PHYSIOLOGICAL CHARACTERISTICS, AMYLASE PROPERTIES AND METAGENOMIC ANALYSES OF BACTERIAL AND FUNGAL COMMUNITIES FROM IKOGOSI WARM SPRINGS

BY

Deborah Ebunoluwa FASESAN

B.Sc. Microbiology (OAU), M.Sc. Microbiology (Ibadan)

Matric. No.: 166310

A Thesis in the Department of Microbiology

Submitted to the Faculty of Science,

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

of the

UNIVERSITY OF IBADAN

AUGUST, 2023

CERTIFICATION

This is to certify that the Research work reported in this Thesis was carried out by DEBORAH EBUNOLUWA FASESAN (Matric Number 166310), under our supervision in the Department of Microbiology, University of Ibadan, Nigeria.

……

SUPERVISOR

A. A. Onilude,

B.Sc. (Ife), M.Sc., Ph.D. (Ibadan),

Professor, Department of Microbiology

University of Ibadan, Nigeria

……

CO-SUPERVISOR

Olubusola A. Odeniyi,

B.Sc., M.Sc., Ph.D. (Ibadan),

Reader, Department of Microbiology,

University of Ibadan, Nigeria

DEDICATION

To the memories of my parents, Prof. and Mrs. S.O. Fasesan, your deposit of prayer yielded so much, even in your absence. Continue to rest in the bossom of the Almighty.

ACKNOWLEDGEMENTS

To the giver of life, in whom I am nothing without– The Almighty God, I acknowledge you for being so mindful of me.

My profound gratitude to Prof. Abiodun Anthony Onilude, my supervisor, truly, you gave your all, you were selfless and caring, and you are a Professor of deep understanding. Dr. Olubusola A. Odeniyi, my co-supervisor, oh! How I worried and disturbed you, yet you never complained, your type is very rare, thank you so much. I want to also express my gratitude to the Head of Microbiology Department, Prof. A.A. Ogunjobi for his support and counsel. To Prof.Sherifat M. Wakil, I say thank you, for all your concerns and help during this project.

My sincere appreciation goes to Prof. Oyekanmi Nash, the Director of Research - Genetics, Genomics and Bioinformatics Department of the National Biotechnology Development Agency, for the push, the constant checks and balances, and most importantly, his genuine interest in Science.

I would also like to express my sincere gratitude to all the teaching staff of the Department of Microbiology, for leaving their doors open to my questions and inquiries. To all the non-teaching staff, as well, for their availability, allowing me to spend endless hours in their offices while tidying up my project, I say thank you so much.

I appreciate my husband, for assisting me in times when I could not do much for myself, for his emotional support and for keeping things together when I couldn't be available. I am also grateful for the gift of Inioluwa- thank you for being you.

I would also like to thank members of staff of the National Biotechnology Development Agency, both in Abuja and Ibadan, for always being mindful of my program, especially Mrs. Temitope Owoeye, Mr. Yinka Aderoju and Mrs. Adejoke Victoria. To the gift of sisters and brothers I made while in the laboratory – Oluwabusola Fatade, Abimbola Olajide, Jude Adeola, and Kehinde Omidoyin. Thank you all so much, there was always a reason to smile despite all we had on our table.

ABSTRACT

Several warm and hot springs exist all over the world whose microbial diversity and physicochemical properties have been explored using both conventional and metagenomic methods to reveal biotechnologically relevant bacterial and fungal microbiomes. The Ikogosi Warm Springs (IWS) in Ekiti State, Nigeria is known for its unique warm and cold water outflow and tourism activities. Unlike other climes**,** there is paucity of information on the bacterial and fungal microbiome of IWS. This work was therefore designed to determine the taxonomic composition of the bacterial and fungal microbiome of IWS as well as prospect for alpha-amylase of potential industrial importance.

Sediment (20) and water (20) samples were collected from IWS during the wet and dry seasons for physicochemical analyses using standard procedures. The bacterial and fungal microbiome of IWS were evaluated using the V1–V3 regions of 16SrRNA and ITS1, respectively. Genomic libraries were constructed for paired-end sequencing and the microbial taxa present were analysed using DADA2 metagenomics pipeline, where alpha and beta diversity statistics and metrics were also computed. Bacteria were also isolated from samples employing culture-dependent method and screened at 50°C for alpha-amylolytic properties using starch-supplemented media. Molecular identification of bacterial isolates, with the highest zone of hydrolysis, were done and alpha-amylase production and enzyme characteristics were determined using UV/visible spectrophotometry. Data were analysed using descriptive statistics, and ANOVA at $\alpha_{0.05}$.

The pH and temperature of IWS ranged 6.36 to 7.93, and 28.3 to 38.0°C, in wet season, and 5.8 to 7.6, and 25.7 to 38.3°C, in dry season. In IWS water, Proteobacteria (84.4, 83.4%), Bacteroidetes (10.0, 16.0%), Firmicutes (2.4, 7.5%), Ascomycota (62.0, 89.0%) and Basidiomycota (37.7, 10.7%), were dominant in the wet and dry seasons; while the sediments (%) had 62.6 and 45.4; 21.4 and 53.5; 15.0 and 0.4; 65.1 and 65.1; 34.8 and 33.5, respectively. Alpha diversity measures indicated no significant phylogenetic diversity between the bacteriome and fungal microbiome of water and sediment, in both seasons. A significant difference occurred in the diversity between the bacteriome of water and sediment samples. Beta diversity showed significant differences in taxonomic similarity and relative abundance in water and sediment of both bacterial and fungal communities in both seasons. *Vogesella*, *Aeromonas*, *Comamonas*, *Brevundimonas* and *Malassezia* were present in both water and sediment samples. Culture-dependent isolated amylolytic bacteria constituted 27.1% of 174 isolates. The best amylolytic isolates were *Bacillus cereus*-MPW3E and *Bacillus siamensis*-SW3F. Optimum alpha-amylase production of these bacilli was observed at 55°C; pH 7.0; Ca^{2+} supplementation; 1% seed inoculum and 48-hour incubation period. The alpha-amylase activities for *Bacillus cereus*-MPW3E and *Bacillus siamensis*-SW3F were optimum at 1% substrate concentration, with pH 6.0 and 7.0, and at 50°C and 55°C, respectively. The Michaelis Menten constant was 0.197 and 0.052 mM, with maximum velocity of 6.305 and 25.974 µmol/min for *Bacillus cereus*-MPW3E and *Bacillus siamensis*-SW3F, respectively.

Microbiome taxonomic diversity of Ikogosi warm springs predominantly contained phyla Proteobacteria, Bacteroidetes, Firmicutes, Ascomycota and Basidiomycota. Thermotolerant alpha-amylase-producing *Bacillus cereus* and *Bacillus siamensis* were also obtained.

Keywords: Warm springs microbial diversity, Firmicutes, Ascomycota, Alpha-amylase.

Word Count: 488

TABLE OF CONTENTS

CHAPTER FIVE: DISCUSSION

CHAPTER SIX: SUMMARY, CONCLUSION AND RECOMMENDATIONS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1 Background to the study

Microorganisms are known to exist in every nook and cranny on earth. They are prevalent in many natural environments, including soils, aquatic organisms, and even in humans (Prescott *et al.,* 2018). Despite the fact that microbes are found everywhere, not all of them have been identified. This is because it can be difficult to cultivate some microorganisms, which limits our ability to fully analyse microbial ecosystems (Prescott *et al.,* 2018).

The maintenance of both terrestrial and aquatic ecosystems depends on microbial diversity. Aquatic environments, including freshwater and marine ecosystems, contain bacteria, viruses, archaea, and microbial eukaryotes (Thompson *et al.,* 2017). The control of biogeochemical cycling processes like nitrogen fixation and carbon cycling depends heavily on this community (Aryal *et al.,* 2015). Microbial communities capable of supporting aquatic life, such as fisheries and other aquaculture industries, also contribute to a healthy aquatic ecosystem. Algal bloom, pollutant cycling, shortage of portable water, and ultimately the demise of aquatic plant and animal life in these water bodies can result from changes in their microbial populations (Kumar *et al.,* 2015).

Microbes have been studied under a microscope and in culture-based methods to characterise the microbial diversity in aquatic ecosystems. These methods, however, are constrained to microbes that can only be cultured under typical laboratory conditions, making it difficult for them to adequately characterise complex microbial systems (López-López *et al.,* 2015). In order to overcome this difficulty, recent molecular-based advancements are being used. These advancements can be used to assess the structure and diversity of microbial associations on yet-to-be-cultured and culturable bacteria existing in these ecosystems (Agrawal *et al.,* 2015). Culture-independent methods that focus on obtaining entire microbial nucleic acid from the environment in order to gain a greater understanding of the composition and behaviour of microbes that exist there is

known as metagenomics (Sharma *et al.*, 2020). Since a wealth of knowledge has been revealed using metagenomics to uncover rare microbes with potentials in industrial biotechnology, many researchers have actively been engaged in using it to find answers to questions in microbial ecology, microbial diversity, and in industrial biotechnology (Tshikhudo *et al.*, 2013; Ghosh *et al.*, 2019).

Sequence-based metagenomics can be used to discover homologous genes, which enables phylogenetic analysis, taxonomic unit clustering, and taxonomic assignment of genes. Additionally, metagenomics can be function-based, in which case active clones from a metagenomic library are screened for interesting products (Ekkers *et al.*, 2012). Researchers have employed functional metagenomics extensively to find novel genes and metabolites. For instance, the research of López-López *et al.* (2015), which used a metagenomic library created from the hot waters of Lobios in Spain, led to the discovery of novel lipolytic enzymes. The identification of protease novel enzymes with a 60 °C peak activity from a mangrove sediment in Brazil, and the discovery of esterases in a field contaminated with hydrocarbons in Brazil (Maester *et al.*, 2016). According to the works cited, metagenomics methodology enables a thorough understanding of the evolution, ecology, and function of sequences that code for significant genes that exist naturally in environmental habitats like plants, soil, animals, and even in unusual environments like hot springs, warm springs, deep-sea vents, etc. (Sharma *et al.*, 2020).

A spring is a location where groundwater naturally emerges from the Earth's subsurface, typically in a defined flow and in quantities large enough to produce a pool or a stream. They can discharge fresh groundwater directly into the ground or into the banks of streams and rivers (Talabi, 2013). Water springs are crucial, as they serve as areas of transition between the terrestrial and aquatic ecosystem. According to Pedron *et al.* (2019), microbial communities in water springs typically contain both culturable and unique species that could serve as potential biotechnological reservoirs. They also often contain endemic microbes that are found only in surface water. Warm and hot thermal springs can be distinguished based on how close they are to the body's temperature, which is 98 degrees Fahrenheit or 37 degrees Celsius. A warm spring is one that is created by groundwater that has been geothermally heated from deep within the earth's crust. However, as groundwater is forced to the earth's surface by some fractures and earth faults, the temperature of the spring rises. The deeper the underground water, the greater the temperature of the spring generated. There are numerous warm and hot

springs around the globe whose microbial diversity has been investigated utilising metagenomic method. These include Xiamen Hotsprings, China (Zhao *et al.*, 2017), Hot springs of Galacia, Spain (López-López *et al.*, 2015), Thermophylae Hotsprings, India (Zarikas *et al.*, 2014), and Ikogosi Warm Springs, Nigeria (Fasesan *et al.*, 2020).

