Analysing the methodology for the use of eDNA in detecting *Batrachochytrium dendrobatidis*

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Abstract

Key words: *Batrachochytrium dendrobatidis* (*Bd*), *Amietia* spp., environmental DNA (eDNA), filtration, DNA extraction, TaqMan Probe qPCR.

Batrachochytrium dendrobatidis (*Bd*) is an aquatic fungal pathogen that has caused mass mortalities in amphibian populations globally. Conventional diagnostic assays for the pathogen are invasive, and involve the collection of toe clips, ventral swabs or mouth parts from tadpoles. Environmental DNA (eDNA) offers a non-invasive and time-efficient alternative to diagnosing the pathogen in aquatic environments. This project aimed to develop a Standard Operating Procedure (SOP) for the detection of *Bd* using eDNA. The protocol was developed under laboratory conditions, and a preliminary method was applied in field application concurrently with laboratory experiments to aid in the further optimisation of the protocol.

Four DNA extraction methods were compared in a variety of matrix types, namely sterile, non-sterile and heat-treated. Two crude extraction methods, namely the Heat Lysis and CTAB method, and two commercial DNA extraction kit methods, namely the DNeasy PowerSoil Kit and Zymo Research Bacterial and Fungal Kit, were selected for comparison using the matrix types. One of each method type was selected for the subsequent filtering test phase. The DNeasy PowerSoil Kit and Zymo Research Bacterial and Fungal Kit delivered very similar results, but the DNeasy PowerSoil Kit was selected as the method of choice due to its better quality DNA and greater consistency in terms of Ct-values overall. The crude extraction methods also delivered similar results, but the Heat Lysis method was selected due to its more consistent results with the lowest standard deviations, while being the more cost-effective, as well as less time consuming method. Overall, the kit methods delivered better Ct-values in the non-sterile matrix, but weaker Ct-values in the sterile matrix compared to the crude methods.

In the filtering phase, three filteration methods, namely the drill filter, syphon pump, and Continuous Low-level Aquatic Monitoring (C.L.A.M) were tested in the field for their practicality in application and possible filter volume limitations. The syphon pump was chosen due to not requiring any electricity, being capable of sampling in remote regions due to its compact size and light weight, as well as performing similarly in terms of filtering capacity when compared to the other methods. Two filter material preservation mediums, 70% ethanol and Lysis Buffer, were tested over three time periods for the two selected DNA extraction methods. The Dneasy PowerSoil Kit coupled with Lysis Buffer preservation medium performed significantly better compared to the other method and preservation medium combinations. Standards for the qPCR assay were set using a G-Block dilution series as well as a filtered zoospore dilution series to determine the efficiency, lowest limit of detection (LOD) and lowest limit of

quantification (LOQ) of the *Bd* primers. The primers delivered an acceptable efficiency of 93.5%, with a LOD of 100 copies/µl and a LOQ of 1000 copies/µl in the G-block series.

A preliminary eDNA assay was applied concurrently with lab experiments to aid in optimising the protocol. This not only gave insight to the limitations of the developed assay, but also provided guidance for future field sampling. A fellow MSc student, Mr. Jacques Potgieter, from the Herpetological Health Lab (HHL) was conducting a study at the Ncandu Nature Reserve, uMsonti Nature Reserve, and Ncandu Private Forest and Grassland Reserve on the distribution of *Bd* using conventional diagnostic measures. To reduce the impact and stress on the amphibian population, the projects were planned in parallel. This collaboration allowed for a direct comparison between the preliminary eDNA assay and conventional diagnostic methods. To test the validity of the assay, an internal control assay, using *Amietia* spp. primers, was run alongside the extracted eDNA samples. This primer set was selected due to the high numbers of *Amietia delelandii* (Delalandi's river frog) present at the sampling sites. The LOD and LOQ of the primers as well as the specificity was tested prior to field sampling. The LOD of the *Amietia* spp. was determined to be 10 copies/µl, the LOQ 100 copies/µl, and the assay only amplified *Amietia* spp. DNA based during the specificity test.

When the preliminary assay was applied in the field, only 70% ethanol had been tested for both the selected extraction methods under laboratory conditions. The DNeasy PowerSoil kit demonstrated a high level of inhibition in downstream application when applying the ethanol and was deemed insufficient for field application. Thus, the Heat-Lysis method, coupled with 70% ethanol was selected as the preliminary method. Three of the four sites tested positive for *Bd* using conventional diagnostic measures, but none for *Bd* eDNA. However, all four of the sites tested positive for the *Amietia* spp. eDNA. Thus, indicating that the protocol is sufficient for eDNA application, but the *Bd* DNA concentrations present in the environment may have been below the detection threshold. After field application of the preliminary method, the Lysis Buffer was tested as a preservation medium and the DNeasy PowerSoil Kit – Lysis Buffer combination delivered significantly better Ct-values compared to the Heat Lysis – Ethanol combination. The sensitivity was significantly increased and the LOD of the final eDNA protocol was determined to be 10 zoospores according to the zoospore filter dilution series.

Based on the *Amietia* spp. results from the field application, much could be gathered from the data regarding which environments present the best conditions for sampling eDNA. It was determined that lentic water bodies with a high level of turbidity and lower water columns present more positive samples and lower Ct-values. It would be recommended to sample eDNA during spring and early summer when flow rates are still low prior to summer rainfall and during the breeding season of the amphibian hosts.

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Bd = *Batrachochytrium dendrobatidis*

Bs = *Batrachochytrium salamandrivorans*

CV% = Coefficient of variation percentage

Ct-value = Cycle threshold value

ddH_2O = Double distilled water

eDNA = Environmental DNA

EID = Emerging Infectious Disease

HHL = Herpetological Health Lab

IPC = Internal Positive Control

LOD = Lowest Limit of Detection

LOQ = Lowest Level of Quantification

NTC = Non-template control

O.I.E. = World Organisation for Animal Health

PCR = Polymerase Chain Reaction

qPCR = Quantitative Polymerase Chain Reaction

SD = Standard Deviation

SOP = Standard Operating Procedure

Chapter 1: Introduction

1.1 Background

Amphibians are considered the most threatened vertebrate class with approximately 40% of all species experiencing declines (Catenazzi, 2015; Cheng *et al*., 2011; Miaud *et al*., 2019). These declines play a significant role in the current global biodiversity crisis which can be described as the sixth mass extinction event in history (Catenazzi, 2015; Collins, 2010). Although mostly due to anthropogenic activities, emerging infectious diseases (EID) such as chytridiomycosis and *Ranavirus* have greatly contributed to this phenomenon in amphibian populations (Cheng *et al*., 2011; Collins, 2010; Miaud *et al*., 2019, West, 2018). Currently there are only three noteworthy amphibian pathogens listed by the World Organisation for Animal Health (O.I.E.) namely, the *Ranavirus*, *Batrachochytrium salamandrivorans* (*Bs*), and *Batrachochytrium dendrobatidis* (*Bd*) (O.I.E., 2020). Understanding the distribution and habitat occupancy of pathogens plays a key role in conservation efforts targeting amphibians (Bancroft *et al*., 2011). Amphibians are known to deliver numerous ecosystems services, provide benefits to society and contribute to ecosystem stability (Hocking and Babbitt, 2014; Hussain and Pandit, 2012; Valencia-Aguilar *et al*., 2013; West, 2018). Thus, the methods for the monitoring and management of these pathogens are extremely important for the conservation of aquatic ecosystems.

Batrachochytrium dendrobatidis is an aquatic fungal pathogen with a cosmopolitan distribution and is often described as one of the leading causes of amphibian declines over the last few decades (Berger *et al.*, 1999; Boyle *et al*., 2004). The early detection of this pathogen is crucial to the management and limitation of zoospore dispersion to other hosts and environments (Bancroft *et al*., 2011; Ghosh *et al*., 2021). Conventional diagnostic measures vary in the degree of invasiveness and may result in the death or cause stress to the sampled specimens (Hyatt *et al*., 2007). The pathogen was conventionally diagnosed directly from the amphibian hosts through toe clipping, the collection of tadpole mouthparts, as well as abrasive ventral swabs (Boyle *et al*., 2004). It should be noted that although skin swabs are considered less invasive compared to other diagnostic measures (Annis *et al*., 2004), this method still requires interaction with the host specimen that may result in stress. As science has progressed, new non-invasive sampling techniques, such as environmental DNA (eDNA), have been developed for numerous species detections (Ficetola *et al.*, 2008; Harper *et al.*, 2019; Huver *et al*., 2015). These techniques can serve as a potential alternative to current diagnostic measures and be further analysed in the context of the target pathogen.

All living organisms release DNA into the environment, which can be derived from skin cells, bodily waste, scales, hair, or other metabolic excretions (Harper *et al*., 2019). These DNA

fragments can be acquired from abiotic environments where the species have previously resided, currently reside, or possibly have migrated, using techniques such as filtration or precipitation for the case of aquatic environments (Harper *et al.,* 2019; Rees *et al*., 2014; Schmidt *et al*., 2013). The data derived from eDNA analysis can then be applied for a wide range of studies, such as palaeontology, pathology, dietary studies, and species distributions (Ficetola *et al.*, 2008; Harper *et al*., 2019; Huver *et al*., 2015). This detection method has been previously utilised to determine the presence of pathogens within aquatic environments (Huver *et al*., 2015), however has not yet been tested for the detection of *Bd* in South Africa. The application of eDNA studies has proven to be accurate, rapid, cost-effective, and does not require any direct interaction with the target species (Huver *et al*., 2015).

Research on eDNA is still relatively new in South Africa with no optimised protocol in place for many aquatic pathogens. Previous eDNA studies conducted in South Africa have reviewed topics such as the presence detection of invasive or threatened species, determining species diversity in various ecosystems, and methodology based studies (Cowan *et al.,* 2021; Crooks *et al*, 2020; Holman *et al*., 2019; Mynhardt and Theron, 2021; Laykay *et al*., 2007; Tekere *et al*., 2011). This study forms part of a larger project with the goal to develop robust protocols for aquatic pathogen detection using eDNA. The aim of this study was to develop a standard operating procedure (SOP) for the detection of *Bd* in aquatic environments. This was achieved through evaluating multiple DNA extraction methods using cultures prepared in a variety of matrix types, analysing and optimising filtering protocols, testing preservation mediums for filter media, and analysing the samples using TaqMan probe qPCR. This project strived to develop a sensitive and accurate protocol under laboratory conditions that can later be tested in field application. A preliminary method was also tested in the field during the course of this project and a new improved protocol was developed thereafter. A follow-up study has already been prepared for the future application of the newly developed eDNA protocol which will be evaluated against conventional diagnostic measures in multiple field locations in the country.

This chapter will provide an extensive literature review on the conservation of amphibians, the target organism, eDNA and the different molecular diagnostics methods that have previously been applied for *Bd* as well as eDNA. Additional information will be provided on eDNA extraction methods and filtering techniques in the subsequent chapters.

1.2. Literature review

1.2.1. Conservation of amphibians

As previously mentioned, amphibians are the most threatened vertebrate class and are in dire need of conservation efforts. There are three orders of amphibians namely Urodela, Anura,

and Gymnophiona (du Preez and Carruthers, 2017). Over 7140 species form part of these orders, and Anurans, represented by frogs and toads, demonstrate the largest distribution (West, 2018). Amphibians occur over a wide variety of habitats ranging from aquatic to terrestrial and rainforest to deserts, which has resulted in an extensive diversification in lifestyle, breeding, parental and feeding habits (Hussain and Pandit, 2012). Anurans are the only order present in South Africa, however an exceptional biodiversity of 169 species occur here (Angulo *et al*., 2011; du Preez and Carruthers, 2017). Of these species, 24 have been reported as threatened according to the Red Data List (du Preez and Carruthers, 2017).

1.2.1.1. Why conserve amphibians?

Amphibians are often overlooked when it comes to conservation because they do not present many direct benefits to society. However, they still fulfil an important role within the environment and present many indirect benefits to the stability of ecosystems, society, and the economy. An extreme diversity can be observed in the amphibian class, which results in an occupation of a wide assortment of habitats and ecological niches (Hussain and Pandit, 2012; Wells, 2007). Due to the different dietary lifestyles of juveniles and adults, they consume a more extensive diet compared to other organisms (Hussain and Pandit, 2012). Resources are also less likely to be depleted due to the dietary changes during their life cycle and intraspecific competition being reduced amongst the age groups (Schriever and Williams, 2013). This allows for more individuals to inhabit the same environment without increasing their impact on the ecosystem (West, 2018). The tadpoles of anurans are herbivores, omnivores, or planktivores, while the adults tend to be insectivores as well as micro-carnivores (Hussain and Pandit, 2012; Wells, 2007). They also serve as a food source to many other predators such as reptiles and birds (Schriever and Williams, 2013; West, 2018). Thus, they serve a crucial role in ecosystem stability through their role in the food web and contribute significantly to the biodiversity present within an area.

Amphibians provide a wide array of ecosystem services and play a significant role in pest management (Hocking and Babbitt, 2014; Tyler *et al*., 2007). Some amphibians can serve as bio-controls for pests such as mosquitos, phytophagous insects, biting flies as well as many other species that serve as vectors to pathogens (Du Rant and Hopkins, 2008; Valencia-Aguilar *et al*., 2013; West, 2018). The larvae of these vector species form part of the tadpoles' diet and adult anurans also prey on the matured insects (Du Rant and Hopkins, 2008; Valencia-Aguilar *et al*., 2013). Mosquitos are known to contribute to the spread of diseases in wildlife as well as in humans (Du Rant and Hopkins, 2008). Although salamanders have a larger impact on mosquito larvae densities, some anuran species still contribute to these

trends (Rubbo *et al*., 2011). For example, a study conducted in Argentina showed tadpoles to decrease mosquito larvae populations in aquatic ecosystems when introduced as a bio-control agent (Valencia-Aguilar *et al*., 2013). Amphibians have also been considered as a possible bio-control for mosquitos in Kenya following many outbreaks from mosquito-borne diseases (Karungu *et al*., 2019). Thus, although it hasn't been tested in a South African context, amphibians may still potentially contribute as a biocontrol to diseases such as malaria.

Amphibians live biphasic life cycles and have permeable skins that are sensitive to changes within the environment (Miaud *et al*., 2019). For this reason, they are often used as environmental indicators for ecosystem health (Hussain and Pandit, 2012; Miaud *et al*., 2019). Not only are amphibians important for environmental health but have contributed to society and the economy through pharmaceuticals, cultural applications, advancements in biological and medical research, as well as the food trade (Valencia-Aguilar *et al*., 2013; West, 2018). *Xenopus laevis*, a South African frog species commonly known as the clawed frog, has historically been exported for medical research (Dazsak *et al.*, 2003; Weldon *et al*., 2007). This species has been used for pregnancy tests in the 1900s, heterologous protein synthesis using oocytes, neural network development, as well as physiological studies of kidneys and muscles (Dazsak *et al.*, 2003; Straka and Simmers, 2012; Tyler *et al*., 2007; Wagner *et al*., 2000).

1.2.1.2. Causes of amphibian declines

Many factors have contributed to the declines of amphibians; however, the majority are often anthropogenic. Habitat degradation/fragmentation, climate change, pollution, overexploitation in both the pet and food trade, and invasive species are just a few causes for these declines (Blaustein *et al*., 2011; Cheng, *et al*., 2011; Collins, 2010; West, 2018). Habitat degradation and fragmentation have shown to have some of the most severe impacts, especially for threatened species (Blaustein *et al*., 2011; West, 2018). Although these two factors are prominent causes of amphibian declines, EID's such as chytridiomycosis and Ranavirus, also significantly contribute to biodiversity losses (Dazsak *et al.*, 2003; Miaud *et al*., 2019). The amphibian trade has also threatened local fauna in some regions through the introduction of invasive species infected with a pathogen (Blaustein *et al*., 2011). These diseases have been observed in five forms of the amphibian trade namely the pet trade, food trade, zoo trade, laboratory experiments, and bio-controls (Dazsak *et al.*, 2003). Amphibians in the pet trade are sometimes released into the wild when they are unable to be cared for and these individuals may carry diseases and pathogens unique to the new environment (Dazsak *et al.*, 2003). Similarly, they are also often exported for laboratory experiments and may be released

into local environments (Weldon and Fisher, 2011). Climate change also has a tremendous impact on the development/growth of pathogens as well as the levels of transmission and can cause changes in the susceptibility of hosts (Stevenson *et al*., 2013).

1.2.2. *Batrachochytrium dendrobatidis* (*Bd*)

Chytridiomycosis has served as the driving force of multiple amphibian declines and extinctions over the last few decades (Mutnale *et al*. 2018; Scheele *et al*., 2019). There are two known species that cause this disease, namely *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bs*) (Mutnale *et al*. 2018). Although *Bs* is known to mostly infect salamanders and newts, it has previously also been detected on some anuran species (Stegen *et al*., 2017), whereas *Bd* is known to be more common in anurans than salamanders (Cheng *et al*., 2011).

Chytridiomycosis has shown to cause the genetic bottleneck effect within amphibian populations which ultimately may result in the collapse and extinction of these communities (Catenazzi, 2015). Declines due to chytridiomycosis have also been reported in remote and protected areas, where environments are still pristine (Berger *et al*., 1999; Cheng *et al*., 2011; Reeder *et al.*, 2012). This pathogen is speculated to be the cause in declines of over 501 amphibian species, which include a possible 90 extinctions (Scheele *et al*., 2019). Other studies even predicted higher numbers, estimating that approximately 200 species are severely affected and more than 700 other species globally are mildly affected by its presence (Mutnale *et al*.; 2018).

Understanding the target organism is crucial to developing a diagnostic assay. This section of the study will provide an in-depth review on the morphology, physiology, pathology, epidemiology, origin and lineages of this organism. This will aid in better understanding the nature of this pathogen, the importance of management and which factors contribute to the spread of the disease.

1.2.2.1. Classification, morphology, and physiology

Batrachochytrium dendrobatidis is a fungal pathogen that forms part of the phylum Chytridiomycota, commonly known as chytrids, and are in the order Rhizophydiales (Hyman and Collins, 2012; Gleason *et al*., 2008). Members of the Chytridiomycota phylum occur over a wide variety of habitats but share similar anatomical characteristics. They are known to have zoospores that are un-walled, possess flagellates, and rely on aqueous environments for survival as well as dispersal (Walker *et al*., 2007). The zoospores of *Bd* embed themselves in

the keratinised epidermal cells of adult amphibians or the mouthparts of larvae (Walker *et al*., 2007). The sporangium is characterised by thread-like rhizoids, and they occur both mono-centrically as well as colonially in cultures (Longcore *et al.*, 1999) (Figure 1.1).

The habitats of members in the different orders within Chytridiomycota tend to vary remarkably based on environmental factors within aquatic environments (Gleason *et al*., 2008). For example, species of the Blastocladiales and Spizellomycerales orders are sampled more frequently from substrates that can vary significantly in terms of temperature and salinity (Gleason *et al*., 2008). Whereas members of the order Rhizophhydiales, such as *Bd,* are sampled more often from waterbodies where the water conditions tend to be more stable and constant (Gleason *et al*., 2008). This plays a key role in selecting the appropriate sampling mediums for eDNA. More information will be provided on the specific environmental factors that contribute to the presence and prevalence of *Bd* in section 1.3.2.

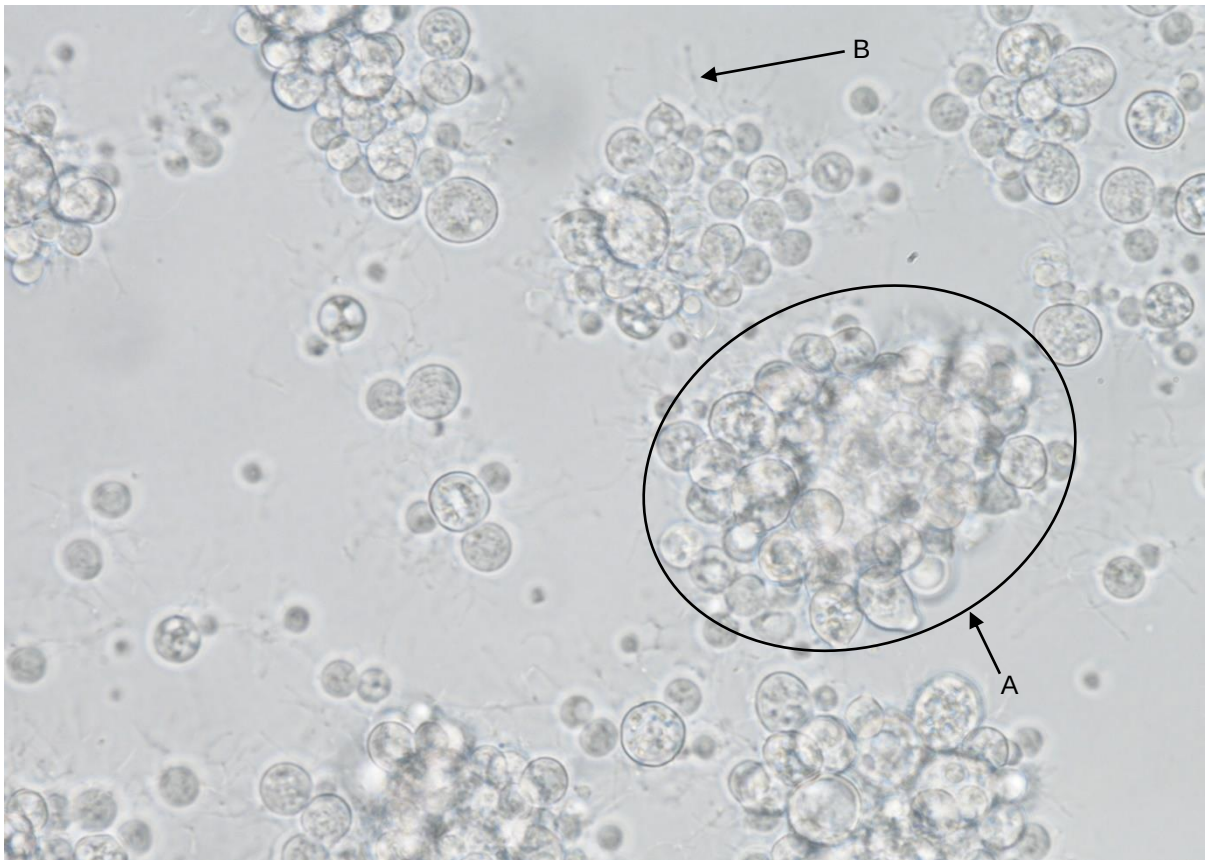

Figure 1.1. Example of *Bd* in liquid culture demonstrating, (A) sporangia mostly in colonial groupings, (B) with rhizoids, thread-like filament structures, anchoring the sporangia.

1.2.2.2. Pathology, symptoms, and epidemiology

Batrachochytrium dendrobatidis is the only species in its phylum known to parasitise on vertebrates (Walker *et al*., 2007). Zoospores encyst themselves in the keratinised epidermal layer on the skin of amphibians and form sporangia (Walker *et al*., 2007; Voyles *et al.*, 2009). This layer is crucial to the homeostasis of the organism due to its significant role in electrolyte, water, as well as respiratory gas exchange (Voyles *et al.*, 2009). Reproduction occurs in the epidermal layer through replication and as the cells grow, they move to the surface of the host's skin (Ramsey *et al*., 2010). In severe cases, this disease causes a disruption in ion retention and osmoregulation, and death results due to the build-up of toxins and cardiac arrest (Ramsey *et al*., 2010; Voyles *et al.*, 2009).

Although this pathogen infects both adults and adolescent amphibians, it does not cause mortalities in tadpoles but mainly affects metamorphic stages and adults (Berger *et al*., 1999). In severe cases of infection some individuals may shed their skin, experience loss in appetite, hyperkeratosis, discoloration or reddening of the skin, and their righting reflex may also be disturbed (Annis *et al*., 2004; Gabor *et al*., 2015; Miller *et al*., 2018; Van Rooij *et al.*, 2015) (Figure 1.2). These clinical symptoms are occasionally used to diagnose the disease (Annis *et al*., 2004).

Figure 2.2. Example of discoloured skin on *Bd* infected *Amietia fuscigula* specimen (Photo credit: Prof. Ché Weldon)

Hormone levels, such as corticosterone, appear to have an effect on some of the clinical symptoms exhibited during infection (Gabor *et al*., 2015). A direct correlation was detected between the level of infection and corticosterone levels which affects the righting reflex of amphibians (Gabor *et al*., 2015). These hormones can also suppress the immune response of amphibians which could make them more vulnerable to the pathogen (Gabor *et al*., 2015). Some amphibian species have more antimicrobial peptides present in the mucus of their skins that make them more resistant (Ramsey *et al*., 2010). This may reduce the impact of the disease by limiting the initial infection as well as limits the growth of current infections (Márquez *et al.*, 2010; Ramsey *et al*., 2010).

The level of resistance and tolerance against the pathogen varies between amphibian species. Some species, such as *Xenopus laevis* (clawed frog) from South Africa or *Rana catesbeiana* from North America, are much more tolerant to *Bd* and often do not exhibit clinical signs despite having high levels of infection (Bancroft *et al.*, 2011; Schloegel *et al*., 2012). Species with a high tolerance level can still serve as possible vectors to the disease, thus, spreading it to more vulnerable species (Schloegel *et al*., 2012). Environmental conditions, behaviour, and physiological characteristics of the amphibians are all contributing factors to the infection intensity in the host (Searle *et al*., 2011).

Mating behaviours such as amplexus, where the male clasps onto the back of the female, have shown a higher potential in the spread of disease (Bancroft *et al.*, 2011). This is due to the longer contact time required between individuals for successful reproduction. Thus, higher levels of infections can also be expected during breeding seasons for these individuals. Species that achieve sexual maturity quickly in their life cycles have also shown an increased risk of infection (Greenberg *et al*., 2017). The clutch size of the eggs can contribute to the species susceptibility. Species with lower clutch sizes might be more prone to population declines, however, individuals with larger clutch sizes are more likely to be infected (Greenberg *et al*., 2017). This is due to the behaviours often associated with larger clutch sizes that increase contact time and level of exposure between individuals (Bancroft *et al.*, 2011). Whereas species with smaller clutch sizes are more affected by declines due to the lower number of individuals produced during reproduction and losses from diseases having a more significant impact on the population size (Bancroft *et al.*, 2011).

Infection levels do not only vary between species but also varies in the different life stages of a single species (Searle *et al*., 2011). The age and size of the individuals showed to influence the risk and level of infection, larger and older individuals demonstrated higher levels of infection due to the increased body surface and the longer exposure due to age (Smith *et al*., 2007; Searle *et al*., 2011).

1.2.2.3. Origin and distribution

Amphibian populations have been experiencing declines since the early 1960s, however this phenomenon had only been recognised at the first World Congress of Herpetology in 1989 (Houlahan *et al*., 2000). *Batrachochytrium dendrobatidis* was first described as a new species in 1998 after multiple juvenile frogs from three species succumbed to the disease in the National Zoological Park, Washington (Longcore *et al.*, 1999). Declines in amphibian populations had also been observed due to the pathogen, since 1989 in Australia (Berger *et al*, 1999). Initially the cause for the population decline in Australia were attributed to habitat degradation, however, for the rapid loss of stream-dwelling amphibians in the high-altitude protected forest areas this did not seem likely (Berger *et al*., 1999). Multiple theories exist for why this pathogen achieved a cosmopolitan distribution, and five regions have been identified as the possible origin of spread, namely Africa, South America, East Asia, Japan, and North America (Weldon *et al.*, 2004; O'Hanlon *et al*., 2018).

The methods of distribution contribute to this project through providing insights to how this pathogen may possibly be dispersed to new geographic regions in the future and the different lineages that currently exist. The evolutionary history of this pathogen is extremely important, because individuals have shown to adapt to various environments across the globe which contribute to their virulence and ability to survive wider tolerance ranges (Stevenson *et al*., 2013). The spread of these more virulent strains can then have devastating impacts on the new environments (Stevenson *et al*., 2013). Understanding the origin of the pathogen allows for better conservation of amphibians as well as determining the possible dispersion and future threats (Doherty-Bone *et al*., 2020). Although the origin was thought to be Africa, new findings indicate a different source, namely Asia (Doherty-Bone *et al*., 2020). Over the last few years, multiple declines have been reported in various amphibian communities across Africa (Doherty-Bone *et al*., 2020). Three origin theories will be discussed in this section, because they each provide valuable insights to the spread of the pathogen globally. The Asia hypothesis is however, the most recent formally accepted origin.

The first attempt to describe the origin was the "Chytrid out of Africa" hypothesis. Originally it was strongly believed that *Bd* originated in Africa through the amphibian trade for *Xenopus laevis* for laboratory experiments and pregnancy tests in the early twentieth century (Dazsak *et al.*, 2003; Farrer *et al*., 2011; Picker, 2013; Soto-Azat *et al*., 2010; Weldon *et al*., 2004). Some of these frogs may have been released or escaped into the wild after the completion of experiments or when companies closed (Lillo *et al*., 2011; Measey and Fouquet, 2006; Picker, 2013). An example of this occurred in France when *X. laevis* was released into the wild after a breeding facility had been closed in the 1980s (Measey and Fouquet, 2006). Another example from the amphibian trade was the transport of *Xenopus gilli* from the Western Cape

to a breeding facility in Spain in 1991 (Walker *et al*., 2008). In 1997, the first case of *Bd* was reported in wild amphibian populations within Europe and it was later discovered that infected *X. gilli* individuals shared an animal room with *Alytes muletensis* that formed part of a re-introduction program for the island of Mallorca (Bosch *et al*., 2001; Walker *et al*., 2008). Multiple lineages of the pathogen have also been recorded in South Africa that suggest that a wide genetic diversity is present (Doherty-Bone *et al*., 2020). It was predicted that infections in South Africa may have been present as early as the 1930s (Soto-Azat *et al*. 2010; Weldon *et al.*, 2004).

The second origin hypothesis postulates that the origin was North America. The North American bullfrog, *Rana catesbeiana,* is a known vector for the spread of *Bd* through the global amphibian food trade (Garner *et al.*, 2006; Schloegel *et al*., 2010). This species is a popular cuisine in countries such as Brazil, where multiple farms have been established for commercial reproduction since the 1930s (Schloegel *et al*., 2010). The North American bullfrog was originally imported from Canada to Brazil and was then exported from Brazil to other continents, such as Europe and Asia (Schloegel *et al*., 2010). Individuals occasionally escaped commercial farms and spread the disease to local amphibian populations (Garner *et al*., 2006).

The third and most recent formally accepted origin hypothesis of *Bd* is Asia. Multiple endemic strains of *Bd* had been discovered in Asian frog populations (Mutnale *et al*., 2018). The high diversity present in the area is believed to be caused by the co-evolution between the pathogen and their amphibian hosts over a long period of time (Mutnale *et al*., 2018). A total of 57 haplotypes were documented for the ITS region of the genome (Mutnale *et al*., 2018). Of these 57 haplotypes a total of 46 are not present in the global database, making them unique to Asia (Mutnale *et al*. 2018). After an extensive genomic analysis of *Bd* specimens, the origin theory changed to Asia (Doherty-Bone *et al*., 2020). It was estimated that *Bd* originated in the Korean peninsula, due to the lineage *Bd*ASIA-1 demonstrating genetic characteristics of a possible ancestral population (O'Hanlon *et al*., 2018). East Asia has shown to host a wide diversity of *Bd,* and multiple other lineages previously identified in other countries share many similar characteristics to those found in Asia (O'Hanlon *et al*., 2018).

Understanding how *Bd* can spread is crucial to conservation efforts and through analysing the history of this pathogen we can better understand the risk of transmission. Amphibians such as *X. laevis*, which is a common anuran species in South Africa, is known to be a carrier of the disease with minimal symptoms and can travel over large distances possibly causing amphibian mortalities (Weldon *et al*., 2007; Measey *et al*., 2017). The risk of invasive species through the amphibian trade has, however, been decreased within South Africa due to the implementation of stricter legislations in recent years (Measey *et al*., 2017). Although the

spread of *Bd* from other countries has been limited, amphibians can still be transported within the country and spread infections to other regions that have not yet been affected (Measey *et al*., 2017). As the pathogen is spread to new areas, it may adapt, and more virulent lineages can develop through genetic recombination (Becker *et al*., 2017). Identifying and managing current infections present in South Africa will significantly contribute to the conservation of anurans in other non-infected regions of the country.

1.2.2.4. Lineage diversity

The genotypes of isolates can vary significantly and influence the level of virulence in a pathogen (Becker *et* al., 2017; Catenazzi, 2015; Farrer *et al*., 2011). Environmental stressors can alter these genotypes and phenotypes due to the highly adaptive nature of pathogens (Becker *et* al., 2017). This potential has given rise to multiple different lineages for *Bd*. The level of virulence can affect how quick the disease spreads amongst populations as well as the mortality rate of individuals (Catenazzi, 2015). Originally, four deeply diverged lineages had been identified within amphibian populations namely, Global Panzootic Lineage (*Bd*GPL), the African lineage *Bd*CAPE, the Brazilian lineage *Bd*BRAZIL and the Swiss lineage *Bd*CH (Farrer *et al*., 2011). Of these four lineages two occur over multiple continents and this may potentially be due to the global amphibian trade (Farrer *et al*., 2011). However, a hyper diverse lineage was discovered in 2018, *Bd*ASIA-1, that is currently regarded as the ancestor to the other lineages (O'Hanlon *et al*., 2018). The Swiss lineage showed to have similar characteristics to that of *Bd*ASIA-1 and is now classified as part of this lineage rather than its own lineage. Two additional lineages have also been identified in Asia, named *Bd*ASIA-2 and *Bd*ASIA-3. *Bd*ASIA-2 has shown to have similar characteristics to *Bd*BRAZIL (O'Hanlon *et al*., 2018). *Bd*BRAZIL is known to be associated with the amphibian trade of *Rana catesbeiana*, and the direction of the intercontinental spread cannot be determined, thus, this lineage is now called *Bd*ASIA-2/*Bd*BRAZIL (O'Hanlon *et al*., 2018; Schloegel *et al*., 2012).

Two lineages are known to occur in South Africa namely *Bd*CAPE and the *Bd*GPL lineage. *Bd*GPL can be found over multiple continents and is considered to be one of the most hyper-virulent of all the lineages and is associated with the majority of amphibian declines especially in tropical areas where a large biodiversity of amphibians occurs (Becker *et al*., 2017). The *Bd*CAPE lineage is considered hypo-virulent in comparison to other strains (Doherty-Bone *et al*., 2020; Farrer *et al*., 2011), however, can still have a devastating impact.

In Tanzania *Nectophrynoides asperginis*, the Tanzanian Kihansi spray toad, was reported as extinct in 2004 (Channing *et al*., 2006). Originally it was thought to be due to the construction of a dam that altered the environment as well as the presence of pesticides or possible

predators (Channing *et al*., 2006). It was later discovered after analysing preserved skin of the specimens that *Bd*CAPE had infested the area in a similar time period as the greatest declines in populations (Weldon *et al.*, 2020; Sewell *et al*., 2021). Before the collapse of the *N. asperginis* population increases had been observed in their numbers possibly due to restoration efforts, but a sudden decline was then observed in June 2003, that ultimately resulted in their extinction (Channing *et al*., 2006; Vandvik *et al*., 2014). Thus, although considered much more hypo-virulent than the GPL lineage, BdCAPE has the potential to cause devastating amphibian losses especially when combined with additional environmental stressors. Therefore, this lineage must be monitored to prevent the spread of infection to possibly more vulnerable areas.

Developing an effective diagnostic assay for *Bd* can significantly improve the conservation of anurans. Current sampling methods for *Bd* diagnostics such as toe clipping, ventral swabs and tadpole mouthparts are invasive to the target organisms and alternative diagnostic measures should be tested. The next section of this report will review a possible solution for developing an effective and ethical diagnostic method to aid in conservation strategies through eDNA.

1.2.3. Environmental DNA

eDNA can be described as DNA fragments that are derived from scales, metabolic waste, deceased organisms, skin, or other bodily excrements that can be sampled from the abiotic environment (Seymour *et al.*, 2018). eDNA has become increasingly popular in biodiversity and ecological studies and serves as a non-invasive, rapid, as well as cost effective alternative to conventional field surveys (Harper *et al*., 2019; Huver *et al*., 2015; Thomsen and Willerslev, 2015). The basic methodology of the protocol is filtering DNA from the environment, preserving and extracting the DNA, and amplifying the fragments through a Polymerase Chain Reaction (PCR) (Deiner *et al*., 2015).

eDNA assays can successfully be applied as an early detection method for identifying species within an area (Dejean *et al*., 2012). Conventional survey methods can be bias, and the biodiversity of an area can often be underestimated (Jerde *et al.*, 2011; Schmidt *et al*., 2013). Many species present within the environment cannot always be observed with the eye or may be missed during field surveying especially rare or cryptic species (Jerde *et al.*, 2011; Schmidt *et al*., 2013). eDNA protocols reduce these errors through analysing the DNA or cells within aquatic environments and do not require the host specimen to be present (Schmidt *et al*., 2013). For this section of the literature review the application of eDNA in previous studies will

be reviewed along with the possible considerations and limitations of these assays as well as species specific factors that contribute to the presence of *Bd*.

1.2.3.1. Application of eDNA from previous literature

Studies were originally conducted on ancient macro-organism communities that have long ago gone extinct, and this is possible due to DNA segments being preserved in sediments, permafrost and ice cores (Ficetola *et al.*, 2008; Thomsen and Willerslev, 2015). These studies later progressed to analysing present day aquatic as well as terrestrial ecosystems (Ficetola *et al.*, 2008; Jerde *et al.*, 2011; Reese *et al*., 2014).

Conventional biodiversity assessments are often extremely time-consuming and require a certain degree of expertise for the taxonomic identification of organisms (Seymour *et al.*, 2018; Thomsen and Willerslev, 2015). eDNA assays have shown to provide accurate estimates of the species present within the environment and collecting water samples is far simpler than other sampling methods (Miaud *et al*., 2019; Schmidt *et al*., 2013). This technique has previously been utilised for the detection of invasive, scarce as well as pathogenic species and has been applied for a miscellaneous of studies such as forensics/law enforcement, dietary investigations, biodiversity assessments as well as plant-pollinator dynamics (Buxton *et al*., 2017; Dejean *et al*., 2012; Ficetola *et al.*, 2008; Ruppert *et al*., 2019). One of the first eDNA studies was conducted on a freshwater environment to detect the presence of the invasive amphibian species, *Rana catesbeiana* (Ficetola *et al.*, 2008). During the study by Ficetola *et al*. (2008), the selected species was detected even when low densities of DNA was present.

eDNA protocols have previous been applied to detect *Ranavirus* as well as *Bd* in other countries, but research is still limited in South Africa (Miaud *et al*., 2019; Vilaça *et al*., 2020). The early detection of pathogens immensely contributes to management and conservation of amphibians and can prevent the future spread to new regions. Similar to Ranavirus, *Bd* does not always exhibit clinical symptoms, thus, methods such as eDNA protocols are accurate, non-invasive, and easy to conduct can significantly aid in the early detection of this pathogen without causing stress to the host organisms (Bancroft *et al.*, 2011; Miaud *et al*., 2019). Importantly, eDNA protocols have to be adjusted based on the target specimen and the environmental conditions present.

1.2.3.2. Batrachochytrium dendrobatidis environmental prevalence factors

The severity of chytridiomycosis often correlate with the environmental conditions as well as the host-specific response to the disease (Márquez *et al.*, 2010). Environmental conditions such as salinity, temperature, pH, elevation, seasonality, and dissolved oxygen concentrations contribute to the level of infections (Gabor *et al*., 2015; Gleason *et al*., 2008; Márquez *et al.*, 2010; Piotrowski *et al*., 2004). Majority of the species under Chytridiomycota occur within freshwater environments that possess a low osmotic potential (Gleason *et al*., 2008). Very few species in this phylum can occur in saline environments and the highest threshold known for chytrids are 2% NaCl (Gleason *et al*., 2008). The pH of the water also has an impact on chytrid distributions, *Bd* is capable of surviving in aquatic environments with a pH range of 6–8, but the optimal range for growth is between 6–7 (Piotrowski *et al*., 2004).

Temperature has a significant impact on the growth of chytrids, and species of this phylum generally have a low resistance to elevated temperatures (Gleason *et al*., 2008). This tolerance does, however, vary considerably amongst the different species. The highest known tolerance for any chytrid is 45°C, but the majority of these species cannot survive in temperatures above 30°C (Gleason *et al*., 2008). *Bd* tends to be more fragile and cannot survive in an environment with a temperature higher than 28°C (Gahl *et al.*, 2011; Piotrowski *et al*., 2004). This organism is capable of reproducing between 5–25°C, however, the optimal growth temperature is between 17–25°C and reproduction halts between 26–28°C (Piotrowski *et al*., 2004; Stevenson *et al*., 2013).

In previous literature, a correlation has been observed between altitude and the level of infection, where higher altitudes generally result in an increase in infections (Bancroft *et al.*, 2011; Gabor *et al*., 2015). Seasonality has also shown to play a role in the intensity of infections, generally increasing during the cooler months of the year (Bancroft *et al.*, 2011; Márquez *et al.*, 2010). However, this is not the case for all countries, for example, a study conducted in South Africa observed a peak in infections in the spring rather than cooler winter months (Conradie *et al*., 2011). It was concluded that the river temperature appeared to be more optimal for the growth of *Bd* during this time period and lower flow levels in the river systems resulted in higher pathogen densities (Conradie *et al*., 2011). Lower flow levels causing increases in infections, are also supported in the study by Bancroft *et al*. (2011) who indicated that species living in ponds rather than river systems were more prone to infections.

These observations demonstrate that the level of infection is dependent on a multitude of environmental factors and can vary between different regions. Thus, the factors may differ between countries, and this provides motivation for *Bd* studies to be investigated on a wider variety of habitats. The aquatic conditions of South African water sources may differ to those

in other countries, thus eDNA protocols previously performed in other countries for *Bd* may have to be adapted to the specific habitats present in South Africa.

Although environmental factors play a crucial role in the distribution and prevalence of the pathogen, biological factors, such as behaviour, also contributes to transmission (Bancroft *et al.*, 2011; Weldon *et al*., 2020). Environmental factors not only affect the pathogen but the biology and behaviour of the amphibian hosts (Conradie *et al*., 2011). Certain traits have been associated with higher intensity of infections and being able to identify these traits within species can aid scientists in determining the risk of infection (Bancroft *et al.*, 2011). For example, the habitat, egg-laying, and mating behaviours, such as amplexus, of individuals may contribute to the level of infection. Individuals more reliant on aquatic environments for breeding or survival may also be more prone to infections (Bancroft *et al.*, 2011; Hossack *et al*., 2013). Furthermore, species found at higher altitudes that have a restricted distribution and lower fecundity have a higher *Bd*-associated extinction risk (Bielby *et al.,* 2008).

1.2.3.3. Limitations and considerations of eDNA

To improve the detection probability, various factors should be considered regarding the target species and environmental conditions present. Water conditions such as temperature, nutrient availability, acidity, dissolved organic matter, and UV radiation all contribute to the persistence of eDNA within the environment (Eichmiller *et al*., 2016; Tsuji *et al*., 2017). Higher temperatures along with lower pH levels and UV-B radiation have shown to increase the level of degradation in cells and decrease the amount of eDNA present (Eichmiller *et al*., 2016; Strickler *et al*., 2015; Tsuji *et al*., 2017). Increased temperatures, neutral pH levels along with a moderate level of UV radiation provide optimal growth conditions for microbial organisms that contributes to eDNA degradation (Strickler *et al*., 2015). UV radiation exposure has been shown to damage DNA and reduces the detectability during PCR (Strickler *et al*., 2015; Ravanat *et al*., 2001). The lowest level of degradation occurs at 5°C or lower (Eichmiller *et al*., 2016; Strickler *et al*., 2015); unfortunately temperature is also a limiting factor for the presence and growth of *Bd*. Although this pathogen can survive at low temperatures, growth will be minimal, which in turn, reduces the amount of eDNA available. Higher concentrations of eDNA have been found in the substrates compared to water columns (Seymour *et al.*, 2018).

The eDNA of different organisms tend to persist for varying periods of time, some DNA fragments may be present for a month after the organism has been removed whereas others may only last for two weeks or less (Eichmiller *et al*., 2016). Generally, eDNA can remain in water for up to two weeks before degradation occurs (Schmidt *et al*., 2013). The zoospores of *Bd* are not extremely resistant and lack the resting spore stage often observed in other chytrid

species (Berger *et al*., 1999). This resistant phase can allow spores to survive harsh conditions and remain dormant for decades before becoming active again (Berger *et al*., 1999). Thus, species that do not possess resting spores are more prone to degradation when in an inactive stage (Berger *et al*., 1999). Aquatic environments provide an array of challenges to eDNA diagnostics due to the presence of oxygen, microbial activities and hydrolysis which all contribute to the fast degradation of eDNA (Seymour *et al.*, 2018). This does, however, present benefits as well due to providing an accurate estimation of recent activities and species within an area (Seymour *et al.*, 2018).

