasis, water, ion, and electrolyte equilibria (Enciso *et al.*, 2008; Voyles *et al.*, 2011). Therefore, batrachochytrids (*Bd*) poses a severe threat to amphibian populations and diversity as it obstructs electrolyte uptake, which results in chytridiomycosis (Kriger & Hero, 2007). Chytridiomycosis leads to epidermal hyperplasia, which compromises the respiration and osmoregulation of amphibians, consequently leading to death at later life cycle stages (Berger *et al.*, 1998; Voyles *et al.*, 2011). Once chytridiomycosis has set in, the amphibian can show signs such as lethargy, abnormal postures, reddening of ventral skin, convulsions, and sloughed skin appearance with occasional lesions (Berger *et al.*, 1998; Voyles *et al.*, 2011). Therefore, an outbreak of *Bd* could cause significant declines and potentially cause abrupt disruptions in an ecosystem.

Processing of the samples collected at NNR indicated that *Bd* is present in the environment, supporting the prediction model developed by Tarrent *et al.* (2013). The prediction model showed a high probability of *Bd* presence in the region of the NNR. Although predicted in the model, the NNR needs to be conclusively verified as to the severity of the prevalence. Sampling was done during April and October to determine the seasonal prevalence of *Bd*. The highland montane streams, namely the Steep Glen River and Union, have significantly higher infection percentages compared to the lowland valley regions. The sampling sites of Bloekombos and Mushroom Rock had no positive traces of *Bd*. Therefore, the NNR may be in a pre-enzootic stage as *Bd* is yet to spread to different parts of the reserve.

During the second survey, some sampling sites could not be retested as they were intermittent streams and pools that are dry from June to December. During the second survey, six sites were screened for *Bd*, of which four sites tested positive (Figure 15). Sites added to the study were Bloekombos, Dampad, and Steep Glen Kloof. Once again, the highland montane streams showed the highest *Bd* prevalence, especially that of Steep Glen River and Steep Glen Kloof. The possible reason for this is that the lowland sites have a higher degree of predation and the water supply tends to decrease in the case of intermittent bodies of water.

Therefore, tadpoles develop at a higher rate than those found at the montane river streams, where predation is minimal, and water supply is constant. This assumption can be supported by looking at the size of the tadpoles found at the corresponding sites. Tadpoles in the lower areas were smaller yet had shown signs of metamorphosis. In contrast, tadpoles at the montane sites exhibited metamorphoses but were double the size of the tadpoles in the lowland areas and have been in a larval state for at least two years, therefore, having prolonged contact with *Bd*. Two previous sites, Bloekombos and Mushroom Rock, had no trace of *Bd*; thus, it is still considered a pristine site.

The presence of *Bd* has been identified in the NNR, with survey one having an overall prevalence of 17% and survey 2 having an overall prevalence of 26%. This indicates that there was an increase in the prevalence of *Bd* during the summer season due to the increased activity of animals in the area. The seasonal difference was a 9% increase in overall prevalence (Figure 16) for NNR, thus supporting the fact that *Bd* becomes more active during the summer months when activities such as mating occur. Although *A. delalandii* breeds throughout the year, this is not the case for other species. Due to the warmer climate, some intermittent pools dry out, potentially forcing amphibians to relocate to a new area or pool. This could cause species aggregation at one water source, consequently increasing the risk of infection coupled with breeding activities in the contaminated water source.

The overall prevalence of NNR is still low compared to other localities in the Drakensberg Mountain Range (HHL, 2019). Although *Bd* is dependent on temperature, and the activity of *Bd* increases as the temperature increases, an increase of 9% is still considered a massive increase in prevalence. All results indicate that the NNR may be in a pre-enzootic phase. This means that *Bd* has been detected at some sites, but there are still areas that remain untainted by *Bd*.

Nature is comprised of natural processes; although biological processes play a pivotal role in the health of an ecosystem, environmental processes are considered key drivers. For instance, looking through the perspective of hydrology and its relationship with *Bd*, as *Bd* is a water-borne parasitic fungi. Like all animals, amphibians require water to survive and would readily occupy any source of water. When looking at high prevalence sites such as the Mont Aux Sources in the Drakensberg having sites with a prevalence of 60%> (HHL, 2019), the infected sites have a similar type of topographical relief as sites in the NNR, namely Steep Glen. Sites at higher altitudes tend to have a higher prevalence mainly due to climate

variations as discussed earlier. Areas of this nature are prone to flooding during the rainy season resulting in flash floods (sudden high-velocity water surges). This natural process could cause infected tadpoles to travel downstream through this hydrological process, consequently infecting low-lying pools and streams.

When looking at Steep Glen in the NNR, it is clear that it is a grassland biome. Like the rest of the Drakensberg, these biomes have a natural water table that supplies rivers like Steep Glen with water throughout the year. The soils of these biomes tend to have a high clay content, meaning that the soil tends to be hydrophobic in cases of sudden rainfall resulting in flash floods. Although, with a consistent presence of water, these soils get saturated and can retain moisture for long periods before drying out. When these soils are saturated, the entire soil column is filled with water up to the natural water table, leaving no open-air pores in the soil column. Once these soils become fully saturated, there is only one way water can flow, which is with the relief of the area, leading to flooding of the pools in the gorge. Therefore, it is clear from the sites of Steep Glen that an increase in *Bd* is inevitable due to the position and natural processes providing a sustainable water supply. It can also be deduced that *Bd* was spread to the lower valley areas through hydrological processes, consequently infecting all the sites in the valley area through flash floods. These sites include Marsh, Riverbend Stream, and Eikenhof Dam. All these sites receive their water from the same river, and infected adult frogs contaminate other pools and streams in the locality as they migrate from habitat to habitat. It is evident that although *Bd* is spread to other sites by tadpoles through the transport of water flow, adult frogs spread *Bd* through migration from the infected site to another site, consequently infecting it. Because the NNR has intermittent pools and streams, all infected frogs relocate to a more permanent area, consequently infecting all amphibian species present in the area. This can be seen when looking at Leopard Kloof, an intermittent pond and marsh; all tadpole samples collected were negative for *Bd*, and two of the three adults were positive for *Bd*. This causes concern as the site Leopard Kloof is only a few kilometers away from the site Bloekombos, which has tested negative on both sampling surveys. Therefore, it is inevitable for *Bd* to spread to Bloekombos and other sites surrounding it, as Leopard Kloof will eventually dry up, and the infected individuals will migrate to the next suitable site.

The overall prevalence of NNR indicates that *Bd* has only recently been introduced to the area, as prevalence was much lower compared to other sites in the Drakensburg Mountain Range. Data shows that *Bd* has yet to infect all areas of the NNR, but sites such as Leopard Kloof show that *Bd* could be spreading via the migration of amphibian species to new localities.

2.8.2 Site-Specific Prevalence

Protea Outlook

Protea Outlook is an intermittent stream that flows from January to May. The site is a montane river stream within the Northern KwaZulu-Natal Moist Grassland biome. The area is relatively undisturbed, and shrubs cover the stream. Protea outlook had a prevalence of 10%, in which one of the tadpole samples tested positive for *Bd*. Due to the site being an intermittent stream, it could not be sampled on the second survey, but it does display a low prevalence. However, it is positive nonetheless, asking the question of where the infected individuals migrated to.

Marsh

The Marsh site is also an intermittent stream that receives water from a gorge higher up in the valley. The site is situated next to one of the larger rivers in the NNR but does not receive its water directly from it. The site was ideal because a marsh is a shallow water source that attracts various insect species as well as nearby amphibian species. These sites provide a natural mating site as tadpoles would reach maturity before the marsh starts to dry up. The Marsh was found to have a *Bd* prevalence of 15%, in which one adult and one tadpole of the species *A. delalandii* were positive.

Eikenhof Dam

During the investigation of the first survey, the Site Eikenhof Dam was found to have both the species *Amietia delalandii* as well as *Xenopus laevis*. Because *X. laevis* is known to be a strong carrier of *Bd* due to its constant aquatic lifestyle, it was decided to sample a few adult toe clips. Eikenhof Dam is also a seasonal dam that only has water during the first few months of the year after the rainy season.

As seen in (Figure 14), *X. laevis* had a much higher prevalence when compared to that of *A. delalandii*. The overall species prevalence for *X. laevis* was 43% opposed to *A. delalandii*, having a mere 8%. Of the seven *X. laevis* adults collected, four were positively screened for *Bd*. In contrast, none of the *A. delalandii* Adults were found to be positive only a single *A. delalandii* tadpole screened positive for *Bd*. Eikenhof Dam showed a *Bd* prevalence dominated by the species *X. laevis*. Because it is a seasonal dam, it can be assumed that *X.*

laevis was the species that infected this sampling site, as none of the adult *A. delalandii* samples tested positive for *Bd*.

Riverbend Stream

The site Riverbend Stream was a shallow rock pool alongside one of the main rivers in the reserve. It was found that the site had a prevalence of 20%, which was conducted during the first survey. The positive sampling site shows that *Bd* has spread further down the valley, a troubling thought as an accelerated distribution throughout the reserve through water flow.

Steep Glen Kloof

The site Steep Glen Kloof was screened during the second survey as it could be a great site that would indicate the presence of *Bd*. The site was not sampled during the first survey as it involves climbing down a small gorge covered with mud and silt. The river that flows through it is a perennial river that flows throughout the year. The area is well covered and nearly inaccessible with no possibility of manmade disturbances such as cattle rearing, making it an ideal locality to screen for *Bd*. The site had a prevalence of 60%, which indicates that *Bd* has been present in that area for some time, as other areas in the region yielded significantly lower prevalence percentages.

Although the prevalence for the site was high, it must be stated that the tadpoles found at the sights were roughly two years old, which allows for longer periods in the water, consequently leading to a higher probability of getting infected by *Bd*. All tadpoles collected were approximately at Gosner stage 39-41 as their hind limbs have fully developed and their forelimbs have started to protrude.

Steep Glen River

Steep Glen River is considered a perfect site as it is a perennial montane stream covered by shrubs leading into a broader gorge. Deep pools of water contained tadpoles and adults who could not be caught because they were too fast and the site was hard to manoeuvre. Steep Glen River was screened on both the first and second survey, as it had a *Bd* prevalence of 40% during the first and 70% during the second survey. Steep Glen River had a 30% increase in prevalence during the summer months in October. This supports that an increase in *Bd* prevalence is directly proportional to an increase in temperature.

Bloekombos

The site Bloekombos is a montane stream with surrounding marshland. It is an excellent habitat for amphibian activity even though it does have disturbances caused by cattle. The site has water throughout the year, which is why sampling was done on both occasions. The results from the first survey indicated a prevalence of 0%; the second survey also indicated a prevalence of 0%. Therefore, it is clear that the site and surrounding area is still negative for *Bd*, as none of the samples had any sign of infection.

Leopard Kloof

The site Leopard Kloof is an intermittent marshland that was not present during the first survey. The site is ideal as it has plenty of tadpoles and adult frogs. The results of the site indicated that *Bd* was present with a prevalence of 15%. Although the site was positive, all tadpoles in the area were negative for *Bd*, but two of the three adult frogs that were caught were positive for *Bd*. Therefore, these two individuals may have spread *Bd* to a new locality through migration from a positive site to none infected site.

Dampad

The site Dampad is part of a small gorge where sampling was done in pools just upstream. The site was ideal as it had plenty of unfertilized eggs indicating that mating activities were present in the area. Although there was a lot of activity, no adult frogs could be found due to the dense vegetation and underwater cavities in which the adult frogs took refuge. Even though no adults were caught, it was found that one sampled tadpole was infected with *Bd*. This indicates that this site has also been recently tainted, with a prevalence of 10%.

Union

The site Union is situated in a hidden gorge with small pools filled with groundwater, providing an ideal habitat for amphibians. The entire site is covered by dense vegetation, minimizing the risk of predation by other animals and birds. The site was sampled during the first survey and had a relatively high prevalence of 40%. During the second survey, the site could not be found and, therefore, could not be sampled.

Mushroom Rock

Mushroom rock is situated above a valley with a small stream produced by groundwater flowing onto a granite outcrop where small pools are formed. This area is on the opposite side of the valley adjacent to the Steep Glen sites making it a good point of reference in terms of the spread of *Bd*. This site was screened on both surveys and was found negative for *Bd* on both occasions indicating that the site is still undisturbed.

2.8.3 NNR Conservation Risks

Environmental Stressors

Although Steep Glen had a higher prevalence than other sites, it should be noted that the Steep Glen sites contained tadpoles that have been in a larval state for longer than a full season. The reason they may have remained in their larval state for so long may be due to the minimal effects of environmental stressors such as predation and drought. Stressors such as drought or predation are lower at this site as opposed to sites lower in the valley. For this reason, tadpoles found at Steep Glen had no reason for accelerated metamorphosis as the need was unnecessary. In contrast, this is not the case for tadpoles in the lower valley area. Lower valley sites are prone to droughts as well as predation from fish and birds. Therefore, tadpoles in the lower valley areas progress faster through the Gosner stages to become adult frogs.

The reason for the higher number of intermittent streams and pools in the valley is that during the rainy season, the area experiences a high influx of water, reaching up to 200 ml of rain in one week. This sudden influx of water results in flash flooding of the lowland valley area as water cannot drain at a rate that keeps riverbanks from overflowing. This results in pools and marshes forming in geological depressions. Once the rainy season has passed, water slowly disperses from the valley, leaving behind intermittent pools and marsh that would hold water for a few months. The intermittent pools provide a temporary habitat for amphibians in the area. However, these habitats are only viable if the groundwater level remains at a level as high as the pool. The groundwater level will gradually lower as temperatures increase; consequently, areas containing intermittent pools will gradually lose water until completely drying out.

The risk that these intermittent streams hold is that amphibians that are positive for *Bd* would have to disperse to another water source. Droughts are a great risk when *Bd* is involved, as

not do *Bd* positive amphibians inhabit alternative water sources but so do healthy amphibians. A scarcity of water would lead to dense aggregations of amphibians at water sources, which can result in spurts of accelerated transmission of *Bd* to healthy individuals as contact with other amphibians of varying species is imminent. Examples of sites that could cause an accelerated transmission of *Bd* include Protea Outlook, Eikenhof Dam, Riverbend Stream, and Bloekombos. All sites include four or more species within the same geographical area. Thus, interspecies transmission is highly possible as all these amphibians share the same or adjacent water sources. This theory can be tested by synchronizing future disease monitoring with extreme weather conditions.

Xenopus laevis from Eikenhof Dam is an example of accelerated interspecies transmission since there was a dense species population. The species is known for being a *Bd* reservoir as they are aquatic amphibians and their populations are resistant to chytridiomycosis (Weldon *et al.*, 2004). The *Bd* prevalence of Eikenhof dam was higher for the species *X. laevis* than *A. delalandii* as none of the adult frogs of *A. delalandii* were found positive, whereas the majority of *X. laevis* were positively infected. The Eikenhof Dam is an example of an intermittent site, which usually dries out within a few months. All the species at the site have the potential to become infected and transmit *Bd* to other water sources in the area when the dam is no longer viable. The risk that this site represents is massive as there would be a definite transmission of *Bd* to other localities infecting other individuals there, thus continuing the cycle.