Ikogosi Warm Springs is one of the many tourist attractions in Nigeria, which is located in Ikogosi town, in longitude 4°56.46°E and latitude 7°36.88°N in Ekiti State, southwest Nigeria (Talabi, 2013). It is distinctive because it is a spring made up of two separate springs with various thermal identities. The cold spring comes from a distinct source deep within the forest and flows outward before merging with the warm spring to form a single flowing stream. The warm spring falls down a rocky hill that is encircled by towering trees (Ojo and Agbede, 2014). The spring records virtually daily human activities brought on by town residents as well as tourist visits, particularly at the meeting point of the cold and warm springs. The spring has been a significant source of interest for many years and is even thought to have some medicinal potentials by some indigenes of Ikogosi town, and is also a well-known source of water supply for residents living in close proximity to the spring.

Ikogosi spring is not unknown to researchers in Nigeria; it has served as the location of numerous samplings for those working in a variety of fields. For instance, Olajuyigbe and Ajele (2005) used a culture-based technique to examine the spring's potential as a source of thermotolerant protease from isolates generated from the spring. Ikudayisi *et al.* (2015) researched the spring's chemical and hydro-geologic analyses, while Hairul and his colleagues (2013) looked into the spring's potential as a tourist destination. The examination of the spring employing culture-independent analysis to thoroughly examine the potentials of the spring is best described as scarce, despite the site playing host to multiple researchers. Therefore, there is a need to critically investigate the industrial potentials of the spring in a bid to place the spring on the pedestal for harnessing microbial bioproducts.

1.2 Statement of problem

The true and complete potentials of microbial communities present in Ikogosi Warm Springs have not been fully ascertained for improved biotechnological innovation, due to limited diversity studies.

1.3 Justification for the study

Owing to the unique thermal and other physicochemical properties of Ikogosi Warm Springs, it would be significant to harness the pool of residential microbes in the spring for potential biocatalysts and other value-added compounds for industrial use. The ability of microorganisms to withstand high temperature makes microbes (particularly extremophiles) important in industries, because their metabolites can be harnessed for use in harsh conditions, especially those found in industrial environments.

1.4 Aim and objectives

This study is aimed at a comprehensive assessment of the genetic content of culturable and 'yet-to-be-cultured' microbes present in sediments and water samples of Ikogosi Springs in relation to the chemical, physical and biological parameters that characterise the spring.

1.5 Specific objectives

These include:

1. Physicochemical characterisation of soil and water samples from different sampling points during different seasons.
2. Isolation and identification of bacteria and fungi from sediment and water samples at different seasons.
3. Screening of bacterial isolates for thermostable alpha-amylase using culture-dependent methodology.
4. Optimisation, characterisation and purification of probable thermostable alpha-amylase present.
5. Targeted metagenomic (16S and ITS) analysis of microbial diversity of the spring at different seasons.

CHAPTER TWO

LITERATURE REVIEW

2.1 Exploring microorganisms in the natural environment

Microbial Ecology studies the diversity, abundance, and distribution of microorganisms within an ecosystem (Fakruddin and Mannan, 2013). The variety, quantity, and dispersion of microorganisms within an ecosystem are studied by microbial ecologists. Likewise, it entails researching their impact and their particular connections to ecosystems (Prescott *et al.,* 2018). It is also the characterisation of microbial communities and identification of elements that contributes to the ecosystem's diversity (DeLong, 2013). The variety found within genetically varied groups of viruses, bacteria, fungus, and archaea is referred to as microbial diversity. In-like manner, it is ecologically important to uncover and comprehend microbial diversity communities, including the number of species and their relative abundance (Vitorino and Bessa, 2018).

According to numerous studies, the variation seen in living organisms can be attributed to many billions of evolutionary changes (Aryal *et al.,* 2015). Microbial diversity can be observed in numerous forms, such as cell shapes and sizes, motility patterns, pathogenicity variations, various ways of adapting to harsh environmental circumstances, physiology, phylogeny, and cell division mechanisms (Prescott *et al.,* 2018). Although Edouard Chatton, in the early 1920s, divided the types of life on Earth into two groups - Prokaryotes and Eukaryotes (Castelle and Banfield, 2018). The prokaryotic member was further separated into two groups: Archaea and Bacteria, which appeared to comprise of millions of different species and dominated practically all kinds of habitats (Torsvik *et al.*, 2002). From well-known habitats, such as water, animals, and soil, to the least common habitats, such as those with harsh environmental conditions (Prescott *et al.*, 2018). Despite this, fewer than 12,000 prokaryote species have been comprehensively described and catalogued, excluding a greater fraction of the variety of prokaryotes that have not been described (Aryal *et al.*, 2015; Vitorino and Bessa, 2018).

Microorganisms, which is one of the most prevalent life forms in the biosphere, are essential to the ecosystem, because they speed up the processes of several biogeochemical cycles, the breakdown of organic matter, and food chains (Aryal *et al.*, 2015). In-depth understanding of microbial diversity research can show us how to cure diseases, protect the environment, grow crops that are healthier and better, and much more (Vitorino and Bessa, 2018).

2.2 Microbial evolution – The birth of microbial diversity

Biological diversity encompasses all of the different types of life that exist in nature, from the smallest ecological unit to the genetic level (Castelle and Banfield, 2018). For more than ten years, molecular-phylogenetic analysis has provided researchers with the possibility to create an intricate map of evolutionary diversification, which has been able to show that diversity is primarily microbial (McMurdie and Holmes, 2013). This is due to the fact that microorganisms have been on Earth considerably longer than other life forms. According to estimates, the first living organisms on Earth emerged around 3.5 billion years ago (Reid and Buckley, 2011). It was believed that the first life form would have used inorganic resources for biosynthesis, also known as lithotrophic metabolism (Ghosh and Dam, 2009). According to Zahn (2017), there are currently over 40 major bacterial divisions and over 12 divisions in the archaea kingdom. Since these microbes—bacteria and archaea—have been hypothesized to have existed before other groups of living things, they may have undergone additional diversification and possess the greatest level of biological variety.

This hypothesis was also postulated by a novel interpretation of the tree of life created by Hug *et al.* (2016), whose tree showed that microbial variety predominated over other forms of life. According to some accounts, the tree of life is one of biology's core organising principles and probably the best-known representation of the evolutionary process (Castelle and Banfield, 2018). The notion behind Darwin's theory of biological evolution was that all life on Earth descended from a common ancestor known as the Last Universal Common Ancestor (LUCA) and that the diversity of these ancestors might be represented as a tree-like structure known as the Tree of Life (Castelle and Banfield, 2018). It illustrates the connections that exist between organisms, explains how they are related to one another, and speculates on their possible origins (Castelle and Banfield, 2018). However, since Charles Darwin's first draft of the tree, the tree has

undergone some notable alterations. The creation of phylogenetic trees using data derived from DNA sequences was one major innovation (Gaucher *et al.*, 2010). Carl Woese and his colleagues used this innovation to create a new classification of microbial life, leading them to define three domains: bacteria, archaea, and eukaryotes (Woese *et al.,* 1990; Gaucher *et al.,* 2010).

2.3 Importance of studying microbial diversity and ecology

Microbial diversity is a vital component of the Earth's overall biological diversity (Joshi *et al.,* 2016). There is still a significant amount of genetic information from microorganisms in the natural ecosystem that has not been explored (Fakruddin and Mannan, 2013). It has been shown that more than 90% of the microbial community in some habitats has not been cultivated using conventional laboratory procedures, supporting the idea that culturable microorganisms only make up a small portion of the overall microbial population present (Fasesan *et al.*, 2020). Therefore, employing a combination of common laboratory procedures and culture-independent methods, diversity studies is becoming more relevant in the identification of numerous undiscovered and undescribed microbial species (Fakruddin and Mannan, 2013; Ghosh *et al.*, 2019).

Microbial ecology and diversity studies are crucial in identifying the genetic differences and functionality of species within an ecosystem, in addition to characterising the numerous types of species that exist in different habitats (Joshi *et al.,* 2016). It also assists in identifying their (the microbial consortium's) ecological function in specific habitats and climate regulation (Ducklow, 2008). Microbial diversity studies have been known to address significant agricultural issues (such as plant health protection, decreased crop yield, soil maintenance, etc.) as well as environmental issues like soil and water bioremediation from organic and inorganic pollutants. This is because of a number of high-level biochemical and molecular advances (Yang *et al.*, 2016).

The metagenomics approach, which has been around for more than 20 years has completely changed the way microbial diversity investigations are conducted (Ghosh *et al.,* 2019), as it entails the direct recovery of genetic material from environmental samples. In addition to helping to reveal novel molecules, compounds, and metabolites with significant applications and functionalities that were previously unknown, this field

can also help to elucidate the structure of microbial populations and metabolic pathways (Simon and Daniel, 2011; Madhavan *et al.,* 2017). Therefore, in order to access untapped genetic resources from the natural environment for biotechnological applications, microbial ecology and diversity research are becoming increasingly important (Yang *et al.,* 2016b).

2.4 Microbial diversity in aquatic systems

Aquatic biodiversity is a significant issue that is currently being dealt with by many researchers, ecologists, and conservation authorities around the world in the era of environmental changes like anthropogenic interventions (intense agricultural activities, deforestation, use of pesticides, etc.), climatic changes, biological disruption, and pollution (Madhavan *et al.,* 2017). Water covers seven tenths of the surface of the Earth. About 96.1% of the world's water is present between continents as oceans, with the remaining 3.5% occurring largely as groundwater and polar ice within continents' borders (Alexopoulos *et al.,* 2009). In comparison to life on land, aquatic systems offer a number of benefits, including buoyancy, movement by water currents, spread of motile components like genetic elements, and mobility of soluble organic and inorganic nutrients are some of the benefits that life in aquatic systems have over land-based living (Sigee, 2005).

According to Cañedo-Argüelles *et al.* (2018), aquatic environments can be roughly categorised into freshwater ecosystems (lakes, springs, ponds, swamps, rivers, and streams) and marine ecosystems (estuaries, oceans, and sea). These habitats are home to a variety of various microorganisms that can either be harmful or beneficial. However, these diverse microorganisms play a significant role in various ecological processes, such as active engagement in a variety of chemical transformations, and are also one of the essential factors in the evaluation of water quality (Fang *et al.,* 2022). The differences between freshwater and marine habitats include their depth, salinity, nutrient content, and average temperature. However, both habitats provide great environment for a variety of microorganisms (Aryal *et al.,* 2015). Microbes in aquatic systems, particularly in water bodies with low nutrient contents, appear to develop on stationary, solid supports (Cañedo -Argüelles *et al.,* 2018). Instead of randomly drifting with the stream, using this strategy would put a microbe in greater touch with nutrients that are available and more abundant (Tuson and Weibe, 2013). As a result, the majority of aquatic

residential bacteria frequently contain attachments that are used to adhere to different solid matrices (Aryal *et al.,* 2015). Freshwater ecosystems have been shown to be essential to human existence and welfare since they are significant sources of irrigation, food, and drinking water (Caedo-Argüelles *et al.,* 2018). On the basis of their physical and chemical characteristics, freshwater ecosystems can be divided into different groups (Sigee, 2005; Singh *et al.,* 2014a). Standing waters, sometimes referred to as lentic habitats, include ponds and lakes; running waters, often referred to as lotic habitats, include rivers, springs, streams, and wetlands, which include places that are notably wet for the most of the year, such as swamps and marshes (Sharma and Giri, 2018).

Eukaryotes, Archaea, and Bacteria are the three major classes of microbes that predominate in freshwater habitats, with viruses making up a smaller subset (Dodds and Whiles, 2010). According to several structural, biochemical, and physiological traits, these microbes can be recognised (Alexopoulos *et al.*, 2009). In freshwater systems, bacteria are among the most conspicuously prevalent microbes. They belong to the Eubacteria kingdom, but this kingdom also includes Actinomycetes and blue-green algae (Sigee, 2005). Members of the Archaea domain are also found in freshwater habitats; however, they are typically restricted to those with harsh conditions. According to Purves *et al*. (2004), the domain eukaryota, which derives its nomenclature from the fundamental eukaryotic organization of its cells, can be divided into four kingdoms: Protista (which includes the microalgae and protozoans, two significant groups of freshwater microbes), Fungi, Plantae, and Animals (Prescott *et al.,* 2018).