DNA tends to have an unequal distribution within the environment (Barnes *et al*., 2021). Understanding the environmental properties, dispersion and origin of eDNA is important in sampling for specific fragments (Barnes *et al*., 2021). Lentic and lotic water systems each present their own challenges. Lentic systems such as ponds, potentially complicate sampling for specific DNA fragments due to reduced water movement that may result in an extremely unequal distribution of DNA within the area (Harper *et al*., 2019). Thus, concentrations can decrease significantly over small distances within lentic systems (Harper *et al*., 2019). The presence of PCR inhibitors may also be higher in lentic systems compared to lotic waterbodies due to certain chemical properties present within these environments (Harper *et al*., 2019). Lotic systems are, however, known to disperse eDNA over a wider area and thus, may reduce the accuracy due to the DNA potentially being sampled at unknown distances from its original source (Jane *et al*., 2015).

Harper *et al.* (2019) proposes that multiple samples should be collected over a wide area within the study site and should also be sampled at different depths to obtain the best representation of the environment. An increase was observed in the detectability for presence/absence studies where more samples were taken for each individual site and when more replicates were preformed over time (Willoughby *et al*., 2016). Furthermore, Golberg *et al.* (2018) suggested that more samples may need to be taken from environments with a higher acidity, UV exposure or temperature, due to possible lower eDNA concentrations present. The eDNA abundancy within an environment also correlates with the number of individuals present (Buxton *et al*., 2017; Strickler *et al*., 2015).

Certain host behaviours, such as mating, have shown to increase the level of infections, tending to occur at specific times during the year and varies between amphibian species (Harper *et al*., 2019). This can aid in selecting a sampling time for field samples in eDNA studies. More eDNA tends to be present towards the end of a breeding season, before the tadpoles have undergone metamorphosis (Buxton *et al*., 2017). This is due to the congregation of adults in one area for breeding as well as the increased presence of individuals through

higher larvae abundances (Buxton *et al*., 2017; Harper *et al*., 2019). When the tadpoles become metamorphosed, they tend to disperse more, and leave the aquatic environment which causes a decrease in the amount of eDNA present (Buxton *et al*., 2017). Thus, selecting an appropriate sampling time is essential to develop a successful diagnostic assay for this project.

1.2.4. Molecular detection

A wide variety of molecular assays exist for presence and absence eDNA studies. The most popular tend to be conventional PCR assays coupled with barcoding or quantitative real-time PCR (Langlois *et al*., 2021). This section of the literature review will discuss the current methods for diagnosing *Bd*, the different types of PCR assays used for eDNA, existing species specific primers for detecting *Bd* as well as distinguishing lineages, and the limitations/considerations of PCR assays.

1.2.4.1. Current diagnostic methods for Batrachochytrium dendrobatidis

Several diagnostic methods exist for *Bd* diagnostic purposes such as histopathology, histo-chemistry, PCR assays, and electron microscopy to name a few (Berger *et al*., 1998; Hyatt *et al*., 2007). Previous diagnostics of the pathogen were done on the toe clips of amphibians through histological as well as immune-histochemical examinations (Boyle, *et al*., 2004; Annis *et al*., 2004). The sporangia of the pathogen can also be observed through microscopy of mouthparts from tadpoles or toe clips and skin samples from adult anurans (Walker *et al*., 2007). Toe clipping does, however, cause severe stress to the organism and is not recommended for long-term studies (Hyatt *et al*., 2007). Swabbing is considered more ethical and is generally done on the ventral side of adults due to the highest density of infections most commonly occurring around the inner thighs as well as between the toes of the hind-feet (Annis *et al*., 2004). In certain fully aquatic species infection may occur on the ventral as well as dorsal surfaces (Annis *et al*., 2004). Boyle *et al*. (2004) developed a diagnostic assay through real-time TaqMan probe qPCR, that uses the ventral swabs along with primers designed for *Bd* in a qPCR assay to determine the presence or absence of the pathogen. Although this method is less invasive than the previous diagnostic assays, it still requires interaction with the host specimen which causes varying degrees of stress to the individuals.

1.2.4.2. PCR assays for eDNA studies

The final stage of eDNA assays involves testing the extracted DNA for either species/genus specific detection or a whole community analysis (Deiner *et al*., 2015). This can be done using different PCR techniques each presenting benefits and limitations based on the study type (Schultz and Lance, 2015). Two of the most common assay types will be discussed for this section of the project namely conventional PCR coupled with DNA sequencing and TaqMan Probe qPCR.

Conventional PCR

During the 1980s, Kary Mullis developed the Polymerase Chain Reaction (PCR) (Dale *et al*., 2012). This process was developed to create copies of specific DNA target regions exponentially (Dale *et al*., 2012). The process consists of three phases namely denaturing, annealing and extension (Dale *et al*., 2012). The reaction mix consists of DNA polymerase, forward primers, reverse primers and nucleotides (Garibyan and Avashia, 2013). The DNA template is first exposed to high temperatures (95°C) to allow the two DNA strands to separate in the denaturation process (Garibyan and Avashia, 2013). The temperatures are then lowered to allow the primers to anneal to specific target areas then increased again for the extension phase (Dale *et al*., 2012; Garibyan and Avashia, 2013). Nucleotides are added from the start of the primers to build a copy of the target region (Dale *et al*., 2012). The entire process is then repeated in multiple cycles to create millions of copies of the desired template (Dale *et al*., 2012). These target regions can then be sequenced to identify specific species.

Different molecular detection techniques are applied in eDNA studies depending on the goal of the experiment, namely DNA barcoding or meta-barcoding. Barcoding is generally applied for a specific target organism, whereas meta-barcoding compares multiple sequences to known species sequences to determine the biodiversity present within an area (Deiner *et* al., 2017; Garlapati *et al*., 2019; Willoughby *et al*., 2016). Originally sequencing was done only through Sanger Sequencing, however, this method was extremely limited in its throughput and could only analyse a single organism at a time (Reuter *et al*., 2015; Shokralla *et al*., 2012). As technology improved High Throughput Sequencing (HTS), such as Next Generation Sequencing (NGS), was developed to sequence multiple samples in parallel (Reuter *et al*., 2015; Shokralla *et al*., 2012). Next Generation Sequencing has become increasingly popular for studying biodiversity and whole community dynamics while being more cost effective and less time consuming than conventional Sanger Sequencing (Shokralla *et al*., 2012). Sanger Sequencing is, however, still an accurate method for identifying single specific organisms and is still often applied (Shokralla *et al*., 2012). Single target organisms can be detected using

species specific primers in conventional PCR, which is then tested using gel electrophoresis, and then confirmed through Sanger Sequencing (Stoeckle *et al*., 2018). This method was often applied during previous eDNA studies, but has shown a low level of sensitivity because of the high quantity of DNA required for successful sequencing (Stoeckle *et al*., 2018).

TaqMan Probe qPCR

TaqMan Probe qPCR uses the same DNA amplification process as conventional PCR, but with the addition of a fluorescent probe. This probe contains oligonucleotides that bind to a sequence within the target region and a reporter dye with fluorescent properties is linked to the 5' end of the oligonucleotides and a quencher dye to the 3' end (Bassler *et al*., 1995; Hawrami and Breuer, 1999). Due to the close proximity of the reporter dye to the quencher, the fluorescents of the reporter gene are neutralised when bound, but when separated the fluorescents of the reporter gene are released and measured through a luminescence spectrometer (Hawrami and Breuer, 1999; Livak *et al*., 1995). When the DNA strand is denatured the oligonucleotide will anneal to the specific target section, but will not extend during the extension phase due to the 3' end of the probe being phosphorylated (Bassler *et al*., 1995). After the probe has annealed to the DNA strand, a fluorescent is released during the extension phase from the primers when the Taq DNA polymerase digests and cleaves the reporter dye from the quencher dye (Bassler *et al*., 1995; Hawrami and Breuer, 1999). This fluorescence remains constant during the reaction and is increased during each cycle which allows for the quantification of the concentration of the DNA present through the level of fluorescence (Hawrami and Breuer, 1999). Real-time quantitative PCR allows the monitoring of the increase in DNA concentration in real-time as the fluorescence is being released (Hunter *et al*., 2017).

Real-time qPCR has become increasingly popular in eDNA studies due to its high level of sensitivity and specificity (Wilcox *et al*., 2013). Although conventional PCR assays are more economic than qPCR, qPCR assays provide additional benefits. This method not only serves as an accurate detection method but can also potentially be applied to quantify the eDNA through standard curves obtained from predetermined DNA concentrations in a dilution series (Lodge *et al*., 2012; Wilcox *et al*., 2013). qPCR assays tend to also be more sensitive and accurate due to their lower detection thresholds compared to conventional PCR assays (Loge *et al*., 2002; Lodge *et al*., 2012). Probe-based qPCR assays, such as the TaqMan Probe, is one of the most applied assay types in eDNA studies (Tsuji *et al*., 2019). These assays monitor the amplification of samples in real time, which makes detecting false positives much easier as compared to other PCR methods (Hunter *et al*., 2017).

The TaqMan probe qPCR assay has previously been tested on *Bd* with great success and has shown to deliver more accurate results compared to histological examinations of toe clips when low numbers of sporangia are present (Boyle *et al*., 2004). The assay can detect *Bd* as early as seven days after an amphibian has been infected, though some studies suggest it may even be as little as four days (Boyle *et al*., 2004; Hyatt *et al*., 2007). This assay also detects the pathogen two weeks earlier than regular histological examinations as well as most other diagnostic assays (Boyle *et al*., 2004; Hyatt *et al*., 2007).

1.2.4.3. Distinguishing lineages of Bd

Specific amplicons can be applied for single organism studies. These amplicons can then be sequenced and used to distinguish lineages within a species. Some lineages are more virulent and can have a more devastating impact on populations or species, thus being able to distinguish between lineages greatly improves conservation efforts. Not only can PCR be applied for diagnosis of *Bd* but it can also be used to distinguish between the different lineages (Ghosh *et al*., 2021). Single nucleotide polymorphisms (SNP) in the reference genome JEL423 located in the mitochondria have previously been used to identify the various lineages (O'Hanlon *et al*., 2018; Sewell *et al*., 2021). Two lineage specific primers have also been developed for the two *Bd* lineages present in South Africa, namely *Bd*CAPE and the *Bd*GPL lineage using mitochondrial DNA (mtDNA) (Ghosh *et al*., 2021).

1.2.4.4. Batrachochytrium dendrobatidis specific primers

For many eDNA studies marker genes are often applied and the products are sequenced to determine the species present, however, species specific primers can also be developed to only amplify the target organism (Stoeckle *et al*., 2018). The ITS regions of rDNA have previously successfully been applied to detect eDNA from fungal species within aquatic environments (Matsuoka *et al*., 2019). This is due to this region of the genome being conservative among the lineages (O'Hanlon *et al*., 2018). The ITS-1 and ITS-2 regions are popular for the diagnostic purposes of *Bd* (Hyatt *et al*., 2007). These regions do not vary significantly between the different lineages in *Bd* and species specific primers have been developed for PCR as well as qPCR assays (Annis *et al*., 2004; Boyle *et al*., 2004).

The ITS-1 region is an ideal primer/probe site for specific fungal diagnostics, because it occurs more than 100 times within the genome which creates a multitude of binding sites for amplification (Boyle *et al*., 2004; Longo *et al*., 2013). This aids in increasing the sensitivity of the protocol. Two different primer sites have been identified with primers designed for *Bd* specific studies (Annis *et al*., 2004; Boyle *et al*., 2004). The protocol developed by Boyle *et al*.

(2004) uses a TaqMan Probe qPCR assay. The target region for the primers is located on the *Bd* ribosomal operon (Kirstein *et al*., 2007). The forward primer targets a section of the ITS-1 region and the reverse primer is located in an adjacent part of the genome within the 5.8S region (Boyle *et al*., 2004). The lowest detection limit of this assay was determined to be 0.1 genomic equivalents (GE), though high levels of variability were seen at the lower concentrations in multiple studies (Blooi *et* al., 2013; Boyle *et al*., 2004). The specificity was tested using three different *Bd* strains and five other species from the order Chytridiales, and only the *Bd* strains amplified (Boyle *et al*., 2004). Additional studies further examined the primers and results showed a high level of specificity when testing various other orders and species in the phylum Chytridiomycota (Blooi *et al*. 2013; Hyatt *et al*., 2007).

The second assay developed was by Annis *et al*. (2004) and applies a single round of conventional PCR on the 5.6S ribosomal RNA. The primers from Annis *et al*. (2004) target the ITS-1 and ITS-2 region of the genome. This protocol has a lowest detection limit of approximately 10 zoospores, however, 1 zoospore could occasionally be detected (Annis *et al*., 2004). The specificity of this assay was tested using different *Bd* isolates, other closely related species, species from the order Chytridiales as well as other fungi that may commonly be found in the environment (Annis *et al*., 2004). The TaqMan Probe assay showed a higher level of sensitivity compared to the conventional PCR method (Goka *et al*., 2009). However, the primers by Annis *et al*., (2004) were applied using nested PCR assay and a significant increase in sensitivity was detected (Goka *et al*., 2009).

It should be noted that in the study by Mutnale *et al*. (2018) the ITS region of the *Bd*-ASIA lineages were analysed and a wide diversity with 57 different haplotypes were identified. Indels have been detected in the regions of the binding site for TaqMan probe as well as the reverse primers, which resulted in some variants being less likely to be detected (Mutnale *et al*., 2018). Although these lineages are currently not present in South African new lineages can always be introduced into new environments or greater threats of inter-lineage recombination can occur between existing lineages in the country (Ghosh *et al*., 2021). This could potentially result in the development of possibly higher virulent lineages (Ghosh *et al*., 2021). However, for the purpose of this study; only two lineages present within South Africa, namely *Bd*CAPE and the *Bd*GPL lineage, both of which can be diagnosed using the current species specific primers (Ghosh *et al*., 2021).

1.2.4.5. Limitations and considerations

The size of the DNA fragments can affect the rate of degradation as well as detection success. For example, eDNA fragments with 300–400 base pairs (bp) can persist in aquatic

environments for a week when presented with the correct environmental conditions, but shorter DNA fragments have shown lower degradation rates and can be detected up to one month after the host has been removed (Dejean *et al*., 2011). Thus, when preforming eDNA studies the use of shorter target regions in the genome may deliver more accurate results. The primers developed by Boyle *et al*. (2004) target a 146-bp region, whereas the primers by Annis *et al.* (2004) amplify a region with a length of 300-bp (Annis *et* al., 2004; Goka *et al*., 2009; Kirshtein *et al*., 2007).

The success in species detection of the samples positively correlates to the number of PCR replicates preformed for each sample (Willoughby *et al*., 2016). When low quantities of target DNA is expected in an environment, multiple filter sample replicates are required along with PCR replicates to achieve a positive result, even when working with an extremely sensitive qPCR assay (Willoughby *et al*., 2016). It should be noted that field sample replicates have a more significant impact on the success of an assay than the PCR replicates, but a duplicate should at the very least be performed for the PCR phase (Willoughby *et al*., 2016).

Primers that are species specific have shown to be more accurate in detecting organisms than sequencing samples from generic primers due to mutations and faults that occur in the PCR process (Willoughby *et al*., 2016). According to Hunter *et al.* (2017), it is very important to always include negative controls when conducting a PCR assay. Examples are no template controls (NTC's) that are made with nuclease free water rather than a DNA template or negative samples made alongside the experimental samples to test possible cross contamination during the DNA extractions. This aids in detecting if false positives have occurred due to contamination, primer dimers, and often with generic primers when no-specific amplifications occur (Hunter *et al*., 2017).

Despite the use of negative controls, false positives may still occur. Thus, creating a standard curve to determine the lowest detection limit (LOD) is necessary for developing a reliable assay (Friedman *et al*., 2014; Hunter *et al*., 2017). A ten-fold dilution series of DNA copy numbers tend to follow a specific trend on a linear graph with an increase of 3.32 Ct-values per dilution (Taylor *et al*., 2010). As the copy numbers decrease and start approaching zero, greater variations can be observed as well as deviations from this regular trend (Hunter *et al*., 2017). It is at this stage the accuracy of the assay becomes more questionable and the possibility of a false positive increases (Hunter *et al*., 2017). A cut-off point should then be selected in this region, after which any amplification would be considered a negative result despite delivering a Ct-value (Friedman *et al*., 2014).

Although false positives significantly affect the reliability of the assay, many factors exist that may also result in false negatives. The sensitivity and specificity of an assay plays a crucial

role in the success of the protocol. The sensitivity of the protocol is determined by the LOD of the assay (Hunter *et* al., 2017; Loge *et al*., 2002). This can be influenced by a variety of factors such as the volume of the samples, the presence of inhibitory agents that may affect the PCR process, and how effectively the target organism can be recovered (Loge *et al*., 2002). These factors may ultimately result in false negatives in the target sites and reduce the accuracy of studies on the prevalence or distribution of specific organisms (Hyman and Collins, 2012).

To reduce the possibility of false negatives, internal positive controls (IPCs) are included in the PCR process (Lance & Guan; 2020). IPCs contain a known number of DNA copies and are run during the field sample assays (Lance & Guan; 2020). If the IPC does not amplify or the Ct-value is increased in comparison to known previous positive samples, it represents a sign of inhibition, thus a possible false negative in the field samples (Lance & Guan; 2020). Standard curves are not only used to determine the lowest level of detection for a specific assay, but can also be used to determine the impact of inhibitors that may be present (Albers *et al*., 2013).

One of the downsides to qPCR assays are due to the increased sensitivity of the protocol that cause inhibitors to have a much more profound impact on the assay compared to a conventional PCR (Albers *et al*., 2013). Although the abovementioned factors play a crucial role in developing an accurate and sensitive protocol, many factors prior to the qPCR phase can determine the success of the assay. Inhibitors are frequently caused during the DNA extraction process, when certain chemicals or mediums are not properly removed and interact with the DNA or PCR reagents (Albers *et al*., 2013; Lance & Guan; 2020; Stoeckle *et al*., 2017). For example, certain inhibitors, such as humic acids found in sediment samples, react with magnesium ions in the PCR polymerase which results in higher Ct-values (Albers *et al*., 2013). Humic acids can also be present in aquatic samples through particle resuspension from riverbeds (Stoeckle *et al*., 2017). Thus, sampling and DNA extraction techniques significantly affect the qPCR process and will further be reviewed in the subsequent chapters.

Chapter 2: Project outline and general methodologies

2.1. Project outline

This chapter provides insights on the problem statement and goals set to achieve during this study. The outline of the project will be discussed, and the general methodologies applied throughout multiple chapters are presented.

2.1.1. Problem statement

Current diagnostic procedures for identifying *Bd* are invasive and time consuming. From the literature presented in the previous chapter, it is clear that eDNA can be used as an effective and non-invasive method to determine the presence of pathogens within aquatic environments with a high level of accuracy. This method serves as a more ethical procedure for pathogen detection by eliminating the handling or euthanasia of animals, previously required to diagnose *Bd,* i.e., toe clips, tadpole mouth parts and abrasive ventral swabs. The World Organisation for Animal Health (OIE) currently lists *Bd* as a notifiable disease of amphibians and encourages the development of multiple diagnostic assays to identify the pathogen. eDNA has successfully been applied in a number of countries for the detection of various pathogens. In South Africa, this research is still very young with no optimised protocol yet in place for pathogen detection. This study forms part of a larger study that aims at optimising and applying eDNA protocols to detect a variety of aquatic and semi-aquatic species. Thus, this project serves as the foundation to analyse a non-invasive, early detection method in a South African aquatic system context to determine which methods will be the most effective for filtering and extracting *Bd* from the environment.

2.1.2. Aim

This project aims to serve as the foundation to developing an additional molecular diagnostic assay for *Bd* using eDNA through optimising each step under laboratory conditions prior to applying the assay to future field sampling.

2.1.3. Objectives

i. Preparation of *Bd* cultures through passaging, agar plate growth and zoospore harvesting.
ii. Determine the impact of three matrix types: sterile, non-sterile and heat-treated cultures on DNA yield, quality and qPCR Ct-values.

iii. Compare two commercial DNA extraction kit methods and two crude DNA extraction methods and select one of each for further optimisation.
iv. Test three filtering protocols to determine the most practical method for remote field sampling.
v. Test two preservation mediums on filter media and compare the selected crude and kit DNA extraction methods over three time periods.
vi. Determine the efficiency of the *Bd* primers using a G-Block dilution series and zoospore filter dilution series.
vii. Set standards for the protocol through determining the lowest limit of detection (LOD), lowest limit of quanitification (LOQ) and cut-off Ct-value of the assay.
viii. Test the specificity and sensitivity of *Amietia* spp. primers for the internal assay control of eDNA samples.
ix. Collect water samples from multiple sites at the Ncandu Nature Reserve, uMsonti Nature Reserve and Ncandu Private Nature Reserve through filtration.
x. Extract the DNA from the filter materials using the selected preliminary protocol.
xi. Determine presence of *Bd* and *Amietia* spp., using genus and species specific primers in qPCR assay.
xii. Compare the effectiveness of the protocol compared to conventional diagnostic measures.

2.1.4. Project structure

This project was set to develop a robust diagnostic assay in a laboratory setting that can be applied to field application in the future. Fieldwork was conducted during the course of the study to test the first preliminary selected method and later further optimised in the laboratory. The methodology of this project consists of two parts. The first part focuses on developing a robust protocol, measuring the effectiveness and setting the laboratory standards to which the field samples can be compared (Figure 2.1). This part comprises of four phases as described in the following section. Phase 1 (Chapter 3 and 4) consisted of growing cultures, harvesting zoospores, creating specific dilutions and preparing different culture matrixes to test. In Phase 2 (Chapter 3), four different DNA extraction methods were tested on the culture matrixes, two commercial DNA extraction kits and two crude extraction methods. One crude method and one kit method were then selected for Phase 3 and further optimised for filter material. In Phase 3 (Chapter 4), three different filter methods were tested for their practicality in field application and the limitations in filter volumes were determined. Multiple filter tests were then conducted using the two selected extraction methods and two different preservation mediums. In Phase 4 (Chapter 3 and 4) samples were quantified using molecular detection techniques

such as Nano-drop spectrophotometry and TaqMan probe qPCR. The most optimal filtering and extraction method was selected and the lowest limit of detection (LOD) and lowest limit of quantificiation (LOQ) of the assay was determined using a serial dilution series (Chapter 4). A synthetic dilution series was also created to determine the genomic equivalence of the zoospores to DNA copy numbers and the primer efficiency was also calculated. This provided a base standard to which field samples could be compared and possibly quantified.

Part 2 of the project focused on the preliminary field application and consisted of two phases (Figure 2.1). During Phase 5 (Chapter 5) field samples were collected using a filter method of choice, and processed and analysed in Phase 6. An internal control assay was developed using a frog species from the selected sampling site. Primers were developed and tested for their efficiency and specificity prior to field work. During the course of this study an opportunity was presented to collect field samples at the Ncandu Nature Reserve and surrounding reserves. The field sampling was run concurrently with the laboratory phase of this project, thus experiments were still ongoing, and a preliminary method of choice was selected for field application. This opportunity was given during the optimal season for sampling and a fellow MSc student, Jacques Potgieter, was studying the occurrence of *Bd* in the Reserve. During his study conventional diagnostic measures were applied such as toe clips and the collection of mouth parts from tadpoles. This provided a direct comparison for the eDNA assay to conventional diagnostic measures and a primary verification to the presence of *Bd* at the reserve. While also providing additional insights to the potential limitations of the preliminary protocol, it also aided in further optimising the eDNA method. Following the application of the methodology to the field samples, laboratory tests continued and the protocol was further optimised. A new study is currently being conducted by a fellow lab member that aims to compare the newly developed eDNA protocol with other conventional diagnostic assays in multiple regions of the country. Thus, the new protocol will be extensively tested in the field during a subsequent study.

This dissertation consists of six chapters, each addressing a major component and includes its own literature review with relevant information to the section. In chapter 3, different DNA extraction methods coupled with the matrix types were compared to determine the most effective methods for the target organism. In chapter 4, the selected DNA extraction methods were tested along with multiple filtering protocols as well as preservation mediums to develop a robust eDNA assay. Additionally, standards were set to which field assays can be compared. In chapter 5, insight was provided on the field application of the preliminary assay using *Amietia* spp. primers as an internal control assay alongside the *Bd* primers. Chapter 6 contains the final conclusions and recommendations for future studies.

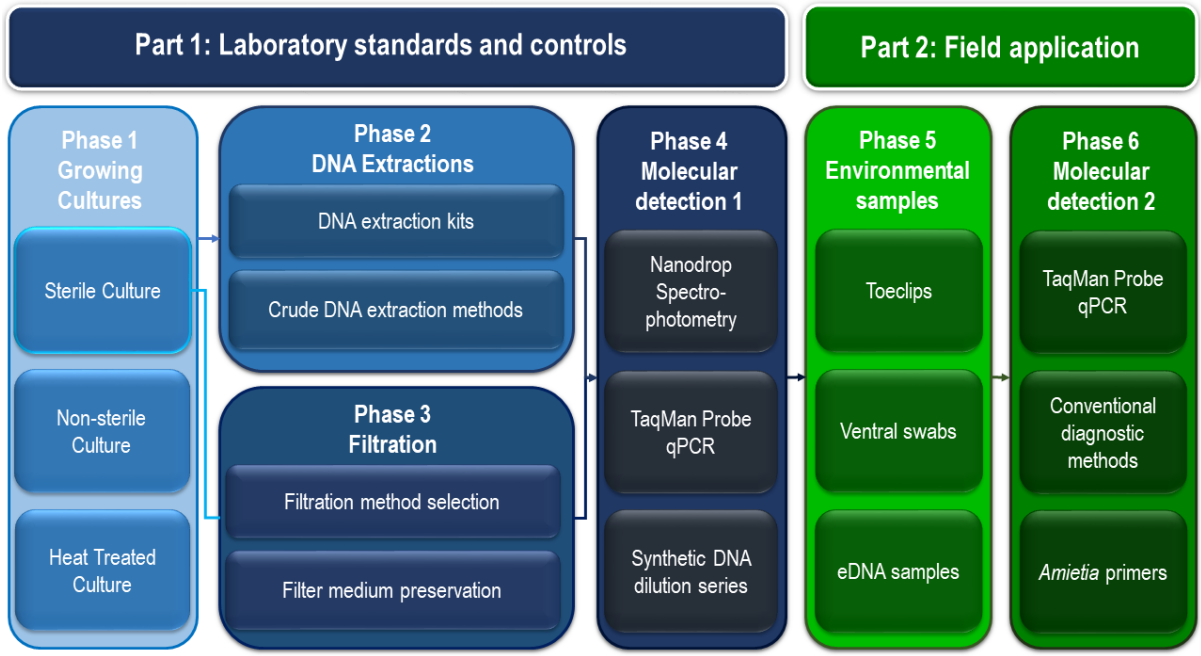

Figure 2.1. Project outline illustrating the workflow for the different sections and phases of the project

2.1.5. Ethics and permits

This study was deemed a *no risk* study and ethics clearance was obtained from the Faculty for Natural and Agricultural Sciences, FNASREC (ethics no.: NWU-01292-21-A9). A collection permit was obtained from Ezemvelo KZN Wildlife for the collection of frogs by fellow student, J. Potgieter as part of his study (permit no.: OP 874/2021). Refer to Annexure 2 for copies of the documentation.

2.2. General methods applied

The methods used for phases 1 and 4 of the project (Figure 2.1) form part of multiple chapters and will be discussed in this chapter of the dissertation rather than be repeated for each subsequent chapter individually. Cultures were grown and diluted to specific concentrations for phases 2 and 3 of the project outline, and the molecular detection for *Bd* was the same for all the phases of the project.

2.2.1. Culture preparation

Stock cultures were prepared through passaging from a single culture with a known strain. This is done to ensure that all the cultures applied for this section of the project are derived from the same lineage. The selected lineage was the Global Panzootic Lineage (*Bd*GPL),

culture strain 08MG04 passage 34 of the Herpetological Health Lab at North-West University. This specific isolate had been collected from the species *Amietia fuscigula* located in the Western Cape province at the Silver Mines, South Africa.

2.2.1.1 Growing flask cultures

Cultures were grown in a 1% tryptone broth, which was prepared using 1l double distilled water (ddH2O) and 10g of tryptone powder. The mixture was swirled in a glass container to allow the powder to completely dissolve and then placed in an autoclave set at 121ºC on a liquid cycle. After the broth had cooled down, the container was placed in a laminar cabinet for a 20 minutes UV decontamination cycle along with the flasks and equipment required to prepare the cultures. Following UV decontamination, the surface of the laminar cabinet was cleaned with 70% ethanol and the selected *Bd* culture was placed in the cabinet. The stock cultures were then prepared using 18ml 1% tryptone broth and 2ml of the selected culture placed in each 100ml culture flask (Figure 2.2A). The flasks were placed in an incubator at 20°C for 2–4 days to suit the optimal growth temperature for *Bd* cultures, between 17–25ºC (Piotrowski *et al*., 2004). Culture growth was monitored with inverse microscopy and after the incubation period the cultures were moved to a fridge set at 4ºC to limit further metabolic activities and prevent rapid nutrient depletion from the broth.

2.2.1.2. Growing cultures on agar plates

The *Bd* cultures consist of sporangia that release zoospores into the aqueous environment. These sporangia contain unknown numbers of zoospores; thus, the concentration of zoospores present cannot be determined as a constant for the DNA extractions. Zoospores can be harvested from agar plates, while leaving the sporangia embedded with rhizoids on the plates.

The 1% tryptone agar was prepared by mixing 1l ddH_2O, 10g tryptone powder and 15g bacteriological agar in a 1l glass container and then placed in the autoclave at 121°C. While the mixture cools to handling temperature, the surface of the laminar cabinet was cleaned with 70% ethanol, and the container as well as petri dishes were placed in the cabinet for a 20 minutes UV decontamination cycle. After decontamination, petri dishes were filled halfway with the liquid tryptone agar and left to solidify for a few minutes. Approximately 3ml of the selected culture was then placed on the gel and left to dry. The petri dishes were sealed using parafilm tape (Figure 2.2B) and placed in an incubator at 20°C for 3 days or until maximum culture size was achieved. Cultures growth was monitored through microscopy as well as to check for contamination (Figure 2.2C and 2.2D).

Figure 2.2. (A) Typical culture flask containing *Bd* broth, (B) Agar plate culture used in the harvesting of zoospores, (C) Monitoring growth of cultures through microscopy, (D) Micrograph of *Bd* culture (400× magnification).

2.2.1.3. Harvesting zoospores and zoospore numeration

Zoospores were harvested by placing 3ml of 1% tryptone broth on the agar plate cultures. The plates were left for 20 minutes to allow enough zoospores to enter the aqueous solution. The solution was transferred, using a Pasteur pipette, from the plates to an Eppendorf tube. Zoospores were counted using a compound microscope and a haemocytometer. A pipette was used to place 200µl of the culture solution on the grid. There were a total of nine 16-block grids on the haemocytometer and zoospores were counted on five of these grids (the four corner grids and the centre grid). An average for the 16-block grid was determined and multiplied by 10 000 to calculate the number of zoospores per ml (zoospores/ml).

2.2.1.4. Preparing dilution series

Following the determination of zoospore numbers per ml, the solution was diluted to 100 000 zoospores per ml. This was done using the following equation:

X = number of zoospores/ml harvested

1000 ml / (X / 100 000) = C (µl of culture required per ml)

1000 ml – C = T (µl of tryptone broth required per ml)

To create a 10-fold dilution series, 100µl from the previous dilution was taken and placed in a new Eppendorf and 900µl of 1% tryptone broth was added to the culture. This step was repeated until a dilution series from 1 – 100 000 zoospores per ml was created.

2.2.2. Molecular detection

The qPCR protocol was the only standardised protocol in the project and was not adjusted during the course of the project. This phase remained the same throughout the study. All the qPCR assays were conducted on a QuantStudio™ 3 Real-Time PCR System and analysed using the QuantStudio™ Design and Analysis Software v1.5.2.

The ∆RN value represents the changes in the fluorescence of the probe and is measured for each cycle to create the amplification plot (Boyle *et al*., 2004). The Ct-value represents the point where the fluorescence crosses the set threshold and the sample is considered positive (Caraguel *et al*., 2011). Previous *Bd* studies have shown to use set thresholds (∆RN) to measure the Ct-values in the amplification curve (Boyle *et al*., 2004; Ghosh *et al*., 2021). According to Archer (2017), the threshold should be located in the exponential phase of the amplification plot. For the *Bd* samples of this project a threshold of 8000 ∆RN was selected, because it is located in the middle of the exponential phase for all the samples and was above the background noise. False positives may still occur if the background noise passes the threshold and presents a Ct-value when looking at the raw numerical data, but on closer inspection of the amplification curves false positives may be identified (Hunter *et al*., 2017). Since the amplification curves should have a sigmoidal shape, the amplification plot was examined for each sample along with their Ct-value to ensure that all values applied in this study were true positive results (Caraguel *et al*., 2011).

The primers developed by Boyle *et* al. (2004) were selected for this project. This is due to the high level of specificity and sensitivity of the primers as well as the short length of the target region (Blooi *et al.*, 2013; Kirshtein *et al*., 2007). eDNA fragments tend to be more degraded and selecting primers for small target regions may increase the accuracy of the assay (Dejean

et al., 2011). Primers and probe were acquired from Whitehead Scientific (Pty) Ltd using the following sequences derived from Boyle *et al*. (2004):

Forward Primer – ITS1-3 Chytr

5' - CCTTGATATAATACAGTGTGCCATATGTC – 3'

Reverse Primer – 5.8S Chytr

5' - AGCCAAGAGATCCGTTGTCAAAT – 3'

Probe Sequence – Chytr MGB2

5' – 6FAM CGAGTCGAACAAAAT MGBNFQ – 3'

SsoAdvanced™ Universal Probes Supermix was purchased from Bio-Rad Laboratories. This supermix consisted of Sso7d-fusion polymerase, $MgCl_2$, passive reference dyes, dNTPs, enhancers and stabilisers. The probe was received lyophilised and was made up to a concentration of 100mM, which was diluted to 10mM for the working stock. A working stock of 10mM was also prepared for the forward and reverse primers. Each qPCR sample consisted of 25µl; 12,5µl Supermix, 0.5µl forward primer (200nM), 0.5µl reverse primer (200nM), 0.25µl probe (100nM), 6.25µl nuclease free water and 5µl of the selected DNA template. Two non-template controls (NTC) that were made from nuclease free water were included in each run along with positive and negative controls. The following amplification conditions were used from Boyle *et al*. (2004); an initial denaturation cycle of 2 minute at 50°C then 10 minutes at 95°C, followed by 50 cycles of 15 seconds at 95°C and 1 minute at 60°C.

Chapter 3: DNA extraction method comparison

3.1. Introduction

Optimising lab protocols prior to field sampling is essential in developing an effective eDNA assay. The sampling methods, DNA extractions methods and PCR phase all have to be optimised in a laboratory setting prior to application to determine the limitations and minimum requirements for the protocol (Piggot *et al*., 2016). After the samples have been collected through filtration, the second step in eDNA diagnostics is the DNA extraction phase from the filter media (Deiner *et al*., 2015). Although many different DNA extraction methods have been tested on fungi, results will always vary based on the type of species being used (Fredricks *et al*., 2005). Environmental factors can also significantly contribute to the selection of a DNA extraction method, and methods often have to be adapted to the target organisms and conditions present (Kuhn *et al*., 2017). The following section will review methods that have previously been applied for the extraction of *Bd* DNA as well as other methods that have not been tested for *Bd*, but have been utilised in eDNA studies or show the potential for eDNA applications. Four DNA extraction methods consisting of two commercial DNA extraction kit methods and two crude extraction methods, were selected for the project. The benefits and limitations of the different methods were analysed to aid in selection of two methods for the subsequent filter tests. Crucial considerations and lab protocols were also discussed as well as the importance of high quality and quantity DNA for diagnostic purposes.

3.1.1. Commercial kits vs crude extraction methods

Multiple considerations exist for DNA extraction protocols that are often determined by the target organism. Fungi possess thick cell walls that present challenges to DNA extractions due to incomplete lysis of the cells (Fredricks *et al*., 2005; Tripathy *et al.*, 2017). To effectively extract DNA, the cell walls as well as membranes around the nucleus need to be disrupted or lysed either chemically, electrically, mechanically or acoustically (So *et al*., 2014). Fungal cells are also known for possessing high levels of polysaccharides which have to be removed along with other proteins, lipids, carbohydrates and organic structures to purify the DNA samples (Kuhn *et al*., 2017; Tripathy *et al.*, 2017). The purification of DNA is extremely important for downstream application and the presence of the mentioned components may result in an inhibitory effect or reduce the efficiency of the qPCR assay (Schrader *et al.*, 2012).

Although DNA extraction kits are one of the most applied methods, it is much more cost-intensive compared to crude extraction methods, especially when large quantities of samples have to be analysed (Kuhn *et al*., 2017; Turan *et al*., 2015). Commercial extraction kit methods are also generalised and can rarely be adapted according to the target organism, but are

reliable due to their consistency and easy application (Kuhn *et al*., 2017). Both crude DNA extraction methods and commercial kits have previously been used for *Bd* and eDNA studies. Kit DNA extraction methods are known for delivering high quality DNA, however some organic compounds are not always effectively removed (Barbier *et al*., 2019). In this instance, crude extraction methods are often favoured because they are a cost-effective alternative to commercial kits and can easily be modified according to the target organisms or environments being sampled (Barbier *et al*., 2019; Piggot, 2016). It should, however, be noted some of these methods tend to be much more time-consuming and often use hazardous reagents such as phenol or chloroform (Barbier *et al*., 2019; Ferencova *et al.*, 2017; Piggot, 2016).

Methods such as the cetyltrimethyl ammonium bromide (CTAB) method and phenol-chloroform-isoamyl methods are some of the most commonly applied crude extraction methods in eDNA studies as well as for fungi (Ferencova *et al.*, 2017; Tsuji *et al*., 2019; Zhang *et al*., 2010). In previous eDNA studies crude extraction methods, such as CTAB, have occasionally shown to perform better than kit methods, such as the Qiagen DNeasy Blood and Tissue Kit and PowerWater Kit (Tsuji *et al*., 2019). The CTAB method has also previously been applied to *Bd* as well as eDNA studies and delivered positive results (Annis *et al*., 2004). Multiple variations of this method exist and the time required to conduct the extractions also varies greatly from a few hours to more than a day (Tripathy *et al.*, 2017). CTAB is used to disrupt the cell membranes, but other disruption methods such as glass beads, thermal exposure, or chemical disruption methods can be applied along with the CTAB for the complete lysis of cells (Zhang *et al*., 2010). Chemicals such as chloroform or phenols are then applied to purify the samples (Tripathy *et al.*, 2017; Zhang *et al*., 2010). Some CTAB methods have been altered to use proteinase K to first lyse the cell walls and then apply chemicals such as phenol or chloroform to remove the protein components (Barnes *et al*., 2020; Chi *et al*., 2009). Phenols break down cellular components that would contaminate the DNA samples and when combined with water can be spun down due to the differences in density of the mediums, leaving the DNA in suspense (Tripathy *et al.*, 2017). Chloroform is used to dissolve the proteins and lipids and separates them from the DNA and is also spun down, leaving the DNA in the upper phase of the solution (Tripathy *et al.*, 2017). This step is normally followed by applying isopropanol and ethanol used to precipitate the samples (Zhang *et al*., 2010).

Another well-known crude DNA extraction method for *Bd* is the PrepMan Ultra method. This was first used by Boyle *et al*. (2004) and applies 40µl of PrepMan Ultra along with Zirconium beads to mechanically disrupt the *Bd* cells. DNA is then extracted through temperature changes, the samples spun down at a high speed and a small volume of DNA (20µl) is recovered (Boyle *et al*., 2004). The extracted DNA then has to be diluted to reduce the impact of the PCR inhibition (Boyle *et al*., 2004). This method has been applied in many *Bd* studies

due to the cost-effectiveness and simplicity (Bletz *et al*., 2015; Hyatt *et al*., 2007; Kosch and Summers, 2013; Talley *et al*., 2015). However, when compared to certain commercial extraction kit methods, such as the Qiagen Blood and Tissue Kit, PrepMan Ultra tends to have a greater inhibitory effect despite the dilution of the product, that negatively affects the qPCR results (Bletz *et al*., 2015; Sabino-Pinto *et al*., 2019). This method has performed similarly to some kits when compared at higher DNA concentrations, however the lower detection rates of the method are more limited due to being less effective at removing inhibitors (Bletz *et al*., 2015; Kosch and Summers, 2013). Although PrepMan Ultra is a very commonly applied method for *Bd,* it may not be as effective for eDNA samples due to this decreased sensitivity at lower concentrations DNA, a condition that is typical for environmental samples.

Although many different crude DNA extraction methods have previously been applied on *Bd*, many still remain untested and could present efficient and cost-effective alternatives. An example of such a method is the Heat Lysis method. This method has previously been used for other fungal pathogens and is considered a very simple and cost-effective method that only applies Chelax-100 beads and nuclease free water (Greeff *et al*., 2012). It is also commonly applied in applications with low copy numbers of DNA, for examples blood samples and fingerprints in forensic investigations, small blood samples in virology tests as well as for cultures in microbiology based studies on fungi and spores (Ferencova *et al.*, 2017; Panda *et al*., 2019; Turan *et al*., 2015). Samples are homogenised in nuclease free water and then transferred to a tube containing the Chelax-100 beads which are negatively charged (Greeff *et al*., 2012). However, different methods exist for applying Chelax-100 resins are also commonly used, and methods for extraction vary from lab to lab based on the target organism (Panda *et al*., 2019; Tripathy *et al.*, 2017; Turan *et al*., 2015). DNA is released from the cells through a boiling process in the presence of Chelax-100 (Greeff *et al*., 2012; Turan *et al*., 2015). These beads have metal chelating properties which prevent the degradation of the DNA at boiling temperature while also binding to metal ions that serve as a catalyst to DNA degradation (Greeff *et* al., 2012; Panda *et al*., 2019; Turan *et al*., 2015; Walsh *et al*., 2013). This method is known to yield high concentrations of DNA because there are minimal transfers of the solution to new tubes (Panda *et al*., 2019; Walsh *et al*., 2013). The majority of the reactions occur within a single tube after the homogenised sample was transferred to the tube containing the beads (Walsh *et al*., 2013). Thus, it can be very beneficial for eDNA studies where low concentrations of the target organisms are generally present in the environment.

A wide variety of commercial DNA extraction kits have previously been tested to extract DNA from *Bd* as well as aquatic eDNA samples (Bletz *et al.*, 2015; Brannely *et al*., 2020; Kirstein *et al*., 2007; Walker *et al*., 2007). Kits tend to be the most widely utilised DNA extraction method for eDNA studies due to their simplicity and effectiveness in removing inhibitors (Tsuji *et al*.,

2019). Some of the most commonly used commercial kit methods for aquatic environments are the DNeasy Blood and Tissue Kit, DNeasy PowerSoil Kit, the Quick-gDNA spin-column Kit, MoBio Qiagen PowerWater Kit, MoBio PowerWater Kit and QIAamp Micro Extraction Kit (Eichmiller *et* al., 2016; Rees *et al*., 2014; Tsuji *et al*., 2019). It should be noted that the most commonly applied methods are not necessarily the most optimal method for every target organism. Some methods provide better DNA yields whereas others may provide higher quality DNA and remove inhibitors more effectively. For the case of *Bd* one of the most applied kit methods is the Qiagen Blood and Tissue Kit (Bletz, *et al*., 2015). However, studies have found other kits to be more effective including a study conducted in the Herpetological Health Lab (HHL) that found the Zymo Research Bacterial and Fungal Kit to be more effective (Du Preez *et al*., 2019). The DNeasy PowerSoil Kit has also previously successfully been used in *Bd* eDNA studies (Brannely *et al*., 2020).

3.1.2. Limitations and considerations

Equipment and lab surfaces need to be sterilised properly prior to DNA extractions and the qPCR process to reduce the risk of false positives and cross-contamination between samples. It is recommended to use 10% bleach to clean all working surfaces, work in a laminar flow, use filter tips for all extractions and PCR processes as well as UV decontaminate equipment prior to use (Deiner *et al*., 2015). Applying proper hygiene protocols in the laboratory is also important for reducing the potential of cross-contamination between labs and samples. Clean clothes and new gloves should be worn when preforming the PCR phase of the samples and this should be performed in a different room than where DNA samples are extracted (Deiner *et al*., 2015).

Natural PCR inhibitors that are often found with eDNA samples are; humic substances, sediment as well as algae (Kuhn *et al*., 2017; Stoeckle *et al*., 2017). Humic acids have shown in multiple studies to have a significant effect on the PCR process, but sediment was shown to be more common in aquatic eDNA samples (Stoeckle *et al*., 2017). The recovery of high quality DNA is crucial to the success of eDNA diagnostic assays due to the low concentrations of zoospores that are present in the environment. Thus, although a DNA extraction method may perform well on sterile cultures, they might not be as effective for environmental samples. It is important to test these limitations in the DNA extraction methods to determine whether they are capable of effectively removing inhibitors from non-sterile samples.

