Assessing the ability of environmental bacteria to inhibit the growth of *Batrachochytrium dendrobatidis*

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PREFACE

Proverbs 3:5-6 (KJV): **5***Trust the Lord with all thine heart; and lean not on thine own understanding.* **6***In all thy ways acknowledge him, and he shall direct thy paths.*

I sincerely thank my supervisor Prof Ché Weldon and Co-supervisor Dr Xavier Harrison, from the University of Exeter, for guidance and support throughout the project.

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ABSTRACT

Amphibians have been facing devastating declines through the decades and it wasn't until 1998 that the once enigmatic declines of amphibians were attributed to *Batrachochytrium dendrobatidis.* This lethal fungal pathogen is responsible for causing the disease chytridiomycosis and with its variation in virulence across lineages, environments and host susceptibility, scientists have been fighting to find a cure for this lethal disease. Through several defence mechanisms, including an adaptive and innate immune system, amphibians have been battling to protect themselves not only against chytridiomycosis but also against other pathogens including bacterial infection. Their adaptive immune response can clear an infection caused by a pathogen while the innate immune system provides a non-specific response against a pathogen and this includes antimicrobial peptides produced by the host as well as the colonization of protective microbes on the skin of amphibians. Research has proved that there is a wide range of bacteria genera that have various effects on the growth of *B. dendrobatidis* (*Bd*) by either inhibiting or promoting the growth of this pathogen. With two known *Bd* lineages in South Africa, namely the Global Panzootic Lineage (GPL) and the CAPE lineage, that differ in virulence, it was imperative to determine the relationship between bacteria and these lineages, but also to determine how bacteria colonize on the skin of adult frogs, and the mouthparts of tadpoles from various species from a wild frog community in a protected area. Molecular analysis revealed a uniqueness to the tadpole microbiome diversity with representative bacteria that was not found on corresponding adults as well as variation in microbiome structure across various species. A further variation in bacteria was observed between bacteria sampled from the environment and bacteria sampled from amphibians. A challenge assay study with 20, environmentally sampled, bacterial isolates' cell-free supernatant were conducted to determine the effects that individual bacterial isolates have on the two *Bd* lineages known from South Africa. After it was discovered that individual isolates had either an inhibiting or augmenting effect on the growth of *Bd* a consortium of bacteria were assembled and challenged against *Bd.* Results obtained indicated that with a consortium of bacteria the effects on *Bd* are significantly different than when bacteria were challenged individually against *Bd.* The results obtained may explain why some amphibian species are more susceptible to diseases, but it also indicates that a lot of work is required to determine why there is a variation in virulence across *Bd* lineages.

Keywords: *Batrachochytrium dendrobatidis,* challenge assays, microbial communities, metabolites, cell-free supernatant, lineages.

TABLE OF CONTENTS

PREFACE ..... **I**

ABSTRACT ..... **II**

LIST OF ABREVIATIONS ..... **VI**

LIST OF TABLES ..... **VII**

CHAPTER 1 INTRODUCTION AND BACKGROUND **11**

1.1 Introduction **11**

1.2 Problem statement and hypothesis **13**

1.3 Study aims and objectives **14**

1.4 Outline of the dissertation **14**

CHAPTER 2 LITERATURE REVIEW **15**

2.1 Amphibian importance **15**

2.1.1 Natural background on amphibians 15

2.1.2 Amphibian importance to humans 16

2.2 Amphibian skin physiology **17**

2.3 Vertebrate immunity **18**

2.3.1 Amphibian immunity 19

2.4 Amphibian declines **20**

2.4.1 Habitat destruction 22

2.4.2 Climate change 23

2.5 Infectious disease: Batrachochytrium dendrobatidis **24**

2.5.1 Life cycle of Batrachochytrium dendrobatidis 25

2.6 Amphibian microbiome 26

2.7 Environmental bacteria 30

CHAPTER 3 MATERIALS AND METHODS 33

3.1 Study sites and species 33

3.2 Amphibian bacteria collection 34

3.2.1 Amphibian sampling 34

3.2.2 Euthanizing tadpoles 34

3.2.3 Bacteria collection 34

3.3 Environmental bacteria collection 35

3.4 Bacterial analysis 35

3.4.1 Amphibian bacterial community analysis 35

3.4.2 Environmental bacteria analysis 36

3.4.2.1 Bacteria culturing 36

3.4.2.2 Bacterial sequencing and identification 37

3.5 *Bd* Challenge assays 40

3.5.1 Preparation of *Bd* cultures 40

3.5.2 Preparation of bacterial cell-free supernatants 41

3.5.3 Challenge assays 41

3.6 Data analysis 42

CHAPTER 4 RESULTS 44

4.1 Bacterial culturing 44

4.2 Molecular results 45

4.2.1 Nanodrop 45

4.2.2 Agarose gel electrophoresis 46

4.2.3 Phylogenetics 49

4.3 Challenge assays 58

4.3.1 Photo records 66

CHAPTER 5 DISCUSSION 69

5.1 Molecular discussion 69

5.1.1 Nanodrop and agarose gel electrophoresis 69

5.1.2 Bacterial diversity 69

5.2 *Bd* challenge assay 76

CHAPTER 6 CONCLUSION AND RECOMENDATIONS 80

BIBLIOGRAPHY 82

LIST OF ABREVIATIONS

AMP's	Antimicrobial peptides
ASV's	Amplicon sequence variants
Bd	*Batrachochytrium dendrobatidis*
DNA	Deoxyribonucleic acid
GPL	Global Panzootic lineage
IPCC	The Intergovernmental Panel on Climate Change
UVB	Ultraviolet B
m.a.s.l.	Meters above sea level
MHC	Major histocompatibility complex
MS-222	Tricaine methanesulfonate
NCBI	National Centre for Biotechnology Information
NWU-AnimCareREC	North-West University Animal Care, Health and Safety Research ethics Committee
OTU's	Operational Taxonomic Units
PCR	Polymerase chain reaction
qPCR	Quantitative Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SANS	South African National Standards
TGhL	Tryptone-gelatin hydro-lysate-lactose

LIST OF TABLES

Table 2-1: Compared number of species in the threat category for South Africa and globally. Adapted from Measey (2011). 21

Table 3-2: 16S PCR conditions used during PCR protocol. 38

Table 4-1: Axenic bacterial isolates indicating gram staining, size, and shape to distinguish morphotypes. 44

Table 4-2: Nanodrop results where a 260:280 ratio between 1.75 and 2 is considered reasonably pure DNA (indicated in green highlight)..... 45

Table 4-3: Selected isolates based on the quality and quantity of DNA. 49

Table 4-4: Species identification based on phylogeny and bioinformatics..... 51

Table 4-5: Amphibian species found at site 1 and 2, separated by adults and tadpoles where the tadpole ecomorphological guild is noted (Botha, 2013). 54

Table 4-6: Statistical analysis of the overall effects of cell-free supernatant on *Bd*CAPE isolate SA 4C and *Bd*GPL isolate MG04. 60

Table 4-7: Mann Whitney test indicating the significance between *Bd*CAPE and *Bd*GPL. 61

Table 4-8: Statistical analysis of the effects of cell-free supernatant on the growth of *Bd*CAPE isolate SA 4C. 62

Table 4-9: Statistical analysis of the effects of cell-free supernatant on the growth of *Bd*GPL isolate MG 04. 64

Table 4-10: Consortium layout, containing one bacterial isolate of each of the various families..... 64

Table 4-11: Statistical analysis of the effects of cell-free supernatant from a consortium of bacteria on the growth of *Bd*CAPE isolate SA 4C. 66

LIST OF FIGURES

Figure 2-1: Amphibian species richness in the Afrotropical realm where darker shades indicate higher concentrations of amphibian species (Measey 2011). 15

Figure 2-2: An illustration of the physical, chemical, cellular and microbiological barriers of amphibian skin (taken from Varga, *et al*., 2019). 18

Figure 2-3: Inverted microscope images of *Bd*, indicating the various stages of the life cycle. 26

Figure 3-1: Map indicating various sites used for the study. 33

Figure 3-2: Streak plate method to obtain single axenic bacterial samples (Sanders, 2012). 37

Figure 3-3: Examples of axenic isolates that resulted from the use of the quadrant streak plate method. 37

Figure 3-4: GeneRuler 1kb DNA ladder used in gel electrophoresis to determine the DNA fragment size. 40

Figure 3-5: Experimental 96-well plate set-up for *Bd* challenge assays with each experimental well containing one bacterial cell-free supernatant isolate, while negative control wells contain heat-killed *Bd* cells and positive control wells contain only *Bd* with no cell-free supernatant added. 42

Figure 4-1: Agarose gel electrophoresis image of samples W1(1) to W3(6) recording DNA fragment size as 1500 bp. 47

Figure 4-2: Agarose gel electrophoresis image of samples W3(7) to W4(9) recording DNA fragment size as 1500 bp. 47

Figure 4-3: Agarose gel electrophoresis image of samples W5(1) to W7(4) recording DNA fragment size as 1500 bp. 48

Figure 4-4: Agarose gel electrophoresis image of samples W7(5) to W10(3) recording DNA fragment size as 1500 bp. 48

Figure 4-5: Phylogenetic tree constructed through the Neighbour-Joining method with a bootstrap value of 5000, from environmental water samples. The

percentage of replicate trees in which the associated taxa clustered together in the bootstrap test can be seen next to the branches..... 53

Figure 4-6: Phylogenetic tree constructed from 16S rRNA metagenetic sequencing obtained from tadpole and adult amphibia swabs. 56

Figure 4-7: Variation in microbial community structure between adult and tadpole swabs along with aquatic and terrestrial swabs. Distances between samples represent ordination of a Principal Components Analysis (PCA) of centred-log-ratio transformed ASVs abundances from 16S rRNA metagenetic sequences. 57

Figure 4-8: Variation in microbial community structure between species where squares represent adult swabs and circles represent tadpole swabs. Distances between samples represent ordination of a Principal Components Analysis (PCA) of centred-log-ratio transformed ASVs abundances from 16S rRNA metagenetic sequences. 58

Figure 4-9: Distribution of cell-free supernatant effects of *Bd*CAPE SA 4C and *Bd*GPL MG 04. The black bars represent the bacterial isolates that were challenged against the *Bd*CAPE lineage isolate SA 4C and the grey bars are the bacterial isolates challenged against the *Bd*GPL lineage isolate MG 04. The positive control represents normal *Bd* growth where no cell-free supernatant was added. 59

Figure 4-10: A) Overall distribution of cell-free supernatant effects on *Bd*CAPE and *Bd*GPL. B) Box-and-whisker plot of the overall distribution of cell-free supernatant effects on *Bd*CAPE and *Bd*GPL to determine the normality of data. 60

Figure 4-11: Box-and-whisker plot of the distribution of the effects of cell-free supernatant on *Bd*CAPE isolate SA 4C. 61

Figure 4-12: Box-and-whisker plot of the distribution of the effects of cell-free supernatant on *Bd*GPL isolate MG 04..... 63

Figure 4-13: Distribution of effects of cell-free supernatant from a consortium of bacteria on the growth of *Bd*CAPE isolate SA 4C. The positive control represents the normal growth of *Bd* where no cell-free supernatant was added. 65

Figure 4-14: Box-and-whisker plot indicating the distribution of effects of cell-free supernatant from a consortium of bacteria on the growth of *Bd*CAPE isolate SA..... 66

Figure 4-15: A) Negative, heat-killed *Bd* zoospores control (40X objective). B) Positive control, *Bd* with no cell-free supernatant (40X objective). C) Partial inhibition where *Bd* growth was halted at the germling stage, observed when *Aquitalea magnosonii* cell-free supernatant was added to *Bd*GPL isolate MG 04 (40X objective). D) Live sporangia with discharge papillae observed when *Caulobacter vibrioides* cell-free supernatant was added to *Bd*CAPE isolate SA 4C (40x objective). E) Example of colonial development where more than one sporangium develops from one zoospore, observed when *Massilia sp*. cell-free supernatant was added to *Bd*GPL isolate MG 04 (40X objective). F) Partial inhibition where large sporangia form but no zoospore developed. Observed when *Caulobacter vibrioides* cell-free supernatant was added to *Bd*GPL isolate MG 04 (10X objective, enlarged to 40X objective). G) *Bd* augmentation observed when *Chakrabartia godavariana* cell-free supernatant was added to *Bd*CAPE isolate SA 4C (40X objective). H) Complete inhibition where cell lysis took place. Observed when *Enterobacter sp*. cell-free supernatant was added to *Bd*CAPE isolate SA 4C (40X objective)..... 68

CHAPTER 1 INTRODUCTION AND BACKGROUND

1.1 Introduction

Three distinct orders of amphibians currently exist, namely Urodela (salamanders), Anura (frogs and toads) and Gymnophiona (caecilians), all characterized by distinct anatomical features (West, 2018). With 8212 described species (AmphibiaWeb, 2020) and a 43% endemicity in South Africa (Measey, 2011), amphibians are described as ancient creatures (Rollins-Smith *et al.*, 2005; Xiao *et al.*, 2011) with remarkable life cycles that range from water-breathing juveniles to air-breathing adults capable of surviving on land and in water (Wake & Vredenburg, 2008). However, when considering the constant exposure to a variety of habitats filled with potentially harmful pathogens, amphibians must have a remarkable defence mechanism to protect them from these pathogens. The main defence mechanism that amphibians have as protection is their skin. It is considered critical for frog skin to maintain its integrity to conduct crucial physiological processes as well as to defend against invading pathogens, because amphibian skin contributes to water uptake, ion transport, respiration, heat transfer, camouflage, and predator prevention (Varga *et al.*, 2019).

These remarkable creatures have fought the battle against extinction for decades but are still facing devastating declines. Population declines have been documented since they started in the western United States, Puerto Rico and North-Eastern Australia in the early 1970s (Stuart *et al.*, 2004). Shortly after scientists released a statement in 1994 explaining that the general biodiversity crisis that amphibians face are possibly the most serious problem that the world faces (Pechmann & Wilbur, 1994), another group of scientists elaborated on a mass mortality event that occurred in 1998 in wild and captive frogs from North and Central America as well as Australia (Berger *et al.*, 1998; Longcore *et al.*, 1999). Histopathological examinations of a blue poison dart frog that died in the National Zoological Park in Washington DC revealed a fungal skin infection, that was then isolated and cultured to reveal a pure chytridiomycete causing a lethal disease known as chytridiomycosis (Longcore *et al.,* 1999). *Batrachochytrium dendrobatidis* is the first described species of chytrid fungus that attacks living vertebrate hosts (Longcore *et al.,* 1999). Symptoms of the infection can include clinical signs such as thickening of the outermost skin layer, reddening, ulcerations as well as excessive skin shedding, lethargy and anorexia (Berger *et al.,* 1998; Hettyey *et al.*, 2019). Breathing and osmoregulation in hosts can further be impaired which can also lead to co-infection and induce immunopathology that can ultimately lead to cardiac arrest and in extreme cases death (Voyles *et al.*, 2009; Hettyey *et al.*, 2019). The lethal disease called

chytridiomycosis is known to be the leading factor of amphibian declines causing the extinction of 40% of targeted species (Farrer *et al.*, 2011).

Scientists have extensively studied this lethal pathogen responsible for declines of a natural bioindicator species (DuRant & Hopkins, 2008). However, scientists aren't the only ones that have been defending amphibians. Adaptive and innate immune systems are defence mechanisms that can be found in vertebrates and are responsible for protection against pathogens (Rollins-Smith, *et al.* 2005; Cortázar-Chinarro *et al.*, 2020). The adaptive immune response is found to be responsible for clearing infection and protecting the host from a reoccurring infection from the same pathogens, while the innate immune response is responsible for the provision of a non-specific response to pathogens (Cortázar-Chinarro *et al.*, 2020). The granular glands found in the dermal layer of frog skin produce bioactive substances such as antimicrobial peptides, upon alarm or injury the active antimicrobial peptides, are released into the skin (Rollins-Smith & Conlon, 2005). It is known that these antimicrobial peptides are part of the innate immune response and are responsible for protection against potentially harmful pathogens with compelling results of inhibition against both gram-positive and gram-negative bacteria as well as fungi, protozoa and viruses (Rollins-Smith, *et al.* 2005; Xiao, *et al.* 2011; Cortázar-Chinarro *et al.* 2020) It has, furthermore, been proposed that antimicrobial peptides in the skin can play a role in protecting the skin from chytrid zoospores or protection of joining skin from spreading the disease following initial infection (Rollins-Smith & Conlon, 2005). An in vitro inhibition assay to test the ability of antimicrobial peptides to protect against *Bd* resulted in conclusive evidence that some antimicrobial peptides are able to protect amphibians against a chytrid infection (Rollins-Smith & Conlon, 2005). These results lead to the realisation that some amphibian species are more resistant to chytridiomycosis due to them having more effective antimicrobial peptides (Rollins-Smith & Conlon, 2005).

To understand the intricate and multilateral relationship between the bacterial microbiome and pathogens, scientists have been constantly relying on the amphibian skin microbiome as an established model system (Harrison *et al.*, 2017). An abundance of cutaneous bacteria, that are likely part of amphibians' innate immune system, has been identified, and it has been proposed that community interactions between amphibians' skin microbes and *Bd* can be manipulated to enhance disease resistance and ultimately reduce the impact of chytridiomycosis (Harris *et al.*, 2006; Woodhams *et al.*, 2007; Harris *et al.* 2009). Research, based on this information, lead to the discovery of a betaproteobacterium, *Janthinobacterium lividum*, identified as a protective microbe that has been isolated from various species of frogs (Harris *et al.*, 2006, Harris *et al.*, 2009, Lam *et al.*, 2010). Randomized, replicated experiments

concluded that *J. lividum* causes prevention of morbidity and mortality that are associated with chytridiomycosis (Harris *et al.*, 2009). Research has been conducted to establish microbial communities on amphibian skin through indirect horizontal transmission, the transmission of bacteria from a host to the environment and then to another host (Rebollar *et al.*, 2016). Studies concluded that through the use of indirect horizontal transmission a colonized probiotic bacterium can be established in a high-risk amphibian population and that this can be achieved by inoculating only a few individuals (Rebollar *et al.*, 2016).

1.2 Problem statement and hypothesis

Across life stages, population and *Bd* infection dynamics, significant differences have been found in the amphibian skin microbiome, while *Bd* in the Pyrenees arose from a single introduction (Bates *et al.*, 2018). Therefore, to counter disease driven amphibian declines a multifaceted approach should be adapted (Langwig *et al.*, 2015). Approaches to prevent amphibian declines include habitat protection, captive breeding and monitoring (Hussain & Pandit, 2012) as well as the development of antifungal probiotic bacteria (Harris *et al.*, 2009). However, long term sustainable solutions are needed to preserve and conserve amphibians in the wild. Several studies have proven that the structure and function of soil microbial communities can be altered with temperature variations (Muletz *et al.*, 2012). With drivers such as climate change to alter environmental bacterial communities, amphibians may be more susceptible to disease, therefore, to intervene via soil bioaugmentation with bacteria that are more resistant to temperature fluctuations, might counter *Bd* infections (Muletz *et al.*, 2012). However, for bioaugmentation to be considered a successful method of mitigating *Bd* (Garner *et al.*, 2016) a deeper understanding of bacterial community structure and stability is needed.

While the use of bioaugmentation has proven to be a successful practice when introducing bacteria into the soil (Muletz *et al.*, 2012), the research question that arises is, what is the relationship between bacteria already occurring in the environment and bacteria found on frog skin? Furthermore, what will the results be if environmental bacteria come in contact with *Bd*? To respond to these questions, an *in vitro* study to test the relationship and the effects that bacteria sampled from the environment might have on *in vitro* cultured *Bd* is needed.

A previous study (Walke *et al.*, 2014) have found an overlap in the microbes found on amphibian skin as opposed to microbes found in the environment, therefore it is hypothesized that *Bd* inhibition will be observed from bacteria sampled from the environment.

1.3 Study aims and objectives

The study aims to get a deeper understanding and to establish a framework for the ecological relationship between environmentally sampled bacteria and a multi-species amphibian community.

The following objectives were established:

1. Identify, and culture single bacterial colonies sampled in a water body containing multiple species of tadpoles. This will create an understanding of the diversity of bacteria that can be found in water habitats.

2. Compare diversity and composition of isolated environmental bacteria to bacteria found in the mouthparts of sampled tadpoles and on the skin of adult frogs from various species. Through this objective, it will be possible to determine whether transmission of bacteria might occur between tadpoles and their surrounding environment.

3. Lastly, *Bd* challenge assays will determine whether environmentally sampled bacteria are able to inhibit the growth of *Bd.* This will be achieved by conducting challenge assays of isolated bacteria on *in vitro* cultured *Bd*.

1.4 Outline of the dissertation

Chapter 1: General introduction and background

Chapter 2: Literature review

Chapter 3: Materials and methods

Chapter 4: Results

Chapter 5: Discussion

Chapter 6: Conclusion

CHAPTER 2 LITERATURE REVIEW

2.1 Amphibian importance

2.1.1 Natural background on amphibians

Biogeography

Anurans occupy the largest distribution over the geographical realms and have the highest diversity in the oriental, neotropical and Afrotropical regions (West, 2018). Amphibian diversity is found to be higher in areas with high precipitation and/or lower evaporative water loss this is due to amphibian's physiology that depends on moist conditions and high humidity (du Preez & Carruthers, 2017). In southern Africa a larger number of species will occupy warmer regions with higher rainfall, therefore, the number of species will increase along a transect drawn from the arid west to the east (du Preez & Carruthers, 2017). This is supported by a biodiversity assessment done to examine the extinction of amphibian species in the Afrotropical realm, this includes Southern Africa (Measey, 2011).

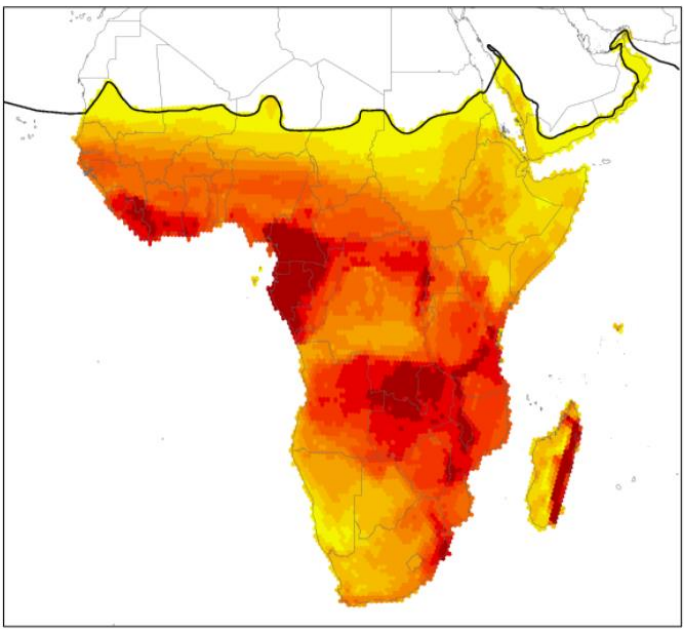


Figure 2-1: Amphibian species richness in the Afrotropical realm where darker shades indicate higher concentrations of amphibian species (Measey 2011).

Amphibians have highly permeable skin and biphasic life cycles, during which most species inhabit aquatic zones as larvae and become semi or wholly terrestrial as adults (Kolby & Daszak, 2016). To date, 169 frog species have been described in southern Africa (du Preez & Carruthers, 2017).

2.1.2 Amphibian importance to humans

When looking at environmental economics and conservation biology, there are five ways to evaluate biodiversity. The first two values are considered direct use values and include consumptive use values and productive use-values. The third and fourth values do not include the removal of a species from their ecosystem and this entails the value of ecosystem services and non-consumptive values such as aesthetics, recreation, cultural and spirituality-oriented amenity values (West, 2018). The last values look into the scientific and educational value of a species and include the development of educational programs (West, 2018). Although amphibians are not widely accepted as the most aesthetically pleasing species on the planet, amphibians are directly beneficial to humans in more than one way.

The most common benefit that amphibians have to humans is the predation on vectors causing human diseases. Mosquitos and biting flies are carriers for many human-related diseases that can be fatal to the hosts such as malaria, yellow fever, African sleeping sickness, eastern equine encephalitis, and dengue fever (DuRant & Hopkins, 2008). Adult amphibians feed on mosquitos and biting flies while tadpoles predate on mosquito larvae thus reducing these vector populations responsible for the spread of human and veterinary diseases (DuRant & Hopkins, 2008). A study found that the larval marbled salamander, *Ambystoma talpoideum* consumed 439±20 mosquitos, *Culex pipiens,* on average in a day (DuRant & Hopkins, 2008). Amphibian larvae and adults in aquatic systems can reach up to 500 000 individuals per hectare and their predation removes a large number of mosquitos out of wetlands, ponds, streams, and rivers (DuRant & Hopkins, 2008). It is estimated that 6 585 000 000 mosquitos get eaten in a single hectare per month by roughly 500 000 individuals of *A. talpoideum,* where red-spotted newts, *Notophthalmus viridescens* are found to consume about 316±35 *C. pipiens* mosquitos a day resulting in 4 740 000 000 consumed mosquitos per hectare in a month (DuRant & Hopkins, 2008). When looking at tadpoles a study found that various species aggressively predates dengue mosquito, (*Aedes aegypti*), eggs, these eggs are known to be protective safe havens for deadly pathogens between mosquito breeding seasons (DuRant & Hopkins, 2008). When evaluating the number of mosquitos that amphibians prey on it is evident that amphibians are exceptional biocontrol agents. However, if amphibians die out in tropical regions where insect-borne diseases have the highest rates, the predatory pressures that amphibians once exerted on disease vectors will be lost and vector populations will increase beyond control.