This site also indicates that the region may be in a pre-enzootic stage of infection, as no *A. delalandii* adults were found positive. This indicates that *X. laevis* might be responsible for the transmission of *Bd* when they migrated to the dam. However, further studies are required as a high infection can cause individuals to become lethargic and fall prey to predators. Even though the *A. delalandii* adults have a lower chance of contracting *Bd* from the site, this is not the case for the tadpoles. Tadpoles are more susceptible to contracting *Bd* due to their aquatic larval stage. Because tadpoles are more exposed to *Bd*, they are more likely to contract it while still in the larval stage.

Infections caused by *Bd* rarely cause mortality in tadpoles. Only once tadpoles have undergone metamorphosis will *Bd* spread to other regions of the body and skin, compromising the bodily functions required by amphibians to survive. The issue with this is that by the time an infected frog dies, it would have spread *Bd* to several other water sources depending on the degree of infection. Sites like Bloekombos are at risk of *Bd* as Leopard Kloof, also being

an intermittent marsh, had two adult frogs of the *A. delalandii* species test positive for *Bd*. This is an issue as Leopard Kloof is just a few kilometers away from Bloekombos, and migration of these infected frogs would lead to the transmission of *Bd* to Bloekombos and other surrounding water sources.

The NNR has various species that are in close proximity to one another, therefore, an outbreak of *Bd* could potentially endanger some species, as not all amphibian species are as robust as others. The amphibian species *A. delalandii* breed year round and would thus be able to reproduce at a rate that would allow the species to survive in the NNR regardless of the degree of infection. Other species found in the NNR are seasonal breeders and are therefore at risk of die-offs in the NNR should an outbreak of *Bd* occur. The NNR can be considered a refuge for amphibian species as *Bd* is still in a pre-enzootic phase. Although *Bd* is present at some sites, it is clear that it has not yet spread to all parts of the reserve, which would serve as an excellent opportunity to monitor and study the disease dynamics of *Bd* in different biomes found in the NNR. Therefore, the conservation of the amphibians in the NNR is crucial as they provide a basis for the state an ecosystem. For this reason, continued monitoring of the amphibian populations in the NNR should be prioritised.

2.8.4 Sample Contamination

One of the most daunting challenges of any field sampling survey focused on microorganisms, such as *Bd*, is contamination. Contamination of attempted *Bd* isolates becomes an issue when non-target microorganisms are not thoroughly removed during the cleaning process of host tissues that takes place when processing in the field and sterile environments such as a laboratory. There are methods to eliminate contamination without removing *Bd* in the process. This involves dragging a sample through nutrient agar with antibiotics. The purpose of this is to remove any impurities, such as other fungal growths and bacteria (Fisher *et al.*, 2018). Even though dragging a sample through the agar should wipe any contaminants, it is not always the case. The purpose of collecting field samples is not only necessary to determine the prevalence of a site but also to isolate and culture *Bd*. It is possible that *Bd* can grow along with some other microbes that the sample was contaminated with, but creating an isolate from that sample would be near impossible. Therefore, all signs of contamination must be removed before attempting to isolate *Bd*.

Identifying a working standard in fieldwork is vital for the best possible outcome. The two methods used for isolating *Bd* incorporated steps aimed at minimizing the risk of contamination that would otherwise result in the loss of the isolate. Both approaches have their benefits, such as being compact in terms of the liquid media. One hundred eppendorf tubes can be stored in a small eppendorf container, whereas the agar approach requires much more space in transportation.

2.8.5 Isolation approaches utilised

The liquid media isolation approach failed in successfully isolating *Bd*. The approach successfully identified the presence of *Bd* in some samples, but these samples could not be isolated successfully due to contamination. The only drawback that makes the liquid media approach obsolete is that there is no way of monitoring the state of the sample once it is placed in the eppendorf tube. Even though thorough cleaning had been done, one cell of contamination can remain and replicate at an unprecedented rate, eventually engulfing the entire sample as well as the *Bd* zoospores and sporangia. These other contaminants/competitors can inhibit the growth of *Bd* to a great extent, rendering the isolation process a failure as *Bd* has been suppressed by the time the samples have reached the laboratory.

Because of the viscous nature of the liquid media, it allows zoospores to move more freely and reproduce at a faster rate, but this is also the case for any contaminants. The contamination percentage of the liquid media approach was 84.4%, meaning that of the 103 samples, 16 had no contamination. Of the 18 positive samples that were identified, none were able to be isolated. This is a troubling issue as contamination as well as *Bd* is at a microscopic level making it nearly impossible to remove these contaminants.

Weeding was done in the lab to remove contaminants but proved ineffective as fungal contaminants created attachment points in the environment, in this case, on the surface of the 24 well plates. When attempting to remove the contaminants using sterilized equipment, fragments of these contaminants break off and create a colony. Extraction of a contaminant also increases the risk of further contamination as spores of the central mass can break off and could spread to other sections of the well that may be closer to the *Bd* colonies.

Another issue was bacteria, as they replicate at an unprecedented rate. It was found that one sample had bacteria that were resistant to antibiotics. Overnight the bacteria bloomed and consequently spread to other wells. To prevent cross-contamination, these wells had to be sterilized by carefully adding 70% EtOH to the contaminated well. From the results acquired, it is clear that the liquid media approach has its shortcoming regarding contamination. Even though contamination was the greatest issue of the approach, it allowed for easy observation of *Bd* zoospores.

The agar isolation approach was the most effective in curbing contamination and successfully producing isolates. The approach took slightly more time for *Bd* to become visible under the microscope but was successful in minimizing contamination to a greater extent. The approach showed signs of contamination while in the field, allowing for quick weeding of a sample before cross-contamination could occur. Using a sterilized scalpel, it was possible to remove entire colonies without the risk of fragments breaking off and settling in another area of the agar. If the contamination was not initially removed through mechanical dragging of the tissues, it could be easily removed later through weeding until all traces of contamination were removed.

The agar approach was found to be the best approach. The contamination percentage of the agar approach was 56.86%, which is considerably lower than that of the liquid media approach. The agar approach also yielded 13 successful *Bd* isolates compared to the liquid media, which yielded no isolates.

Although liquid media allows for easier identification of *Bd*, it has significant shortcomings, such as the uncontrollable spread of contamination, which is the primary issue regarding culture isolation. The agar approach allows the opportunity to observe contamination easily in the field and could be easily extracted to avoid further spreading of contamination. In contrast, the liquid media approach does not allow the researcher to observe contamination while in the field, as it could result in complete contamination of the sample by the time it reaches the laboratory. It is clear that the agar approach has a higher success rate when dealing with the control of contamination and should therefore be considered the preferred approach.

2.8.6 Amphibian Species of Ncandu Nature Reserve

Ncandu Nature Reserve is a unique environment, although it is geographically homogenous to certain extents when looking at amphibian diversity. According to the Atlas and Red Data

Book of the Frogs of South Africa, Lesotho, and Swaziland by Minter *et al.* (2004), 19 possible amphibian species could occur in the geographical region of the NNR. During our surveys, a total of 11 species were successfully identified. Most of the amphibians found in the NNR are predominantly suited for grassland habitats, the dominant vegetation type of NNR. The NNR has various localities at varying altitudes, which include three microbiomes that provide amphibians with a self-sustaining environment to survive. It was found that there was a higher density of amphibian species in the Low Escarpment Moist Grasslands. No species of amphibians were found within the Southern Mistbelt Forest biome, as no natural pools are present due to the topography of the Southern Mistbelt Forest biome. By looking at the distribution of amphibian species, we can see that areas have up to 5 species within a 300-meter radius. Therefore, an outbreak of *Bd* could easily infect an entire community using the same water source.

Other species that had unique habitats were that of *Breviceps verrucosus* and *Breviceps adpersus*, which occupied the steep valley slopes of gorges. These gorges lead into hidden forests, which produce heat, and when cold air comes over the mountain, the hot and cold air produce condensation on the slopes. This allows the *Breviceps* species to have access to enough water making the slopes of the NNR an ideal habitat for these amphibians. It should also be noted that *B. verrucosus* was not among the known species to inhabit the area. This makes it an important population in terms of taxonomy and species distribution maps. The species *Amietia delalandii* was the most common amphibian, occurring at all the sampling sites, thus making it the preferred target species for comparative analysis of *Bd* occurrence in the area. Therefore, the probability of finding tadpoles was greater when sampling in the field.

2.8.7 Conclusion

The Ncandu Nature Reserve is a homogenous ecosystem rich in species diversity, making it a biologically significant area. Results indicated that *Bd* is present but still has a low prevalence in most of the region, which means that the reserve is still in a pre-enzootic stage. It is imperative to continue monitoring this region as an outbreak of *Bd* could lead to mass die-offs in the region, altering the ecological processes within. The results show that *Bd* is present at 8 of the 11 sites, but due to the low prevalence of *Bd*, it can be assumed that *Bd* has not been in the area that long when compared to other sites that are found in the Drakensberg mountain range. It could be problematic when taking into consideration the intermittent pools as infected individuals would naturally migrate to other ponds and streams, consequently spreading *Bd* to new localities.

The amphibian populations within the NNR are dense and within close proximity to one another. Sites like Bloekombos have a high probability of becoming positive as the adjacent site Leopard Kloof is an intermittent site. Therefore, infected species could migrate to Bloekombos as it is a permanent water source. Overall, it is clear that *Bd* has spread to most regions of the NNR, but the overall prevalence of *Bd* in the NNR remains low. It is clear that the NNR is in a pre-enzootic stage of *Bd* prevalence as not all sites are positive, even though these sites house a dense population of amphibian species. The sites Steep Glen Kloof, Steep Glen River, and Union indicated a high *Bd* prevalence, but the tadpoles sampled at these sites have been at a larval stage for at least two years. Prolonged exposure to a *Bd* infected aquatic environment would explain why the tadpoles at these sites had a greater probability of being infected with *Bd*. In conclusion, the NNR is a vibrant biological region that is in a pre-enzootic stage of *Bd* infections. Therefore, biomonitoring of *Bd* should be considered a conservation priority.

Chapter 3: Fitness of *Bd* in the presence of competition

3.1 Introduction

Contamination has been a major contributing factor to failure in culturing and the isolation of *Bd*. Contamination is a major issue when attempting to successfully isolate *Bd* from an infected host. Two types of contamination occur when trying to isolate *Bd* cultures; these include bacteria and other fungi, specifically filamentous fungi. The isolation process is complex when attempting to isolate any organism from a field sample. This is because there are hundreds, if not thousands, of microorganisms in any environment from which one attempts to isolate a pure culture. The most unpredictable contaminant to influence the isolation of *Bd* is bacteria. The best recipe for the effective elimination of bacteria was developed by Longcore *et al.* (1999) in which she added 400 mg of streptomycin and 200 mg of penicillin-G to a 1 L Agar solution after being autoclaved. It is crucial to ensure that the samples are appropriately dragged through the agar, as only a singular bacterium can replicate into thousands within a few hours. Although antibiotics do remove most bacterial contamination, apart from antibiotic-resistant bacteria, they are not effective at removing other fungal competitors, such as filamentous fungi and some yeasts. The replication of *Bd* occurs at a much slower rate than that of filamentous fungi. This causes complications as *Bd* colonies can only be transferred when adequate growth along with active zoospores are visible.

The fitness of fungi plays a pivotal role in the competition for survival. "Fitness can be defined as the survival and reproductive success of an allele, individual or group" (Bolnick & Otto, 2013; Pringle & Taylor, 2002). Early reproduction may have a higher influence on total fitness than late reproduction: a single genet that can establish quickly in a novel habitat may overwhelm a second genet that establishes slowly, even if the two genets eventually have comparable propagation rates (Pringle & Taylor, 2002). For *Bd*, fitness is specifically determined by its transmission rate and primarily modulated by thermal variation (Agha *et al.*, 2018).

Water is a substance that all living organism require and also where some thrive. There is plenty of evidence that supports that some microbes can survive in bodies of freshwater and seawater for very long periods of time. Mostly found in the sediment microbial cells can subsist under strong nutrient and energy limitations for hundreds if not thousands of years (Hoehler &

Jørgensen, 2013; Jørgensen & Marshall, 2016). Sediment is where all these thousands of microbes lay dormant in a state of sub-zero metabolism known as cryptobiosis or anabiosis (Hallsworth, 2022). When these microbes are placed in a favourable nutrient rich environment they have the potential to reproduce at a faster rate. Microbes including *Bd* have the ability to suppress metabolism to conserve energy and only when conditions are favourable do they bloom or start reproducing. There are some fungi that have been identified in Lake Vostok which is a sub-glacial Antarctic freshwater lake. The lake itself has sub-zero temperatures, high pressure, and nutrient-depleted making it inhospitable and yet these fungi are able to survive showing that microbes are resilient even in adverse conditions. Therefore, the fitness of microbes can be extremely high as they are able to adapt to any environment, whether it is favourable or inhospitable.

Every organism has needs that have to be satisfied; this includes resources, territory, and reproduction. The microscopic world is no different; organisms compete for resources to survive, each equipped with their own attributes, whether it be the secretion of toxins/metabolites or the ability to reproduce at an accelerated rate and dominate the other organism. Therefore, microbes such as *Bd* are faced with a formidable foe as *Bd* primarily feeds on keratin and do not have any known competitors within the keratinized areas of amphibians. When placed *in vitro* with a filamentous fungi, which was sampled at the same site as the isolate. *Bd* is at a great disadvantage as other fungi do not infect frogs but instead grow and compete against other microbes for resources in the water. Because of competition for resources microbes develop defence mechanisms such as secreting inhibitory toxins or enzymes (Atlas, 1998; Pinto *et al.*, 2018). This competition leads to amensalism in which one microbial population produces a substance that is inhibitory to other populations, therefore giving the substance secreting population a competitive edge that is beneficial (Atlas, 1998).

There is always a winner and a loser in competition within a confined environment. Each living organism has a fight or flight response regarding competition. This interaction is vital to observe as it provides a basis of behaviour for a specific organism. To date, no behavioural studies have been done on *Bd* that ask whether they tend to fight and dominate a competitor such as filamentous fungi or bacteria or avoid and flee the environment to the best of their abilities. The fact that *Bd* has motile zoospores shows that it could exhibit an apparent fight or flight response in the presence of a competitor.

3.2 Causes of contamination

Parasitic fungi like *Bd* is a distinct minority against a myriad of diversified organisms (Tsao, 1970), *Bd* grows well on keratin but are sensitive to stressing factors such as isolation. Tadpoles feed on decaying organic matter, thus consuming other microbes growing on that substrate. These microbes include bacterium, yeasts and other fungi that do not affect the tadpoles. Although, tadpoles efficiently consume these microbes traces such as spores can remain between their keratodont rows.