Degradation rates vary between different types of cells and environmental factors have the greatest impact on the degradation rates of the samples (Moushomi *et al*., 2019). Understanding the target organism can aid in identifying possible limiting factors of the

protocol being developed. The type of marker gene being used in a study is also affected by degradation rates, for example nuclear DNA has shown to be less successful than mitochondrial DNA (mtDNA) for eDNA studies (Moushomi *et al*., 2019). This is because nuclear DNA occur in fewer copies than mtDNA in the cells and is thus, greatly impacted by DNA degradation. It should however be noted that ribosomal markers in the nuclease occur in higher numbers and are often as successful as the mtDNA (Moushomi *et al*., 2019). In the case of *Bd,* the ITS region of the genome is amplified using the selected primers that occur in multiple parts of the genome. The impact of cellular degradation for *Bd* needs to be evaluated in this part of the current study as well as whether the selected DNA extraction methods are able to successfully extract DNA from degraded cells.

3.2. Hypothesis, aim and objectives

3.2.1. Hypothesis

H0 – No significant difference will be observed in the yield, quality and Ct-values of the DNA extraction methods in the different matrix types, and a decision will have to be made based on other variables such as the consistency, practicality and repeatability of the results.

H1 – Significant differences will be observed in the yield, quality and Ct-values between the DNA extraction methods in the different matrix types, and will highlight which methods would be the most appropriate commercial DNA extraction kit method and crude method to select for subsequent tests.

3.2.2. Aim

Determine the most optimal DNA extraction methods for *Bd* that can be applied for the subsequent filtering protocol tests by comparing two crude extraction methods and two kit extraction methods in various matrix types.

3.2.3. Objectives

- Preparation of *Bd* cultures through passaging, agar plate growth and zoospore harvesting.
- Determine the impact of three matrix types: sterile, non-sterile and heat-treated cultures on DNA yield, quality and qPCR Ct-values.
- Compare two commercial DNA extraction kit methods and two crude DNA extraction methods and select one of each for further optimisation.
- Determine whether any statistically significant differences can be detected between the various extraction methods.
- Determine whether matrix types have a statistically significant effect within specific DNA extraction methods.

3.3. Materials and methods

For this section of the project three different culture matrixes were prepared to test the effectiveness of the DNA extraction methods under various conditions. The first two matrixes each represented a different environmental type that may affect the concentration or quality of the extracted DNA. This was crucial for understanding the possible limitations of the DNA extraction methods while also providing insights to the possible inhibitory factors on the qPCR process. The final matrix provided insight to the degradation of *Bd* cells over time and whether this rate is constant between methods. Four DNA extraction methods were compared, two crude extraction methods and two kit methods. One crude method and kit method was then selected at the end of this section for further validation in the subsequent chapter.

3.3.1. Preparing culture matrixes

Cultures were prepared using the methods described in Chapter 2 section 2.2.1.1 – 2.2.1.4 and diluted using 1% tryptone to a concentration of 100 000 zoospores/ml for all the samples. A final volume of 1 ml was prepared with the selected concentration. Samples were prepared in triplicate for each matrix and DNA extraction method. The three matrixes that were tested were a sterile, non-sterile and heat-treated matrix as described in the following section.

3.3.1.1. Sterile culture

The sterile culture represents the purest form of a culture and only consists of a known concentration of zoospores, suspended in 1% tryptone broth. All samples were diluted to 100 000 zoospores/ml. The values from this culture were used as the set standard to which the other matrixes were compared.

3.3.1.2. Non-sterile culture

The non-sterile cultures were prepared through spiking borehole water collected from the NWU botanical gardens with a known concentration of zoospores. Other micro-organisms and possible inhibitors were therefore present, and represented an environmental sample. When sampling from the environment, qPCR inhibitors and contaminants may be present that could potentially decrease the sensitivity of the protocol (Albers *et al*., 2013; Lance & Guan; 2020; Stoeckle *et al*., 2017). This matrix tested the effectiveness of each DNA extraction method to remove possible inhibitory agents and contaminants.

3.3.1.3. Heat-treated culture

Unlike most chytrids, *Bd* does not possess a resting spore phase (Berger *et al*., 1999). The resting spore phase is a mechanism that allows organisms when presented with unfavourable conditions to enter a resting phase until more optimal conditions allows it to resume with life unaffected (Berger *et al*., 1999). Thus, *Bd* spores may potentially be more likely to perish when presented with unfavourable conditions. This can significantly affect the amount of eDNA available in the environment. If degradation of the cells occurs too quickly this will reduce the amount of DNA available for analysis. Field samples may at times have to be transported to the lab prior to filtering and this section aims to determine whether a significant loss in DNA can be observed over a period of as little as a week. Fieldwork is often conducted over multiple days and could easily last up to a week prior to when samples can be processed.

Certain types of cells are more prone to degradation than others which is why this matrix is important in determining whether a significant change can be observed for *Bd* over time. Understanding the degradation rates of *Bd* cells can aid us in determining how long these cells may persist in the environment over time and if the extraction methods would be able to effectively extract DNA from the degraded cells. This section determined the possible limitation of DNA degradation in the detection of the target pathogen and could be used to measure the effectiveness of preservation mediums in the subsequent filter tests.

Bd zoospores are heat sensitive and cannot survive above temperatures of 28ºC (Piotrowski *et al*., 2004), however, they do not immediately degrade after exposure. Zoospores require specific exposure times at different temperatures before they are deceased; at 37ºC spores will perish after 4 hours, at 47ºC it would take 30 minutes and at 60ºC they only need to be exposed for 5 minutes (Johnson *et al*., 2003). To prepare this matrix, the culture was heat-treated by placing the Eppendorf with the culture in a water bath for 30 minutes at 47°C. The culture was then placed in an incubation fridge at 20°C for a week prior to extraction to allow cell degradation to occur.

3.3.1. DNA extraction methods

Two commercial DNA extraction kit methods and two crude extraction methods were selected for this part of the project, namely the DNeasy PowerSoil Kit, Zymo Research Bacterial and Fungal Kit, Heat Lysis (Greeff *et al*., 2012) and CTAB (Cetyltrimethyl ammonium bromide) method. These methods were selected based on their previous success with fungal pathogens or eDNA studies.

3.3.1.1. DNeasy PowerSoil Kit

The DNeasy PowerSoil Kit (Qiagen Catalogue no: QIA/12888-100) has previously been applied to eDNA studies for *Bd* and shown to deliver high quality DNA with accurate results (Brannelly *et al*., 2020). In water samples where high levels of inhibitors are present the DNeasy PowerSoil Kit has repeatedly shown to effectively remove all inhibitors (Tsuji *et al*., 2019).

Initially the prepared cultures were spun down at 10 000 × g for 5 minutes and 650µl of the broth removed. The remaining 450µl was then transferred to the bead tube provided with the kit. This was done because the samples were too small to be weighed and a pellet could not be formed when spun down. The DNeasy PowerSoil Kit recommends using a vortex for the first step of the DNA extraction, but a bead beater can also be applied as a substitute. For this method a bead beater was used for 5 minutes at full speed. The manufacturer's instructions were followed for the rest of the protocol.

3.3.1.2. Zymo Research Bacterial and Fungal Kit

The Zymo Research Bacterial and Fungal Kit (Zymo Research Catalogue no: D6005) method was selected due to its previous application on *Bd* at the North-West University Herpetological Health Lab (HHL) lab (Du Preez, 2019). This method was compared to the DNeasy Blood and Tissue Kit which is one of the most commonly utilised methods for eDNA studies, and delivered better results (Du Preez, 2019).

Cultures were prepared the same way as the DNeasy PowerSoil Kit and samples were spun down at 10 000 × g for 5 minutes and 650µl of the broth removed. The remaining 450µl was then transferred to the bead tube provided with the kit. One step in the protocol had to be changed due to the "Zymo-Spin IV Filters" being absent in the kit. For this step the samples were meant to be spun through the filters to aid in removing excess cellular debris. This step was replaced by centrifuging the solution at 8 000 × g for 1 minute and transferring the supernatant to a new tube. The rest of the protocol was followed as described by the manufacturer's instructions.

3.3.1.3. Heat Lysis

The Heat Lysis method is a quick and cost-effective protocol that does not apply hazardous chemicals such as phenol or chloroform. This method is derived from the study by Greeff *et al*. (2012) and was slightly modified for the target organism of this project. This method has not previously been applied for *Bd*, but was selected due to its success on other fungal

pathogens. Fungal cells are known to have thicker cell walls that complicate DNA extractions (Greeff *et al*., 2012). This method has proven to be effective in this regard and disrupts the cell walls through the use of a hand-held homogeniser. Homogenising tips were made from epoxy placed in an Eppendorf with a syringe needle in the middle. After the tips had dried, they were removed and sanded down to allow them to rotate with ease within an Eppendorf. These tips were then attached to a Dremel tool and used to homogenise the samples. This method was extremely cost-effective and only required Chelax-100 beads and nuclease-free water.

Samples were centrifuged at 10 000 × g for 5 minutes and supernatant was removed leaving 50µl of the sample because a clear pellet could not be seen and 250µl of nuclease free water was added. The samples were then homogenised for 1 minute 30 seconds. The tip of the homogeniser was cleaned using 10% bleach, followed by 70% ethanol and then ddH_2O each for a 30sec cycle between every sample. Homogenised samples were placed in an Eppendorf containing 0.04 ± 0.005g Chelax-100 beads. The samples were briefly vortexed and placed in a heating block at 56ºC for 20 minutes. The tubes were then vortexed briefly and then placed in the heating block again at 95ºC for 30 minutes. Samples were then vortexed and rapidly cooled on ice for 5 minutes before being centrifuged at 17 000 × g for 5 minutes at 4ºC. The DNA was in suspense and 150µl of the supernatant was transferred to a new tube and stored at -80ºC for analyses.

The 1% tryptone broth from the cultures had shown to have a significant impact on the Nano-drop spectrophotometry readings for this method. In the next phase of this project, as previously elaborated on in the project outline of Chapter 2, various filter methods were tested and *Bd* zoospores were extracted from the filter media. To create the filter samples, water samples were spiked with the zoospores and all the liquid media was filtered out prior to the DNA extraction. Thus, no 1% tryptone would be present during the extraction phase and inhibitory effect from the 1% tryptone would not be expected in the Heat Lysis extraction method should it be applied. To test the full capacity of the Heat Lysis DNA extraction method in extracting *Bd* DNA, the sterile as well as heat-treated samples, agar plates were re-suspended using 6ml nuclease free water rather than 1% tryptone. The samples were also diluted to the 100 000 zoospore/ml concentration using nuclease free water. The non-sterile matrix used the same environmental matrix as the other DNA extraction methods in this chapter, thus the Heat Lysis was still tested for its efficiency in terms of environmental inhibitory agents. A blank was created along with all the matrixes to use in the measurements of the Nano-drop spectrophotometer. After the zoospores had been re-suspended in the nuclease free water, the solution was divided into two sterile Eppendorfs. The one solution was used to create the three replicate samples to test the DNA extraction method and the

second was used to create the blank to which the samples would be compared for the Nano-drop measurement. To prepare the blank the contents of the Eppendorf were diluted with the same volume of nuclease free water as the test samples. The liquid was then drawn into a syringe and a 0.2µm filter was placed on the tip. The size of *Bd* zoospores ranges approximately from 1–5µm, thus when filtering the media through the syringe no zoospores will be present in the filtered medium (Farthing *et al.*, 2020; Walker *et al*., 2007). The blank was then subject to the same extraction process as the three tested samples.

3.3.1.4. CTAB

The CTAB method makes use of CTAB and proteinase K in a thermal lysis process to extract the DNA and then uses chloroform, and isopropanol for the purification of DNA. CTAB methods in general have shown to effectively extract DNA from plant and fungal cells and is known to be a cost-effective method that removes inhibitory agents (Zhang *et al*., 2010).

Most of the liquid in the culture had to be eliminated prior to applying the CTAB buffer and this was done through centrifuging the culture for 5 minutes at 10 000 × g. The top 950µl of the 1ml culture was removed and 300µl of the CTAB buffer was added. Using a hand-held homogeniser, the sample was homogenised for 1 minute to aid in the lysis of the cells. The tip of the homogeniser was cleaned between each sample in three 30 seconds cycles, the first was in 10% bleach, the second in 70% ethanol, thirdly in double distilled water. The samples were placed in a freezer at -50°C for 30 minutes, followed by 15 minutes at 65°C in a water bath. Following the incubation steps, 3µl proteinase K was added, the sample was vortexed and again incubated at 65°C for 60 minutes. Samples were cleaned by adding 300µl of chloroform and gently being mixed using the pipette tip. This was followed by centrifugation for 15 minutes at 3 800 × g and the upper 260µl was transferred to a new tube. A second wash with chloroform was performed using 87µl of chloroform, the samples were vortexed and followed by centrifugation for 5 minutes at 12 000 x g. The upper 200µl was transferred to a new tube and 133µl of ice-cold isopropanol was added. Samples were incubated for 15 minutes at 4°C before being placed in a centrifuge at 16 000 × g for 5 minutes. The supernatant was removed and 100µl of ice-cold 70% ethanol was added and vortexed. The supernatant mix with ethanol were then placed in a centrifuge for 5 minutes at 16 000 × g. After centrifugation the supernatant was removed leaving a pellet. A bio-flow cabinet and heating block were prepared and the surface sterilised with 70% ethanol. Samples were placed with open caps in the heating block at 65ºC until the pellet had dried completely. In the final step 100µl TE buffer was used to re-suspend the pellet. DNA was ready for analysis after an hour and stored at -80°C.

3.3.3. Molecular detection

Nano-drop spectrophotometry was applied along with TaqMan Probe qPCR to determine the quality, quantity, Ct-values and level of variance within as well as between the different matrixes. For more information on the qPCR protocol and reagents applied refer to Chapter 2 section 2.6.2. Lower Ct-values are more desirable, because they indicate earlier amplification of the samples and, due to the samples all containing the same number of zoospores in these trials, it can also provide a direct indication of the quality of the samples. All the methods and variables were tested in triplicate and run in duplicate in the qPCR cycle. This provided six data points per method and variable combination.

The quality of the extraction method was measured using the Nano-drop spectrophotometer A260/A280 measurement. A value between 1.8 and 2.1 is considered good quality DNA. Values beneath 1.8 may contain protein contamination, which could possibly cause inhibition in the qPCR phase and values above 2.1 may contain RNA contamination (Koetsier and Cantor, 2019; Psifidi *et al*., 2015). The quantity of the samples was measured in ng/µl.

All surfaces and equipment were cleaned using 10% bleach, followed by 70% ethanol and UV decontaminated for 20 minutes in a laminar flow hood prior to use. Filter tips were used for all the protocols applied in the study. It should be noted that each matrix was run on a different plate. The reagents, quantities, methods and applied *Bd* strain stayed the same throughout the entire molecular phase of this project. Comparisons of results were based on the assumption that the PCR efficiency remained the same between different samples and different plates. In the study by Blooi *et al.* (2013), the same primers designed by Boyle *et al*. (2004) were also utilised. Inter- and intra-plate variations were tested for the qPCR assay and it was determined that the assay was highly replicable across plates with a high efficiency for each run (Blooi *et al*., 2013). Thus, a high level of reproducibility in results was demonstrated for the primers and could allow for comparison of Ct-values between the plates. Similar findings were made by Hyatt *et al* (2007) where the reproducibility of Ct-values between different institutions and qPCR machines were tested through applying *Bd* primers in multiple dilution series. The Ct-values were compared based on their mean, standard deviations and CV%, and the results indicated a high level of reproducibility for the method across machines (Hyatt *et al.*; 2007). For this study the primer efficiency was also tested using synthetic DNA (G-block) (see Chapter 4). This test was conducted prior to the DNA extraction method tests and showed the primers to have an excellent efficiency.

3.3.4. Statistical analysis

The mean, standard deviations (SD) and coefficient of variability percentages (CV%) were calculated using Excel 2016 for each extraction method within each matrix and the methods overall. The SD and CV% of the Ct-values were determined using all six data points obtained from the qPCR run. The CV% was used to determine the intra-assay variability and reproducibility of the datasets using the equation CV% = 100 * (SD/Mean).

The sample averages were used from the qPCR replicates for each subsequent analysis. GraphPad Prism v8.0.2 was used to determine mean differences and statistical significance of the data. A series of one-way ANOVAs were used to determine the statistical significance for each of the following variables Nano-drop quantity, Nano-drop quality and qPCR Ct-values. The sterile and non-sterile matrix were analysed separately and then an overall analysis was run for both variables. A Bonferroni multiple comparison test was preformed post hoc to determine the significance of each individual method in comparison to the other methods within each matrix as well as overall. The Bonferroni multiple comparison test has previously been applied in other studies for the statistical comparison between Ct-values of DNA extraction methods (Auricchio *et al*., 2013; Psifidi *et al*., 2015). The data was considered statistically significant when the p-value was less than 0.05.

The matrixes within each DNA extraction method were compared to determine whether any significant differences could be observed in the DNA extraction methods and if losses in DNA occurred over time between the sterile and non-sterile matrix. This analysis was done using a Two-way ANOVA, with Bonferroni post hoc multiple comparison test. The data was considered statistically significant when the p-value was less than 0.05.

3.4. Results

For this section of the project the sterile and non-sterile matrixes are discussed separately and then an overall analysis is provided for the data. Each matrix has three figures providing a visual representation of the quantity, quality and Ct-values as well as relations of the methods to each other, along with a summary for the mean, SD and CV% of each method for all three of the variables. A table with all the mean differences, p-values and statistical significance from the Bonferroni post hoc multiple comparison test between the methods is also included for each matrix analysis.

3.4.1. Sterile matrix results

3.4.1.1. Overall assessment

The results from the overall One-Way ANOVA analysis for the DNA quantity, indicated that the values were statistically significant ($p = 0.0234$). This was due to the high quantity values presented by the Zymo Research Bacterial and Fungal Kit, but this was at the cost of its quality which was the lowest amongst the methods (Figure 3.1; Table 3.1). The quantity was only statistically significant when the Zymo Research Bacterial and Fungal Kit was compared to the DNeasy PowerSoil Kit in the post hoc Bonferroni's multiple comparison test ($p = 0.0372$) (Table 3.2). A high level of variability could be observed for the quantity values for the majority of the methods as seen by the high CV% in Table 3.1.

The Heat Lysis method delivered the highest quality results of all the methods, but none of the p-values in the One-way ANOVA ($p = 0.1313$) nor post hoc Bonferroni multiple comparison test indicated any statistical significance (Figure 3.2; Table 3.1; Table 3.2). Both crude extraction methods delivered better Ct-values compared to the kit extraction methods in the sterile matrix. However, the One-Way ANOVA analysis of the Ct-values indicated that overall there was no statistical significance between the different methods ($p = 0.0851$), nor were there any significant differences in the multiple comparison test.

3.4.1.2. Commercial DNA extraction kit method comparison

The quality of the DNeasy PowerSoil Kit was higher than the Zymo Research Bacterial and Fungal Kit, but not statistically significant ($p = 0.7893$) (Figure 3.2; Table 3.1; Table 3.2). Despite the high quantity readings by the Zymo Research Bacterial and Fungal Kit, this was not reflected in the Ct-values of the samples (Table 3.1).

A single outlier was identified in the Zymo Research Bacterial and Fungal Kit for one of the qPCR results in the sterile matrix. The outlier was identified using the GraphPad Prism

software and this value was excluded from the analysis. The two kit methods delivered similar Ct-values. The DNeasy PowerSoil Kit having marginally better values with a mean difference of 0.64, which was not significant (p = >0.9999) (Table 3.2). Although the mean Ct-values were not significant, the Zymo Research Bacterial and Fungal Kit had a higher SD (0.71) and CV% (3.25) that indicates a greater variability and inconsistency amongst the data, but not outside an acceptable range (Figure 3.3; Table 3.1).

3.4.1.3. Crude extraction method comparison

The crude extraction methods delivered similar Ct-values with the CTAB method delivering slightly lower Ct-values with a mean difference of 0.3927 compared to the Heat Lysis method that did not vary significantly (p = >0.9999) (Figure 3.1; Table 3.1; Table 3.2). The Heat Lysis method delivered a slightly higher quality and lower quantity compared to the CTAB method (Figure 3.1; Table 3.1). Neither of these readings differed significantly between the methods and had a p-value of >0.9999 for both variables (Table 3.2). The CTAB method had the greatest variation amongst its Ct-values (SD = 2.00 and CV% = 10.28%) compared to all the DNA extraction methods in this matrix and the CV% was above what is considered a good level of variation for this analysis, indicating less reliable results. The samples all amplified in duplicate and little variation (less than 0.2) could be seen between the duplicates, indicating the variation in the Ct-values were between samples and the qPCR process was done accurately. The most consistent Ct-values of this entire matrix were observed in the Heat Lysis method (SD = 0.2 and CV% = 1.03) (Table 3.1).

Sterile matrix results for DNA extraction methods

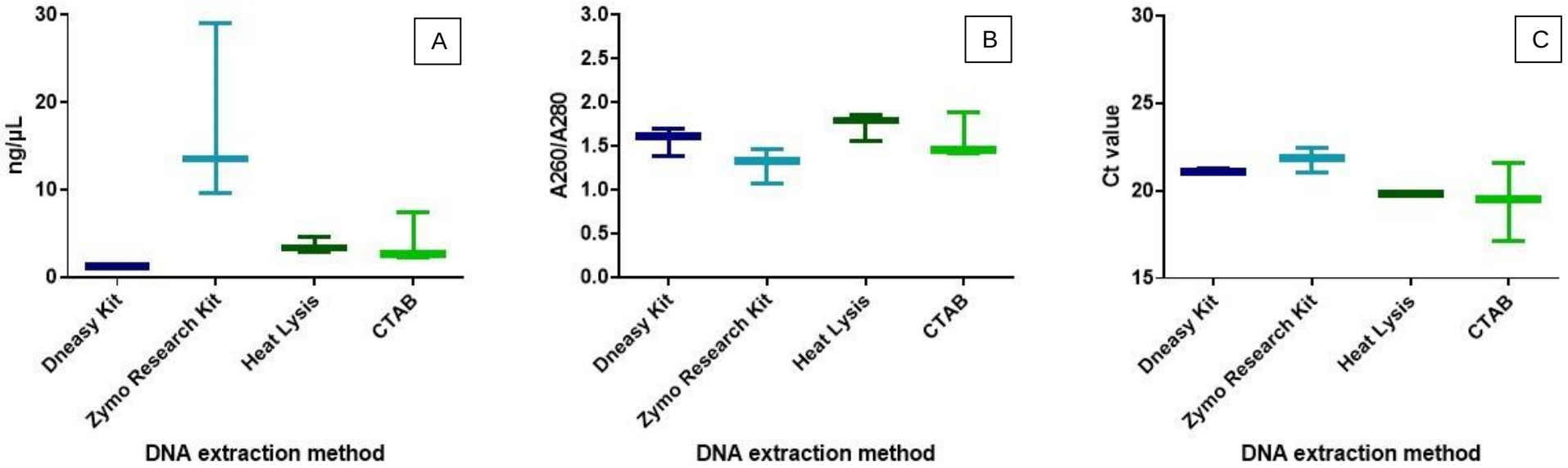

Figure 3.1. Sterile matrix quantity (ng/µl), quality (A260/A280) and Ct-values for four DNA extraction methods, (A) Quantity (ng/µl) comparison and sample range for all DNA extraction methods, (B) Quality (A260/A280) comparison and sample range for all DNA extraction methods, (C) Ct-value comparison and sample range for all DNA extraction methods

Table 3.1. Sterile matrix, quantity (ng/µl), quality (A260/A280) and Ct-value means, standard deviations (SD) and coefficient of variability (CV%) for DNA extraction methods.

DNA extraction method	Mean quantity ± SD (CV%)	Mean quality ± SD (CV%)	Mean Ct-value ± SD (CV%)
Dneasy PowerSoil Kit	1.22 ± 0.04 (3.30)	1.56 ± 0.17 (10.56)	21.13 ± 0.29 (1.39)
Zymo Research Bacterial and Fungal Kit	17.36 ± 10.30 (59.32)	1.29 ± 0.20 (15.43)	21.74 ± 0.71 (3.25)
Heat-lysis method	3.59 ± 0.88 (24.49)	1.73 ± 0.16 (9.18)	19.81 ± 0.20 (1.03)
CTAB method	4.06 ± 2.90 (71.57)	1.58 ± 0.27 (16.82)	19.42 ± 2.00 (10.28)

Table 3.2. Sterile matrix methods comparison, using a One-Way ANOVA Bonferroni post hoc multiple comparisons test between DNA extraction methods for quantity (ng/µl), quality (A260/A280) and Ct-values (p-values in red are significant).

Quantity (ng/µl)		
Method comparison	**Mean difference**	**P-value**
DNeasy PowerSoil Kit vs Zymo Research Bacterial and Fungal Kit	-16.14	0.0372
DNeasy PowerSoil Kit vs Heat Lysis method	-2.367	>0.9999
DNeasy PowerSoil Kit vs CTAB method	-2.833	>0.9999
Zymo Research Bacterial and Fungal Kit vs Heat Lysis method	13.77	0.0826
Zymo Research Bacterial and Fungal Kit vs CTAB method	13.30	0.0971
Heat Lysis method vs CTAB method	-0.4667	>0.9999
Quality (A260/A280)		
Method comparison	**Mean difference**	**P-value**
DNeasy PowerSoil Kit vs Zymo Research Bacterial and Fungal Kit	0.2767	0.7893
DNeasy PowerSoil Kit vs Heat Lysis method	-0.1667	>0.9999
DNeasy PowerSoil Kit vs CTAB method	-0.0200	>0.9999
Zymo Research Bacterial and Fungal Kit vs Heat Lysis method	-0.4433	0.1646
Zymo Research Bacterial and Fungal Kit vs CTAB method	-0.2967	0.6563
Heat Lysis method vs CTAB method	0.1467	>0.9999
Ct-value		
Method comparison	**Mean difference**	**P-value**
DNeasy PowerSoil Kit vs Zymo Research Bacterial and Fungal Kit	-0.6400	>0.9999
DNeasy PowerSoil Kit vs Heat Lysis method	1.3190	>0.9999
DNeasy PowerSoil Kit vs CTAB method	1.7110	0.6691
Zymo Research Bacterial and Fungal Kit vs Heat Lysis method	1.9590	0.4492
Zymo Research Bacterial and Fungal Kit vs CTAB method	2.3510	0.2369
Heat Lysis method vs CTAB method	0.3927	>0.9999

3.4.2. Non-sterile matrix results

3.4.2.1. Overall assessment

When assessing the results from the One-Way ANOVA overall, the DNA quantities between the methods were considered statistically significant (p = 0.0001). This was once again due to the high quantity presented by the Zymo Research Bacterial and Fungal Kit which was significant compared to all the other methods (Figure 3.2; Table 3.3; Table 3.4). The DNeasy PowerSoil Kit delivered the lowest quantity but with the highest quality DNA on average for this matrix (Figure 3.2; Table 3.3), but the quality value was not statistically significant when compared in the One-Way ANOVA nor the post hoc Bonferroni multiple comparison test (p = 0.0695) (Table 3.4).

In contrast to the sterile matrix, the kit methods delivered better Ct-values in the non-sterile matrix compared to the crude methods. These differences were overall statistically significant in the One-Way ANOVA (p = <0.0001), as well as between methods for the Bonferroni multiple comparison test (Table 3.4). The aim of the non-sterile matrix was to test whether the methods were capable of effectively removing inhibitors from the environment. The DNeasy PowerSoil Kit had marginally better Ct-values compared to the Zymo Research Bacterial and Fungal Kit, but was significantly better than both the crude extraction methods in the Bonferroni multiple comparison (Table 3.4). The Zymo Research Bacterial and Fungal Kit only delivered significant results compared to the Heat Lysis method (p = 0.0163).

3.4.2.2. Commercial DNA extraction kit comparison

Despite the statistical significance of the quantity, this was not reflected in the Ct-values and the Dneasy PowerSoil Kit, similar to the sterile matrix results, delivered marginally better Ct-values with a mean difference of 0.2603, which was not statistically significant (p = 0.2643) (Table 3.4). The DNeasy PowerSoil Kit SD (0.34) and CV% (1.62) for the Ct-values was marginally smaller than the Zymo Research Bacterial and Fungal Kit (SD = 0.36 and CV% = 1.68) (Figure 3.2; Table 3.3). The only significant difference observed for the two kit methods were in the quantity of the Nano-drop spectrophotometry readings where the Zymo Research Bacterial and Fungal Kit delivered a significantly higher quantity (p = 0.0002).

3.4.2.3. Crude extraction method comparison

The Ct-values between the crude extraction methods were similar, the CTAB method preforming marginally better than the Heat Lysis method with a mean difference of 0.2768 that is not statistically significant (p = 0.2643) (Table 3.4). The standard deviation amongst the two

methods were the same for this matrix (SD = 0.28) and the Heat Lysis method CV% was only 0.01% lower than the CTAB method (Table 3.3). The CTAB method delivered higher quality and quantity results compared to the Heat Lysis method, but the values were not statistically significant with a p-value of >0.9999 for both these variables (Table 3.4). Thus, both methods performed equally well for this matrix type.

Non-sterile matrix results for DNA extraction methods

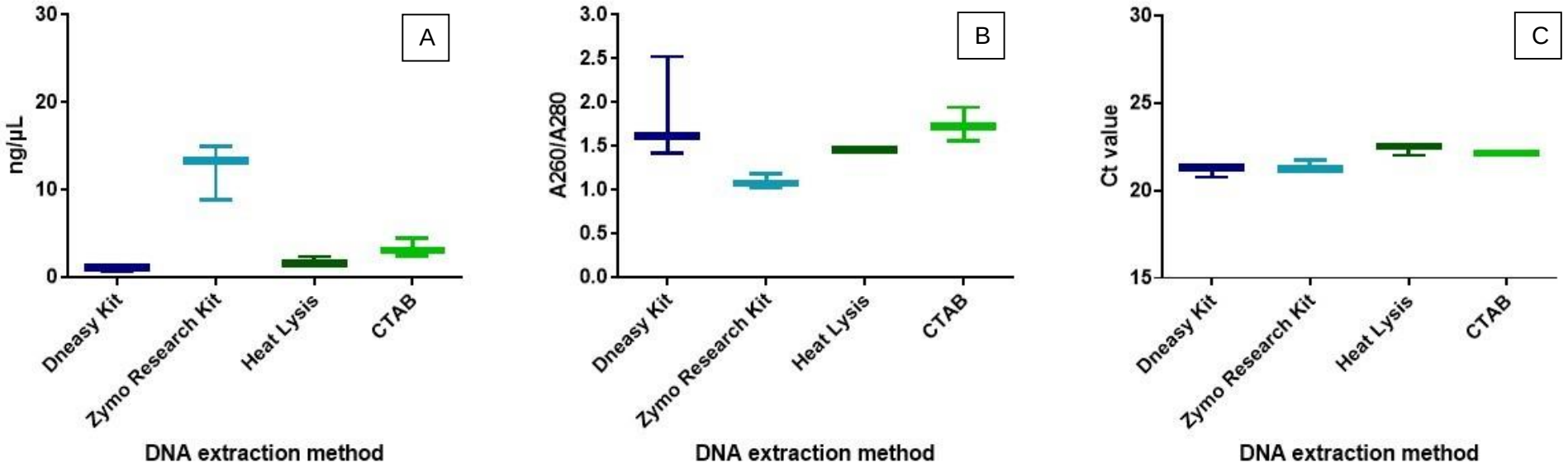

Figure 3.2. Non-sterile matrix quantity (ng/µl), quality (A260/A280) and Ct-value results for all DNA extraction methods, (A) Quantity result comparison and sample range for all DNA extraction methods, (B) Quality result comparison and sample range for all DNA extraction methods, (C) Ct-value result comparison and sample range for all DNA extraction methods.

Table 3.3. Non-sterile matrix, quantity (ng/µl), quality (A260/A280) and Ct-value means, standard deviations (SD) and coefficient of variability (CV%) for DNA extraction methods.

DNA extraction method	Mean quantity ± SD (CV%)	Mean quality ± SD (CV%)	Mean Ct-value ± SD (CV%)
Dneasy PowerSoil Kit	0.98 ± 0.28 (29.06)	1.85 ± 0.59 (32.04)	21.13 ± 0.34 (1.62)
Zymo Research Bacterial and Fungal Kit	12.37 ± 3.17 (25.67)	1.09 ± 0.08 (7.51)	21.39 ± 0.36 (1.68)
Heat-lysis method	1.74 ± 0.52 (29.95)	1.45 ± 0.02 (1.38)	22.37 ± 0.28 (1.27)
CTAB method	3.31 ± 1.01 (30.50)	1.74 ± 0.19 (10.96)	22.09 ± 0.28 (1.28)

Table 3.4. Non-sterile matrix methods comparison, using a One-Way ANOVA Bonferroni post hoc multiple comparisons test between DNA extraction methods for quantity (ng/µl), quality (A260/A280) and Ct-values (p-values in red are significant).

Quantity (ng/µl)		
Method comparison	**Mean difference**	**P-value**
DNeasy PowerSoil Kit vs Zymo Research Bacterial and Fungal Kit	-11.39	0.0002
DNeasy PowerSoil Kit vs Heat Lysis method	0.7667	>0.9999
DNeasy PowerSoil Kit vs CTAB method	-2.333	0.7781
Zymo Research Bacterial and Fungal Kit vs Heat Lysis method	10.62	0.0003
Zymo Research Bacterial and Fungal Kit vs CTAB method	9.057	0.0011
Heat Lysis method vs CTAB method	-1.567	>0.9999
Quality (A260/A280)		
Method comparison	**Mean difference**	**P-value**
DNeasy PowerSoil Kit vs Zymo Research Bacterial and Fungal Kit	0.7567	0.1098
DNeasy PowerSoil Kit vs Heat Lysis method	0.3967	0.9601
DNeasy PowerSoil Kit vs CTAB method	0.1067	>0.9999
Zymo Research Bacterial and Fungal Kit vs Heat Lysis method	-0.3600	>0.9999
Zymo Research Bacterial and Fungal Kit vs CTAB method	-0.6500	0.2089
Heat Lysis method vs CTAB method	-0.2900	>0.9999
Ct-value		
Method comparison	**Mean difference**	**P-value**
DNeasy PowerSoil Kit vs Zymo Research Bacterial and Fungal Kit	-0.2603	>0.9999
DNeasy PowerSoil Kit vs Heat Lysis method	-1.232	0.0038
DNeasy PowerSoil Kit vs CTAB method	-0.9550	0.0180
Zymo Research Bacterial and Fungal Kit vs Heat Lysis method	-0.9715	0.0163
Zymo Research Bacterial and Fungal Kit vs CTAB method	-0.6947	0.0942
Heat Lysis method vs CTAB method	0.2768	>0.9999

3.4.4. Overall results

For the overall analysis the sterile and non-sterile matrix results were first analysed in a combined analysis to determine the overall performance of the DNA extraction methods. The heat-treated matrix comparison for the matrixes is then discussed to demonstrate the loss in DNA over time within the different methods.

3.4.4.1. Overall combined sterile and non-sterile comparison

Overall, the same trends were observed when comparing the sterile and non-sterile results. The quantity values were significant due to the high values delivered by the Zymo Research Bacterial and Fungal Kit for both matrixes that was statistically significant compared to all the other methods (overall p = <0.0001) (Table 3.6). The quantities of the Heat lysis method, DNeasy PowerSoil Kit and CTAB method were very similar and yielded no statistically significant differences for any of the matrixes (Figure 3.3; Table 3.5; Table 3.6).

The overall quality in the One-way ANOVA was statistically significant (p = 0.0126). Although the Zymo Research Bacterial and Fungal Kit delivered the highest quantity results, it was at the expense of its quality, which was the lowest overall (Figure 3.3; Table 3.5). The CTAB method (p = 0.0380) and DNeasy PowerSoil Kit (p = 0.0201) both performed significantly better in their quality readings compared to the Zymo Research Bacterial and Fungal Kit (Table 3.6). Large variations could be observed in the SD and CV% values for all the methods in the quality and especially the quantity values overall (Table 3.5).

The Ct-values did not differ significantly overall for the One-way ANOVA (p = 0.1306). Neither could any significant differences be detected in the post hoc Bonferroni multiple comparison between the different methods (Table 3.6). Overall the crude extraction methods delivered better Ct-values than the kit methods, but the difference was only marginal. Both the kit methods presented more consistent results overall, with much lower SD and CV% values compared to the crude extraction methods. This was mainly due to the increased Ct-values presented by both the crude extraction methods in the non-sterile matrix. The DNeasy PowerSoil Kit delivered the most consistent data in terms of its SD (0.29) and CV% (1.39) (Table 3.5). The CTAB method delivered the highest level of variability when analysing the SD (1.95) and CV% (9.38), however the CV% still fell in what is considered an acceptable level of variation in the data set (CV% < 10).

3.4.4.2. Commercial DNA extraction kit method comparison

The Zymo Research Bacterial and Fungal Kit had significantly higher quantity values ($p = <0.0001$), however both had significantly lower quality values compared to the DNeasy PowerSoil Kit ($p = 0.0201$) (Table 3.6). The Dneasy PowerSoil Kit delivered marginally better Ct-values compared to the Zymo Research Bacterial and Fungal Kit with a mean difference of 0.4388. Overall these methods delivered very similar Ct-values for both matrixes which were not significant in the post hoc Bonferroni multiple comparison test ($p = >0.9999$) (Table 3.6). The Dneasy PowerSoil Kit did, however, deliver more consistent results in terms of the SD (0.29) and CV% (1.39) compared to the other kit (SD = 0.55 and CV% = 2.53).

3.4.4.3. Crude extraction method comparison

No significant differences could be detected in the quantity, quality nor Ct-values of the two methods and both performed equally for both matrixes as seen in the post hoc Bonferroni multiple comparison test (Table 3.9). The CTAB method presented marginally better Ct-values with a mean of 0.3176 compared to the Heat Lysis method, however, the Heat Lysis method delivered more consistent results in terms of SD (1.35) and CV% (6.42) overall compared to the CTAB method (SD = 1.95 and CV% = 9.38) for the Ct-values. Thus, both methods performed extremely similarly according to these variables and a method had to be selected based on other factors such as the variability and practicality of the methods (elaborated on in the discussion).

Overall results for DNA extraction methods

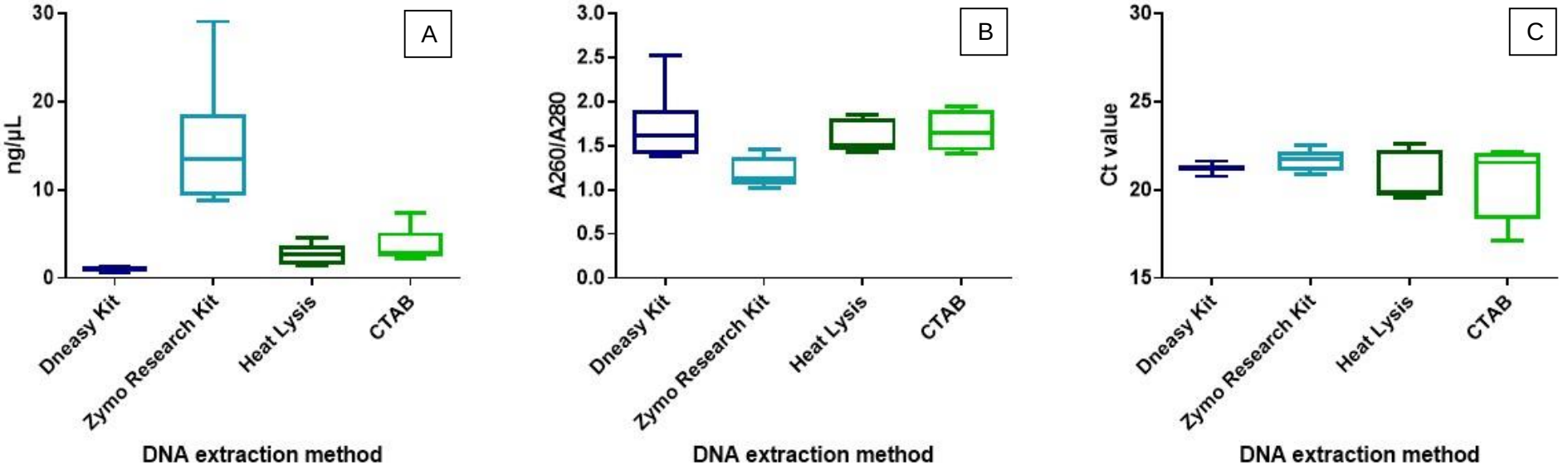

Figure 3.3. Combined sterile and non-sterile matrix quantity (ng/µL), quality (A260/A280) and Ct-value results for all DNA extraction methods, (A) Quantity result comparison and sample range for all DNA extraction methods, (B) Quality result comparison and sample range for all DNA extraction methods, (C) Ct-value result comparison and sample range for all DNA extraction methods.

Table 3.5. Overall quantity (ng/µl), quality (A260/A280) and Ct-value means, standard deviations (SD) and coefficient of variability (CV%) for DNA extraction methods.

DNA extraction method	Mean quantity ± SD (CV%)	Mean quality ± SD (CV%)	Mean Ct-value ± SD (CV%)
Dneasy PowerSoil Kit	1.10 ± 0.23 (20.55)	1.71 ± 0.42 (24.53)	21.13 ± 0.29 (1.39)
Zymo Research Bacterial and Fungal Kit	14.86 ± 7.34 (49.41)	1.19 ± 0.17 (14.59)	21.57 ± 0.55 (2.53)
Heat-lysis method	2.67 ± 1.20 (45.02)	1.59 ± 0.18 (11.56)	21.09 ± 1.35 (6.42)
CTAB method	3.68 ± 1.99 (53.94)	1.66 ± 0.22 (13.50)	20.75 ± 1.95 (9.38)

Table 3.6. Combined sterile and non-sterile comparison, using a One-Way ANOVA Bonferroni post hoc multiple comparisons test between DNA extraction methods for quantity (ng/µl), quality (A260/A280) and Ct-values (p-values in red are significant).

Quantity (ng/µl)		
Method comparison	**Mean difference**	**P-value**
DNeasy PowerSoil Kit vs Zymo Research Bacterial and Fungal Kit	-13.76	<0.0001
DNeasy PowerSoil Kit vs Heat Lysis method	-1.567	>0.9999
DNeasy PowerSoil Kit vs CTAB method	-2.583	>0.9999
Zymo Research Bacterial and Fungal Kit vs Heat Lysis method	12.20	0.0001
Zymo Research Bacterial and Fungal Kit vs CTAB method	11.18	0.0004
Heat Lysis method vs CTAB method	-1.017	>0.9999
Quality (A260/A280)		
Method comparison	**Mean difference**	**P-value**
DNeasy PowerSoil Kit vs Zymo Research Bacterial and Fungal Kit	0.5167	0.0201
DNeasy PowerSoil Kit vs Heat Lysis method	-0.1150	>0.9999
DNeasy PowerSoil Kit vs CTAB method	0.04333	>0.9999
Zymo Research Bacterial and Fungal Kit vs Heat Lysis method	-0.4017	0.1055
Zymo Research Bacterial and Fungal Kit vs CTAB method	-0.4733	0.0380
Heat Lysis method vs CTAB method	-0.07167	>0.9999
Ct-value		
Method comparison	**Mean difference**	**P-value**
DNeasy PowerSoil Kit vs Zymo Research Bacterial and Fungal Kit	-0.4327	>0.9999
DNeasy PowerSoil Kit vs Heat Lysis method	0.5863	>0.9999
DNeasy PowerSoil Kit vs CTAB method	0.9093	0.8256
Zymo Research Bacterial and Fungal Kit vs Heat Lysis method	1.019	0.5741
Zymo Research Bacterial and Fungal Kit vs CTAB method	1.337	0.1879
Heat Lysis method vs CTAB method	0.3176	>0.9999

3.4.4.4. Sterile, non-sterile and heat-treated matrix comparison results

Quantity (ng/µl)

The quantity readings did not differ significantly (p=0.2332) overall for the different matrixes or methods when analysing the Two-way ANOVA results. When comparing the quantity results between the sterile matrix and heat-treated matrix, the Dneasy PowerSoil Kit and Heat Lysis method experienced an increase in quantity and the Zymo Research Bacterial Fungal Kit and CTAB method experienced a decrease in the quantity values (Figure 3.4; Table 3.7; Table 3.8). However, the only significant change detected was for the Zymo Research Bacterial and Fungal Kit method (p = 0.0330) (Table 3.8). All the other readings in the post hoc Bonferroni multiple comparison test did not yield any significant difference in the DNA yield between the sterile, non-sterile and heat-treated matrixes for any of the methods (Table 3.8).

Quality (A260/A280)

The quality readings differed significantly in the overall Two-way ANOVA analysis (p = 0.0007). When reviewing the kit methods, no significant differences could be detected between any of the matrixes in the post hoc Bonferroni multiple comparison test (Table 3.9). In contrast, significant differences were found for both the crude extraction methods between the different matrixes. For the Heat Lysis method, a significant difference could be observed between the non-sterile and heat-treated qualities (p = 0.0090) (Table 3.9). This is due to the very high readings presented by the heat-treated matrix which had an average above 2.1, indicating possible RNA contamination (Table 3.7). Significant differences were observed between the sterile and heat-treated matrix (p = 0.0065) as well as non-sterile and heat-treated matrix (p = 0.0009) of the CTAB method (Table 3.9). This may have been caused due to the odd readings presented by the Nano-drop spectrophotometer that indicated a drop in quality and quantity for the heat-treated matrix.