2.5 Factors affecting microbial diversity

According to Fakruddin and Mannan (2013), the microbial ecosystem is commonly affected by biotic and abiotic components. Biotic components include macro-organisms like plants and animals, protozoans, and others, for example, transposons, plasmids, and phages (Zhao *et al.*, 2012). Abiotic components are elements of the environment that have an impact on the structure and activity of microbial communities (Li *et al.*, 2015). Temperature, pH, salinity, organic and inorganic nutrients, etc. are a few of them (Li *et al.,* 2015, Adedire *et al.,* 2021, Adedire *et al.,* 2022). One of the main goals of microbial diversity and ecology studies is to explore these environmental components that influence microbial communities (Adedire *et al.,* 2021).

2.5.1 Temperature

Temperature is one of the core environmental component that significantly affects the organization of microbial communities (Li *et al.*, 2015; Li *et al.,* 2021). It plays a crucial role in determining the distribution of different functional categories of microorganisms in a given environment, either directly or indirectly (Zhou *et al.,* 2016).Since a rise in temperature can speed up metabolism, growth, and the time it takes for a population to double in size, it can help create and maintain a greater variety of species (Adedire *et al.,* 2021). An increase in temperature in terrestrial ecosystems is typically associated with an increase in the productivity of the ecosystem, which may allow for the support of more species (Wang *et al.*, 2009). A wider variety of plant species may result from higher temperatures (Zhou *et al.,* 2016). Increased microbial variety may result from higher plant diversity because more plants may serve as supplies of nutrients, substrates, or physical attachments for more microorganisms (Lange *et al.*, 2015).

Additionally, temperature might have an impact on how different species interact. According to studies, rising temperatures are associated with higher rates of ecological association and contact, including predation, competition, and parasitism, all of which have a pronounced impact on species diversity (Chen *et al.*, 2012). Microbial diversity can be indirectly impacted by temperature when combined with other environmental parameters as nutrient availability, pH, carbon availability, and water availability (Zhou *et al.*, 2016). For instance, greater temperatures may cause more decomposition, which may therefore indirectly alter nutrient availability and help to maintain and expand the diversity of both plants and microorganisms (Wang *et al.,* 2009).

The microbial membrane and the extracellular and intracellular proteins produced by bacteria can both be strongly impacted by changes in temperature inside their growing environment (Roncarati and Scarlato, 2017). This is due to the ability of most microorganisms to adapt and adjust the kind of lipids being generated in response to temperature fluctuations, as well as the capacity to induce heat shock and cold shock proteins that may aid in the refolding of proteins or the development of protective cocoons during such alteration (Roncarati and Scarlato, 2017). However, this method does not alter the temperature at which microorganisms thrive best (Aragno, 1981). The temperature at which growth is most rapid is known as the "optimum temperature," while “maximum temperature” is the temperature above which growth is inhibited, and

"minimum temperature" is the temperature below which no growth occurs. The "optimum growth temperature" is the temperature at which the growth rate is highest (Aragno, 1981). In general, microorganisms can be categorised based on the temperature range in which they can thrive (Wiegel, 1990). They comprise of mesophilic, thermophilic, hyperthermophilic, and psychrophilic microbes (Coleman and Smith, 2007).

Microbes with an optimal temperature range of 0 to 20 °C are psychrophilic in nature. These microbes are evolved to live in many kinds of cold conditions (Cavicchioli *et al.*, 2006). In addition, some psychrophilic bacteria have been linked to food spoilage (Junge *et al.*, 2019). These microbes can be found in marine habitats like oceans and seas, glacial ice settings, and the Arctic. Obligate psychrophiles and facultative psychrophiles are two general categories for psychrophilic microorganisms (Ferroni and Kaminski 1980). Microorganisms known as facultative psychrophiles can grow at temperatures below 20°C but can also do so at higher temperatures (Gooch, 2011). Examples include *Pseudomonas fluorescens*, *Vibrio marinus*, *Listeria monocytogenes*, and *Pseudomonas maltophilia* (Prescott *et al.*, 2018). They are also known as psychrotolerant organisms, and they can be found in a variety of low-temperature habitats but not in the very coldest ones (Helke and Weyland, 2004). Obligate psychrophiles are organisms that cannot develop at temperatures above 20°C and whose optimum growth temperature is around 15°C (Metpally and Reddy 2009). Examples include the obligatory yeast-like microbes: *Aureobasidium pullulans* and *Candida rugosa* (Buzzini *et al.*, 2012), and the bacterial *Arcobacter* and *Herminiimonas* (Canion *et al.*, 2013), and others.

Mesophilic bacteria are microorganisms that thrive between 20 and 45°C and have their optimum growth conditions between 30 and 39 °C (Singh and Das, 2019). They often inhabit soil and water habitats and participate in a variety of biological processes, including the breakdown of organic materials. They are also thought to be pathogens like *Staphylococcus aureus*. Additionally, they contain a significant population of microorganisms that benefit their host. For example, probiotic microbes like *Lactococcus lactis* and *Lactococcus acidophilus*, which are examples of lactic acid bacteria (Nuryshev *et al.*, 2016).

Thermophilic microorganisms are heat-loving microorganisms that thrive at temperatures between 50 and 80 °C and proliferate over 45 °C (Ferrera and Reysenbach,

2007). They have been found in all three domains of life - Bacteria, Archae, and Eukarya. They can be found in hot habitats including self-heating compost piles, coal piles, hot springs, and other extreme geological areas (Ferrera and Reysenbach, 2007). In general, they can be divided into facultative and obligatory thermophiles based on the minimum and maximum temperature ranges they can tolerate. As a result of their lowest and maximum growth temperatures falling within the mesophilic and thermophilic range, organisms that can grow at temperatures between 37°C and 55°C are known as facultative thermophiles (Bausum and Matney, 1965). *Bacillus licheniformis, B. coagulans,* and *Paenibacillus* sp. are a few examples of facultative thermophilic bacteria that have all been identified from heat-treated vegetables (Ruzaike *et al.,* 2015). However, obligate thermophiles are microbes with a growth temperature range of 55°C to 80°C (Lasa and Berenguer, 1993). Examples of obligate thermophilic bacteria include *Thermoanaerobacter* sp. and *Bacillus stearothermophilus*, both of which have an optimal temperature at around 60°C (Noll, 2001), *Thermomyces lanuginosus* (previously known as *Humicola lanuginos*, and the famous *Thermus aquaticus* used in PCR applications, have been documented to have an optimum growth temperature at 75°C (Prescott *et al.*, 2018).

Another category of thermophiles are the hyperthermophilic microorganisms. They include bacteria and archaea with optimal growth temperatures above 80°C (Vieille and Zeikus, 2001). These group of organisms have been isolated from habitats with exceptionally high temperatures between 80 and 115 °C. They have been found in deep-sea hot sediments, hydrothermal vents, sewage sludge systems, and geothermal power facilities, among other places (Vieille and Zeikus, 2001). A member of the *Sulfolobus* sp. was identified as the first hyperthermophilic bacterium, which was found in the hot, acidic springs in Yellowstone Park in 1972 (Brock *et al.*, 1972). The archaea *Pyrolobus fumarii*, the most thermophilic microorganism, has been reported to flourish in the 90 to 113°C temperature range.

In terms of how temperature affects microbial membranes, psychrotolerants and psychrophiles appear to have a higher proportion of unsaturated membrane lipids and a lower proportion of branched-chain lipids than species living in habitats with a moderate (mesophiles) or high (thermophiles) temperature range (Nedwell, 1999). Additionally, in low-temperature conditions, cold-shock proteins, such as the enzyme desaturase,

which transforms saturated fatty acids into unsaturated fatty acids, start to preferentially produce long-chain fatty acids, straight-chain fatty acids, and iso fatty acids rather than short-chain fatty acids, branched-chain fatty acids, and anteiso-fatty acids (Suutari and Laakso, 1994). Finally, bacteria that can survive at extreme temperatures (high temperature or very cold temperature) do so by using specific adaptive techniques. According to numerous studies, charged amino acids (like glutamic acid, lysine, and arginine) predominate on the surfaces of thermophilic proteins while uncharged amino acids (like glutamine, asparagine, threonine, and serine) are less abundant (Cambillau and Claverie, 2000). These proteins' surface-located amino acids appear to be flexible, which allows them to interact freely with one another (Hiteshi and Gupta, 2014). This mechanism improves the thermotolerance of the proteins present in thermophilic microorganisms. The creation of surface salt bridges, which results from the high concentration of charged amino acids at the surface, is an illustration of such a free protein interaction (Cambillau and Claverie, 2000).

2.5.2 Hydrogen Ion Concentration (pH)

It is common knowledge that microorganisms are typically found in abundance in nature - from sediment to thermal springs and to ocean floors. According to Lopez-López *et al*. (2015), they have been found to catalyse a number of biogeochemical processes, including weathering, the cycling of carbon and other elements, and numerous redox reactions. A number of environmental elements, including pH, influence how their metabolism functions during these activities (Jin and Kirk, 2018). One of the many factors that have been shown to significantly affect microbial populations in a variety of biogeochemical environments is hydrogen ion concentration (Ye *et al.*, 2012). it is one of the many variables that have been shown to strongly affect microbial populations across a variety of biogeochemical environments (Thompson *et al.*, 2017). Generally, microbes can be divided into three groups: acidophiles, neutrophiles, and alkalophiles, based on their response to pH (Glenn and Dilworth, 1991).

Acidophiles are microorganisms that can survive in extremely acidic environments, even at pH levels as low as 1. They can be discovered in sulfuric pools, volcanic craters, animal stomachs, acid mine drainage, and hydrothermal sources (Sharma *et al.*, 2016). While acidophiles are found in all three domains of life, bacteria and archaea happen to be the two domains in which they are most characterised (Madigan and Martinko, 2005).

Bacteria from the genera *Acidithiobacillus*, *Alicyclobacillus*, and *Leptospirillum* are examples of acidophilic bacteria (Johnson, 1998), while *Ferroplasma*, *Sulfurococcus* and *Sulfolobus* are examples of acidophilic Archaea (Sharma *et al.*, 2016).

Neutrophilic microbes are microorganisms that can grow in a pH range of 5 to 8.5 but show their best growth at neutral or close to neutral pH. The majority of well-known bacteria, including *Salmonella* sp., *Escherichia coli*, and *Staphylococcus* sp., are typically neutrophils. However, some of these microorganisms, such as the intestine pathogenic strains, can thrive in acidic environments (Jain and Sinha, 2009). These category of microbes, are often ubiquitous, and are among the most extensively investigated microbes. They can be found in terrestrial and aquatic habitats, free of extreme conditions such as high pH and low pH. While some are aquatic environment residents, others are soil-dwellers like *Rhizobium* and *Bradyrhizobium*. Examples of their habitats include soil, lakes, rivers, sediments, plants and even in the air (Glenn and Dilworth, 1991).

Alkalophiles are microorganisms that can grow in an alkaline environment, particularly at pH values above 9, and are frequently shown to thrive well at a pH range of 10 to 13 (Horikoshi, 1999). Alkalophiles can be found in a variety of environmental settings, including alkaline soda lakes, high-pH soils susceptible to industrial processes, insect hindguts, and alkaline hydrothermal vents (Grant *et al.*, 2004, Jin and Kirk, 2018). *Amphibacillus xylanus*, *Pseudomonas alcaliphila*, *Bacillus pseudofirmus*, and *Bacillus alcalophilius* are a few examples of alkaliphilic bacteria (Janto *et al.*, 2011).