Microbial contamination is primarily caused by human error and poor aseptic techniques. Batrachochytrids have a longer incubation period than other aquatic fungi and bacteria, making it vitally important to process a *Bd* sample thoroughly, as other fungi and bacteria would replicate at a faster rate than *Bd*. However, isolating a specific organism collected from the wild becomes difficult as some traces of competitors can remain depending on the condition of the sampling environment. The condition of sampling sites, in regard to substrate or pollution, contribute to possible traces of microbes. There tends to be less sediment and biological debris such as decaying plants at a site that has a constant and fast flow of water, sites that have this characteristic have less microbes as most sediment is transported downstream. Areas like dams and pools have higher sediment deposits and provide a nutrient rich environment, the area is also favourable as flow of water is slow or the water is stagnant (Sandrin *et al.*, 2009).

It only takes a single spore of a microbe to encyst and reproduce along with the target organism. The growth rate of the contaminant is accelerated when processed samples are placed in a nutrient-rich environment, such as isolation media. When a spore is placed in an *in-vitro* environment, it will reproduce at an exponential rate as there is no form of competition nor any environmental stressors that would inhibit growth. For this reason, it is tedious and in some ways impossible to isolate *Bd* from an infected sample without taking the necessary precautions aimed at eliminating contamination.

It is also essential to monitor and observe samples in the isolation process as some spores can become airborne and infect other samples through cross-contamination (Fleischer *et al.*, 2006). As microbes are living organism they generate heat that creates a humid environment, resulting in condensation within the sample casing. Spores, primarily bacterium can become suspended accumulate on the lid of casing, they can then spread to adjacent samples through the process of condensation. Cross-contamination can occur through a human error when samples are incorrectly handled or tools are not appropriately sterilised with EtOH and flaming

before attending to another sample. Therefore, it is important to work aseptically to ensure that there is no contamination caused through human error. Thus techniques such as weeding and transferring of samples has to be performed aseptically in the field and laboratory.

Cross-contamination can occur among samples if competitors grow exponentially and spread to adjoining samples during the experiments or isolation prior to field work. An example is when bacteria multiply in one well and grow to a point where they can jump to another well containing a sample. However, improper interaction with the contaminated well can result in cross-contamination through human error (Wurtz et al., 2016). Once bacteria have been identified, they need to be removed as soon as possible before cross-contamination occurs. In contrast, should the sample container become contaminated, any potential isolates need to be removed and stored separately to minimize the risk of losing an isolate. Contamination is a risk that all researchers face and should therefore be aware of, contamination can occur easily, and therefore the practice of aseptic techniques is required to mitigate and minimise any contamination.

3.3 Competition Experiment

Through fieldwork experiences and the amount of contamination encountered, it was interesting to see *Bd*'s response to competition with filamentous fungi. Field sampling yielded three filamentous fungi competing with *Bd* at a biological level. These fungi were found to grow at a higher rate than *Bd*. Therefore, *Bd* is outcompeted *in vitro* in the presence of faster-growing fungi. Therefore, the objective of this chapter is to determine the effect a competing fungi has on the fitness of *Bd* and if so how fast does is a competitor successfully inhibit *Bd*. The secondary objective is to determine if *Bd* exhibits an avoidance (fight or flight) response when in the presence of a competitor and if inhibition occurs during an avoidance response using zoospore density.

3.4 Methodology

3.4.1 Prototype formulation

The behaviour of *Bd* as well as other microbes is not something that has been delved into extensively, and behaviour is one of the major characteristics when studying a species. To observe this phenomenon, a device that can separate *Bd* from a competitor was necessary to ensure that no zoospores of either organism are accidentally placed in the same area. A device of this nature does not exist, as behavioural studies of this specific nature have not been done on microscopic organisms to ascertain if they exhibit a fight or flight response to a competitor.

Several prototypes were made using various methods and materials that would enable *Bd* to exhibit a behavioural response to the presence of a contaminant. The shortcoming with all the prototypes prior to the final one was contamination by bacteria. The first prototype was created using plastic containers acting as chambers and plastic tubing providing a means of accessibility between chambers (chamber diameter = 4.5 cm, Chamber height = 2 cm). Tubes were connected to adjoining chambers and glued to prevent leaking. Although having the desired shape it was too large for organisms like *Bd* and would take a long time before a pattern emerges, therefore, the size of the prototype had to be significantly reduced. The size of the following prototype was significantly smaller (chamber diameter = 2 cm, chamber height = 1.5cm). This was the ideal size for the prototype and an experiment was used to determine the behavioural pattern of *Bd* but after incubation for 24 hours at 20°C the media was found to be completely contaminated by bacteria. In an attempt to mitigate contamination, the prototype was submerged for 24 hours in 70% EtOH, after which it was thoroughly rinsed using autoclaved distilled water to remove any EtOH residue. The prototype was then subjected to Ultra Violet (UV) light for 30 min to kill any remaining bacteria. This process proved obsolete in mitigating bacteria contamination and making the prototype's plastic brittle due to exposure to UV light. The only alternative was to place the prototype in an autoclave, which would have resulted in the entire prototype melting.

The second prototype was designed through modifying a standard 24 well plate as it allowed a closer means of exchange between chambers and also allowed the possibility of multiple configurations of connected chambers for variation in experimental design (Figure 24). However, bacterial contamination still persisted in spite of using the same disinfection procedures described for the first prototype. The only way to completely sterilize it would be

sterilization by autoclave. The second prototype, despite having harder, more durable plastic, could not maintain its structural integrity at the high temperatures of the autoclave.

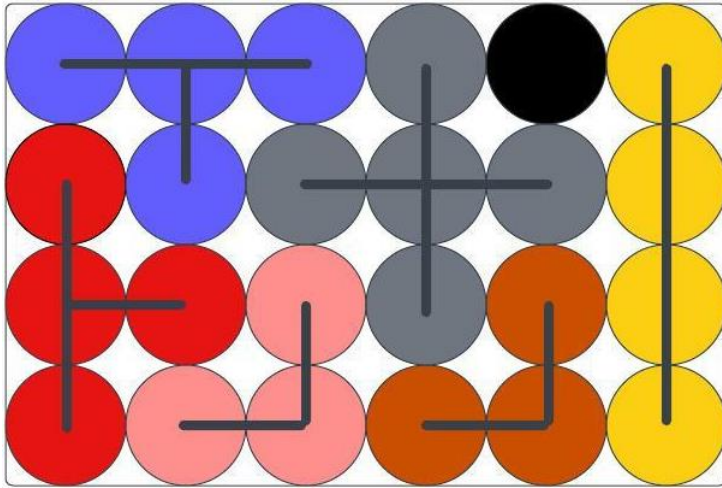


Figure 24 Prototype number 2: 24 Well plate modified for behavioural observations.

Considering that a working, cost effective prototype was not viable, it was necessary to construct one using glass. A glass prototype is capable of withstanding the temperatures of an autoclave. This would completely sterilise the prototype in theory if the causes of contamination of the previous prototypes were due to the plastic material they were made of, which led to the design of the final prototype. The final prototype, named the Tri-tube Behavioural Chamber (TBC), was designed in a way that the center chamber is the origin of the test organism (inoculation site) and the other three chambers acted as choice destinations. All three chambers are equidistant from the center chamber allowing the test organism to make behavioural decisions without the issue of distance being a deciding or limiting factor. Due to the size of the organisms, it was important to design the TBC in a way that it would not take an excessive amount of time to see an apparent behavioural pattern. This meant reducing the size of the prototype to the smallest degree possible.

Each chamber can hold up to 4.71 ml, with pathways between chambers having a distance of 4 mm (Figure 25). Reduced length of the channels allows *Bd* to travel faster between the adjacent chambers to best determine the behaviour in the presence of a competitor. Various alterations, material substitutions and dimensions were tested before developing the final prototype (Figure 26).

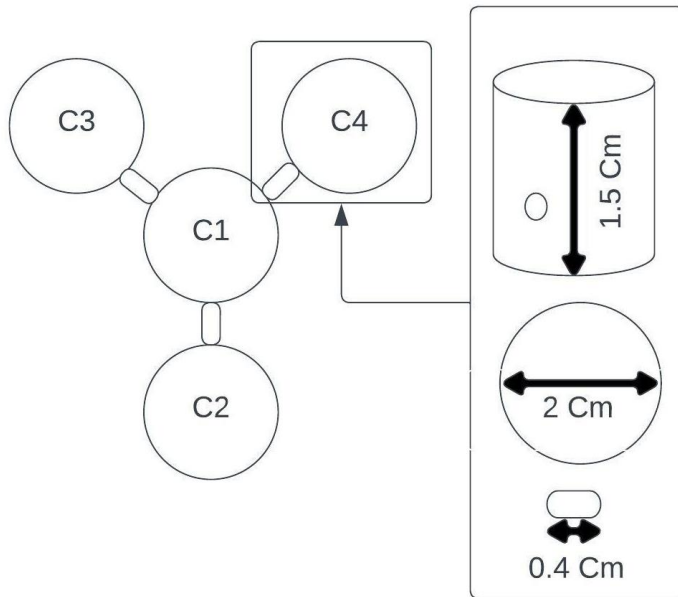


Figure 25 Design and dimensions of the Tri-Tube Behavioural Chamber.



Figure 26 The progression of the Tri-Tube Behavioural Chamber.

3.4.2 Preparation of *Bd* Sample

An active growing *Bd* culture was prepared by passaging and incubating an archived culture from the Herp Health Lab. The isolate used in this experiment was SANC10 (*BdGPL*). Exactly 1ml of is the isolate was withdrawn and spread on a fresh agar plate (see chapter 2 for preparation). Once *Bd* was transferred to the agar plate, it was left to dry in a Class II Biosafety Cabinet till no liquid media was present. Once the liquid media had dried, the agar plates were sealed using parafilm and placed upside down in an incubator at 20°C for three days.

Once active zoospores were observed, the agar plate was placed in the sterile biosafety cabinet and flooded with fresh liquid media (tryptone broth) (see chapter 2 for preparation). It was left for at least 10 minutes to allow active zoospores to resuspend. Using a sterile 200 ml culture flask, 100ml of broth was added, followed by carefully decanting the broth containing zoospores into the sterile culture flask. The culture flask was then labelled and incubated for approximately one week to allow for sufficient growth.

3.4.3 Preparation of fungal contaminants

The filamentous fungi used for this experiment was isolated from the NNR at the site Steep Glen Kloof which had a thick layer of silt deposits and organic matter which was the ideal locality for sampling filamentous fungi. The three filamentous fungi isolates were identified by their morphology, such as the type of hyphae, conidia and conidiophores that the isolates displayed (Kendrick, 2017). The three isolates were identified as belonging to the phylum Ascomycota, class Eurotiomycetes, order Eurotiales, and the family *Trichocomaceae*. These isolates were only identified to family level using morphological characteristics. Further distinction between species would have required DNA sequencing and this was not the objective of this study. From henceforth the three isolates will be referred to as Morph sp.1, Morph sp.2, and Morph sp.3.

For the purpose of the experiment, fresh cultures needed to be prepared from the three morphospecies. Samples were placed in the biosafety cabinet along with nutrient agar. Using an inoculation needle, fungal mycelia from the outer actively growing zones of fungal formations were gently scraped and streaked onto the new agar plates. The agar plates were then sealed with parafilm and placed upside down in an incubator for 3 to 5 days.

After sufficient growth, the fungal plates were placed into the biosafety cabinet, and actively growing areas were scraped and placed in a sterile 3ml eppendorf tube filled with liquid media. Samples were then placed in a vortex to suspend the filamentous fungi in the liquid media. Samples were then returned to the incubator for two days.

3.4.4 Experimental Setup

In the *in vitro* experiment, *Bd* was placed in the same well as filamentous fungi. This experiment aimed to infer the growth rate of *Bd* colonies when challenged with a competitor. The experiment consisted of two filamentous fungi treatments consisting of two dosages, 20 μ l, and 100 μ l; the reason for the lower dose treatment was to observe whether contaminant dosage influences the survival probability of *Bd*. Using one 24 well plate allowed for six replicates for each fungal contaminant, along with six control replicates with the same inoculation dosage. A total of four experiments were run using four 24 well plates, two with 20 μ l and two with 100 μ l, each had the same initial *Bd* concentration of zoospores per 1000 μ m². With two repetitions of the experiments, each contaminant had 12 repetitions each in their corresponding dosages. This experiment shows how *Bd* competes against filamentous fungi in an *in vitro* environment, which demonstrates *Bd*'s ability or inability to compete for needed resources.

The first step was to ensure the laminar flow cabinet was sterile and four 24 well plates have undergone the UV cycle to ensure a clean bacteria free environment. A live culture of SANC10 was then wiped down with alcohol and placed into the laminar flow cabinet. Using a pipette 1 ml of culture was then transferred to each well along with 1.5 ml fresh 1% tryptone to ensure a nutrient rich environment. The 24 well plates were then sealed using parafilm and placed under the Nikon eclipse TS 100 inverted microscope. Using the Nikon 0.55x DS camera under 10x magnification and the NS Elements program, an overlay grid of 1000 μ m² was used to determine zoospore density. After four days of documented growth the 24 well plates were placed back into the sterile laminar flow cabinet to be subjected to the 20 μ l and 100 μ l dosage of the filamentous fungi. Before placing the filamentous fungi cultures into the laminar flow cabinet, they were placed in the vortex for 30 seconds to homogenise the samples. 20 μ l of each sample was added to designated wells on two of the 24 well plates, and the process was repeated for the 100 μ l dose. Once all filamentous fungi samples have been added to the 24 well plates with respect to the specified dose, they were sealed with parafilm. After the 24 well plates were spiked observation of zoospore density was resumed using the overlay grid of 1000 μ m². This was done to determine the rate at which *Bd* density increases or decreases in the presence of competitors within a 1000 μ m².

3.4.5 Observation

There are four different phases in which microbial growth occurs, namely the lag phase, exponential phase, stationary phase, and death phase (Buchanan, 1918; Huang, 2013;

Widdel, 2007) (Figure 27). The lag phase is where no or minimal growth in the number of living cells occurs. The exponential phase is a dramatic increase in the number of living cells. The stationary phase is where living cells reach a plateau in the number of living cells at which the rate of production and death of cells are roughly equal to each other. The death phase is where there is an exponential decrease in the number of living cells, either by inhibition of competitors or an inadequate supply of nutrients.