Ct-values

All four of the DNA extraction methods experienced a significant increase in Ct-values, thus a decline in the DNA present, when comparing the sterile matrix to the heat-treated matrix (Figure 3.4; Table 3.10). Thus, a significant loss in DNA occurred over time when no preservation medium was applied. Although all the methods experienced an increase in their Ct-values compared to the sterile matrix this increased value demonstrated variation between

the methods and indicated that the degradation rates may have differed. The level of degradation varied from 1.737–4.090 Ct-values (Table 3.10). The Heat Lysis method was suspended in nuclease free water, as previously described in the methodology, due to the inhibiting effect of the tryptone. This medium experienced the greatest decline in DNA over time with an increase in Ct-values of 4.1 (Table 3.10). For all the other methods suspended in the tryptone broth this variation was only between 1.737–2.875. This indicates that the nuclease free water may be a weaker preservation medium compared to the tryptone.

When comparing the sterile matrix to the non-sterile matrix Ct-values for each individual method, differences could be observed between the kit methods and crude extraction methods. A significant increase in Ct-values was observed in the non-sterile matrix compared to the sterile matrix for both the crude extraction methods (Table 3.10). Whereas, the kit methods did not experience a significant change in non-sterile matrix compared to the sterile matrix (Table 3.10). This possibly indicates that the kit methods are more effective at removing the inhibitors present in non-sterile samples compared to the crude extraction methods. It should, however, be noted that the Ct-values for the non-sterile matrix, as previously indicated, does not differ significantly between the crude and kit extraction methods and all four methods performed similarly in this matrix (Table 3.4).

Matrix comparison results for DNA extraction methods

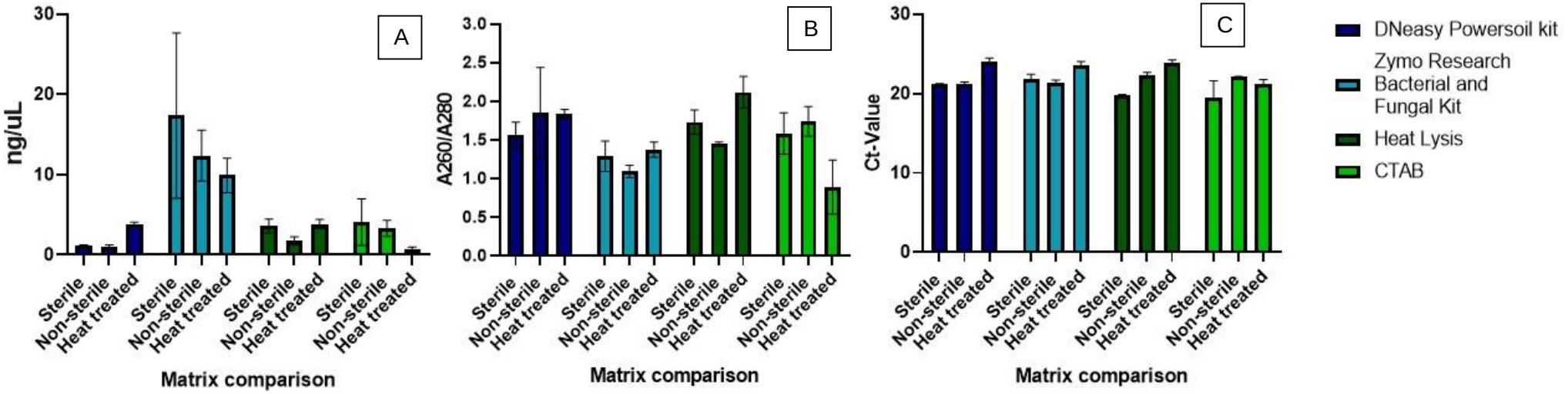

Figure 3.4. Matrix quantity (ng/µL), quality (A260/A280) and Ct-value results for all DNA extraction methods, (A) Matrix quantity result comparison and sample range for all DNA extraction methods, (B) Matrix quality result comparison and sample range for all DNA extraction methods, (C) Matrix Ct-value result comparison and sample range for all DNA extraction methods.

Table 3.7. Heat-treated matrix quantity (ng/µl), quality (A260/A280) and Ct-value means, standard deviations (SD) and coefficient of variability (CV%) for the DNA extraction methods.

DNA extraction method	Mean quantity ± SD (CV%)	Mean quality ± SD (CV%)	Mean Ct-value ± SD (CV%)
Dneasy PowerSoil Kit	3.77 ± 0.32 (8.41)	1.84 ± 0.06 (3.19)	24.00 ± 0.43 (1.78)
Zymo Research Bacterial and Fungal Kit	9.90 ± 2.15 (21.71)	1.37 ± 0.10 (7.29)	23.57 ± 0.45 (1.92)
Heat-lysis method	3.83 ± 0.57 (14.83)	2.12 ± 0.20 (9.65)	23.90 ± 0.35 (1.47)
CTAB method	0.64 ± 0.31 (48.07)	0.89 ± 0.35 (39.36)	21.16 ± 0.58 (2.75)

Table 3.8. Sterile, non-sterile and heat-treated matrix comparison for Quantity values (ng/µl) using a two-way ANOVA Bonferroni post hoc multiple comparisons test between matrix types (p-values in red are significant).

DNeasy PowerSoil Kit		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	0.2467	>0.9999
Sterile vs Heat-treated	-2.543	>0.9999
Non-sterile vs Heat-treated	-2.790	0.9386
Zymo Research Bacterial Fungal Kit		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	4.993	0.2322
Sterile vs Heat-treated	7.457	0.0330
Non-sterile vs Heat-treated	2.463	>0.9999
Heat Lysis		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	1.847	>0.9999
Sterile vs Heat-treated	-0.2433	>0.9999
Non-sterile vs Heat-treated	-2.090	>0.9999
CTAB		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	0.7467	>0.9999
Sterile vs Heat-treated	3.413	0.6581
Non-sterile vs Heat-treated	2.667	>0.9999

Table 3.9. Sterile, non-sterile and heat-treated matrix comparison for Quality (A260/A280) using a two-way ANOVA Bonferroni post hoc multiple comparisons test between matrix types (p-values in red are significant).

DNeasy PowerSoil Kit		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	-0.003667	0.5204
Sterile vs Heat-treated	-2.875	0.5657
Non-sterile vs Heat-treated	-2.871	>0.9999
Zymo Research Bacterial Fungal Kit		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	0.3760	>0.9999
Sterile vs Heat-treated	-1.796	>0.9999
Non-sterile vs Heat-treated	-2.172	0.5204
Heat Lysis		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	-2.554	0.5351
Sterile vs Heat-treated	-4.090	0.2027
Non-sterile vs Heat-treated	-1.536	0.0090
CTAB		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	-2.670	>0.9999
Sterile vs Heat-treated	-1.737	0.0065
Non-sterile vs Heat-treated	0.9328	0.0009

Table 3.10. Sterile, non-sterile and heat-treated matrix comparison for Ct-values using a two-way ANOVA Bonferroni post hoc multiple comparisons test between matrix types (p-values in red are significant).

DNeasy PowerSoil Kit		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	-0.003667	>0.9999
Sterile vs Heat-treated	-2.875	0.0003
Non-sterile vs Heat-treated	-2.871	0.0003
Zymo Research Bacterial Fungal Kit		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	0.3760	>0.9999
Sterile vs Heat-treated	-1.796	0.0215
Non-sterile vs Heat-treated	-2.172	0.0048
Heat Lysis		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	-2.554	0.0010
Sterile vs Heat-treated	-4.090	<0.0001
Non-sterile vs Heat-treated	-1.536	0.0571
CTAB		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	-2.670	0.0006
Sterile vs Heat-treated	-1.737	0.0269
Non-sterile vs Heat-treated	0.9328	0.4196

3.5. Discussion

3.5.1. Overall analysis

Large variations were presented in the Nano-drop spectrophotometry readings, which may be due to the low concentration of DNA present in all of the samples. Sample concentrations are considered low when they are less than 50ng/µl (Koetsier and Cantor, 2019), and all the samples for all the methods in this chapter yielded concentrations below this value. This results in greater variations in the data sets, that provides the larger CV% values for both the quantity and the quality readings. The quantity readings indicated the greatest level of variation for the data sets and did not always correlate with the Ct-values presented. In the study by Psifidi *et al*. (2015), eleven DNA extraction methods were compared based on the quality and Ct-values. The quality of the sample is often more important that the quantity of the DNA present. qPCR assays only require a small quantity of DNA to successfully amplify, but the presence of inhibitors can severely affect the Ct values (Walsh *et al*., 2013). Thus, the quality of the samples and the Ct-values generated carried a greater weight in the method selection of this study. The CV% of the Ct-values for the majority of the DNA extraction method and matrix type combinations, fell within the normal range for intra-assay variation when compared to previous literature (Abera *et al*., 2014; Balamurugan *et al*., 2010; Hyatt *et al*., 2007). The only exception to this range was the CTAB method in the sterile matrix, which presented a high CV%, indicating a lower level of reproducibility.

For this study, the sterile and non-sterile matrixes present the greatest representation for the effectiveness of the DNA extraction methods. Due to all the samples possessing the same known number of zoospores, the differences in the Ct-values between the methods can provide an indication of how effectively DNA is extracted as well as how well inhibitors are removed from the samples. This provides insight into the possible limiting factors within each of the tested methods. The sterile matrix tests how effectively the methods extract the DNA as well as the Ct-values that would normally be expected without inhibitors. Whereas the non-sterile matrix tests whether the methods are capable of effectively removing inhibitors, by observing if increases in Ct-values occur due to the environmental samples.

In the overall combined comparison of the Ct-values including both the non-sterile and sterile matrix, the crude extraction methods delivered lower Ct-values compared to the kit methods. Lower Ct-values indicated higher numbers of DNA copies which is favourable. This is due to the crude extraction methods performing better in the sterile matrix. Indicating that when no-inhibitors are present the crude extraction methods are capable of extracting DNA more effectively compared to kits. Commercial kit methods are known for yielding lower quantities of DNA due to the multiple cleaning steps applied, but often yield high quality DNA (Barbier *et*

al., 2019). The benefits of this can be seen when analysing the non-sterile matrix. The non-sterile matrix contained inhibitors that would typically be associated with an environmental sample. Both the commercial DNA extraction kit methods performed better than the crude extraction methods with regards to Ct-values, potentially indicating the better removal of inhibitors. Despite the low quality readings from the Zymo Research Bacterial and Fungal Kit, this method still presented better Ct-values than the crude extraction methods. Significant increases in Ct-values were detected for both crude extraction methods for the non-sterile matrix when compared to the sterile matrix.

The main purpose of the heat-treated matrix was to determine whether significant decreases in DNA can be observed for the methods over the period of a week if no preservation medium is applied. According to previous literature, deceased cells can persist for longer than two weeks if presented with the ideal environmental conditions (Schmidt *et al*., 2013). These degradation rates may, however, differ between species and vary based on the environmental conditions present (Alan *et al*., 2021).

All four of the DNA extraction methods experienced a significant increase in Ct-values over the period of a week. The normal standard curve for a dilution series has a gradient of 3.3 (Taylor *et al.*, 2010). This implies that for every 3.3 Ct-value increase observed, a 10-fold loss in DNA is experienced. The samples stored in the 1% tryptone broth experienced losses less than 3.3 Ct-values, however these losses were still considered significant when compared to the sterile matrix. In the Heat Lysis method, heat-treated matrix was prepared using nuclease free water due to the inhibiting effect of the 1% tryptone on this method and experienced much greater declines in the Ct-values compared to the other methods. The 1% tryptone was found to be a better preservation medium compared to the nuclease free water. The concentration of eDNA in the environment is extremely low and decreases of this magnitude can potentially reduce the efficiency of the eDNA protocol being developed. This experiment indicated that significant quantities of DNA can be lost over a period of a week, thus, the use of preservation mediums in filter material was tested in Chapter 4.

3.5.2. Commercial DNA extraction kit method selection

Although the Dneasy PowerSoil Kit delivered the lowest quantity DNA overall, it delivered the highest quality DNA. This method also showed in the non-sterile matrix to effectively remove inhibitors from the environment which yielded the highest Ct-values of all the methods. The Zymo Research Bacterial and Fungal Kit constantly presented high quantity and low quality values, which may be caused due to the presence of protein contamination (Koetsier and Cantor, 2019). Ideally the A260/A280 should be between 1.8–2.1 to indicate high quality DNA

(Koetsier and Cantor, 2019; Psifidi *et al*., 2015). Protein contamination can have a significant effect on the A260/A280 reading when the concentration of the samples is low (Koetsier and Cantor, 2019). As previously mentioned in the literature review, kits are well known for yielding high quality DNA but may not always effectively remove some organic compounds (Barbier *et al*., 2019). This may have been the case for the Zymo Research Bacterial and Fungal Kit, and additional cleaning steps or kits can potentially be applied at the end of the protocol to aid in removing these compounds to improve quality values.

Despite the lower quality of the samples, the Zymo Research Bacterial and Fungal Kit still delivered Ct-values that did not differ significantly from the DNeasy PowerSoil Kit for any of the matrixes nor overall. Thus, this method still removed qPCR inhibitors effectively when compared to the DNeasy PowerSoil Kit. The Dneasy PowerSoil Kit, however, delivered the most consistent results in terms of the Ct-values within all the matrixes with a lower SD and CV% compared to all the methods and overall. This method also delivered good quality results in terms of the A260/A280 readings, which were between 1.8–2.1 for two of the three matrixes, and thus does not require any additional cleaning steps. This correlates with the findings of previous research that claimed the DNeasy PowerSoil Kit delivers lower quantity values, but effectively removes inhibitors and produces high quality results (Tsuji *et al*., 2019). The effective removal of inhibitors is crucial to avoiding false negatives in eDNA samples (Stoeckle *et al*., 2017). This makes the DNeasy PowerSoil Kit more favourable compared to the the Zymo Research Bacterial and Fungal Kit for *Bd* DNA extractions. Thus, based on the results, the selected kit method for this study is the DNeasy PowerSoil Kit.

3.5.3. Crude extraction method selection

Overall the Ct-values, quantity, and quality did not vary significantly between the Heat Lysis and CTAB methods, however the Heat Lysis method delivered more consistent results within the sterile matrix type and overall for this study. Thus, the Heat Lysis method demonstrated more reliable and quantifiable results when presented with specific conditions compared to the CTAB method. Certain reagents, such as Proteinase K in the CTAB method and other organic solutions, may reduce the effectiveness of subsequent reactions and some residues of these chemicals may remain when transferring samples during the various steps (Chi *et al*., 2009). The higher level of variability may also be caused due to the multiple transferring steps of the supernatant in the CTAB method compared to the Heat Lysis method which requires minimal transfers (Chi *et al*., 2009; Walsh *et al*., 2013).

It should, however, be noted that the CTAB method delivered more consistent results in the non-sterile matrix, which were similar to the Heat Lysis method. The non-sterile matrix

provides the most accurate analysis for what can be expected from the field results and due to the very similar results for both methods in terms of quality, quantity and Ct-value, both would be equally suitable for this application. A different method of evaluation had to be used in order to select the method and the practicality and cost of each method was evaluated. The CTAB method took twice as long to perform compared to the Heat Lysis method and also applied chloroform, which is known to be a hazardous substance (Barbier *et al*., 2019; Ferencova *et al.*, 2017; Piggot, 2016). The CTAB method is more cost-intensive regarding the number of reagents required per sample.

The Heat Lysis method appears to be more reliable, delivers more consistent results within each matrix, is less time consuming, more cost-effective, and does not apply hazardous chemicals. For these reasons the Heat Lysis method was selected as the method of choice for the crude extraction methods.

3.5.4. Future recommendations

The Nano-drop spectrophotometry readings indicated high CV% values that did not always correlate with the Ct-value readings. This was especially true for the quantity readings which varied greatly amongst all of the samples. Although the quality readings sometimes correlated with the variability in the data, great levels of variability were still detected within these datasets that falls above the acceptable CV% limit. Nano-drop spectrophotometry has shown to be less accurate at lower concentrations of DNA and produces results with high levels of variability at these levels (Koetsier and Cantor, 2019). This was very prominent for the study being conducted. Low concentrations of DNA were tested throughout this project and for this reason the Nano-drop readings were omitted in the subsequent chapter. The Ct-values for the different samples delivered much more reliable results for comparison between the methods and is also the end parameter that will be applied to field application. Thus, optimisation of Ct-values is considered a higher priority for this study.

Nano-drops have shown to not only measure the DNA or RNA present, but also contaminants and other structures present (Garcia-Elias *et al*., 2017). It is recommended to use a different method for the quality and quantity measurements for future studies. Other fluorometric techniques, such as the Qubit 2.0 Fluorometer are often recommended for a more accurate analysis over Nano-drop spectrophotometry (Garcia-Elias *et al*., 2017).

In this study the different matrixes were done on different qPCR runs and matrixes were compared on the assumption that the efficiencies did not vary significantly between the plates. This, however, is not always the case. Even though the primers selected for this project have

proven accuracy and delivered similar efficiencies and results across plates (Blooi *et al*., 2013; Hyatt *et al.,* 2007), the efficiency may still vary between plates. It would be recommended for future studies to run all the samples on the same plate, if possible, in order to remove inter-plate variations more effectively and allow for a more accurate comparison between samples.

3.6. Conclusion

Each step of the eDNA protocol must first be optimised prior to applying the assay to field sampling. After water samples have been collected from the field, the next step in the protocol is extracting the DNA from the filter media. Not all DNA extraction methods are equally effective at extracting DNA from target organisms. A variety of extraction methods must first be tested to set the base standard to which future tests can be compared and determine the most effective method to deliver high quality and quantity DNA for the qPCR process. This chapter aimed to test two extraction kit methods and two crude extraction methods under various conditions to determine the most appropriate two methods to apply to the subsequent filter tests for the protocol.

Overall, the DNeasy PowerSoil Kit delivered a lower quantity but a higher quality of DNA compared to the Zymo Bacterial and Fungal Kit. Although quantity is important, quality can have a more significant impact on the Ct-values and the lower quality may potentially indicate protein contamination in the samples. Despite this the Zymo Research Bacterial and Fungal Kit delivered Ct-values that did not differ significantly from the DNeasy PowerSoil Kit, but the Dneasy PowerSoil Kit delivered more consistent results throughout all of the matrixes and overall. This makes the DNeasy PowerSoil Kit a more reliable and consistent method and can potentially be used to quantify the results from the field with a higher level of accuracy.

The crude extraction methods were mainly compared based on the sterile and non-sterile samples due to the different resuspension methods applied in the heat-treated matrix. The CTAB and Heat Lysis method performed very similarly in terms of their Ct-values, quality and quantity. The Heat Lysis method was selected as the method of choice based on the lower standard deviations in the results which indicate a higher consistency between the samples. This method was also more cost-effective, less time intensive, and did not include the use of hazardous chemicals.

Although the Ct-values, quality and quantity varied significantly between the kit and crude extraction methods, the variations were much less significant when comparing only kit and only crude extraction methods and the methods of choice were not slected based on statsitcial significance. Thus, the H-1 hypothesis was rejected. The methods of choice were rather selected based on their standard deviation, CV% and practicality, which aligns with the H-0 hypothesis.

It would be recommended in the future to use a different method to measure the quantity and quality of the DNA samples. Great variations were observed in the Nano-drop spectrophotometry readings and was mainly caused due to the low quantity of DNA applied in the study, thus a more sensitive method of measurement is required. Also to improve the

accuracy for the matrix comparison Ct-value analysis it would be recommended to run all samples on the same plate rather than compare the matrix types from different plates.

Chapter 4: Filter tests and protocol standards

4.1. Introduction

The first step in eDNA diagnostics is the collection of samples from the environmental source. When developing an eDNA protocol there are many key factors that need to be considered such as the method of sampling, the preservation of DNA and the DNA extraction method (Pilloid *et al*., 2013; Renshaw *et al*., 2015). In Chapter 3 one of these factors were addressed through testing multiple DNA extraction methods to determine their effectiveness on the target organism. This chapter aims to analyse the different environmental sampling methods, preservation mediums and extraction protocols that can be utilised to develop the most optimal diagnostic assay under laboratory conditions. The selected DNA extraction methods will be applied and optimised for the extraction of DNA from filter material. The lowest detection limit as well as standards will also be set to limit the possibility of false positives or negatives in the field.

The concentration of eDNA tends to be incredibly low within the environment, thus, selecting the appropriate filtration method to concentrate the DNA on filter media is essential to the successful detection of organisms (Li *et al*., 2018). No filtration, preservation or DNA extraction protocol is universal and must be adjusted according to the target organism and their specific habitats. In this section of the chapter, the different sampling methods that exists for eDNA, and the types of preservation methods that can be used will be reviewed. The possible limiting factors when sampling eDNA as well as setting standards for the assay will also be addressed.

4.1.1. Methods of filtration

There are two well-known methods for sampling eDNA from aquatic environments, namely filtration and precipitation (Rupert *et al*., 2019; Tsuji *et al*., 2019; Wittwer *et al*., 2018). Both these sampling methods present numerous benefits and limitations. Precipitation is a very cost-effective method of collecting eDNA, however, uses a much smaller quantity of water compared to filtration (Piggot *et al*., 2016; Tsuji *et al*., 2019). In this method, a small quantity of water, for example 15ml, is precipitated using ethanol or isopropanol (Hinlo *et al*., 2017; Tsuji *et al*., 2019). An alternative method makes use of sodium acetate along with ethanol (Ficetola *et al*., 2008; Peixoto *et al*., 2021; Piggot, 2016). Precipitation is effective when sampling from remote or difficult to reach places but is very limited due to the small sample volume that is processed (Tsuji *et al*., 2019). In regards to the current study, this method is unlikely to be very effective at pathogen detection because of the low concentration of eDNA available in the environment. Previous studies have indicated that precipitation can deliver

excellent results where high eDNA copy numbers are present but recommend filtration when limited copy numbers are expected (Minamoto *et al*., 2016).

Filtration has previously been applied in multiple *Bd* and amphibian eDNA studies (Brannely *et al.*, 2020; Peixoto *et al.*, 2021; Walker *et al*., 2007). In the filtration method, a filter medium is used through which water flows, the DNA is then trapped and extracted from the filter media (Hinlo *et al*., 2017). Filtration is one of the most applied methods for eDNA studies due to its capability to filter larger volumes and often yields more DNA than precipitation, (Majaneva *et al*., 2018; Piggot, 2016; Renshaw *et al*., 2015; Rupert *et al*., 2019; Tsuji *et al*., 2019). There are, however, several factors to consider when using filtration, such as the type of filter material, pore sizes and filter volumes. Methods have to be adjusted based on the target organism. All of the previously mentioned considerations are further addressed in section 4.1.3.

4.1.2. Filter preservation

Due to the rapid degradation rates often observed in DNA, it is recommended to filter the samples directly from the source and use a preservation medium, rather than transporting water samples to be analysed at a later stage (Kumar *et al*., 2020). Studies have shown that DNA degradation can occur in as little as 48 hours, thus an appropriate preservation medium or method must be selected to prevent possible DNA loss (Kumar *et al*., 2020). When reviewing the results from Chapter 3, all four the DNA extraction methods showed to have a significant loss in DNA over a period of a week and a preservation medium would be required for the successful sampling of the target organism. A wide array of methods and chemicals can be applied to preserve eDNA after filtration. Some of the most common methods are ethanol fixation, lysis buffers, freezing and silica gel/beads (Majaneva *et al*., 2018). Selecting the appropriate method can significantly affect the number of DNA copy numbers that can be extracted from the filters.

Silica gel beads have been applied in various previous eDNA studies for the preservation of filter media (Allison *et al*., 2021; Haakon *et al*., 2019; Majaneva *et al*., 2018). This preservation method has shown to successfully preserve eDNA with minimal loss over a month and delivers very consistent results (Allison *et al*., 2021; Majaneva *et al*., 2018). To prevent any significant decreases in DNA for a longer period of time the samples would have to be stored at -20°C (Allison *et al*., 2021). To apply this method, dry filter materials are stored on silica gel beads either directly or in a petri dish and placed in the zip lock bag containing the silica beads (Haakon *et al*., 2019; Majaneva *et al*., 2018).

Chilling or freezing samples is often used for eDNA preservation of filter materials and water samples (Kumar *et al*., 2020). Freezing samples will reduce the rate of degradation but does not prevent degradation (Kumar *et al*., 2020; Takahara *et al*., 2015). Freezing potentially affects the amount of eDNA present through the freezing and thawing process resulting in a loss of DNA (Kumar *et al*., 2020; Takahara *et al*., 2015; Williams *et al*., 2016). The sampling sites selected for this project occur within remote regions that often do not have electricity available, and previous studies have also stated that freezing is not an ideal method for sampling remote regions (Renshaw *et al*., 2015; Wegleitner *et al*., 2015).

Ethanol, ranging from 70% to 95%, has proven to be sufficient for eDNA preservation in multiple studies and has even previously been used for the preservation of *Bd* swabs and amphibian tissue samples (Piggot, 2016; Rees *et al*., 2014; Soto-Azat *et al*., 2009; Talley *et al*., 2015). This method has also shown over time to preserve DNA better than freezing samples and can limit DNA degradation for up to 172 days (Allison *et al*., 2021; Thomas *et al*., 2019). This preservation medium is considered a cost as well as time effective method of eDNA preservation (Hinlo *et al*., 2017). This is due to it not requiring the addition of other ingredients and ethanol is easily accessible (Hinlo *et al*., 2017).

The Longmire's Lysis Buffer has been used in many studies with remarkable success (Kumar *et* al., 2020; Mauvisseau *et al.,* 2021; Renshaw *et* al., 2015; Wegleitner *et al*., 2015). Originally this buffer was developed for the long-term preservation of tissue samples (Williams *et al*., 2016). In more recent years this method has been applied for eDNA applications (Williams *et al*., 2016). This buffer coupled with cellulose nitrate filters and the DNeasy Blood and tissue Kit has shown to be one of the most utilised methods for the successful capture of eDNA (Kumar *et al*., 2020). The Lysis Buffer has also shown to deliver reliable results with lower variation when compared to freezing and other buffers such as Sarkosyls buffer (Mauvisseau *et al.,* 2021). Previous studies using Longmire's Lysis Buffer have showed that eDNA can remain on filter material for up to 14 days at room temperature without any significant loss in DNA (Renshaw *et* al., 2015). Other studies have shown DNA can be preserved at room temperature for up to 150 days without any significant losses (Wegleitner *et al*., 2015).

4.1.3. Limitations and considerations

Many filtration methods require a source of power whether it be electrical or battery powered. During fieldwork electricity may not always be available or appliances cannot be charged between samples. Water can be collected in containers and filtered at a later stage if required, but as previously stated this is not preferable due to the risk of DNA loss (Kumar *et al*., 2020). In a previous study, *Bd* infected individuals were placed in a water bath and filtered to

determine the effectiveness of filtration as a diagnostic method compared to conventional assays (Hyatt *et al*., 2007). The number of zoospores detected decreased by 70% compared to conventional methods and the significant decrease in zoospores may be attributed to the spores attaching themselves to the surface of the container (Hyatt *et al*., 2007). It was recommended that if water is sampled in containers that they be centrifuged or mixed prior to filtering to increase the number of zoospores captured (Hyatt *et al*., 2007). Not all filtering methods require electricital equipment to be performed and alternatives exist to allow for the filtration in the field. Previous studies have used 50ml or 100ml syringes to draw water through filter material and present a very cost-effective alternative to expensive machinery or devices (Walker *et al*., 2007; Muha *et al*., 2019).

The filtration method selection depends on the target organism as well as the filter pore size required and varies between studies (Rupert *et al*., 2019). Smaller filter sizes collect higher concentrations of eDNA, however, increase the time required for filtering due to clogging occurring much quicker and water filtering slower through the material (Barnes *et al*., 2021; Lacoursière-Roussel *et al*., 2016; Li *et al*., 2018). Finding the appropriate ratio between pore size and filtration time is important to improve the effectiveness of the protocol. The pore size depends on the size of the target organism, for example bacteria and other micro-organisms tend to be smaller than 0.5µm are usually filtered with 0.2µm filter pores (Djurhuus *et al*., 2017), whereas macro-invertebrate eDNA particles and cells tend to be bigger and are filtered with pore sizes ranging from 1–10µm (Tsuji *et al*., 2019; Turner *et al*., 2014). *Bd* zoospores vary in size from 1–5µm and in previous studies pore sizes for filtration of the target organism have ranged from 0.2–1µm in aquatic environments (Farthing *et al.*, 2020; Hyatt *et al*., 2007; Kirstein *et al*., 2007; Sieber *et al*., 2020; Walker *et al*., 2007). The type of filter material being used can also affect the concentration of eDNA collected (Tsuji *et al*., 2019). Cellulose nitrate filters have shown to have great success in capturing eDNA from aquatic environments and is one of the most applied filter types (Kumar *et* al., 2020; Majaneva *et al*., 2018).

Although eDNA assays can provide valuable insights to the presence of certain species, false negatives and positives have also been reported in multiple studies and this is due to a wide variety of factors (Schmidt *et al*., 2013). Cross-contamination may occur between sites if the equipment being used is not properly decontaminated prior to each sampling (Goldberg *et al*., 2016). Handled and processed samples must also be kept separate from where the PCRs are being conducted (Deiner *et al*., 2015; Goldberg *et al*., 2016). Standards must be set to limit the possibility of false positives and negatives.

4.1.4. Setting standards

It is important to understand the lowest detection limit (LOD) of the assay. Previous studies have shown that the lowest detection between assays tend to vary significantly (Roussel *et al*., 2015). This may ultimately determine whether the assay is sensitive enough to be utilized for an eDNA assay, as well as prevent the possibility of false positives or negatives. qPCR assays are considered one of the most popular detection methods for eDNA due to its cost-effectiveness and its ability to properly set standards (Langlois *et al*., 2021). To develop a robust detection method for eDNA multiple factors must be considered including the sensitivity of the assay, the specificity of the primers to detect the target organisms and primer efficiency (Langlois *et al*., 2021). Contamination from nucleic acids can potentially cause false positives at lower concentrations (Taylor *et al*., 2019). Thus, it is important to understand what the lowest limit of detection (LOD) for the developed assay is as well as to include non-template controls (NTC's) to ensure that when amplifications occur, they are true amplifications and not false positives (Taylor *et al*., 2019).

Some DNA studies are very stringent when setting their standards such as in the study by Kirshtein *et al*. (2007) conducted on *Bd*, which stated that any Ct-value above 35 was considered negative and excluded whether all PCR replicates amplified or not. Previous unpublished studies conducted on ventral swabs of amphibians at the Herpetological Health Lab (HHL) deemed Ct-values above 37 as negative. These were however for swabs and a different standard may need to be set for the case of eDNA.

According to MIQE guidelines to determine the LOD, 95% of the samples should amplify per concentration in the standard curve, however this is also considered extremely strict for eDNA assays (Hunter *et al*., 2017). Some studies follow the guidelines set by the MIQE, because it delivers results with the highest level of confidence but limits the protocol regarding the low DNA copy numbers (Guan *et al*., 2019; Polinski *et al*., 2015). The LOD according to MIQE guidelines does not necessarily mean that Ct-values below this limit are false positives (Kralik *et al.*, 2017). It implies that values amplified below this threshold are less likely to be amplified in multiple replications. Thus, the repeatability of the sample to produce a positive result is reduced at lower concentrations and may be excluded or deemed negative (Kralik *et al.*, 2017). When the concentration of DNA is very low, as for eDNA, variation from pipetting may even result in no template being present in a sample during the qPCR step (Hunter *et al*., 2017). Thus, samples that are expected to have low concentration may potentially not comply with the MIQE standards and many less stringent methods have been developed for eDNA assays (Hunter *et al*., 2017).

Studies utilising eDNA tend to follow their own method for determining the cut-off point as well as the LOD and various approaches have been used in previous studies. In the study by González *et al*. (2021) a sample was considered positive if both PCR replicates amplified and had a Ct-value below 40. In the study by Roux *et al*. (2020) the LOD was set as the concentration where 50% or more of the samples delivered a positive Ct-value. Other studies require as little as a single positive amplification out of three to eight replicate qPCR samples and run twice on the machine to be considered positive (Biggs *et al*., 2015; Rees *et al*., 2014). In the studies by Takahara *et al*. (2013) and Agersnap *et al*. (2017), the lowest detection limit was determined as where at least one well for each replicate sample delivered a positive value in the qPCR assay. The LOD has also been defined as the lowest level where one or more replicates of the qPCR assay delivered a positive amplification (Davison *et al*., 2019; Ostberg *et al*., 2018).

There is a difference between the LOD of an assay and the Lowest Limit of Quantification (LOQ). The LOD refers to the lowest number of DNA copies that can be amplified with a level of confidence, whereas the LOQ refers to the level at which a sample can be confidently quantified with a high level of certainty and accuracy (Davison *et al*., 2019; Forootan *et al*., 2016). Both these factors are important in eDNA diagnostics. The LOQ tends to vary between studies, some referring to the CV% of the samples to determine the lowest dilution within an acceptable range of variation (Forootan *et al*., 2016; Klymus *et al*., 2020). Others define it as the value where the 100% of the samples in a dilution amplify (Agersnap *et al*., 2017; Davison *et al*., 2019; Ostberg *et al*., 2018; Roux *et al*., 2020).

4.2. Hypothesis, aim and objectives

4.2.1. Hypothesis

H0 – No significant differences will be observed between the DNA extraction methods coupled with preservation mediums over time, and other factors will need to be considered such as practicality and repeatability in the method selection.

H1 – Significant differences will be observed between the DNA extraction methods coupled with preservation mediums over time and a clear method can be selected based on the statistical data.

4.2.2. Aim

Determine the most appropriate filtration method to apply to the DNA extraction methods as well as test two DNA extraction methods coupled with preservation mediums over multiple time periods to determine the most optimal protocol for future field sampling.

4.2.3. Objectives

- Test three filtering protocols to determine the most practical method for remote field sampling.
- Test two preservation mediums on filter media and compare the selected crude and kit DNA extraction methods over three time periods.
- Determine the efficiency of the *Bd* primers using a G-Block dilution series and zoospore filter dilution series.
- Set standards for the protocol through determining the lowest limit of detection (LOD), lowest limit of quanitification (LOQ) and cut-off Ct-value of the assay.

4.3. Materials and methods

For this chapter, three filtration methods were tested during a field experiment and one method was selected for further application and optimisation for laboratory experiments. Samples often cannot be processed immediately following collection and a preservation medium must be applied to the filters. Two preservation mediums were selected, and DNA was extracted using the selected DNA extraction methods from Chapter 3, namely the DNeasy PowerSoil Kit and Heat Lysis method. The preservation mediums were tested in three intervals over a period of a week to determine whether any significant losses in DNA would occur over time. The possible qPCR inhibitory factors of the preservation mediums on the extraction methods were also tested. The lowest detection limit of the assay was determined using a G-Block dilution series as well as zoospore dilution series on filter material.

4.3.1. Filter methods

The three selected filtration methods were the drill filter, Continuous Low-level Aquatic Monitoring (C.L.A.M.), and the syphon pump method. These methods were mainly tested in the field to determine time investment, optimum water volume before filter clogging occurred, and the practicality of the methods for application in remote regions. The benefits and limitations of the methods were compared to determine appropriateness for future field sampling and subsequent testing and optimising of the sampling assay in the laboratory. Five samples were taken using each of the methods in an attempt to filter approximately 1l of water per sample or until clogging of the filter occurred.

4.3.1.1. Drill filter

This method was selected based on its previous eDNA applications for aquatic pathogen detection (Haakon *et al*., 2019). This filtration method was able to successfully detect the host specimen in every sample and occasionally the selected pathogen (Haakon *et al*., 2019). The lack of detection of the pathogen was, however, not attributed to the filtration method but rather because the prevalence of the pathogen within the environment was below the detection limit of the assay (Haakon *et al*., 2019). This method uses a drill to power a pump that creates a vacuum that draws water through the filter media. The following components were used to construct the filter; a filter cup (Biotechnology Hub Africa. Cat# 145-2045), tubing, a drill powered pump (Gardena, Electric Drill Pump, no 1490-20) and a power drill (Figure 4.2). Filter cups were changed with every sample taken. It is considered a cost-effective alternative to commercial filtration devices and could potentially be applied directly in the field.

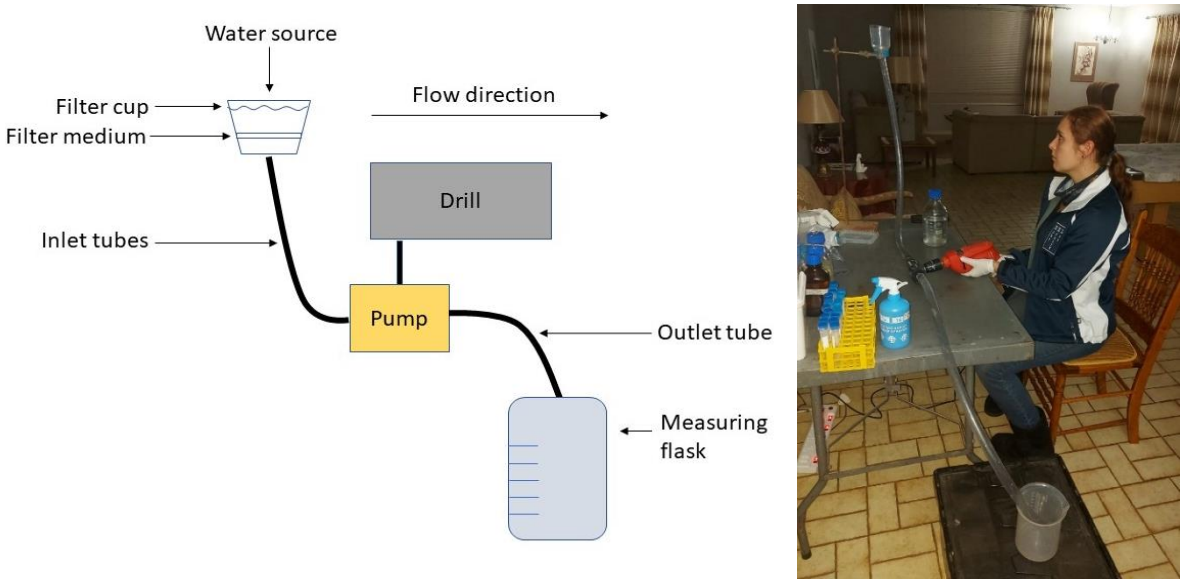

Figure 4.1. Diagram for drill filter method and application in the field.

4.3.1.2. Continuous Low-level Aquatic Monitoring (C.L.A.M.)

The C.L.A.M. is a commercial filtration product developed by Aqaulitical. Originally this product was developed to test for organic solutions in the upper phase of water bodies (Ensminger *et al*., 2017). This method applied a solid phase extraction (SPE) disk to capture organic particles which are then recovered from the discs through elution (Ensminger *et al*., 2017). According to the manufacturers this method can filter up to 100l of water, but filters water at a low flow rate of 5-80ml/minute (Aqualitical, 2021). This product has a built-in battery that can last up to 36 hours (Aqualitical, 2021). Due to filter material being encased, it is recommended to freeze the discs to preserve samples (Aqualitical, 2021).

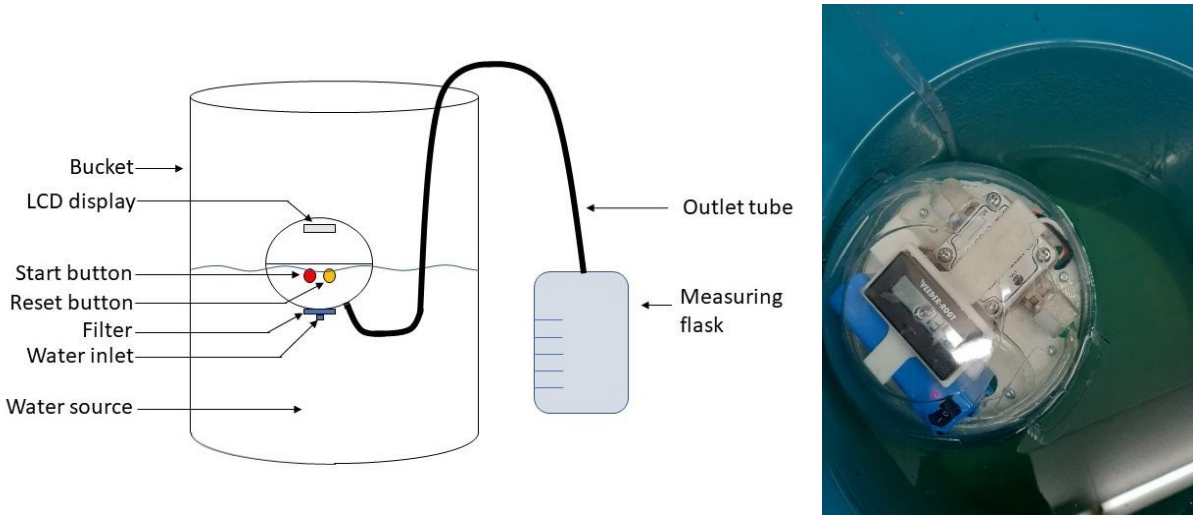

Figure 4.2. Diagram for Continuous Low-level Aquatic Monitoring (C.L.A.M) method and application in the field.

4.3.1.3. Syphon pump

The syphon pump method was the most simplistic of the three methods and requires no electricity for filtration. The design of this method was inspired by the method used by Walker *et al*. (2007), which applied a 50ml syringe to create a vacuum to filter the water. The syphon pump method consisted of the MAC AFRIC Manual Liquid and Air Siphon pump, two pipes and a filter cup (Figure 4.3). This method was the most cost-effective and very easy to acquire. Using the handheld pump, a vacuum was created which drew the water through the filter material. This method could filter larger quantities of water quicker and with less effort compared to the syringe method. This method was excellent for direct application in the field due to its light weight and portable size that could easily be carried over great distances to remote regions. It should be noted this protocol required much more manual labour compared to the previous two methods described.

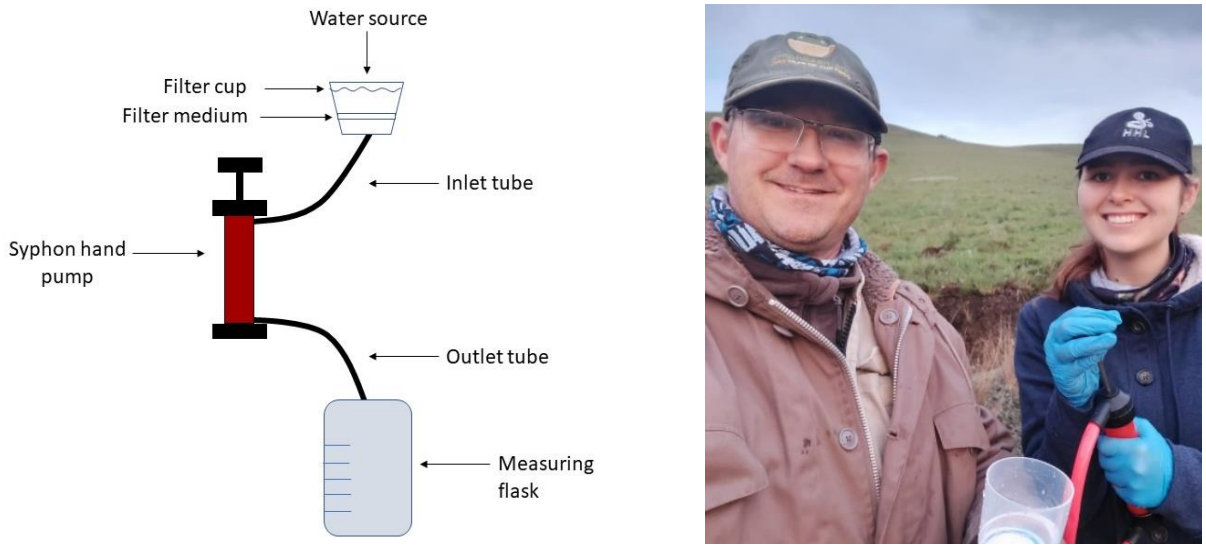

Figure 4.3. Diagram for syphon pump filtration method and application in the field.

4.3.2. Filter tests

As discussed in Chapter 3, all the methods demonstrated significant declines in their Ct-values for the heat-treated matrix. This implies that significant decreases may occur over a period of a week for *Bd* DNA. The selection of an appropriate preservation medium is essential to the success of the project. Due to the low concentration of DNA present in the environment, any decreases in the Ct-values may result in false negatives.

Three variables were tested for this section of the project. The first was to determine which DNA extraction method would perform the best on the filtration mediums. The second tested which preservation medium, the Lysis Buffer or 70% ethanol would preserve the filter material

the best. The third variable was time and tested the effectiveness of the preservation mediums over the course of a week. The different preservation mediums were tested with both the DNA extraction methods over three time periods namely, 24 hours, 3 days, and 7 days. Based on these results a DNA extraction method was selected for the final test. The third test was to determine the LOD and LOQ of the qPCR assay in terms of DNA copy numbers as well as create a standard curve from a synthetic DNA (G-Block) dilution series. The LOQ of the assay would determine whether the assay is sensitive enough to allow for possible quantification of field samples in the future. A dilution series was then made using zoospores and the selected protocol to determine the lowest number of zoospores that can be detected with the assay.