Amphibians are further considered as biocontrol agents when it comes to agriculture. Frogs and toad with a relatively large body volume are effective consumers and will feed indiscriminately on larger pest invertebrates such as locusts (West, 2018). In crop fields,

amphibians will feed with minimal prey selection, based on availability and profitability, likely consuming invertebrates feeding on crops.

2.2 Amphibian skin physiology

Amphibians are described as ancient creatures with extraordinary life cycles that metamorphose from water-breathing juveniles to air-breathing adults capable of existing on land and in water (Rollins-Smith *et al.,* 2005; Wake & Vredenburg, 2008, Xiao *et al.*, 2011). However, with these intricate means of life, amphibians are constantly exposed to various habitats that are filled with potentially harmful pathogens that might come into contact with their moist skin (Xiao *et al.*, 2011).

The respiration of amphibians is unique in the sense that cutaneous respiration is more important, and better developed than respiration through the lungs. Their excellent vascularized skin places amphibians in intimate contact with their surrounding environment (Wake & Vredenburg, 2008). The close relatedness between amphibians and their environment makes them especially vulnerable to changes that may occur in the water and air quality which may be the result of diverse pollutants (Wake & Vredenburg, 2008).

It is important for a frog's overall health to maintain its skins integrity to conduct both crucial physiological processes and for the defence against invading fungal and bacterial pathogens. Amphibian skin contributes to water uptake, ion transport, respiration, heat transfer, camouflage, and avoidance of predation (Varga *et al.*, 2019). Due to the relatively thin and permeable nature of amphibian skin, it is particularly vulnerable to cutaneous injury, and frog skin is considered an important first line of defence against harmful agents that may disrupt skin function or cause cutaneous or systematic disease, interrupting physiological functions that could ultimately lead to death (Varga *et al.*, 2019). Amphibian skin contains physical, chemical, and microbial barriers that act together to defend against pathogen assault (Figure 2-1). The skin of amphibians is composed of an epidermal and dermal layer that contains immune cells throughout. The epidermis is comprised of stratified squamous epithelial cells that can be divided into three distinct layers; *stratum corneum*, *stratum spinosum* and *stratum germinativum* (Varga *et al.*, 2019). Connective tissue formed by collagenous fibres is the main component of the dermis layer and can be divided into two layers namely spongious dermis and compact dermis, these two layers are connected by collagenous columns (Varga *et al.*, 2019). Three glands can be found within the dermal layer, this includes granular glands, mucosal glands, and small mixed glands, which secretes compounds, including mucus and antimicrobial peptides. Finally, commensal bacteria colonize frog skin to form the microbial barrier.

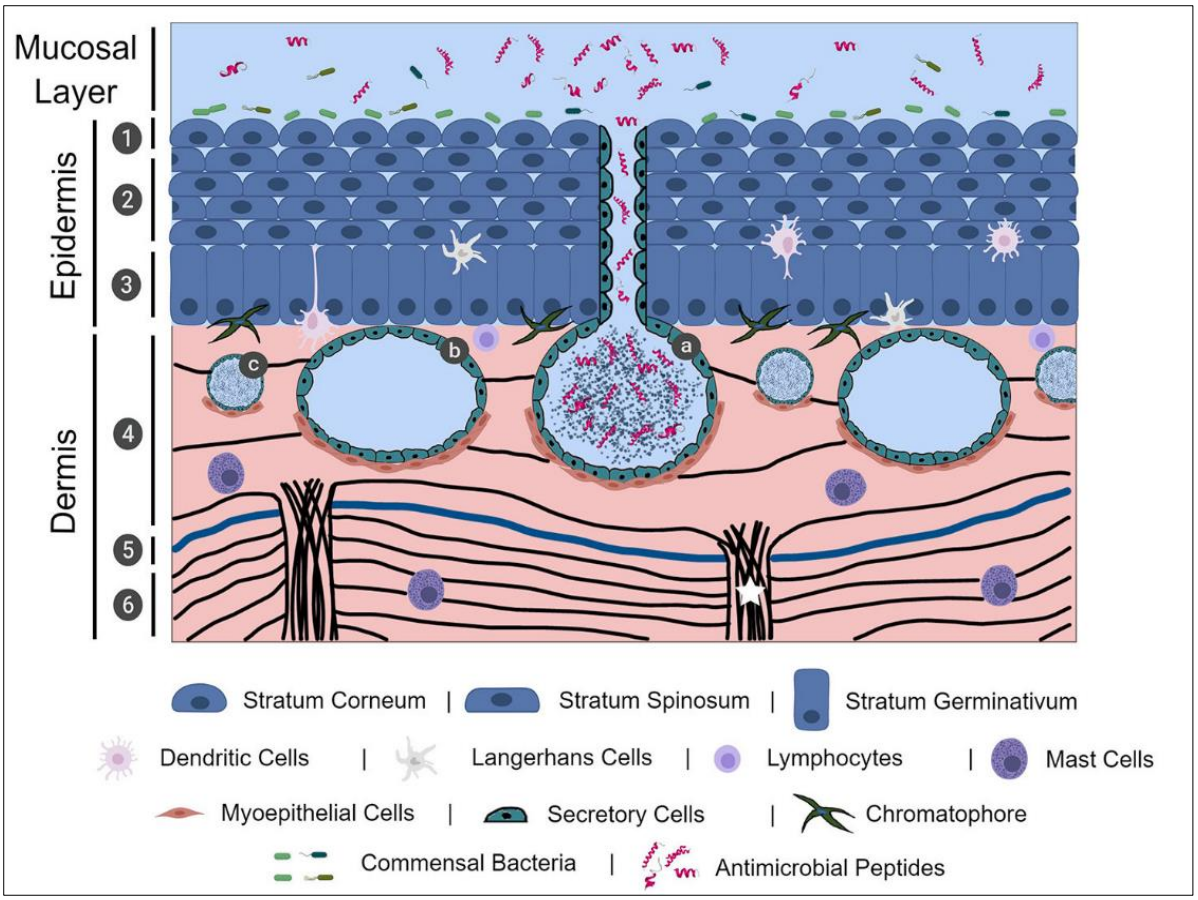


Figure 2-2: An illustration of the physical, chemical, cellular and microbiological barriers of amphibian skin (taken from Varga, *et al*., 2019).

2.3 Vertebrate immunity

The immune system of vertebrates consists out of many specialized cell and molecules that interact to protect the organism against pathogens (Frank, 2002). The vertebrate immune system can be divided into two types of immunity, namely the nonspecific immunity and the specific immunity (Frank, 2002). The nonspecific immune response is known for recognising a pathogen as an invader rather than a part of the host, within the nonspecific immunity various proteins act I unison to disrupt the surface of invading cells (Frank, 2002). Through phagocytic cells such as macrophages and neutrophils an invading cell can be engulfed completely (Frank, 2002). However, the nonspecific immune systems of vertebrates can not entirely clear an infection on its own and it is possible for a pathogen to completely avoid the nonspecific immunity of vertebrates (Frank, 2002). In contrast with the nonspecific immunity is the specific immunity that recognises small regions of pathogen molecules (Frank, 2002). Specific immunity recognises and binds to a small molecule site within a larger pathogen molecule, this is called an epitope, while an antigen is a pathogen molecule able to stimulate a specific immune response and contains one or more epitopes (Frank, 2002).

In addition to the nonspecific and specific immune responses are also B cells and antibodies that can fight infections caused by pathogens (Frank, 2002). B cells develop into lymphocytes circulating the blood and lymph systems, these B cells can either express immunoglobulins which forms B cell receptors, or the immunoglobulins can circulate as antibodies (Frank, 2002).

Furthermore, the vertebrate immune system contains major histocompatibility complex molecules that binds short peptides within a cell that was broken up by intracellular proteins (Frank, 2002). Adding to immunity of vertebrates are T cells, lymphocyte cells that matured in the thymus (Frank, 2002). T cell receptors are known to have varying binding specificity and each T cell can only bind to particular peptide major histocompatibility complex combinations presented on the surface of cells.

2.3.1 Amphibian immunity

With a better understanding of vertebrate immunity, it is time to look in amphibian immunity. Amphibians have both adaptive and innate immune system that is used as protection against pathogens (Rollins-Smith, *et al.*, 2005, Cortázar-Chinarro *et al.*, 2020). The adaptive immune responses in cold-blooded vertebrates include antibody and T lymphocyte-mediated responses that develop after pathogens have been detected by the antigen-presenting cells including macrophages and dendritic cells (Rollins-Smith, *et al.*, 2005). The adaptive immune response is therefore responsible for the clearing of an infection and further protects the host against a reoccurring infection from the same pathogen (Cortázar-Chinarro *et al.*, 2020). The innate immune system is responsible for providing a non-specific response to pathogens. Upon the exposure of a pathogen, an early induced inflammatory response is recognised by non-specific effectors (Cortázar-Chinarro *et al.*, 2020). The vertebrate innate immune response includes macrophages and neutrophils, complement-mediated lysis of pathogens, natural killer cells, and secretion of antimicrobial peptides (AMP's) (Rollins-Smith *et al.*, 2005; Xiao *et al.*, 2011; Cortázar-Chinarro *et al.*, 2020). AMP's are recognised as short, cationic, amphipathic and Alpha helical. These molecules can be found in a variety of taxa such as molluscs, fish, amphibians, birds, and mammals including humans (Cortázar-Chinarro *et al.*, 2020). The antimicrobial peptides of amphibians are synthesized as larger proteins with a signal sequence as well as an acidic propiece that are cleaved to release the mature and active peptide (Rollins-Smith *et al.*, 2005). In amphibians, the antimicrobial peptides are synthesized and stored in the granular glands of the dermal layers (Bovbjerg, 1963; Mills & Prum, 1984). The granular glands, also known as poison glands, are syncytial structures that are surrounded by a smooth muscle layer innervated by sympathetic nerves (Rollins-Smith *et al.*, 2005). In the event of alarm or injury, the sympathetic nervous system is activated followed

by the stimulation of the adrenergic receptors. Of which results in the release of the contents of the granular glands to the skin's surface (Rollins-Smith *et al.*, 2005).

Antimicrobial peptides produced by amphibian skin display compelling activity against both Gram-positive and Gram-negative bacteria as well as fungi, protozoa, and viruses (Rollins-Smith *et al.*, 2005; Cortázar-Chinarro *et al.*, 2020). A large group of linear amphipathic helical peptides are the main families of antimicrobial skin peptides (Rollins-Smith *et al.*, 2005; Cortázar-Chinarro *et al.*, 2020). These amphipathic helical peptides are found to be cationic with a considerable amount of positively charges residues and hydrophobic regions providing them with the ability to bind to a negatively charged molecule and/or membrane lipids and disturb the membrane structure (Rollins-Smith *et al.*, 2005; Cortázar-Chinarro *et al.*, 2020).

2.4 Amphibian declines

It has been well established that amphibians have direct importance to humans not only as bio-control agents of disease vectors (DuRant & Hopkins, 2008) and agricultural pests but also being excellent environmental indicators (Kolby & Daszak, 2016). However, even with remarkable physiological parameters against pathogens, our beloved amphibians, responsible for maintaining ecosystem balance are facing devastating declines that could lead to a mass extinction event.

When reviewing historical data on amphibian declines, it can be found that declines began presumably as early as the 1970s (Stuart *et al.*, 2004). Data suggests that these amphibian declines started in the western United States, Puerto Rico, and North-eastern Australia (Stuart *et al*., 2004). In 1990 the National Research Council's board sponsored a workshop to examine widespread amphibian population declines, scientists then proceeded to examine the population of *Bufo canorus* for 20 years at Tioga Pass, California (Sherman & Morton, 1993). Their survey produced results of population declines in *B. canorus* due to predation disease and below average spring-snow fall indication that population declines started as early as 1976 (Sherman & Morton, 1993). In 2004 a complete evaluation of then-recognised species was performed to assess the status and trends of amphibian populations. The results were obtained through the use of the top three established categories of the threat of extinction, namely, vulnerable, endangered, or critically endangered. The evaluation found that amphibians are more threatened than either birds or mammals, with 32.5% of amphibian species being globally threatened (Stuart *et al*., 2004). During the species trend evaluation by Stuart *et al.* (2004), it was found that 43.2% of amphibian species are experiencing some kind of decrease in population numbers, however only 0.5% species are increasing, and 27.2%

species were stable as of 2004. Furthermore, it was estimated that 427 species (7.4%) were listed as critically endangered (Stuart *et al*., 2004).

In 2010 an assessment was conducted for South-Africa specifically, and data from 2004 were compared to data collected in 2010. In a comparative summary, where the number of amphibian species from different threat categories for South Africa is compared globally in 2004 and 2010, it can be seen that amphibian extinction is far more prevalent globally than it is in South Africa (Table 2-1 (Measey, 2011)).

Table 2-1: Compared number of species in the threat category for South Africa and globally. Adapted from Measey (2011).

Red List Category	% of species Global 2004	% of species South Africa 2004	% of species Global 2010	% of species South Africa 2010
Extinct	0.6	0	0.6	0
Extinct in the wild	0.2	0	0.03	0
Critically endangered	7.4	3.4	7.7	4.2
Endangered	13.3	6.8	12	5.9
Vulnerable	1.6	6.8	10.5	4.2
Near Threatened	6.3	4.3	6.1	4.2
Least concern	38.3	71.8	37.7	81.5
Data deficient	22.5	6.8	25.4	0

Various studies have found six major drivers of amphibian declines, including overexploitation, habitat reduction, climate change and emerging infectious diseases (Pechmann & Wilbur, 1994, Stuart *et al*., 2004, Wake & Vredenburg, 2008, Muletz *et al.*, 2012). In addition to the six major drivers Blaustein *et al.* (2011) proposed nine stressors adding UVB radiation, contaminants and amphibian collection, that are affecting amphibians in various stages of their life cycles and explains that amphibians are continuously “bombarded” by these stressors, either directly or indirectly.

Research regarding amphibian declines was introduced at the First World Congress of Herpetology in 1989 (West, 2018). Mainly it is anthropogenic activities that are to blame for the global scale declines that amphibians are undergoing, and declines are predominantly attributed to habitat destruction and epidemics of an infectious disease (Muletz *et al.*, 2012). Furthermore, it was found that global warming is another factor contributing to the devastating

declines in amphibian populations as well as environmental pollution, overexploitation, and the introduction of various invasive species. This literature review will be looking extensively at the category most threatening to amphibians in South Africa as well as globally, namely habitat destruction (Measey, 2011; Muletz *et al.*, 2012). A second global threat that will be researched expansively is global warming, seeing that global warming can be attributed to various human activities (IPCC, 2007) such as logging and wood harvesting as well as residential and commercial development threatening an estimate of 33% of all amphibians (Measey, 2011).

2.4.1 Habitat destruction

Hussain & Pandit (2012) explain the term land-use change as the altering of the way that humans use the land, while land cover change is known as the modification of the physical or biotic nature of a site. These phenomena can occur either separately or together and are commonly referred to as land-use change. It has been recorded that land-use change can facilitate local and ultimately regional extinction of populations and species (Hussain & Pandit, 2012). The extinction of populations can be facilitated through the killing of organisms, removal of habitat and the prevention of access to breeding sites (Hussain & Pandit, 2012).

It has been stated that land-use change is the best well-documented cause for amphibian declines, reducing the amphibian abundance as well as diversity in the affected regions (Hussain & Pandit, 2012). Habitat loss has been identified as the major threat to amphibians in the Afrotropical region, affecting approximately 90% of threatened species (Stuart *et al.* 2004). Logging, being one of the most prominent means of land-use change, are responsible for exposing terrestrial amphibians to considerably changed microclimates, soil compaction, and dehydration as well as a reduction in habitat complexity (Hussain & Pandit, 2012). Furthermore, aquatic amphibians are exposed to environments with increased siltation and reduced wood debris (Hussain & Pandit, 2012). In the Afrotropic 70% of threatened species can be attributed to the expansion of croplands, removal of vegetation (mainly logging), and urbanization or industrial development (Stuart *et al.* 2004). Regarding the loss of habitat due to urban development, a study conducted in Florida found a reduction of amphibians after the degradation of upland, dry season refuges and the modification wetlands that were used as breeding sites (Delis *et al.*, 1996). With regards to the Afrotropical region, a less significant but serious threat in South Africa is tree plantations and planned mining activities that might become an increased threat in West Africa (Stuart *et al.* 2004).

2.4.2 Climate change

According to The Intergovernmental Panel on Climate Change (IPCC), an accord has been reached that climate change is happening and can largely be attributed to human activities (IPCC, 2007). Generally, it has been estimated that during the next century global warming will proceed with temperatures in the range of 2°C to 4°C, however, rises as high as 7°C is anticipated for large portions of the United States and Europe, with even higher temperature fluctuations projected for Eurasia, Canada and Alaska (IPCC, 2007). Although these temperature fluctuations may seem insignificant to the average person, these climate changes could have distressing effects on montane species with scarcely distributed populations (Wake & Vredenburg, 2008). Montane species that are specialized to cool microclimates are of specific concern when considering the ability of a species to adapt to climate change (Bernardo & Spotila, 2006). It is highly probable that these species lack the physiological ability to endure warming-induced stress as well as the capability to disperse away from their natural habitat (Bernardo & Spotila, 2006). The mentioned limitations of montane species make them especially susceptible to population decline, genetic erosion and ultimately to extinction (Bernardo & Spotila, 2006).

A study performed in 2006 (Bernardo & Spotila, 2006), considered whether populations of montane salamanders (*Desmognathus*), dispersed through various thermal environments, show variations in physiological tolerance and whether metabolic stress is evident as a response to the natural temperature ranges that these species experience. The findings of the study provided insight suggesting that these species are living near the limit of their physiological tolerances (Bernardo & Spotila, 2006). This conclusion was reached when results indicated that low elevation *Desmognathus* populations display one of the highest levels of metabolic depression yet discovered for amphibians as well as the highest for a non-aestivating species (Bernardo & Spotila, 2006). Ultimately, the results of the study indicated that global warming would cause excessively higher extinction rates in organisms that are physiologically specialized to colder habitats (Bernardo & Spotila, 2006). In addition to this, it was found that 32% of Afrotropical species inhabiting tropical forests are globally threatened whereas, 43% of known species in tropical montane forests are threatened (Stuart, *et al.* 2004).

Contrary to tropical forest species, South Africa has 47% of the world's 484 savannah-associated amphibians and 44% of the world's 94 arid-habitat species (Stuart *et al.* 2004). It is less likely for savannah and arid habitat species to become threatened, however as a result of habitat loss as these habitats shrink and dry out, being particularly vulnerable to climate change, the number of threatened species is expected to increase (Stuart, *et al.* 2004).

The theory has been proposed (Pounds *et al.*, 2006) that climate change together with cloud cover has been interdependently acting in favour of the growth of infectious chytrid fungus. At Monteverde and other intermediate elevation areas of Central and South American highlands, where the most *Atelopus* extinctions have occurred (Wake & Vredenburg, 2008), the statement has been made (Pounds *et al.*, 2006) that climate change is shifting temperatures closer to the expected optimal conditions for *Batrachochytrium dendrobatidis.* Although it might be controversial and not yet tested, it has been hypothesized that climate change has altered temperatures in such a manner that night-time temperatures are fluctuating closer to the thermal optimum for the pathogen, while increased day-time cloudiness prevents frogs from finding thermal refuges from *B. dendrobatidis* (Pounds *et al.*, 2006 & Wake and Vredenburg, 2008 & Blaustein *et al.*, 2011).

2.5 Infectious disease: Batrachochytrium dendrobatidis

In 1994 (Pechmann & Wilbur, 1994) a statement was made that the general biodiversity crises that amphibians face are possibly the most serious problem that the world faces. Pechmann and Wilbur (1994) compared amphibian declines to anthropogenic activity, however, enigmatic (Stuart *et al.*, 2004) population declines were found that were not attributed to any human activities indicating that something other than anthropogenic activity was responsible for declining amphibian declines. If the same literature review has been conducted a mere two to three years later, it would have been evident that *Batrachochytrium dendrobatidis* (*Bd*) was the leading cause for these seemingly mysterious amphibian declines.

Berger *et al.* (1998) and Longcore *et al.* (1999) elaborate on the mass mortality event that occurred in wild and captive frogs from North and Central America as well as Australia. A pure isolate of chytridiomycete was cultured from a captive blue poison dart frog that died at the National Zoological Park in Washington, DC (Longcore & Pessier, 1999). The autopsy on these frogs, together with histological examination revealed a fungal infection of the skin. While previously known as important decomposers of non-living organic material such as pollen and rotting vegetation is *B. dendrobatidis* (*Bd*) *now* the first described species of chytrid fungi to attack living vertebrate hosts (Kolby & Daszak, 2016).

Bd has been detected in over 500 amphibian species worldwide, where approximately half of the species are experiencing population declines (Skerratt *et al.*, 2007; Hyatt *et al.*, 2007) and linked to various species extinctions (Hyatt, *et al.* 2007; Burrowes *et al.*, 2004). In South Africa, 29% of frog species are listed as threatened (Tarrant *et al.*, 2013). These taxa are distributed throughout five provinces, namely Western Cape, KwaZulu-Natal, Eastern Cape, Northern Cape, and Limpopo. *Bd* is known to be the driver of the extinction of up to 40% of species in

targeted communities (Farrer *et al.*, 2011). It has spread rapidly through diverse environments while, recent phylogenetic analysis of the global *Bd* population indicated South-East Asia is likely the source of *Bd* (O'Hanlon *et al.*, 2018).

Regarding symptoms, metamorphs and adults may experience clinical signs including thickening of the outermost skin layer, reddening, ulcerations as well as excessive shedding of the skin, lethargy, and anorexia (Berger *et al.,* 1998, Hettyey *et al.*, 2019). Furthermore, it was found that breathing and osmoregulation can be impaired by chytridiomycosis (Hettyey *et al.*, 2019). Chytridiomycosis can also facilitate co-infection and induce immunopathology which can ultimately lead to cardiac arrest (Hettyey *et al.*, 2019; Voyles *et al.*, 2009). Tadpoles are known to become infected, but they do not develop or die from chytridiomycosis (Berger, *et al.,* 1998), and a study in Queensland found that tadpoles, infected with *Bd,* are often still present even months after juveniles and adult frogs have already disappeared (Marantelli *et al.*, 2004). In free-living *Rana Muscosa* tadpoles in California it was found that although the tadpoles had large fat bodies that appeared to be healthy, the infected tadpoles had oral disk abnormalities including, damaged and missing tooth rows as well as a loss of pigment and flattening of epithelial cells (Marantelli, *et al.*, 2004).

2.5.1 Life cycle of Batrachochytrium dendrobatidis

Batrachochytrium is placed in the order Rhizophydiales because it has a chitin-walled zoosporangium (Berger *et al.*, 2016). The life cycle of *Bd* begins when an aquatic uniflagellate zoospore, the unwalled, waterborne, motile, flagellated stage is released from a mature zoosporangium (Berger, *et al.,* 2005 & Kolby and Daszak, 2016 & Berger, *et al.,* 2016). *Bd* zoospores (Figure 2-3 A) are known to be spherical but can be prolonged and amoeboid when they are first released (Berger *et al.*, 2005). A membrane surrounds the zoospores, they are motile but spread mainly through water via passive movement (Berger, *et al.,* 2016). Upon contact with amphibian skin the zoospores burrow several layers into the skin where keratin is produced (Kolby & Daszak, 2016), the zoospores encyst on the skin and infect deeper cells through a germ tube (Berger, *et al.,* 2016). After the zoospore has encysted, fine branching rhizoids (Figure 2-3 A) grow from one or more areas, the young sporangia are now known as a germling (Berger, *et al.,* 2005). The rhizoids also increase the surface area for enzyme excretion and nutrient absorption (Berger, *et al.,* 2016). With the growth of the thalli, the cytoplasm becomes more complex and multinucleated through mitosis (Berger, *et al.,* 2005). The entire contents of the thalli cleave and mature to form rounded flagellated zoospores (Berger, *et al.,* 2005). In many chytrids it is observed that the zoosporangia may not release the zoospores in unfavourable environmental conditions such as too dry conditions. Berger,

et al. (2005) explains that *Bd* may also retain from zoospore release until the sufficient moisture levels are met.