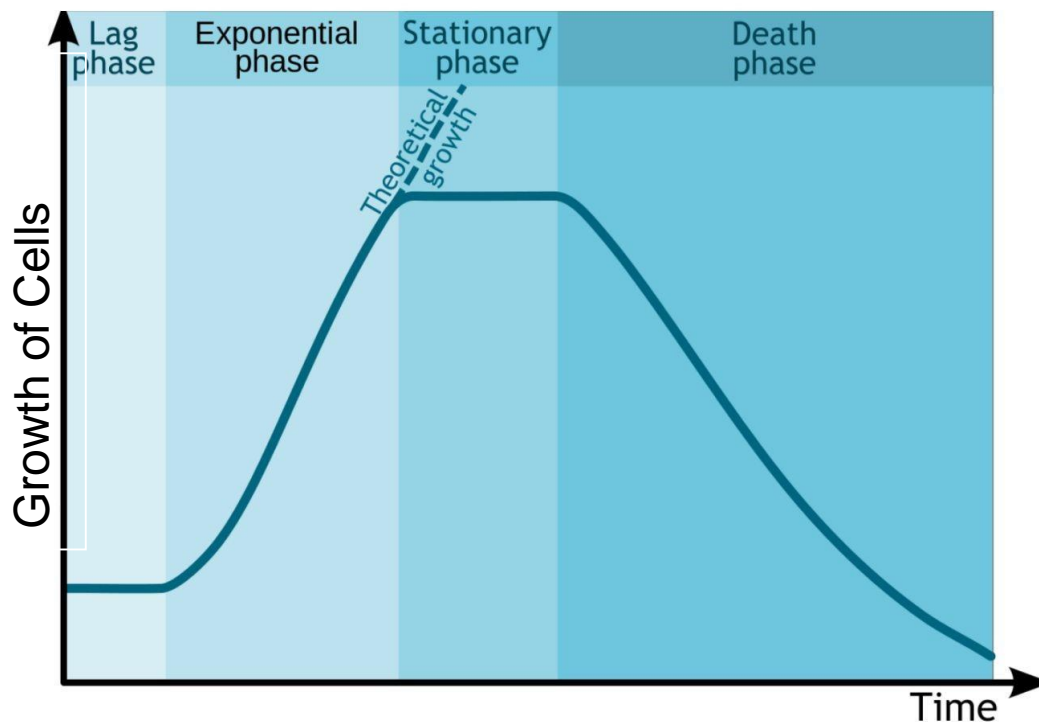


Figure 27 Phases of Cell Growth adapted from Michal Komorniczak/Wikimedia Commons/CC BY-SA 3.

Observation was done using a Nikon Eclipse TS 100 inverted microscope that allows specimens suspended in a liquid state to be easily observed. The program used to quantify active zoospores is NS Elements; this program allows the user to view specimens on a monitor with the aid of a Nikon TV Lens 0.55x DS microscope camera. Using a grid system sized 1000 μm^2 at 10x magnification and a counting system, the user can physically count the active zoospores much like the process of counting zoospores in a haemocytometer. Three squares were counted, and the average zoospore count was calculated for the most accurate quantification. These steps were repeated for each of the four 24 well plates every 24 hours until no active zoospores were present or two weeks have passed. The daily average and standard deviation was noted daily and placed into excel worksheets that will provide a clear pattern of competition and inhibition of either *Bd* or the competing filamentous fungi. Once

complete inhibition of either *Bd* or the filamentous fungi has occurred and there has been no change for two days, the experiment was completed.

3.4.6 Preparation of Fungal Slides

Filamentous fungi grow fast and dense, so in order to see how these fungi affect *Bd* it needs to be placed under a compound microscope. In order for this to be done, slides need to be prepared. After the experiments have been completed, the four 24 well plates were topped with 70% EtOH to ensure no live biological waste is discarded. Fragments of the filamentous fungi competitors were then extracted to prepare slides. For fixation purposes, lactophenol blue was required to preserve the specimens for prolonged storage.

Lactophenol blue was prepared as follows:

- 2 Parts Lactic Acid
- 1 Part Phenol
- 1 Glycerine
- 1 Part distilled water
- ¼ Part methylene blue

Using sterilised forceps, a sample of each specimen was removed and placed on a glass slide. The sample was then teased to spread sample to properly observe the formations between the *Bd* cells and the filamentous fungi. Once properly teased, one drop of lactophenol blue was placed on the slide to cover the sample. A cover slip was then carefully placed onto the slide and sealed with an adhesive. Slides were subsequently observed using the Nikon AZ 100M compound microscope at 40x magnification and high definition micrographs were taken with a Nikon Digital sight DS – F12 mounted camera (Figure 33).

3.4.7 Setup of TBC experiments

Thorough disinfection of the TBC was necessary before starting each experiment. The TBC was cleaned of debris using a soft scrubbing brush followed by submergence in 70% EtOH for 10 min ensuring that EtOH flushes each of the connection channels. The TBC was then thoroughly rinsed using distilled water, placed upside down on a glass petri dish, and covered in foil. The TBC was then autoclaved on a dry cycle. Once the TBC was autoclaved, it was placed directly in the laminar flow cabinet and undergoes a 20 min decontamination UV cycle.

For all the experiments to be accurate, there must be repetitions using the same device, and a standardized concentration of *Bd* needs to be used. A haemocytometer was used to quantify the amount of zoospores present in the culture. The standardized concentration of 140 000 zp/ml was used for each experiment. *Bd* cultures were incubated until the zoospore density reached the required range. Concentration was diluted with broth media when the density was above the required concentration. Once standard values for *Bd* were in the specified range, the culture was placed into the laminar flow cabinet. The four chambers of the sterile TBC were filled with fresh tryptone broth (liquid media) just beneath the level of the openings of channels connecting the chambers. Once the chambers were filled, the center chamber was inoculated with 1 ml of the stock *Bd* culture. The TBC was then sealed using parafilm and left for 24 hours in the laminar flow cabinet, allowing *Bd* to settle and create a biofilm on the bottom surface. After the 24 hours have passed, tryptone broth was slowly added by allowing the broth to run down the inside surface of the chambers in order to prevent zoospores from resuspending and causing accidental transmission to the adjacent chambers. The three surrounding chambers were filled first to create a positive meniscus at the brim of the channel to the other chambers. Once all the surrounding chambers containing no zoospores are filled to the channel opening, the center chamber was slowly filled until it had a positive meniscus. The TBC was then left for an hour as a precaution in case there were any zoospores in suspension within the upper column of the chamber.

Surrounding chambers were then filled by pipetting tryptone broth into the channel to prevent sudden turbulence in the chamber when the central chamber and surrounding chambers connected with tryptone broth. Pipetting was done clockwise to have equal volumetric/viscous forces distribution when all the chambers become connected. Once all chambers were connected, more tryptone was carefully added to raise the broth above the channels to have a homogenous pressure throughout the TBC. Once all steps were completed, 20 µl of the Morph sp.1 was added to one of the outside chambers. Therefore, this setup involves two outer chambers acting as choice chambers, one outer chamber containing the Morph sp.1 competitor and the central chamber containing *Bd*. The reason for using only 20 µl of the Morph sp.1 was because it has the ability to grow at an exponential rate in a nutrient rich environment.

After all the preparation has been completed, the TBC was sealed using parafilm covering the opening of the chambers. The TBC was then removed from the laminar flow cabinet and

examined under the Nikon Eclipse TS 100 inverted microscope to see if there were any zoospores in the choice chambers as well as initial zoospore density per 1000 μm^2 . Should accidental spillage occur, the TBC preparation procedure needs to be repeated. Zoospore density was also assessed under the inverted microscope after preparation using the Nikon 0.55x DS camera at 10x magnification on an overlay grid of 1000 μm^2 . This is done to determine the *Bd* concentration on the final day. Daily observation was then done to determine *Bd*'s behavioural responses when faced with a contaminant. The experiment ended after a period of five days, which was based on how fast *Bd* was able to colonise all chambers. Control experiments needed to be done without the presence of the *Morph* sp.1 competitor to establish that *Bd* would spread to all chambers should it have no competition. A total of three control experiments were done to show if *Bd* would migrate to all the chambers in the given time. The *Morph* sp.1 experiments were run in triplicate as well with the same time constraints as the control experiments. The effect of inhibition on *Bd* can be quantified by using the initial zoospore concentration and comparing it with the final zoospore concentration using the Student t-test to determine the effect of inhibition. Colonisation success was measured by surface area with each of the four wells representing 25%. Each well was divided into 25% quadrants as well. For example, should *Bd* fully colonise two chambers and only colonise 50% of a third chamber, the colonisation for the two fully colonised chambers would be 25% each and the third chamber would have 12.5%, therefore having an overall colonisation of 62.5%.

3.5 Results

3.5.1 Competition experiment results

It is evident that there was competition to a great degree in terms of nutrient acquisition. There was a clear trend of *Bd* zoospore growth without the filamentous fungi competitors up to day 4, at which point *Bd* zoospore activity dropped significantly once the filamentous fungi competitors were introduced (Figure 28).

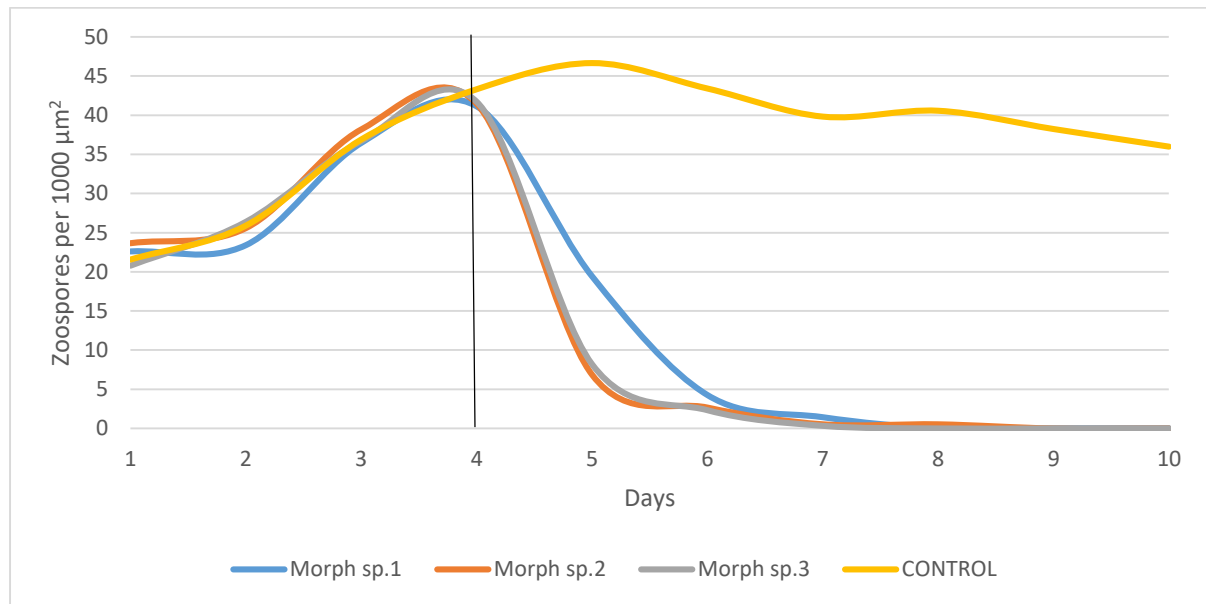


Figure 28 reactional trend of 3 competitors introduced to *Bd* the black line through day 4 of the graph represents the day of introduction of filamentous fungi competitors with a dosage of 100 μ l.

Batrachochytrid zoospore activity remained in the lag phase for a day, followed by the exponential phase in which *Bd* growth increased from approximately 20-23 zp/1000 μ m² to 41-43 zp/1000 μ m² before the introduction on day 4 (Figure 29). From the previous graphic, it is clear that there was an adverse reaction to the growth of *Bd* when a competitor is present (Figure 28). (Figure 29) shows the first repetition of how *Bd* competes in the presence of three different filamentous fungi competitors, which had a dosage of 20 μ l.

Each of the competitors inhibited zoospore activity 24 hours after their introduction to the wells on day 4. Although wells containing Morph sp.3 and Morph sp.2 had a more immediate response, wells containing the Morph sp.1 contaminant had the most rapid decline in the death phase moving from 42.72 zoospores to 3.77 within 24 hours (Figure 29). The control wells reached a peak on day eight and remained in a stationary phase having a zoospore count of 38.38 zp/1000 μ m² at the end of the trial.

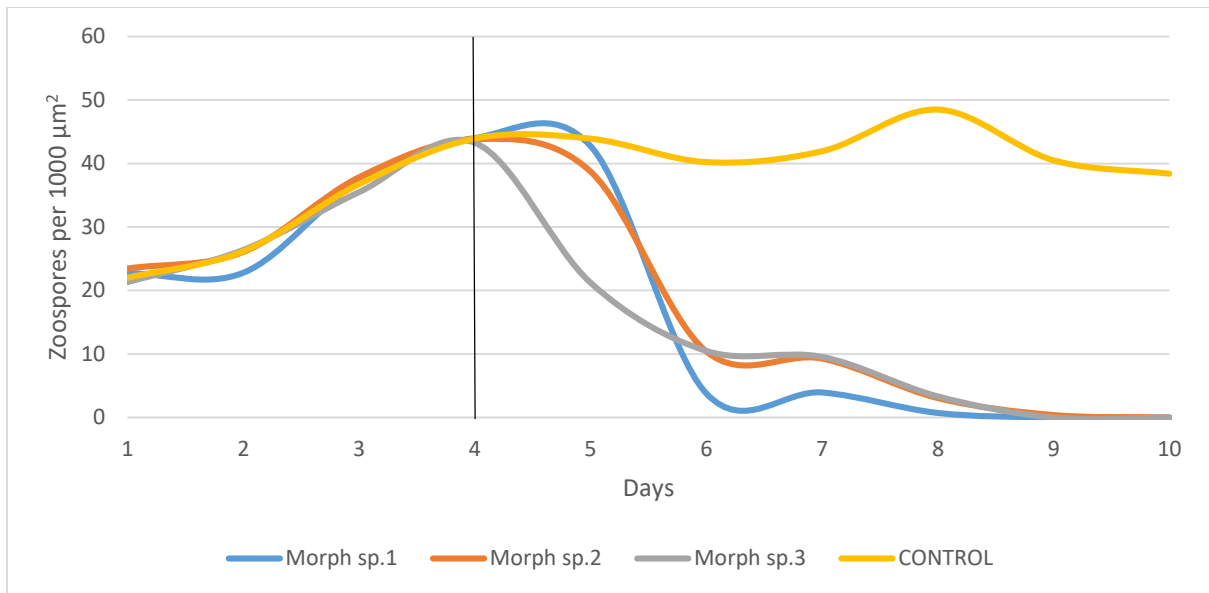


Figure 29 Relational trend of 3 competitors introduced to *Bd* the black line through day 4 of the graph represents the day of introduction of filamentous fungi competitors with a dosage of 20 μ l.

The competition experiment with the Morph sp.1 dosage of 100 μ l had a more abrupt effect on *Bd* zoospores than the lower dose of 20 μ l. All competitor wells showed a rapid decline in *Bd* zoospore activity from the introduction of Morph sp.1. There was a definite lag phase during the first day leading to the start of the second, where exponential growth occurred. Once day four was reached, the 20 μ l and 100 μ l doses of Morph sp.1 were introduced. The 100 μ l dose resulted in the immediate decline of *Bd* zoospores, whereas the 20 μ l dose only resulted in a decline after approximately one day (Figure 30). By looking at the control group, it is clear that *Bd* was still in the exponential phase of growth when the Morph sp.1 was introduced and immediately started to inhibit the growth rate of *Bd*.