4.3.2.1. Filtration preservation test and DNA extraction comparison

Longmire's Lysis Buffer and 70% ethanol were selected as the preservation mediums of choice for this project due to its previous success in eDNA literatures (Alison *et al.*, 2021; Hinlo *et al.*, 2017; Kumar *et al*., 2020; Renshaw *et al*., 2015; Talley *et al*., 2015; Wegleiter *et al*., 2015). The Longmire's Lysis Buffer was made using the procedure described by Longmire *et al*. (1997). To create 1l of the buffer 200ml Ethylenediaminetetraacetic Acid (EDTA concentration: 0.5 and pH: 8.0), 50ml Tris Hydrochloride (Tris-HCl concentration: 2M and pH: 8.0), and 2ml Sodium Chloride (NaCl concentration: 5M) was combined and then ddH_20 was added until a volume of 975ml was achieved (Longmire *et al*., 1997). Finally, 25ml of 20% SDS (Sodium Dodecyl Sulphate) was added, the contents were shaken until thoroughly mixed before being autoclaved (Longmire *et al*., 1997).

To prepare the water samples, Erlenmeyer flasks were washed, autoclaved, and rinsed multiple times with ddH_2O before use. Each flask was then filled with 150ml of ddH_2O and autoclaved. Cultures of 1ml with a concentration of 100 000 zoospores per ml were prepared in triplicate for each experiment. The water samples in the Erlenmeyer flasks were spiked with the cultures and one negative control was created containing only autoclaved ddH_2O made for each test. The negative control was used to determine whether cross-contamination occurred between samples. The water samples were filtered using the syphon pump method which was selected after multiple field tests had been conducted. More information on the selection of the filtration method can be reviewed in the results (section 4.4.1) and discussion (section 4.5.1). Nalgene Single Use Analytical Funnels with cellulose nitrate filters and 0.45µm pore size (catalogue number: 145-2045) were purchased in individual sterile packages to limit cross contamination between samples (Figure 4.4-A). The water samples were swirled in the filter cups occasionally during the filtration process to ensure that only a minimum number of zoospores are lost due to adhering to the sides of the filter cup. Tweezers and scissors were

autoclaved between each experiment and rinsed with 70% ethanol and flamed between each sample to prevent cross contamination. The filter material was cut in halve using the sterilised tweezers and scissors for easier processing in the subsequent phase and each half was folded twice within itself to reduce the loss of DNA into the preservation medium. The filters were placed in 15ml falcon tubes containing approximately 1.5ml of the selected preservation medium. The tubes were sealed with parafilm and left for their selected preservation time (<24h, 3d, or 7d). Less than 24 hours for this project referred that the samples were extracted on the same day as the culture preparation and filtration. The extractions were done as soon as possible after filtration had been completed.

Initially the first processing step for the filter material would have been homogenising using a plant homogeniser. However, due to the size and the type of material the filter consisted of (cellulose nitrate), the homogeniser was unsuccessful in finely cutting the material. It was then decided that the filter material would directly be placed in the Eppendorf tubes containing the DNA extraction beads manually cut into small fragments using sterile scissors. The filter processing step is described in detail in the subsequent paragraphs.

The filter material processing step followed the same procedure for both the DNA extraction methods, with the only difference being the types of beads and solution applied in each method. For the DNeasy PowerSoil Kit the PowerBead tube and reagents provided in the kit were used. For the Heat Lysis method, 500 ± 0.01 mg glass beads and nuclease free water were applied. Initially different volumes of nuclease free water were tested with the glass beads for the Heat Lysis method to determine the most optimal combination. A supernatant with a volume of 450µl would need to be available to be transferred to a new tube after the bead beating phase, to allow for a direct comparison to the kit method volumes. Samples with 600µl and 700µl of nuclease free water were prepared for the Heat Lysis method and DNA was extracted from the filter materials. The Ct-values varied minimally and were not significant between the two water volumes. A volume of 650µl was selected for the protocol, because cross contamination is more likely to occur when using the 700µl due to probable spillage. It was also difficult to extract the full selected volume for comparison to the DNeasy PowerSoil Kit from the 600µl sample.

To process the filter material, tweezers were used to transfer the filter material to the prepared Eppendorf bead tubes and the filter material was finely cut using fine dissection scissors (Figure 4.4-B and Figure 4.4-C). The tools were rinsed in 70% ethanol and flamed between each sample and were autoclaved between each experiment to ensure that no cross-contamination occurred. For the DNeasy PowerSoil Kit, the first reagent as specified in the manufacturer's instructions was added prior to bead beating, and the Heat Lysis method only

applied nuclease free water. The prepared samples were vortexed for 5 seconds, then placed in a bead beater at full speed for 10 minutes, briefly vortexed again for 5 seconds and placed in a bead beater for an additional 5 minutes (Figure 4.4-D). The second vortex was done to redistribute the filter material that had been compressed by the beads into the bottom of the tube to ensure the full processing of all the filter material pieces. After bead beating, the samples were spun down in a centrifuge at 10 000 x g for 30 seconds and 450µl of supernatant was transferred to a new Eppendorf tube. The rest of DNeasy PowerSoil Kit protocol was followed according to the manufacturer's instructions. For the Heat Lysis method, after the 450µl supernatant was transferred, the samples were spun down for 5 minutes at 10 000 x g, 400µl of the supernatant was removed and 250µl of nuclease free water was added to the pellet to serve as an additional cleaning step. The Heat Lysis protocol was then followed as described in Chapter 3 section 3.2.3. from the start of the handheld homogenising step.

In the study by Walker *et al*. (2007), *Bd* was extracted from filter materials through a similar method as the kit method, but rather than finely cutting the filter material, the whole filter was placed into the Powerbead tube and processed in a bead beater for 2 minutes. In the current project a slightly modified version was used. The finely cut filter material allowed for better homogenisation of the material because the beads could move effectively between the filter pieces during the bead beating phase. Filters were also placed in the bead beater for 15 minutes rather than 2 minutes to ensure that the maximum amount of DNA can be retrieved from the filter materials.

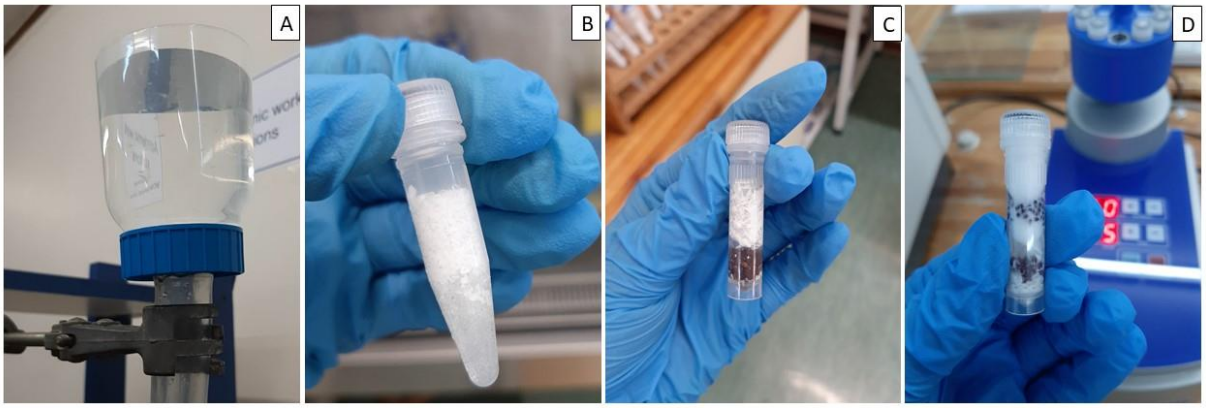

Figure 4.4. (A) Filter cup with blank water sample for filtration of NTC, **(B)** Finely cut filter material with glass beads and nuclease free water for the Heat Lysis extraction method, **(C)** Finely cut filter material in the Powerbead tube for the DNeasy PowerSoil Kit extraction method, **(D)** Filter material in the Powerbead tube after being placed in the bead beater for 15 minutes.

4.3.3. Primer efficiency and setting standards

The specificity of the selected primers had already been tested extensively in multiple studies (Blooi *et al*., 2013; Boyle *et al*., 2004; Kirstein *et al*., 2007) and thus, was not tested during this project. However, due to qPCR machines often varying in readings, a serial dilution series was tested using a G-Block to determine the efficiency of the primers for the specific qPCR machine. The cut-off point for the Ct-values, LOD and LOQ of the assay was also determined using the dilution series. It would be recommended for any future studies conducted on different qPCR machines to first conduct a standard curve of their own rather than relying on the results from other assays.

It is important to consider the lowest detection limit of an assay. This lowers the risk of false positives, which is defined as background signals that are misinterpreted by a qPCR machine as a positive value (Hunter *et al*., 2017). eDNA is often associated with low copy numbers of DNA and can cause many uncertainties regarding true positive and negative results. Thus, determining the sensitivity, efficiency and specificity of the primers as well as calculating lowest limit of detection (LOD) of the PCR machine is a crucial part in developing a reliable assay. The accurate quantification of field results is also dependant on the lowest limit of quantification (LOQ) of the assay.

For this project a G-Block containing the target region of the *Bd* genome was purchased (from Integrated DNA Technologies). The DNA fragments were 742 base pairs long and the following sequence was used to create the G-Block (Figure 4.5).

Name - Bd-gblock 1 gBlocks® Gene Fragments 742 base pairs

```
5'- GTT TTT TAA TTT AAA AGA AGC TGG TCA AAC TTG GTC ATT TAG AGG AAG TAA AAG TCG TAA CAA GGT AAC
CGT AGG TGA ACC TGC GGT TGG ATC ATT ATT AGT TGT TGG GGT GGA TGG GAC TTT TAT TGA TGT GTA AAT GTT
GAT GGA ATG ACC CAT TGT TTT TTT CAA AAA ACA CCC TTG ATA TAA TAC AGT GTG CCA TAT GTC ACG AGT CGA
ACA AAA TTT ATT TAT TTT TTC GAC AAA TTA ATT GGA AAT TGA ATA ATT TAA TTG AAA AAA ATT GAA AAT AAA
TAT TAA AAA CAA CTT TTG ACA ACG GAT CTC TTG GCT CTC GCA ACG ATG AAG AAC GCA GCG AAA TGC GAT ACG
TAA TGT GAA TTG CAA ACC TTT GTG AAT CAT TAA ATC TTT GAA CGC ACA TTG CAC TCG TAA AAG AGT ATA CAT
GTT TGA GAA TTA TAA AAA TAC ATT GTC CGA ATT GAC TGG ACA GAT ATG AAC CAT GCC AAA ATT GTT TGA CAA
TTT GAC AGG TTT TAA AAG TAG TAG TAA AAA AGA GTG ATA CAA AAA GTA GTA ATA CAA CCC CGT CAC ACC ATA
CAA AAA TAT AAT CTC AAA TCA TGC AAG ATT ACC CGC TGA ACT TAA GCA TAT CAA TAA GCG GTG GAA AAG AAA
CTA ACA AGG ATT CCC CTA GTA ACG GCG AGT GAA GCG GGA ATA GCT CAA ATT TGA AAT CTC ACA ATA GTG CGA
ATT GTA GTT TAG AGA AAC CCC ATT T -3'
```

Figure 4.5. Base pair sequence from 5' to 3' for *Bd* G-block.

A dilution of 1000 000 DNA copies/µl were made as a working stock for analysis. A 10-fold dilution series from 1–1 000 000 DNA copies/µl was created using 100µl of the previous concentration along with 900µl of nuclease free water. A triplicate was made of each series and four qPCR replicates were run for each sample; thus, 12 data points were created per concentration. This was used to create a standard curve that can be used to determine the genomic equivalence of samples.

To determine the loss of DNA from the filtration process as well as the lowest detection limit of the developed assay, a filter dilution series was created using 10–100 000 zoospores. This aided in determining the LOD and LOQ of the assay in terms of zoospores. To prepare the zoospore dilution series an initial sample of 100 000 zoospores in 1ml of 1% tryptone broth was prepared. To create the dilution,100µl of the previous concentration along with 900µl of 1% tryptone broth was combined. This step was repeated until the 10–100 000 zoospore series was created. The filter samples were prepared using the same methods as for the filter preservation tests. Thus, 150ml sterile water samples were spiked using the different concentrations and filtered using the syphon pump method. The DNA was extracted using the method of choice from the filter preservation medium tests. The filter dilution series consisted of triplicate samples for each ten-fold dilution and was run in duplicate for the qPCR assay.

As previously stated in the literature review many eDNA studies consider a sample positive if both replicates provide a Ct-value below 40, whereas others select a cut-off point based on the number of qPCR replicates that amplify. For this study the cut-off point in the Ct-values were determined through analysing the level of variance between the PCR replicates to the trend line determined in the standard curve, as well as reviewing the number of replicates amplified below 40 cycles. As per the articles by Davison *et al*. (2019) and Takahara *et al*., (2013) one of the qPCR replicates for all the samples in each concentration should amplify as well as follow the trend line created to be considered for the LOD. The LOQ was determined as the level where 100% of all the replicates amplified, followed the trend line of the standard curve and presented an acceptable level of variation in terms of their SD values (Davison *et al*., 2019; Ostberg *et al*., 2018).

4.3.4. Molecular detection

All surfaces and equipment were cleaned using 10% bleach, followed by 70% ethanol and UV decontaminated for 20 minutes in a laminar flow hood prior to use. Filter tips were used for all the protocols applied in the study. Samples were prepared in triplicate for the filter tests as well as filter dilution and the samples were processed in duplicate for the TaqMan Probe qPCR assay. This provided six data points per method and variable combination, and the average Ct-value was determined per sample prior to the statistical analysis. All duplicate samples were in an acceptable level of variance prior to determining the averages. For more information on the qPCR protocol refer to Chapter 2 section 2.6.2.

As mentioned in the previous chapter multiple studies have shown the primers used in this study to have a high efficiency level and a high level of reproducibility between different qPCR plates (Blooi *et al*., 2013; Hyatt *et al*., 2007). Due to the same reagents, samples and machine

being used in the study as well as a set threshold, similar amplifications should theoretically be observed over the two plates. For this project, the qPCR phase for the filter samples took place over two time periods, before and after a preliminary field work session. Due to the considerable number of samples being assessed not all the samples could be run on the same plate. Two samples from the previous plate (one from the DNeasy PowerSoil Kit and one from the Heat Lysis method) were selected to serve as possible Inter Run Calibrators (IRCs) to determine whether there was any significant change in the qPCR runs and determine whether the sample Ct-values would have to be adjusted before being compared. An IRC can be defined as a sample that is included on all plates to be analysed for comparison to allow for the calibration to reduce the impact of inter run variation (Hellemans *et al*., 2007). The Ct-values between the IRCs in this project varied less than 0.2 Ct from the original readings and the values were not further adjusted, because the average acceptable level of variations between replicates in a sample is ±0.3 Ct according to MIQE guidelines (Bustin *et al*., 2009; Flatschacher *et al*., 2022). The samples were compared with the assumption that the efficiency between the plates remain the same.

4.3.5. Statistical analysis

Ct-value comparison for filter tests

Each DNA extraction method and preservation medium combination represented a single method for the statistical analysis and were named as followed; Heat Lysis – Ethanol, Heat Lysis – Lysis Buffer, DNeasy PowerSoil – Ethanol and DNeasy PowerSoil – Lysis Buffer. The mean, standard deviations, and CV% (CV = 100 * SD/Mean) of the Ct-values for each method combination were calculated on Excel 2016. The SD and CV% of the Ct-values were determined using all six data points per method combination obtained from the qPCR run. These variables were used to determine the intra-assay variability and reproducibility of the data sets. A statistical analysis was performed with the aid of GraphPad Prism v8.0.2. The average Ct-value for each sample was used in the ANOVA analysis. A One-way ANOVA with a Bonferroni multiple comparison post hoc test was run for the Ct-values between methods for each time period individually. This was done to determine whether any significant differences can be observed in the Ct-values between the methods. A Two-way ANOVA with a Bonferroni multiple comparison post hoc test was then run overall between the methods as well as used for within every method between the different days. This was done to determine whether any significant changes can be observed over time for a single method as well as determine the overall effectiveness of each method compared to the other methods. A method was considered statistically significant if it had a p-value of less than 0.05.

Primer efficiency

The efficiency of the primers was determined through the following equation:

Efficiency (%) = $(10^{(-1/-m)} - 1) * 100$

The m represents the gradient of the standard curve drawn from the serial dilution series. An efficiency of 90–110% is considered acceptable (Taylor *et al.*, 2019). The R^2 value of the graph was also determined along with the efficiency to determine the reliability of the graph. The R^2 value should preferably be above 0.98, however, some outliers may need to be removed to achieve this value (Taylor *et al.*, 2019).

4.4. Results

4.4.1. Filtration method selection

The selected filtration methods were tested in the field to determine the volume of water that can be filtered. All three methods performed similarly and the main factor limiting the filtration volume was the turbidity of the water that resulted in the clogging of filters. Water from ponds with high turbidity were the most difficult to filter and 250 – 500ml per sample could be filtered with difficulty for all methods. Water with low turbidity, such as those found in streams with clear running water, could easily filter 750 – 1000ml per sample before clogging occurred. The C.L.A.M could likely continue filtering after this volume, however the rate of filtrations as well as efficiency was greatly reduced. The majority of water samples, however, had some degree of turbidity and although 750ml of water can be filtered with difficulty, filtration volume was often lower to prevent the filters from potentially tearing or equipment from possibly being damaged.

The syphon pump method was the only filtration method that could be applied directly at all the filter sites and did not require an external power supply apart from physical force. Both the other methods were limited in their direct field application and water samples often had to be collected in sterile autoclaved water containers for filtration. The syphon pump method was selected as the filtration method of choice for testing the preservation mediums due to its simplicity and versatility in the field. More details are provided on the benefits and limitations for the different methods in the discussion section of this chapter.

4.4.2. Preservation medium test

None of the NTCs, nor any of the negative controls tested positive in the qPCR assay. The positive controls, which served as inter-run calibrators, for each plate varied minimally and fell within the normal expected range for sample variation, ±0.3 Ct. Thus, the samples between the different plates could be compared relatively with the assumption that the efficiency remained the same between the different plates and no adjustments were made to the Ct-values.

4.4.2.1. Overall comparison

Overall, the Ct-values between the methods and preservation medium combinations were significant when reviewing the Two-ANOVA results ($p = 0.0005$). The same tendency could be observed for all the methods over the three tested time periods. The DNeasy PowerSoil – Lysis Buffer combination had significantly lower Ct-values overall for all three days according

to the results from the Bonferroni multiple comparison post hoc test in the two-way ANOVA (Figure 4.6, Table 4.1 and Table 4.2). This method combination also delivered the second lowest SD (0.75) and CV% (3.35%), overall indicating a low level of variance in the data (Table 4.1). The Heat Lysis – Ethanol combination demonstrated the lowest SD (0.69) and CV% (2.71%) of all the tested combinations. The method combination with the highest level of variance in Ct-values (SD = 1.06 and CV% = 3.55%) was the Dneasy PowerSoil – Ethanol combination. The Dneasy Powersoil – Ethanol combination demonstrated a high level of inhibition based on the Ct-values and was the least reliable method due to its high level of variability. Overall, the DNeasy PowerSoil – Ethanol method preformed significantly worse in comparison to the other methods for both the One-way ANOVA Bonferroni multiple comparisons tests and Two-way ANOVA overall analysis (Table 4.2 & 4.3). The most prominent differences between the methods could be observed in the samples processed during the less than 24-hours test, thus immediately after culture preparation and filtration. For the subsequent days the differences in Ct-values decreased, but the overall significant differences between the methods still remained the same over time for all periods.

4.4.2.2. Individual method comparison between the different days

The Heat Lysis – Ethanol method delivered the most consistent results on average over time with the lowest SD (0.69) and CV% (2.71) for the overall results. When reviewing the results from the Two-way ANOVA Bonferroni multiple comparison test, this method also demonstrated the least significant changes over time (>0.9999 for all comparisons) (Table 4.4). It should be noted that although the averages of the Ct-values remained more consistent overall and for each day, the greatest increase in CV% could be observed for this method as time increased. For example, on Day 7, this method had the highest SD (1.19) and CV% (4.64) of all the methods, but the average Ct-values from Day 7 varied minimally compared to Day 1. This implies that overall, this method delivers consistent results over time in terms of its average, but the level of variation between the samples increased as time progressed. Thus, if this method is used samples should be processed as soon as possible after sampling to reduce variability in the data sets.

The Heat Lysis – Ethanol combination experienced an increase in Ct-values over time, however the increase was gradual and insignificant. When compared to the Heat Lysis – Lysis Buffer combination, this combination experienced the greatest increase in Ct-values over time and the difference between day 1 and 7 was significant when analysing the Two-way ANOVA Bonferroni multiple comparison test ($p = 0.0177$) (Table 4.2). Despite this greater increase in Ct-values, the differences between the Heat Lysis – Lysis Buffer and Heat Lysis – Ethanol

combinations were not significant for any of the days observed nor overall when analysing the one-way and two-way ANOVA results (Figure 4.6; Table 4.1; Table 4.2). Thus, both methods preformed similarly during all of the tested experiments. It would, however, still be recommended to rather apply the Ethanol preservation medium for this extraction method due to its more consistent results and lower loss in DNA over time despite not performing significantly better than the Lysis Buffer.

The Dneasy Powersoil Kit method combinations followed an irregular trend in Ct-values compared to the Heat Lysis methods over time. Both the Heat Lysis methods demonstrated a gradual increase in the Ct-values over time, whereas the DNeasy PowerSoil Kits demonstrated fluctuating Ct-values. The Dneasy Powersoil – Lysis Buffer combination experienced a significant increase in the Ct-values from <24h to Day 3. The Ct-values for Day 7 were highly similar compared to the Day 3 results, however, there was no significant difference between the <24h and the Day 7 results. Thus, an increase in Ct-values can be observed within three days when using the Lysis Buffer preservation medium, however, this increase may not necessarily always be significant. The rate of DNA loss may possibly decrease after 3 days explaining why there is no significant difference between Day 3 and Day 7. Due to the greater loss in DNA within the first three days it would be recommended to process filter material within three days of sampling to ensure the highest quantity results.

No significant differences could be observed over time for the DNeasy PowerSoil – Ethanol combination, however this method demonstrated greater fluctuations in Ct-values compared to the DNeasy PowerSoil – Lysis Buffer combination as seen in the SD value for the overall mean.

Overall, the preservation mediums performed similarly for the Heat Lysis methods, but greater differences could be observed between the DNeasy PowerSoil combinations. For three of the four tested method combinations the SD and CV% in the samples per day increased the longer the samples were preserved. This indicates a greater level of variation amongst the samples over time. Thus, quantification of field samples may be less accurate the longer the samples are preserved. Slightly greater increases in the Ct-values were observed for both the methods using the Lysis Buffer preservation medium over time when compared to the Ethanol preservation medium (Table 4.4). This indicated that the Lysis Buffer may be slightly less effective compared to the 70% ethanol at preserving DNA over time, but both methods still delivered similar results for the Heat Lysis extraction method.

4.4.2.3. Impact of preservation mediums on Ct-values

When comparing the results from <24h to those of the sterile matrix from Chapter 3, a significant inhibitory effect was observed for three of the four extraction method and preservation medium combinations (Table 4.5). The only method combination which did not indicate a significant loss in DNA in terms of Ct-values was the Dneasy PowerSoil – Lysis Buffer combination ($p = 0.1975$) (Table 4.5). Thus, this method effectively removes the Lysis Buffer preservation medium and leaves high quality DNA, which differs minimally from a sterile culture DNA extraction. Although ethanol was previously indicated to be more effective at preserving DNA over time, it had the greatest inhibitory effect on the DNeasy PowerSoil Kit and caused the greatest level of variability between days (Table 4.1; Table 4.4; Table 4.5).

The Lysis Buffer indicated a lower inhibitory effect for both the DNA extraction methods compared to the ethanol for the less than 24-hour period (Table 4.5). Although the Heat Lysis – Lysis Buffer method had lower Ct-values compared to the ethanol for this time period, the difference was insignificant. Thus, the DNA was extracted with a similar level of efficiency for both preservation mediums in this method. It should, however, be noted that both preservation mediums in the Heat Lysis method still indicate a significant level of inhibition based on the increase in Ct-values compared to the sterile matrix (Table 4.5). Based on these results the DNeasy PowerSoil – Lysis Buffer combination was the most successful combination and removed inhibitors with great efficiency. This method was selected as the method of choice for this project and was further tested in the following section to determine the LOD and LOQ of the assay.

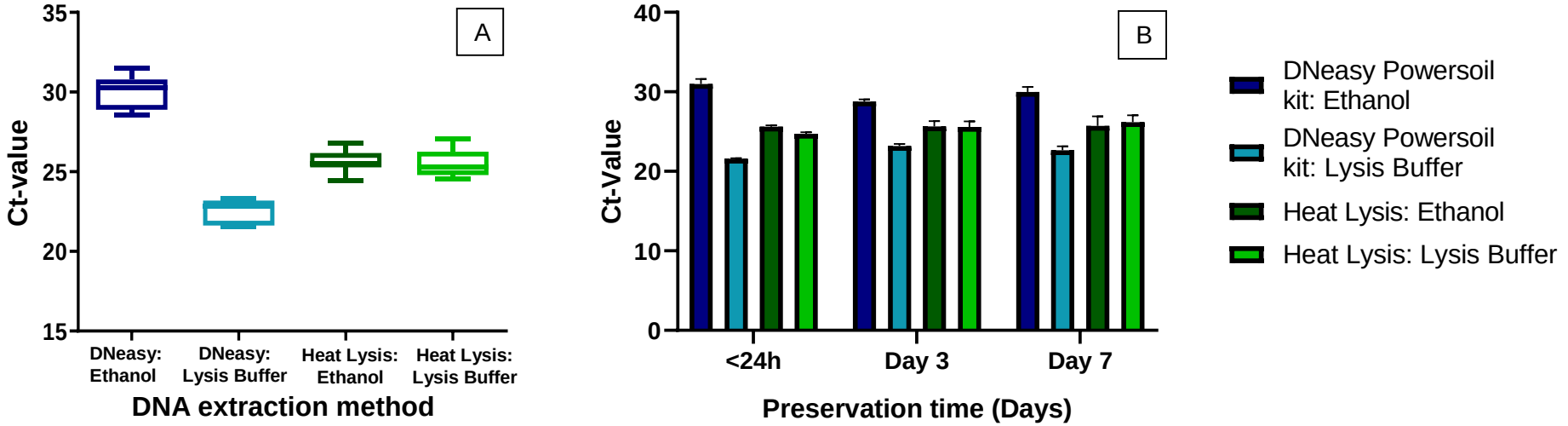

Figure 4.6. (A) Overall Ct-values for different DNA extraction methods and preservation mediums, (B) Ct-values for DNA extraction methods and preservation medium combinations over time.

Table 4.1. Overall mean, standard deviations, and CV% of Ct-values for the selected DNA extraction methods and preservation mediums over three time periods: <24h, 3days and 7 days.

DNA extraction method	Preservation medium	Preservation time	Mean Ct-value per time period ± SD (CV%)	Overall mean Ct-value ± SD (CV%)
DNeasy PowerSoil Kit	Lysis buffer	<24h	21.60 ± 0.05 (0.24%)	22.48 ± 0.75 (3.35%)
		3 days	23.19 ± 0.26 (1.11%)	
		7 days	22.65 ± 0.49 (2.17%)	
	70% Ethanol	<24h	30.99 ± 0.62 (2.00%)	29.91 ± 1.06 (3.55%)
		3 days	28.79 ± 0.26 (0.92%)	
		7 days	29.97 ± 0.65 (2.16%)	
Heat Lysis method	Lysis buffer	<24h	24.71 ± 0.21 (0.84%)	25.49 ± 0.85 (3.55%)
		3 days	25.58 ±.0.69 (2.71%)	
		7 days	26.19 ± 0.86 (3.27%)	
	70% Ethanol	<24h	25.61 ± 0.16 (0.63%)	25.66 ± 0.69 (2.71%)
		3 days	25.64 ± 0.69 (2.68%)	
		7 days	25.72 ± 1.19 (4.64%)	

Table 4.2. Overall Ct-value comparison between DNA extraction methods and preservation medium combinations, using Two-way ANOVA with Bonferroni multiple comparison test (p-values in red are significant).

Overall Ct-values		
Method comparison	**Mean difference**	**P-value**
DNeasy PowerSoil Kit – Ethanol vs. DNeasy PowerSoil Kit – Lysis Buffer	7.435	<0.0001
DNeasy PowerSoil Kit – Ethanol vs. Heat Lysis – Ethanol	4.256	<0.0001
DNeasy PowerSoil Kit – Ethanol vs. Heat Lysis – Lysis Buffer	4.423	<0.0001
DNeasy PowerSoil Kit – Lysis Buffer vs. Heat Lysis – Ethanol	-3.179	<0.0001
DNeasy PowerSoil Kit – Lysis Buffer vs. Heat Lysis – Lysis Buffer	-3.012	<0.0001
Heat Lysis – Ethanol vs. Heat Lysis – Lysis Buffer	0.1671	>0.9999

Table 4.3. Average Ct-value statistical comparison between DNA extraction methods and preservation medium combinations per time interval (<24h, Day3, Day7), using One-Way ANOVA analysis with Bonferroni multiple comparison test (p-values in red are significant).

<24h Ct-values		
Method comparison	**Mean difference**	**P-value**
DNeasy PowerSoil Kit – Ethanol vs. DNeasy PowerSoil Kit – Lysis Buffer	9.39	<0.0001
DNeasy PowerSoil Kit – Ethanol vs. Heat Lysis – Ethanol	5.38	<0.0001
DNeasy PowerSoil Kit – Ethanol vs. Heat Lysis – Lysis Buffer	6.28	<0.0001
DNeasy PowerSoil Kit – Lysis Buffer vs. Heat Lysis – Ethanol	-4.02	<0.0001
DNeasy PowerSoil Kit – Lysis Buffer vs. Heat Lysis – Lysis Buffer	-3.11	<0.0001
Heat Lysis – Ethanol vs. Heat Lysis – Lysis Buffer	0.90	0.0836
Day 3 Ct-values		
Method comparison	**Mean difference**	**P-value**
DNeasy PowerSoil Kit – Ethanol vs. DNeasy PowerSoil Kit – Lysis Buffer	5.60	<0.0001
DNeasy PowerSoil Kit – Ethanol vs. Heat Lysis – Ethanol	3.14	0.0005
DNeasy PowerSoil Kit – Ethanol vs. Heat Lysis – Lysis Buffer	3.21	0.0004
DNeasy PowerSoil Kit – Lysis Buffer vs. Heat Lysis – Ethanol	-2.46	0.0026
DNeasy PowerSoil Kit – Lysis Buffer vs. Heat Lysis – Lysis Buffer	-2.39	0.0031
Heat Lysis – Ethanol vs. Heat Lysis – Lysis Buffer	0.07	>0.9999
Day 7 Ct-values		
Method comparison	**Mean difference**	**P-value**
DNeasy PowerSoil Kit – Ethanol vs. DNeasy PowerSoil Kit – Lysis Buffer	7.31	<0.0001
DNeasy PowerSoil Kit – Ethanol vs. Heat Lysis – Ethanol	4.24	0.0016
DNeasy PowerSoil Kit – Ethanol vs. Heat Lysis – Lysis Buffer	3.77	0.0034
DNeasy PowerSoil Kit – Lysis Buffer vs. Heat Lysis – Ethanol	-3.07	0.0123
DNeasy PowerSoil Kit – Lysis Buffer vs. Heat Lysis – Lysis Buffer	-3.54	0.0051
Heat Lysis – Ethanol vs. Heat Lysis – Lysis Buffer	-0.47	>0.9999

Table 4.4. Average Ct-value statistical comparison between days within each DNA extraction method and preservation medium combination, using Two-Way ANOVA analysis with Bonferroni multiple comparison test (p-values in red are significant).

DNeasy PowerSoil Kit – Ethanol		
Day comparison	**Mean difference**	**P-value**
<24h vs. Day 3	2.203	0.0005
<24h vs. Day 7	1.021	0.1469
Day 3 vs Day 7	-1.183	0.0732
DNeasy PowerSoil Kit – Lysis Buffer		
Day comparison	**Mean difference**	**P-value**
<24h vs. Day 3	-1.588	0.0108
<24h vs. Day 7	-1.056	0.1268
Day 3 vs Day 7	0.5322	0.8711
Heat Lysis – Ethanol		
Day comparison	**Mean difference**	**P-value**
<24h vs. Day 3	-0.03167	>0.9999
<24h vs. Day 7	-0.1117	>0.9999
Day 3 vs Day 7	-0.0800	>0.9999
Heat Lysis – Lysis Buffer		
Day comparison	**Mean difference**	**P-value**
<24h vs. Day 3	-0.8665	0.2733
<24h vs. Day 7	-1.488	0.0177
Day 3 vs Day 7	-0.6212	0.6573

Table 4.5. The impact of preservation mediums on the Ct-values for each DNA extraction method and preservation medium combination, using average Ct-values from the sterile matrix of the Heat Lysis method and Dneasy PowerSoil Kit in comparison to the Ct-values from less than 24 hours from the filter tests (<24h). Analysis done using One-way ANOVA with Bonferroni multiple comparison post hoc test (p-values in red are significant).

DNA extraction method	**Matrix / Preservation medium type**	**Mean Ct-value ± SD (CV%)**	**Mean difference to sterile matrix**	**P-value**
Dneasy PowerSoil Kit	Sterile matrix	21.13 ± 0.29 (1.39)	N/A	N/A
	Lysis buffer	21.60 ± 0.05 (0.24%)	0.4688	0.1975
	70% Ethanol	30.99 ± 0.62 (2.00%)	9.860	<0.0001
Heat Lysis method	Sterile matrix	19.81 ± 0.20 (1.03)	N/A	N/A
	Lysis buffer	24.71 ± 0.21 (0.84%)	4.896	<0.0001
	70% Ethanol	25.61 ± 0.16 (0.63%)	5.800	<0.0001

4.4.3. Primer efficiency and setting standards

4.4.3.1. G-Block dilution series

When analysing all of the Ct-values generated from the G-block dilution series the graph had an excellent efficiency of 98,8%, however, the R^2 value was low (0.9461), which was mainly due to a few odd amplifications that occurred in the lower concentration range of the dilution series (Figure 4.7 and Table 4.6). This section of the graph was further analysed to determine what the true lowest detection limit of the qPCR assay was.

Three of the twelve qPCR replicates for 1 DNA copies/µl delivered a Ct-value. These values were all above 37 Ct and fell below the normal expected trend line for the graph (Figure 4.7 and Table 4.6). Only one of the 12 qPCR replicates for 10 copies/µl delivered a Ct-value and the Ct-value was similar to those observed for the 1 copy per µl concentration. Thus, more samples amplified at a lower DNA copy number, and presented similar Ct-values, all above 37 cycles. For this reason, these values were considered unreliable. Additionally, some of the replicates at 100 copies/µl also presented values above 37 cycles and these values were much higher than the expected trend line for this concentration. For this reason, values above 37 were excluded from the standard curve and a new curve was drawn for further interpretation (Figure 4.8 and Table 4.7). Previous unpublished data in the Herpetological Health Lab (HHL) have also indicated that values above 37 cycles are considered negative for ventral swabs of amphibians.

As stated in the methodology, outliers may need to be removed to achieve acceptable R^2 in the data. Two qPCR samples of the twelve qPCR replicates for the 1000 DNA copies per µl had a significant effect on the R^2 value. When these values were excluded the R^2 of the graph improved significantly to 0.9775, which is slightly below the desired variation, but still considered an acceptable level of variation (Figure 4.8 and Table 4.7). The efficiency for the new graph was lower than the original, however, was still within the acceptable range (90 – 110%) with a value of 93.5% (Figure 4.8 and Table 4.7). After Ct-values above 37 cycles had been removed only 50% of the samples delivered a positive amplification for 100 copies/µl. Two of the four qPCR replicates amplified for all three sample replicates, thus, qualified as the LOD for the project based on the standard described in the methodology. The LOQ of the primers is approximately 1000 copies/µl due to 100% of the samples amplifying at this quantity.

Table 4.6. Summary of all Ct-values generated from *Bd* G-Block dilution series from the TaqMan Probe qPCR assay.

Concentration 10^ DNA copy numbers per µl	Positive amplifications (%)	Ct-value mean ± SD (CV%)
0	25	38.73 ± 0.56 (1.45%)
1	8	37.36 ± 0 (0%)
2	75	35.93 ± 1.66 (4.61%)
3	100	32.13 ± 1.18 (3.68%)
4	100	27.71 ± 0.48 (1.74%)
5	100	24.18 ± 0.27 (1.13%)
6	100	21.41 ± 0.78 (3.65%)

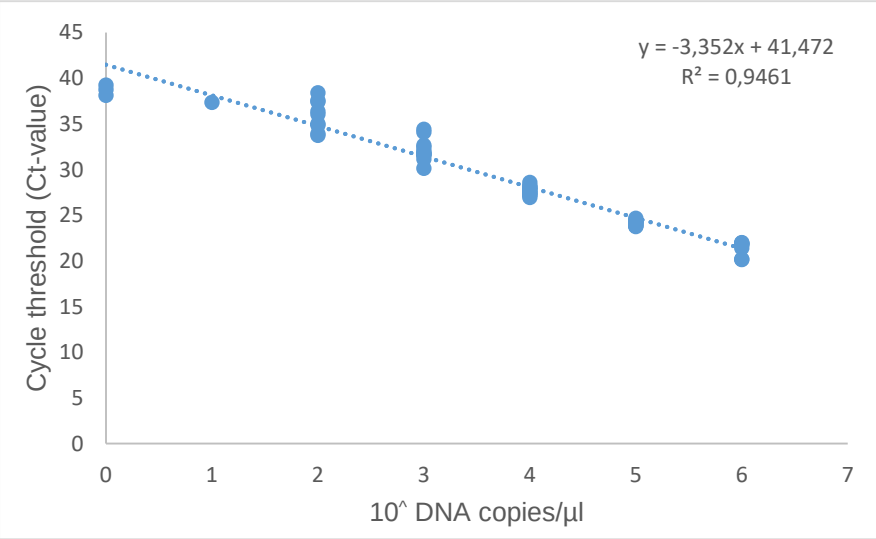

Figure 4.7. *Bd* G-Block dilution series standard curve generated from all Ct-values presented during TaqMan Probe qPCR assay.

Table 4.7. Summary of remaining Ct-values from *Bd* G-Block dilution series after processing and analysis.

Concentration 10^ DNA copy numbers per µl	Positive amplifications (%)	Ct-value mean ± SD (CV%)
0	0	N/A
1	0	N/A
2	50	35.00 ± 1.07 (3.06%)
3	100	31.71 ± 0.69 (2.20%)
4	100	27.71 ± 0.48 (1.74%)
5	100	24.18 ± 0.27 (1.13%)
6	100	21.41 ± 0.78 (3.65%)

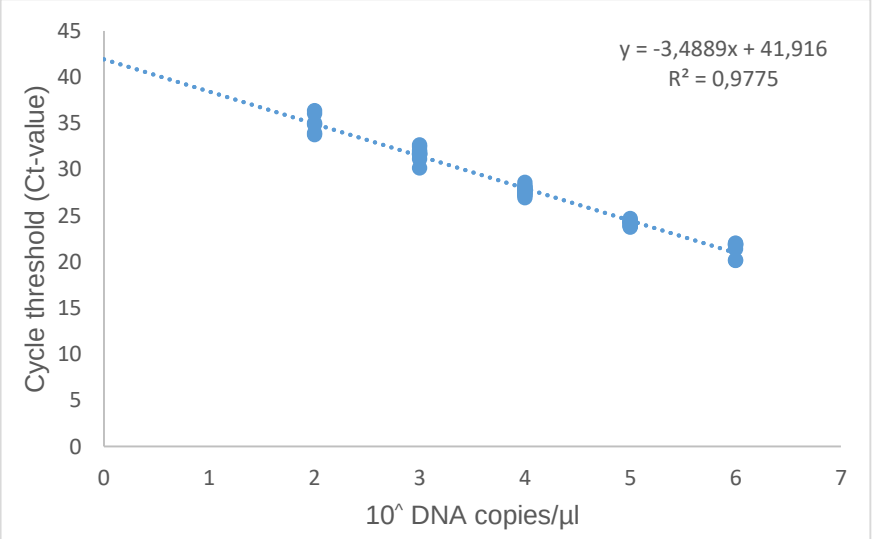

Figure 4.8. *Bd* G-Block dilution series standard curve generated from remaining Ct-values after processing and analysis.

4.4.3.2. Filter dilution series

The primer efficiency for the filter dilution series was 92.84%, which falls in the acceptable range for primer efficiency (90–110%) and correlates with the efficiency of the G-Block (Figure 4.9). The R^2 value for the graph is 0.9817, which is considered excellent and indicates a low level of variability in the values. All three of the samples and six qPCR replicates delivered positive results below 37 cycles for the 10 zoospores concentration, thus this was deemed positive and the lowest detection limit of the assay (Table 4.8). No outliers were detected in the samples and based on the high R^2 value variability between replicates were low. This may be due to the DNA from the zoospores being more stable than smaller DNA fragments such as those from the G-Block. The LOD and LOQ based on the zoospore quantities for this project is 10 zoospores.

Table 4.8. Summary of all Ct-values generated from *Bd* G-Block dilution series from the TaqMan Probe qPCR assay.

10^ Zoospores per ml	Positive amplification (%)	Ct-value mean ± SD (CV%)
0	0	N/A
1	100	35.73 ± 0.69 (1.92%)
2	100	33.06 ± 0.28 (0.86%)
3	100	29.61 ± 0.19 (0.66%)
4	100	26.26 ± 0.79 (3.02%)
5	100	21.60 ± 0.34 (1.56%)

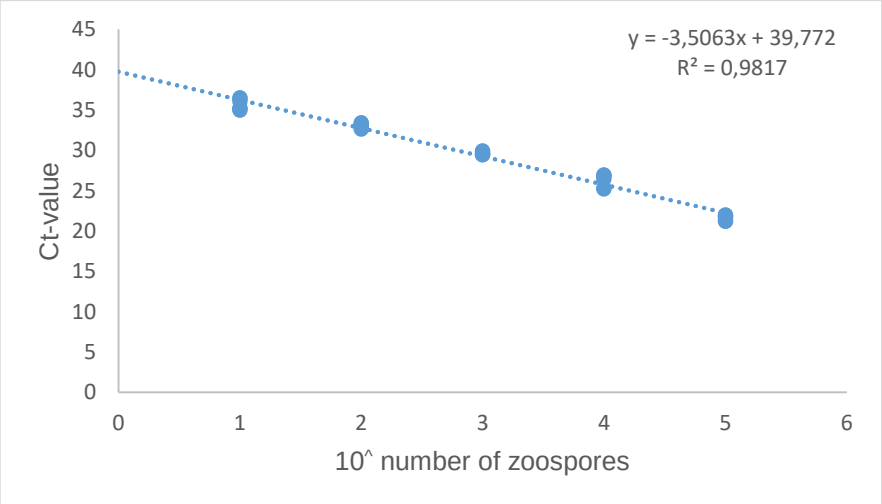

Figure 4.9. Filtered zoospore dilution series standard curve generated from all Ct-values presented during TaqMan Probe qPCR assay.

4.4. Discussion

4.4.1. Filter method and volume selection

4.4.1.1. Volume selection

Filter volumes varied from 250ml to 1l between samples and methods during the field application test. Clogging generally occurred at the same volume between the different methods and the main factor that determined the filter volume was turbidity. Most of the methods were able to filter 750ml of water, but filtration often became slow towards the end. Based on the filter results from the field, it was decided that only 500ml would be filtered per sample to reduce the risk of damaging the filters, while also providing a constant volume to compare field samples with. This correlated with a previous eDNA study for *Bd* conducted in Norway, that used similar pore sizes and filtered 500ml per sample (Taugbøl *et al*., 2021). Depending on the turbidity of the water at times they were unable to filter 500ml, but were still able to obtain positive results at the various sites (Taugbøl *et al*., 2021). Filtering during the subsequent field application were done until the selected volume was reached, or until the filters have clogged prior to reaching 500ml.

4.4.2.1. Filter method selection

Each filtration method presented many benefits and limitations that aided in the method selection for the laboratory experiments. The following section provides a summary of the performance of each method and why the syphon pump method was selected as the method of choice.

Many of the streams being sampled were too shallow to submerge the C.L.A.M. and water had to be placed in a bucket for filtration. The C.L.A.M. method filtered the water samples quickly and clogging occurred relatively at the same time as the other two methods. It should be noted in clear water conditions this method may be capable of filtering more water compared to the other methods. There were, however, many drawbacks to this method despite its good performance. This device is much more expensive compared to the other methods and the design caused a few problems during the project. The charging port was located on the same hemisphere as the water filter and was thus submerged in the water. Although a screw cap is present over the port, water damp could still be found in the port occasionally after filtration. Charging the device takes exceptionally long (approximately 12 hours to fully charge). During the project the battery stopped working, thus, the device only worked while being plugged in and due to the design of the dome, the battery could not be accessed or replaced.