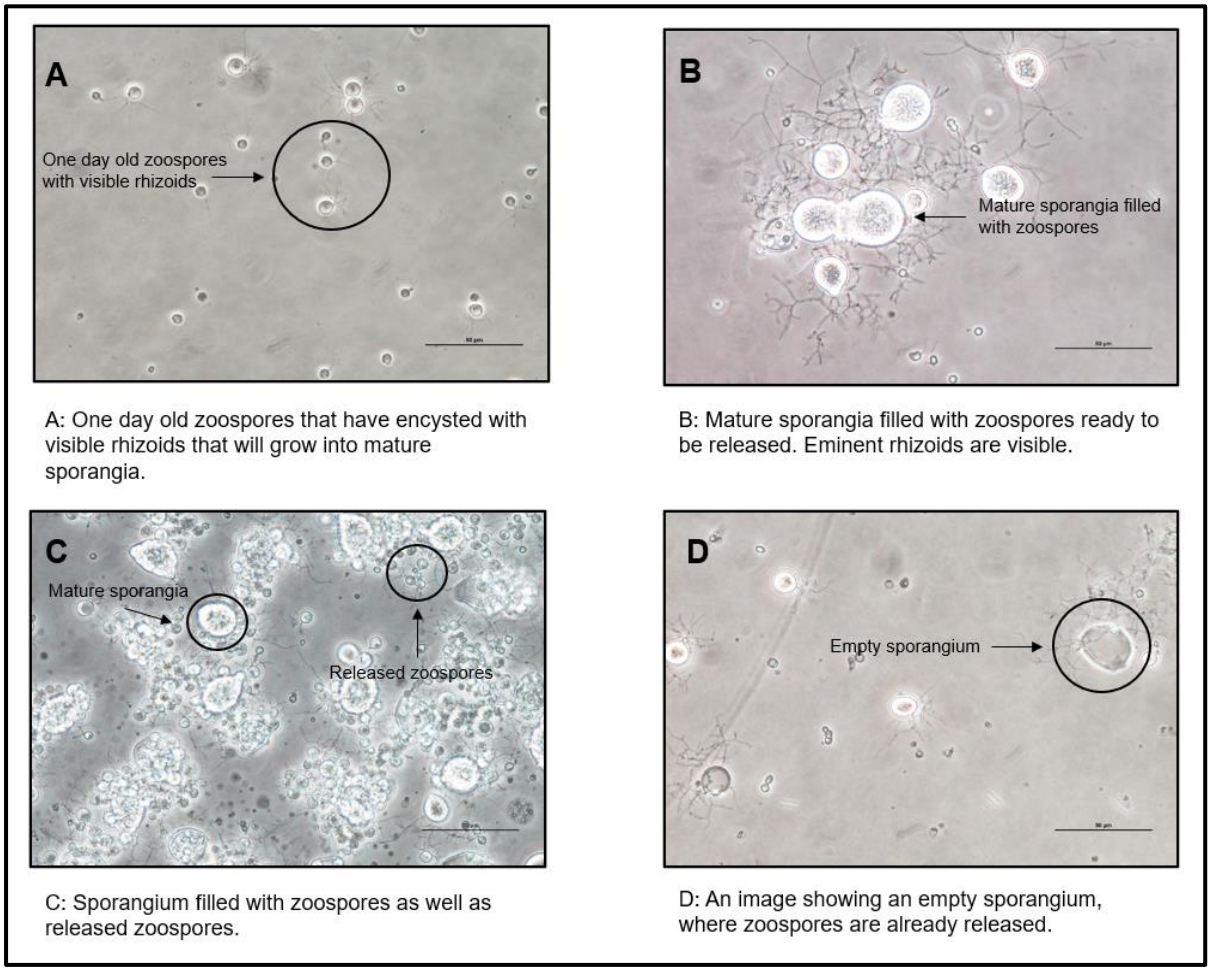


Figure 2-3: Inverted microscope images of *Bd*, indicating the various stages of the life cycle.

At optimum temperature and nutrient conditions, the growth cycle for zoospores to mature zoosporangia takes approximately five days (Kolby & Daszak, 2016). If the infected amphibian host occurs in a terrestrial location the released zoospores are likely to cause reinfection in the host, and/or the zoospores can be shed onto surrounding vegetation or into the soil (Kolby & Daszak, 2016).

2.6 Amphibian microbiome

A diverse suite of bacterial communities, collectively known as a microbiome are inhabiting a variety of multicellular organisms. An array of studies, across multiple taxa, have indicated that these bacterial communities perform various beneficial functions for their hosts, including protection from infectious pathogens (Griffiths, *et al.*, 2018). The amphibian skin microbiome is rapidly becoming an established model system to understand the multilateral relationship that exists between microbiome and pathogens (Harrison *et al.*, 2019).

Amphibians display a substantial amount of variation towards vulnerability to infection and susceptibility to lethal symptoms (Rubio *et al.*, 2018). A study done by Harris *et al.* (2006) explored the possibilities as to why some amphibian species exhibit lethal symptoms while others don't. Several of these possibilities of variation towards susceptibility of lethal symptoms include, variation in pathogenicity of *Bd,* the ability of amphibians to secrete antimicrobial peptides as well as factors such as skin shedding rate, life history, and behaviour (Harris *et al.*, 2006). It has been found that some species of amphibians can prevent disease caused by *Bd* through a mutualistic relationship with communities of cutaneous antifungal bacteria (Harris *et al.*, 2006; Bletz *et al.*, 2017; Rubio *et al.*, 2018). The pathogenicity of chytridiomycosis can be altered via a combination of the cutaneous bacteria, their metabolites as well as compounds that are secreted by amphibian skin (Woodhams *et al.*, 2007; Harris *et al.*, 2009; Rubio *et al.*, 2018).

An abundance of cutaneous bacteria, which are likely part of amphibians' innate immune system, has been identified. It has also been proposed that community interactions between amphibian skin microbes and *Bd* can be manipulated to enhance disease resistance caused by *Bd* and ultimately reduce the impact of chytridiomycosis (Harris *et al.*, 2006; Woodhams *et al.*, 2007; Harris *et al.*, 2009). A betaproteobacterium, *Janthinobacterium lividum* was one of the first identified protective microbes and has been isolated from various species of frogs (Harris *et al.*, 2006; Harris *et al.*, 2009; Lam *et al.*, 2010) as well as salamanders (Brucker *et al.*, 2008). Not only has this betaproteobacterium been isolated from amphibians, but it has also been isolated from a variety of environmental habitats including Antarctic soil, stream water sampled in Pennsylvania, and soil and water bodies from Italy (Muletz *et al.*, 2012). It has been found that *J. lividum* produces antifungal and anti-*Bd* metabolites namely violacein and indole-3-carboxyaldehyde (Brucker *et al.*, 2008, Harris *et al.*, 2009; Muletz *et al.*, 2012). A study revealed that adding *J. lividum* to the skins of a population of *Rana muscosa,* sampled in the Sierra Nevada mountains, resulted in the reduction of morbidity and mortality that are associated with chytridiomycosis (Harris *et al.*, 2009). Apart from *J. lividum* recent studies have found an immense range of genera inhibiting various lineages of *Bd,* these inhibitory genera include *Acinetobacter, Chryseobacterium, Serratia, Enterobacter, Staphylococcus,* and *Stenotrohomonas* (Antwis & Harrison, 2018; Harrison *et al.*, 2020).

Water frogs (*Telamatobius),* native to the Andean highlands of South America, are extremely vulnerable to chytridiomycosis and exhibit pathological conditions and rapidly die after displaying clinical symptoms (Rubio *et al.*, 2018). Rubio and co-workers (2018) explored the presence and dispersal of *J. lividum* in the high-Andean grassland streams with existent, but at the time Vulnerable and Data Deficient species namely, *Telamatobius marmoratus* and *T.*

intermedius, respectively. Not only did the study involve the prevalence of *J. lividum,* but also information regarding the relationship between *J. lividum* and *Bd* prevalence. The study concluded that *J. lividum*-positive frogs had 10-fold fewer *Bd* zoospores on their skin than *J. lividum*-negative frogs (Rubio *et al.*, 2018). Due to the metabolites, (Brucker *et al.*, 2008; Harris *et al.*, 2009; Muletz *et al.*, 2012) produced by *J. lividum,* either through natural augmentation or through probiotic baths the infection load was reduced as well as the morbidity and mortality normally associated with chytridiomycosis (Rubio *et al.*, 2018). As a further result, the study found that *J. lividum* is widespread in the study area contrary to results of previous studies (Burkart *et al.*, 2017; Catenazzi *et al.*, 2018) that found an extreme rarity of *J. lividum* in lower elevation cloud forests (Rubio *et al.*, 2018). However, the presence of *J. lividum* confirmed that these bacteria can grow at low temperatures (Rubio *et al.*, 2018). The use of *J. lividum* as a probiotic has been extensively researched due to its diverse presence on various amphibian species and environments (Bletz, *et al.*, 2013). The presence of an anti-*Bd* bacterium on various amphibian species will ultimately determine its suitability as a probiotic to establish community treatment (Bletz, *et al.*, 2013). *Pseudomonas* is another genus that has been commonly found on amphibians that also consists over the ability to inhibit the growth of *Bd* that can potentially be used as a community probiotic (Bletz, *et al.*, 2013). It is important for a bacterium to not only inhibit *Bd* growth but also to be widely distributed over amphibian communities to be considered a good probiotic for community treatment (Bletz, *et al.*, 2013). Therefore, it is of highest priority to explore the possibility of other bacterial species to inhibit *Bd* growth to ultimately treat amphibians with a universal probiotic.

Strong inter-individual variation in microbiome structure has been established and the consequences of these variations have remained largely understudied. Griffiths and colleagues (2018) gave insight into the possible causes of variation of microbiome structure between individuals and explained that the variation in presence and persistence of protective microbes may be influenced by host genotype both within and among populations (Griffiths *et al.*, 2018). For example, polymorphism of the major histocompatibility complex (MHC) Class IIb gene, co-varies with the gut microbiome in vertebrates (Bolnick *et al.*, 2014). The antimicrobial peptides expressed on frog skin, and governed by the innate immune response, might be a driving factor in the structural changes of the microbiome. Furthermore, the genetic structure of a population could also yield differences in the microbiome at the landscape level if it is representative of the non-random distribution of functional genetic variation at immune loci (Griffiths *et al.*, 2018). Across large geographic scales, a shift in the environmental reservoir of bacteria can also cause variation in the relative abundances of bacteria that can colonize their host (Griffiths *et al.*, 2018). When looking within populations, variation in life-

history traits such as age, there might be a factor that modifies the ability of bacteria to coexist on the skin, or favour differential selection by the host from the environment (Griffiths *et al.*, 2018). A study in the Drakensberg Mountains of South Africa on a disease-challenged high-altitude amphibian species, *Amietia hymenopus,* aimed to investigate these driving factors of the microbiome (Griffiths *et al.*, 2018). It was found that the *A. hymenopus* populations had a relatively low *Bd* infection rate and that the population structure was largely influenced by the river network as well as geographic distance. Furthermore, results indicate the shared and unique sequence variants found at the sample locations (Griffiths *et al.*, 2018).

The authors concluded that variation in these genes could be an influencing factor in the composition of microbiomes by controlling the horizontal transmission of microbes from the environment (Griffiths *et al.*, 2018). In the sampled species, *A. hymenopus,* it is most likely that the microbiome of the tadpole mouthparts is transmitted from the environment during feeding or behaviourally selecting areas in the river. It is mentioned that behaviours can also influence symbiont transmission and that there might be a link between genetic variation and microbe acquisition (Griffiths *et al.*, 2018). Site-level environmental differences might have more influence in the structuring of the tadpole microbiome than the host genotype because host genotype does not extend to landscape-level microbiome structures and simply exerts some selection pressure or behavioural influence on horizontal transmission (Griffiths *et al.*, 2018).

To maintain a long-term symbiotic relationship between host and microbe, symbiosis relies on a successful means of transmission or acquisition from each generation to the next (Walke *et al.*, 2014). Across species the means of obtaining symbiosis is predominantly through parent to offspring via vertical transmission, however, another researched method of transmission may be to obtain the symbiont from the environment (Walke *et al.*, 2014). A major disadvantage of vertical transmission of microbiome (from parent to offspring) is a reduction in genome size as a result of gene loss and the absence of horizontal gene transfer (Walke *et al.*, 2014). Genome reduction decreases the evolution of symbiotic bacteria limiting the function of the symbiont and the evolution of the symbiosis. While vertical transmission can result in gene loss, transmission of bacteria through the environment often exhibit larger, more expanded genomes.

2.7 Environmental bacteria

Harrison *et al.* (2019) explain that various studies have pursued to measure the impact of pathogenetic infection on host-microbiome structure, but all these studies have assumed that the magnitude of disruption is uniform for all infected hosts and not regulated by their initial state. In addition, there exists strong evidence that host-mediated microbial communities are influenced by abiotic conditions including microbial diversity of the habitat that surrounds the host (Harrison *et al.*, 2019). Therefore, the strength of interactions between the host microbiome and invading pathogens may be modified through spatial or temporal variation in microbiome diversity mediated by the environment (Harrison *et al.*, 2019).

A study was conducted on European common frogs (*Rana temporaria)* to test the influence of the complexity of the environmental bacterial reservoir on host-microbiome diversity and to further test when a host-microbiome is exposed to a pathogen if the magnitude of the disruption to the microbiome is dependent of the microbiome diversity (Harrison *et al.*, 2019). The study was performed by exposing the microbiome of European common frogs to *Ranavirus* (Harrison *et al.*, 2019). The results of this study concluded that the structure and diversity of the amphibian skin microbiome are dependent on the bacterial diversity present in the environment. These results support previous studies that showed that the environment acts as a standing reservoir of microbes for host colonization and is, therefore, a driving factor of host-microbiome diversity across a range of taxa (Harrison *et al.*, 2019). However, the study has found Operation Taxonomic Units (OTU's) on the amphibian skin that was undetectable in the environment, especially more complex environments, however, these results may suggest that these bacteria may be present in a low abundance that would make them difficult to detect (Harrison *et al.*, 2019). The presence of these potentially rare OTU's on amphibian skin suggests that host-microbiome structure is not simply a direct function of random colonization from the environment but that there is a selection by the host of rare OTU's (Harrison *et al.*, 2019).

A study to explore bacterial community structure on the skin of cohabiting amphibian species found an overlap (Figure 2-5) of bacterial communities on amphibian skin and their surrounding pond environment, namely water and substrate (Walke *et al.*, 2014). However, the results suggest that opposed to skin being colonised by microbes in the environment, amphibian skin rather harbours microbes in low abundance in the environment (Walke *et al.*, 2014). In conclusion to the study, it was found that both host factors and symbiont are important determinants of microbiome found on amphibian skin, however, amphibian hosts likely have a strong influence on the composition of microbes inhabiting the skin (Walke *et al.*, 2014). A long-term study system in the Pyrenees of France and Spain where *Bd* infections

have been monitored continuously, the quantification and sequencing of bacterial communities of amphibian skin indicated a similar overlap of OTU's where 7.8% of OTU's are common to all types of samples, namely larvae, metamorphs and environmental samples (Bates *et al.*, 2018).

Bioaugmentation can be defined as the process where locally occurring populations of antifungal bacteria on different hosts are increased (Rebollar *et al.*, 2016). The concept of bioaugmentation dates back to the 1800s where it was originally used in agriculture, however, today it is implemented to improve crop yields through the promotion of plant-growth and plant-protective microorganisms (Muletz *et al.*, 2012). In recent decades the benefit of native bacteria in metazoans to prevent disease has been gradually reported resulting in the suggestion that bioaugmentation can be used as preventative measures against disease in metazoans (Muletz *et al.*, 2012). The application of bioaugmentation has been proposed in fish, poultry, and corals and recently this concept has been investigated in the prevention of chytridiomycosis in amphibians (Muletz *et al.*, 2012).

A laboratory-based experiment pursued the notion of bioaugmentation to determine whether *J. lividum* can be introduced into environmental soil and whether this form of transmission will have the same beneficial effect as bacterial baths (Muletz *et al.*, 2012). It was argued that optimum conditions will be created through the interaction of moist, nutrient-rich amphibian skin with bacterially rich soil and that this will likely provide ample opportunities for bacterial transmission to amphibian skin (Muletz *et al.*, 2012). This experiment proved to be successful in introducing *J. lividum* to soil and transmitting *J. lividum* from soil to *Plethodon cinereus* however, it was mentioned that little information regarding ecological processes of amphibian skin microbial communities is known, specifically how anthropogenic activities can affect these processes (Muletz *et al.*, 2012). It has been found that when soil ambient temperatures are increased that the structure and function of soil microbial communities are altered, furthermore, temperature variation may also compromise amphibian skin microbial communities (Muletz *et al.*, 2012). Therefore, drivers such as climate change may alter the susceptibility of amphibians to *Bd* leading to an increase of chytridiomycosis in the affected environments (Muletz *et al.*, 2012; Muletz-Wolz *et al.*, 2017).

Another option for the establishment of microbial communities on amphibian skin is through indirect horizontal transmission, defined as the transmission of bacteria from a host to the environment and then to another host (Rebollar *et al.*, 2016). Through the use of indirect horizontal transmission, the colonization of a probiotic bacterium can be established in a high-risk amphibian population by inoculating only a few individuals (Rebollar *et al.*, 2016). It is argued that indirect horizontal transmission will be successfully attained when the larval stage

is inoculated since larval skin communities have a lower density and the larval host immune system has not yet developed fully (Rebollar *et al.*, 2016). However, it is hypothesized that optimum transmission of the probiotic will be achieved if both direct and indirect horizontal transmission could be possible, therefore, Rebollar, *et al.* (2016) designed an experiment where tadpoles were exposed to both treatments with direct and indirect horizontal transmission and only indirect horizontal transmission. It was found that transmission of *J. lividum* from inoculated to uninoculated tadpoles were achieved in both treatments, however, the treatment proved to be more effective when tadpoles were separated and not allowed to have direct contact (Rebollar *et al.*, 2016). Furthermore, it was concluded that inoculating tadpole skin with *J. lividum* did not significantly alter the bacterial diversity of the tadpoles and that contrary to expectations, a combination of direct and indirect horizontal transmission proved to be a less effective method of transmission (Rebollar *et al.*, 2016).

To make use of probiotics by exposing the host to microbes with known protective properties are considered a useful tool to protect amphibians from infectious diseases, and to apply antifungal bacteria as a preventative measure against *Bd* has been proposed and researched by various scientists (Muletz *et al.*, 2012; Bletz *et al.*, 2017). However, it is difficult to predict the impacts that amphibian skin microbes have on the fungal disease, seeing that it is unknown how microbiome functions will be affected by ecological and environmental factors (Muletz-Wolz *et al.*, 2017). Furthermore, distinct lineages of a pathogen can exhibit different susceptibilities to the exact same bacterial assemblage (Antwis *et al.*, 2015; Antwis & Weldon, 2017; Muletz-Wolz *et al.*, 2017; Antwis & Harrison, 2018; Harrison *et al.*, 2020). It has been proven that bacterial inhibition varies between lineages of *Bd* and can inhibit one lineage while augmentation is observed in another lineage (e.g. Antwis & Harrison, 2018). Findings of a previous study suggested that bacteria identified *in vitro* as *Bd*-inhibitory did not consistently display the same effectiveness against *Bd in vivo* (Muletz-Wolz *et al.*, 2017). Temperature is another factor that should be taken into consideration when formulating a probiotic, seeing that previous results concluded that bacteria consistently inhibiting the growth of *B. salamandrivorans* did not present the same consisted inhibition in the growth of *B. dendrobatidis* (Muletz-Wolz *et al.*, 2017).

This concludes that the application of bacterial probiotics in an attempt to mitigate *Bd* is complex and can be modified by a range of host, pathogen, and environmental factors. Therefore, it is imperative to do extensive research to better understand the complex relationship between bacteria and pathogen but also the relationship between amphibians and bacteria.

CHAPTER 3 MATERIALS AND METHODS

3.1 Study sites and species

Ukutula Lodge and Conservation Centre, situated in the Brits district of the North West Province, is a world-class research and conservation facility that specializes in genetic mammal research and predator conservation that also includes a Genetic Bio-bank. The Ukutula reserve consists of unspoiled bushveld, with a variety of antelope, giraffe, zebra and more than 130 bird species. The Brits district is located at 1127 meters above sea level (m.a.s.l.) and has an average annual temperature of 18°C and annual precipitation of 620 mm (Climate-data.org). The Brits district has a summer rainfall season with the greatest amount of precipitation occurring during January when this district experiences an average of 110 mm rainfall. Considering that in January the Brits districts experience their highest rainfall and seeing that this coincides with the mating season of amphibians (Du Preez & Carruthers, 2017), it was decided to conduct fieldwork in January 2019.

Subsequently, three sites were chosen on the Ukutula reserve with the following coordinates site 1 S 25.51850° E 027.66153°, site 2 S 25.51777° E 27.66437° and site 3 S 25.52014° E027.67355°, these sites were chosen based on the appearance of the water body (e.g. flowing water or stationary water) and the presence of frogs or signs thereof, such as foam nests of Southern foam nest frogs (*Chyromantis xerampelina*).

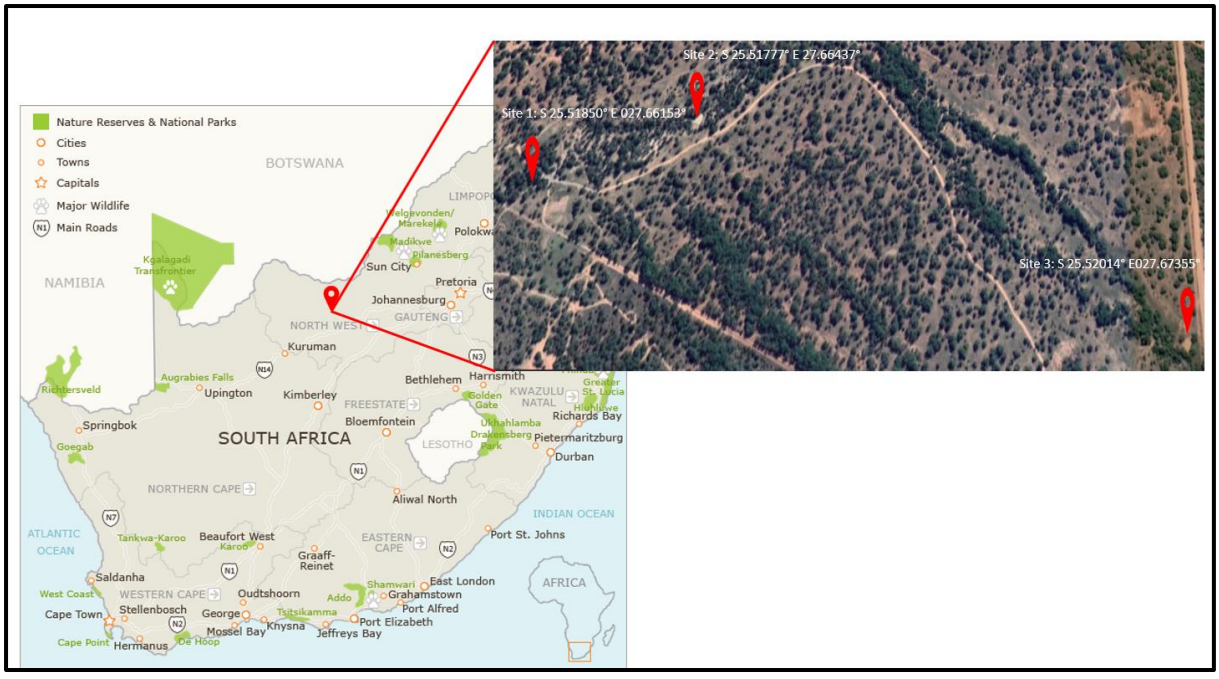


Figure 3-1: Map indicating various sites used for the study.

3.2 Amphibian bacteria collection

3.2.1 Amphibian sampling

Dipnetting was used to collect tadpoles from the selected sites. The method prevents biased sampling towards any particular species and the samples represent a fair indication of the species present at the time of sampling. Tadpoles were placed in clear sterile plastic bags containing water from the respective sampling sites (Antwis & Weldon, 2017). A diversity of tadpole species was required to establish a framework of bacteria colonizing the amphibian community. Adult frogs were collected during active night sampling. Disposable gloves were changed between each collected individual, and frogs were placed in separate plastic bags containing same pond water.

3.2.2 Euthanizing tadpoles

Ethical consideration

Approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) has been received for a Category 1 study under the following ethics nr: NWU-00059-19-A5.

The tadpoles were euthanized through submersion in a Tricaine methanesulfonate (MS-222) solution, following the national guidelines for amphibian euthanasia (SANS 2008). During the process of tadpole euthanizing, the tadpoles were placed in a buffered solution of MS-222 at a concentration of 200 mg/l. Tadpoles were immersed in the MS-222 solution until death could be confirmed as noted in the SANS guidelines. After the death of each sampled individual tadpole was confirmed the mouth part of each tadpole was swabbed as described in subsection 3.2.3.

3.2.3 Bacteria collection

Bacteria were collected by swabbing the mouthparts of tadpoles and the skin of frogs. A sterile MW100 DrySwab (Medical Wire Equipment, United Kingdom) was used to gently swab the keratodonts, rostrodonts, and oral cavity of tadpoles, or the dorsal and ventral skin of frogs (Kadekaru *et al.*, 2016; Kadekaru & Une, 2018; Harrison *et al.*, 2019). Each cotton swab sample was placed in a sterile microtube and stored at -20°C until molecular analyses were conducted at the Institute of Zoology, London.

3.3 Environmental bacteria collection

Since the majority of tadpole species were collected at site 1, it was decided to collect environmental samples at site 1 to compare the bacteria found in the environment to the bacteria found in amphibians. Water sampling was conducted at site 1 approximately 2 m from the shoreline where careful consideration was taken to sample only from the photic zone and not to disturb the benthic zone of the lentic sampling site (Willey *et al.,* 2014). Ten replicate water samples were collected in 100 ml sterile Schott bottles and stored at 4°C (Jiang *et al.*, 2006) for 2 days until bacteria culturing could begin.