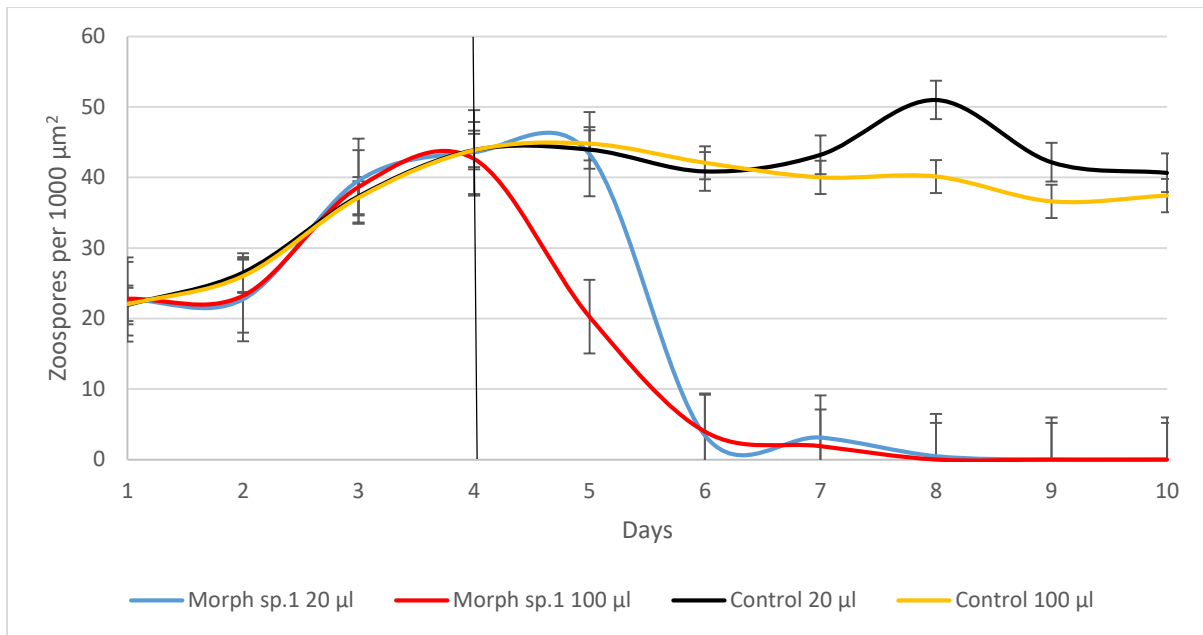


Figure 30 Inhibition of *Bd* at 20 µl dose compared to 100 µl dose of *Morph sp.1* the black line through day 4 of the graph represents the day of introduction of *Morph sp.1*.

The *Morph sp.2* competitor had an immediate effect on *Bd* growth from both the 20 µl and 100 µl doses (Figure 31). Although the effect was immediate, it had a less aggressive effect on *Bd* zoospores during the death phase when compared to the *Morph sp.1* competitor. The 20 µl and 100 µl have a near identical trend when considering the dosage.

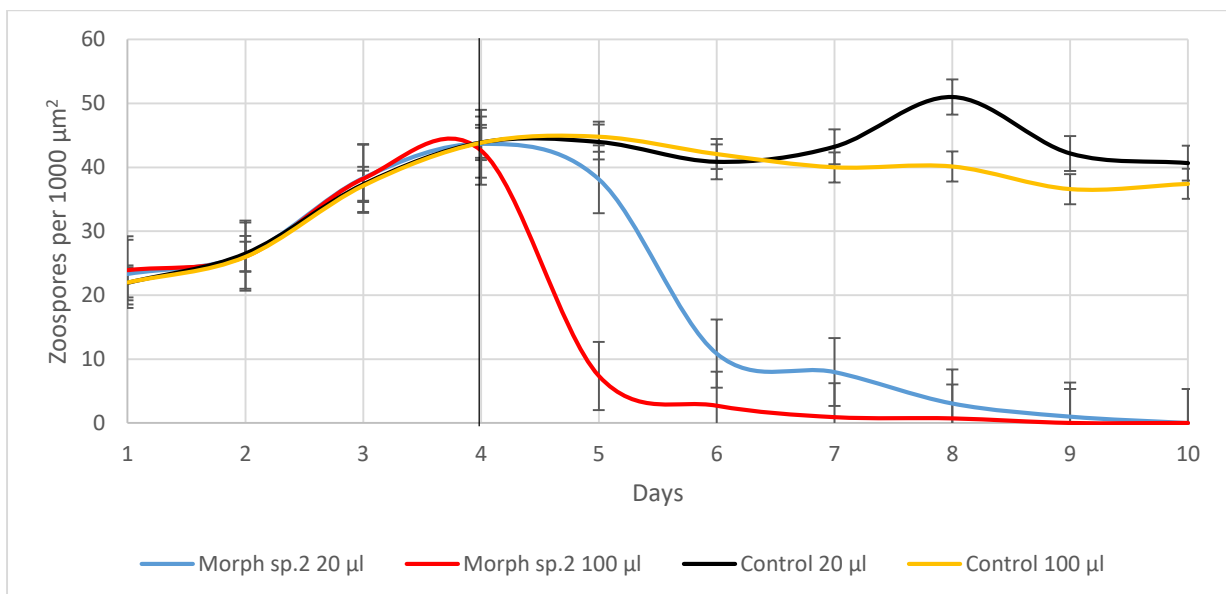


Figure 31 Inhibition of *Bd* at 20 µl dose compared to 100 µl dose of *Morph sp.2* the black line through day 4 of the graph represents the day of introduction of *Morph sp.2*.

The Morph sp.3 competitor, like the Morph sp.2 competitor, had an immediate effect on *Bd* growth from the point of introduction. Compared to the other competitors, it is clear that Morph sp.3 has a less aggressive effect and is similar to the trend of Morph sp.2. Although it is less aggressive, it does cause complete inhibition of the *Bd* growth rate by day 10 for the 20 μ l and day 9 for the 100 μ l (Figure 32).

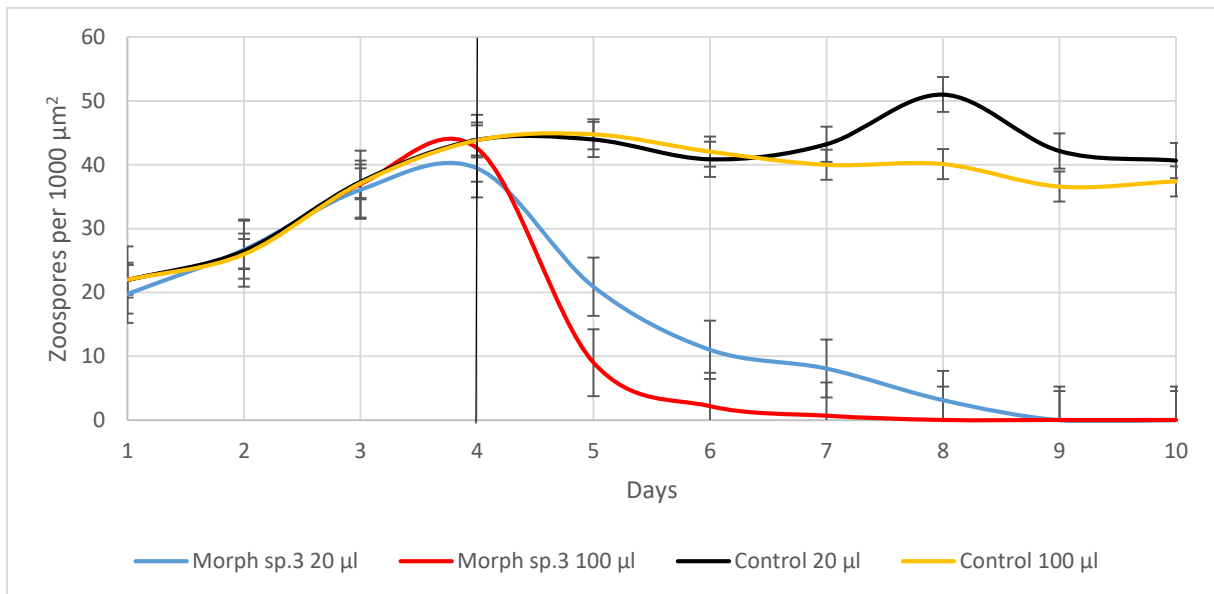


Figure 32 Inhibition of *Bd* at 20 μ l dose compared to 100 μ l dose of Morph sp.3 the black line through day 4 of the Graph represents the day of introduction of Morph sp.3.

All slides prepared from the experiments show how *Bd* is intertwined with the competitors. From the images provided, it can be seen why zoospore activity ceases rapidly as they come in contact with competitors.

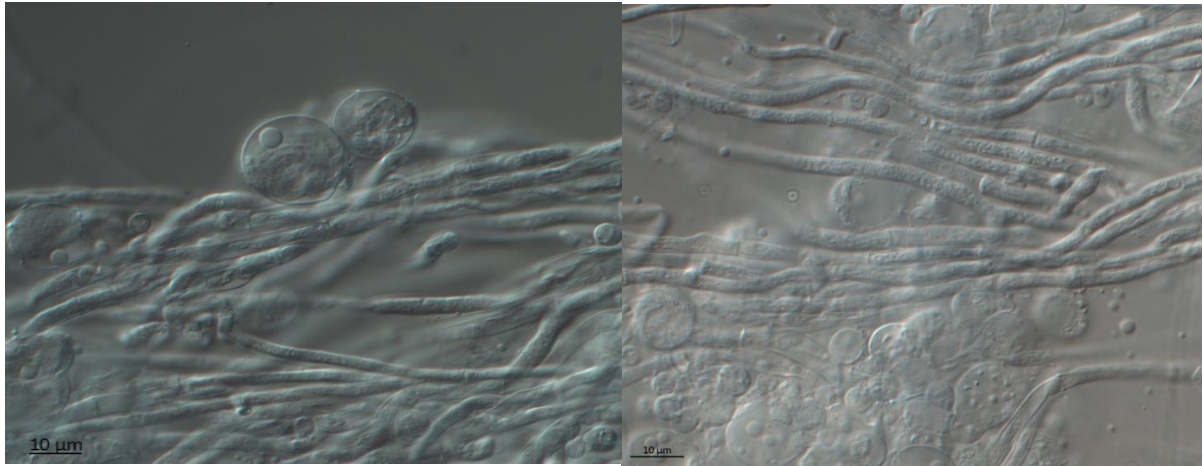


Figure 33 Examples of entanglement of Bd by (Left) Morph sp.1 and by (Right) Morph sp.2.

3.5.2 Behavioural tendency of *Bd* towards Morph sp.1

An illustration of *Bd*'s ideal behaviour when there are no environmental competitors can be seen in (Figure 34). *Bd* grew within the center chamber for the first two days before migrating to other chambers in the following days. Replicate 1 migrated to two chambers by day three, whereas replicate 2 and 3 only migrated to 2 chambers after four days. Although *Bd* migrated to two chambers faster than the other two replicates, all repetitions migrated to 3 chambers on day 5. It is clear that when there are no competitors *Bd* has the ability to colonise available chambers at a steady rate.

By looking at the control group, it shows that *Bd* does have a consistent distribution rate. By looking at the mean average distribution percentage, each chamber represents 25% for a cumulative total of 100%. On days 1 and 2, *Bd* remained in the center chamber gradually growing, meaning that *Bd* had a 25% distribution on days 1 and 2. On day 3, the distribution of *Bd* increased to 37.5%, and on day 4 had a distribution of 50% in which *Bd* had spread to more than 2 chambers. By day 5, *Bd* had spread to all chambers and had a mean distribution average of 70.83% by the end of the trial (Figure 34).

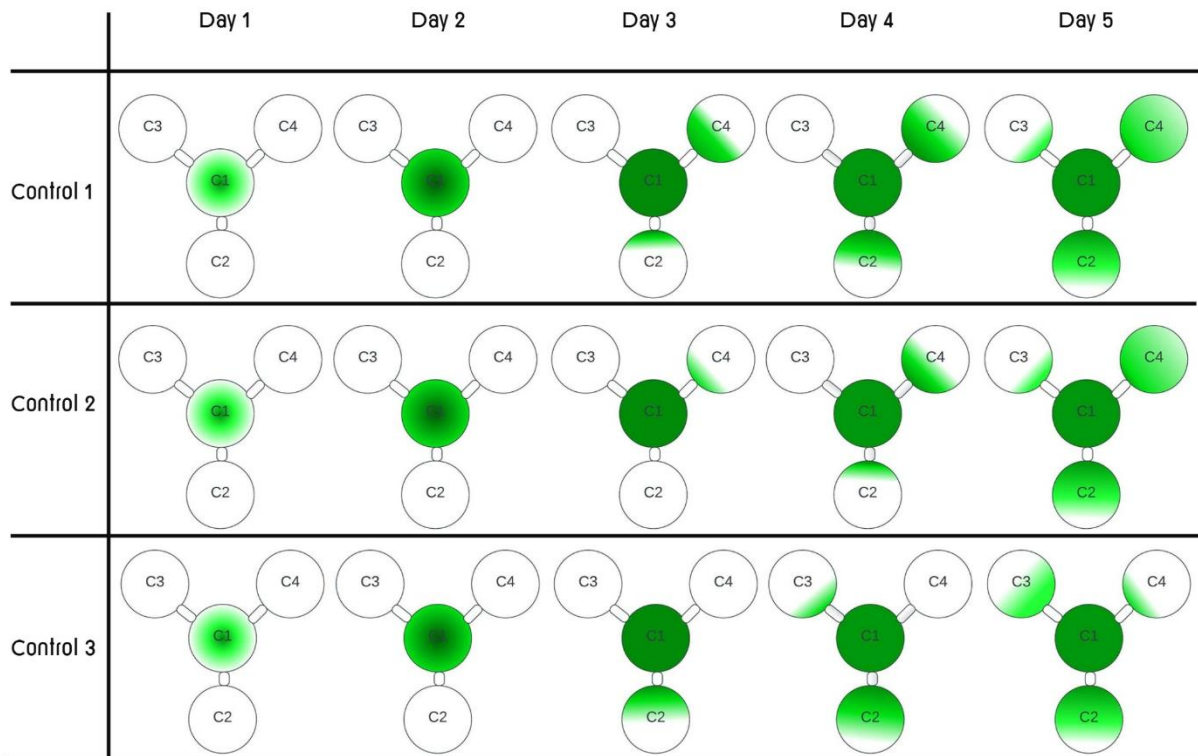


Figure 34 Colonisation behaviour of *Bd* without a competitor present, with distribution based on colonisation of surface area. Controls 1, 2 and 3 represent the progression of three replicates through time.