Previous research has connected pH limits to specific physiological mechanisms in microorganisms, such as how their metabolism and cellular structure respond to the pH of their environment (Jin and Kirk, 2018). For instance, extraordinarily high or low pH levels have been associated with the disruption of solute transport through microbial membranes as well as energy conservation (Jin and Kirk, 2018). While research has shown that in most bacteria, a low pH situation causes changes in the lipid content of the membrane. They reduce the fluidity of their membrane, which is thought to boost their acid tolerance (Jain and Sinha, 2009). Saturated to unsaturated fatty acid ratio generally affects membrane fluidity. According to Dunkley *et al.* (1991), there seemed to be a hint that the cyclopropane fatty acids of the phospholipids may increase the membrane's stiffness and thus decrease its permeability to protons in acidic conditions.

Additionally, prior research has demonstrated that *E. coli* under study, demonstrated improved protection from low pH with an increase in the percentage of cyclopropane fatty acids of mono-unsaturated fatty acids (Brown *et al.*, 1997). Furthermore, a decrease in the fluidity of the membrane may be linked to modifications in the proton flux, causing cells that have evolved to survive in an acidic environment to be less permeable to protons than unadapted cells (Yuk and Marshall, 2004).

2.5.3 Salinity

The concentration of salt in the soil or aquatic habitats is referred to as salinity. There are dissolved salts in almost all water bodies, and none are fully salt-free (Lovett *et al.*, 2007). According to Edbeib *et al.* (2016), the principal soluble salts in soil are cations such as calcium, magnesium, and sodium ions as well as anions like chloride, carbonate, and nitrate ions. However, the amount of dissolved salts present in the environment can influence the microbial population that develops there. For instance, bacteria would lose water and nutrients if their habitat contains high quantities of dissolved salts that they cannot tolerate. Their cells would shrink as a result of the efflux of water and nutrients, which would result in an irreversible loss of cellular structure and function and cell death (Edbeib *et al.*, 2016).

Microorganisms can be categorised as mild/slight halophiles, moderate halophiles, or extreme halophiles based on how they respond to salt (Ventosa *et al.*, 1998). Microbes known as slight halophiles typically flourish in environments where sodium chloride (NaCl) concentrations range from about 0.3M (2.0%) to roughly 0.8M (5.0%). The highest number of recognised bacteria that may be found among the microflora of the rumen, intestines, and even marine microbes belong to this group of halophiles (DasSarma and Arora, 2002). Halotolerant microorganisms are those that can survive both in the presence of relatively high salt concentrations and also in the absence of salt. As an illustration, *Staphylococcus aureus* can thrive on media with an 8% NaCl concentration or more (Ventosa *et al.*, 1998).

In general, there are a lot of halophiles in places like saline soil, naturally occurring salt lakes (such the Great Salt Lake and the Saline Lake in Inner Mongolia), and even in salted meals (DasSarma and Arora, 2002). The Dead Sea and the Great Salt Lake are the most extensively researched and largest hypersaline habitats, respectively (Edbeib

et al., 2016). *Bacillus hunanensis, Dethiosulfovibrio salsuginis*, and *Roseovarius aquimarinus* (Katayama *et al.*, 2014) are a few examples of microorganisms that are just mildly halophilic, as all of them have a salt tolerance threshold between 0.2M and 0.5M. With a salt tolerance range of 0.5M to 2.5M, *Marinobacter piscensis*, *Spiribacter salinus*, and *Halobacillus sediminis* are a few examples of moderately halophilic microorganisms (Kim *et al.*, 2015). Extremely halophilic microorganisms including *Halococcus salifodinae*, *Salinibacter ruber*, and *Limimonas halophila* have been shown to flourish in environments with salt concentration levels between 4M and 5.9M (Abdeljabbar *et al.*, 2013).

To keep their cytoplasm and environment in a stable osmotic equilibrium, halophiles predominantly use two different types of strategies. One of these is the cellular accumulation of potassium and chloride ions in their cytoplasm in molar quantities (Oren, 2013). The vast majority of halophiles that employ this strategy have highly acidic proteomes and are incapable of surviving in environments with low salt concentrations. Because this technique demands extensive modifications to their enzymes, they are best known among the very halophilic Archaea, such as the *Halobacterium salinarium* (Weinisch *et al.*, 2018). Among certain halophile groups, this tactic is not very well-liked. The more prevalent strategy is the intracellular synthesis and/or accumulation of organic solutes (Oren, 2013). Contrary to the former, the relatively high concentration of these organic solutes does not significantly impede typical enzymatic reactions because the organism's proteome only has to be slightly altered. Generally speaking, organisms having this feature can adapt to a wide range of salt concentrations (Ventosa *et al.*, 1998). In addition to maintaining osmotic equilibrium, these organic solutes also shield macromolecular structures against denaturation, deactivation, and inhibition (Andronov *et al.*, 2012). Glycine betaine, which is widely distributed throughout all kingdoms, ectoine, which has only been well-known among prokaryotes, sugars, sugar alcohol, amino acids, and polyols are a few examples of organic osmolyte solutes. The low-salt-in approach is another name for this technique. Halophilic methanogens like *Methanohalophilus* are examples of such halophilic bacteria that employ this tactic (Ruginescu *et al.*, 2020).

According to a number of studies, salinity affects and influences the composition of microbial communities. For instance, Pankhurst *et al.* (2001) noted that bacteria are typically less sensitive to salt than their fungal counterparts. Therefore, it appears that in

saline soil, the ratio of bacteria to fungi is higher than the ratio of fungi to bacteria. Salinity tolerance in microorganisms has consequently led to changes in the composition of communities in saline soils as compared to non-saline soils. Halophiles have their protein surface enhanced with aspartate with low quantities of asparagine and lysine, according to a comparison of the proteomes of halophilic mesophiles, thermophiles, and archaea (Fukuchi *et al.,* 2003). In contrast to non-halophiles, whose proteins would become rigid and aggregate in such conditions, halophilic microbes' proteins are excessively negatively charged, making them resistant to aggregation in a highly saline environment and instead flexible and soluble in such environment (Gunde-Cimerman *et al.,* 2018).

2.6 Methods for describing and measuring diversity

Measuring and comprehending the pattern of microbial diversity's distribution across temporal and spatial scales is one of the fundamental goals of microbial ecology (Birtel *et al.,* 2015). Similarly, microbial populations are quickly evolving into model systems in ecology studies to examine how the diversity and abundance of microbial taxa can influence particular ecosystem processes (Prescott *et al.,* 2018).

The methods used to analyse microbial diversity have significantly increased over the past ten years, and new methods are now accessible that give scientists better access to microorganisms in their natural habitats (Hill *et al.,* 2000). However, there had been a number of methods for accessing microbial diversity before the development of this recent methodology. The two main approaches for describing and assessing microbial diversity are as follows: Culture-dependent (Conventional and Biochemical Method) and culture-independent approach (Ghosh *et al.,* 2019).

2.6.1 Culture - dependent approach / conventional and biochemical method

The study of different types of environmental bacteria has always been a top goal for early scientists (Tshikhudo *et al.,* 2013). As a result, numerous culture-dependent methodologies as well as conventional and biochemical techniques for analysing the microbial community were developed (Ghosh *et al.,* 2019). Among them are a variety of physiological measurements like microbial tolerance, their choice for feeding, and even the usage of a number of metabolic aspects like the exploitation of diverse nitrogen and carbon sources, their varied sources of energy, and their dependence on growth

factors (Fakruddin and Mannan, 2013). The traditional method of bacterial identification is still one of the reliable and consistent ways to determine the species of bacteria. Gram staining, for instance, is still the traditional technique of choice for classifying bacteria based on the structural characteristics of the cell wall, thereby identifying bacteria as either Gram-positive or Gram-negative, and it continues to be useful today (Tshikhudo *et al.*, 2013). Additionally, this technique has been around for a while and is typically simple, quick, and affordable to use. However, this method has some limitations, including the inability to identify microbes that do not exhibit any biochemical patterns typical of any known genus, the inability to culture and identify microbes that cannot be grown in a laboratory, and the laborious nature of this method when used to identify slow-growing microbes. Some of these conventional methods include the Serial dilution technique and the sole carbon source utilisation (SCSU) method (Fakruddin and Mannan, 2013; Ghosh *et al.*, 2019; Adedire *et al.*, 2022).

2.6.1.1 Dilution and plate count of microbes

Plating bacteria on differential and selective media, which is then followed by viable counts, is the most common procedure for evaluating microbial diversity (Hill, 2000). The density of microbial content present in a sample can be accurately assessed using this method. Plate counts can be accomplished using membrane filters, spread plate, or pour plate methods. The term "Colony Forming Unit" refers to the quantity of live cells in an observed sample, and its information is still significant in the field of Microbiology (Trevors, 1998). Also, this method provides useful information regarding culturable heterotrophic bacteria present in the sampled environment while being affordable and relatively quick (since this method helps in the maximal recovery of distinct culturable microbial species) (Trevors, 1998; Hill, 2000). When microorganisms are cultivated on solid media, their characteristics can be utilised to some extent for their identification. According to Tshikhudo *et al.* (2013), morphological features considered include their size, shape, elevation, surface, colour margin and boundaries, as well as their rate of growth in a specific medium. Some drawbacks of this methods however, include, the provision of precise growth conditions, (such as light, temperature, and pH), the likelihood of inhibition on culture media, or the growth of unwanted colonies. As a result, this approach is unable to fully reflect the environment's microbial diversity (Fakruddin and Mannan, 2013).

2.6.1.2 Sole Carbon Source Utilisation (SCSU)/ Community-Level Physiological Profiles (CLPP)

This technique is an improvement on the standard conventional method for bacterial taxonomic identification. This approach is based on the use of diverse carbon sources to identify bacteria down to the species level (Hills *et al.*, 2000). It employs the use of a commercial taxonomy system, such as the Analytical Profile Index (API) and BIOLOG biochemical identification systems. Garland and Mills first presented the BIOLOG identification technique in 1991. Based on their metabolic characteristics, the API and BIOLOG kit were developed to recognise pure bacterial cultures (Fakruddin and Mannan, 2013, Hills *et al.*, 2000). The BIOLOG system, which incorporates a variety of 95 different carbon sources, has been extensively utilised to study soil bacteria (Garland, 1996). The API kit is made up of substrates that are dehydrated and placed in wells; microbes are then introduced into these wells, and the enzymatic activity and sugar fermentation pattern of the microorganisms are thereafter studied (Smith *et al.*, 1972). The API 50CHB medium is an example of an API kit. It is used to characterise *Bacillus* and other species that are related by examining the fermentation pattern of 49 carbohydrates by the inoculated bacteria. This profiling method may provide a wealth of information for evaluating the microbial population, and it is also rather simple to apply and repeatable. Although this method still favours fast-growing microorganisms, it still has the same bias as culture plating. For instance, it is sensitive to inoculum density, as it impacts how wells develop their colour and how quickly bacteria grow, therefore, it must first be standardised (Garland and Mills, 1991; Garland, 1996; Hills *et al.*, 2000).

2.6.2 Culture-Independent method/ approach

Long-term studies have shown that culture-dependent methods are a powerful tool for characterising and identifying microorganisms. Nevertheless, Lopez-López *et al.* (2015) found that multiple research has revealed that only around 1% of prokaryotes can be cultivated on common media under standard conditions. As a result, this approach had not been able to give comprehensive information on the variety of complex microbial communities (Schloss and Handelsman, 2005; Adedire *et al.*, 2022). As a result, various scientists have suggested methods using culture-independent techniques to determine the identity of microorganisms in natural habitats. Metagenomics, a relatively recent branch of Science, is one such recommendation (Otlewska *et al.*, 2014).