3.4 Bacterial analysis

3.4.1 Amphibian bacterial community analysis

The molecular analysis of the tadpole mouthpart bacteria was conducted through collaboration with the Institute of Zoology, London. The following summary explains the method that was used to obtain identifications of bacteria from amphibian swabs.

Extraction of DNA from tadpole mouthpart swabs was conducted using the Qiagen DNeasy PowerSoil HTP 96 Kit, following the standard protocol. For 16S sequencing library preparation followed the methods outlined in Harrison *et al*. (2019) using the indexed primer sets from Kozich *et al*. (2013). Briefly, single PCR amplification was used to amplify the 16S v4 region as well as add indexes and Illumina compatible adaptor sequence. PCR's were conducted in triplicate and visualized on an agarose gel before being pooled. Pooled samples were cleaned using Ampure XP beads following the protocol for standard PCR cleanup.

Libraries were quantified by running them on an Illumina MiSeq Nano reagent kit (Harrison *et al*. 2019), and index representation from this run was used to prepare an equimolar sample pool. Finally, sequence data were generated using an Illumina MiSeq v2 500 cycle reagent kit (Harrison *et al*. 2019).

Polymerase chain reactions (PCR's) were conducted through the use of indexed primer sets to amplify the v4 section of the 16S rRNA gene (Kozich *et al.*, 2013). Three 10 µl replicates were set up for each sample using 5× HOT FIREPol® Blend Master Mix (Solis Biodyne).

For the PCR setup 6 µl master mix, 3 µl of 2 µM primers and 1 µl gDNA was added to each well of the PCR plate and a PCR was run at the following conditions: 15 minutes at 95°C and then 28 cycles under the following, 20 seconds at 95°C, 1 minute at 50°C and 1 minute at 72°C, the qPCR was finished with 10 minutes at 72°C and held at 4°C

PCR products were visualised on agarose gels to detect and remove possible failed reactions.

To remove any PCR master mix contaminants a clean-up method using Ampure XP beads and 70% Ethanol was used, and sequencing was performed using 250 bp paired-end reads on Illumina MiSeq using a v2 chemistry 500 cycle cartridge (Harrison *et al.*, 2019). The raw 16S data was processed in the DADA2 pipeline, form R, and *phyloseq* was used for downstream sequence processing (Griffiths *et al.*, 2018 & Harrison *et al.*, 2019). The amplicon sequence variants (ASV's), present in the no-template controls, were removed. To focus on the difference in high abundance ASVs and to remove any bias introduced by small differences and low-abundance reads ASVs were removed in datasets with fewer than 100 reads (Harrison *et al.*, 2019).

3.4.2 Environmental bacteria analysis

3.4.2.1 Bacteria culturing

Environmental samples were diluted 10^{-3} times and spread on R2A media plates using standard methods described by Sanders (2012) . Briefly, 100 µl of diluted sample was transferred with a sterile micropipette to an agar plate, the sample was gently spread across the surface of the agar using a metal spreader sterilised with 70% ethanol drained and ignited with a Bunsen burner (Sanders, 2012). Each agar plate containing individual samples were then sealed with Parafilm and incubated inverted at 37°C for 4 to 5 days or until morphologically distinct bacterial colonies were observed (Bell *et al.*, 2013). R2A media is considered a low nutrient medium specifically intended for culturing bacteria from potable water, but it is also commonly used for the culturing of amphibian-associated bacteria (Walke *et al.*, 2015). After morphologically distinct colonies were observed through a dissection microscope the streak plate method was used to select and isolate individual bacterial colonies (Sanders, 2012). A sterile inoculation loop was used to pick up single bacterial colonies and streak the colonies on the agar plate in a quadrate pattern (Figure 3-2).

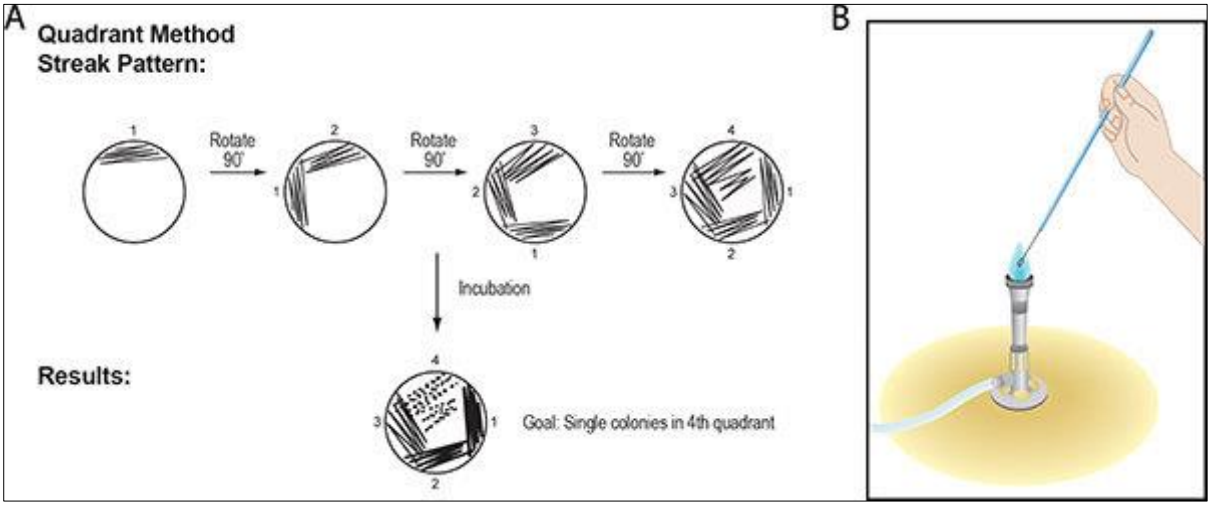


Figure 3-2: Streak plate method to obtain single axenic bacterial samples (Sanders, 2012).

The streak plate method was repeated with each sample until axenic (non-contaminated) organisms were observed (Bell *et al.*, 2013). Purity of axenic isolates were pre-determined based on morphology where clear single coloured colonies could be observed. The axenic isolates (Figure 3-3) were stored at 4°C until molecular analyses could be performed.



Figure 3-3: Examples of axenic isolates that resulted from the use of the quadrant streak plate method.

3.4.2.2 Bacterial sequencing and identification

Bacterial isolate DNA extraction

For the extraction of bacterial DNA, the Qiagen DNeasy Blood and Tissue kit was used. The following steps, with minor modifications, were used to extract the bacterial DNA.

A maximum of 5 $\times 10^6$ cultured cells was centrifuged for 3 min at 500 rpm and was then resuspended in 200µl PBS. 25µl Proteinase K was added to the resuspended cells. 200µl Buffer Al was added and mixed thoroughly through vortexing. The sample was then incubated at 60°C for 15 min. 200µl ethanol of a concentration between 96 and 100% was added and mixed thoroughly through vortexing. The mixture was then pipetted into a DNeasy Mini spin column and placed in a 2ml collection tube, which was centrifuged at 8000 rpm for 1 min, after which the flow-through in the collection tube was discarded. The spin-column was then placed in a new 2ml collection tube, 500µl Buffer AW1 was added to the spin column and centrifuged a second time at 8000 rpm for 1 min, again the flow-through in the collection tube was discarded. The spin-column was then placed in a new 2ml collection tube, then 500µl Buffer AW2 was added to the spin column and centrifuged at 14000 rpm for 3min, after which the flow-through in the collection tube was discarded. The spin-column was then transferred to a 1.5 ml microcentrifuge tube where the DNA was eluted by adding Buffer AE to the centre of the spin column membrane. The sample was then incubated for 1min at room temperature (15-25°C) and was centrifuged the last time at 8000 rpm for 1 min. The protocol was repeated for each of the isolated bacterial samples.

After DNA extraction was completed, a nanodrop analysis was performed on each sample to determine the quality and quantity of the extracted DNA (Table 2).

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using universal bacterial primers 27F (5'-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CACGGATCCTACGGGTACCTTGTTACG ACT-3') to amplify the conserved region of the 16S rRNA gene from the extracted DNA of each bacterial isolate (Antwis *et al.*, 2014; Bletz *et al.*, 2017). In a PCR tube 1µl of each of the primers were added to 2 µl DNA template, 12.5 µl DreamTaq Master Mix was added to the mixture, and lastly, 8.5µl Nuclease free H_2O was added to the PCR tube. A 16S PCR program was run using the following conditions (Table 3-2):

Table 3-1: 16S PCR conditions used during PCR protocol.

4°C	-	2 min	
95°C	-	5 min	
95°C	-	30 s	

53°C	-	1 min	× 35 cycles
72°C	-	1 min	
72°C	-	1 min	
10°C	-	HOLD	

The amplification of DNA was verified by Gel electrophoresis. A 1% electrophoresis gel was prepared from 0.7g agar and 70ml TAE buffer. 2µl GelRed loading dye was mixed with 3µl of DNA template and a total of 3µl loading dye and DNA was placed in each well of the electrophoresis gel. A GeneRuler 1kb DNA ladder (Figure 3-4) was loaded to determine the band size of each sample. The Gel electrophoresis program ran at 90V for 45 min and the gel was analysed with Bio-Rad Image Lab™ software. After analysing the quantitative and qualitative gDNA Nanodrop as well as the gel electrophoresis results a total of 20 isolates was selected based on DNA quality and quantity and was send to Inqaba Biotechnical Industries (Pty) Ltd for sequencing. After sample sequences were received, chromatograms were analysed with FinchTV software and through the use of MEGA7 software (Kumar & Hedges, 1998) using the Neighbour-Joining method (Saitou & Nei, 1987) and to infer the variability of the estimate a bootstrap value of 5000 was used (Calestani, 1985).

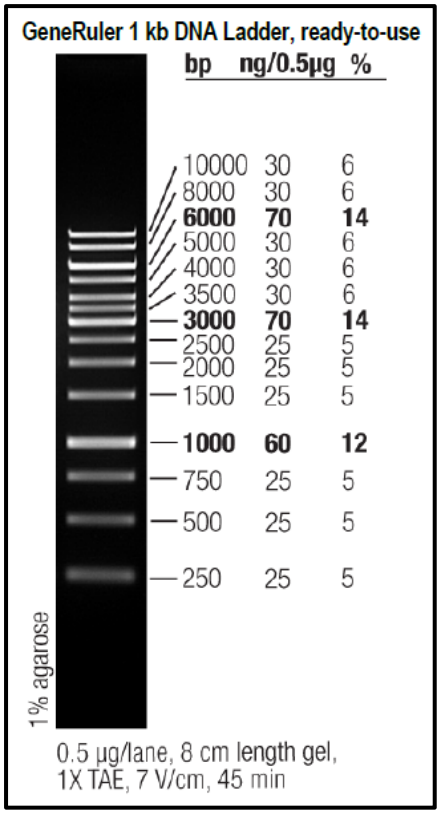


Figure 3-4: GeneRuler 1kb DNA ladder used in gel electrophoresis to determine the DNA fragment size.

3.5 *Bd* Challenge assays

3.5.1 Preparation of *Bd* cultures

Two *Bd* isolates of different lineages and with different virulence were selected from pre-isolated cultures kept in 1% Tryptone broth in sterile conditions at 4°C. The first isolate MG04, from the global hypervirulent panzootic lineage *Bd*GPL (Berger *et al.*, 2016), was isolated at Silver Mine in the Western Cape South Africa in 2008. The second isolate SA 4C, a *Bd*CAPE lineage, was isolated in 2010 in Greytown Kwazulu-Natal in South Africa. These two isolates were selected due to their known virulence with *Bd*GPL being a global panzootic lineage and *Bd*CAPE being a lineage unique to South Africa, but also because the selecting pressures that affect their virulence were not known (Farrer *et al.*, 2011). The 2ml of the *Bd* isolates were passaged in 18 ml sterile Tryptone-gelatin hydro-lysate-lactose (TGhL) broth media (8g tryptone, 1g hydrolysed gelatin, 2g lactose to 1L deionised water, autoclaved) (Bell *et al.*, 2013 & Rollins-Smith *et al.*, 2002), and incubated at 23°C for approximately 7 days until growth was confirmed. After 7 days of incubation 2ml of *Bd* culture was transferred to TGhL agar plates where they were incubated at 23°C for another 3 days (Bell *et al.*, 2013). After 3 days of incubation, the presence of motile zoospores was confirmed through a light microscope and the agar plates were flooded with 3ml sterile TGhL broth, this was done to create a suspension

of zoospores that were collected and counted on a haemocytometer and resuspended in sterile TGhL broth to a concentration of 1×10^4 ml^{-1} (Bell *et al.*, 2013).

3.5.2 Preparation of bacterial cell-free supernatants

After axenic bacterial cultures were established, a single colony of each bacterial isolate was inoculated into 1ml sterile TGhL media in a 24-well plate tissue culture microplate (TPP 92024) and incubated at 23°C for 48 hours (Bell *et al.*, 2013). Each culture was transferred to a 1.5 ml microtube a centrifuge at 9500 rpm for 5 min to form a cell pellet, the supernatant was then filtered through a sterile 0.22µm syringe filter (Acrodisc syringe filter, Pall corporation) to remove all bacterial cells and leave the bacterially produced metabolites in the TGhL media (Bell *et al.*, 2013).

3.5.3 Challenge assays

Challenge assays were set up in 96-well tissue culture microplates (TPP 92096), the experimental set up consisted of experimental wells, positive controls, negative controls, and media only wells (Bell *et al.*, 2013). The experimental wells contained 50µl of *Bd* zoospores at a concentration of 1×10^4 ml^{-1} as well as 50 µl of a bacterial cell-free supernatant sample. Positive control wells contained 50 µl *Bd* zoospores and 50µl TGhL broth while negative control wells contained 50µl heat-killed *Bd* zoospores (60°C for 60min) and 50µl TGhL broth, additional medium only control wells contained 100µl TGhL broth (Bell *et al.*, 2013). The complete microplate contained 10 replicates of experimental wells, 10 replicates of positive control wells, 12 replicates of negative control wells and 22 replicates of medium only wells (see Figure 3-5 for experimental set up). Immediately after plate set-up was completed spectrophotometer readings were taken with a 492nm filter, this was considered as the day 0 reading. The microplate was then incubated at 23°C and spectrophotometer readings taken every 24h for 10 consecutive days (until maximum growth was observed) (Bell *et al.*, 2013). To record culture appearance and to exclude any wells where bacterial or fungal contamination might occur, the microplate was inspected every day with an inverted microscope and on day 6 photos were taken with a Nikon DS-Fi3 Microscope Camera and photos were analysed with NIS- Elements D5.02.00 64.bit software.

The experimental plate set-up was repeated for both lineages of *Bd* (*Bd*GPL and *Bd*CAPE) with only one bacterial cell-free supernatant per replicate. Based on the identification of bacteria from the isolates, the challenge assay was conducted with groups/consortiums of bacterial species from various families.

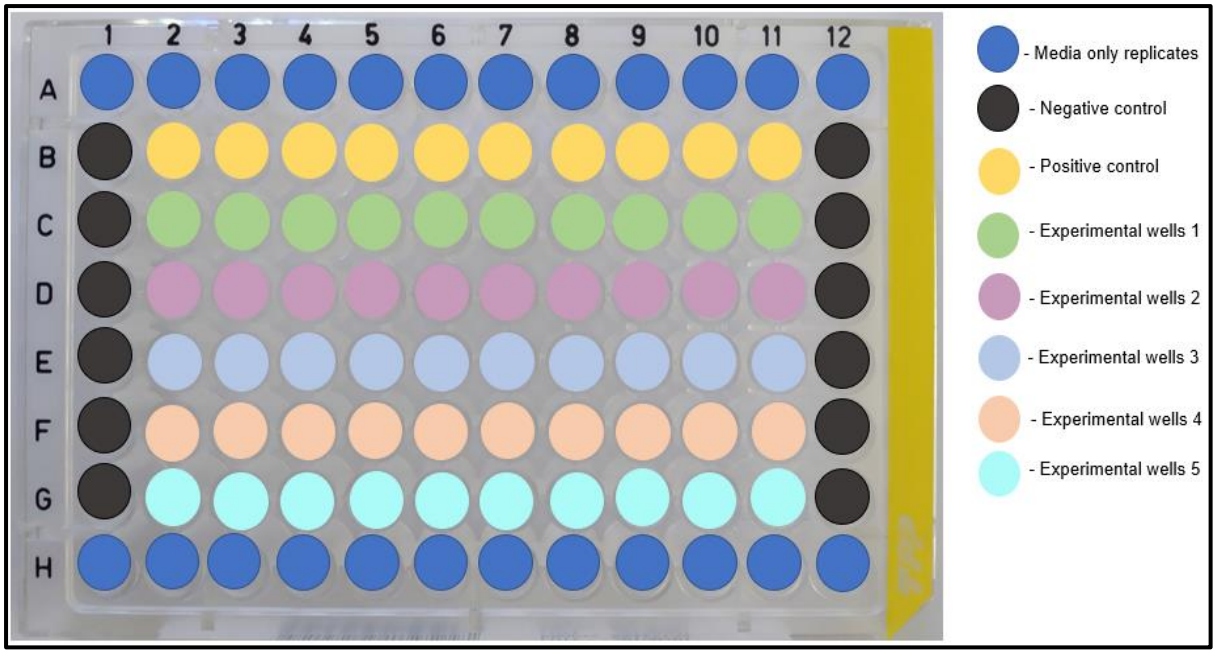


Figure 3-5: Experimental 96-well plate set-up for *Bd* challenge assays with each experimental well containing one bacterial cell-free supernatant isolate, while negative control wells contain heat-killed *Bd* cells and positive control wells contain only *Bd* with no cell-free supernatant added.

3.6 Data analysis

After spectrophotometer readings were completed a mean absorbance value of the replicates of the controls as well as the experimental wells were calculated. To remove baseline absorbance, the mean negative control absorbance for each day was subtracted from the mean sample and positive control values on that day, this was done to obtain a corrected absorbance value (Bell *et al.*, 2013). After obtaining the corrected absorbance values for each sample, column bar graphs were plotted to visualize the various impacts that the cell-free supernatants of different bacterial species have on different lineages of *Bd.*

Statistical analysis for challenge assays was done with GraphPad Prism 8. Firstly, box-and-whisker plots for each experiment was drawn to determine if data is normally distributed, distribution of data was further confirmed through a Shapiro-Wilk normality test performed on each dataset (Yap & Sim, 2011). A Kruskal-Wallis test was done to determine statistical significance between bacterial isolates and a one-way ANOVA was done to determine statistical significance between lineages of *Bd.*

16S Metagenetic Sequencing Analysis: Bioinformatic processing of raw FASTQ files from 16S rRNA metagenetic sequencing was conducted in R v4.0.3 (R Core Team, 2020) using the package *DADA2* (Callahan *et al.*, 2016) to identify Amplicon Sequence Variants (ASVs). Taxonomy of ASVs was assigned using the SILVA reference database (Callahan *et al.*, 2016).

Contaminant ASVs were identified using the R package *decontam* (Davis *et al.*, 2017) based on the prevalence of ASVs in biological samples relative to negative sequencing controls and removed from subsequent analyses. Statistical analysis and visualisation of drivers of microbial community structure were all conducted in *R* using the packages *phyloseq* (McMurdie & Holmes, 2013) and *vegan* (Oksanen *et al.*, 2020). Among-sample differences in microbial community structure were visualised using Principal Components Analysis (PCA) of centred-log-ratio (CLR) transformed raw ASV abundances. To test for differences in microbial community structure among sample types (adult, tadpole, and environmental) or among species within age classes, a PERMANOVA analysis were used with CLR-transformed matrix of ASV abundances as the response variable. PERMANOVA models were run for 999 permutations to derive p values for effects of interest (Harrison *et al.,*2019).

CHAPTER 4 RESULTS

4.1 Bacterial culturing

Bacterial culturing yielded 44 axenic bacterial isolates, gram-staining revealed whether they were gram-positive or gram-negative and the size and shape were also noted, making it possible to distinguish morphotypes (Table 4-1). The majority of isolates were gram-negative with a bacilli-like cell shape. In addition, the recorded cell shapes consisted of cocci, streptococci, and diplococci while only one isolate had a coccobacillus cell shape and two isolates were vibrios cell shaped.

Table 4-1: Axenic bacterial isolates indicating gram staining, size, and shape to distinguish morphotypes.

Isolate	Gram +/-	Shape	Size µm
W1(1)	-	Bacilli	1
W1(2)	-	Cocci	1
W1(3)	-	Bacilli	1.5
W1(4)	-	Cocci	1
W2(1)	+	Diplococci	1
W2(2)	-	Diplococci	0.5
W2(3)	-	Diplococci	1
W2(5)	-	Bacilli	2
W2(8)	-	Vibrios	2.5
W3(2)	+	Streptococci	1.5
W3(3)	-	Cocci	0.5
W3(4)	+	Bacilli	2
W3(5)	-	Streptococci	2
W3(6)	-	Bacilli	1
W3(7)	-	Bacilli	1
W3(9)	+	Streptococci	1.5
W3(11)	-	Bacilli	1
W4(1)	-	Bacilli	2.5
W4(2)	-	Bacilli	2
W4(3)	-	Bacilli	1
W4(4)	-	Streptococci	1.5
W4(7)	-	Vibrios	1
W4(8)	+	Cocci	1
W4(9)	-	Bacilli	1.5
W5(1)	-	Bacilli	1.5
W5(2)	-	Bacilli	1
W5 (4)	-	Bacilli	1.5
W6(1)	-	Bacilli	2

W6(3)	+	Cocci	1.5
W6(4)	-	Bacilli	2.5
W6(5)	-	Coccobacilli	1
W7(1)	-	Bacilli	1
W7(2)	+	Bacilli	4
W7(3)	-	Bacilli	1
W7(4)	+	Diplococci	1.5
W7(5)	+	Cocci	1
W8(1)	-	Cocci	1
W8(2)	-	Cocci	1
W9(2)	-	Bacilli	2
W9(10)	-	Bacilli	2.5
W10(1)	+	Cocci	1
W10(2)	+	Bacilli	0.5
W10(3)	-	Bacilli	4

4.2 Molecular results

4.2.1 Nanodrop

A nanodrop analysis determined the quality and quantity of the extracted DNA. Table 2 contains the results obtained through a UV spectrophotometer at 260nm where any protein or phenol contamination can be detected at an absorbance of 280nm while a 260:280 ratio between 1.75 and 2 is considered as a reasonably pure DNA sample (Dale *et al.,* 2014). Based on the quantity and quality of DNA, 20 samples were selected for further molecular analysis, including sequencing to identify the isolates. The 20 selected samples can be seen highlighted (Table 4-2). Importantly, samples with a 260:280 ratio between 1.75 and 2 as well as with a reasonable concentration of DNA (ng/µl) were selected.

Table 4-2: Nanodrop results where a 260:280 ratio between 1.75 and 2 is considered reasonably pure DNA (indicated in green highlight).

Sample	ng/µl	A260/A280	A260/A230
W1(1)	5.2	1.87	0.59
W1(2)	13.03	1.67	0.58
W1(3)	23.3	1.79	1.19
W1(4)	7.43	1.52	0.95
W2(1)	8.06	1.57	0.59
W2(2)	9.3	1.79	0.51
W2(3)	6.4	1.8	0.37
W2(5)	9.1	1.84	0.53
W2(8)	11.4	1.78	0.68
W3(3)	4.5	1.54	0.31

W3(6)	9.6	1.74	1.55
W3(7)	3.8	1.71	0.4
W3(9)	1.6	1.84	1.81
W3(11)	11.06	1.84	1.28
W4(1)	4	1.94	0.86
W4(2)	3.5	2.15	0.57
W4(3)	1.56	2.21	0.65
W4(4)	0.3	-1.5	-0.13
W4(6)	3.1	1.71	0.45
W4(9)	2.5	3.4	0.26
W5(1)	11.26	2.03	1.95
W5(2)	6.9	2.05	0.74
W6(1)	3.6	1.83	3.29
W6(3)	3.4	1.71	0.34
W6(4)	6.9	1.87	0.91
W6(5)	4.43	2	2.68
W7(1)	11.4	1.9	0.71
W7(3)	5.66	1.91	0.69
W7(4)	3.5	1.46	0.54
W7(5)	4.8	1.86	0.63
W7(8)	0.4	0.61	-0.4
W8(1)	11.3	1.63	0.74
W8(2)	4.7	1.2	0.69
W9(1)	92.4	1.36	0.57
W9(2)	18.3	1.7	0.84
W10(1)	6.5	1.27	0.76
W10(2)	12.76	1.63	0.51
W10(3)	2.7	1.17	0.26

4.2.2 Agarose gel electrophoresis

Gel electrophoresis is considered an invaluable determinant of DNA molecule size (Dale *et al.,* 2014). It uses the charge of a molecule (positive or negative) to allow molecules to migrate to the electrode of the opposite charge (Dale *et al.,* 2014). Figures 1 to 4 shows the gel electrophoresis results where each sample can be seen in a separate well and can be compared to the GeneRuler 1kb DNA ladder to determine the size of each DNA sample. After each pure DNA sample went through amplification protocols the agarose gel electrophoresis was used to confirm the quality of DNA recorded through Nanodrop (Table 4-2). In Figures 4-1 to 4-4 it can be seen that isolates with darker bands yielded purer DNA through DNA extraction, which can be confirmed by the 260:280 ratio recorded through Nanodrop (Table 4-2). From the results obtained through agarose gel electrophoresis and according to the 1kb GeneRuler, the DNA fragments are 1500bp this indicates that the gene that was amplified during PCR was indeed the 16S rRNA gene. In Figure 4-1 it can be seen that sample W3(3)

had no band showing in the agarose gel electrophoresis, this can also be seen for samples W4(3) and W4(4) in Figure 4-2. Seeing that this occurred in only three samples and no contamination was observed in the no-template control, the DNA template of these samples could have been damaged during the PCR protocol or a technical error caused a too low DNA template or primer volume being omitted to the PCR tube.