An illustration of the behaviour *Bd* has when a stressor is present can be seen in (Figure 35). The stressor *Morph* sp.1 was placed in one of the outer chambers, and *Bd* in the central chamber. *Bd* remained in the central chamber for all repetitions, and on day three, replicate 1 and 3 migrated to another chamber, while replicate 2 remained in the central chamber till day three. In all three repetitions *Bd* migrated to at least one chamber on the fourth day. On the final day, *Bd* from all three repetitions migrated to another chamber. By the end of the repetitions, *Bd* was able to occupy the two empty choice chambers but did not migrate to the chamber containing the competitor.

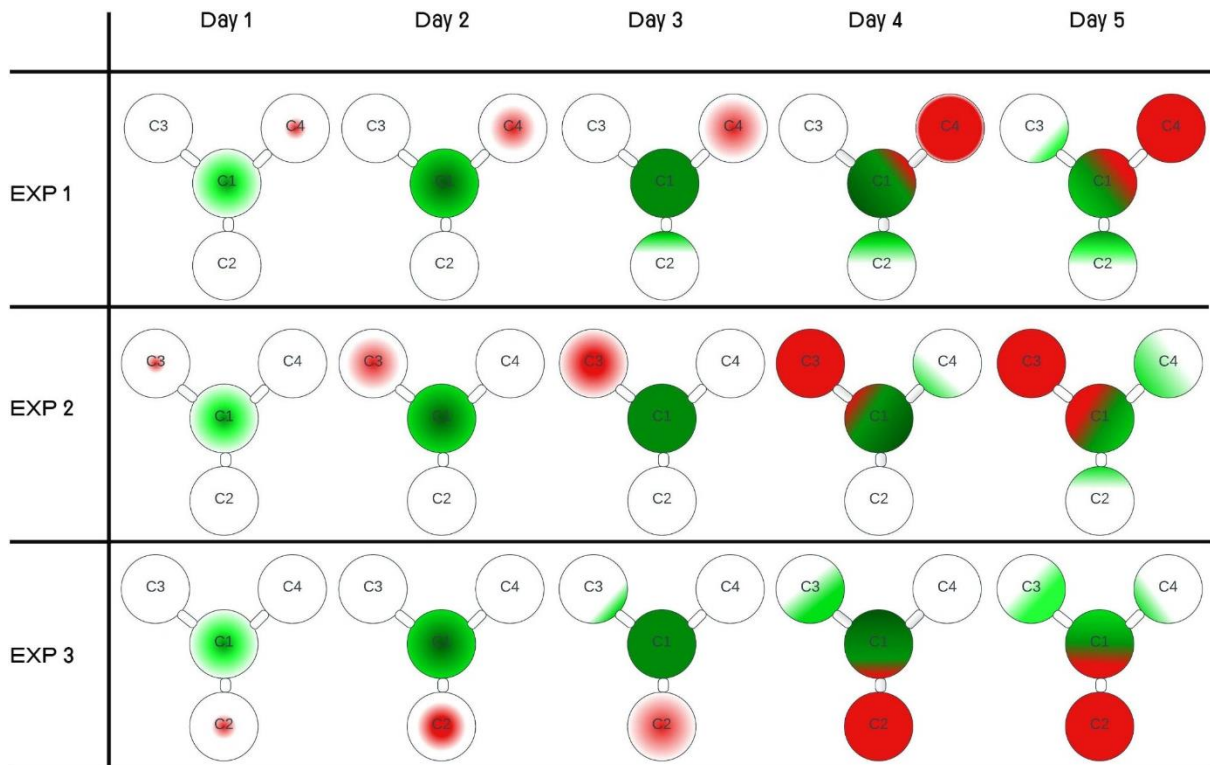


Figure 35 Colonisation behaviour of *Bd* in the presence of a competitor, with distribution based on colonisation of surface area. Experiments 1, 2 and 3 represent the progression of three replicates through time. The progression of *Bd* is indicated with green graphics and that of the competitor with red graphics.

Figures 34 and 35 illustrates the collective behaviour of *Bd* with and without a competitor. From the patterns seen, it is clear that the *Morph* sp.1 competitor does affect *Bd*'s ability to migrate and colonise. None of the EXP repetitions migrated into the chamber containing the competitor but did migrate to the other chambers by the end of the five days. From this, it is clear that *Bd* has a avoidance (flight) response to the competitor rather than a fight response. The competitor had a significant effect on *Bd*'s ability to colonise having a P value of 0.00123 based on the initial zoospore count compared to the final zoospore count on day 5.

3.6 Discussion

Contamination has been a significant obstacle in the success of isolating batrachochytrids (*Bd*). These contaminants include bacteria and other filamentous fungi that inhibit the growth of *Bd*. When in a stable environment, *Bd* has the ability to colonise and spread to a great extent should there be no competition for nutrients. Contaminants are categorized as unwanted bio-matter, but these contaminants are competitors. There has always been a form of competition in every aspect of nature, from large vertebrates to microscopic singular cells and viruses. These filamentous fungi are no different; every organism has a fundamental purpose: survival. In the case of *Bd* and filamentous fungi, it is a battle for limited nutrients and rate of reproduction. The filamentous fungi have a remarkable ability to adapt and replicate at an exponential rate. Our filamentous fungi isolates only had slight variation in morphology, but all distinctly belonging to the family *Trichocomaceae*. One thing all members in this family have in common is the ability to colonise aggressively with adaptability to extreme environmental conditions (Houbraken & Samson, 2011).

In contrast, *Bd* has a slow life cycle that has to be completed before the next generation of zoospores are released (Berger *et al.*, 2005). *Trichocomaceae* colonise rapidly due to their morphological nature, having branched conidiophores producing conidia into an environment that settles and attaches to a surface, starting a new colony. Microorganisms such as fungi have the means to inhibit competing populations by secreting toxic or inhibitory substances to gain a competitive advantage (Atlas, 1998; Fredrickson & Stephanopoulos, 1981); this relationship is referred to as amensalism. Amensalism may lead to preemptory colonisation of a habitat, and once a population has established itself, it may prevent other populations from surviving in that habitat (Atlas, 1998). In contrast, there are fungal communities that have a cooperative relationship in which they secrete toxins that do not affect each other but instead affect common competitors intruding on their environment (Fifani, 2021).

Two different experiments were conducted to determine the rate at which the filamentous fungi competitors inhibit *Bd* and whether *Bd* exhibits a behavioural response to competition. This has relevance on how long *Bd* will survive should a contaminant/competitor be present during the isolation process of *Bd* from host tissue. This pattern accurately represents the growth of *Bd* that could be used to assess its response to the presence of competitors during the exposure experiment.

The second experiment was that of the behaviour trials using the prototype design to determine if *Bd* exhibited an avoidance response in the presence of a competitor. The Tri-tube Behavioural Chamber (TBC) was designed to provide *Bd* with a choice to a “fight” or “flight” response toward a competitor. The experiment was done to see if *Bd* has the ability to escape a competitor if presented with the opportunity.

3.6.1 Fungal interaction promoting amensalism towards *Bd*

There is a clear disadvantage for the survival of *Bd* in the presence of other filamentous fungi (Figures 28 & 29). When faced with competition *Bd* is significantly inhibited through inhibitory substances produced by competitors. The experiment started with the absence of competitors and showed a uniform increase in *Bd* zoospore activity, entering the exponential phase. *Bd* proliferated until day 4, at which point the competitors were introduced. This resulted in an immediate onset of the death phase for *Bd*, missing the stationary phase altogether. From the trend exhibited by the control group, *Bd* should have remained at approximately 40 zoospores per 1000 μm^2 . The dosage of 100 μl caused a faster rate of inhibition on *Bd* than the 20 μl dosage which was expected, but the competitors have the same eventual result on the *Bd* zoospore growth, which is complete inhibition. The best representation of how the competitors outcompete *Bd* is by looking at the 20 μl dosage as it shows how inhibition of *Bd* increases as the competitor grows. The 20 μl dosage (figure 29) has a more gradual inhibition effect on *Bd* when compared to the 100 μl dosage (Figure 28).

Environmental filamentous fungi that share the same environment as *Bd* infected tadpoles would not affect or compete with *Bd*, as *Bd* infects the sub-dermal layer of tadpoles and thus utilizes a separate resource. These filamentous fungi do not infect tadpoles as tadpoles consume these fungi along with other microbes growing on decaying bio-matter. Therefore, *Bd* remains unaffected by these filamentous fungi competitors while infecting a tadpole. In contrast, the same cannot be said for *Bd* when placed *in vitro* with one of these competitors, as the tissue of its host no longer provides protection from environmental fungi. The filamentous fungi used in this study was found in the natural environment and had to survive in harsh circumstances leading to the natural production of toxic and inhibitory substances to protect themselves against other microbes present in the area. For this reason, filamentous fungi have the competitive edge when placed in an *in vitro* environment with *Bd*, which has no need for inhibitory substances as it has no natural competitors within an infected host.

3.6.2 Competitor specific trails against *Bd*

From the Morph sp.1 trial, it is clear that the inhibition of *Bd* zoospores by competitors is dose dependent. The 100 µl dosage resulted in an immediate decline in *Bd* zoospore activity. In contrast, the 20 µl dose still experienced the exponential phase of *Bd* growth till day 5, after which a significant decline was experienced (Figure 30). Both dosages were introduced during the exponential phase of *Bd* growth to determine how fast filamentous fungi competitors have an effect on *Bd*. By looking at the control group, it is clear that *Bd* goes into the stationary phase from day 5 to day 7, followed by a momentary peak of zoospore activity before starting to decline after the peak of day 8.

The Morph sp.1 competitor successfully inhibited the growth of *Bd* in both dosages by day 9, at which point no zoospore activity was found. The trend of the 20 µl and 100 µl dosages are nearly identical even though inhibition effects between the two dosages are a day apart. Both dosages had a slight increase in zoospore activity on day 7, which was when the control group experienced the momentary peak of zoospore activity. The result shows that although the Morph sp.1 competitors severely inhibited *Bd*, it still reached a peak of zoospore activity, indicating that the life cycle of *Bd* was still the same regardless of the inhibition caused by its competitor.

Unlike the Morph sp.1 competitor, the Morph sp.2 competitor had an immediate effect on zoospore activity from the moment of introduction of both the 20 µl and 100 µl dosages. The 100 µl dosage resulted in immediate aggressive inhibition of *Bd* growth, having a *Bd* zoospore count of less than 10. The 20 µl dosage had an immediate effect on the growth of *Bd* but was less aggressive and only reached a zoospore count of less than ten after day 7 (Figure 31). There is also a near identical trend in the *Bd* zoospore activity, indicating that the effect Morph sp.2 has on *Bd* is uniform with respect to the dosage.

The Morph sp.3 competitor had similar effects on *Bd* as Morph sp.2 but was less aggressive, making it the least aggressive competitor among the three competitors. The Morph sp.3 competitor immediately affected the zoospore activity, but the 100 µl dosage had a more significant inhibition effect than the 20 µl (Figure 32). By day 5, zoospore activity was less than 10 for the 100 µl but was above 20 zoospores for the 20 µl dosage.

All three competitors inhibit the growth of *Bd* to a great extent. The Morph sp.1 competitor was the most aggressive despite the delay of the 20 μl dosage. By looking at the 20 μl dosage of all competitors, Morph sp.2 and Morph sp.3 have an immediate effect on the activity of *Bd* zoospores. However, Morph sp.1, despite only affecting zoospore activity a day after introduction, causes the most rapid decline of *Bd* zoospores. By day 5, Morph sp.1 had reduced *Bd* zoospore activity to 3.7 zoospores per 1000 μm^2 , whereas Morph sp.2 and Morph sp.3 had approximately 10.3 -10.5 zoospores per 1000 μm^2 making Morph sp.1 the most aggressive competitor of the three (Figure 29).

When taking a closer look at how these competitors have inhibited the growth of *Bd*, it is understandable why they have done it so effectively. All three competitors are filamentous fungi that entirely encapsulate *Bd* colonies (Figures 33), consequently limiting zoospore motility and interfering with their ability to create attachment points to encyst to become a zoosporangium that can produce more zoospores. The energy it requires to encyst and produce zoospores are great and require full functionality of their metabolism and cell functions. All three competitors reproduce at a higher rate than *Bd*, possibly secreting more inhibitory substances that prevent *Bd* from being able to reproduce successfully. Moreover, should a zoosporangium be able to finish the cycle and release zoospores, the likelihood of those zoospores being able to encyst and complete their life cycles while exposed to the inhibitory substance secreted by filamentous fungi is unlikely.

By looking at the result of the competition between *Bd* and filamentous fungi competitors, it is evident that should one be in the process of isolating *Bd* from a field sample, there is only a limited amount of time before *Bd* is outcompeted by the contaminant/competitor for nutritional resources. This study shows that when isolating *Bd* it becomes vulnerable to inhibition by other competitors/contaminants that can cause full inhibition in a matter of days if either is not removed. It is true that *Bd* is a resilient fungi, but it does not compete against other fungi for survival within an infected host. Therefore, *Bd* does not have an enzyme or substance that can shield itself or inhibit another competitor as it never had the need to develop that characteristic in the host it is infecting. In order to identify and isolate *Bd* for the purposes of conservation it is vital to properly clean potential *Bd* samples during the field processing and observe all samples daily.

This study shows that *Bd* can be inhibited within a day by a competing microbe when placed in an *in vitro* environment. Therefore, the use of liquid media as a preservation source after field processing is not preferred as competitors would have a competitive edge against any

possible *Bd* isolate derived from the sample taken in the field. This could result in the inhibition of isolates or even false negatives as *Bd* had no chance of proliferating in the presence of an inhibitory substance secreted by a competing microbe population.

3.6.3 Avoidance response of *Bd* in the presence of competitors

Batrachochytrids can be easily inhibited by a competitor as seen in the previous experiment, but what would their behaviour be towards a threat if they had an option to act on. Every living organism has the propensity to reproduce and survive. For this to happen, the organism has to compete naturally against other organisms to survive. Survival of the organism is based on its inherent abilities to survive, which are either fight or flight. A fight or flight mechanism is engrained in the genetics of an organism even microbes in their own way (Molloy, 2005). But when looking at it from a microbial perspective, some populations exhibit avoidance (flight) behaviour toward a competitor that secretes a substance that causes an inhibition effect (fight) on the avoiding populations. Identifying an avoidance response in nature is easy, but this is not the case at a microscopic level. To see a definite behavioural response to a competitor at a microscopic level, one has to be able to observe it. This question led to the design of the TBC as one can see specific behavioural patterns and establish if a microorganism such as fungi has an avoidance response to other fungi.

Batrachochytrids are highly influenced by other fungal competitors, as discussed in the inhibition experiment. Looking at how *Bd* behaves and colonises areas in the control group, we can see how *Bd* evenly distributes itself gradually throughout the TBC until day five. By looking at the repetitions, it is clear that *Bd* exhibits a similar distribution rate based on the environmental conditions it is faced with.