2.6.2.1 Metagenomics analysis and bioinformatics

Metagenomics has transformed Biological Sciences, particularly Microbiology, over the past few decades. Since Handelsmann initially coined the term "metagenomics" in 1998, it has gained popularity in the context of addressing real-life issues in the fields of Agriculture, Life Sciences, Earth Sciences, and Biotechnology (Thomas *et al.*, 2012). Without the requirement for cultivation on conventional cultivation media, it is the study of microbial communities that were directly sampled from their natural habitat (Schloss and Handelsman, 2005, Fasesan *et al.*, 2020). This method enables the study of all types of microorganisms, even those that haven't been cultivated yet. The method used in metagenomics studies begins with the extraction of total DNA from an environmental sample, which is followed by the direct or indirect sequencing of their genetic material, followed by the interpretation of the generated dataset using various bioinformatics suites and software (Escobar-Zepeda *et al.*, 2015). Metagenomics investigations have been utilised to examine a wide range of natural environments, including the human gut, soils, faeces, springs, and oceans (López-López *et al.*, 2015). Many metagenomes have been sequenced from a number of these habitats to address practical issues (Thomas *et al.*, 2012). Some researchers have made an effort to divide metagenomics screening techniques into two major groups: Sequence-based methodology; function-based methodology (Fakruddin and Mannan, 2013)

2.7 Metagenomics – An overview

2.7.1 Metagenomics and PCR

Without the use of Polymerase Chain Reaction (PCR), metagenomics research are essentially impossible. Kary Mullis and colleagues created the popular molecular biology technique known as PCR in 1983. It enables researchers to enzymatically generate and also amplify particular DNA fragments by more than a billion-fold (Rahman *et al.*, 2013, Prescott *et al.*, 2018). The three stages of this process are denaturation, annealing, and the elongation/extension stage.

The basis for the DNA replication process used in PCR is the fact that at high temperatures (up to 95°C), the G-C and A-T bonds in the double-stranded DNA helix is separated, forming two single-stranded DNA molecules (denaturation stage). Complementary primer pairs, forward and reverse, can then bind to the 3' ends of the

flanking regions of each single stranded DNA molecule when the reaction mixture's temperature is decreased to between 50 and 65 °C, this process is known as the annealing stage. When the temperature of the reaction mixture is increased again to about 72°C, the enzyme DNA polymerase is able to extend and synthesise new DNA molecules from each template DNA by the addition of free dNTPs (dioxyribonucleotide triphosphates), which are complementary to the template DNA in the 5' to 3' direction. This stage of the process is known as the elongation stage. In a thermocycler, this cycle is repeated numerous times to produce enormous copies of the target DNA molecule (Singh *et al.,* 2014b).

According to Rahman *et al.* (2013), the PCR technique is renowned for its high specificity, precision, and robust sensitivity. The detection of genetic fingerprints, the cloning of genes, the identification of "yet-unculturable-microbes," and the identification of slow-growing microbes are only a few of the biological research applications that it can be utilised for. In a 16S rRNA-based metagenomics study, PCR enables the amplification of the 16S ribosomal gene, allowing for the creation of a metagenomic library. This library is then subjected to sequencing, and the resulting data are then interpreted and used to describe the diversity of the bacterial environment (García-López *et al.*, 2020). PCR is used to test metagenomic libraries in function-based metagenomics studies. In order to clone and study gene expression, it is also utilised to amplify specific gene segments (Johny and Bhat, 2017). Several modifications to the standard PCR method have been created over time. some of these include; Real-Time (quantitative) PCR, nested PCR, gradient PCR, reverse-transcription-PCR, touchdown-PCR, colony-PCR, band-stab PCR, hot-start PCR, multiplex-PCR, etc (Rahman *et al.,* 2013).

One of the PCR versions that has found a lot of use in biological research is real-time qPCR. It can be used to measure either RNA or DNA in a sample and is based on the precise identification and quantification of PCR products that are being synthesised after each cycle process (Kralik and Ricchi, 2017). Fluorescence is used for this measurement, which can come from double stranded DNA binding dyes or fluorescently-labeled oligonucleotide probes or primers (Klein *et al.,* 2012). Multiplex qPCR is a variation of qPCR. By utilising several fluorogenic probes, this technique of amplification enables the detection and differentiation of multiple amplicons in a single reaction tube (Arya *et*

al., 2005). In Metagenomics studies, it is used for quantitative microbiome (the total genetic material of microbes living in a particular host) profiling from environmental samples as well as from clinical samples (Jian *et al.*, 2020).

Nested PCR is another modification of conventional PCR, which is usually used in studies where the concentration of the target DNA is very low in samples (Fan *et al.,* 2009). Nested PCR is an excellent alternative that is used when the standard PCR technique is unable to amplify minute DNA targets in samples (Fan *et al.*, 2009; Yu *et al.,* 2015). In nested PCR, two rounds of PCR reaction take place: the first round of PCR reaction targets a large region of DNA while using a pair of primers; the second round of PCR reaction narrows its amplification to a specific region of the product of the first round, which is now used as its template, making use of another set of primers (Yu *et al.,* 2015). It has been used to determine the bacterial profile from clinical samples such as the ones collected from the middle ear fluid of children suffering from ear infections (Sillanpää *et al.*, 2017).

Colony PCR is one of the most commonly applied techniques which is used to rapidly screen (bacterial or yeast) colonies which had grown on selective media after a transformation procedure, either to confirm the presence of the desired genetic construct or to amplify a part of the genetic construct harboured by such colonies (Bergkessel and Guthrie, 2013). For example, it is frequently used for screening genetically engineered *E. coli* colonies for the presence of plasmid DNA after it has undergone transformation protocol (Jamal *et al.,* 2017). After the cloning experiment, the organism is normally plated on selective media, and a successful procedure will be indicative of colony growth from one to a few hundred of colonies on the plate. However, the desired genetic construct might or might not be present among those colonies, hence the need to further screen each colony for the presence of the genetic construct/recombinant plasmid. Colony PCR, is, therefore, a valuable technique for rapidly and effortlessly screening sizable numbers of colonies to identify true positives from false positives, as amplification is directly from a colony rather than from a DNA sample (Bergkessel and Guthrie, 2013). Colony PCR is commonly used in function-based metagenomics studies to screen for genes of interest. For example, Itoh *et al.* (2016) employed the use of colony PCR to screen for positive clones from an *E. coli* library constructed from a soil metagenome, harbouring the phenylacetaldehyde reductase gene.

2.7.2 Metagenomics and screening approaches

Metagenomics approach can be based on direct sequence analysis (sequence-based metagenomics) or might be based on cloning of DNA and/or the expression of DNA, otherwise known as function-based metagenomics (Johny and Bhat, 2017).

2.7.2.1 Sequence-based screening

The two primary methodologies for sequencing-based metagenomics screening are, targeted amplicon sequencing analysis and whole metagenome approach/whole metagenome shotgun sequencing/shotgun metagenomics (Fricker *et al.*, 2019). Shotgun metagenomics screening has the ability to shed light on both taxonomic classifications and new genes with unique functions in uncultured microbial communities from complex sources (Escobar-Zepeda *et al.*, 2015). According to Madhavan *et al.* (2017), the whole metagenome sequencing method shears entire DNA into tiny pieces that are then individually sequenced. In the process of assembling different sequences, also known as reads, each read is compared to the others, overlapped, and used to create longer contiguous reads. These longer contiguous reads are then further assessed for gene prediction and functional annotation to make sense of the data generated (Sharpton, 2014). Multiple reads are generated as a result, and these reads align to various genomic sites for the variety of genomes present in the sample (including non-microbes). While reads would align with loci that are taxonomically useful, such the 16S rRNA genes, some of the reads would also align to coding sequences, making information about the biological activities of the diverse genomes also accessible (Sharpton, 2014).

In targeted metagenomic screening strategy, there is a need to design oligonucleotide primers from conserved areas of already known genes or recognised protein families (Simon and Daniel, 2011). The use of phylogenetic markers like 16S rRNA in sequence-based analysis is a highly effective method for drawing conclusions about the physiology, ecology, and taxonomic classification of all individuals living in the studied natural environment (Schloss and Handelsman, 2005). The DeLong group, who were the first to produce the first genomic sequence coupled with a 16S rRNA gene from an uncultured archaeon, introduced this use of phylogenetic markers in a sequence-driven strategy (Stein *et al.*, 1996). The bacterial 16S rRNA gene is the target amplicon most frequently employed for taxonomic classification in metagenomics/microbiome study (Kittelmann *et al.*, 2013). In addition, universal primers have been constructed and

described for eukaryotic small subunit ribosomal RNA genes and internal transcribed spacers (ITS) for fungal metagenomics research (Fricker *et al.*, 2019). RNA polymerase subunit B (rpoB) genes, DNA gyrase subunit B (gyrB), and recA genes are examples of additional protein-coding genes and molecular markers that have been utilised in targeted metagenomics for the assessment of microbial diversity studies (*Case et al.*, 2007).

2.7.2.2 Function-based screening

Metagenomics holds great potential for assessing rich genetic resources from uncultured microbes, by bypassing the conventional means of culturing microorganisms and aiming directly for their genomic DNA from the environment (Ngara and Zhang, 2018). Function-based screening entails obtaining the total genomic DNA of microbial communities from their natural environments and expressing them in a foreign host to check for the expression of target genes in the cloning host (Genilloud *et al.*, 2011). It also includes the capability of identifying, isolating, and characterising expressed genes from a metagenomic library that has been created. The bioprospecting of metagenomes for prospective biotechnological products typically use this screening technique (Simon and Daniel, 2011). Due to a number of features, including better plasmid yields, quicker growth rates, and superior transformation efficiency, genetically altered *Escherichia coli* strains have been the most often employed cloning hosts in most function-based techniques (Kostylev *et al.*, 2015).

In function-based methods, vectors are also essential to the successful transformation of external environmental DNA pieces. They are typically used to produce functional-expression libraries and serve as a vehicle in replicating the appropriate DNA segment in the intended host. Plasmids, fosmids, cosmids, and bacterial artificial chromosomes (BACs) are a few examples of the various types of vectors that have been described (Vester *et al.*, 2015). Plasmids are frequently utilised if the size of the DNA insert is less than 15 kb, fosmids are used for DNA that is between 25 and 45 kb in size, and BACs are used for large DNA fragments with sizes between 100 and 200 kb (Vester *et al.*, 2015). Following insertion and transformation, screening assays are performed to identify and choose the colony of interest (containing the DNA insert) based on phenotypic identification. When colonies are grown on chromophore- or fluorophore-induced substrates, for instance, they can be checked to see if the gene encoding the

enzyme produces color (Vester *et al.,* 2015). Halo formation and irregular colony formation are two additional phenotypic identification techniques that can be used to identify positive clones (Ekkers *et al.,* 2012). Many metagenomes have been bioprospected using function-based screening to find new biocatalysts with better and more acceptable biotechnological and industrial features (Kostylev *et al.,* 2015).

2.7.3 Metagenomics and phylogenetic markers

The taxonomic evaluation and functional diversity of the microbial community in its natural environment is one of the countless applications of metagenomics research (Simon and Daniel, 2011). Studying conserved marker genes found in these microbial communities is a frequent way to accomplish this. This allows for the proper taxonomical classification of the microbial communities. (Kittelmann *et al.,* 2013).
Several phylogenetic markers, some of which contain nuclear ribosomal genes like 16S rRNA genes and nuclear ribosomal Internal Transcribed Spacer (ITS) regions, have been described and employed in investigations of microbial diversity and microbial ecology studies. Others marker genes include rpoB genes, gyrB, and heat shock protein (dnaK) (Case *et al.,* 2007).