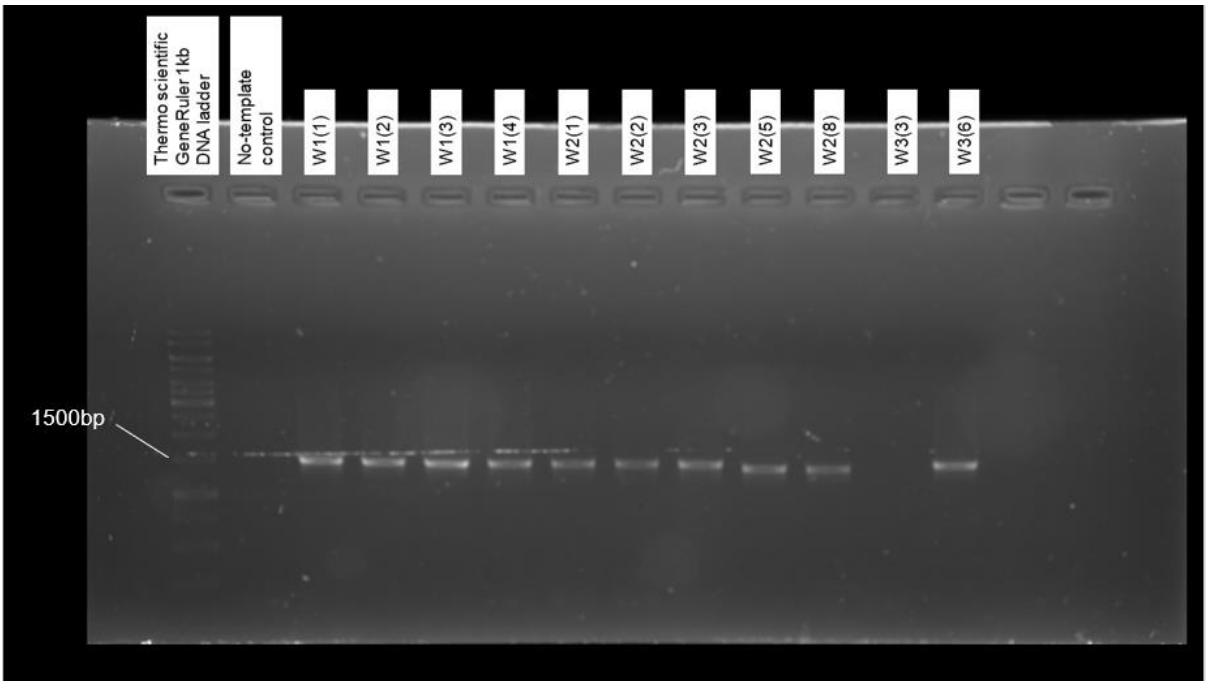


Figure 4-1: Agarose gel electrophoresis image of samples W1(1) to W3(6) recording DNA fragment size as 1500 bp.

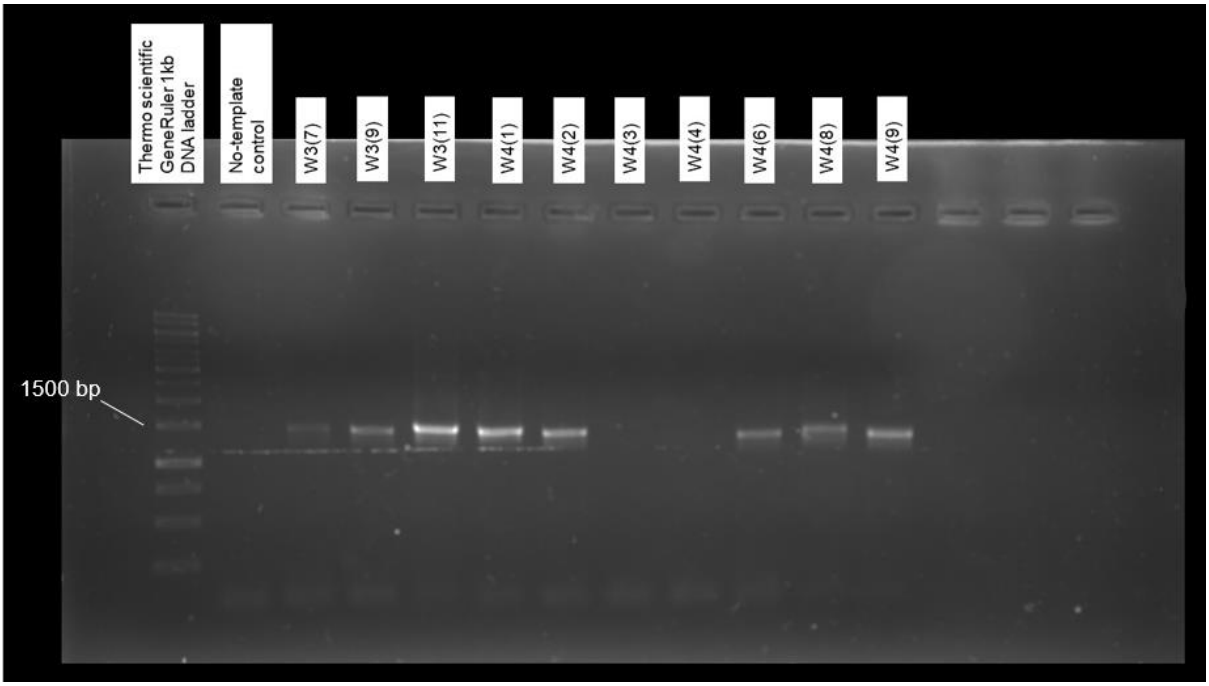


Figure 4-2: Agarose gel electrophoresis image of samples W3(7) to W4(9) recording DNA fragment size as 1500 bp.

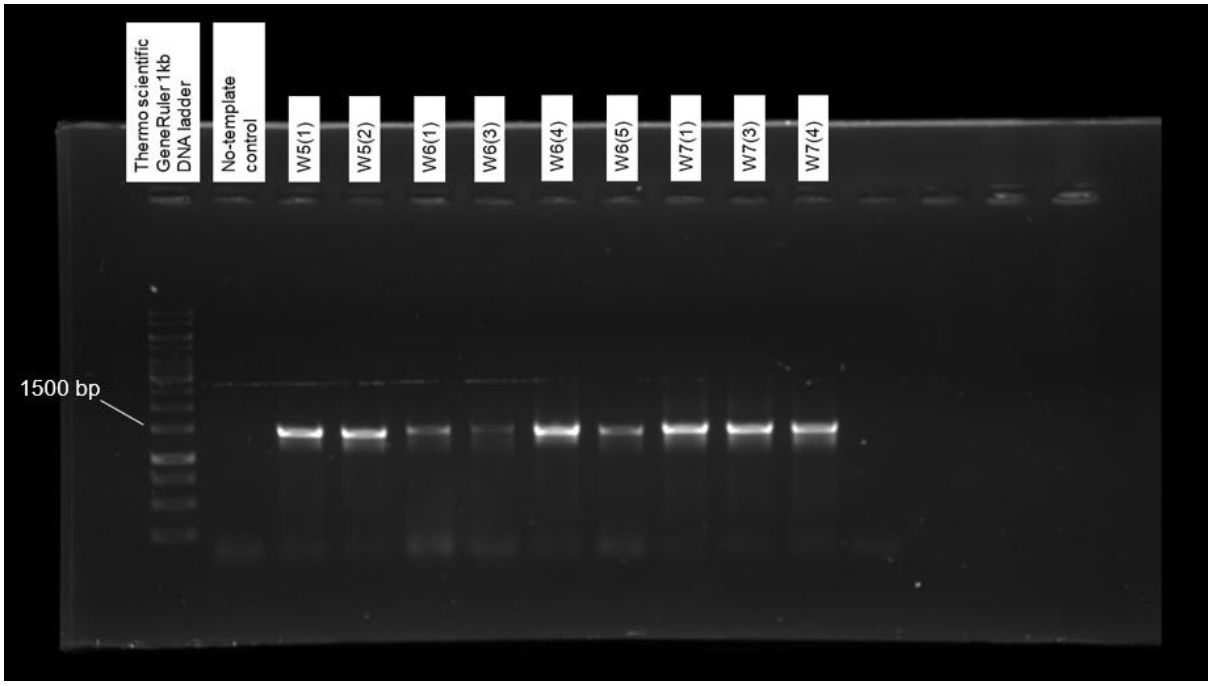


Figure 4-3: Agarose gel electrophoresis image of samples W5(1) to W7(4) recording DNA fragment size as 1500 bp.

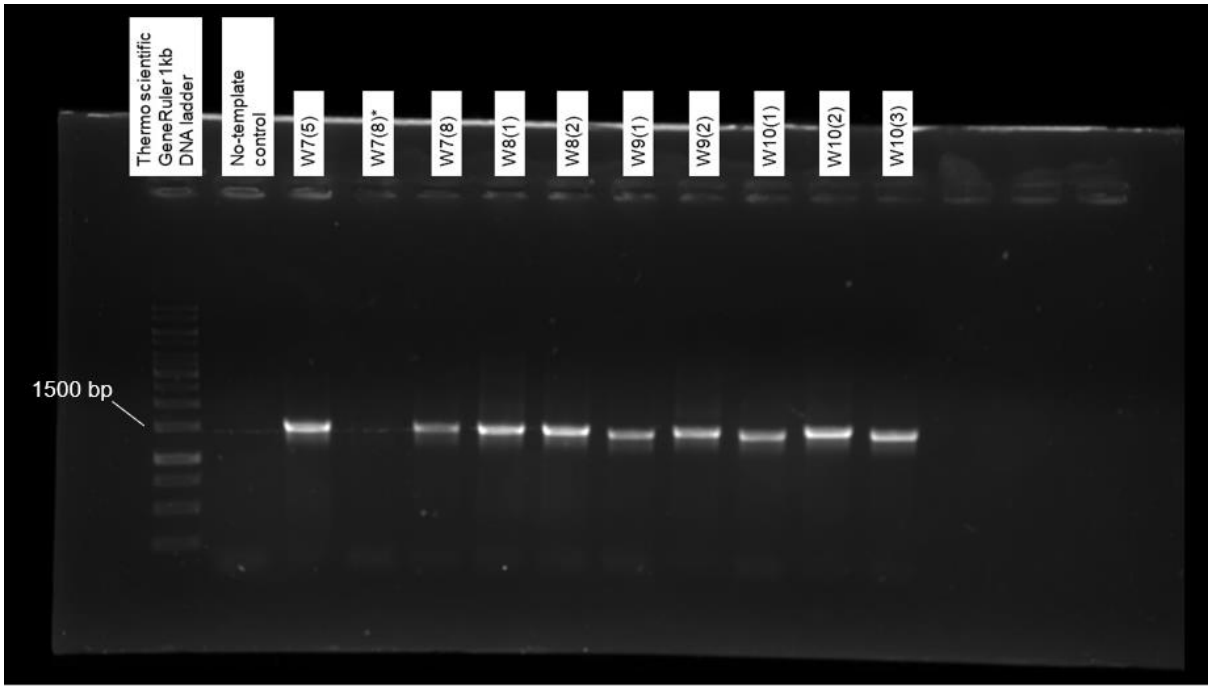


Figure 4-4: Agarose gel electrophoresis image of samples W7(5) to W10(3) recording DNA fragment size as 1500 bp.

After carefully analysing both nanodrop and gel electrophoresis results, 20 DNA samples were selected to be sequenced for identification (Table 4-3). These samples were selected based on their morphotype as well as the quality and quantity of DNA extracted from these bacterial isolates.

Table 4-3: Selected isolates based on the quality and quantity of DNA.

Sample	ng/µl	A260/A280	A260/A230	Gram stain (+/-)	Shape
W1(1)	5.2	1.87	0.59	-	Bacilli
W1(3)	23.3	1.79	1.19	-	Bacilli
W2(2)	9.3	1.79	0.51	-	Diplococci
W2(3)	6.4	1.8	0.37	-	Diplococci
W2(5)	9.1	1.84	0.53	-	Bacilli
W2(8)	11.4	1.78	0.68	-	Vibrios
W3(6)	9.6	1.74	1.55	-	Bacilli
W3(7)	3.8	1.71	0.4	-	Bacilli
W3(11)	11.06	1.84	1.28	-	Bacilli
W4(1)	4	1.94	0.86	-	Bacilli
W4(2)	3.5	2.15	0.57	-	Bacilli
W5(1)	11.26	2.03	1.95	-	Bacilli
W5(2)	6.9	2.05	0.74	-	Bacilli
W6(4)	6.9	1.87	0.91	-	Bacilli
W6(5)	4.43	2	2.68	-	Coccobacilli
W7(1)	11.4	1.9	0.71	-	Bacilli
W7(3)	5.66	1.91	0.69	-	Bacilli
W7(5)	4.8	1.86	0.63	-	Bacilli
W9(2)	18.3	1.7	0.84	-	Bacilli
W10(1)	6.5	1.27	0.76	-	Bacilli

4.2.3 Phylogenetics

Environmental bacteria

Through phylogenetic analysis and bioinformatics, the identity of bacterial species sampled in the water samples could be determined. Table 4-4 shows each species identity as well as their taxonomy which can be confirmed through the NCBI accession nr. In Table 4-4 it is evident that bacteria from two major phyla, namely *Proteobacteria* and *Bacteroidetes,* has been isolated. Within the phylum Proteobacteria, there are five major classes namely, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria and Epsilonproteobacteria (Willey *et al.,* 2014). Bacteria from three of these classes have been isolated and identified (Table 4-4). It is, however, important to note that only 20 samples were selected for the phylogenetic analysis and that there might be more species within the water samples representative of other phyla.

The evolutionary history of the environmental bacterial samples were inferred by using the Neighbour-joining method (Saitou & Nei, 1987).The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test can be seen next to the branches (Figure 4-5). The phylogeny is well-supported with the majority of bootstrap proportions above 80 and only one with a bootstrap value of 52. The phylogenetic tree is drawn to scale, with branch lengths the same units as those of the evolutionary distances used to infer the phylogenetic tree. He evolutionary were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The rate variation among sites were modelled with a gamma distribution (shape parameter = 5). All positions containing gaps and missing data, within the 20 nucleotide sequences were eliminated and there were a total of 1245 positions in the final dataset.

Table 4-4: Species identification based on phylogeny and bioinformatics.

Sample nr	Kingdom	Phylum	Class	Order	Family	Species	NCBI Ascension nr.
W1(1)	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	*Enterobacteriaceae*	*Enterobacter sp*	EU693571.1
W1(3)	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	*Enterobacteriaceae*	*Enterobacter asburiae*	MT557022.1
W2(2)	Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	*Neisseriaceae*	*Aquitalea magnusonii*	NR_114200.1
W2(3)	Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	*Neisseriaceae*	*Aquitalea magnusonii*	NR_114200.1
W2(5)	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	*Sphingomonadaceae*	*Chakrabartia godavariana*	NR_165009.1
W2(8)	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	*Caulobacteraceae*	*Caulobacter vibrioides*	MK318611.1
W3(6)	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	*Enterobacteriaceae*	*Enterobacter asburiae*	MT557022.1
W3(7)	Bacteria	Proteobacteria	Gammaproteobacteria	Sphingomonadales	*Sphingomonadaceae*	*Sphingomonas sp*	MN081029.1
W3(11)	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	*Enterobacteriaceae*	*Enterobacter asburiae*	MT557022.1
W4(1)	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	*Flavobacteriaceae*	*Flavobacterium sp*	MG597180.1

Table 4-4 Continued

Sample nr	Kingdom	Phylum	Class	Order	Family	Species	NCBI Ascension nr.
W4(2)	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	*Sphingomonadaceae*	*Chakrabartia godavariana*	NR_165009.1
W5(1)	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	*Sphingomonadaceae*	*Chakrabartia godavariana*	NR_165009.1
W5(2)	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	*Sphingomonadaceae*	*Chakrabartia godavariana*	NR_165009.1
W6(4)	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	*Flavobacteriaceae*	*Flavobacterium sp*	MG597180.1
W6(5)	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	*Acetobacteraceae*	*Roseomonas stagni*	KC157035.1
W7(1)	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	*Enterobacteriaceae*	*Kosakonia sp*	MT322962.1
W7(3)	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	*Enterobacteriaceae*	*Kosakonia oryzae*	MK872359.1
W7(5)	Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	*Aeromonadaceae*	*Aeromonas jandaei*	MT384383.1
W9(2)	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	*Oxalobacteraceae*	*Massilia sp.*	KJ794111.1
W10(1)	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	*Erythrobacteraceae*	*Porphyrobacter sp.*	MN833052.1

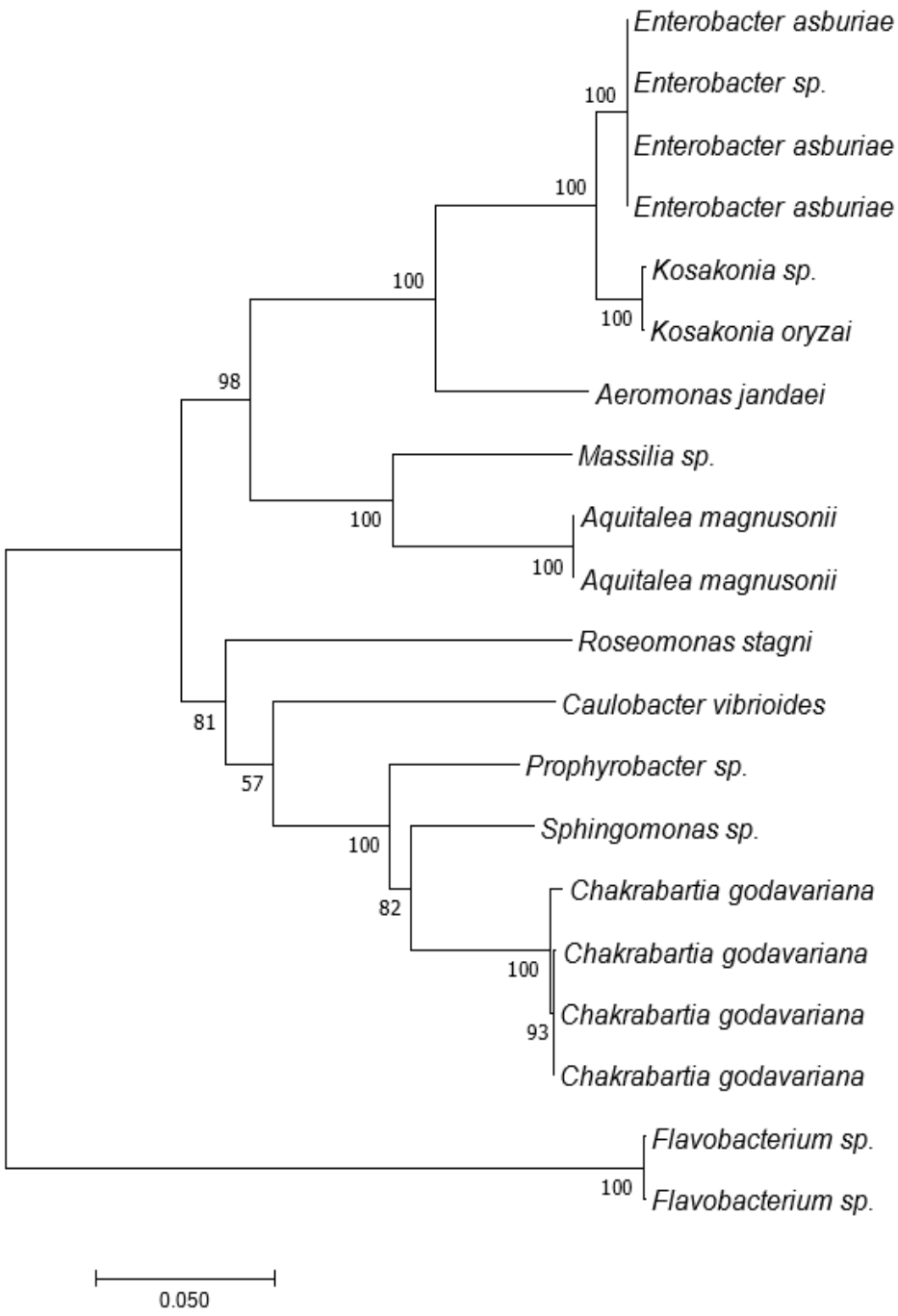


Figure 4-5: Phylogenetic tree constructed through the Neighbour-Joining method with a bootstrap value of 5000, from environmental water samples. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test can be seen next to the branches.

Amphibian diversity

The majority of amphibians found at site one was tadpoles, and interestingly tadpole species were found that did not have representative adults and contrariwise at site one and two (Table 4-5). However, this can easily be explained through the phenomenon of adults being more

motile and terrestrial, being able to move between various localities, and tadpoles being bound to aquatic habitats and persisting at sites after adults have completed breeding activities. Through the sampled tadpole species, it is evident that representative species of various ecomorphological guilds were present at the sampling sites with the majority of tadpoles being categorized in the Benthic ecomorphological guild (Table 4-5).

Table 4-5: Amphibian species found at site 1 and 2, separated by adults and tadpoles where the tadpole ecomorphological guild is noted (Botha, 2013).

		Site 1	
	Ecomorphological guild	**Adult**	**Tadpole**
Kassina senegalensis	Lentic-nectonic	✓	✓
Ptycadena mossambica	Benthic	✓	✓
Phrynomantis bifasiatus	Suspension feeder		✓
Strongylopus grayii	Benthic		✓
Amietia delalandi	Rheophilic		✓
Chyromantis xerampelina	Benthic	✓	✓
Ptycadena anchietae	Benthic	✓	✓
Xenopus laevis	Suspension feeder		✓
Phrynobatrachus natalensis	Lentophytophilic	✓	✓
Sclerophrys gutturalis		✓	
Total number of individuals		18	107
		Site 2	
		Adult	**Tadpole**
Strongylopus grayii	Benthic		✓
Kassina senegalensis	Lentic-nectonic	✓	✓
Chyromantis xerampelina	Benthic		✓
Phrynobatrachus natalensis	Lentophytophilic	✓	
Pixycephalus edulus	Excitus parageios	✓	
Ptychadena anchietae	Benthic	✓	
Tomopterna cryptotus	Excitus parageios	✓	
Total number of individuals		16	36

Amphibian bacterial diversity

Phylogeny data analysis from the 16S rRNA metagenetic sequencing revealed bacterial species representative of 11 phyla found in adult frogs and tadpoles (Figure 4-6). A few bacterial species of importance have been isolated, including *Candidatus amphibiichlamydia* from the phylum *Verrucomicrobiota* first isolated from exotic invasive bullfrogs (*Lithobates catesbeianus*) in Belgium (Martel *et al.*, 2013). With its known inhibitory effects on *Bd* (Harris *et al.*, 2006, Harris *et al.*, 2009, Lam *et al.*, 2010) it was of great significance when

Janthinobacterium was isolated from the amphibian microbiome. Furthermore, it can be seen that two of the phyla represented in the amphibian microbiome are also present in the surrounding water, and various bacterial species isolated from the water samples were also isolated from the amphibian swabs.