Although *Bd* showed a uniform distribution pattern when faced with a competitor, it was clear that there was a form of inhibition in the distribution of *Bd* (Figure 35). On days 1 and 2, *Bd* remained in the central chamber as it did for the control group. On day 3, *Bd* had a distribution of 29.17%, which is 6.25% less than the mean distribution average for the control group. Day 4 indicated that there was apparent inhibition of *Bd* by the competitor as it had a mean average distribution of 35.42% compared to the 50% mean distribution average of the control group. By the end of the trial, *Bd* did manage to occupy three of the four chambers, the chamber not being occupied being the competitor chamber. On day 5, *Bd* had a mean distribution average of 43.75%, which did not occupy more than 50% of the colonised

chambers. By looking at the concentration of *Bd* zoospores before and after the trial we can determine that *Bd* was significantly inhibited by Morph sp.1. Therefore, it is clear that *Bd* faces clear inhibition from competitors, consequently delaying its distribution and its survival. By looking at the percentages on the various days, it is clear that the competitor both inhibits and repels *Bd*, as *Bd* did not enter the competitor's chamber. It shows that *Bd* exhibits a flight response to the competitor by avoiding colonisation of the competitor chamber rather than a direct competition approach resembling a fight response. Although *Bd* could colonise the two control chambers, it was evident that the rate of spread was retarded compared to the control group. The competitor, although having been placed into the chamber with a dosage of 20 µl, could still grow at a rapid rate.

The competitor entered the central chamber at the start of day 4, but signs of inhibition were already present on day 3 when the first signs of *Bd* distribution were identified. Therefore, it could be possible that the competitor secretes an inhibitory substance that significantly inhibits *Bd*, leading to a slower distribution rate (Atlas, 1998; Fredrickson & Stephanopoulos, 1981). Both the competitor and *Bd* have the same need for resources to survive. However, the fact that *Bd* has a life cycle in which zoospores form zoosporangia and produce their own zoospores takes too long and cannot compete with the rate of growth the competitor exhibits.

3.6.4 Conclusion

These experiments show just how defenceless *Bd* is when faced with a competitor and that isolation of *Bd* should be kept under stringent supervision when there are traces of other contaminants and/or competitors. The competitors use the same nutritional resources as *Bd* and grow at a much faster rate than *Bd*, while secreting inhibitory substances giving the competitor a competitive edge.

The competition experiment shows that even the smallest amount of competition by other fungi has the propensity to inhibit *Bd*'s growth, leading to complete inactivity. Competitors showed that at the moment of introduction to the environment, which was during the exponential phase of *Bd* growth, led to an immediate decline commencing the death phase of *Bd*, rendering *Bd*'s capability to reproduce null and void. The TBC experiment shows that *Bd* exhibits a competitor avoidance response when faced with a situation where there is a scramble for resources. The competitor had a significant effect on *Bd* behaviour and its ability to spread as the competitor seems to produce a substance that causes inhibition

of *Bd* zoospores influencing the motility and the rate at which the zoospores can encyst and release the next generation of zoospores.

These experiments show that *Bd* can be significantly inhibited when *in-vitro*. This creates a troublesome predicament for the isolation of *Bd* from field samples as *Bd* needs time to complete its life cycle to reproduce more active zoospores. When placed in the liquid media, *Bd* has no limitations to grow and spread. However, there are also no limitations for other competitors and/or contaminants to spread, leading to the inhibition of potential *Bd* isolates. Batrachochytrids are very vulnerable after field processing as some active zoospores are removed through the processing of field samples during mechanical removal of bacteria and other impurities. Mostly encysted zoosporangia remain on the excised host tissue, depending on the original infection intensity. Therefore, taking a longer time to reproduce zoospores should there only be a few active zoosporangia. During this stage, it is vital to cause no disturbance to the sample as it could interfere with the reproduction of *Bd*. Although *Bd* may be vulnerable when faced with a competitor, such as filamentous fungi capable of reproducing at an exponential rate, one should establish the state of *Bd* and either attempt to remove the competing stressor or transfer the *Bd* infected sample. These experiments made it clear that *Bd* is easily outcompeted within an *in vitro* environment. One should be thorough in the field processing to ensure that minimal to no contamination is transferred along with the sample. Although causing a predicament in the isolation and culturing of *Bd*, it does give hope for possible natural mitigation of *Bd* pathogenicity, as possible solutions could be derived from the synthesis and utilization of a competitor on an infected host.

Chapter 4: General Discussion

4.1 Distribution and occurrence of *Bd* in the Ncandu Nature Reserve

The chapters in this thesis all focus on different components concerning the ecology of *Batrachochytrium dendrobatidis* (*Bd*) in the Ncandu Nature Reserve (NNR). Most of South Africa is infected with *Bd*, with the province of KwaZulu Natal (KZN) having the highest *Bd* concentration, as well as two lineages and a hybrid (Farrer *et al.*, 2011). The two lineages, *BdCAPE* and *BdGPL*, are widespread across the country, but large gaps in distribution still exist for the northern parts of the Drakensberg mountain range. Due to the possibility of recombinant *Bd* lineages, more research and biomonitoring of amphibian species is needed in the area. Chapter 2 of this thesis primarily focused on determining the distribution and prevalence of *Bd* in the NNR using two isolation methods with *Amietia delalandii* as the focal species. The NNR forms the northernmost part of the Drakensberg mountain range, which is notorious for high *Bd* prevalence based on the prediction model that Tarrent *et al.* (2013) developed. Surveys conducted during the summer and winter periods yielded positive results for *Bd* at most localities, with summer having a higher overall prevalence. Seasonal variation revealed that *Bd* proliferates during summer periods, although still maintaining a high prevalence in the winter periods. The NNR is exhibiting a pre-enzootic state in *Bd* distribution as some localities are negative for the presence of *Bd*, whereas others sites, such as Steep Glen and Kloof, have a high prevalence. The two sites at Steep Glen are a cause for alarm as water in that locality flows down into the valley. Although the stream velocity is low for most of the year, not fully reaching the main river, the rainy season in the area brings about flash floods resulting in accumulated biomatter and infected tadpoles reaching the main river and lower valley areas. Numerous intermittent streams arise due to flooding; these streams may contain infected tadpoles or adults from upstream. An example of an intermittent site is Leopard Kloof, a marsh with high volumes of amphibian activity. The issue of the site being an intermittent positive site is that they tend to dry up, leading to the migration of infected individuals to another site, like Bloekombos, that may not yet be infected as they are in extremely close proximity to one another. Infected sites such as Leopard Kloof are adjacent to non-infected sites like Bloekombos shows that *Bd* is spreading to other regions. The NNR is also rich in amphibian biodiversity (Parris, 2006; Werner *et al.*, 2007), having 11 identified species of amphibians, with some localities having up to 5 species. Therefore, it should be a conservational priority to monitor the prevalence and distribution of *Bd* within the NNR, as all species are at risk of getting infected by *Bd*.

4.2 Isolation approaches and the implication of contamination

In chapter 2, two isolation approaches were tested to determine isolation success based on how many isolates of *Bd* could be obtained. The first approach was liquid media isolation, in which processed samples are stored in an eppendorf tube with liquid media containing tryptone and antibiotics. The second approach is agar isolation, in which processed samples are embedded into agar containing tryptone and antibiotics (Longcore *et al.*, 1999). Both have their merits, for liquid media, it is cost-effective and requires minimal storage space as opposed to agar isolation, being more costly in terms of agar plates and effective storage. But when considering an isolation approach, it should be the one with the highest isolation success. It was identified that the agar approach had a much higher isolation success as opposed to the liquid media approach, which yielded no isolates. The primary cause of isolation failure was the contamination of tissue samples used for the isolation of *Bd*. Chapter 2 determined not only isolation success but contamination percentage as well. It was decided to document contamination to understand why *Bd* could not be isolated. Contamination of both approaches was primarily from filamentous fungi that were not removed during the field processing. These filamentous fungi grow rapidly *in vitro* and concerning liquid media, engulfs entire samples making it impossible to isolate *Bd*. When comparing the two isolation approaches in relation to the number of contaminated samples, it was found that the liquid media had 84% contamination of all samples as opposed to agar, which had 57%. This shows that agar is the better approach for the isolation of *Bd*.

The possible reason for the massive difference in contamination is that when using the liquid media approach, samples processed in the field are directly placed into eppendorf tubes and remain there until laboratory processing. Therefore, any contaminants that were not successfully removed during the field processing had the opportunity to proliferate in the eppendorf tubes resulting in complete contamination of samples. In contrast, the agar approach involved processing samples and embedding them into the agar. This is beneficial as contamination could be observed and removed while in the field, thus not allowing contaminants to proliferate and spread. Because samples could be actively observed and any contamination could be removed by weeding, the contamination percentage was significantly lower than that of the liquid media approach.

It was noted that although contamination arose from samples, most contamination was found to be growing around the samples. All contaminants are just unwanted microbes like yeasts,

bacteria, and fungi. These microbes all compete with *Bd* for survival in an *in vitro* environment. It was noted that a filamentous fungi was present in one of the wells along with a potential isolate. The fungi was not in close proximity to *Bd* as it was on the opposite end of the well, but as the mass grew larger, *Bd* zoospores started to decrease to a point where no active zoospores were left. This raises the question of how and why *Bd* stopped growing without being overgrown by a competing fungi. We designed the experiments from chapter 3, specifically the competition experiment, to address this question. The competition experiment consisted of 3 filamentous fungi from the family *Trichocomaceae*, referred to as Morph 1,2 and 3. The experiment focused on what effect filamentous fungi competitors have on the growth of *Bd* and, if so, what the impact of that effect was. The experiment was done using a dosage of 100 µl and 20 µl with multiple replicates. It was observed that within 24 hours of introducing Morph sp.1 to the well zoospore activity drastically decreased. The 20 µl group had the best indication of how fast *Bd* can be inhibited by a competing population, even though the dosage was minute compared to the environment *Bd* was in. These filamentous fungi have a great propensity to proliferate within a short period of time while secreting inhibitory substances that inhibit the growth of *Bd* in an *in vitro* setting.

Based on the findings of the competition experiment, it is evident that even the smallest trace of a microbe has the propensity to proliferate in an *in vitro* setting. For this reason, using liquid media as an isolation approach is not recommended as a first choice of an isolation medium. A single microbe that may not have been removed during field processing can inhibit any potential isolate before they have a chance to release zoospores as they proliferate as soon as they are placed into the eppendorf tube. This can lead to false negatives as infected samples did not have the time to produce zoospores due to the inhibition effects of other microbes, such as filamentous fungi secreting inhibitory substances. Therefore, the use of agar is recommended as contamination can be seen and quickly removed through weeding. In contrast, contamination in liquid media can only be observed once samples are transferred from the eppendorf tubes to 24 well plates. In addition, the viscous nature of agar allows for a slower spread of contaminants in contrast to liquid media, which provides an unobstructed way of effectively dispersing inhibitory substances throughout the environment.

Chapter 3 included the Tri-Tube Behavioural Chamber (TBC) the design was inspired by choice tubes for insects that help determine behaviour. The contamination encountered and data from the competition experiment incited the idea that *Bd* could avoid a competitor and, in so doing, make a strategic decision to flee and colonise adjacent areas. The TBC provided *Bd*

with a choice and enabled observation of the behaviour *Bd* incites in the presence of competition. The experiment identified that *Bd* exhibits an avoidance strategy but is still significantly inhibited even though the competitor is not in the same chamber. Therefore, it can be concluded that *Morph* sp.1 from the *Trichocomaceae* family secretes an inhibitory substance that significantly affects *Bd*'s capability to function optimally and reproduce successfully in an *in vitro* environment. This substantiates the claim that liquid media is not recommended for the preservation of field samples for the purpose of isolation as the smallest trace of competition may inhibit potential *Bd* isolates, leading to possible false negatives as *Bd* was never able to proliferate enough to be successfully observed and identified.

4.3 Implications of *Bd* isolation in the context of ecology

Research has been the backbone of conservation; it provides an understanding of the natural processes that makes nature behave the way it does. Therefore, it is imperative to practice good science when it comes to research, and for this to happen, proper standard operating procedures and techniques need to be created and maintained. The impact *Bd* has on the amphibian populations of the world is great, but proper research needs to be conducted to obtain accurate results. The NNR is one of the few protected areas in Kwa-Zulu Natal that have been screened for *Bd*, leaving a huge gap in the distribution of *Bd* in South Africa. Ezemvelo regulates more than 120 protected areas, including the Ncandu Nature Reserve (NNR). Even though there are guidelines on regulating and managing nature reserves, little attention is paid to amphibian conservation within these reserves. Of the 120 known reserves, approximately 9 Nature reserves have been screened of *Bd*.

The prediction model developed by Tarrent *et al.* (2013) is a good starting point, but further research in other localities is required to increase the accuracy of the prediction model. Localities should be prioritised based on amphibian density and diversity, which would allow greater impact of monitoring and expanding the known database for *Bd* prevalence. Areas within the Drakensberg Mountain Range have been extensively and continuously screened, making the need for screening and monitoring of protected areas further south and along the coast to the Western Cape of great importance. When screening a new locality, it is imperative to do it thoroughly using techniques suited to the environment yielding the best possible results. The liquid media approach is viable, but it is not recommended for localities with high sediment and bio-matter as microbes in these areas occur in higher quantities than in localities with minimal sediment and clean flowing water. The importance of accurate isolation of *Bd* is

vital to advance research and knowledge we have on *Bd* regarding possible recombinant lineages and the distribution of these lineages. Therefore, protected areas like the NNR need to be monitored for *Bd* as amphibians provide vital ecological processes within an ecosystem. Without proper isolation practices, false negatives could lead to false distribution data that would otherwise prove beneficial for ecological purposes.

4.4 Conclusion

The content included in this thesis includes the distribution prevalence of *Bd* in the Ncandu Nature Reserve (NNR) and what are the best isolation methods to use for *Bd*. The study also revealed that *Bd* is highly vulnerable and exhibits avoidance behaviour in the presence of other competitors. However, *Bd* is still significantly affected by inhibitory substances secreted by competitors. This pathogen still threatens amphibians, and more research is required in South Africa and elsewhere in Africa regarding the overall distribution of lineages. The NNR is considered an undisturbed environment as there is little anthropogenic influence. Yet, *Bd* was present at most sites regarded as almost pristine. The NNR is still a vibrant ecosystem that is home to 11 identified amphibian species which are at risk of *Bd* infections. Based on the distribution data, the NNR is in a pre-enzootic state as not all areas are infected with *Bd*, but the spread of *Bd* is highly likely. It is important for future studies that *Bd* is isolated through the agar approach to obtain the best possible data when lineage determination is central to the research question. The NNR has a low infection prevalence in some areas, but the sites Steep Glen River and Kloof are a catalyst for further distribution of *Bd* to the lower valley areas. The site Bloekombos is at risk due to *Bd* positive adults being found within close proximity to the water source. Through isolation of *Bd*, it is clear that competing microbes like filamentous fungi have the propensity to completely inhibit the growth of *Bd* within hours. Therefore, it is recommended that the agar approach be utilised to isolate *Bd*, as it provides a way of observing contamination while in the field, leading to a higher probability that *Bd* can be isolated. In conclusion, the NNR is in a pre-enzootic state, and monitoring of *Bd* in the area should be considered a conservational priority.