2.7.3.1 16S ribosomal RNA as a Molecular Marker

The ability to define nucleic acid sequences of genomic DNA has transformed existing studies involving microbial ecology as well as diversity studies. In all microbes, the ribosomal DNA (rDNA) gene is present and has been reported to accrue mutations overtime at a relatively slow but constant rate (Tshikhudo *et al.,* 2013). In prokaryotes, the ribosome is a 70S complex that consists of two subunits: the small ribosomal subunit (30S) and a large ribosomal subunit (50S), the 30S subunit is made up of 21 proteins and the 16S ribosomal rRNA that is 1,542 base pairs long. However, the 50S subunit consists of 33 proteins and two ribosomal RNA known as the 23S (of 2,904 base pairs) and 5S (with a length of 120 nucleotides) (Shajani *et al.,* 2011).

The 16S rRNA gene has been widely employed to characterise bacterial and archaeal communities from a variety of niches, including from animal, clinical, and environmental habitats. This is due to the fact that this gene has some traits that make it valuable as a phylogenetic marker (Garcia-López *et al.,* 2020). One of these characteristics is their ubiquity among prokaryotes, especially in bacteria. Furthermore,

the 16S rRNA gene contains nine hypervariable regions, designated V1 - V9, (that exhibit significant sequence variation across various bacterial species), and highly conserved regions (that can be used to create universal primers across all bacterial taxa), allowing for the successful differentiation of bacterial taxa (Garcia-López *et al.*, 2020).

Lastly, Woese (1987) noted that its magnitude and slow rate of evolution make it a useful tool for phylogenetic classification of bacteria. Woese and Fox were the first to suggest using rRNA genes to infer phylogenetic relationships between microorganisms, and it was their work that served as the foundation for the three domains of life (Case *et al.*, 2007). Microbial ecologists' approach to studying prokaryotes was fundamentally changed in 1990 by the direct amplification and sequencing of 16S rRNA genes (Rosselli *et al.*, 2016). Examining specific hypervariable sections of the gene -which are only a few hundred nucleotides long, falls within the range at which they can be extensively sequenced, via the development of high throughput sequencing tools. As a result, the 16S rRNA gene has generated a significant amount of data for the profiling of bacteria and archaea and has been referred to as the gold standard for bacterial profile research (Tshikhudo *et al.*, 2013; Wang *et al.*, 2015a).

The majority of current investigations into sequence-based bacterial communities mainly rely on 16S rRNA gene high throughput sequencing (Wang *et al.*, 2015). A community taxonomic survey must frequently use one or two hypervariable regions within the gene because the read lengths of sequences produced by high throughput sequencers are typically short (Garcia-López *et al.*, 2020). Some common regions that have been used in various studies include hypervariable regions 1 and 2 (V1 –V2) with an approximate length of about 330bp (basepairs), V1 –V3 of about 490 bp long, V4 – V5 of about 390bp in length, and V3 – V4 regions with an approximate length of 460bp (Chen *et al.*, 2017; Zhou *et al.*, 2017).

Analysis of the 16S rRNA gene has shown that it can consistently identify bacteria at the genus and species level up to 90% and 86% because the hypervariable regions used in taxonomic classification are not uniformly informative across different families, genera and species, and sometimes even between them (Yang *et al.*, 2016a). Despite this non-uniformity, studies showed that classification at the genus and family level has been indeed remarkably accurate, establishing the statement that the 16S rRNA gene is still a very influential and potent approach for identification of bacteria in complex samples

(Yang *et al.,* 2016). The use of 16S rRNA sequencing has, therefore, been applied in large microbial ecology projects such as the Human Microbiome Project (Huttenhower *et al.*, 2012) and Plant Microbiome Studies (Mendes *et al.,* 2011).

2.7.3.2 Internal Transcribed Spacer (ITS) regions as a molecular marker

Eukaryotic cells, like those in the kingdom of fungi, have a complex region called the rDNA that is organised into the 18S rDNA gene coding sequence, an internal transcribed spacer region (ITS)1, a 5.8S rDNA gene coding sequence, an additional ITS (known as the ITS2), and the 28S rDNA sequence coding gene (Bellemain *et al.*, 2010). The coding sequences of 18S, 5.8S, and 28S evolve slowly and are largely conserved throughout fungi, giving a means of establishing relationships phylogenetically among species, as shown in the 16S and 23S rDNA of prokaryotic cells (Gosavi, 2016). The ITS regions, ITS 1 and 2, which are located in the midst of these rDNA gene coding areas, are similar to the variable regions in bacteria that develop more quickly, resulting in varied sequences among fungal species and genera (Gosavi, 2016).

The ITS region is a desirable target for environmental sample analysis, especially in substrates where the amount of DNA is scarce, due to the enormous number of ITS copies per fungus cell, often as high as 250 (Bellemain *et al.,* 2010). The 18S rRNA is typically used for high-resolution taxonomic investigations of fungus, but the ITS is the area of preference for total fungal analysis studies in environmental samples (Bromberg *et al.*, 2015). The recommended DNA barcoding method for identifying fungi in simple or complicated environmental samples is the ITS. Environmental DNA barcoding is another name for this technique (Bellemain *et al.*, 2010). The ITS was recommended as the principal barcoding marker for fungi after discussion by a number of mycologists from various nations in 2007. Since then, thousands of fungal ITS sequences have been sequenced and deposited in numerous databases, providing a vast amount of reference data for classifying fungal taxa (Nilsson *et al.*, 2009).

According to the classic Sanger sequencer's analysis, the whole ITS region has a size that typically spans from 450 to 700 bp. In recent times, owing to the availability of high throughput sequencers, it is common to target either the ITS 1 or ITS2 or fungal analysis studies as the entire ITS region is too long for high throughput sequencers or even for 454 sequencing methods (Bellemain *et al.*, 2010). Many environmental metagenomics

studies have been investigated using ITS regions for fungal mycobiome/fungal profiling in the natural environment. Some of these include, fungal diversity studies from coarse and fine air filter samples using both regions of the fungal ITS (Fröhlich-Nowoisky *et al.,* 2009) and the mycobiome diversity studies of eight islands in Antarctic terrestrial habitats (Baeza *et al.,* 2017).

2.7.4 Metagenomics and sequencing techniques

DNA sequencing is a technique used to resolve the exact order of nucleotide bases that makes up a strand of DNA. Reads or sequences are common terms for the output of DNA sequencing produced by a sequencer, as was previously mentioned (Pereira *et al.*, 2013). Standard metagenomics study combines the field of genomics, bioinformatics and biology, in investigating total microbial genomes that had been directly extracted from the natural environment (Kumar *et al.*, 2015). However, the field of metagenomics is currently advancing quickly due to the use of next-generation sequencing (NGS) technologies, which has allowed researchers to further exploit microbial communities that were previously unknown (Malla *et al.*, 2019). Next-generation sequencing, also known as high throughput sequencing, uses platforms that enable the independent, concurrent, and parallel sequencing of millions of DNA strands from various sources, producing millions of reads (Shuikan *et al.*, 2019). In the early stages of microbial diversity investigations prior to the development of NGS, Sanger sequencing was frequently employed (Escobar-Zepeda *et al.*, 2015).

2.7.4.1 First generation/Sanger sequencing technology

One of the first-generation sequencing techniques employed in the study of genomics is known as Sanger sequencing. It was largely adopted in the sequencing of 3.2 billion base pairs of the human genome under the project Human Genome Project in 2000 that lasted till 2013 (Hood and Rowen, 2013). In order to assess the microbial community of the Sargasso Sea, Venter and his associates employed Sanger sequencing (Venter *et al.*, 2004).

Frederick Sanger and his colleagues created Sanger sequencing technology, sometimes referred to as the "chain termination method," in 1977 (Sanger *et al.*, 1977). This method of sequencing can be summarised in three main steps. First, a sort of chain termination

PCR occurs, and its reagents are similar to those of the standard PCR, except for the addition of modified dioxyribonucleotides (dNTPs) known as dideoxyribonuleotides (ddNTPs) which are fluorescently labelled and are added in low ratio to the regular dNTPs (Kchouk *et al.*, 2017). Unlike the dNTPs, these ddNTPs lack the 3'-OH group necessary for the formation of a phosphodiester bond, hence, when the DNA polymerase randomly incorporates a ddNTP rather than a dNTP, during the elongation stage of the chain termination PCR, elongation terminates. The result is pieces of DNA of random lengths (in thousands to millions) of the DNA sequence of interest. The second stage involves the separation of these fragments of DNA (by size) that had been terminated by ddNTPs. This separation is usually done by capillary gel electrophoresis by running all fragments through the capillary, which is usually present in the sequencing machine. Lastly, the gel is read to determine the sequence of the DNA of interest, which is usually done by a computer to read individual bands of the capillary gel in an orderly manner. This is read by making use of the fluorescently-labelled ddNTPs and are thereafter visualised by an imaging system such as UV light or X-ray (Kchouk *et al.*, 2017).

The Sanger sequencing method still has major drawbacks, despite having previously been utilised for significant genome projects (Shuikan *et al.,* 2019). The inability to detect low abundance microbes from multiple metagenomes, low discovery power to detect rare variants and the inability to sequence millions of fragments from complex and mixed samples are just a few of these drawbacks (Shuikan *et al.,* 2019). However, it does have the advantage of being able to produce longer reads (with an average read length of 400 - 900bp) than most NGS platforms (Kchouk *et al.,* 2017). Sanger sequencing technology was used on platforms like the automated DNA sequencers ABI Prism 310 and ABI 3700 by PE Biosystems, which were launched in 1996 and 1998, respectively (Ari and Arikan, 2016). An example of a first-generation sequencing platform is shown in Figure 2.1.

Figure 2.1: The ABI Prism® 3700 Sequencer developed by PE Biosystems
Source: Felder, (1998).

2.7.4.2 Next-Generation sequencing platforms

Although the underlying principles of NGS and Sanger sequencing are similar, NGS has been able to address Sanger sequencing's shortcomings and, as a result, has been able to lessen its influence in metagenomics as the primary source of sequence data (Malla *et al.,* 2019). The ability to sequence multiple complex samples at once, the ability to sequence multiple regions of genes simultaneously, the quicker turnaround time for large sample volumes, and the superior sensitivity to capture rare variants are just a few of the many benefits of NGS over Sanger sequencing (Shuikan *et al.*, 2019). Any typical metagenomics or microbial ecology study using NGS technology adheres to a set of predetermined procedures, like sample collection, metadata collection (additional data describing collected samples), extraction of high-quality environmental DNA, library construction, sequencing, read pre-processing, functional binning, and quantitative analysis (Kumar *et al.*,2015).

Whole metagenome sequencing and deep amplicon sequencing are two types of sequencing (NGS) that can be used in microbial diversity studies. When determining the functionality of the microbial community, whole metagenome sequencing is used. Read assembly, functional binning, functional annotating, and functional assignment to predicted protein-coding regions are all steps in the subsequent processing of generated sequence data (De Mandal *et al.,* 2015). Deep amplicon sequencing is frequently used to profile communities within different ecosystems by employing marker genes like the 16S rRNA gene. According to Murray *et al.* (2012), the processing of generated reads from amplicon sequences includes denoising, chimera identification, operational taxonomic (OTU) clustering, taxonomic analysis, diversity, and statistical analysis. The choice of the NGS platform to utilise for metagenomic investigations is crucial in deciding the study's outcome because there are numerous NGS platforms available in the market, each with a different set of capabilities (Murray *et al.*, 2012). Examples of NGS platforms that exist include Roche 454, Illumina, IonTorrent, Applied Biosystems, Pacific Biosciences, and Oxford nanopore platforms, all of which are in the category of second, third and fourth generation sequencing technology (Kumar *et al.*, 2015, Shuikan *et al.*, 2019).