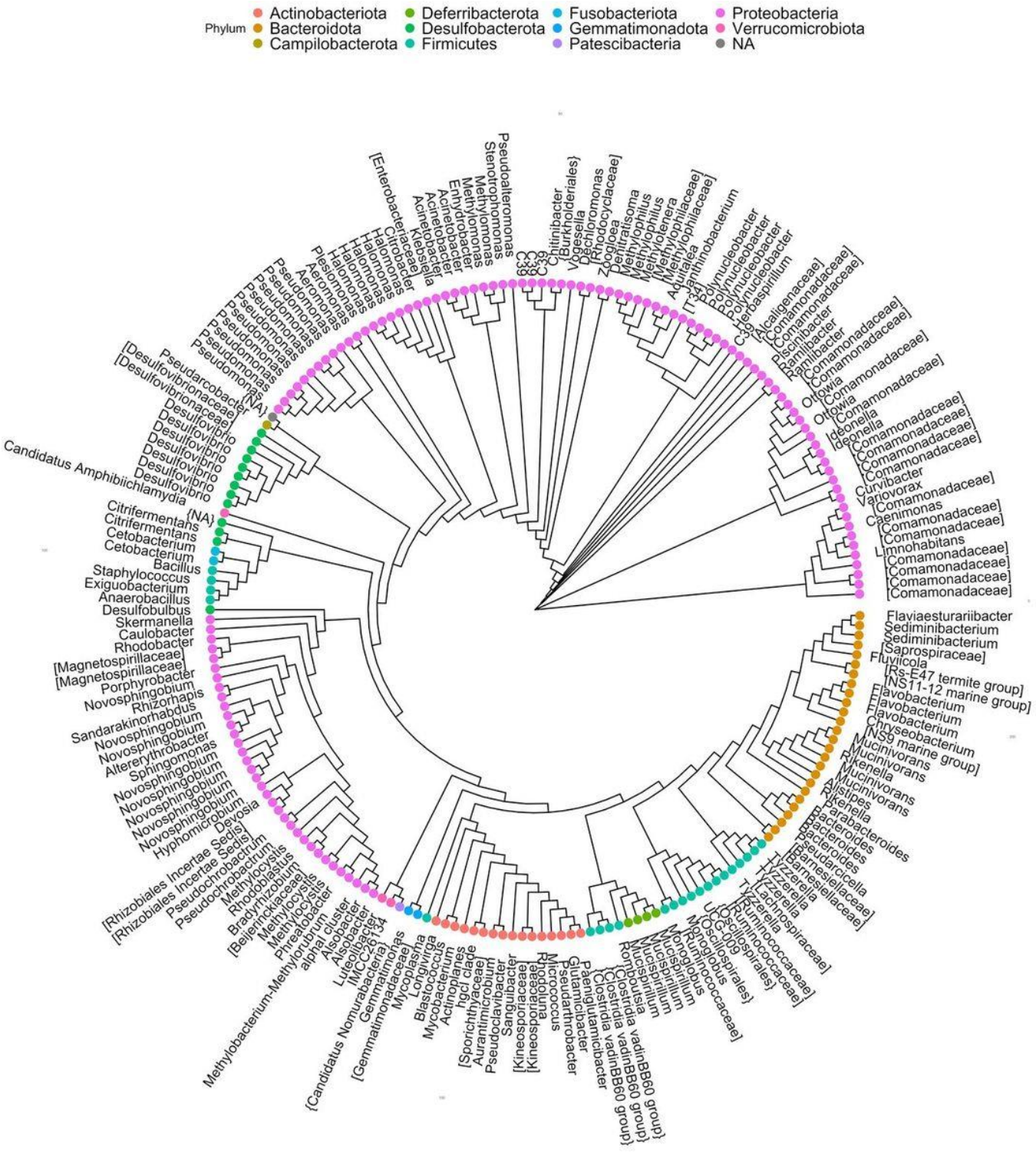


Figure 4-6: Phylogenetic tree constructed from 16S rRNA metagenetic sequencing obtained from tadpole and adult amphibia swabs.

The abundance variation of bacteria present in adult frogs, tadpoles and environmental swabs were plotted on a PCA graph (Figure 4-7). From the data, it was noted that bacteria found on adult frogs were more closely related to the bacteria found in the water and terrestrial samples, while the tadpoles consisted of a more unique microbiome with a few overlapping bacteria

found in the water swabs. PERMANOVA analysis revealed significant differentiation among samples types in microbial community composition ($F_{3,127}$ = 4.42, p = 0.001, r^2 = 9.4%).

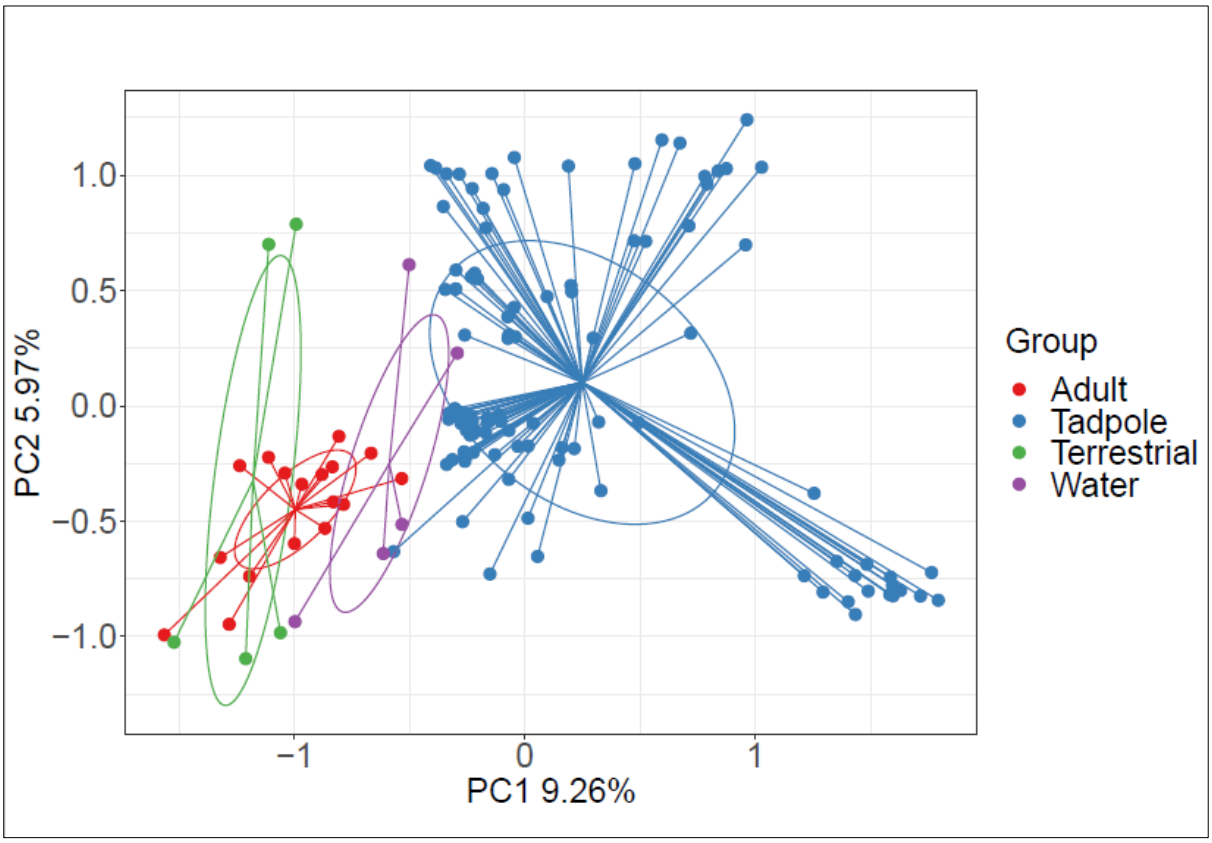


Figure 4-7: Variation in microbial community structure between adult and tadpole swabs along with aquatic and terrestrial swabs. Distances between samples represent ordination of a Principal Components Analysis (PCA) of centred-log-ratio transformed ASVs abundances from 16S rRNA metagenetic sequences.

To quantify variation in microbiome structure among species, only species with at least five replicate swabs were included (Figure 4-8). These data reveal clear variation among species, but also a distinction can be made between bacterial communities from the adult swabs (represented by squares) and the tadpole swabs (represented by circles). PERMANOVA analysis supported these patterns, identifying significant effects of species ID ($F_{1,105}$ = 3.36, p = 0.001, r^2=14.54%) and age ($F_{1,105}$ = 7.01, p = 0.001, r^2=5.05%)

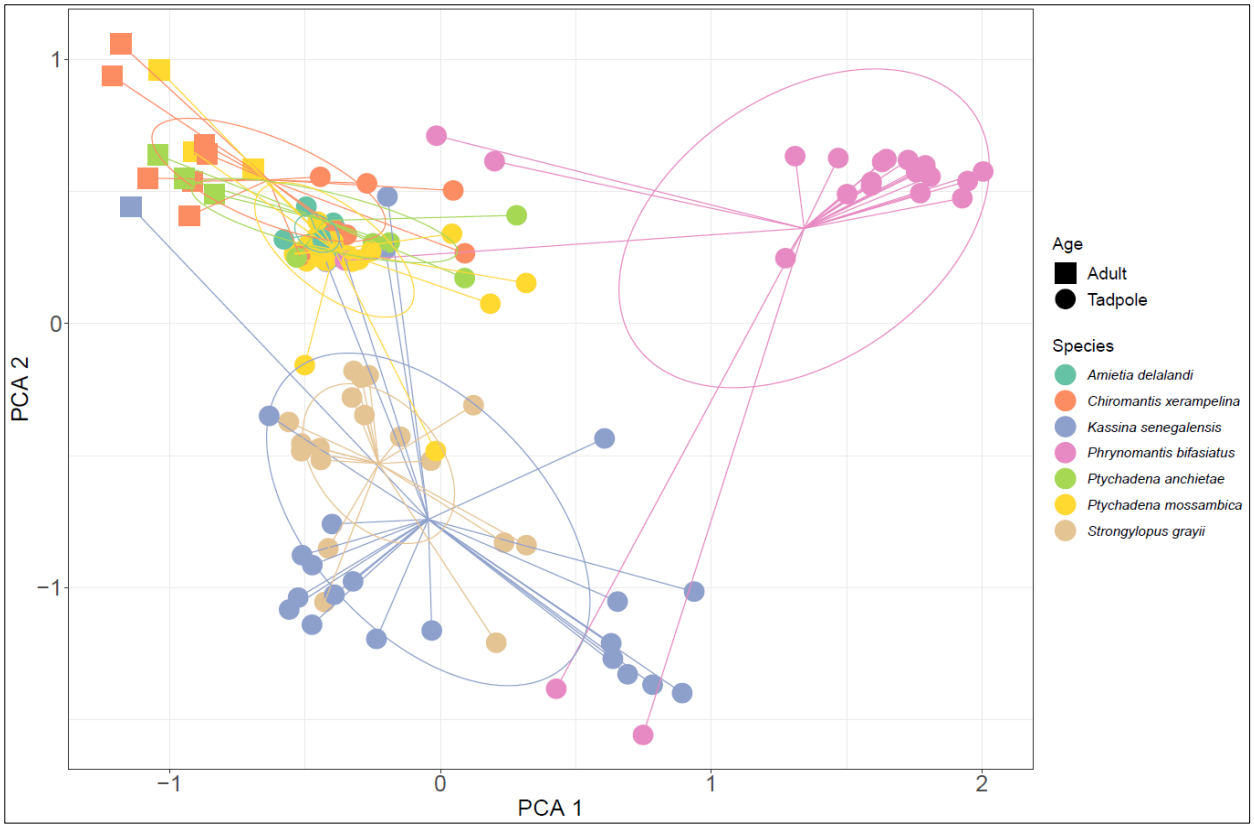


Figure 4-8: Variation in microbial community structure between species where squares represent adult swabs and circles represent tadpole swabs. Distances between samples represent ordination of a Principal Components Analysis (PCA) of centred-log-ratio transformed ASVs abundances from 16S rRNA metagenetic sequences.

4.3 Challenge assays

After *Bd* challenge assays were conducted the effects of bacterial cell-free supernatant could be visualized through graphs and statistical analysis. Figure 4-9 represents the mean absorbance for each bacterial isolate taken over ten days where the error bars represent the standard deviation for each isolate. From Figure 4-9 it is clear that the majority of isolates inhibited the growth of *Bd* (negative values) while only two of the bacterial isolates augmented *Bd* growth (positive values). Augmentation of growth of *Bd* was observed across phylogeny and was not only concentrated in one genus.

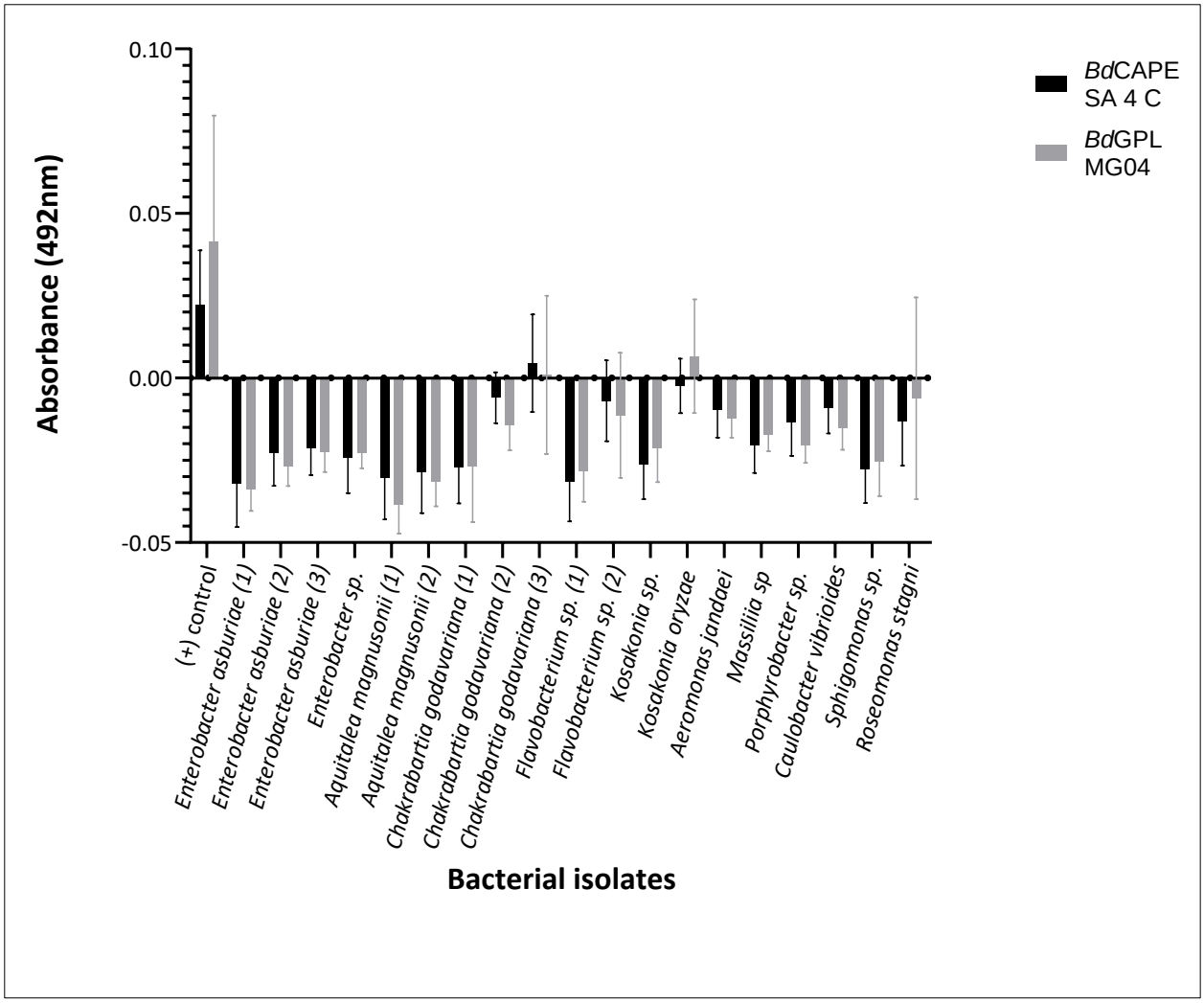


Figure 4-9: Distribution of cell-free supernatant effects of *Bd*CAPE SA 4C and *Bd*GPL MG 04. The black bars represent the bacterial isolates that were challenged against the *Bd*CAPE lineage isolate SA 4C and the grey bars are the bacterial isolates challenged against the *Bd*GPL lineage isolate MG 04. The positive control represents normal *Bd* growth where no cell-free supernatant was added.

Following the challenge assays, it was important to determine whether there was a significant difference in *Bd* growth between the two lineages *Bd*CAPE and *Bd*GPL. Figure 4-10 A indicated that both *Bd*CAPE and *Bd*GPL were generally inhibited by the cell-free supernatant from the bacterial isolates while a box-and-whisker plot (Figure 4-10 B) indicated that the data were asymmetrically distributed.

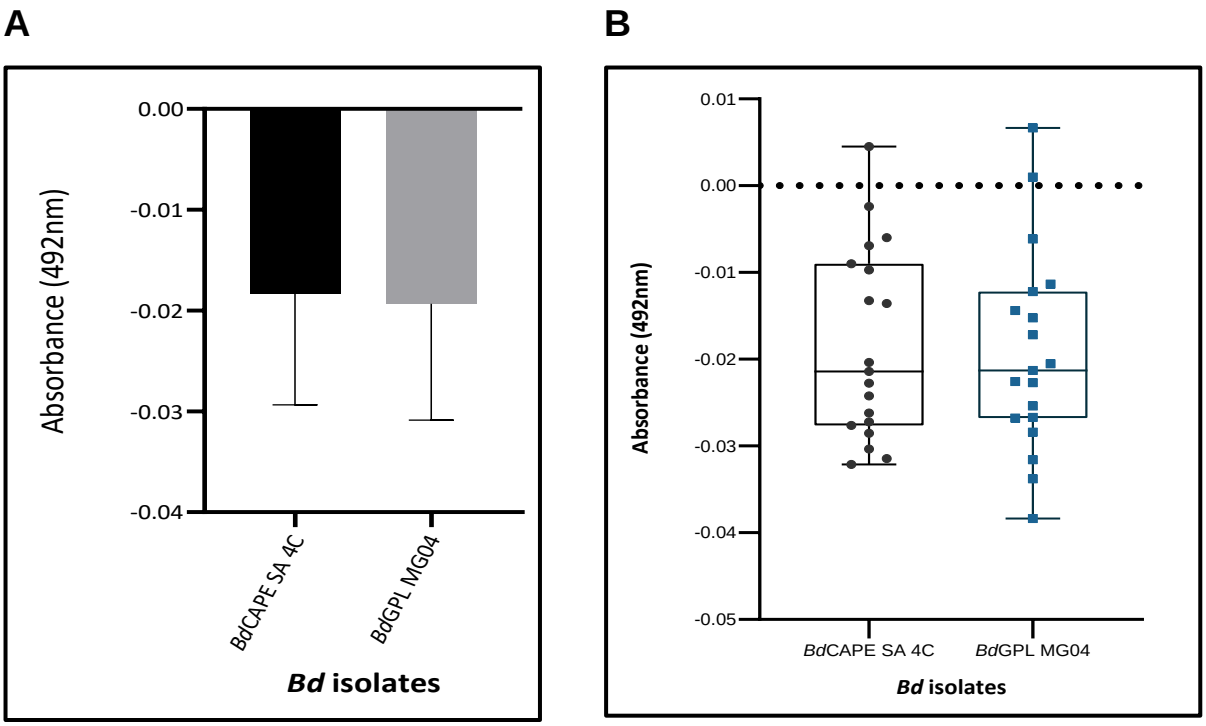


Figure 4-10: A) Overall distribution of cell-free supernatant effects on *Bd*CAPE and *Bd*GPL. B) Box-and-whisker plot of the overall distribution of cell-free supernatant effects on *Bd*CAPE and *Bd*GPL to determine the normality of data.

The Shapiro-Wilk normality test showed a p-value of 0.15 for *Bd*CAPE isolate SA 4C and a p-value of 0.75 for *Bd*GPL isolate MG 04 (Table 4-6), indicating that there was no lineage-dependent statistical significance concerning the overall effects of the cell-free supernatant.

Table 4-6: Statistical analysis of the overall effects of cell-free supernatant on *Bd*CAPE isolate SA 4C and *Bd*GPL isolate MG04.

	***Bd*CAPE SA 4C**	***Bd*GPL MG04**
P value	0.1568	0.7193
Passed normality test (alpha=0.05)?	Yes	Yes
P-value summary	ns	ns
Shapiro-Wilk test		
W	0.9274	0.9687
P value	0.1554	0.7492
Passed normality test (alpha=0.05)?	Yes	Yes
P-value summary	ns	ns

Furthermore, the Mann-Whitney (Table 4-7) test confirmed that there is no statistical significance between the effects of the cell-free supernatant on the *Bd* lineages used for the challenge assays.

Table 4-7: Mann Whitney test indicating the significance between *Bd*CAPE and *Bd*GPL.

Mann Whitney test	
P-value	0.8626
Exact or approximate P-value?	Exact
P-value summary	ns
Significantly different (P < 0.05)?	No

A box-and-whisker plot of the effects that the cell-free supernatant has on the *Bd*CAPE isolate SA 4C (Figure 4-11) indicated that the majority of bacterial isolates had spectrophotometer data that were asymmetrically distributed and in order to determine statistical significance a Kruskal-Wallis test needed to be performed.

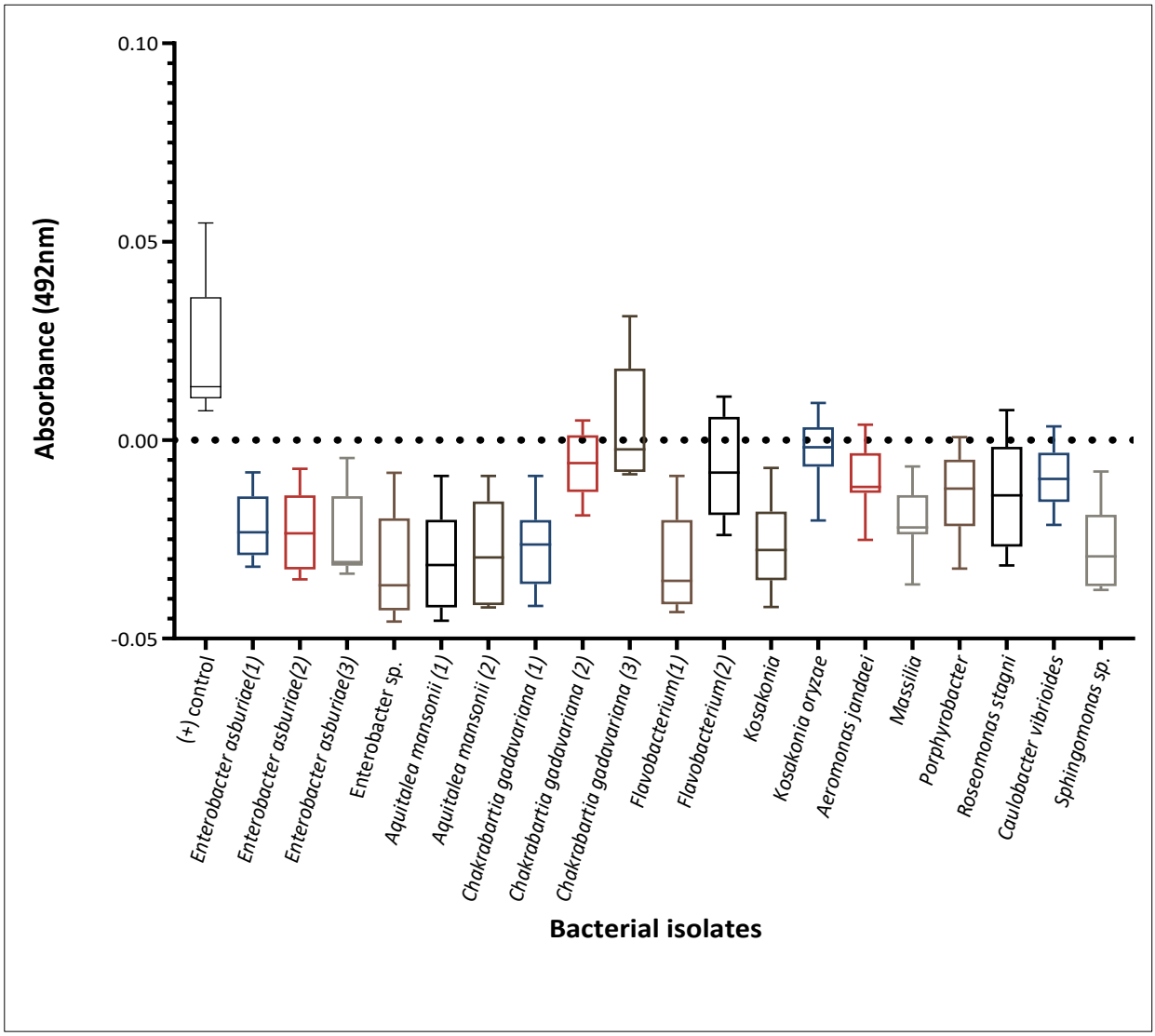


Figure 4-11: Box-and-whisker plot of the distribution of the effects of cell-free supernatant on *Bd*CAPE isolate SA 4C.

The Kruskal-Wallis, and it can be seen that there is a statistical significance between the medians of spectrophotometer absorbance data obtained during the challenge assays where cell-free supernatant of various bacterial species was challenged against *Bd*CAPE isolate SA 4C (Table 4-8).

Table 4-8: Statistical analysis of the effects of cell-free supernatant on the growth of *Bd*CAPE isolate SA 4C.

Kruskal-Wallis test	
P-value	<0.0001
Exact or approximate P-value?	Approximate
P-value summary	****
Do the medians vary significant? (P < 0.05)	Yes
Number of groups	21
Kruskal-Wallis statistic	133.1

In Figure 4-12 show that the majority of data obtained during the challenge assays, where the cell-free supernatant of bacterial species was challenged against the *Bd*GPL isolate MG 04, was also asymmetrically distributed.