The limited access to electricity was likely the greatest challenge for this method as well as for the drill filter during field application. Electricity was unavailable during a part of the excursion and water samples had to be collected in clean containers to be processed later when electricity was available again. eDNA is known to immediately start decaying after being released into the environment (Goldberg *et al*., 2016). It is recommended that samples are filtered within 24 hours after collection to reduce significant losses in DNA (Goldberg *et al*., 2016). As seen from the results in Chapter 3, significant declines may occur within a week when *Bd* cells are deceased. It would be preferable to sample directly from the field and immediately apply a preservation medium to reduce the loss of DNA.

Although this method is less labour intensive and water is filtered relatively quickly, the design of the product caused a few problems during the project and thus this method was not selected. This method should, however, not be ruled out completely for future eDNA studies and has a lot of potential if the design were to be adjusted and components were accessible for replacement when required. Due to difficulties caused by covid-19 at the time, eDNA filters for the C.L.A.M. could not be imported from Aqualitical in North America. These filters were recently developed specifically for the C.L.A.M. and allows the operator to easily remove the filter media from the disc. Only SPE discs that were originally purchased with the product were available for analysis and these were extremely difficult to open in order to remove the filter media and samples could not be frozen in the field. These filters can however be tested in future eDNA studies and should not be ruled out as a potential method. It can provide many benefits due to it being less labour intensive and able to sample water for longer periods compared to the drill filter. However, this method was impractical for this study's application and may be better suited for environments where electricity is readily available.

The drill filter method had the slowest performance due to the drills constantly overheating, thus filtration was often interrupted even when three different drills were being used per session. Initially this method was designed to be performed directly in the field using a battery-operated drill but unfortunately the battery only lasted for 2–3 samples for a single site. Thus, all samples will have to be processed where electricity can be present, which complicates sampling at remote sites. Due to the size and weight of the device, it was difficult to carry when hiking to remote areas. The pump that was connected to the drill overheated and became damaged after a few sites had been tested and required replacement. Thus, although this method is cost-effective, it had the slowest performance, became damaged after a few applications, and requires an electrical source to filter all the samples.

The syphon pump had the simplest design of all the methods, and for this reason could directly be applied in the field. It does not require any electricity and was the only method that could

directly be used in the field for all the sites in this project. The device was very light and could be carried with ease to remote areas within the study site. This method filtered at the same rate as the C.L.A.M. and could filter the same volume of water as the other methods. Due to its simple and light design, as well as effectiveness in the field this method was selected as the filter method for the protocol. This is also a highly cost-effective method and components are easy to replace. The only drawback of this method is it requires a lot of manual labour, and the pump needs to be oiled occasionally for maintenance.

Cross contamination using the syphon pump is also less likely to occur between samples compared to the C.L.A.M., because the device does not need to be in contact with the water samples and a clean sterile filter cup is used for each sample. Applying single-use equipment, such as the filter cups, have shown to decrease the possibility of cross-contamination between samples significantly (Golberg *et al*., 2016). Samples are taken directly in the field and a new cup is used with every sample. These filter cups are bought and stored in individual sterile packages and are only opened on site. Water is collected using the filter cups directly, thus water containers do not need to be applied and cleaned between each site.

4.4.2. Filter preservation medium selection

The DNeasy PowerSoil Kit with Lysis Buffer preservation medium presented the lowest Ct-values that were statistically significant when compared to the other methods. In the study by Brannelly *et al*. (2020) the Qiagen Powersoil Kit had been selected as the method of choice for eDNA of *Bd*. During the study it was determined that the lowest detection limit of their assay was approximately 100 zoospores, however, in the present study the lowest detection limit was 10 zoospores based on the filter dilution series. This may be accounted for by the filter processing steps that were additionally applied in our assay as well as the application of a buffer for eDNA preservation.

In Chapter 3 section 3.5.1 when reviewing the sterile and non-sterile matrix, the kit methods appeared to remove inhibitors more effectively compared to the crude extraction methods. This became more apparent when analysing the filter material samples. The Ct-values of the Heat Lysis method demonstrated a significant increase compared to the sterile matrix Ct-values from Chapter 3 for both preservation combinations. The Dneasy PowerSoil – Lysis Buffer combination did not experience any significant increase in Ct-values compared to the sterile matrix indicating the effective removal of inhibitory agents. This further supports the previous notion from Chapter 3 that kit methods may potentially remove inhibitors more effectively compared to crude extraction methods.

Greater increases in Ct-values were observed over time when using the Lysis Buffer preservation medium compared to the 70% ethanol. It should also be noted that 70% ethanol did not deliver significantly better results for any of the days compared to the Lysis Buffer for the Heat Lysis method. Thus, overall, both methods performed very similarly in terms of Ct-values over time and the preservation medium did not drastically affect the results for this method. However, the Heat Lysis – Ethanol combination did deliver the most consistent results of all the methods combinations over time. Although the ethanol preserved the zoospores slightly better than the Lysis Buffer, it had a significant inhibitory effect on the DNeasy PowerSoil Kit. Thus, each preservation medium presents their own benefits and limitations, and the application depends on the DNA extraction method being applied.

The DNeasy PowerSoil Kit – Lysis Buffer method delivered significantly better results over all the days, and is the method of choice for this project. It would be recommended to process samples as quickly as possible, preferable within three days of collection, to prevent great losses in DNA. Samples should preferably not be left for longer than a week before processing when using the Longmire's Lysis Buffer. The CV% of the Ct-values for all the DNA extraction method and preservation medium combinations, fell within the normal range for intra-assay variation (Abera *et al*., 2014; Balamurugan *et al*., 2010; Hyatt *et al*., 2007). Greater variances were, however, detected in the datasets over time for three of the four methods and thus, quantification of field samples may become less accurate the longer they are preserved for both preservation medium types. It would be recommended to process samples as soon as possible after sampling in order to obtain the most accurate results.

The Heat Lysis – Ethanol method presented the most consistent results overall over time and no significant differences could be observed between any of the days. This method combination is also the most cost-effective of all the methods tested. Additional cleaning kits could possibly be applied to this method to improve the Ct-values in the future. Additional studies have also shown that improving the qPCR phase of a study can compensate for the shortcomings of the extraction method, such as increasing the number of qPCR replicates in the study (Piggot *et al*., 2016). This can be tested in future studies if a more cost-effective alternative would be preferred. It should, however, be noted that the DNeasy PowerSoil Kit is a more time-effective method compared to the Heat Lysis method, because the time required to perform the Heat Lysis method increases with each added sample. This is due to the individual sample homogenising step in the protocol.

4.4.3. Primer efficiency and setting standards

When analysing all the data presented by the G-Block samples concentration from and below 1000 copies/µl demonstrated a great increase in variability and some extreme outliers were removed. In the study by Taylor *et al*. (2019), it was mentioned that the level of variability between samples increase greatly after 30 Ct-values. This is due to subsampling errors that often occur even with excellent pipetting skills at lower DNA concentrations when small volumes of samples must be applied (Taylor *et al*., 2019). This correlated with the data presented in the present study. It should be noted that when compared to previous literature, including a study that applied the same *Bd* primers, the CV% of the Ct-values for the different concentrations fell within the normal range for intra-assay variation in a dilution series (Abera *et al*., 2014; Balamurugan *et al*., 2010; Hyatt *et al*., 2007).

It is important to establish what the lowest detection limit is of an assay to avoid the possibility of false positives in the eDNA assay. This is often caused by background fluorescence being too high or non-specific amplifications such as primer dimer formations (Hunter *et al*., 2017). Sometimes these false positives may also be identified when samples with specific concentrations do not follow the expected trend line of a graph (Hunter *et al*., 2017). This was observed in the raw data for the G-Block dilution series where random samples amplified at the lowest concentrations and fell below the expected trend line. Additionally, more samples amplified at the lowest concentration compared to the second lowest concentration. None of the NTC's amplified on any of the runs, and samples were prepared from the lowest concentration to the highest using separate strip tubes for each concentration, which indicates the amplifications were unlikely due to cross-contamination of samples. The samples all had a Ct-value above 37 and were deemed unreliable. For this reason, all values above 37 will be marked as negative and excluded in future field studies.

The general methodology applied to determine the LOD by the MIQE is often considered too strict for eDNA applications and may potentially result in false negatives due to limiting the detection of low concentration DNA (Hunter *et al*., 2017). These guidelines recommend that 95% of samples should test positive to be deemed reliable for a LOD (Taylor *et al.*, 2019). According to the MIQE standard the LOD for the assay would be approximately between 100–1000 copies/µl. Various methods of determining the LOD have been developed for eDNA assay as previously discussed in section 4.1.4.

Based on the results from both the G-Block DNA as well as the filter dilution series, a cut-off point of 37 Ct was selected for the most reliable results in the field application. The LOD for the synthetic DNA was determined to be 100 DNA copies/µl, because 50% of the replicates for all the samples amplified below 37 Ct and two replicates amplified for each sample created.

The LOD for the filter material test is approximately 10 zoospores because all 6 qPCR replicates amplified for this concentration below 37 cycles. The zoospore LOD correlates with findings in previous *Bd* studies where the lowest detection swabs were determined to be 10 zoospores, and for eDNA of *Bd* from filter media the lowest detection limit was found to be 100 zoospores using the PowerSoil Kit (Brannely *et al*., 2020; DiRenzo *et al*., 2018; Sabino-Pinto *et al*., 2019). Thus, this assay is very likely to be sensitive enough for field application of *Bd* eDNA. The LOQ for the assay is approximately 1000 copies/µl, which is a very high value. This may imply that despite the assay being sensitive enough to detect *Bd,* the quantification of field samples in the future might be inaccurate and would not be recommended.

4.4.4. Future recommendations

The results comparison for this chapter was done on the assumption that the efficiency between the different runs is the same. The ΔCt between the reference sample and unknown samples should theoretically remain the same across plates if the same reagents and methods are applied (Hellemans *et al*., 2007). Thus, if the IRC provides the same Ct-value on the different plates, the plates can directly be compared or adjusted according to the reference sample variation (Hellemans *et al*., 2007). Previous studies have also shown that the *Bd* primers used in this study have a high efficiency and level of reproducibility in the results between different qPCR plates (Blooi *et al*., 2013; Hyatt *et al*., 2007). This statement was proven to be true based on the replicate samples that were run between the different plates that differed with less than 0.2 Ct-values or less than 0.1 Ct-values when comparing the means between replicates. For this project the difference between the replicate positive controls had a marginal variance that still fell within the natural acceptable range that can be expected for qPCR sample variation (±0.3 Ct-values) (Bustin *et al*., 2009; Flatschacher *et al*., 2022). This allowed for the relative comparison between the different plates in this study. The reality is however that efficiency of runs may vary between different plates. It would be recommended for future studies that an alternative method can be applied such as the absolute quantification method. This can be done by running a dilution series along with the samples to quantify the unknown targets according to the adjusted efficiency from the newly generated standard curve (Sivaganesan *et al*., 2010). Samples can then be compared based on quantified copy values rather than Ct-values between plates.

4.5. Conclusion

The filter method of choice for the project is the syphon pump due to its simplicity and practicality for field application in remote regions. The syphon pump had a very light design that could easily be carried over long distances as well as did not require any electricity and only manual labour to filter water through sterile Nalgene 0.45µm pore size, cellulose nitrate filter funnels.

The H-1 hypothesis tested true for this chapter of the study, which stated that significant differences would be detected in the Ct-values between the different extraction methods and preservation medium combinations. The method of choice for protocol was based on statistical significance. The Dneasy PowerSoil – Lysis Buffer combination indicates the lowest level of inhibition and delivered significantly better Ct-values compared to all the method combinations. Thus, the Dneasy PowerSoil – Lysis Buffer combination was selected as the method combination of choice due to its higher sensitivity and effective removal of inhibitors. Although the Heat Lysis – Ethanol combination delivered the most consistent results overall and had the lowest loss in DNA over time, the Dneasy PowerSoil – Lysis Buffer combination delivered significantly better Ct-values and was thus better at removing inhibitors. The Heat Lysis – Ethanol method was not selected as the method of choice for this project, however, can potentially be further optimised in future studies to reduce inhibition if a more cost-effective alternative method would be desired.

According to the G-Block dilution series the LOD of the assay is approximately 100 copies/µl and the LOQ is 1000 copies/µl. An additional filter dilution series using 150ml sterile water samples spiked with zoospores was also tested and demonstrated a LOD of 10 zoospores for the developed protocol. This showed great potential for future application in the field and results were comparable to the sensitivity of *Bd* protocols designed for swab methods.

Chapter 5: Field application

5.1. Introduction

The aim of this dissertation was to develop a Standard Operating Procedure (SOP) under laboratory conditions that could later be applied for field application. However, a preliminary method was tested in the field during the experimental phase and this greatly contributed to identifying the possible limitations that could be optimised in the method development. This preliminary application also provided guidance to increasing future field sampling success, through analysing various habitat types. To reduce the impact and stress on the amphibian population, this project was planned in parallel with an MSc project of a fellow student from the Herpetological Health Lab (HHL), Mr. Jacques Potgieter. A primary verification of *Bd* infections was required along with filter samples to reduce the possible presence of false positives or negatives in the qPCR assay. As previously discussed, many conventional diagnostic measures for *Bd* are extremely invasive and require direct sampling from amphibians. Examples of these measures include toe clips or ventral swabs, which may cause stress or trauma to the individuals, or tadpoles have to be euthanized for the collection of mouthparts for analysis (Annis *et al*., 2004; Boyle *et al*., 2004;).

Mr. Potgieter was conducting a project on the presence of *Bd* at three adjacent nature reserves in the northern Drakensberg, namely the Ncandu Nature Reserve, Ncandu Private Forest and Grassland Nature Reserve and the uMsonti Nature Reserve (Potgieter, 2022). During two field expeditions in April 2021 and October 2021 he detected the pathogen using conventional diagnostic measures (Potgieter, 2022). This not only provided data on the presence of *Bd* at the reserves, but also allowed him to test for seasonal variations in the pathogen prevalence. During the April 2021 excursion, filtering methods were tested for their practicality in field application, and this aided in the selection of the syphon pump as the filtering method of choice (see Chapter 4). eDNA filter samples were collected at the reserve during the second expedition, in October 2021, to allow for direct comparison between the preliminary eDNA testing compared to the conventional diagnostic measures.

The October expedition took place during the optimal season, which is spring, for sampling *Bd* in South Africa as previously determined by Conradie *et al*. (2011). Only a preliminary eDNA method had been selected for field application at this stage due to laboratory experiments being performed concurrently. During field sampling, ethanol had already been tested as a preservation medium of filter materials for both the DNA extraction methods (Heat Lysis and DNeasy PowerSoil Kit) and the preliminary method was selected based on the data collected at this stage.

This sampling opportunity served as the primary field verification of this project and provided valuable insight to the protocol developed as well as the limitations and strengths in eDNA sampling methods. The eDNA assay was further optimised after fieldwork and a future project has already been arranged for after the completion of this project to test the newly optimised method compared to conventional diagnostic measures in various environmental gradients within the country.

According to the guidelines for eDNA sampling set out by Goldberg *et al*. (2016), the sampling period has an extremely important impact on the success of the assay. During a *Bd* eDNA study conducted in Norway, sampling time had a significant impact on the presence of the pathogen in the water samples (Taugbøl *et al*., 2021). Springtime, similar to what was observed in the study by Conradie *et al*. (2011) in South Africa, appeared to be the optimal season for sampling *Bd* (Taugbøl *et al*., 2021). The time of the expedition was also selected to co-incide with the breeding season for the selected amphibian host specimen (*Amietia delalandii*). During this period, flow rates were still low due to rainfall only starting during this time, and temperatures were still low enough for the better preservation of eDNA (Conradie *et al*., 2011; Buxton *et al*., 2017; Harper *et al*., 2019).

This chapter will provide a literature review regarding the current known distribution of *Bd* in South Africa, the physical and biological characteristics of the selected study site, and the selection of an internal assay control specimen. The experimental phase consisted of testing genus specific primers of the internal control assay, determining the specificity as well as sensitivity of the primers acquired, applying the preliminary protocol to field application and comparing to the results of the conventional diagnostic measures.

5.1.1. Distribution of *Batrachochytrium dendrobatidis* in South Africa

Although multiple studies have been conducted on filtering *Bd* from aquatic environments, research remains limited and must still be evaluated on a variety of habitats to determine its effectiveness (Hyman and Collins, 2012). Some of the previous environments where *Bd* has been detected in South Africa will be evaluated in this section along with environmental parameters that contribute to the distribution. The lineages *Bd*CAPE and *Bd*GPL have been associated with the most amphibian mortalities of all the known lineages (Ghosh *et al*., 2021). Both these lineages occur in South Africa and require management and mitigation measures to prevent possible future spread to vulnerable amphibian communities (Ghosh *et al*., 2021).

Based on the application of MAXent for global species distribution modelling of *Bd*, South Africa has previously been identified as a high-risk area for *Bd* infections (Bie *et al*., 2021).

The greatest prevalence of the pathogen worldwide was detected in the Montane grassland biome as well as the Shrublands biome (Olsen *et al*., 2013). Lower levels of infections generally occur in the following biomes based on global data: Woodlands, Scrub as well as the Mediterranean Forests biomes (Olsen *et al*., 2013). Previous studies have indicated that high elevation areas have shown to be more favourable for the prevalence of the pathogen (Bancroft *et al*. 2011; Gabor *et al*., 2015; Zimkus *et al*., 2020).

Specifically, in South Africa, species distribution models on *Bd* positive samples have determined that the pathogen mainly occurs in the coastal as well as high rainfall regions in the eastern sections of the country (Tarrant *et al*., 2013; Zimkus *et al*., 2020). High elevation, low temperatures, and high precipitation appeared to be the major drivers to the pathogen prevalence (Tarrant *et al*., 2013; Verster 2022). It should be noted that a wide variety of habitats outside these regions have previously tested positive for *Bd*, ranging from extremely arid environments to high elevation and rainfall areas (Zimkus *et al*., 2020). This is further proven by the presence of the pathogen, both *Bd*CAPE and *Bd*GPL, in the Orange River Basin, including the Vaal River system and associated tributaries (Conradie *et al.*, 2011; Ghosh *et al*., 2021; Verster 2022).

Many studies have previously been conducted on *Bd* within the Drakensberg area of the country (Meyer, 2009; Ghosh *et al*., 2021; Griffiths et al. 2018). The Drakensberg in South Africa represents the highest escarpment in the country and the climate is typically associated with colder temperatures and above average precipitation compared to the rest of South Africa (Nel and Sumner, 2008). Great changes in elevation occur over the mountain range and, orographic precipitation with thunderstorms is typically associated with the region (Nel and Sumner, 2008). This is in contrast to the climate associated with the Orange River Basin which occurs in a semi-arid environment (Tarrant *et al*., 2013). The Drakensberg is, however, known to serve as a water source for the Orange River (Ghosh *et al*., 2021). The temperature of the valley rivers in the basin does also comply with the natural survival range of the pathogen (Tarrant *et al*., 2013), potentially explaining its presence in the river system.

The majority of the frog species that have tested positive for *Bd* in the Drakensberg belong to the genus *Amietia* and the three most common species were *Amietia hymenopys, A. delalandii* and *A. vertebralis* (Tarrant *et al*., 2013; Zimkus *et al*., 2020). Despite the high prevalence of the pathogen in specific regions of the country, threatened amphibian species populations have shown to be affected minimally thusfar (Tarrant *et al*., 2013). The management of this pathogen is however, still of great concern and could still potentially spread to more vulnerable populations.

Many regions of the Drakensberg have not yet been tested for *Bd*. One such area is the Ncandu Nature Reserve located in the northern section of the Drakensberg. Understanding the distribution of a pathogen can aid in the management and spread thereof. For this reason, the Ncandu reserve, along with adjacent private reserves were selected for this project and the project by Mr Potgieter. The following section will review the environmental characteristics and amphibian diversity of the selected sampling sites.

5.1.2. Ncandu Nature Reserve, uMsonti Nature Reserve, and Ncandu Private Forest and Grassland Reserve

The Ncandu Nature Reserve was initially an acclaimed State Forest in 1925 but was later reclassified in 1989 as a nature reserve and falls under the responsibility of Ezemvelo Kwazulu-Natal Wildlife (Rambarath *et al*., 2017). The reserve lies adjacent to two other nature reserves, namely the Ncandu Private Forest and Grassland Reserve (further referred to as the Ncandu Private Nature Reserve) and the uMsonti Private Nature Reserve.

The Nacandu Private Nature Reserve was officially declared a reserve in October 2013 and the private landowners serve as the managing authority to the Protected Area (PA), known as the Ncandu Reserve Private Owners Association (Rambarath *et al*., 2017). According to the Government Gazette (Vol 7) No. 1054 of 19 November 2019 (Kwazulu-Natal Nature Conservation Board, 2013), the reserve consists of four farm portions located in the Newcastle Local Municipality, namely Steep Glen, Union, Trespass, and Galway. This protected area received its name from the Ncandu River flowing through the valley of the reserve (Rambarath *et al*., 2017).

The uMsonti Private Nature Reserve lies adjacent to the Ncandu Private Nature Reserve. The reserves mentioned are bordered to decrease the impact of habitat fragmentation and improve the conservation value of the area (Conservation Outcomes, 2021). The uMsonti Nature reserve is owned and managed by two private owners and consists of five farms namely, Leopard Kloof, Albany, Normandien, Woodburn and Buffelshoek (Conservation Outcomes, 2021). Only portions of the aforementioned farms form part of the reserve, except for the farm Leopard Kloof for which the entire extent is part of the reserve (Conservation Outcomes, 2021).

Limited research has been conducted in the above mentioned reserves and the information for this section is mainly derived from the Ncandu Nature Reserve Integrated Management Plan 2008–2013 (Rambarath *et al*., 2017), uMsonti Private Nature Reserve: Management Plan version 2 (Conservation Outcomes, 2021) and a government gazette (Kwazulu-Natal

Nature Conservation Board, 2013). An additional minor desktop assessment was conducted in this chapter using GIS to identify additional environmental features and characteristics of the sampled areas within the reserves.

5.1.2.1. Location

The study sites are located on the eastern Drakensberg Escarpment in the province of Kwazulu-Natal (KZN) and borders the Free State Province (Rambarath *et al*., 2017). The extent of *Bd* over the Drakensberg is still being researched (Zimkus *et al*., 2020) and the mentioned reserves had not yet been tested for *Bd* prior to this and Mr. Potgieter's study. The closest town to the reserve is Newcastle, which is approximately 32km to the northwest of the study area (Rambarath *et al*., 2017).

5.1.2.2. Climate

Little information exists on the climate of the Ncandu Nature Reserve and the weather measured from 22 km away, close to Newcastle, was applied in the integrated management plan (Rambarath *et al*., 2017). The annual rainfall of the measured area is approximately 801 mm, with the wet season being between November and March (Rambarath *et al*., 2017). It should be noted that orographic precipitation caused by the escarpment is likely to increase the rainfall of the reserve compared to the measured area (Rambarath *et al*., 2017). Orographic precipitation is described as the process where clouds move over objects such as mountain ranges, which modifies the intensity as well as frequency of rainfall occuring in the area (Roe, 2005). Changes in vegetation and biodiversity have previously been noted in the areas where orographic precipitation occurs (Roe, 2005). The altitude of the Ncandu Nature Reserve ranges from 1460m – 1900m above sea level (Rambarath *et al*., 2017).

Although limited climate data exists for the Ncandu Nature Reserve, data has previously been collected from the farm Normandien in the uMsonti Nature Reserve (Conservation Outcomes, 2021). This may potentially provide a more accurate description of the climate for the area. The climate data from 2010 – 2017 indicated that the farm had an average annual precipitation of approximately 1012mm (Conservation Outcomes, 2021). This demonstrates the impact of orographic precipitation on the annual rainfall. The elevation of the uMsonti Nature Reserve varies from 1300m – 1850m (Conservation Outcomes, 2021). Due to the close proximity of the reserves and elevations falling within a similar range, climatic data between the areas may be alike. The weather at the farm Normandien indicated an average minimum temperature of 0°C in the winter to an average maximum temperature of 27°C in the summer, with an annual average of 14°C. Thus, the selected sampling sites occur at a high elevation and cooler

temperatures which are favourable for the occurrence of *Bd* as well as for the presence of eDNA fragments in the environment, based on previous studies (Bancroft *et al*., 2011; Eichmiller *et al*., 2016; Gabor *et al*., 2015; Strickler *et al*., 2015).

The data provided, further supports the notion that October is an excellent sampling period for the collection of *Bd* and eDNA. This is due to this period being just before the official rainfall season for the area and as previously discussed high rainfall may result in higher flow rates that reduces sampling efficiency (Conradie *et al*., 2013). Previous literature have also indicated that behavioural factors, such as breeding, can greatly contribute to higher infection rates amongst individuals (Bancroft *et al*. 2011; Harper *et al*., 2019; Kinney *et al*., 2011). Based on field observations, the area received enough rainfall during this period for the breeding season of amphibians to commence. This was confirmed as a pair of *A. delalandii* were observed in amplexus during the field sampling.

5.1.2.3. Hydrology

Multiple tributaries feed the Ncandu River that is located in the valley of the Ncandu Nature Reserve (Rambarath *et al*., 2017). This river feeds into the main Ncandu River artery as well as the Amcor Dam and serves as an important water source to the catchment area (Rambarath *et al*., 2017). The water in the river system is often utilised for agricultural purposes downstream (Rambarath *et al*., 2017). Many other tributaries in the Ncandu Nature Reserve also form part of the Horn River system (Conservation Outcomes, 2021). The rivers of the uMsonti Nature Reserve form part of the Buffalo secondary catchment area and serves as a significant source to the Horn River (Conservation Outcomes, 2021). Examples of the Ncandu River, along with other river systems and the supporting streams/tributaries observed during the excursion can be viewed in Figure 5.1.

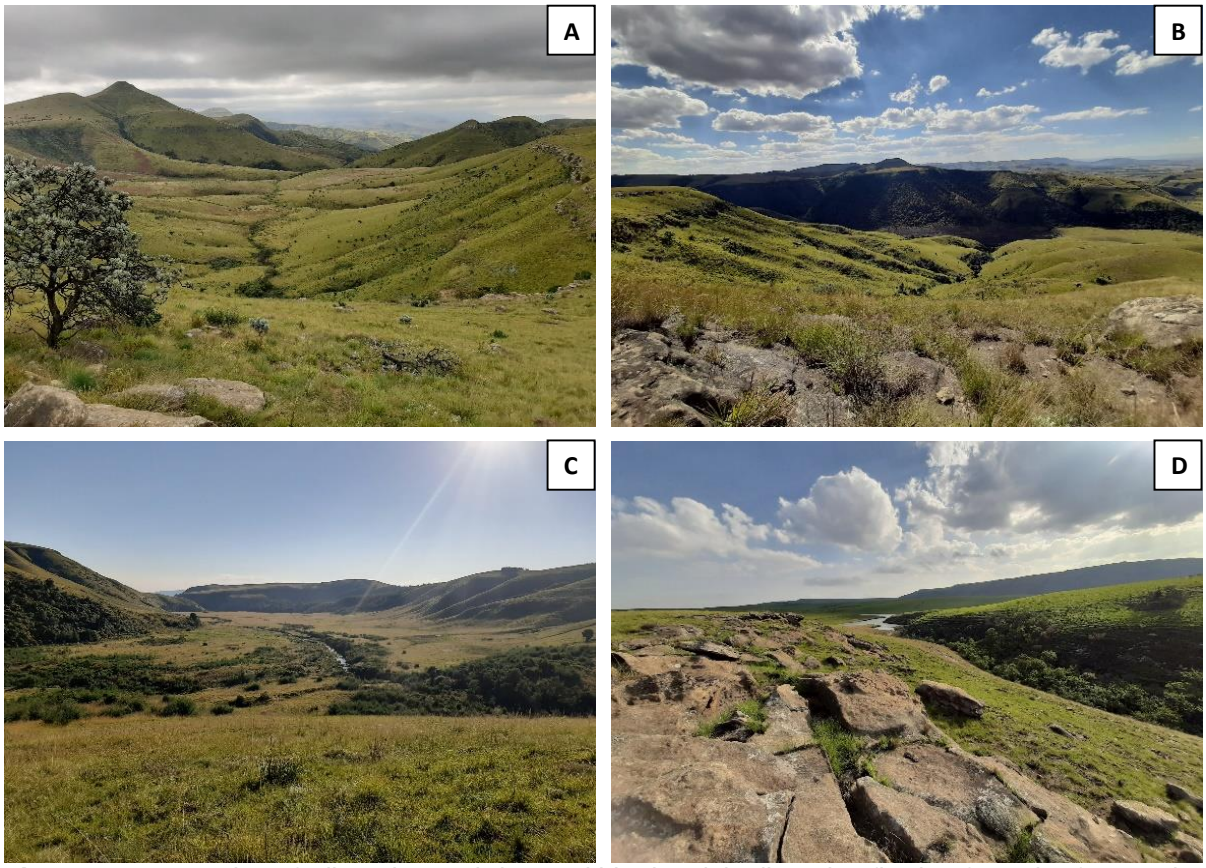

Figure 5.1. Examples of tributaries and rivers within the; (A) tributaries flowing from the farm Union (Ncandu Private Nature Reserve) connecting to the main Ncandu arterial river system, (B) tributaries from the farm Steep Glen (Ncandu Private Nature Reserve) connecting to the main Ncandu arterial river system, (C) the Ncandu river (Ncandu Nature Reserve), and (D) river from the farm Leopard Kloof connecting to a local dam (uMsonti Nature Reserve).

5.1.2.4. Amphibians of the Ncandu Nature Reserve

As previously stated in the literature review, the species present as well as interspecific interactions play a crucial role in the level of infection of the individuals (Bancroft *et al*., 2011). Very few studies have been done within this Nature Reserve and the amphibian diversity present still needs to be analysed. During the first trip to Ncandu, the amphibian diversity was analysed by an Honour's student in the HHL lab, Mrs. Chenelle Scheepers (Scheepers, 2022). Based off the species diversity and distribution data, a secondary target specimen was selected for the eDNA verification test.

A species had to be selected that is not only common to the area, but also common throughout southern Africa for future tests involving eDNA assays for *Bd*. In the Ncandu Nature Reserve Integrated Management Plan, it is mentioned that only three frog species occur in the reserve (Rambarath *et al*., 2017), however during the excursions Scheepers (2022) found a total of

ten amphibian species, with a possible eleventh species, present. The following amphibians were identified in her project through the capturing of frogs or identifying species through their vocalisations: *A. delalandii*, *Cacosternum boettgeri*, *Kassina senegalensis*, *Sclerophrys capensis*, *Sclerophrys gutturalis*, *Semnodactylus wealii*, *Strongylopus grayii*, *Tomopterna natalensis*, *Xenopus laevis*, *Breviceps verrucosus* and potentially *Breviceps adspersus* (Figure 5.2). One of the most prevalent anuran species found in the aquatic environments was *A. delalandii*, the Common river frog.

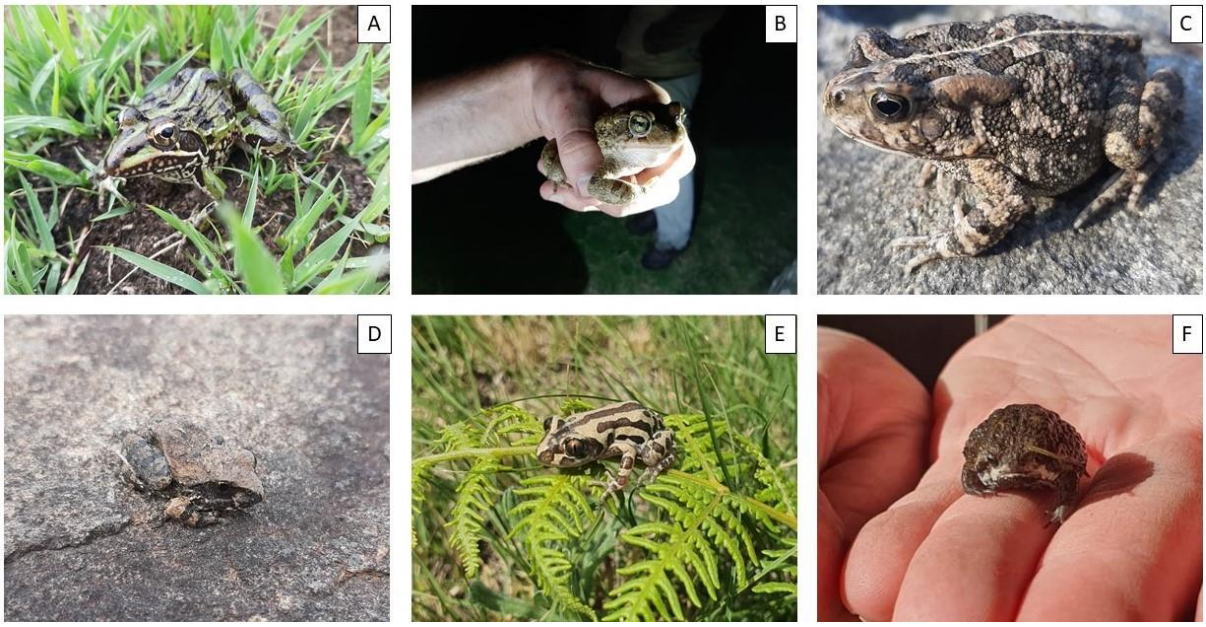

Figure 5.2. Examples of amphibians at the Ncandu Nature Reserve; (A) *Amietia delalandii*, (B) *Sclerophrys capensis*, (C) *Sclerophrys gutturalis*, (D) *Tomopterna natalensis*, (E) *Kassina senegalensis*, (F) *Breviceps verrucosis*. (Photo credit for E and F: Jacques Potgieter)

5.1.3. Selection of an internal assay control specimen

Previous *Bd* studies on the African continent have shown Pyxicephalidae frogs, specifically *Amietia* spp., to be the second most common frog family and genus to test positive for *Bd* (Zimkus *et al*., 2020). As previously stated in section 5.1.1. *A. delalandii* is also one of the most common species to be tested for *Bd* in the Drakensberg area (Tarrant *et al*., 2013; Zimkus *et al*., 2020). *Amietia delalandii* can be found throughout southern Africa and occurs on the banks of streams where the water flows slowly as well as a diverse array of wetland ecosystems (Du Preez and Carruthers, 2017). In a previous *Bd* study conducted in multiple regions across South Africa, *Amietia* spp. were the most commonly sampled amphibian species (Ghosh *et al*. 2020). These frogs are often easy to find and have a wide distribution across southern Africa (Du Preez and Carruthers, 2017) which makes them excellent target specimens for this type of research.

This frog species is known to be relatively resistant towards *Bd* which could potentially increase their survivability, but these individuals often still have a low-level of infection present as seen in the high frequency of infected individuals (Antwis and Weldon, 2017). The prevalence of the pathogen for this amphibian species has been shown to be approximately 38.8% (Antwis and Weldon, 2017). This may be due to the skin mucosa of this species that sometimes inhibits the growth of *Bd* (Antwis and Weldon, 2017). Based on the species diversity results from the Ncandu Nature Reserve it was decided that *Amietia* spp. will serve as the secondary verification method also referred to as an internal assay control standard for eDNA sampling and detection due to its high occurrence at the reserve as well as its previous successful use for *Bd* detection.

5.2. Hypothesis, aim and objectives

5.2.1. Hypothesis

H0 – The selected protocol is unsuccessful at detecting *Bd* in the environment or no significant differences in performance can be detected compared to conventional diagnostic measures.

H1 – The selected protocol is successful at detecting *Bd* in the environment and significant differences in performance can be detected compared to conventional diagnostic measures.

5.2.2. Aim

To test the selected preliminary protocol in field sampling and comparing the results with conventional diagnostic measures to determine potential limitations in the assay that can be further optimised in the laboratory post fieldwork.

5.2.3. Objectives

- Test the specificity and sensitivity of *Amietia* spp. primers for the internal assay control of eDNA samples.
- Collect water samples from multiple sites at the Ncandu Nature Reserve, uMsonti Nature Reserve and Ncandu Private Nature Reserve through filtration.
- Extract the DNA from the filter materials using the selected preliminary protocol.
- Determine presence of *Bd* and *Amietia* spp., using genus and species specific primers in qPCR assay.
- Compare the effectiveness of the protocol compared to conventional diagnostic measures.

5.3. Materials and methods

The methodology for this section consists of three sections. Section 5.3.1 describes the method of filtration applied as well as the preliminary method selected for the eDNA analysis. Information was also provided on the selected sampling sites and an additional GIS desktop study was undertaken to provide more information on the sampling locations along with observations made during the field sampling. Section 5.3.2 reviewed the methodology of the verification methods that have been applied to confirm the accuracy of the preliminary selected assay. The primary verification to the eDNA samples were done through conventional diagnostic measures performed in the study by Mr Potgieter (Potgieter, 2022). An internal assay control was tested using genus specific primers for the selected amphibian host specimen, *Amietia* spp. The LOD and LOQ of the assay was determined as well as the specificity of the primers. Section 3.3 discussed the statistical analysis methods applied to quantify and compare the results.

5.3.1. Field sampling

5.3.1.1. Filter samples

Filter samples were collected using the selected syphon pump method tested in Chapter 4 and Nalgene Single Use Analytical Funnels with cellulose nitrate filters, pore size 0.45µm (catalogue number: 145-2045). Filter cups were bought in separate sterile packages to ensure no cross contamination between samples, and were opened on site. One filter cup was used per sample and water was directly collected into the sterile filter cups. Filter material was only handled with tweezers and scissors that were rinsed in 70% ethanol and sterilised by flame between each sample. Samples were stored in sterile 15ml falcon tubes containing 1.5ml of the selected preservation medium and sealed with parafilm. The falcon tubes were placed upright in a falcon tube holder to limit cross-contamination by ensuring none of the samples from different sites or within a single site touched. When sampling was completed the syphon pump was rinsed with 70% ethanol that has proven to effectively kill zoospores and sporangia rapidly (Johnson *et al*., 2003), in order to prevent the possible spread of the pathogen to new sites.

5.3.1.2. Number and sample size

Various authors have recommended that multiple large water samples should be gathered over a wide area to obtain the most accurate results in aquatic water bodies (Deiner *et al*., 2015; Rupert *et al*., 2019). Previous studies have shown that, when attempting to detect elusive species, more replicates are required, but the target organism should be detectable

within five filter samples per site (Willoughby *et al*., 2016). Thus, five filter samples of 500ml were taken, a total of 2.5l per study site. The volume was selected based on the results from filter tests discussed in Chapter 4, section 4.4.1.1. In total four sites were tested, thus, 20 filter samples were collected during the excursion. Samples were collected randomly along the surface waters of the sites and included a variety of habitats such as lentic and lotic water systems.

5.3.1.3. Permits

Permit No. OP 874/2021 was obtained from Ezemvelo KZN Wildlife in accordance with the Nature Conservation Ordinance No. 15 of 1974, Chapter 7 (Annexure 2). This permit also allowed Jacques Potgieter to sample amphibians for the primary verification in this project. The Ncandu Reserve, uMsonti Nature Reserve, and Ncandu Private Forest and Grassland Reserve all form part of Ezemvelo KZN Wildlife. Permission was also obtained from two of the farm portion owners, Mr. Johan Scheepers and Mrs Susan Basson, to sample from private owned portions in the nature reserves.

5.3.1.4. Sampling sites

During the first excursion seven different sites were tested for *Bd* using conventional diagnostic measures. Five of these sites tested positive, however, during the second excursion, many of the previously confirmed positive sites could not be sampled again. Some sites became inaccessible due to the rainfall from the previous week, others had not yet been fully replenished from the dry season or no amphibian specimens were present, and one site became largely transformed due to the presence of cattle. Thus, a number of new sites had to be selected during the second excursion for the application of the preliminary eDNA protocol. A variety of habitats were sampled during this section of the project. This allowed us to test the possible impact of environmental types, such as lentic and lotic as well as permanent and temporary water bodies on eDNA distribution and quantity.

Filter sampling was conducted within all three different reserves and four sites were sampled in total; two sites from the main Ncandu Nature Reserve (Steep Glen River and Steep Glen Trench), one site from the farm portion Leopard Kloof in the uMsonti Nature Reserve (Leopard's Creek) and one site from the farm portion Union in the Nacandu Private Nature Reserve (Mushroom Rock) (Figure 5.3). The subsequent section will provide insight on the GIS methodology for all the maps made, and additional information for each of the selected sampling sites based on field observations and a desktop GIS analysis.

Methodology for GIS desktop analysis

The additional GIS desktop study was done to determine the vegetation types and wetland types (if any) present at eDNA sampling sites. Vegetation types can provide valuable insights to the climate present in the environment. All the maps created were done using ArcGIS v.10.8. The extent of the uMsonti Private Nature Reserve was determined based on the maps from Conservation Outcomes (2021), and the extent of the farm portions of the Ncandu Private Nature Reserve were based off the maps from Rambarath *et al*. (2017) and Conservtaion Outcomes (2021). The data layers used in this study were acquired from the SANBI BGIS database as well as Esri and the metadata of the layers applied can be viewed under Annexure 1, Table A1.1. More information on the data layers applied can be found under Annexure 1 along with the vegetation map and NFEPA rivers and National wetlands version 5 map used for the analysis. All maps were created in the coordinate system: GCS_WGS 1984 (Geographic coordinate system – World Geographic System 1984). The location map for the four selected sampling sites can be viewed in Figure 5.3 of this chapter.

GIS analysis

According to the GIS analysis the three reserves mainly consist of the Low Escarpment Moist Grassland vegetation type, with large patches of Southern Mistbelt Forest in the valleys and around the water drainages on the slopes flowing towards the valley. The Southern Mistbelt Forest, has shown to be typically associated with the Low Escarpment Moist Grassland vegetation type. The eastern parts of the uMsonti reserve have sections part of the Northern Kwazulu-Natal Moist Grassland vegetation type. All of the selected sampling areas are found within the extent of the Low Escarpment Moist Grassland vegetation type, with the two Steep Glen sites occurring within close proximity to the Southern Mistbelt Forest on the slopes (Annexure 1: Figure A.1.1). These vegetation types are associated with the escarpments of the Drakensberg and occur in climates with high rainfall (Mucina *et al*., 2006). During the excursions fog was often observed during the day, which is also commonly associated with this vegetation type (Mucina *et al*., 2006). Based on previous literature this type of weather is favourable for the distribution of *Bd* due to the higher precipitation and lower temperatures (Tarrant *et al.,* 2013; Zimkus *et al*., 2020).

Limited data seems to be present on the wetlands and river systems found in the reserve when reviewing the GIS data (Annexure 1: Figure A.1.2). Although not present on the map, a part of the Ncandu river flows through the reserve and feeds into the main artery of the Ncandu river north of the reserve. Research on the wetlands and water systems of the Ncandu reserve is still understudied and more information is required. However, based on the National Wetland

map v5, the Leopard's Creek sampling site occurs within close proximity to a wetland area. The other sites all occur in streams which were observed in the field but have not yet been included in SANBI BGIS databases.

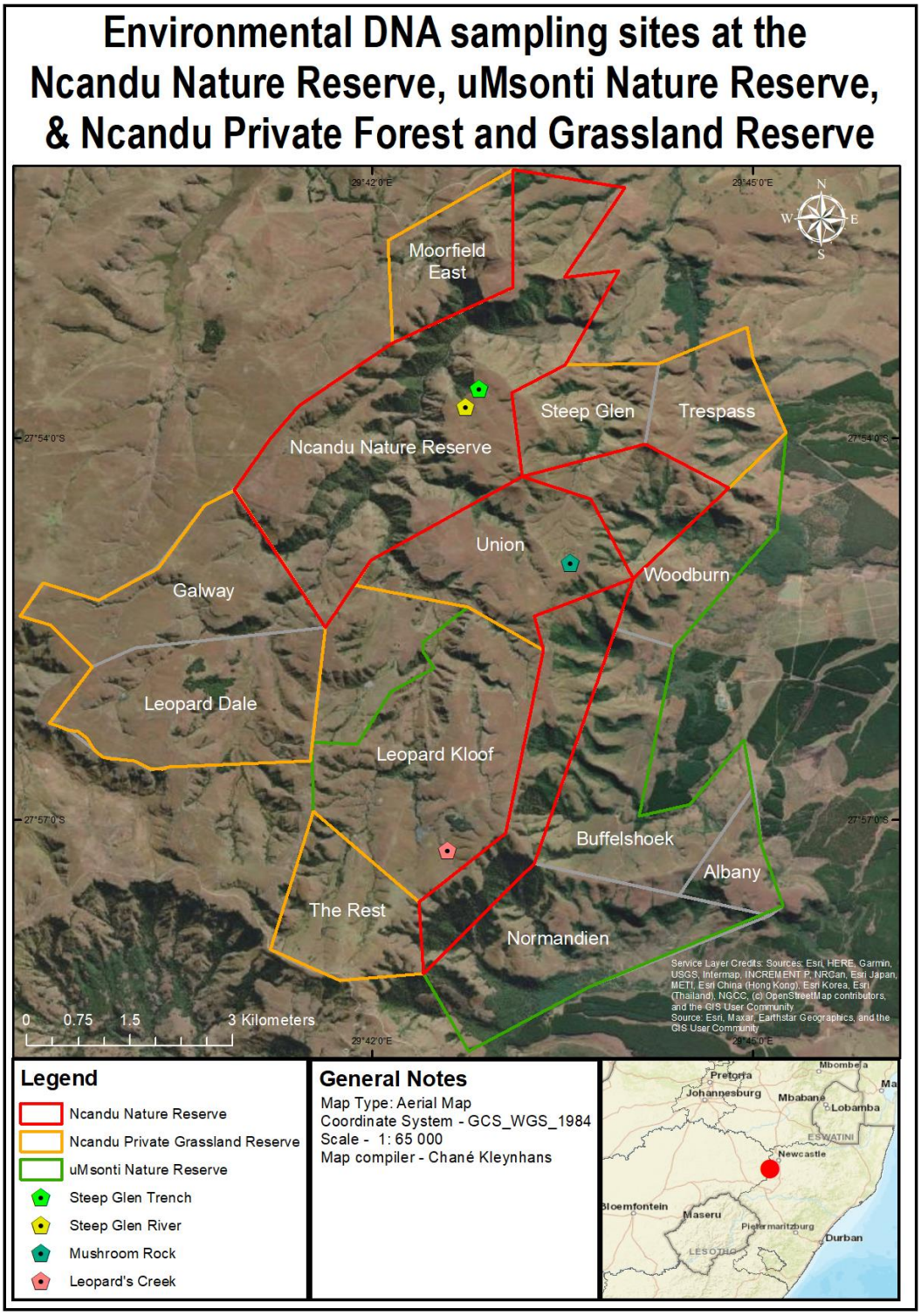

Figure 5.3. Location of the eDNA sampling sites at the Ncandu Nature Reserve, uMsonti Nature Reserve and Nacandu Private Forest and Grassland Nature Reserve in Kwazulu-Natal, South Africa (Refer to Annexure 1, Table A.1.1. and Table A.1.2. for the metadata of the GIS layers applied).