2.7.4.3 Second-Generation sequencing platforms

The first NGS technology, which materialised commercially in 2005 was produced by 454 life Sciences (Roche 454 platform) which began several changes in NGS, since then, several other NGS platforms have surfaced that produce both long and short reads (Besser *et al.*, 2018). Next-generation sequencers that have now emerged in addition to the Roche 454 include the Illumina Systems, ABI SOLiD system, and Ion Torrent. The 454/Roche and Illumina sequencing systems, followed closely by the ABI SOLiD sequencers, are the two most widely utilised sequencing platforms for metagenomics studies (Kumar *et al.*, 2015).

According to Petrosino *et al.* (2009), the 454 instrument is well-known in the scientific community and can boasts of more than 100 publications on its website, including papers on bacterial metagenomics. The original 454 sequencing systems had read lengths of 100 to 150 bp initially, but in 2008, an enhanced 454 sequencer (the 454 GS FLX) was released, increasing the read length to 700 bp. This platform is a good sequencer for applications like the sequencing of small genomes and amplicon sequencing due to the comparatively lengthy read length (Ari and Arikan, 2016). However, because the output of reads produced by a 454 platform is several orders of magnitude lower than that of reads produced by an Illumina platform, 454 Roche may not be appropriate for studies that relies on ultra-deep coverage (Petrosino *et al.*, 2009).

Illumina, formerly known as Solexa, entered the market in 2007 and quickly overtook it as the most widely used NGS platform due to its capacity to provide high-quality readings. Miniseq, MiSeq, NextSeq, HiSeq2500, and NovaSeq 5000/6000 are some of the different versions of this platform that are available (Besser *et al.*, 2018). The Illumina systems have the ability to sequence DNA fragments from either one side (producing single-end (SE) reads) or from both sides (producing paired-end (PE) reads), as well as multiplexing a large number of samples at once (Shuikan *et al.*, 2019). The Miseq platform is a benchtop sequencer that can produce PE reads with a maximum read length of 2 x 300 base pairs. These reads can be combined to produce reads with a length of 600 base pairs. Several community-targeted sequencing studies, including the 16S rRNA sequencing from complex samples, have used this platform (Kumar *et al.*, 2015). Figure 2.2 depicts a pictoral representation of an HiSeq 2500 sequencing platform.

Figure 2.2: The HiSeq® 2500 Sequencer developed by Illumina Inc.

Source: Illumina, (2014)

2.7.4.4 Third-Generation sequencing platforms

The second generation of sequencing technology has rightfully been referred to as the generation of sequencing that mapped the course for change in the genomics sector. Third-generation sequencing technologies were established in an effort to lessen their known shortcomings, including short read lengths, time-consuming processes, and biases during library amplification (Ari and Arikan, 2016). These include the MinION sequencers from Oxford Nanopore, the PacBiosequencing (also known as PacBio) platforms from Pacific Biosciences, and the HeliScope sequencing platform created by Helicos Biosciences (Fichot and Norman, 2013; Malla *et al.*, 2019). The time needed for DNA preparation and the errors and biases brought on by PCR are reduced by third-generation sequencing platforms since they do not require PCR amplification prior to sequencing. According to Besser *et al.* (2018), the average read length produced by these platforms is considerably longer than that of second-generation sequencers. However, they developed an upgraded sequencing platform with recent chemistries capable of generating read lengths of up to 10,000 base pairs, with the drawback of significantly higher error rates (Malla *et al.*, 2019).

Pacific Biosciences was the first to produce commercially long reads, as long as 1500bp. Although their high error rate can be reduced by deeper sequencing, this sequencing platform is not commonly used for microbial ecology studies because of its high sequencing costs. Therefore, short-reads produced from Illumina platforms are used in the majority of diversity and profiling studies. Investigation of the bacteria microbiota in cheese (Jin *et al.*, 2018), and the analysis of the microbiome diversity of the bacterial community present in the Idah River in Kogi State, Nigeria (Adedire *et al.*, 2022) have all been conducted using this platform, despite these setbacks.

In 2014, Oxford Nanopore Technology's MinION was made available for purchase. It is the smallest sequencer now available, weighing only 90g, and can be connected to a computer's USB3 interface to collect data (Lu *et al.*, 2016). The MinION has been used to sequence the genomes of pathogens like the influenza virus (Wang *et al.*, 2015a) and the Ebola virus (Malla *et al.*, 2019). Additionally, this platform has some drawbacks, including high error rates (even higher than PacBio) and its inability to correctly sequence GC-rich regions (Besser 2018). A pictoral representation of a third-generation sequencing platform is shown in Figure 2.3

Figure 2.3: The MinION Sequencer developed by Oxford Nanopore Technologies.

Source: Le, (2023)

2.7.5 Metagenomics and reference databases

To make precise biological inferences, sequences produced by any sequencing platform must be interpreted meaningfully. Reference databases are repositories where particular categories of data are organised and stored so that they are manageable, accessible, and up to date. (Santamaria *et al.*, 2012). They provide researchers access to the fundamental data needed to draw conclusions regarding the physiology, epidemiology, and evolutionary background of representative sequences (Kim *et al.*, 2013). There are several well-curated databases, which are publicly available for use in microbiome/ metagenomic analysis, and are of specific types. For example, databases containing information about small ribosomal subunits (SSU) includes SILVA, Ribosomal Database Project (RDP) and Greengenes (Balvočiūtė *et al.*, 2017). Some other databases that are dedicated for the identification of ITS for fungal barcoding include ITSoneDB (Santamaria *et al.*, 2018), UNITE (User-friendly Nordic ITS Ectomycorrhiza Database) and BOLD (Barcode of life database) (Nilsson *et al.*, 2019).

2.7.5.1 UNITE reference database

Researchers make use of the UNITE database, which is open to the public, for identification of fungi. By gathering and making accessible taxonomic, regional, and even ecological metadata from these sequences, it was developed in 2003 to identify fungus using nuclear ribosomal ITS sequences (Nilsson *et al.*, 2019). This database has been meticulously curated (as a result of the collaboration between Taxonomists, Bioinformaticians, and Fungal Ecologists), with significant processing and interpretation in addition to approximately one million fungal ITS sequences that are available for referencing. The International Nucleotide Sequence Databases Collaboration (INSDC) is where these publicly accessible ITS sequences were derieved (Nakamura *et al.*, 2012).

The INSDC is a collaborative effort existing between three large databases; namely DNA Data Bank of Japan (DDBJ) in Mishima, Japan, the European Nucleotide Archive (ENA) in Hinxton, UK and the GenBank in Maryland, USA (Nakamura *et al.*, 2012). All these are in response to tonnes of data been produced by emerging and existing sequencing technologies (Cochrane *et al.*, 2011). These databases collaborate by collecting, sharing and updating data between each other by synchronising data daily.

These data include raw sequences, alignments, assemblies, functional annotations as well as metadata related to submitted sequences (Cochrane *et al.*, 2011).

In UNITE database, sourced fungal ITS sequences from the INSDC is further passed through some measures of quality filtering to remove unwanted sequences such as chimeric and non-ITS sequences, these filtered ITS sequences are then made available for users either in an interactive or as static forms (Nilsson *et al.*, 2019). The UNITE website and database is available at https://unite.ut.ee .Other types of databases that are freely available for fungal identification include *Aspergillus* Genome Database (AspGD), Candida Genome Database (CGD), Doctor Fungus, Institut Pasteur – FungiBank, FUSARIUM-ID, FungiDB, *Fusarium* Database, Mycobank, etc. (Prakash *et al.*, 2017).

2.7.5.2 SILVA reference database

Despite the large accumulation of genomic sequences across all databases, 16S rRNA genes are still regarded as the gold standard for taxonomic identification for nucleic-acid-related evaluations in microbial ecology studies, microbial diversity studies and phylogenetic construction analysis (Glöckner *et al.*, 2017). Therefore, in metagenomics studies, the assignment of thousands of sequences to a particular taxonomy entity is crucial and cannot be overemphasised (Park and Won, 2018). However, the identification of sequences is unavoidably affected by the choice of the selected database. There are several databases that make the assignment of taxonomy to 16S rRNA sequences possible. Some of such public databases include SILVA (https://www.arb-silva.de) , Greengenes (https://greengenes.secondgenome.com), and the Ribosomal RNA Database project- RDP Project (Balvočiūtė *et al.*, 2017).

SILVA is one of the world's open-access databases and web resources for rRNA gene sequences, which offers all-inclusive, quality filtered, and constantly updated datasets of small ribosomal subunits (SSU - 16S/18S) and large ribosomal subunits (LSU - 23S/28S) of Archaea, Bacteria and Eukaryota domains (Glöckner *et al.*, 2017). SILVA was made public in February 2007, with 353,366 sequences of SSU and LSU 46,979 sequences of LSU as its datasets, and has since released more SSU and LSU sequences with better improvements and features (Quast *et al.*, 2012). SILVA indicates its latest release by version numbers, and all sequences in this database are derived from the

EMBL-EBI/ENA, which are then quality filtered, linked to detailed and related information and most importantly linked to taxonomic classification (SILVA, 2019). Such related information about these sequences are called metadata which includes taxonomic classification by other databases, publication ID, organism name, name of author, title, submission and probably modification date (Park and Won, 2018).

SILVA's web resources include different online tools such as sequence-based search and online aligner, up-to-date documentation sheets, tutorials for SILVA tools as well as frequently asked questions (FAQs) (Park and Won, 2018). Datasets in SILVA are publicly made available as releases, instead of the dataset been updated. Every release is numbered according to the release number of EMBL-EBI/ENA from which the sequences were extracted into SILVA database (Quast *et al.,* 2012). As of September 2016, there were 5,616,941 SSU and 735,238 LSU rDNA sequences in the archives of SILVA (Glöckner *et al.*, 2017). However, the current release of SILVA as of December 2019 (SILVA version 138), contains 9,469,656 SSU and the LSU sequences released in the spring of 2020 (SILVA, 2019).

2.7.5.3 Ribosomal Database Project (RDP)

Ribosomal Database Project **(**RDP) database is an organised and highly-tailored database that contain collections of aligned, annotated and taxonomic information of the small subunit (SSU) ribosomal RNA genes from bacteria, archaea and fungi. RDP obtained its taxonomic classifications from the European Nucleotide Archive (ENA) (Cochrane *et al.,* 2012), while it obtains most of its ribosomal sequences from the International Nucleotide Sequence Database Collaboration (INSDC) databases. In cases where names of organisms in the INSDC are not updated, the most reliable up-to-date bacterial nomenclature are obtained from the German Collection of Microorganisms and Cell Culture (Nakamura *et al.,* 2012).

The RDP is one of the most highly recommended databases that also offer web resource tools and data analysis for researchers in the fields of Microbial ecology, Microbiology, Human health, Taxonomy and Genetics. As at September 1999, the RDP database (version 7.1) contained small subunit (SSU) rRNA aligned sequences exceeding 10,700 in number and unaligned sequences above 21,000. Version 9.21 of the database was released in August 2004 which contained 101,632 gene sequences of the bacterial SSU rRNA. In September 2008, the database released an updated version known as version

10.3, containing 643, 916 SSU rRNA genes from bacteria and 33,082 archaea sequences (Cole *et al.*, 2009).