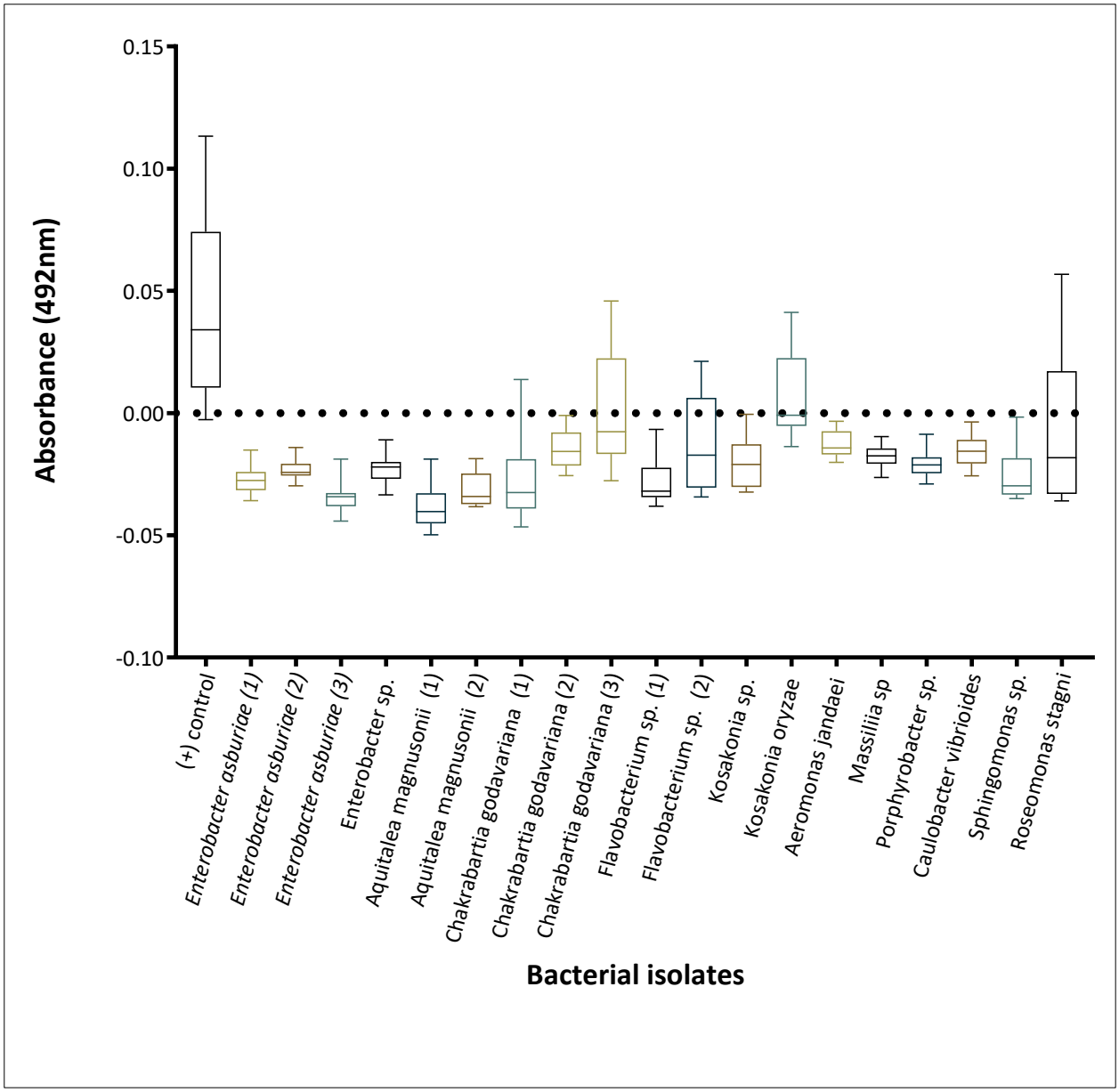


Figure 4-12: Box-and-whisker plot of the distribution of the effects of cell-free supernatant on *Bd*GPL isolate MG 04.

Through the Kruskal-Wallis test, it was possible to determine the significance of the medians of the spectrophotometer absorbance data when cell-free supernatants of bacterial isolates are challenged against *Bd*GPL isolate MG 04 and in Table 4-9 it is evident that there was significant variance between the medians.

Table 4-9: Statistical analysis of the effects of cell-free supernatant on the growth of *Bd*GPL isolate MG 04.

Kruskal-Wallis test	
P-value	<0.0001
Exact or approximate P-value?	Approximate
P-value summary	****
Do the medians vary significant? (P < 0.05)	Yes
Number of groups	21
Kruskal-Wallis statistic	142.7

Since there was no statistical significance of the effects of the cell-free supernatant on *Bd*CAPE and *Bd*GPL isolates it was decided to only use *Bd*CAPE for the consortium challenge assays since *Bd*CAPE is considered mostly endemic to Africa (Farrer *et al.*, 2011, O'Hanlon *et al.*, 2018). Figure 4-13 indicates the distribution of the effects that the cell-free supernatants of a consortium of bacteria have on the growth of *Bd*CAPE isolate SA 4C. The negative values represent the instances where the cell-free supernatant inhibited the growth of *Bd*CAPE isolate SA 4C, and the positive values indicate the augmentation of the *Bd* isolate. The groups/consortiums were made up out of one bacterial species per family (see Table 4-10 for family identification) where consortium one consisted out of two families and one species per family was added up until consortium 7.

Table 4-10: Consortium layout, containing one bacterial isolate of each of the various families.

Consortium nr	**Bacterial combination**
1	*Chakrabartia godavariana* + *Flavobacterium sp.*
2	*Chakrabartia godavariana* + *Flavobacterium sp* + *Aquitalea magnusonii*
3	*Chakrabartia godavariana* + *Flavobacterium sp* + *Aquitalea magnusonii* + *Aeromonas jandaei*
4	*Chakrabartia godavariana* + *Flavobacterium sp* + *Aquitalea magnusonii* + *Aeromonas jandaei* + *Massiliia sp*
5	*Chakrabartia godavariana* + *Flavobacterium sp* + *Aquitalea magnusonii* + *Aeromonas jandaei* + *Massiliia sp* + *Porphyrobacter sp*
6	*Chakrabartia godavariana* + *Flavobacterium sp* + *Aquitalea magnusonii* + *Aeromonas jandaei* + *Massiliia sp* + *Porphyrobacter sp.* + *Enterobacter sp*

7	*Chakrabartia godavariana + Flavobacterium sp + Aquitalea magnusonii + Aeromonas jandaei + Massiliia sp + Porphyrobacter sp. + Kosakonia oryzae*

From the distribution of the effects, the consortium of cell-free supernatants had on the *Bd*CAPE isolate SA 4C it is evident that the majority of consortia had an inhibitory effect (negative values) on the growth of *Bd* while only one consortium had an augmenting (positive values) effect on the growth of the isolate.

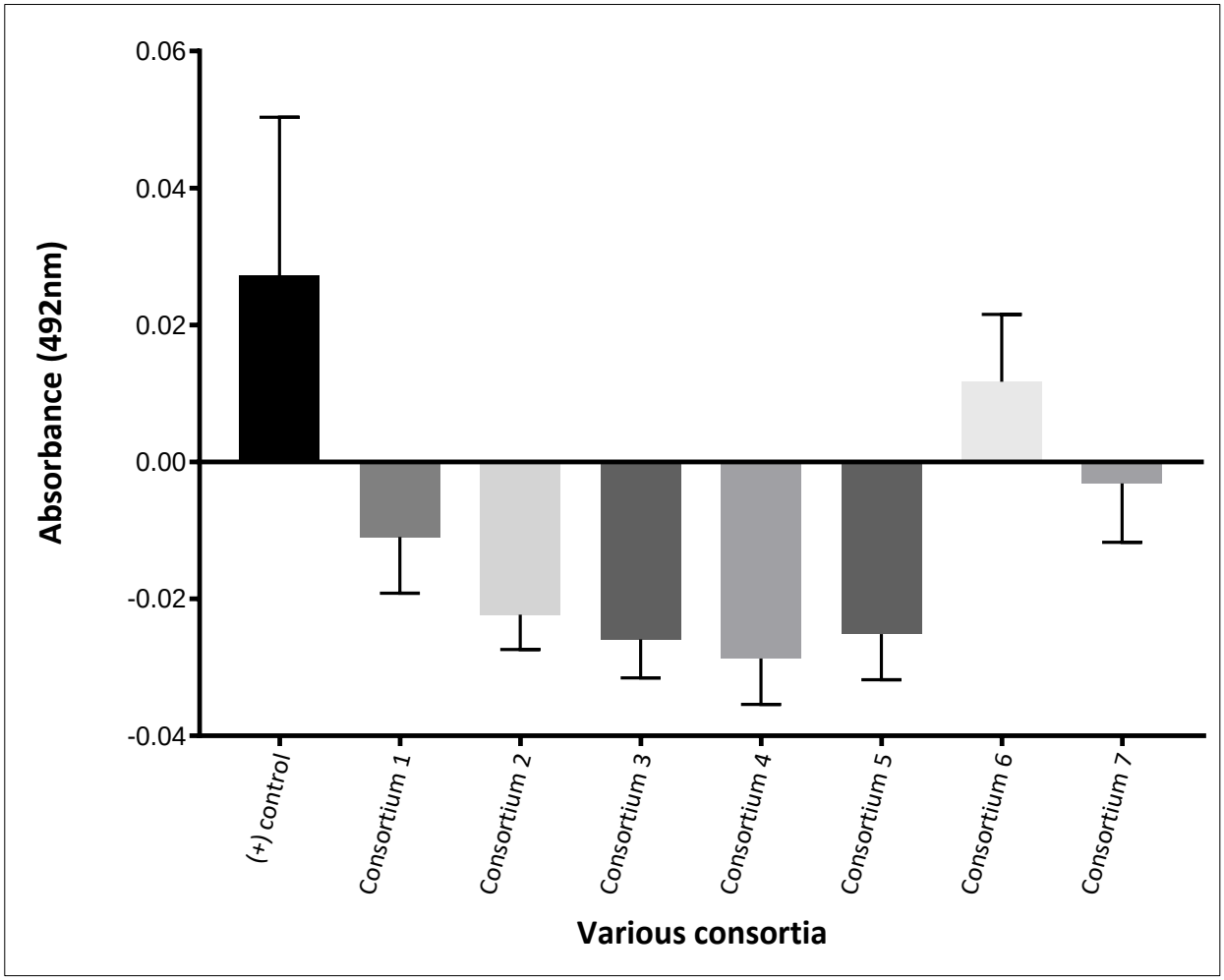


Figure 4-13: Distribution of effects of cell-free supernatant from a consortium of bacteria on the growth of *Bd*CAPE isolate SA 4C. The positive control represents the normal growth of *Bd* where no cell-free supernatant was added.

From the box-and-whisker plot in Figure 4-14, it is evident that the spectrophotometry absorbance data obtained from the challenge assays were mainly asymmetrically distributed and that a non-parametric test was needed to determine the statistical significance of the data.

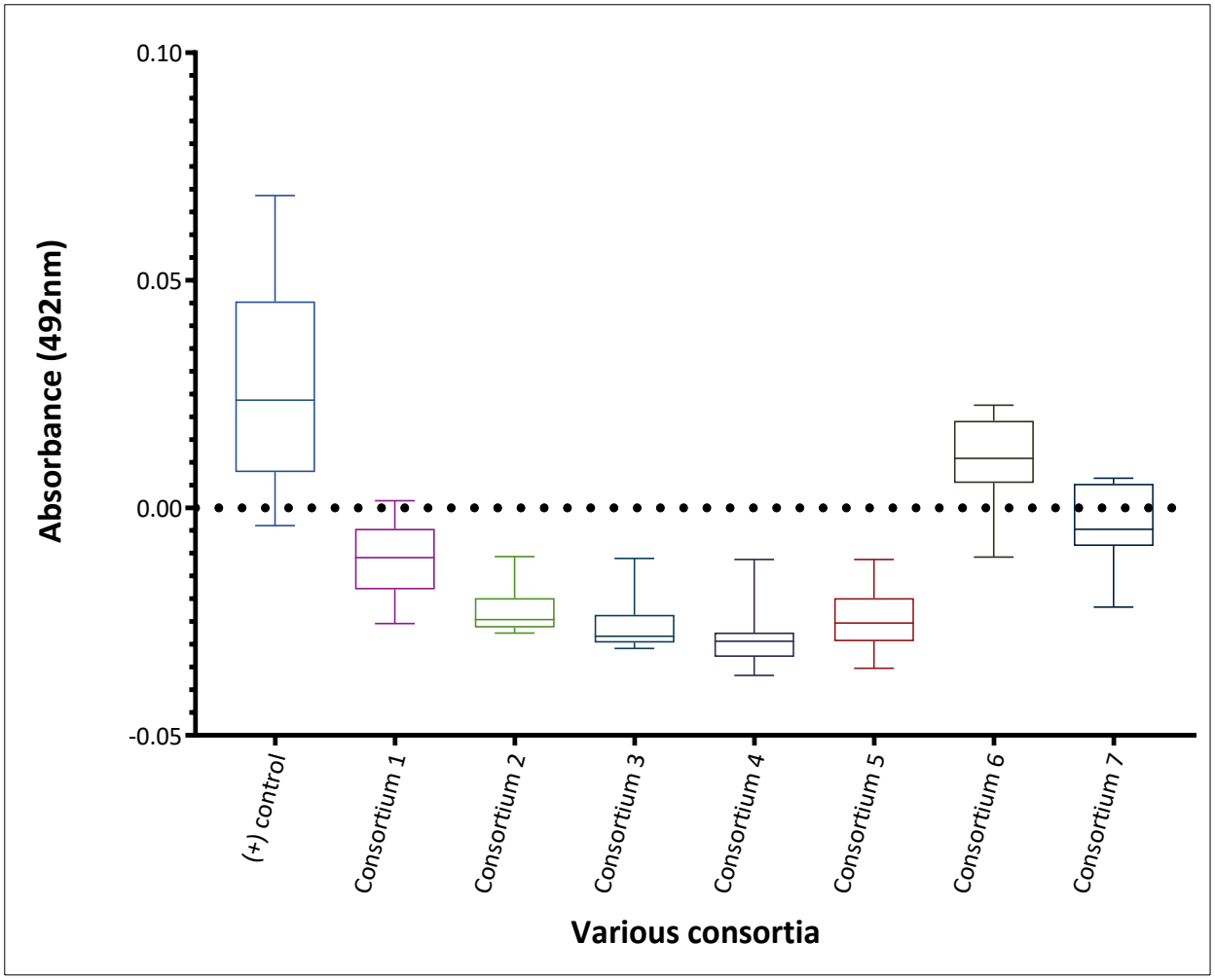


Figure 4-14: Box-and-whisker plot indicating the distribution of effects of cell-free supernatant from a consortium of bacteria on the growth of *Bd*CAPE isolate SA.

It is evident that the medians of the various consortia of bacteria varied significant, with a p-value <0.0001 (Table 4-11).

Table 4-11: Statistical analysis of the effects of cell-free supernatant from a consortium of bacteria on the growth of *Bd*CAPE isolate SA 4C.

Kruskal-Wallis test	
P-value	<0.0001
Exact or approximate P-value?	Approximate
P-value summary	****
Do the medians vary significant? (P < 0.05)	Yes
Number of groups	9
Kruskal-Wallis statistic	83.67

4.3.1 Photo records

It has been found that an average *Bd* life cycle lasts about four to six days (Berger *et al.*, 2005), therefore, at day 6 when one *Bd* life cycle should be complete, photos were taken of each well

containing the positive-, negative-, and experimental controls. Figure 4-15 indicates the various stages that were observed throughout the experimental layout. It can be seen that the cell-free supernatant of various bacterial species had varying effects on the growth of *Bd.* The effects that bacterial cell-free supernatants had on *Bd* ranged from augmenting (Figure 4-15 G) to complete inhibition where cell lysis was observed (Figure 4-15 H). Various sporangia sizes can also be observed, where large mature sporangia with discharge papillae are visible (Figure 4-15 D), as well as sporangia still in the germling stage (Figure 4-15 C) and large empty sporangia with no developing zoospores inside (Figure 4-15 F).

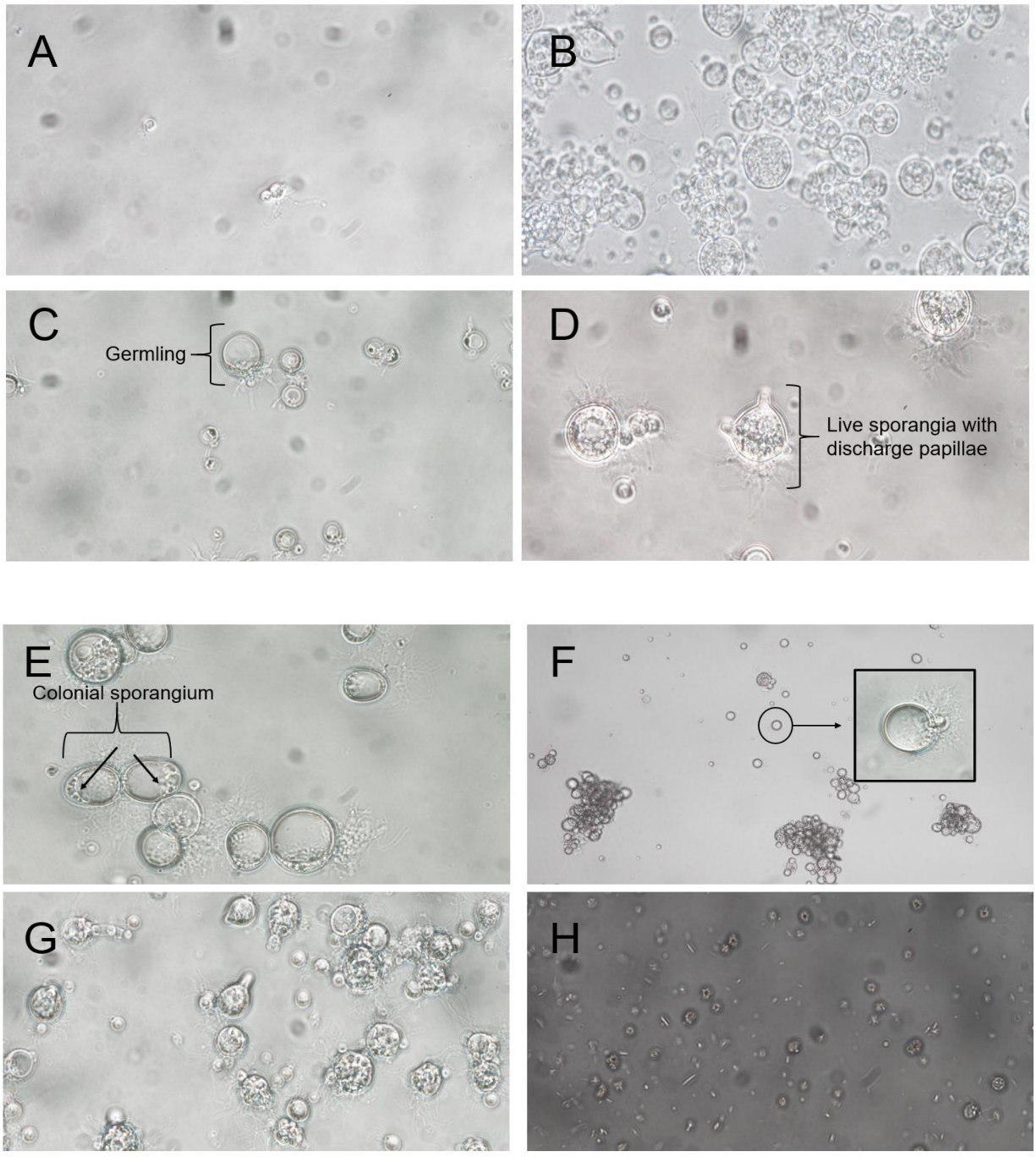


Figure 4-15: A) Negative, heat-killed *Bd* zoospores control (40X objective). B) Positive control, *Bd* with no cell-free supernatant (40X objective). C) Partial inhibition where *Bd* growth was halted at the germling stage, observed when *Aquitalea magnosonii* cell-free supernatant was added to *Bd*GPL isolate MG 04 (40X objective). D) Live sporangia with discharge papillae observed when *Caulobacter vibrioides* cell-free supernatant was added to *Bd*CAPE isolate SA 4C (40x objective). E) Example of colonial development where more than one sporangium develops from one zoospore, observed when *Massilia sp*. cell-free supernatant was added to *Bd*GPL isolate MG 04 (40X objective). F) Partial inhibition where large sporangia form but no zoospore developed. Observed when *Caulobacter vibrioides* cell-free supernatant was added to *Bd*GPL isolate MG 04 (10X objective, enlarged to 40X objective). G) *Bd* augmentation observed when *Chakrabartia godavariana* cell-free supernatant was added to *Bd*CAPE isolate SA 4C (40X objective). H) Complete inhibition where cell lysis took place. Observed when *Enterobacter sp*. cell-free supernatant was added to *Bd*CAPE isolate SA 4C (40X objective).

CHAPTER 5 DISCUSSION

A comprehensive analysis of the bacterial community structure of amphibian hosts and their environment including both adults and tadpoles was performed. Through the results, it was possible to determine how amphibians interact with their surrounding environment and also gave insight as to how amphibians acquire different bacteria. The use of bacteria as a probiotic to inhibit the growth of *Bd* has been extensively researched (e.g. Antwis & Harrison, 2018). The challenging of bacterial cell-free supernatant of different bacterial species against isolates from different lineages of *Bd* revealed various effects with regards to inhibition and augmentation. The results obtained through this study allowed for a better understanding of the relationship between frogs and bacteria as well as the relationship between bacteria and one of the biggest natural threats of amphibians, the amphibian chytrid fungus.

5.1 Molecular discussion

5.1.1 Nanodrop and agarose gel electrophoresis

Considered as one of the most commonly used methods for estimating nucleic acid concentration, is the measurement of sample absorbance at 260nm, where the 260/280 and 260/230 ratios determine the DNA purity and presence of contaminants that may occur during DNA extraction (García-Alegría *et al.*, 2020). When observing the results obtained through the Nanodrop analysis it can be seen that some DNA samples adhered to the desired A260/280 ratio of 1.75 – 2 while other samples had definite protein or phenol contamination. The contamination observed through these results was most likely obtained during the DNA extraction protocol. Furthermore, it was confirmed that attention should be given to factors that might alter the yield and purity of the DNA sample Nanodrop results. These factors include solution homogeneity, which can be obtained by mixing the DNA sample with a pipette, delay time of sample measurement, loading sample volume and cross-contamination between sample measurements (Li *et al.*, 2017).

Both the Nanodrop and agarose gel electrophoresis proved to be valuable methods in determining DNA quality and quantity. There was a definite correlation between the Nanodrop and agarose gel electrophoresis results aiding in the selection of samples to achieve desired results in identifying isolates through DNA sequencing and bioinformatics.

5.1.2 Bacterial diversity

<u>Environmental bacteria</u>

Lentic systems are typically still standing water systems where lakes and ponds are representative examples of lentic systems (Joshi *et al.*, 2016; Willey *et al.,* 2014). Various zones within the lentic systems are responsible for hosting various microbes and in return, these microbes are responsible for different biological processes, including degrading of organic carbon compounds and the production of hydrolytic enzymes such as amylase, lipase, protease, phospholipase and catalases among other important industrial enzymes (Findlay, 2010; Joshi *et al.*, 2016; Willey *et al.,* 2014). Microorganisms are present in almost all ecosystems and it has therefore been suggested that microbes account for most of the diversity of life on earth (Joshi, 2016).

The Alphaproteobacteria class can be divided into seven orders and 20 families (Willey *et al.,* 2014) where four of these families are represented by the bacteria that were isolated during the study. *Caulobacteraceae* is known as one of two groups of alphaproteobacteria that has a life cycle featuring a prostheca where reproduction takes place through budding (Willey *et al.,* 2014). *Caulobacteraceae* contains two of the most studied genera *Caulobacter* and *Hyphomicrobium* (Willey *et al.,* 2014). It can be seen that *Caulobacter vibrioides* was isolated from the environmental water samples. *Caulobacter* is described as a vibrioid-shaped cell that can alternate between a polarly flagellated rod and cells that possess a prostheca that can attach to a substrate (Willey *et al.,* 2014). Previous studies have found that *Caulobacter* is most commonly found in aquatic ecosystems where the limiting nutrient is phosphorous (Gonin *et al.*, 2000). Phosphorous is considered an essential element and is specifically imported in the form of inorganic phosphate, however, when bacteria are starved for phosphate, they increase their ability to up-take inorganic phosphate and to utilize organic phosphate sources (Gonin *et al.*, 2000). It has further been found that *Caulobacter* can adhere to other microorganisms to possibly absorb the nutrients released by these organisms (Willey, *et al.,* 2014).

The next species, in the alphaproteobacteria class isolated during the study, is representatives of the family *Sphingomonadaceae,* a ubiquitous bacteria that can frequently be found in aquatic environments such as drinking water and remarkably also in supposedly sterile drug solutions including distilled water (Vaz-Moreira *et al.*, 2011). The genus *Sphingomonas* was first described by Yabuuchi and colleagues in 1990 (Takeuchi *et al.*, 2001) and members of the family are considered strict chemoheterotrophs that are characterized by a yellow pigment (Takeuchi *et al.*, 2001, Vaz-Moreira *et al.*, 2011). With a frequently observed oligotrophic character, the members of *Sphingomonadaceae* are widespread throughout nature and can be found in soils, corals, eutrophic waters, and plant surfaces (Vaz-Moreira *et al.*, 2011). *Sphingomonadaceae* members have a unique malleability in man-made environments, they contain the ability to degrade xenobiotic compounds and due to their allegedly oligotrophic nature they have the capacity to

survive chlorinated water and to produce biofilms (Vaz-Moreira *et al.*, 2011). Two species from the family *Sphingomonadaceae* was isolated from the environmental water samples one of the species *Chakrabartia godavariana* is a novel species described in 2019 (Jani *et al.*, 2019).