Selected sampling sites

The first site on the Leopard Kloof farm portion was a wetland area named Leopard's Creek (Figure 5.4A). This site was not tested during the first expedition. Two *A. delalandii* individuals were observed in amplexus at this site and tadpoles were collected. As previously stated in the literature review of Chapter 1, breeding behaviours significantly contribute to the spread and prevalence of *Bd* (Bancroft *et al*. 2011). Many adult amphibians were also found throughout the water body and this site was selected based on the high density of individuals. Water samples were collected a day after tadpole mouthpart sampling. Other water bodies were first assessed before selecting this site and this site was deemed the most appropriate. This site had the most abundant adult *A. delalandii* population compared to the other sites present in the area and mating behaviours were also observed. This site was also selected because it formed part of a seasonal waterbody, based on the observations from the landowners. Thus, water is only present during the wetter seasons of the year, but despite this, many individuals were present at the site. Thus, it could be tested whether seasonal aquatic systems can produce positive results for eDNA tests in the future.

The second site selected on the Union farm portion was named Mushroom Rock due to the unique geological structures present at the site (Figure 5.4.B). This area previously showed to have a potential positive individual for *Bd* during the first excursion taken in March 2021. This sample was however inconclusive and could not be confirmed to be positive. Many amphibian eggs were present in the water along with tadpoles. Although adult amphibians were present during the first excursion, no matured individuals were observed during the second excursion. The tadpoles appeared small and remained at the bottom of the pool. Water was sampled along the surface and where minimal turbidity was experienced. The site occurred at the origin of the stream and could provide valuable information on how eDNA concentrations may be affected by the reduced accumulation in ponds due to limited inflow of additional eDNA from upstream.

The final two sites selected are named the Steep Glen River and Steep Glen Trench (Figure 5.4.C and 5.4.D). These sites are not located on the Steep Glen farm portion, but rather form part of the main Ncandu Nature Reserve. They were reached through traveling over the Steep Glen Hills, which provided the sample site names. Many streams originate from these high-altitude regions in the reserve and flow into the main arterial river, the Ncandu River, located in the valley of the reserve. Due to the water originating from this site and the undisturbed nature of the environment the water is pristine and suitable for human consumption. Many *A. delalandii* adults and tadpoles were present, along with *X. laevis* which could also be observed occasionally. The Steep Glen River was the only site that previously tested positive for *Bd*

during the first excursion. The Steep Glen Trench was selected due to its close proximity to Steep Glen River which may have increased the likelihood for *Bd*, while still forming part of a separate river system which presented a different habitat for comparison.

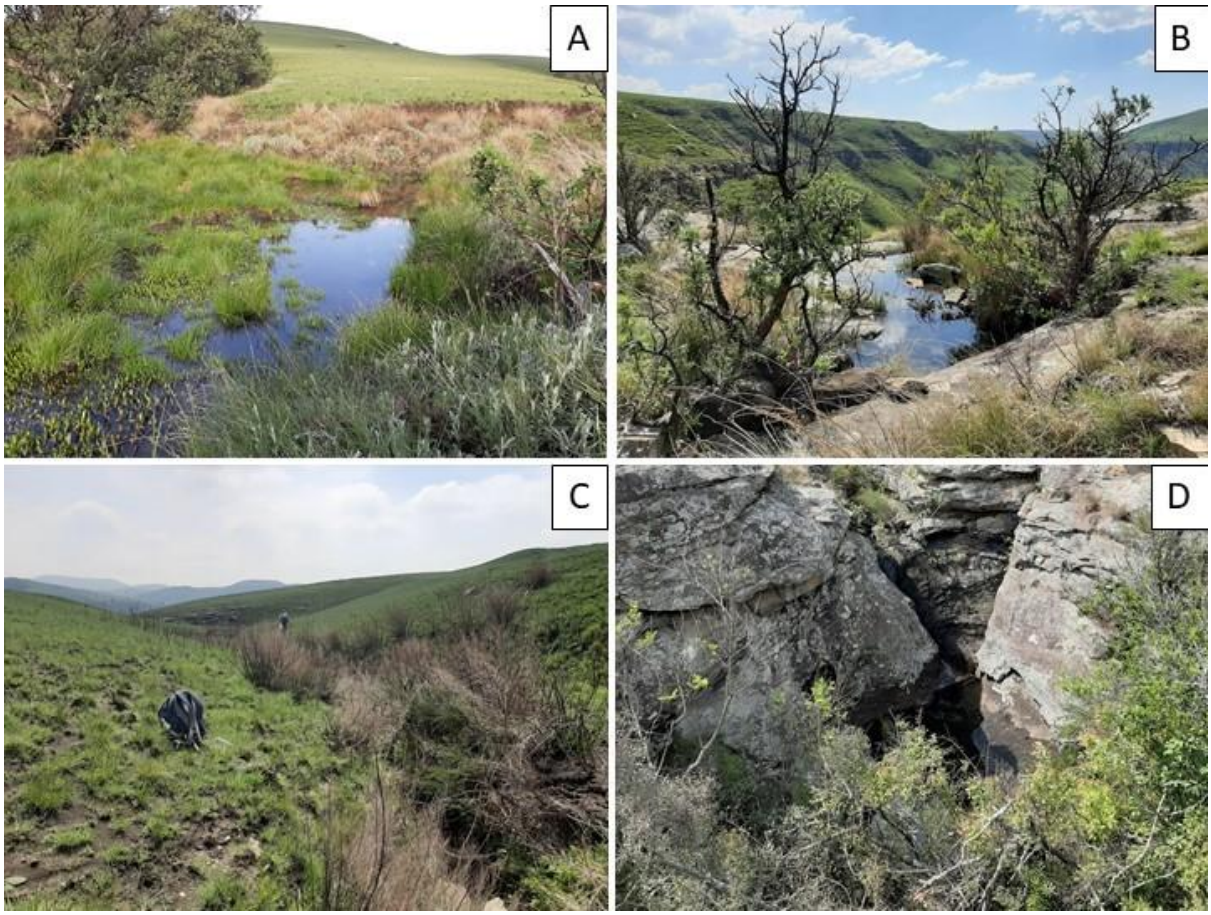

Figure 5.4. (A) Site 1: Leopard's Creek, (B) Site 2: Mushroom Rock, (C) Site 3: Steep Glen River, (D) Site 4: Steep Glen Trench.

5.3.1.5. DNA extraction

At this stage of the project the DNA extraction methods had all been tested under laboratory conditions for the different matrix types and two methods were selected (DNeasy PowerSoil Kit and Heat Lysis method). Filtering protocols had also been tested and the syphon pump selected was the filtering method of choice. Similarly, ethanol had been tested as preservation medium of filter materials (day one and three) for both DNA extraction methods. The primer efficiency, LOD and LOQ had been determined for both the *Bd* G-block and *Amietia* spp. DNA samples. Additionally, the specificity of the *Amietia* spp. primers had also been tested at this stage. Based on the previous results, the 70% ethanol had a significant downstream inhibitory effect on the Dneasy PowerSoil Kit when compared to the Heat Lysis method, which greatly

reduced the sensitivity of the assay. Thus, the preliminary method of choice at this stage was the Heat Lysis method using the 70% ethanol preservation medium due to the lower qPCR inhibitory effect of the extracted DNA. Lower variations in Ct-values were also detected using the Heat Lysis method which would allow for a more accurate quantification of field sample DNA copy numbers. Samples were submerged in 1.5ml 70% ethanol in sterile 15ml falcon tubes. The DNA extraction was as described in Chapter 4 section 4.3.2.1.

5.3.1.6. Molecular detection

For details on the qPCR protocol and reagents applied for the *Bd* sample analysis refer to Chapter 2 section 2.2.2. The Ct-values for the *Bd* samples were considered positive if one of the two PCR duplicates presented a Ct-value below the selected cut-off point of 37 cycles. As previously described in Chapter 4 section 4.4.3., the LOD for the primer was 100 copies/µl and the cut-off Ct-value was determined to be 37 cycles. Samples can be measured according to the values from the standard curve generated, but due to the LOQ of the primers being 1000 copies/µl, it is not recommended to quantify samples below this value.

5.3.2. Result verification

5.3.2.1. Primary verification

The primary verification of the field samples was done through the use of conventional diagnostic measures conducted as part of the completed MSc research project of Jacques Potgieter (Potgieter, 2022). In total 11 sites were analysed over the two excusion periods, seven during the first excursion in April 2021 and six during the second in October 2021, of which two were analysed on both excursions. The following is a short description of each of the methods applied in his study.

Mouth parts of tadpoles

Tadpoles were collected, 10 individuals at each selected site, and euthanized using Tricaine Methanesulphonate (MS222). Scissors and tweezers were sterilised using 70% ethanol and a flame. The jaw of the tadpole was gently squeezed using the tweezers to allow the mouthparts to protrude. The mouthparts were then carefully removed using the scissors and placed on a 1% tryptone agar plate containing antibiotics. The sample was further split into four sections, and each section was dragged through the agar to clean and sterilise the segments of bacteria or other possible contaminants (Fisher *et al.* 2018). The segments were then transferred to fresh agar also containing antibiotics and incubated for a few days at 20ºC

to allow *Bd* to grow if present. The parts were regularly monitored for contamination as well as the presence of *Bd* using microscopy. The number of positive individuals were used to determine the level of prevalence for the infection of a specific site.

Toe clips

Toe clips were collected from adult amphibians and treated in a similar manner as the mouthparts, except whole toe clips were used rather than being split into segments (Fisher *et al*., 2018). These toe clips were dragged through antibiotic infused 1% tryptone agar plates and transferred to fresh agar plates with antibiotics for incubation at 20ºC. Samples were monitored using microscopy.

A total of fifteen indivudals were analysed using toeclips. Matured amphibians could only be captured at four of the sites analysed, namely the Marsh (four anurans captured), Bloekombos (one anuran captured), Eikenhof Dam (seven anurans captured) and Leopard's Creek (three anurans captured).

5.3.2.2. Internal assay control

The internal assay control for this project referred to the use of additional primers for a second target specimen within the environment. This test determined whether a negative result for the *Bd* eDNA was due to a lack of zoospores present in the environment or whether this was due to the protocol itself being insufficient. If the protocol was able to detect the internal assay target specimen from the environmental samples, but not *Bd*, it may have been due to too low DNA copy numbers of *Bd* in the environment, which fell below the assay detection limit. If neither one of the specimens were detected and it was confirmed that one or both occurred within the environment, then the protocol was deemed inadequate.

As previously discussed in the literature review, *A. delalandii* was selected as the most appropriate internal assay control specimen for this project. This is due to the widespread occurrence of this genus at the study site as well as southern Africa, and their potential to host high intensities of *Bd* infections. *Amietia* spp. specific primers can potentially be applied for almost any subsequent *Bd* eDNA study in South Africa, however, there are no known primers for the detection of this genus specifically. Therefore, a request for primer development was made to YouSeq Ltd. and an *Amietia* spp. qPCR kit was provided (Catalogue number: YS-QP-IC-Amietia.spp). A total of 100 samples were provided with each kit. Due to these primers being untested in practice the efficiency and specificity had to be analysed. Standards such as the LOD and qPCR thresholds were set prior to field application.

Amietia *spp. primer efficiency*

A positive control with a known concentration of 1 000 000 DNA copies was provided with the kit, and was prepared according to the manufacturer's instructions to a final volume of 100µl. A 10-fold dilution series was created for 10–1000 000 copies using 90µl of the template resuspension buffer and 10µl of the previous dilution concentration to create a final volume of 100µl. A duplicate of each dilution series was made, and each sample was run in duplicate for the qPCR.

Amietia *spp. primer specificity*

According to YouSeq Ltd, the primers developed would amplify for the following species from the Pyxicephalidae family with distributions obtained from Channing and Rödel (2019): *A. delalandii* (western Mozambique, South Africa, Lesotho, Zimbabwe, Malawi, and Zambia), *Amietia fuscigula* (South Africa), *Amietia vandijki* (South Africa), *Amietia vertebralis* (Lesotho and South Africa), *Amietia poyntoni* (Lesotho, South Africa, and Namibia), *Amietia johnstoni* (Malawi), *Amietia moyerorum* (Malawi and Tanzania), *Amietia ruwenzorica* (Uganda and Democratic Republic of Congo), and *Amietia wittei* (Kenya, Tanzania, and Uganda).

Due to no analytical specificity data for these primers, four different *Amietia* species namely *A. delalandii*, *A. veterbralis*, *A. poyntoni*, and *A. fuscigula* were selected to test the specificity of the primers. An outlier species, *S. grayii* was included in the screening. This outlier was selected due to its presence at the Ncandu Nature Reserve and because it also belongs to the Family Pyxicephalidae (Du Preez and Carruthers, 2017). Tissue samples were obtained from old samples collected and stored by the African Amphibian Conservation Research Group at the North-West University. Samples were stored in a freezer at -80°C and small aliquots were used for DNA extractions. The tissue samples were from different body parts of the specimens and differed in size and weight. Some parts contained hard cartilage that was difficult to split for a specific quantity of tissue sample. Thus, the whole aliquot was weighed and then used during extraction. Each species was extracted during their own session to prevent cross-contamination between the different samples. Tools were autoclaved between sessions and the DNA was extracted according to the Heat Lysis method (Chapter 4 section 4.3.2.1). The tissue samples were finely cut using scissors that were sterilised using 70% ethanol and flamed between each sample. The Heat Lysis method was applied for the DNA extraction because the tissue samples were stored in 95% ethanol which has shown to have an inhibiting effect on the DNeasy PowerSoil Kit as previously discussed.

Molecular detection

Nano-drop spectrophotometry readings were taken to ensure that sufficient amounts of DNA were present as well as of workable quality. The qPCR assay was conducted on the QuantStudio^{TM}3 – 96-Well 0.2-ml Block. The reactions each consisted of a total volume of 20µL containing 10µl Tetra 2x qPCR MasterMix, 1µl *Amietia*SPP specific primer with FAM Probe, 1µl Internal control primer with VIC/HEX Probe and 8µl of the selected DNA sample. All reagents (except for the DNA) formed part of the manufacturer's kit. The primers were designed to target a section of the mitochondrial 12S rRNA gene. According to the manufacturer's instructions, this sequence is unique to *Amietia* spp. and was designed to have a broad detection profile specifically for the genus. Primer- and probe sequences have a 95% homology with *Amietia* sequences found on the NCBI database.

Two non template controls (NTCs) were included along with a positive control (supplied with the kit) for the specificity test. The default standard curve settings were applied along with the following conditions: 1 minute at 95ºC for initial denaturation, followed by 45 cycles at 10 seconds at 95ºC then 1 minute at 60ºC. The ∆RN threshold for the Ct-values were set at 15 000 for all the runs conducted. This value was determined through analysing the background florescence and selecting a value in the middle of the exponential phase on the amplification curve, above the background florescence (Archer, 2017). A Ct-value lower than 35 is considered a positive for this specific assay according to the manufacturer's instructions. However, as stated in Chapter 4, due to the low copy numbers expected from field samples for eDNA analysis, eDNA assays tend to apply a higher threshold to reduce the chances of false negatives. The standard curve data from the sensitivity test allowed us to determine the true LOD of the assay. For example, the *Bd* primer cut-off is set at 37, but this cut-off will vary between the different primers. Thus, the cut-off for the *Amietia* spp. primers may differ and needs to be determined for the assay. Some previous eDNA assays have used the standard if both replicates provide a Ct-value below 40 with a sigmoidal amplification curve the sample was also considered positive (González *et al*., 2021). For field samples two NTCs using nuclease free water were included along with a standard curve from the already tested PCR efficiency test for absolute quantification of the Ct-values to DNA copy numbers.

eDNA protocols have not only been applied to determine the presence of specific species but can also be used to determine the abundance (Takahara *et al*., 2012). This is due to the positive correlation between the biomass or abundance of organisms present to the concentration of eDNA (Takahara *et al*., 2012). Although eDNA concentrations can be applied to determine the level of infection, it is important to consider that factors such as the flow rate can alter the distribution as well as concentration of eDNA present (Yamamoto *et al*., 2016).

For this project, a dilution series was run with the field samples in the *Amietia* spp. qPCR assays to allow for the relative quantification of samples. The LOQ of the *Amietia* spp. primers was also determined to provide insight to the accuracy of the quantified samples.

5.3.3. Statistical analysis

The mean, standard deviations and CV% for the qPCR Ct-values of the samples were calculated on Excel 2016.

Primer efficiency for the *Amietia* spp. was determined through the calculation, where m represents the gradient of the standard curve:

Efficiency (%) = $(10^{-1/-m}-1) * 100$

The efficiency of the graph has to be between 90–110% to be acceptable (Taylor *et al.*, 2010). The level of variance for the standard curve was also analysed through the R^2 value. This value should preferably be above 0.98 to represent data with a low level of variance (Taylor *et al.*, 2010).

The LOD of the samples were determined as the concentration where at least one replicate of every qPCR tested positive as well as followed the natural trend line of the standard curve (Davison *et al*., 2019; Harper *et al*., 2018; Takahara *et al*., 2013). The LOQ was determined as the point where all of the samples for a specific concentration amplified and delivers a Ct-value with limited variation from the standard curve (Davison *et al*., 2019).

The prevalence percentage was determined for the conventional diagnostic measures for each of the sites as well as eDNA samples for analysis. Prevalence was determined using the equation:

Prevalence (%) = N (positive samples)/ N (total) * 100

A statistical analysis was performed on GraphPad Prism v8.0.2. A One-way ANOVA along with a Bonferroni multiple comparison post hoc test was run between the Ct-values and quantified DNA copies of the different sites to determine whether any significant differences in the DNA quantities existed between the sites.

5.4. Results

5.4.1. *Amietia* spp. primer assessment

5.4.1.1. Amietia *spp. primer efficiency*

The primer efficiency for the kit was excellent with a value of 100.25% (Figure 5.5). All the samples amplified in duplicate at 10 copies/µl, with Ct-values less than 40 cycles. Thus, based on the standards set out in the methodology, this is considered the LOD of the primers. Although 100% of the samples amplified at 10 copies/µl, a greater level of variability could be seen in the data compared to the other concentrations (Table 5.1). Based on the methodology the LOQ of the assay was determined to be 10 copies/µl, but the most reliable quantifications are expected from 100 copies/µl copies and above due to the very low SD and CV% values in these concentration ranges which indicates a greater level of accuracy. The Ct-values above 35 cycles still follow the normal trend line set in the graph and the R^2 values were still excellent. Thus, this concentration can still be quantified, but with a lower level of confidence compared to the samples with a concentration of 100 copies/µl. For quantification purposes of biomass load estimation, values below 35 cycles can potentially be quantified with great accuracy based on the data from the standard curve.

The standard curve for the *Amietia* spp. primers had an R^2 value of 0.9939, which indicated a very high level of consistency in the data, despite the higher variability in the lower concentrations present (Figure 5.5). It was decided that the cut-off for these primers would be 40 cycles based on the data presented. For field application all samples would require a positive amplification below 40 cycles for both qPCR replicates to be considered a true positive, which correlates with the previously described literature as discussed in the methodology.

5.4.1.2. Amietia *spp. primer specificity*

All the samples delivered a sufficient amount of DNA for the qPCR assays based on the Nano-drop spectrophotometry results (Table 5.2). However, due to the small quantities of the samples used, variability could be seen between the quantity and the quality of the samples analysed. Thus, similarly to Chapter 3 section 3.5.1 Nano-drop readings would be less reliable for quality measurements, due to the low DNA concentrations. Despite the small quantities, excellent Ct-values were derived from the samples. All three replicates for the four *Amietia* species delivered positive Ct -values below 35 for both replicates. Thus, the primers were successful at amplifying all the target species (Table 5.2). For the outlier group, none of the outlier samples tested positive. A single sample of one of the duplicate qPCR replicates from one outlier sample presented a Ct-value, but the amplification curve for the sample was further

analysed and did not present a sigmoidal curve. The fluorescence was caused due to excessive background noise in the sample. Thus, the sample was deemed negative, and this shows the importance of inspecting the amplification curves for the samples when conducting a diagnostic assay and not just evaluating the results based on Ct-values generated. All amplifications presented in the field samples were inspected individually to ensure that all values generated are true positives and no false negatives were presented.

Table 5.1. Summary of all Ct-values generated from the *Amietia* species specific primer dilution series from the qPCR assay

Log 10 DNA copy numbers	Sample amplification (%)	Ct-value mean
1	100	37.21 ± 1.12 (3.00)
2	100	33.91 ± 0.22 (0.66)
3	100	30.94 ± 0.23 (0.73)
4	100	27.29 ± 0.23 (0.85)
5	100	23.95 ± 0.13 (0.55)
6	100	20.70 ± 0.13 (0.64)

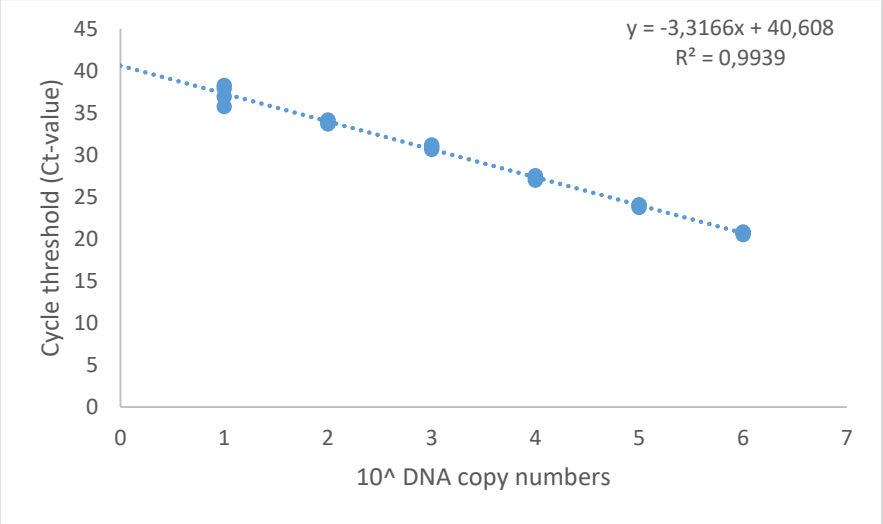

Figure 5.5. *Amietia* spp. primer positive control dilution standard curve generated from all the Ct-values presented during the qPCR assay

Table 5.2. Mean mass (g), quantity (ng/µl), quality (A260/A280) and Ct-values for *Amietia* spp. primer specificity test of four *Amietia* species (*A. delalandii, A. poyntoni, A. fuscigula,* and *A. vertebralis*) and one outlier group (*Strongylopus grayii*) (Sections in green represent samples that tested positive using *Amietia* spp. primers).

Anuran species	Mass (g)	Mean Quantity (ng/µl)	Mean Quality (A260/A280)	Mean Ct-value ± SD (CV%)
Amietia delalandii	0.0183	15.07 ± 0.15 (1.01%)	1.51 ± 0.04 (2.75%)	17.70 ± 0.27 (1.51)
	0.0052	2.90 ± 0.26 (9.12%)	2.28 ± 0.18 (7.79%)	22.51 ± 0.37 (1.64)
	0.0145	3.50 ± 0.26 (7.56%)	1.71 ± 0.14 (7.95%)	20.30 ± 0.12 (0.58)
Amietia poyntoni	0.0131	28.37 ± 0.15 (0.54%)	1.80 ± 0.03 (1.47%)	17.17 ± 0.17 (0.98)
	0.0035	4.67 ± 0.12 (2.47%)	1.67 ± 0.13 (8.06%)	24.12 ± 0.06 (0.26)
	0.0031	4.03 ± 0.15 (3.79%)	1.59 ± 0.13 (8.20%)	26.37 ± 0.27 (1.02)
Amietia fuscigula	0.0177	4.30 ± 0.26 (6.15%)	1.88 ± 0.15 (7.99%)	22.22 ± 0.17 (0.76)
	0.0085	3.30 ± 0.10 (3.03%)	2.03 ± 0.11 (5.41%)	20.09 ± 0.10 (0.49)
	0.0038	6.40 ± 0.40 (6.25%)	1.61 ± 0.13 (7.78%)	20.00 ± 0.24 (1.20)
Amietia vertebralis	0.0096	5.53 ± 0.47 (8.54%)	1.66 ± 0.16 (9.79%)	21.42 ± 0.14 (0.65)
	0.0055	2.35 ± 0.07 (3.01%)	1.71 ± 0.17 (9.51%)	25.50 ± 0.20 (0.79)
	0.0054	2.57 ± 0.06 (2.25%)	1.90 ± 0.17 (8.70%)	27.14 ± 0.12 (0.43)
Strongylopus grayii	0.0089	2.8 ± 0.17 (6.19%)	1.28 ± 0.18 (14.21%)	Not detected
	0.0396	8.37 ± 0.15 (1.83%)	1.69 ± 0.12 (7.00%)	Not detected
	0.0083	4.8 ± 0.10 (2.08%)	1.39 ± 0.08 (5.76%)	Not detected

5.4.2. Field application

5.4.2.1. Primary verification: Conventional diagnostic measures

The results in Table 5.3 are from Potgieter (2022) on the *Bd* distribution at the Ncandu Nature Reserve, uMsonti Nature Reserve and Ncandu Private Nature Reserve. Potgieter (2022) not only focused on the distribution of *Bd* at the reserves, but also the prevalence over time. The following statements are made based on the data provided and individual observations made while accompanying him during the excursions.

During the April 2021 field excursion, a total of seven sites were sampled, of which five sites (71%) tested positive for *Bd* (Table 5.3). The most successful sites were the Steep Glen River and Union site, both presenting a pathogen prevalence of 40%. Pathogen prevalence was relatively low compared to the samples from the October excursion, ranging from 10–40%. In total 17.1% of all the tadpoles tested for *Bd* were positive and 20% of the toe clips sampled were positive. Toeclips were more successful as compared to mouthparts, however less sites could be tested using toeclips due to the absence of matured amphibian individuals.

Fewer sites were accessible for testing during the October 2021 excursion. Only six sites could be tested compared to the seven from April 2021. Many of the original sites tested could not be accessed again during the second excursion and only two of the original sites could be sampled, namely Steep Glen River and Mushroom Rock. The Eikenhof dam became transformed during the second trip due to cattle invading the area from a nearby settlement. Union was located in an obscure location and could not be accessed during the second trip. The previous week before sampling, landowners reported that the area received 70mm of rain. Most rainwater drained into the main arterial river system of the valley, the Ncandu River. This complicated accessing the original river bend sampling site due to raised water levels and was, thus, excluded. Despite the rainfall, many of the marshes and small rivers, such as the Marsh and Protea Outlook, locations had not yet been completely replenished after drying up during the winter months. Thus, no individuals were available for sampling at these sites. New sampling sites were selected during the October excursion based on availability and suitability at the time of sampling.

During the October 2021 excursion, six sites in total were sampled and five sites (83%) tested positive for *Bd* (Table 5.3). The most successful sampling sites were Steep Glen River (Pathogen prevalence: 70%) and Steep Glen Trench (Pathogen prevalence: 60%). Once again, toeclips from amphibians presented a higher pathogen prevalence compared to tadpole mouthparts (Toeclips: 60%, Tadpole mouthparts: 25%). Pathogen prevalence overall increased for the October excursion (Excursion 1: 71%; Excursion 2: 83%) and tadpole

mouthpart prevalence ranged from 10–70% for the positive sites. Thus, the *Bd* prevalence at the sites tested increased in spring compared to autumn.

The October excursion provided more positive results for both the toe clips (Excursion 1: 20%; Excursion 2: 60%) and the tadpole mouth parts (Excursion 1: 17.1%; Excursion 2: 25%) compared to the April excursion. The Steep Glen River site also experienced an increase in pathogen prevalence in October compared to April (Increase from 40% to 70%). This indicates that the spring sampling period yields greater results compared to the autumn sampling period.

Although higher infection rates could be detected during the October excursion fewer water sources were available to sample during this trip. The April excursion occurred at the end of the wet season thus more water sources were available for sampling. However, intensity of infections was higher during the October excursion that may potentially be attributed to the breeding season of amphibians or lower flow rates as previously discussed in the literature review. Toe clips were more difficult to acquire but had a higher success rate compared to the tadpole mouthparts. However, adult amphibians were not present at the majority of the sites; thus, the tadpoles provided a better indication of the *Bd* distribution within the reserve during our time of sampling.

Table 5.3. Prevalence (%) of *Bd* using conventional diagnostic measures (toe clips and tadpole mouthparts) for April and October 2021 at the Ncandu Nature Reserve, Ncandu Private Forest and Grassland Nature Reserve and uMsonti Nature Reserve (Sections in green represent sites that tested positive for *Bd*).

	Excursion 1: April 2021		**Excursion 2: October 2021**	
Site name	**Positive toe clips (%)**	**Positive tadpole mouthparts (%)**	**Positive toe clips (%)**	**Positive tadpole mouthparts (%)**
Protea Outlook	N/A	10%	N/A	N/A
Marsh	$^{4}/_{5}$ = 25%	10%	N/A	N/A
River bend stream	N/A	20%	N/A	N/A
Eikenhof dam	N/A	N/A	$^{4}/_{7}$ = 57%	10%
Steep Glen River	N/A	40%	N/A	70%
Steep Glen Trench	N/A	N/A	N/A	60%
Bloekombos	$^{0}/_{1}$ = 0%	0%	N/A	N/A
Dampad	N/A	N/A	N/A	10%
Leopard's creek	N/A	N/A	$^{2}/_{3}$ = 67%	0%
Union	N/A	40%	N/A	N/A
Mushroom Rock	N/A	0%	N/A	0%
Number of sites analysed	2	7	2	6
Number of samples	5	70	10	60
Overall prevalence (%)	20%	17.1%	60%	25%
Positive sites (%)	**71%**		**83%**	

Source: Potgieter (2022)

5.4.2.2. eDNA samples – Batrachochytrium dendrobatidis *and* Amietia spp.

Overall eDNA prevalence (%)

None of the samples for any of the sites tested positive for *Bd* eDNA, however, the samples did test positive for the host specimen. Thus, the pathogen load in the environment may have been too low for detection using the preliminary method of choice. The Ct-values and prevalence of the *Amietia* spp., were further interpreted to provide guidance on how to improve sampling success for eDNA in general for the future. The subsequent results discussed represent the data collected from the *Amietia* spp. eDNA.

The results presented by the different sites varied greatly and may be attributed to the different conditions being sampled. Despite these varying results, there was no significant difference between the Ct-values, nor the DNA copy numbers of the different sites based on the One-way ANOVA ($p= 0.1158$), nor were there any significant differences in the Bonferroni multiple comparison post hoc test between the sites (Table 5.6). Each of the sites were further investigated individually to determine the possible factors that may contribute to increasing the eDNA prevalence of a sample.

Leopard's Creek is a temporary wetland, thus, water is not always present throughout the year based on observations from landowners. Despite this, Leopard's Creek presented the highest eDNA prevalence of all the eDNA sites sampled and had the second lowest Ct-values on average (Table 5.4, Figure 5.6, and Table 5.5). This site presented positive Ct-values for *Amietia* spp. for both duplicate qPCR samples in all the samples tested, thus had a 100% eDNA prevalence in the samples (Table 5.4, Figure 5.6, and Table 5.5). There was, however, a difference between the Ct-values of the different water samples and the site had an overall SD of 1.74 and CV% of 5.13%, which was the second highest of all the sampled sites (Table 5.5). Thus, although the samples could be quantified based on the high efficiency previously proven by the standard curve (Table 5.1 and Figure 5.5), the great level of variability caused by the environment itself may not present the most reliable estimate of intensity of infection or quantity of eDNA for an area.

The lowest number of positive samples were collected from Mushroom Rock, with an eDNA prevalence of 20% for *Amietia* spp., as well as the highest Ct-values on average (Table 5.5). Thus, the lowest quantity of eDNA occurred at this site. This site was based in a pool very close to the origin of a stream. Thus, very few individuals were upstream of the pool area and also no matured amphibian specimens were present on site, but eggs were observed in the stream and multiple tadpoles resided in the pool area. Water was sampled along the surface and due to the depth of the pool, the base was minimally disturbed. The samples of this site

filtered very quickly and had a low turbidity. The potential impacts of the water conditions were further investigated in the discussion section of this Chapter.

The lowest Ct-values on average detected, thus, the highest eDNA concentrations, were at the Steep Glen Trench site. Although none of the p-values in the Bonferroni multiple comparisons post hoc test indicated any significance, the Steep Glen Trench samples had the greatest influence on the p-values of all the sites. This was the only site that did not present a p-value above 0.9999 in terms of Ct-values when compared to the other sites (Table 5.6). Many large tadpoles entering a metamorphic stage were present at this site. The eDNA prevalence at the site was 80% (Figure 5.6 and Table 5.5). Three of the five samples (Sample 1–3) were taken in this pool area and the last two were taken from the river stream flowing into the pool. The site experiences minimal movement in the flow of the water, which might explain the high Ct-values presented due to the greater accumulation of DNA over time. This site also had the highest *Bd* prevalence according to the results of Potgieter (2022) (Table 5.3). The first sample collected at this site was taken along the surface of the water and had a low level of turbidity, however tested negative. The movement in the water while sampling disrupted the loose soil at the base of the pool which increased the turbidity of the site. The two samples that were subsequently taken had a higher turbidity, but were both very successful and presented the highest Ct-vales for the site. This may suggest that DNA can accumulate more in the sediment of the pool where most of the host individuals resided, and the disruption of the water may have increased the detection success. The larger size of the individuals may potentially also have had an impact on the results. These notions were further investigated in the discussion section of this chapter.

At the Steep Glen River, the first three samples were taken along the stream, and the last two samples were taken from a nearby downstream pond. Three of the five samples tested positive for the site, which gave a 60% eDNA prevalence overall. This site presented the greatest level of variation in the Ct-values, mainly caused due to the difference between the Ct-values of the lentic systems and the lotic systems (Table 5.4, Figure 5.6, and Table 5.5). The impact of lentic vs lotic systems will be further elaborated on in the subsequent section.

Lentic vs lotic systems

At the Steep Glen River, the first three samples were taken along the stream, and the last two samples were taken from a downstream pond. Only one of the samples from the stream tested positive and both samples from the pond tested positive (Table 5.4). The Ct-values from the pond were also lower compared to the Ct-values presented by the stream samples. Thus, indicating a higher concentration of eDNA. As previously stated in the methodology, Ct-values

correlate with DNA quantity and thus, it can be concluded that higher quantities of DNA were sampled in the lentic system compared to the lotic system.

This phenomenon was further evident in the results from the Steep Glen Trench site. For this site, two samples were taken from the stream and three samples were taken from the downstream pond. The two samples (Samples 4 and 5) collected from the stream upstream from the pool, both tested positive for the site with marginally higher Ct-values compared to Samples 2 and 3 from the pool area (Table 5.4). Thus, both lotic and lentic systems presented positive results for the site, but a higher concentration of eDNA was observed for the lentic system. This may potentially suggest that eDNA accumulates in higher concentrations in lentic systems due to the stream transporting the DNA downstream into the pools/ponds where reduced movement in water may be experienced. However, the differences between the Ct-values are marginal and both water systems can still be utilized for eDNA studies in the future based on the results presented here.

Only adult amphibians were present in the streams at both sites and it was observed that tadpoles tend to reside in the calmer pools rather than moving lotic streams. This may also have potentially increased the eDNA concentration of the environment due to the greater density of individuals present. These notions will be further investigated and elaborated in the discussion section of this chapter.

Table 5.4. qPCR results from eDNA filter material samples of *Batrachochytrium dendrobatidis* and *Amietia* spp. for October 2021 at the Ncandu Nature Reserve, uMsonti Nature Reserve and Ncandu Pivate Nature Reserve in Kwazulu-Natal, South Africa (Sections in green represent sample Ct-values that tested positive).

Site name	Sample number	Lentic/Lotic	*Bd* Ct-value	*Bd* mean DNA copy number	*Amietia* spp. Ct-value ± SD (CV%)	*Amietia* spp mean DNA copy number ± SD (CV%)
Leopard's Creek	1	Lentic	Not detected	N/A	33.60 ± 0.03 (0.08%)	133 ± 2 (1.79%)
	2	Lentic	Not detected	N/A	35.58 ± 1.34 (3.75%)	42 ± 33 (80.47%)
	3	Lentic	Not detected	N/A	33.69 ± 0.37 (1.10%)	127 ± 32 (25.03%)
	4	Lentic	Not detected	N/A	35.60 ± 0.91 (1.10%)	37 ± 22 (58.40)
	5	Lentic	Not detected	N/A	31.35 ± 0.07 (0.23%)	616 ± 30 (4.48%)
Mushroom Rock	1	Lentic	Not detected	N/A	36.41 ± 1.07 (2.94%)	22 ± 15 (67.32%)
	2	Lentic	Not detected	N/A	Not detected	N/A
	3	Lentic	Not detected	N/A	Not detected	N/A
	4	Lentic	Not detected	N/A	Not detected	N/A
	5	Lentic	Not detected	N/A	Not detected	N/A
Steep Glen River	1	Lotic	Not detected	N/A	37.65 ± 0.07 (0.20%)	8 ± 0.42 (5.08%)
	2	Lotic	Not detected	N/A	Not detected	N/A
	3	Lotic	Not detected	N/A	Not detected	N/A
	4	Lentic	Not detected	N/A	32.71 ± 0.39 (1.18%)	247 ± 64 (26.06%)
	5	Lentic	Not detected	N/A	34.25 ± 0.05 (0.14%)	85 ± 3 (3.19%)
Steep Glen Trench	1	Lentic	Not detected	N/A	Not detected	N/A
	2	Lentic	Not detected	N/A	30.54 ± 0.11 (0.37%)	1076 ± 84 (7.78%)
	3	Lentic	Not detected	N/A	31.30 ± 0.10 (0.32%)	637 ± 44 (6.86%)
	4	Lotic	Not detected	N/A	32.43 ± 0.09 (0.27%)	295 ± 18 (6.09%)
	5	Lotic	Not detected	N/A	33.16 ± 0.08 (0.23%)	179 ± 9 (5.22%)

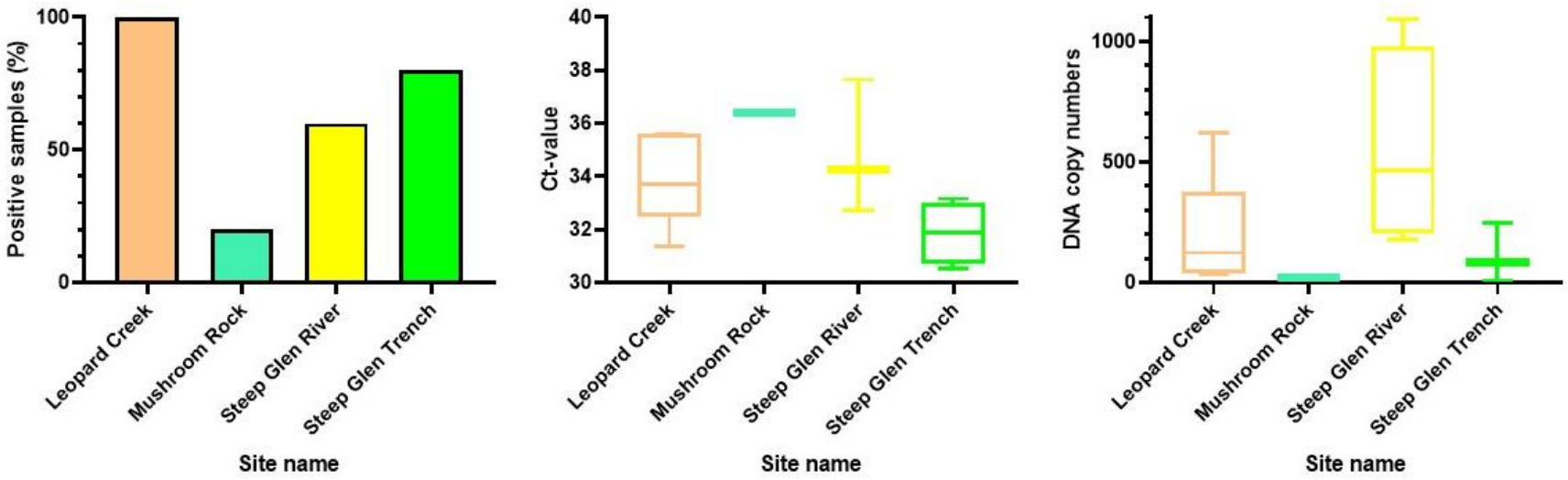

Figure 5.6. (A) Percentage (%) of positive samples per sampling site for *Amietia* spp. primers, (B) Average Ct-values and Ct-value range of the positive samples per sampling site for *Amietia* spp. primers, and (C) Average DNA copy numbers and DNA copy number range of the positive samples per sampling site for *Amietia* spp. primers.

Table 5.5. The percentage of positive samples for *Amietia* spp. primers, average Ct-values, and mean quantified DNA copy numbers as calculated using the generated standard curve for each of the sampling sites tested using for *Amietia* spp. primers located at the Ncandu Nature Reserve, Ncandu Private Nature Reserve and uMsonti Nature Reserve.

Site name	Percentage of positive samples (%)	Mean Ct-value of positive samples ± SD (CV%)	Mean DNA copy numbers of positive samples ± SD (CV%)
Leopard Creek	100%	33.96 ± 1.74 (5.13%)	191 ± 229 (120%)
Mushroom Rock	20%	36.41 ± 1.07 (2.94%)	22 ± 15 (67%)
Steep Glen River	60%	34.87 ± 2.27 (6.51%)	113 ± 113 (99%)
Steep Glen Trench	80%	31.86 ± 1.08 (3.39%)	547 ± 374 (68%)

Table 5.6. Overall average Ct-value comparison of the positive samples between the different sampling sites tested using for *Amietia* spp. primers located at the Ncandu Nature Reserve, Ncandu Private Nature Reserve, and uMsonti Private Nature Reserve, using One-way ANOVA with Bonferroni multiple comparison test for Ct-values and DNA copy numbers (no significant p-values were present in the data).

Site comparison	Mean difference of Ct-values	P-value	Mean difference of copy numbers	P-value
Leopard Creek vs. Mushroom Rock	-2,446	>0.9999	169	>0,9999
Leopard Creek vs. Steep Glen River	-0,9060	>0.9999	-360	0,5993
Leopard Creek vs. Steep Glen Trench	2,107	0.6907	78	>0,9999
Mushroom Rock vs. Steep Glen River	1,540	>0.9999	-529	0,8436
Mushroom Rock vs. Steep Glen Trench	4,553	0.3003	-91	>0,9999
Steep Glen River vs. Steep Glen Trench	3,013	0.3372	438	0,4915

5.5. Discussion

5.5.1. *Amietia* spp. primer test

Different primers and methods are known to yield different qPCR thresholds (Langlois *et al*., 2021; Roussel *et al*., 2015). For the case of the *Bd* primers, odd or unspecific amplification with greater standard deviations were seen to occur after 37 cycles and thus, this was the set cut-off. For the case of the *Amietia* spp. primers, a low level of variation was detected even in the lower concentration ranges as seen in the high R^2 value of the standard curve and all the samples amplified at the lowest concentration tested. Although the manufacturer's instructions suggested a Ct-value cut-off of 35, samples still amplified after this value for the duplicate qPCR values per sample in the dilution series. Based on previous eDNA studies the Ct-value cut-off is generally set at 40 if both duplicates of a sample produce a positive Ct-value with sigmoidal curve (González *et al*., 2021). Thus, the cut-off for the primer set used in this study was determined as 40 cycles based on the standards used in previous eDNA literatures.