4.5 Recommendations and Future research

Sample sites should be expanded to further reaches of the NNR, focusing on permanent pools and streams leading into and out of the NNR. Sites such as Steep Glen River and Kloof need to be screened near the water source to determine if *Bd* only occurs at the mouth of the gorge.

The isolation approach using liquid media, although being viable it is not recommended, especially in localities high in bio-matter and sediment, as microbes can severely inhibit the growth of *Bd* in an *in vitro* setting.

Seasonal climates affect the prevalence of *Bd*; therefore, it is recommended that surveys need to be conducted during the summer months after the rainfall season to obtain the best indication of *Bd* prevalence and distribution.

The Tri-Tube Behavioural Chamber provides a way to observe *Bd*'s behaviour in the presence of a competitor. Although the experiment was highly consistent, it is recommended to construct another TBC with the same specifications to run both a control and a competitor replicate simultaneously to get a more accurate result, mitigating possible inconsistencies in terms of zoospore activity. Because even though zoospores density was diluted to the same concentration, colonies may be in different stages of growth. For example, *Bd* colonies from the first experiment may have a concentration of 150 000 zp/ml and are currently in a death phase, whereas the next week the culture may have a concentration of 300 000 zp/ml but are in an exponential phase of growth. Thus one would dilute the 300 000 to 150 000 but this colony is still in an exponential phase and will continue growing but the other colony in the death phase would continue declining resulting in inconsistencies.

Hydrology plays a major role in the ecology of an environment. Further studies on the relationship between hydrological processes and the distribution of infected individuals is needed. The study of these processes may provide significant data for the accuracy of predictive modelling.

An in-depth comparison of the best isolation approach, looking at environmental factors such as quality of water, bio-matter presence, and sediment load and the effect these factors have on isolation success. This would set the standard operating procedure for the best method to be utilised when isolating *Bd* within a microbe rich environment.

Further research should be done using the Tri-Tube Behavioural Chamber as its use in extensive future research could include.

- Rate of colonisation of *Bd*.
- Extensive behavioural experiments using *Bd* as done in this thesis.
- Baiting experiments in which the choice of *Bd* could be actively observed.

The TBC is not limited to *Bd*; therefore, studies using bacteria can also be used in which each well would contain a different concentration of antibiotics to see if bacteria spread to the given well or are repelled by it.

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Appendix A : Survey 1 Prevalence Data

| | | Sample | Bd |
|------------------|------------------|----------|-------------------|
| Protea outlook | Amietia Tadpoles | 115 | Positive |
| | | 116 | Negative |
| | | 117 | Negative |
| | | 118 | Negative |
| | | 119 | Negative |
| | | 120 | Negative |
| | | 121 | Negative |
| | | 122 | Negative |
| | | 123 | Negative |
| | | 124 | Negative |
| | | 125 | Negative |
| | | Marsh | Amietia Toe Clips |
| 133 | Negative | | |
| 134 | Positive | | |
| Amietia Tadpoles | 135 | | Negative |
| | 136 | | Negative |
| | 137 | | Negative |
| | 138 | | Negative |
| | 139 | | Negative |
| | 140 | | Negative |
| | 141 | | Negative |
| | 142 | | Negative |
| | 143 | | Negative |
| | 144 | | Positive |
| | River | | Amietia Tadpoles |
| 146 | | Negative | |
| 147 | | Positive | |
| 148 | | Positive | |
| 149 | | Negative | |
| 150 | | Negative | |
| 151 | | Negative | |
| 152 | | Negative | |
| 153 | | Negative | |
| 154 | | Negative | |
| Eikeenh | Amietia | 155 | Negative |

Eikenhof Dam

| | | | |
|--|-------------------|-----|----------|
| | | 156 | Negative |
| | | 157 | Negative |
| | | 158 | Negative |
| | | 159 | Negative |
| | | 160 | Positive |
| | Xenopus Toe clips | 161 | Negative |
| | | 162 | Positive |
| | | 163 | Positive |
| | | 164 | Negative |
| | | 165 | Positive |
| | | 166 | Negative |
| | Amietia Tadpoles | 167 | Negative |
| | | 168 | Negative |
| | | 169 | Negative |
| | | 170 | Negative |
| | | 171 | Negative |
| | | 172 | Negative |
| | | 173 | Negative |
| | | 174 | Positive |

Steep Glen River

| | | | |
|--|--|--------|-----------|
| | | Sample | <i>Bd</i> |
| | | 175 | Negative |
| | | 176 | Negative |
| | | 177 | Positive |
| | | 178 | Negative |
| | | 179 | Negative |
| | | 180 | Positive |
| | | 181 | Negative |
| | | 182 | Negative |
| | | 183 | Negative |
| | | 184 | Positive |
| | | 185 | Negative |
| | | 186 | Positive |

Bloekombos

| | | | |
|--|-------------------|--------|-----------|
| | | Sample | <i>Bd</i> |
| | Amietia Toe Clips | 187 | Negative |
| | | 188 | Negative |
| | | 189 | Negative |
| | | 190 | Negative |
| | Amietia Tadpoles | 191 | Negative |
| | | 192 | Negative |
| | | 194 | Negative |
| | | 195 | Negative |
| | | 196 | Negative |
| | | 197 | Negative |
| | | 198 | Negative |

Union

Mushroom Rock Outlook

| | | |
|------------------|--------|-----------|
| | 199 | Negative |
| | 200 | Negative |
| | Sample | <i>Bd</i> |
| Amietia Tadpoles | 201 | Negative |
| | 202 | Negative |
| | 203 | Negative |
| | 204 | Positive |
| | 205 | Positive |
| | 206 | Negative |
| | 207 | Positive |
| | 208 | Positive |
| | 209 | Negative |
| | 210 | Negative |
| | Sample | <i>Bd</i> |
| Amietia Tadpoles | 211 | Negative |
| | 212 | Negative |
| | 213 | Negative |
| | 214 | Negative |
| | 215 | Negative |
| | 216 | Negative |
| | 217 | Negative |
| | 218 | Negative |
| | 219 | Negative |
| | 220 | Negative |

Appendix B: Survey 2 Prevalence Data

| Bloekombos | | | |
|------------|-----|-----|-----|
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |

| Dampad | | | |
|--------|-----|-----|-----|
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | POS | NEG | NEG |
| NEG | NEG | NEG | NEG |

| DAMPAD | | Bloekombos | |
|--------|-----|------------|-----|
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |

| Leopard Kloof | | | |
|---------------|-----|-----|-----|
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |

| MUSHROOM ROCK OUTLOOK | | | |
|-----------------------|-----|-----|-----|
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |

| | | | |
|-----|-----|-----|-----|
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |

| LEO KLOOF | | MUSHROOM ROCK | |
|-----------|-----|---------------|-----------|
| NEG | NEG | NEG | NEG |
| NEG | NEG | POS (Toe) | POS (Toe) |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |

| STEEP GLEN RIVER | | | |
|------------------|-----|-----|---------|
| NEG | POS | NEG | NEG |
| NEG | NEG | NEG | NEG |
| POS | NEG | NEG | NEG |
| NEG | NEG | NEG | POS |
| NEG | NEG | NEG | POS/CON |
| NEG | NEG | NEG | NEG |

| STEEP GLEN KLOOF | | | |
|------------------|-----|-----|-----|
| POS | NEG | NEG | NEG |
| NEG | NEG | NEG | NEG |
| POS | NEG | NEG | POS |
| NEG | NEG | NEG | NEG |
| NEG | POS | NEG | NEG |
| NEG | NEG | NEG | NEG |

| STEEP GLEN RIVER | | STEEP GLEN KLOOF | |
|------------------|------|------------------|------|
| NEG | POS | POS | POS |
| NEG | NEG | NEG | POS |
| NEG | POS | CONT | CONT |
| CONT | POS | CONT | CONT |
| CONT | CONT | CONT | CONT |
| CONT | CONT | POS/CON | CONT |

Appendix C: Summarised Raw data used for Inhibition experiments

| | Day 1 | | Day 2 | | Day 3 | | Day 4 Cont. Intro | | Day 5 | | Day 6 | | Day 7 | | Day 8 | | Day 9 | | Day 10 | |
|-------------|----------|----------|----------|----------|----------|----------|-------------------|---------|-------|---------|-------|---------|----------|----------|----------|----------|----------|----------|----------|----------|
| | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV |
| Morph sp. 1 | 22,666 | 1,425947 | 22,666 | 1,425947 | 41,88889 | 2,147106 | 43,17 | 2,63 | 43,89 | 1,69 | 3,06 | 1,05 | 2,333333 | 0,686055 | 0,277778 | 0,31427 | 0 | 0 | 0 | 0 |
| Morph sp. 2 | 23,2 | 1,76077 | 26,6 | 1,487673 | 38,83333 | 1,69754 | 43,56 | 2,05 | 37,56 | 1,58 | 11,33 | 1,49 | 6,666667 | 1,299726 | 3,055556 | 0,801085 | 1,611111 | 0,501087 | 0 | 0 |
| Morph sp. 3 | 18,2 | 1,100193 | 27 | 1,399747 | 36,66667 | 1,352277 | 35,61 | 1,89 | 20,56 | 1,38 | 11,56 | 1,61 | 6,611111 | 1,04613 | 2,944444 | 0,565383 | 0 | 0 | 0 | 0 |
| CONTROL | 21,83333 | 1,58859 | 26,83333 | 1,260595 | 37,91667 | 1,642003 | 43,83 | 1,88 | 44 | 1,57 | 41,5 | 2,11 | 44,5 | 2,085621 | 53,5 | 1,456776 | 43,83333 | 1,289663 | 42,91667 | 1,014174 |

| | Day 1 | | Day 2 | | Day 3 | | Day 4 | | Day 5 | | Day 6 | | Day 7 | | Day 8 | | Day 9 | | Day 10 | |
|------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|---------|
| | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV |
| Morph sp.1 | 22,73267 | 1,349176 | 22,79933 | 1,279774 | 37,22222 | 1,622958 | 44 | 1,442589 | 42,72222 | 1,364021 | 3,777778 | 0,937879 | 3,944444 | 0,686055 | 0,722222 | 0,665003 | 0 | 0 | 0 | 0 |
| Morph sp.2 | 23,46667 | 1,442307 | 26,06667 | 1,548127 | 37,83333 | 2,323339 | 43,83333 | 1,25691 | 38,72222 | 1,320112 | 10,38889 | 1,465955 | 9,277778 | 1,103645 | 3,055556 | 0,801085 | 0,388889 | 0,549972 | 0 | 0 |
| Morph sp.3 | 21,33333 | 0,94124 | 26,4 | 1,564828 | 35,5 | 1,236392 | 43,27778 | 1,272357 | 21,22222 | 1,139432 | 10,5 | 1,386924 | 9,555556 | 0,95144 | 3,333333 | 0,808577 | 0 | 0 | 0 | 0 |
| CONTROL | 22 | 1,473446 | 26,16667 | 1,058698 | 36,75 | 1,28423 | 43,94444 | 1,250841 | 43,92 | 1,52 | 40,22222 | 1,463686 | 41,94444 | 1,05291 | 48,5 | 1,50828 | 40,5 | 1,251991 | 38,38889 | 1,05969 |

| | Day 1 | | Day 2 | | Day 3 | | Day 4 | | Day 5 | | Day 6 | | Day 7 | | Day 8 | | Day 9 | | Day 10 | |
|-----------|----------|----------|----------|----------|----------|----------|----------|----------|-------|---------|-------|---------|----------|----------|----------|----------|-------|----------|--------|----------|
| | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV |
| Morph sp. | 22,59933 | 1,356929 | 23,39933 | 1,256678 | 36,46667 | 1,099339 | 41,13333 | 1,186893 | 19,42 | 1,25 | 4,25 | 1,16 | 1,416667 | 0,751631 | 0 | 0 | 0 | 0 | 0 | 0 |
| Morph sp. | 23,66667 | 1,558817 | 25,6 | 0,884148 | 38,2 | 1,114683 | 41,46667 | 1,595846 | 6,87 | 1,43 | 2,67 | 0,92 | 0,533333 | 0,446142 | 0,533333 | 0,446142 | 0 | 0 | 0 | 0 |
| Morph sp. | 20,8 | 1,257481 | 26,4 | 0,833623 | 36,73333 | 1,005912 | 41,73333 | 1,066753 | 8,2 | 1,32 | 2,33 | 0,7 | 0,333333 | 0,471405 | 0 | 0 | 0 | 0 | 0 | 0 |
| CONTROL | 21,58333 | 1,252652 | 25,91667 | 1,365766 | 36,91667 | 1,404806 | 43,33 | 1,19 | 46,67 | 1,65 | 43,42 | 1,65 | 39,83333 | 1,349096 | 40,58333 | 1,147481 | 38,25 | 0,816497 | 36 | 1,247219 |

| | Day 1 | | Day 2 | | Day 3 | | Day 4 | | Day 5 | | Day 6 | | Day 7 | | Day 8 | | Day 9 | | Day 10 | |
|-----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|---------|----------|---------|
| | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV | MEAN | ST. DEV |
| Morph sp. | 22,99933 | 1,166187 | 23,066 | 1,101515 | 40,83333 | 2,17208 | 44,16667 | 1,162932 | 21,08333 | 4,476267 | 3,666667 | 0,787525 | 2,388889 | 0,371785 | 0 | 0 | 0 | 0 | 0 | 0 |
| Morph sp. | 24,13333 | 1,186893 | 26,46667 | 0,917547 | 38,22222 | 1,587302 | 43,77778 | 0,930388 | 21,94444 | 1,302538 | 2,722222 | 1,01691 | 1,277778 | 0,392837 | 0,888889 | 0,450352 | 0 | 0 | 0 | 0 |
| Morph sp. | 23,13333 | 1,104539 | 25,93333 | 1,436565 | 37,22222 | 1,200106 | 43,44444 | 1,102934 | 20,55556 | 1,184349 | 2 | 0,780033 | 1 | 0,722518 | 0 | 0 | 0 | 0 | 0 | 0 |
| CONTROL | 22,41667 | 1,562229 | 26,08333 | 1,144971 | 37,33333 | 1,42716 | 44,33333 | 1,386924 | 42,88889 | 1,311515 | 40,72222 | 1,275515 | 40,16667 | 1,449116 | 39,66667 | 1,185834 | 34,94444 | 1,32625 | 38,83333 | 1,04613 |