Within the *Acetobacteracea* family, only one representative species has been identified through molecular analysis. Various members of this are aerobic and are able to conduct incomplete oxidation of sugars and alcohols to produce organic acids as a final product of metabolism (Reis & Teixeira, 2015). The optimum pH range for members of the *Acetobacteracea* family is 5.0-6.5. However, some species in this family can grow in extremely acidic conditions with a pH close to 3.0-3.5 (Reis & Teixeira, 2015). The optimal range for *Acetobacteracea* is significant seeing that research has proven that *Bd* can grow and reproduce at pH levels ranging between 4-10 (Chestnut *et al.*, 2014). Representatives of the genera *Roseomonas* have been considered as opportunistic bacteria associated with chronic pneumonia (Reis & Teixeira, 2015, Furuhata *et al.*, 2008). During recent studies, *Roseomonas* have frequently been isolated from various natural environments, including aquatic environments (Furuhata *et al.*, 2008). Through molecular analysis of bacteria isolated from pond water in Japan in 2008, *Roseomonas stagni* was described as a novel strain of *Roseomonas* (Furuhata *et al.*, 2008).

The fourth family of alphaproteobacteria represented by the isolated bacteria is the family *Erythrobacteraceae*. Members of the family *Erythrobacteraceae* has a global distribution and can be found inhabiting a variety of environments including various freshwater as well as marine water and hot springs, various soils including desert sand and even plants and animals (Xu *et al.*, 2020). The members of *Erythrobacteraceae* are gram-negative bacteria with a variety of pigmentation that occur throughout the various genera, these pigmentations include pink-, red-, orange- or yellow-pigmented isolates and are aerobic chemoorganotrophs and the majority requires NaCl for growth (Xu *et al.*, 2020). Based on 16S rRNA gene phylogeny the family *Erythrobacteraceae* contains seven genera including *Porphyrobacter* (Xu *et al.*, 2020). *Porphyrobacter* along with two other genera from this family are classified as aerobic bacteriochlorophyll (BChl)-containing bacteria (Hiraishi *et al.*, 2002). Regarding the class alphaproteobacteria, various species from different families have been identified through this study and the bacterial isolates from alphaproteobacteria is mainly considered to be commonly found in water environments.

Metabolically betaproteobacteria are similar to alphaproteobacteria but differ in the use of substances to diffuse from organic decomposition in anoxic habitats (Willey *et al.,* 2014) Some of the bacteria found in the betaproteobacteria class use hydrogen, ammonia, volatile fatty acids and other similar substances during their metabolic processes (Willey *et al.,* 2014). Within the betaproteobacteria there are 12 families as well as considerable metabolic diversity and species can be chemoorganotrophs, photolithotrophs or chemolithotrophs (Willey *et al.,* 2014). Through

the isolation and identification of water sampled bacterial species, it was found that two families, representative of the class betaproteobacteria, is present including *Neisseriaceae* and *Oxalobacteraceae*.

The family *Neisseriaceae* contains species of bacteria that are closely related to human disease with *Neisseria meningitides* causing bacterial meningitis and septicaemia and *Neisseria gonorrhoeae* being the causative agent of gonorrhoea (Wong *et al.*, 2015). During a study in northern Wisconsin, USA, a gram-negative, rod-shaped, non-spore-forming betaproteobacteria was isolated from humic-lake samples (Lau *et al.*, 2006). It was found that this bacteria grew optimally at 35°C and pH 6.0, it does not utilize sucrose but instead used glucose, some organic acids and most amino acids during metabolic processes, it was then described as the novel species named *Aguitalea magnosonii* (Lau *et al.*, 2006).

Members of the genus *Massilia* from the family *Oxalobacteraceae* have been isolated from clinical samples but were also isolated and detected in various environmental samples, including air, aerosols, and dust samples as well as freshwater, soils and phyllosphere samples, which is significant seeing that these bacterial species has also been isolated during the study from the water samples (Ofek *et al.*, 2012) Species from the genus *Massilia* is characterized as aerobic, flagellated, non-spore-forming rods and have recently been isolated from the rhizosphere and roots of a variety of plant species (Ofek *et al.*, 2012). Regarding the isolates identified from the water samples, it is considered that both *A. magnosonii* and *Massilia* are bacterial species that are fairly recently but commonly associated with freshwater ecosystems.

Considered as the largest subgroup of Proteobacteria is the class Gammaproteobacteria with a unique variety of physiological types (Willey *et al.,* 2014). The gammaproteobacteria class can be divided into an extraordinary 28 families, it can be seen that two of the families in the gammaproteobacteria class is represented through isolated species. The families, representative of the gammaproteobacteria class include *Enterobacteriaceae,* which uses the Embden-Meyerhof pathway to catabolize carbohydrates (Willey *et al.,* 2014), and *Aeromonadaceae.* Two species from the class *Enterobacteriaceae* have been identified during the phylogenetic analysis, namely, *Enterobacter asburiae* and *Kosakonia oryzae*. Even though *E. asburiae* has been isolated from various environmental sources such as soil and water, *E. asburiae* is a bacteria that raise concern in the medical industry due to the fact that it has also been isolated from human sources and has been identified as an opportunistic human pathogen that is causative of pneumonia, soft-tissue infections as well as wound and other infections (Mardaneh & Dallal, 2016). This organism was described in 1986 by Brenner and colleagues and is defined as a facultative anaerobic, non-spore-forming gram-negative rod-shaped bacterium that is motile through a peritrichous flagellum (Mardaneh & Dallal, 2016). Another major concern surrounding *E. asburiae* is the fact that this

organism is capable of producing the Bush group 1 β-lactamase enzyme at high levels which renders this bacterium resistant to most β-lactam antibiotics (Mardaneh & Dallal, 2016). *K. oryzae* is a gram-negative, non-spore-forming, motile rod-shaped bacteria with a peritrichous flagellum that grows aerobically but can reduce N_2 to NH_3 at low pO_2 (Li *et al.*, 2017) It was surprising that *Kosakonia oryzae* was isolated from water samples since species from the genus *Kosakonia* are considered nitrogen-fixing bacteria closely related with plants and the promotion of crop production (Li *et al.*, 2017). However, the pond where the water samples were taken is surrounded by various plants and tree species which can explain the occurrence of plant-associated bacteria being found in a water environment.

Another representative species from the class gammaproteobacteria and the family *Aeromonadaceae,* that was isolated from the water samples, is the species *Aeromonas jandaei.* The genus *Aeromonas* is a group of gram-negative, catalase and oxidase-positive, glucose-fermenting motile bacteria and has been reported to inhabit most aquatic ecosystems (Pu *et al.*, 2019). Various species of *Aeromonas* have been studied intensively due to their clinical importance in their ability to cause gastroenteritis, wound infection, septicaemia and other infections in fish, reptiles, amphibians and humans (Pu *et al.*, 2019). It has also been found that species from the genus *Aeromonas* are responsible for causing bacterial infections, specifically red-leg disease caused by *A. hydrophila* (Bradford, 1991). During recent studies, *A. jandaei* has been isolated from Nile tilapia (*Oreochromis niloticus*) juveniles at a commercial farm in Thailand (Dong *et al.*, 2017) as well as Siamese crocodiles (*Crocodylus siamensis*) obtained from a farm in China (Pu *et al.*, 2019). In both studies, the sampled species died from symptoms reflecting infectious disease (Dong *et al.*, 2017) and septicaemia and respiratory inflammation (Pu *et al.*, 2019).

Regarding the species isolated from gammaproteobacteria and their potential pathogenic nature, it is worth looking into the effects that these bacteria could have in the study area where they were isolated from. Seeing that this is a site rich in amphibian diversity these bacteria could potentially have detrimental effects on the amphibian population of Ukutula. The study aimed to establish a better understanding of the ecological relationship between environmental bacteria and a multi-species amphibian community and for the purpose of this study, the main focus was to establish how environmental bacteria interact with the amphibian pathogen *B. dendrobatidis.* However, it will be immensely valuable to see how amphibians react to environmental bacteria especially bacteria with potentially pathogenic traits.

A second phylum identified during phylogenetic analysis is the phylum *Bacteroidetes.* The phylum *Bacteroidetes* are considered a very diverse phylum even though all the members of this phylum are known as chemoheterotrophs (Willey *et al.,* 2014). A major contribution of these bacteria is

the digestion of cellulose, agar, chitin, proteins, as well as a variety of other macromolecules and, are commonly found in terrestrial and marine environments (Willey *et al.,* 2014). Members from the genus *Flavobacterium* are characterized by rapid gliding motility and are mostly free-living soil or aquatic bacteria (Willey *et al.,* 2014).

A previous study has found that the Proteobacteria and Bacteroidetes are among the most abundantly found phyla in water, substrate, and amphibian skin (Walke *et al.*, 2014). Considering that the majority of bacteria isolated from the water samples belongs to phyla Proteobacteria the assumption can be made this is the dominant phyla for this study regarding not only the environmental bacteria (Figure 4-5) but also the bacteria isolated from the amphibians skin and tadpole mouthparts (Figure 4-6).

Amphibian bacterial diversity

The unique symbiotic relationship between amphibians and bacteria are maintained by the transmission of symbiont to host in each new generation (Walke *et al.*, 2014). However, in some systems symbionts can be obtained through the surrounding environment (Walke *et al.*, 2014). Therefore, it was initially hypothesized that bacteria isolated from water samples will overlap with the bacteria found on amphibian skin and tadpole mouthparts and that environmental bacteria will have the ability to inhibit the growth of *Bd.* However, results have suggested that amphibian skin maintain bacteria that are relatively low in abundance in the environment (figure 4-7). This is also evident in the phylogenetic analysis where a larger bacterial diversity has been isolated from the amphibians as appose to the bacteria isolated from the environmental samples. These results are coherent with results from a study where bacteria from two anuran species were compared to each other and their surrounding environment (Walke *et al.*, 2014). The sampled bacterial communities from the tadpole mouthparts clustered separately from the adult amphibians as well as the environmental swabs, with a small overlap between the tadpole bacteria and the water bacteria (Figure 4-7). The overlap between the amphibian bacteria and the environmental bacteria can further be confirmed through the phylogenetic results (Figures 4-5 & 4-6) where only two of the bacteria phyla isolated from the amphibian swabs, namely Proteobacteria and Bacteroidetes, were also isolated from the water samples. This configuration of bacterial distribution has been observed by Walke *et al.* (2014), as well as in studies conducted on squid and sponge systems (Nyholm & McFall-Ngai, 2004; Webster *et al.*, 2010).

With the complex composition of amphibian skin that includes mucous skin secretions, host-produced antimicrobial peptides, as well as microbially produced metabolites, amphibians likely have a strong influence on determining the composition of microbes inhabiting their skin (Walke *et al.*, 2014). The varying amounts of mucus secretions, produced by amphibians, to aid in skin-

shedding, preventing desiccation and escaping from predators may also affect the regulation of microbial growth (Walke *et al.*, 2014). With various studies determining the presence and composition of antimicrobial peptides on amphibian skin (Woodhams *et al.*, 2006**;** Woodhams *et al.*, 2007; Daum *et al.*, 2012) the peptides may also have a role in determining the growth of certain bacteria, leading to the assumption of amphibian species-specific and population-specific microbiota (Walke *et al.*, 2014). Another leading factor in determining the composition of amphibian skin microbes is skin sloughing. However, this is a controversial factor seeing that one study found that skin sloughing reduces skin microbe abundance substantially (Myers *et al.*, 2012), while a bioaugmentation study has found that in *Rana muscosa* some bacteria persist on the skin after sloughing with no available environmental inoculum (Harris *et al.*, 2009).

The above-mentioned factors are attributing to the colonization of bacteria on amphibian skin and in tadpole mouthparts and how these bacteria differ or are similar to bacteria found in the surrounding environment. However, results suggest that there is also a significant variation in the microbiome of various frog species and a very distinguishable variation between adults and tadpoles. A study to investigate the population predictors of microbiome structure in amphibians found a link between the Gosner stage and the mouthpart microbiome composition indicating that ontogenetic variation is not just the result of increasing mouthpart size but more specific to the changing composition of the community that surrounds the tadpole (Griffiths *et al.*, 2018). Throughout the larval development, behavioural changes are observed, and ontogenetic variation can likely arise when individuals are exposed to various environmental conditions influencing their microbial composition (Griffiths *et al.*, 2018). Results indicate that this might be the reason for various bacteria observed in tadpoles and adults with an overall small overlap (Figures 4-7 & 4-8).

Ecomorphological tadpole guilds found during the study

Originally four morphological types of tadpoles were recognized largely based on microhabitat, tadpole development, and external morphology (Orton, 1953) until internal oral structures were brought into consideration and six developmental guilds of endotrophic larvae and 18 ecomorphological guilds of exotrophic larvae were described (Altig & Johnston, 1989). Upon tadpole sampling, it was clear that the sampling site was habitat to a diverse amphibian community with tadpoles from different ecomorphological guilds present (Table 4-5). It is important to note that individuals of the same genus are not necessarily grouped in the same guild and that two species from the same genus can be grouped into separate guilds (Botha, 2013). A study on exotrophic anurans from South Africa identified and described 10 ecomorphological guilds based on previous classifications (Altig & Johnston, 1989) as well as feeding behaviour (Botha, 2013). According to the ecomorphological guilds established by Botha

(2013), the sampled tadpole species can be categorized into five separate ecomorphological guilds namely, Benthic, Lentic-nectonic, Suspension feeder, Rheophilic, and Lentophytolytic (Botha, 2013). Four of the sampled tadpole species are positioned in the Benthic guild, these species include *Ptycadena mossambica, Strongylopus grayii, Chyromantis xerampelina* and *Ptycadena anchietae.* The individuals in the Benthic guild are distinguished based being found in either lentic or lotic systems as well as physical features, including dorsolateral eyes, anteroventral oral discs, rounded heads, and a moderate jaw sheath (Botha, 2013). Two representatives of the suspension feeder guild have been sampled namely, *Phrynomantis bifasiatus* and *Xenopus laevis* and are characterized as filter feeders with an absent jaw sheath and labial teeth and laterally positioned eyes (Botha, 2013). *Phrynobatrachus natalensis* was sampled and categorized within the lentophytolytic guild. This guild is considered a new delineation with individuals found in mid-water lentic systems with a ventrally situated oral disc (Botha, 2013). The lentic-nectonic guild consists out of species that are normally found in mid-water whose eyes are laterally situated and *Kassina senegalensis* was sampled as a representative of this guild (Botha, 2013). *Amietia delalandii* is characterised by dorsolateral eyes and has a long developmental time while found in lotic water systems, therefore, these species are categorized in the Rheophilic guild (Botha, 2013). Although ecomorphological guilds were not originally considered during the sampling it becomes evident that with each guild occupying different regions in a water body it is plausible that the individuals from each guild are exposed to different environmental factors including different bacteria within the water. Based on the distribution of bacteria from various tadpole species (Figure 4-8) it is probable that the ecomorphological guild to which a species belongs is also the determinant for the bacteria they are being exposed to. This is easily seen with *P. bifasiatus* having a completely separate set of bacteria in their mouthparts (Figure 4-8) and also being the only sampled species in the suspension feeder guild. However, *K. senegalensis* and *S. grayii* are categorized in different guilds but overlap in bacteria found in their mouthparts are evident (Figure 4-8) and can be explained due to both species being found in mid-water and therefore being exposed to more or less the same type of bacteria. *P. mossambica*, *C. xerampelina* and *P. anchietae* are all placed in the Benthic guild and has a distinguishable overlap in bacteria together with *A. delalandii* from the Rheophilic guild (Figure 4-8). With both guilds being found in lotic water systems it supports the theory that these species are exposed to the same bacteria found in the water.

5.2 *Bd* challenge assay

The life cycle of *Bd* is considered a simple progression from a motile zoospore to a thallus producing a single zoosporangium that cleaves into new zoospores exiting the sporangium through one or more papillae (Berger *et al.*, 2005). The only variation observed in this simplified

life cycle is the formation of more than one sporangium from one zoospore called colonial development (Longcore *et al.*, 1999). During the challenge assays, this simplified *Bd* life cycle was observed through the spectrophotometer absorbance readings. The positive control can be observed where no bacterial cell-free supernatant was added and *Bd* was allowed to grow optimally at 22°C (Berger *et al.*, 2005).

Results suggested that there was no statistical significance between the data obtained from the spectrophotometer absorbances of *Bd*GPL and *Bd*CAPE. These results can be regarded as significant due to previous results obtained from a study where it was found that there exists significant variation in the ability of bacteria to inhibit the growth of different *Bd* isolates (Antwis *et al.*, 2015). However, this study consisted of various *Bd*GPL isolates that were challenged against mucosome (containing bacteria and skin secretions) from anuran skin (Antwis *et al.*, 2015). Furthermore, the Kruskal-Wallis test for both the *Bd*CAPE (Table 4-7) and *Bd*GPL (Table 4-8) indicated there is a statistical significance between the bacterial isolates. This can be confirmed by Figure 4-9 where positive and negative values can be observed regardless of the *Bd* lineage. These results lead to the assumption that the cell-free supernatant of different bacteria has various effects on the growth of *Bd,* suggesting that *Bd* inhibition is dependent on the cell-free supernatant and that these cell-free supernatants will affect *Bd* regardless of the lineage used for the challenge assays.

On day six the recorded photographs provided visualization for the effects of the cell-free supernatant on the *Bd* isolates (Figure 4-15). Figure 4-15 A & B records the negative and positive controls, respectively, which are typical of the respected treatments and provides a reference for other observed effects. In Figure 4-15 G, *Bd* augmentation is photographed, coupled with figure 14 D where a live sporangium with discharge papillae is observed, however, when compared to the positive control (Figure 4-15 B) it is evident that growth is not as optimal as it is without any cell-free supernatants added. This is supported by Figure 4-9 where no bacterial cell-free supernatant added to either *Bd*CAPE or *Bd*GPL augmented *Bd* growth in such a manner that the mean absorbance surpassed that of the positive control. With the addition of cell-free supernatants, it is seen in some case that partial inhibition of *Bd* growth takes place, this can be seen in Figure 4-15 C where zoospores have encysted and fine branches of rhizoids have developed, the thalli have grown and the cytoplasm becomes more complex however the sporangium didn't fully mature and is still in the germling stage (Berger *et al.*, 2005). Another effect of the cell-free supernatant is where the sporangium matured passed the germling stage but remain empty with no zoospores developing inside of the sporangium, this can be observed in Figure 4-15 F. In extreme cases, the addition of bacterial cell-free supernatants caused total *Bd* inhibition where cell lysis took place and can be seen in Figure 4-15 H. In Figure 4-15 D and

F it can be seen that when *Caulobacter vibrioides* was added to two different lineages of *Bd* different results was observed. In *Bd*CAPE the addition of *Caulobacter vibrioides* cell-free supernatant caused augmentation (Figure 4-15 G) while in *Bd*GPL partial inhibition (Figure 4-15 F) were observed. These results were however expected due to results obtained from a study where the cell-free supernatants of different bacterial species were challenged against *Bd* in a 96-well plate layout and similar results were recorded (Bell *et al.*, 2013). An interesting occurrence of *Bd,* with the addition of bacterial cell-free supernatant, is colonial development observed in Figure 4-15 E. This type of development takes place when internal septa form in the early development of the thalli and lead to the development of a group of sporangia from a single zoospore (Longcore *et al.*, 1999). It is, however, unclear if this type of development is enhanced by the addition of a certain bacterial species' cell-free supernatant and if this is a common occurrence for the *Bd* isolate used for the study, but seeing that colonial development was only observed when *Massilia sp.* cell-free supernatant was added to *Bd*GPL it is plausible that the addition of this bacteria is responsible for this type of development.

After the effects that individual bacterial cell-free supernatants can have on the growth of *Bd* was determined*,* it was important to study the effects that a consortium of bacterial cell-free supernatants have on *Bd.* Metabolites are described as the final products of cellular processes (Rebollar *et al.*, 2016), however, the networking between metabolites are not limited to a single cell but occurs in complex assortments (Yim *et al.*, 2006). The effects of a consortium of bacteria have been proven to be stronger at inhibiting *Bd* growth (Antwis & Harrison, 2018; Harrison *et al.*, 2020). In any given environment, whether soil, water or the gastrointestinal tract of mammals, the entangled microbe communities communicate with each other through small-molecule signals, which science is yet to understand and decipher (Yim *et al.*, 2006). The low concentration antibiotics that are produced by microorganisms have the ability to influence the metabolism as well as the physiology of co-habiting microbes (Yim *et al.*, 2006). The interaction between microbes has been proven where single soil bacterial strains challenged against plant fungi caused moderate growth reduction but when a bacterial mixture was challenged against the same fungi the inhibition in growth was much stronger (De Boer *et al.*, 2007). These results led to the argument that cohabitation of microbes triggers antibiotic production and that the reduction in the growth of the fungi was a side-effect of the secondary metabolites produced by the bacteria (De Boer *et al.*, 2007). During the current challenge assays, similar results were obtained, however, bacteria that seemingly inhibited the growth of *Bd* caused augmentation when grouped with bacteria from other families. It has been found that bacteria from the family *Enterobacteriaceae,* which are commonly found in the gastrointestinal tract, produces microcins, a class of small molecules with antibiotic activity (Yim *et al.*, 2006). Indeed, when an *Enterobacter sp.* was added to the consortium augmentation instead of inhibition in *Bd* growth was observed (Figure 4-13). It

is possible that the antibiotic metabolites produced by *Enterobacter sp.* inhibited the production of metabolites from other bacterial species leading to augmentation rather than inhibition on the growth of *Bd.* With regards to inhibition, consortium 4 showed the most inhibition of *Bd.* Interestingly, consortium 4 contained only cell-free supernatants of bacterial isolates that when challenged singularly against *Bd* also inhibited the growth of *Bd.* Although the metabolites produced by the bacteria is unknown it is evident that the addition of bacterial cell-free supernatant has varying effects on the growth of *Bd* and that a lot of research needs to be done to determine the intricate relationship that evidently exists between *Bd* and bacteria.

It is very important to note that according to Bell *et al.* (2013) when inhibition of *Bd* growth is greater than the maximum possible value for nutrient depletion the inhibition is considered genuine and not just the effect of nutrient depletion. Furthermore, growth enhancement or augmentation of *Bd* cannot occur as a result of nutrient depletion and should therefore be considered as a genuine cause of the addition of bacterial cell-free supernatant (Bell *et al.*, 2013).

CHAPTER 6 CONCLUSION AND RECOMENDATIONS

With amphibian skin microbiome becoming the model system to understand multi-faceted relationships between microbiome and pathogens (Harrison *et al.*, 2019) it is important to understand how the microbiome on amphibian skin is assembled and how amphibians acquire their various microbiomes. Research has found that amphibians likely acquire their microbiome from transmission from the environment (Walke *et al.*, 2014; Griffiths *et al.*, 2018) and it has been found that similar bacteria can be found on amphibians and in their surrounding environment (Walke *et al.*, 2014; Bates, *et al.* 2018).

Therefore, a study was conducted to determine whether the acquisition of bacteria from the environment was applicable at any given geographical site. Molecular analysis of bacteria isolated from water samples and bacteria sampled from adult amphibian skin and tadpole mouthparts revealed that it is plausible that amphibians might acquire their microbiome from the environment. Results revealed a correlation between the bacteria found in the environment and the microbiome of adult frogs. These results are consistent with previous studies where it was found that amphibian skin harbours bacteria that are found in relatively low abundance in the environment (Walke *et al.*, 2014). However, results also indicated that tadpoles have a more unique microbiome and that there is only a small overlap in bacteria found in water and bacteria found on tadpole mouthparts. It was further concluded that across amphibian species there is also clear differentiation between their microbiome, which can likely be attributed to their ecomorphological guilds where various tadpole species are found in different sections of a water body and having different feeding behaviours.

Previous studies have proven that bacteria can be transmitted to hosts through both direct and indirect transmission (Rebollar *et al.*, 2016). Therefore, a few individuals can be inoculated with a bacterium and transmit said bacterium to their surrounding environment as well as to other individuals (Rebollar *et al.*, 2016). A plausible theory as to microbiome acquisition is that various amphibian species are exposed to various environments and therefore are acquiring different bacteria through direct and indirect transmission. Interactions between species are further responsible for exposing one another to each other's microbiome and therefore transmitting some of their bacteria to each other. This relates back to ecomorphological guilds and the likelihood of various species interacting with each other.

The results obtained from the *Bd* challenge assays concluded that bacteria cell-free supernatant does have inhibiting and augmenting effects on the growth of *Bd.* Furthermore, it was important to note that effects caused by nutrient depletion should be considered when conducting challenge

assays due to nutrient depletion having an inhibitory effect on *Bd* (Bell *et al.*, 2013). The challenge assay results unfortunately did not contain information on the metabolites produced by the bacteria, therefore it is recommended that future studies determine the metabolites produced by the bacteria to narrow down the effects that specific metabolites have on *Bd* growth. A limitation of the study includes the small variation in bacteria used for the *Bd* challenge assays. With only 20 bacterial species used, the possibilities of the effects that other bacteria might have on *Bd* is endless. It is further recommended that bacteria from various sources such as soil and plants could also be used to further explore the effects of bacteria on the growth of *Bd.*

It is imperative to do extensive research to better understand the complex relationship between bacteria and pathogen but also the relationship between amphibians and bacteria. With a better understanding of the relationship that exists between amphibians and their surrounding environmental bacteria the possibility of introducing a bacterial species with inhibitory effects into an environment can be narrowed down to select the best possible candidate.

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