The primers delivered an excellent efficiency of 100.25% and only tested positive for the target organisms and none of the outlier samples. This indicated a high level of specificity and sensitivity. The LOD and LOQ for the assay is at 10 copies/µl due to all the samples amplifying at this concentration. Although the LOD of the assay is 10 copies/µl, this concentration presented a much higher level of variability compared to the other DNA concentrations. Thus, although samples can be quantified at this concentration, the most accurate quantifications can be expected from 100 copies/µl and above. Based on the excellent results of the standard curve, field samples can theoretically be quantified with a high level of confidence.

5.5.2. Conventional diagnostic measures

According to data provided by Potgieter (2022) the prevalence for the sites outside of Steep Glen had a lower prevalence. In the wetland area at Leopard's Creek although many tadpoles were present and toe clips from the adults in the wetland indicate positive results, none of the tadpole mouthparts yielded any positive *Bd* results.

Multiple factors could have resulted in this phenomenon. The river in the wetland area is a temporary river system and thus, dries out during the winter when the rainfall is at its lowest. Infections may thus, not yet have properly established in the area. The adults that were infected were found in amplexus, thus *Bd* likely spreads directly through frog-to-frog transmission and less likely through the water source. Breeding has shown in previous studies to increase the level of

infection of individuals as well as the amount of eDNA available in the environment (Bancroft *et al*., 2011; Harper *et al*., 2019). During a study conducted on seasonal variation of *Bd* in Crawfish frogs, it was found that the breeding season increased infections amongst matured individuals and only 1% of juveniles tested positive during the experiment (Kinney *et al*., 2011). Infection rates tended to increase in adults as the breeding season continued as well as at the end of the breeding season before summer (Kinney *et al*., 2011). Juveniles that test negative at the end of the breeding season were more likely to be infected only during the coming winter months when residing in their aquatic burrows or during the following breeding season (Kinney *et al*., 2011). This may explain the lack of infection in the tadpoles at the Leopard's Creek site. Previous *Bd* studies have also indicated that the duration of the tadpole life stage can significantly affect the infection probability, and older tadpoles tend to have higher infection rates (Smith *et al.* 2007; Skerratt *et al*., 2008). Perhaps later in the season the tadpoles at Leopard's Creek may present positive results as the infection rates increase while the breeding season continues and allows the pathogen to properly establish in the area. Thus, the spread of *Bd* seems to be high in adult amphibians during the breeding season when behaviours such as amplexus often occur, but tadpoles may require more time to first accumulate infections through the environment.

The Steep Glen Trench and Steep Glen River systems are permanent water bodies that allow individuals to reside in this system throughout the year and may result in the higher accumulation of infections over time. This may be the reason why infection rates were higher at these sites compared to all the other sampled sites. Based on the study from Kinney *et al*. (2011), it can be gleaned that individuals are still infected while residing in aquatic environments, but higher levels of infections occur when individuals actively interact. Although Mushroom Rock presented a potentially infected individual during the first excursion to Ncandu, none of the tadpoles had any *Bd* infections during the second sampling period. No adult amphibians were present at the site, but many tadpoles and eggs were found. This site occurred at the origin of the stream, thus, *Bd* was unlikely to spread through the water source to the tadpoles and due to the lack of matured individuals present, *Bd* may only have been present in low concentrations in the environment.

Many of the individuals present at the Steep Glen River and Steep Glen Trench during the October 2021 excursion were large and near metamorphosed stages in their life cycles. These sites presented the highest pathogen prevalence of all the sampling sites as well as for both excursions. Previous literature have indicated that older and larger individuals tend to have higher infection loads compared to younger individuals (Bancroft *et al.*, 2011; Smith *et al*., 2007; Searle *et al*. 2011). This may explain the high prevalence observed in these sampling sites. This phenomenon

might also explain why the toe clips were more successful as compared to the mouthparts, as toe clips were sampled from matured individuals thus infections may have accumulated over time.

Although fewer sites were available for sampling during the second excursion, the sites that tested positive presented higher levels of infections compared to the first excursion during April. This corresponds to the results of Conradie *et al*. (2011), which indicated higher infection rates present during the spring months of the year in South Africa. This also correlates with another *Bd* eDNA study that found the prevalence of the pathogen to be the highest during spring months (Taugbøl *et al*., 2021).

It can be recommended for future sampling in eDNA to target sites that have more matured tadpoles compared to small and young individuals so as to increase the sampling success and provide a better estimate of pathogen presence within an environment. Based on the prevalence of the pathogen over time at the reserve it is also recommended to sample during the springtime of the year as previously suggested by Conradie *et al*. (2011) and Taugbøl *et al*. (2021).

5.5.3. eDNA analysis

A previous study has shown that when experiments fail to detect target species, eDNA results are less likely to be published, thus, creating a shortcoming in research because the limitations of the protocols are not described even though value can still be found in these results (Beng and Corlett, 2020). This section of the discussion will review what was gathered from the *Amietia* spp. eDNA results as well as the possible limitations of the preliminary method to provide insight to what can be done in future *Bd* eDNA protocols to increase the detection probability.

eDNA samples from all the sampling sites tested positive for *Amietia* spp., but none tested positive for *Bd.* Unfortunately, eDNA assays are limited in areas where low abundances of the DNA occur (Beng and Corlett, 2020; Sieber *et al*., 2020; Roussel *et al*., 2015). This may be due to the reduced quantity of DNA present for sampling within the environment (Roussel *et al*., 2015). Thus, although the assay was able to detect the *Amietia* spp. present within the environment, the quantity of *Bd* DNA present in the environment may have been below the detection threshold of the preliminary assay. Only two of the four sites sampled using conventional diagnostic measures had *Bd* present on the tadpoles and one of the other sites had positive matured amphibians. As previously mentioned, *A*. *delalandii* was the most prevalent amphibian species present, and is known to be a vector for *Bd* but generally carries a low intensity of infection (Antwis and Weldon, 2017). It should be noted that the final eDNA diagnostic method developed at the end of Chapter

4 is up to ten times more sensitive than the preliminary assay that was tested in this Chapter. The lowest detection limit of the newly developed assay is as low as 10 zoospores, thus, future eDNA sampling for *Bd* is likely to be more successful.

Although the assay was unable to detect the target pathogen, it was very successful at detecting the target amphibian and a lot could be learned from these results. All the sampling sites tested positive for *Amietia* spp., but the level of success for detection varied between the different sites tested. This may have been due to the type of environments sampled such as lentic or lotic systems, the level of turbidity in the water samples, the number/size of individuals present as well as their movement through the sampling site (Barnes *et al*., 2021; Harper *et al*., 2019).

Mushroom Rock was the least successful site regarding the protocol success when analysing the *Amietia* spp. samples and only a single sample tested positive of the five (20% detection rate). During sampling, there was very little disturbances in the water and the majority of the individuals resided at the bottom of the pool. No adult amphibians were present at the site during sampling, however, many small tadpoles and eggs could be found. The water samples were taken more along the surface of the water, at the point where the water flowed into the pool, which had a lower turbidity and resulted in faster filtration. Due to only eggs being upstream, the DNA concentration may have been low for *Amietia* spp*.* The DNA present in the pool may also have been slightly displaced by the movement of the water flowing into the system. Running water has shown in previous studies to deliver lower quantities of eDNA due to displacement (Rees *et al*., 2014). Despite the low detection of the site, it did still test positive for the target organism, indicating that the protocol was sensitive enough to detect low quantities of eDNA.

Although eDNA is often considered an accurate method of detection in various studies, many limitations still exist. For example, false positive and negatives can easily occur during an assay. To reduce the possibility of these false readings in field samples, positive and negative controls are included during the qPCR phase (Taylor *et al*., 2019). For this study NTCs were tested along with a positive standard curve to serve as controls to the samples. None of the NTCs amplified and the positive control standard curve amplified at a similar rate to the predetermined standard curve.

Various other factors can, however, contribute to the success of eDNA results prior to the qPCR phase. False negatives are also known to occur during the sampling phase of the project, due to environmental conditions or the sampling technique applied (Beng and Corlett, 2020). Various previous eDNA studies have shown that the level at which the water is filtered can have a

significant effect on the detection probabilities (Barnes *et al*., 2020; Lacoursière-Roussel *et al*., 2016). For the case of Mushroom Rock, the samples were collected at the surface of the water where low levels of turbidity occurred. In a previous eDNA study for *Bd* conducted in Texas, USA, samples with a higher level of turbidity were more likely to test positive for the target specimen (Barnes *et al*., 2020). The positive samples were also taken in shallower water compared to the other sites (Barnes *et al*., 2020), which would be closer to the substrate where the hosts reside. Highly turbid water may contain increased levels of humic acids and clay particles, which have shown in previous studies to bind to DNA particles in aquatic environments and prevent degradation (Huerlimann *et al*., 2020). Thus, larger quantities of eDNA can potentially be preserved in these environments which could increase the detection probabilities. Although turbid water is considered more favourable in this condition, it should also be noted that the quantity of humic acids and other inhibitory agents present may also affect the qPCR phase if not properly removed during the DNA extractions (Kuhn *et al*., 2017; Stoeckle *et al*., 2017). The water collected at all three of the other sites sampled filtered much slower as compared to the Mushroom Rock site, indicating a higher level of turbidity. In this study, higher turbidity levels in the water resulted in more positive results when reviewing the *Amietia* spp. eDNA data.

During the laboratory phase of the project many *Bd* cultures of the same strain were grown in different flasks. It was observed that the sporangia and zoospores tend to be at their highest densities at the base of the flask. Many zoospores were free moving in the liquid media and could be observed in all levels; however, the majority of zoospores and sporangia were present near the base of the flask. Previous eDNA studies indicate similar findings for higher *Bd* concentrations in shallower water columns (Barnes *et al*., 2020). Thus, the water level at which sampling occurs would not only be applicable to *Amietia* spp. eDNA, but based on this information provided could potentially apply to *Bd*.

Some studies have found that eDNA can be used to quantify the biomass of the target specimen present in the environment (Lodge *et al*., 2012; Takahara *et al*., 2012). However, many other studies have shown that great variability exists in these data sets and the results should only be analysed relatively rather than precise units (Barnes *et al*., 2021; Loge *et al*., 2002; Sieber *et al*., 2020). In terms of quantification of the field samples in this study, great levels of variation existed between the replicate samples of the different sites. The pools showed to be more successful for analysis, but variations still existed in the eDNA quantities between samples, only providing a relative quantification of the biomass for the area. This is clearly evident in the Leopard's Creek samples, where all of the samples were collected in the same area, all presenting positive results,

but demonstrated the greatest level of variation in terms of DNA copy numbers. This proved the heterogeneous distribution of eDNA within the same environment.

Although eDNA concentrations could theoretically have been applied to determine the level of infection, it is also important to consider that factors such as the flow rate can alter the distribution as well as concentration of eDNA present (Rees *et al*., 2014; Yamamoto *et al*., 2016). When analysing the different parts of the pools and lentic vs lotic systems variations could be detected in the data generated by this project. This was evident for the case of lentic and lotic systems at the Steep Glen sites. As previously discussed in the results, the two Steep Glen sites, both presented positive and negative Ct-values for the streams and ponds, however the positive Ct-values from the pools tended to be lower, thus containing more eDNA, compared to those from the streams. This may be due to the tadpoles being more likely to reside in the ponds rather than moving river bodies or can be due to DNA flowing in from the stream and accumulating in the pond area. Adults were often found in the flowing streams, but all the tadpoles generally resided in the calm waters present in the pond. This does however not mean lotic samples can't be used for eDNA studies, as seen in the positive results for both the lotic systems tested at the Steep Glen sites. Lentic systems might, however, be more successful than lotic systems as demonstrated by the lower Ct-values and higher number of our positive samples.

It is important to consider the DNA load may increase in the pools due to DNA flowing from the streams into the pools and the higher loads may not always accurately represent the density of the individuals present (Jane *et al*., 2015; Rees *et al*., 2014; Yamamoto *et al*., 2016). Thus, quantification of eDNA may be limited in its accuracy and DNA may be found far from the original source (Jane *et al*., 2015).

Despite Leopard's Creek being a seasonal water body, the samples collected from the site delivered the most positive results for the *Amieta* spp. eDNA. All the samples and qPCR duplicates at this site tested positive for *Amietia* DNA. This may possibly be due to the high number of individuals present and the high level of activity amongst the adults and tadpoles at this site. The results also demonstrate that both seasonal and permanent waterbodies can be used for eDNA assays and other factors such as flow rates and turbidity may have a greater impact on the results.

Various other environmental conditions may additionally contribute to the success of the eDNA assay. The amount of light present, rainfall, flow rates, pH, temperature, and various other factors may alter the persistence and degradation of eDNA in the environment (Beng and Corlett, 2020).

The field sampling took place just before the start of the wet season, however, a week prior to sampling, the area received approximately 70mm of rain. This may also have increased the flow rates and volume of the water present which would be less favourable for eDNA sampling (Conradie *et al*., 2011). As previously discussed for the Leopard's Creek *Bd* positive results, the spread of *Bd* seems to be high in adult amphibians during the start of the breeding season when behaviours such as amplexus often occur, but tadpoles may require more time to first accumulate infections. Thus, *Bd* may require more time to accumulate in the water sources to increase the detection probability using eDNA. Factors such as rainfall should however also be considered when selecting a sampling time. Higher rainfall would result in higher flow rates and larger volumes of water which would be less favourable for eDNA. However, the dilution effect can potentially be countered to some degree by an increased congregation of adults at the breeding sites and subsequent shedding of *Bd* DNA. This is based on the fact that greater host densities positively correlate with higher eDNA quantities (Buxton *et al*., 2017; Lodge *et al*., 2012; Strickler *et al*., 2015; Takahara *et al*., 2012).

Two sampling periods may be recommended for field sampling and surveys can be planned to favour tadpole or adult biased shedding. The first is early in the spring at the start of the host breeding season, when flow rates are still low and rainfall is still limited, but a consequent increased matured host activity is present. In contrast, the second sampling period can be during the early summer which will allow for the level of infection to accumulate amongst the tadpoles and in the water body, but flow rates may be increased during this period compared to early spring. If early summer sampling is timed well, increased flow rates may, however, still be avoided.

5.5.4. Future recommendations

Sampling success often depends on the number of sample replicates taken from each site (Beng and Corlett, 2020; Willoughby *et al*., 2016). The number of replicates provided excellent results for the *Amietia* spp. samples of the project, however, potentially may not have been enough to detect *Bd*. False negatives are not an uncommon phenomenon in eDNA studies and are caused due to a wide variety of factors but can generally be improved by increasing the number of replicates per site provided that DNA concentration within the environment is within the detection limits of the assay (Beng and Corlett, 2020). It was suggested by Willoughby *et al*. (2016) that when the probability of detection is extremely low, approximately 10 samples should be taken per sampling site to increase the odds of detection to more than 0.95. This would, however, increase the cost of the protocol significantly if it were to be applied in the future. Increasing the number

of qPCR replicates could also improve the detectability in the future (Beng and Corlett, 2020), however this may also increase the cost of the protocol depending on the number of samples being analysed. The detection probability of an assay is greatly influenced by factors such as the concentration of the DNA present as well as chemical properties of the water (Piggot *et al*., 2016). Apart from visual inspection of turbidity, the water quality and properties were not measured during this project, however, can potentially be considered in future studies.

Based on the previously discussed results, turbidity and the level sampled in the water column greatly affects detection probabilities. Thus, it can be recommended for future studies to sample the water from the lower phase of the water column where higher levels of turbidity occur and more individuals reside or to sample from shallower sections of the water where water can be collected closer to the target organisms. In the study by Barnes *et al*. (2020) it was recommended to sample from sediments in the future due to the hosts and pathogen being more likely to occur here. It should however be noted that another study found sediments to be insufficient for detecting *Bd*, and the results indicated a five-fold lower detection rate compared to conventional swabbing and filtered water eDNA analyses (Brannelly *et al*., 2020). The increased turbidity at the Steep Glen Trench site, after the water was disturbed during the first sample taken, also greatly improved the Ct-values of the samples. These findings correlated with the data from Barnes *et al*. (2020), which suggests that lower water columns with higher turbidity should be targeted for sampling. Thus, based on these results it is recommended to filter water closer to the base of the water column where the individuals reside and in water systems with higher turbidity levels. Lightly stirring the water to increase suspended DNA and turbidity may also improve detection rates in some low turbid lentic systems. It may also be recommended to sample in areas where more DNA may potentially have accumulated, such as further downstream pools as well as where more host specimens occur (Jane *et al*., 2015). Considering the low eDNA concentrations of the target pathogen in the environment it is recommended that sampling should favour lentic systems rather than lotic to increase the chances of detection. It should however be considered that if a positive result is detected for the pathogen, the source of infection may potentially be higher up stream and not just limited to the pool area (Jane *et al*., 2015; Rees *et al*., 2014).

As previously stated in the discussion sampling larger water quantities from sites may deliver more positive results especially when sampling from different water body types such as lentic and lotic systems (Brannely *et al*., 2020; Rees *et al*., 2014). The volume for the water samples were kept constant for this project, however it is recommended for future studies to continue filtering

until the filters have clogged to collect the maximum amount of DNA from the environment. This would be particularly useful when identifying *Bd* status in an area is preferred over comparative habitat occupancy data where standardized filtered volume is a requirement. Both objectives can be achieved when standard volumes are supplemented with filter capacity restricted volumes. Another suggestion by Hunter *et* al., (2019) is to use a multi-filter approach to increase the filtered water volume by first using filters with large pore sizes to allow most particles to pass through but remove large excessive debris, prior to filtering with the 0.45µm pore size filters.

It would be recommended to target water bodies that contain larger or more matured tadpoles, as seen with the increased infection rates as well as higher eDNA rates demonstrated in this study. Spring has also shown to be the most favourable season for sampling due to the lower flow rates, increased activity among individuals, and higher *Bd* infections rates present.

5.6. Conclusion

Although the eDNA protocol failed to detect the target organism, much could be gathered from the data collected. The protocol was successful at detecting the *Amietia* spp. in the environment which proves the protocol is capable of detecting eDNA, however it can be further improved for the target pathogen. Due to the low concentrations of the pathogen in the environment, a multitude of adjustments may need to be made to the protocol or the sampling technique. To summarise, the following would be recommended for future eDNA sampling. Either the number of samples per site should be increased or the volume of water filtered should be increased. Higher filter volumes can be obtained through a multiple filter approach or filtering water until filters have clogged rather than setting specific water quantity limits. The number of qPCR replicates can be increased to improve detection probabilities. Sampling should be done during the spring months and shallower pools containing older or more matured tadpoles would be the most ideal to maximize detectability at a site. However, when occupancy data at the landscape level is being collected every candidate site (evidence of amphibians) needs to be sampled regardless of tadpole demographics. Increased turbidity in water samples have shown to increase eDNA detection rates.

The H-0 hypothesis was supported by the results from this section of the project, that is the preliminary method failed to detect the target pathogen. Despite not detecting the target pathogen, this section of the project still created a foundation for future projects in *Bd* eDNA. The improved protocol from Chapter 4 along with recommendations provided in this study can aid in improving this method for future field sampling.

Chapter 6: Future prospects, recommendations and concluding remarks

6.1. Future prospects and recommendations

Overall, all four of the DNA extraction methods tested in this project, presented similar Ct-values when considering both the sterile and non-sterile matrix. However, when only reviewing the non-sterile matrix the kit methods delivered significantly better results compared to the crude extraction methods. As such, the kits could remove environmental inhibitors more effectively. During the filter preservation medium tests, the DNeasy PowerSoil Kit – Lysis Buffer combination out preformed all the other method and preservation combinations in terms of Ct-values. Based on the results the commercial DNA extraction kit methods appear to perform better when presented with certain inhibitors, however, it should also be considered that each kit has their own limitations and drawbacks. For example, the DNeasy PowerSoil Kit – Ethanol combination demonstrated the greatest level of downstream inhibition of all the methods tested. Thus, the effectiveness of the kit method can severly be affected by other variables and do not always present the best method for all scenarios. Prior to testing the Lysis Buffer, the Heat Lysis – Ethanol combination was applied as a preliminary method in the field and despite being unable to detect *Bd*, was still capable of detecting the internal assay control specimen at all of the sites. It would be recommended for future studies to use the DNeasy PowerSoil Kit – Lysis Buffer combination due to its higher sensitivity, but if a more cost-effective alternative is required additional cleaning steps can be applied to the Heat Lysis method to potentially improve its performance.

The method of choice, as determined by the results from this project will, however, remain the DNeasy PowerSoil Kit – Lysis Buffer combination. Although this method combination was not applied for fieldwork during this project, it still indicated a very high level of sensitivity that is capable of detecting as little as 10 zoospores under laboratory conditions. In the study by Brannely *et al*. (2020), this kit method was also selected as the most optimal DNA extraction method, however their study had a LOD of 100 zoospores. Based on these results the newly developed protocol may have the potential to deliver excellent results in field application, and a subsequent study is already in progress.

eDNA assays are less sensitive in environments where abundancies of the target organisms are low (Roussel *et al*., 2015). Clogging is one of the greatest limitations to the volume of water that can be filtered from the environment. At the majority of the field sites for this project it was only possible to filter between 500 – 800ml of water before clogging, and a final volume of 500ml was

selected to allow for the direct comparison between sites. Increasing the filter volume might improve the probability of collecting a sufficient number of DNA copies for the assay to deliver a positive result. Multi-filter protocols can be used to reduce clogging. A filter with large pore sizes, such as 20µm, can first be applied to reduce the amount of large debris in the samples but still allow the majority of particles, such as the larger *Amietia* spp. cells, to pass through. These water samples can then be filtered with the 0.45µm pore size filter cups. It would also be recommended, rather than having a fixed filter volume to continue filtering until filters have clogged to ensure the maximum volume of water is achieved for the sample.

Based on the data from the preliminary field application, many recommendations could be provided on where future sampling should occur within the target environment to improve detection probabilities. For this study sampling sites with lower water columns in lentic systems with high levels of turbidity increased the detection probability as well as delivered lower Ct-values indicating higher DNA concentrations. Sampling periods can be timed according to the breeding season of amphibians, when higher agglomerations of amphibians are present at the sites as well as prior to the summer rainfall period in South Africa, during the spring, when flow rates are still low. This combination of increased individuals present, along with low flow rates in the aquatic systems would likely deliver the highest probability for detecting the host and *Bd* eDNA.

Based on the quantified results from the *Amietia* spp. field samples, great levels of variability could be detected in the DNA copy numbers between each individual sample taken at a site in terms of DNA copy numbers. Thus, specific quantification of a site may be very limited and not accurately describe the population density present at a site. An overall comparison of DNA copy numbers between sites may provide a relative comparison of host densities per site, but would still not be recommended for accurate estimates. Additionally, quantification of samples is extremely limited for *Bd*, due to the different strains and isolates containing varying copy numbers in the ITS region of the genome (Rebollar *et al*., 2017). Thus, quantifying the pathogen load using the standard curve method may not be recommended for future studies in *Bd* eDNA.

The main aspects tested and optimised in this project were DNA extraction methods, filtration methods and filter preservation mediums of *Bd* eDNA. The molecular detection phase of this project was standardised, but additional improvements can be made in future studies to further increase the sensitivity. PCR technologies have improved over the last decade and other alternative methods exist. Nested PCR has recently been applied for *Bd* diagnostics in eDNA and have shown to deliver excellent results (Mutnale *et al*., 2018). This may also present a more cost-effective alternative (Mutnale *et al*., 2018). Nested PCR is known to deliver results with a high

level of sensitivity and specificity, which is a crucial part in molecular detection assays (Stoeckle *et al*., 2018). Other methods such as LAMP can also be tested on field samples and compared to the TaqMan Probe qPCR. A LAMP protocol has been developed for *Bd* in the HHL lab by Du Preez (2019) and has shown to deliver excellent results.

Alternative primers can also be used or developed to accommodate possible variances in the ITS region of the *Bd* genome, as seen in the 57 haplotypes present in the Asian *Bd* lineages. Changes in the genome may occur over time reducing the accuracy of the assay. For future studies, the new lineage specific primers developed by Ghosh *et al.* (2021) which differentiate between lineages can be tested and primer and probe concentrations can be optimised to improve detectability.

Minimum primer concentrations were applied during this project, but the Ct-values of samples may potentially be improved by increasing the primer and probe concentrations. In the study by Barnes *et al*. (2020) similar primer concentrations were used as during this project and positive results for *Bd* eDNA were only obtained from one of the four sites sampled. Primer concentrations can have a significant effect on the sensitivity of a protocol and increasing the concentration may deliver better results. In the article by Boyle *et al.* (2004), primer concentrations were set at 900nM. In the current study lower concentrations were used, therefor it is recommended that the concentrations of Boyle *et al*. (2004) should be tested in the future along with the developed protocol of this study. However, caution should be taken since the use of concentrations that are too high may cause the formation of primer dimers that may result in false positives in the assay. A dilution series using different primer concentrations can be applied to create a standard curve of the previously extracted DNA to observe the optimal concentrations as well as their limitations.

6.2. Concluding remarks

The aim of this project was to develop a Standard Operating Procedure (SOP) under laboratory conditions that could be applied to future field sampling of *Bd* eDNA. The DNeasy PowerSoil Kit – Lysis buffer preservation medium delivered the best results and highest level of sensitivity amongst all the methods tested. This method can detect as little as 10 zoospores in 150ml water with a 100% confidence based on the zoospore filter dilution series. Based on the laboratory data this method may provide promising results in future field applications. A follow-up study has already been launched by the HHL to conduct field application and validation of the protocol. This project will include various localities within South Africa and the results will be compared with

those from conventional diagnostic techniques. This new project also provides the opportunity for optimising the molecular detection phase, as previously discussed in the recommendations, to increase the sensitivity even further.

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Annexure 1

GIS desktop analysis maps and metadata

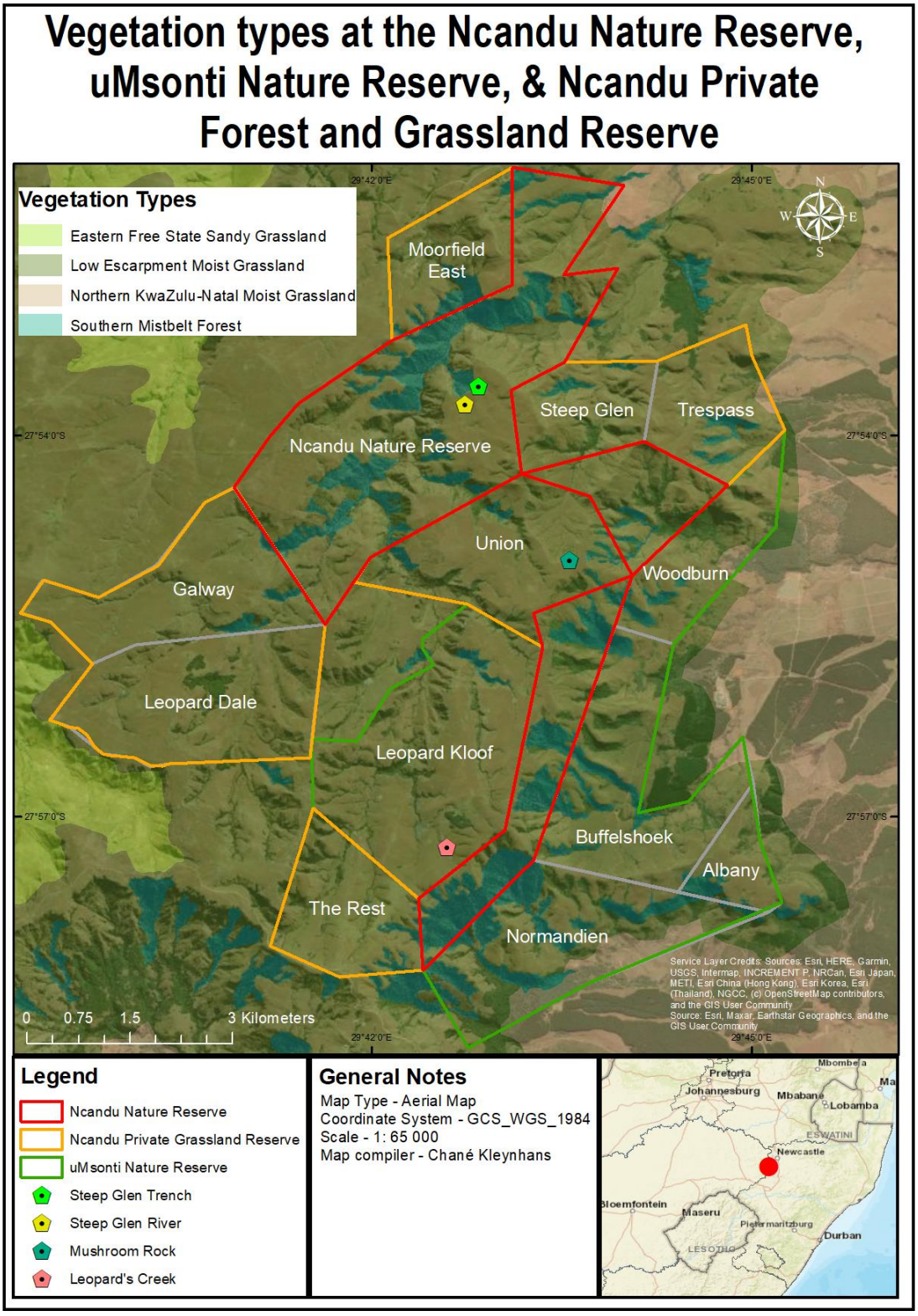

Figure A.1.1. Vegetation type map of the Ncandu Nature Reserve, uMsonti Nature Reserve, and Ncandu Private Forest and Grassland Reserve (Refer to Table A.1.1. and Table A.1.2. for the metadata of the GIS layers applied).

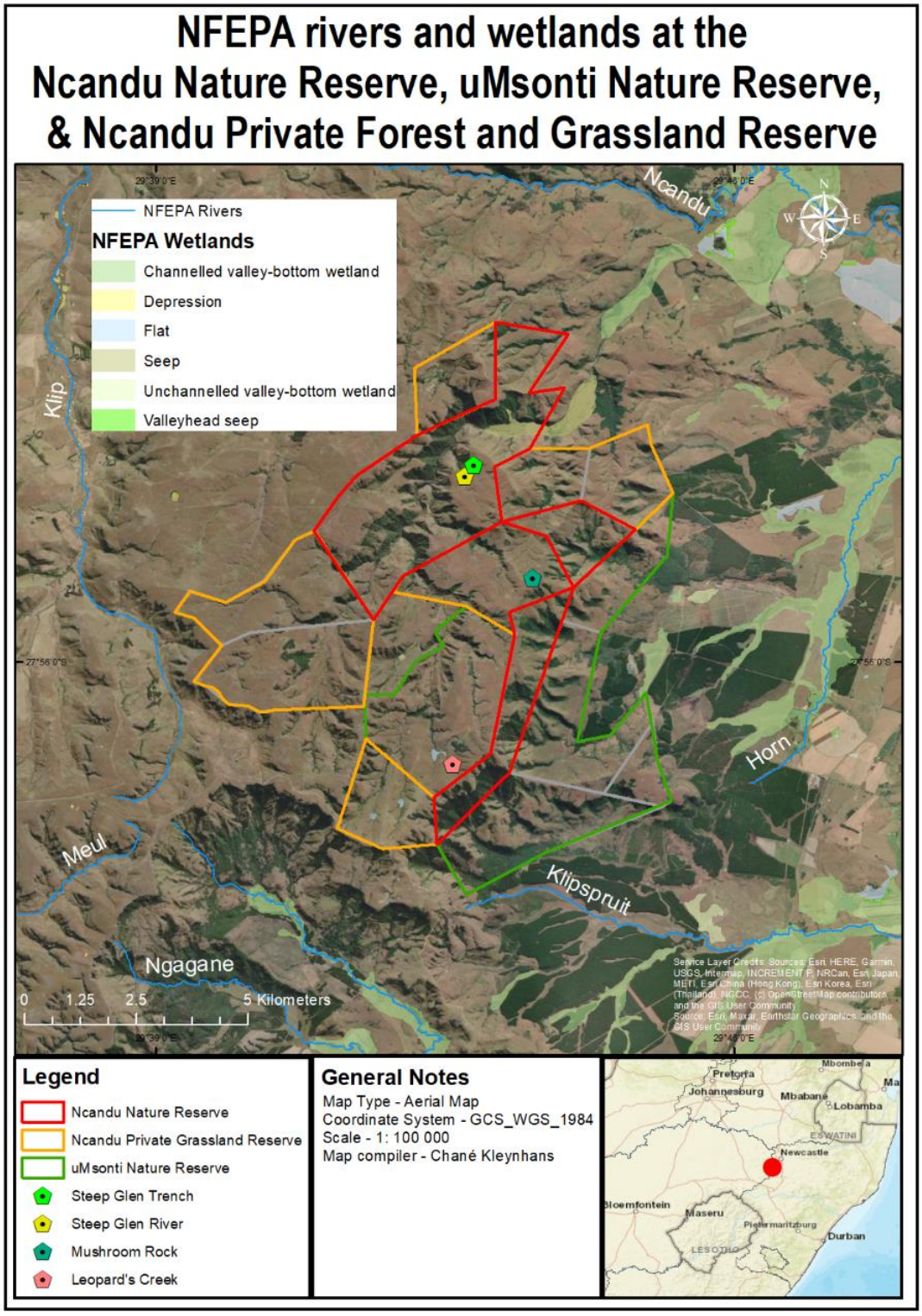

Figure A.1.2. Map of the NFEPA classified rivers and wetlands in the area of the Ncandu Nature Reserve, uMsonti Nature Reserve, and Ncandu Private Forest and Grassland Reserve (Refer to Table A.1.1. and Table A.1.2. for the metadata of the GIS layers applied).

Table A.1.1. Metadata for GIS desktop analysis maps of Ncandu Nature Reserve, uMsonti Nature Reserve and the Ncandu Private Forest and Grassland Nature Reserve

Map Layer name	File name as per data creator	Data type	Data creators	Date of data capture	Internet source	Coordinate system	Scale	Attribute list field label applied	Link
NFEPA Wetlands	NFEPA_Wetlands.shp	Vector: Polygon	CSIR	July 2011	SANBI BGIS	WGS84	1:500 000	NWCS_L4	https://bgis.sanbi.org/SpatialDataset/Detail/395
NFEPA Rivers	NFEPA_Rivers.shp	Vector: Line	CSIR	July 2011	SANBI BGIS	WGS84	1:500 000	NAME	http://bgis.sanbi.org/SpatialDataset/Detail/397
Vegetation Types	VEGMAP2018_AEA_07012019_beta.shp	Vector: Polygon	SANBI	2016 – 2018	SANBI BGIS	GCS_WGS_1984 AEA_RSA_WGS84	1:250 000	NAME_18	http://bgis.sanbi.org/SpatialDataset/Detail/669
World Imagery	World Imagery	Raster	ESRI, Maxar, Earthstar, Geographics, and GIS User Community	Created: 12 Dec 2009 Updated: 8 Jul 2022	ESRI / ArcGIS	WGS84	Scale not given	N/A	https://www.arcgis.com/home/item.html?id=10df2279f9684e4a9f6a7f08febac2a9
World Street Map	World Street Map	Raster	ESRI, HERE, Garmin, USGS, Intermap, INCREMENT P, NRCAN, Esri Japan, METI, Esri China (Hong Kong),	Created: 12 Dec 2009 Updated: 3 Aug 2021	ESRI / ArcGIS	WGS84	Scale not given	N/A	https://www.arcgis.com/home/item.html?id=3b93337983e9436f8db950e38a8629af

			NOSTRA, © OpenStreetMap Contributers and GIS User Community						

Table A.1.2. Metadata for GIS desktop analysis of Ncandu Nature Reserve, uMsonti Nature Reserve and the Ncandu Private Forest and Grassland Nature Reserve created using ArcGIS

Layer name as seen on map	Coordinate System	Description and sources applied
Steep Glen Trench	GCS_WGS_1984	Coordinates: 27.8935763 S, 29.7144282 E Altitude: 1 771m Coordinates were obtained from Google Earth along with altitudes and were imported into ArcGIS with the described coordinate system.
Steep Glen River	GCS_WGS_1984	Coordinates: 27.8955803 S, 29.7130928 E Altitude: 1 774m Coordinates were obtained from Google Earth along with altitudes and were imported into ArcGIS with the described coordinate system.
Mushroom Rock	GCS_WGS_1984	Coordinates: 27.9164411 S, 29.7260354 E Altitude: 1 809m Coordinates were obtained from Google Earth along with altitudes and were imported into ArcGIS with the described coordinate system.
Leopard's Creek	GCS_WGS_1984	Coordinates: 27.9550591 S, 29.7101925 E Altitude: 1 814m Coordinates were obtained from Google Earth along with altitudes and were imported into ArcGIS with the described coordinate system.
Nccandu Nature Reserve	GCS_WGS_1984	The maps present in Conservation Outcomes (2021) and Rambarath *et al*. (2017) were applied to draw the extent of Ncandu Nature Reserve.
Ncandu Private Forest and Grassland Nature Reserve	GCS_WGS_1984	The maps present in Conservation Outcomes (2021) and Rambarath *et al*. (2017) were analysed to determine the location and extent of farm portions.
uMsonti Nature Reserve	GCS_WGS_1984	The maps present in Conservation Outcomes (2021) were analysed to determine the location and extent of farm portions.

Annexure 2

Ethics and permits

NORTH-WEST UNIVERSITY
NOORDWES-UNIVERSITEIT
YUNIBESITI YA BOKONE-BOPHIRIMA

Private Bag X1290, Potchefstroom
South Africa 2520
Tel: 018 299-1111/2222
Fax: 018 299-4910
Web: http://www.nwu.ac.za
Senate Committee for Research Ethics
Tel: 018 299-4849
Email: nkosinathi.machine@nwu.ac.za

ETHICS APPROVAL LETTER OF STUDY

Based on the review by the **Faculty of Natural and Agricultural Sciences Ethics Committee (FNASREC)**, the Committee hereby clears your study as no ethical risk. This implies that the FNASREC grants permission that, provided the general conditions specified below are met, the study may be initiated, using the ethics number below.

Study title: Analysing the methodology for the use of eDNA in detecting Batrachochytrium dendrobatidis
Study Leader/Supervisor: Prof C Weldon
Student: C Kleynhans

Ethics number:	N	W	U	-	0	1	2	9	2	-	2	1	-	A	9
	Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application type: Single **Risk Category:** **No Risk**

Commencement date: 28/08/2021
Expiry date: 31/01/2023

General conditions:
The following general terms and conditions apply:

- *The commencement date indicates the date when the study may be started.*
- *In the interest of ethical responsibility, the NWU-SCRE and FNASREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the FNASREC or that information has been false or misrepresented;*
    - *submission of the annual (or otherwise stipulated) monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and / or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *FNASREC can be contacted for further information or any report templates via Roelof.Burger@nwu.ac.za 018 299 4269*

The FNASREC would like to remain at your service as scientist and researcher, and wishes you well with your study. Please do not hesitate to contact the FNASREC or the NWU-SCRE for any further enquiries or requests for assistance.

Yours sincerely,

Prof Roelof Burger
Chairperson Faculty of Natural and Agricultural Sciences Ethics Committee (FNASREC)

EZEMVELO KZN WILDLIFE
Conservation, Partnerships & Ecotourism

ORIGINAL

ORDINARY PERMIT

Fee:	R 50,00	Permit No:	OP 874/2021
Receipt No:	994/2021	Contact:	Permits Office

This permit is issued in pursuance of the provisions of the Nature Conservation Ordinance No 15 of 1974 , Chapter 7 and the Regulations framed thereunder.

The permit is issued to:

ID Number: 7502075129088

Prof. Che Weldon
North-West University (NWU)
School of Biological Sciences
Private Bag X6001
Potchefstroom
2520

Residential Address
North-West University (NWU)
School of Biological Sciences
Building E6 Potchefstroom
Campus ,NWU Potchestroom
2531

In the capacity of Researcher

To Capture, Collect and Release the following species of Amphibians Invertebrates and Reptiles

FROGS *(ANURA)*

20 (Twenty) Adults per species Collect skin swab (non-invasive), collect toe clip release animals throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ncandu Forest Reserve.

FROGS *(ANURA)*

20 (Twenty) per species collect complete organism throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ncandu Forest Reserve.

Snakes *(SERPENTES SPP)*

10 (Ten) Adults per species Collect blood, skin swab and cloacal swab release animals throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ncandu Forest Reserve.

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 10 March 2021

for CHIEF EXECUTIVE

Permit Holder

EZEMVELO KZN WILDLIFE PERMITS OFFICE
PO Box 13053, Cascades, 3202, Pietermaritzburg, KwaZulu-Natal.
Tel +27 33 845 1320 / 1324. Fax: +27 33 845 1747. Fax to Email: 086 529 3320
Email: permits@kznwildlife.com. Website: www.kznwildlife.com

EZEMVELO KZN WILDLIFE
Conservation, Partnerships & Ecotourism

ORIGINAL

SKINKS ,GECKOS AND LIZARDS *(SQUAMATA)*

10 (Ten) Adults per species Collect blood and cloacal swab release animals throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ncandu Forest Reserve.

LIZARDS *(LACERTILIA SPP)*

10 (Ten) Adults per species Collect blood and cloacal swab release animals throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ncandu Forest Reserve.

CHAMELEON *(CHAMAELONIDAE)*

1 (One) ADULT Capture and release throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ncandu Forest Reserve.

AGAMA SP *(AGAMA SP)*

10 (Ten) Adults per species Collect blood and cloacal swab release animals throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ncandu Forest Reserve.

NILE MONITOR LIZZARD (Water legua *(VARANUS NILOTICUS)*

10 (Ten) Adults per species Collect blood and cloacal swab release animals throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ncandu Forest Reserve.

TERRAPIN

10 (Ten) Adults per species Collect nasal fluid and blood release animals throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ncandu Forest Reserve.

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ORIGINAL

TERMS AND CONDITIONS UNDER WHICH THIS PERMIT IS ISSUED

1. It is valid only:
   (i) from : 01 March 2021
   to : 28 February 2022
   (ii) in the original
   (iii) if all **4** pages are signed by the permit holder named above
   (iv) to the permit holder named above and the following Nominees :
   Dr M Greeff-Laubscher
   Miss C Kleynhans
2. By signing the permit or licence the holder accepts, and agrees to comply with the conditions under which it is issued.
3. Permit to be returned to E KZN Wildlife, P O Box 13053, Cascades, 3202, upon expiry for renewal or cancellation.
4. Permit shall be carried by holder, or the specified nominees, at all times during use.
5. Outside of E KZN Wildlife areas, use of this permit is subject to landowner's or controlling authority's written permission.
6. Prior to collecting in areas under the control of the E KZN Wildlife the holders shall contact the Officer-in-Charge of the area at least 48 (Forty-eight) hours before commencing, and shall comply with any conditions which the Officer may impose at his discretion. The officer may refuse collection or capture at his or her discretion.
7. At least one representative specimen (preferably at least one male and one female) of each species collected from each locality must be lodged with a recognised South African museum/herbarium. Holotype specimens, and half the number of paratype specimens, of any new species MUST BE DEPOSITED with a recognised South African museum/herbarium, and may only leave South Africa on a loan basis. These specimens are to be deposited in the SA museums within a year of publishing the description of the new species. The holder shall provide the Chief Executive Officer, KZNNCS with the name of the museum at which the specimens have been lodged, and the accession number of each specimen. This condition relates to unavoidable by-catch of non-target organisms as well.
8. A copy or copies of any publication arising from the authority herein contained will be made available to E KZN Wildlife.

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 10 March 2021

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Permit Holder

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9. Should renewal of this permit be desired, a minimum of one month's notice is required.

10. (i) Reserving accommodation within E KZNWildlife areas is entirely the responsibility of the permit holder. Booking is obtainable at the Central Booking Office, Telephone 033 8451000.(ii) Any assistance required from Board staff will be subject to other demands on the Officer's time and must be arranged in advance with him/her.

11. Holders shall provide the Chief Executive, with a named list of every specimen collected (including the class, order, family, gender and age), the geographical co-ordinates (to seconds accuracy) of each collection locality and dates of collection, as laid out in the following table. A Global Positioning System with the WGS84 Datum should be used wherever possible to determine the geographical co-ordinates of the collection sites; please state the method used.

12. SPECIMEN - COLLECTION DATE - SPECIES - LOCALITY - LATITUDE - LONGITUDE
(museum (ddmmyy) (Seconds (Seconds
Accession) Accuracy) Accuracy).
Holders are requested to provide additional information, such as the habitat in which each specimen was collected and abundance or relative abundance data (providing standardised sampling methods are used) with the list.

13. No collecting is permitted within the road reserve which is a strip 30 (thirty) metres either side of a public road, no matter how small or remote the road may be.

14. No collecting is permitted in the wilderness areas within the Protected Area. For confirmation of boundaries of the wilderness area contact the Officer in Charge.

15. No specimens collected or captured or exported under this permit may be sold.

16. If possible, dead by-catch is to be distributed to the relevant experts for those groups in South Africa under the same conditions of this permit. Please enclose a copy of this permit and the locality and field data associated with the by-catch with the by-catch specimens.

17. This permit/licence/certificate is issued subject to compliance with all other relevant legislation and does not preclude the permit holder from adherence thereto.

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 10 March 2021

for CHIEF EXECUTIVE

Permit Holder

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