In October 2013, version 11.1 was released. The release contained 2,809,406 aligned and annotated archaeal and bacterial information of the SSU rRNA sequences, as well as a handful of gene sequences from the large subunit (LSU) of fungi (approximately 62,860 sequences). In August 2020, the latest version of RDP taxonomy was released, (version 11.5) which contained 3,356,809 16S rRNA bacterial sequences and 125,525 of the LSU fungal sequences (Ribosomal Database Project, 2020). The exponential growth and increase in the number of sequences in the data repository is due to the rapidly increasing number of sequencing projects over the years. This results from an increase in the popularity/use of high throughput sequencing platforms, thus generating large volume of data (Cole *et al.*, 2014).

2.7.6 Bioinformatics tools and pipelines for metagenomic analysis

Recent developments in NGS/High Throughput Sequencing (HTS) have resulted in the generation of massive amounts of data in need of analysis and interpretation (Lu *et al.*, 2016). As enormous amounts of datasets are continuously being churned out, more computational and bioinformatics resources and tools are required to mine and extract useful and genuine information concerning the microbial population under study (Malla *et al.*, 2019). Bioinformatics tools are softwares designed to aid researchers in analysis, interpretation and comparison of vast amounts of genomic datasets (Branton *et al.*, 2008). The outcome derived from any metagenomics investigation depends on computational tools and pipelines that can scrutinise and analyse huge datasets which can source for and focus on several aspects, such as the taxonomic and functional composition of representative sequences of the community under examination (Ladoukakis *et al.*, 2014). To this end, a wide range of metagenomic analysis pipelines are available. These include QIIME, MG-RAST, MOTHUR and DADA2 (Caporaso *et al.*, 2010; Callahan *et al.*, 2016), etc., while examples of some stand-alone bioinformatics tools used in metagenomics analysis include FastQC, Trimmomatic, Phyloseq etc. (Andrews, 2010; McMurdie and Holmes, 2013).

2.7.6.1 QIIME - A metagenomic pipeline

Bioinformatics analysis pipelines are designed in such a way that they can integrate several algorithms and steps into one single program to generate a wide array of results (Siegwald *et al.,* 2017). Such steps include pre-processing of reads, detection of chimera, clustering of sequences into Operational Taxonomic Units, taxonomic delegation to representative sequences, diversity and statistical analysis (Kim *et al.*, 2013). QIIME (an acronym for Quantitative Insights Into Microbial Ecology) is a bioinformatics software that specialises in microbial community analysis by performing several analysis from DNA datasets generated from several sequencing platforms, such as Sanger, Illumina, Roche 454 and others. It can be used to investigate sequences generated from archaeal, bacterial, fungal and even from viral communities (Caporaso *et al.,* 2010).

QIIME makes use of Unix/ Linux Commands in writing and executing scripts on the command line/terminal in a UNIX-like environment, as this pipeline's mode of interaction is via the command-line interface (Ursell *et al.,* 2015). QIIME is currently one of the most popular bioinformatics pipelines used in analysing rRNA amplicons and it is capable of producing quality graphics and statistical analysis and aids in performing quality control analysis of raw reads, clustering sequences into operational taxonomic units (OTUs), assigning taxonomy to sequences, constructing phylogenetic trees, performing diversity analysis and has interactive visualisation tools for viewing microbial data (López-García *et al.,* 2018).

QIIME1 has incorporated several OTU clustering tools within its workflow, and it has three methods of performing clustering. Normally, OTUs are created by initially performing pairwise sequence alignment (against a reference sequence collection or each other - between reads). It is the percentage of the agreed pairwise sequence alignment between these reads that is computed as the percentage similarity (Johnson *et al.,* 2019). However, a popular percentage similarity threshold used is 97%. This percentage originated from an experimental study which indicated that most strains have 97% 16SrRNA sequence similarity (Nguyen *et al.,* 2016), therefore, strains that are 97% or greater than the set threshold value are clustered together as same species (Schloss and Handelsman, 2005). With the OTU method, the researcher cluster reads accordingly and then a single sequence is designated as the representative of other reads in the

cluster, this single sequence is thereafter assigned into a particular taxonomy (Chaudhary *et al.,* 2015), it is this selected taxonomy that automatically becomes the same for all reads in the same clusters. This method has been used by several pipelines which include QIIME1 and MOTHUR (Caporaso *et al.,* 2010, Johnson *et al.,* 2019). However, the current version of QIIME termed QIIME2 in addition to OTU picking strategies has now included the use of precise "sequence variants" methods (also known as Amplicon Sequence Variants) for inferring biological sequences, which is a major improvement over the use of Operational Taxonomic Units (Bolyen *et al.,* 2019). Several metagenomic studies have employed the use of this pipeline in analysing several communities. Some of such studies includes studies from López-García *et al.,* (2018) and Rajan *et al.,* (2019).

2.7.6. 2. DADA2 pipeline

The Divisive Amplicon Denoising Algorithm 2 (DADA2) pipeline is an open-source bioinformatics software located within the R package. DADA2 pipeline was built with the intention of modelling and correcting (from amplicon reads) errors associated with Illumina sequencing (Callahan *et al.,* 2016). It has however, been used for processing and analysing amplicon reads generated from other sequencing platforms such as Roche 454 sequencing platforms, PacBio CCS sequencers and Ion Torrent (Callahan *et al.,* 2019; Siegwald *et al.,* 2019). This pipeline can be applied to any genetic locus such as regions from the 16S rRNA gene for bacteria analysis, the ITS loci for fungal analysis and the 18S ribosomal gene for eukaryotic analysis (Pauvert *et al.,* 2019). The pipeline accepts paired-end demultiplexed fastq files, commences its workflow with quality filtering of raw reads and ends with the merging of cleaned paired-end reads (Callahan *et al.,* 2016).

DADA2 relies heavily on the R package as it is embedded in the R environment (Callahan *et al.,* 2019). R is a combined collection of software amenities that is freely available for manipulation of data, construction of publishable graphics as well as statistical calculations. It is a well-developed programming language and environment which has gained rapid attention in the world of bioinformatics. The R environment provides tools for data handling, data storage, data analysis, different formats of documentation, and a wide range of statistical computation (R Core Team, 2020).

DADA2 pipeline when compared with other platforms was confirmed to perform better in some instances, some of which include: the ability of the pipeline to perform better in the face of technical noise, that is, it was able to reduce false positives as a result of sequencing errors and in some cases, remove them (Caruso *et al.,* 2019). Furthermore, DADA2 makes use of the ASV method, ASVs are used to indicate/describe distinct DNA sequences retrieved from NGS marker gene analysis, after the removal of spurious sequences that might have been created during PCR amplification and sequencing. They are, therefore, referred to as deduced sequences of accurate biological origin (Callahan *et al.*, 2017). The ASV method can deduce precise amplicon sequence variants from data generated from amplicons, therefore, it can distinguish biological variances by as little as 1 or 2 nucleotides. Hence, is also known as the high-resolution sample inference from Illumina amplicon data (Callahan *et al.,* 2017).

ASVs are used to indicate/describe distinct DNA sequences retrieved from NGS marker gene analysis, after the removal of spurious sequences that might have been created during PCR amplification and sequencing. They are therefore referred to as deduced sequences of accurate biological origin. Studies have shown that ASV methods record better sensitivity, specificity and a more encompassing computational identification of microbial communities than OTU clustering methods (Callahan *et al.,* 2017, Caruso *et al.,* 2019).

2.8 Bioprospecting microorganisms from the natural environment for industrial enzymes

Bioprospecting can be described as the methodical and organised exploration of biological diversity for novel products that may be of biotechnological importance, and value to society (De Oliveira *et al.,* 2015). Presently, there is an increasing need for appropriate biocatalysts which possess extraordinary activities as an alternative to chemically-synthesised biomolecules. The advantage of bioprospecting over chemically-synthesised biomolecules includes novelty in products such as a rare variety of biomolecules, metabolites and metabolic pathways, and can also chart a pathway for a new design or inspiration (Madhavan *et al.,* 2017).

2.9 General characteristics of enzymes

Enzymes are biological macromolecules which increases the rate of a biochemical reaction in living systems, they are also called biocatalyst and can speed up rate of reactions from hundreds of thousands to million times faster, without them being converted in the process (Robinson, 2015). Enzymes comprises of chains of amino acids connected by amide bonds to form a three-dimensional structure. They range in molecular weight from kilodalton to megadalton and are usually produced by living cells, such as microbes, plants and animals (Gurung *et al.,* 2013).

Enzymes due to their protein nature are prone to denaturation, when subjected to extreme conditions such as extreme temperature, pressure and pH, heavy metal ions, and solvent composition (Gurung *et al.,* 2013). Protein denaturation occurs when there is a disruption of the 3-Dimensional structure of proteins, therefore rendering them functionally inactive (Robinson, 2015). Denaturation can occur when there is a dislocation of several bonds (hydrogen bonds, electrostatic forces and hydrophobic bonds) that plays a critical role in the protein's stability. It can also occur when the hydrophobic groups of the protein are exposed, which results in loss of solubility. For example, when extreme heat or excessive increase in temperature is introduced to protein medium, the protein molecules begin to vibrate due to increased kinetic energy (Kishore *et al.,* 2012). This excessive vibration then results in an irreversible damage by bringing about disorderliness in the intramolecular bonds of the protein such as in hydrophobic interactions (the largest driving force in protein structure), as well as in hydrogen bonds which eventually leads to unfolding of the protein molecule (Kishore *et al.,* 2012).

When protein is subjected to certain pH levels, such as extremely basic or acidic pH, it can result in the unfolding of the protein's helical structure, by damaging both tertiary and secondary structures of the protein, due to the interference of the excessively introduced ions on the ionisable groups of the protein's amino acids (Konermann, 2012). The same mechanism in denaturation is also observed when excessive salts is introduced to protein medium. Heavy metals play the role of a denaturant when introduced into protein media in various ways such as: Their ions forming bonds with free sulfhydryl groups of the available proteins, the heavy metal ions forming complexes with the side chain groups available in proteins, or act on the side chains of amino acids by oxidising them. All of these mechanism results in the irreversible disruption of disulphide and ionic bonds present in the protein (Tamás *et al.,* 2014). Heavy metals when in contact

with an enzyme whose cofactor is a metal ion, are capable of displacing the cofactor ion and replacing it with their own ions, hence rendering the enzyme biologically non-functional. The introduction of high concentration of organic solvents to enzymes can also result in the loss of tertiary structure of the enzyme. This is due to the severe break in the inter and intramolecular bonds within the protein, such as hydrogen bonds (Konermann, 2012).

Enzymes are highly specific in nature – they are precise in their reaction and in their choice of substrates (Bhatia, 2018), therefore, they are known to catalyse a number of closely related reactions or a particular chemical reaction. (Gurung *et al.,* 2013). Their specificity was explained in 1894 by Emil Fischer who postulated that both enzyme and substrates had definite matching shapes which made them fit perfectly into each other. This postulation is what is now referred to as the "Lock and Key" model (Fischer, 1894).

Studies have shown that only minor and specific parts of enzymes are directly implicated in enzyme catalysis (Robinson, 2015). These small portions are known as the enzyme's active site. The site contains distinctive combination of amino acids which binds to substrates, and brings about catalysis in a reaction. In 1958, another model was proposed by Daniel Koshland Jr. He described the active site as a flexible site capable of changing its shape, which is induced by the introduction of a substrate to the enzyme's active site, in such a way that the active site changes in conformation to the best form to encourage chemical reaction (Bhatia, 2018). Substrates are bound to the active site using several forms of interactions such as Van der Waals force, electrostatic interactions, hydrogen bonds etc.

Most enzymes work in tandem with other molecules known as coenzymes, to facilitate catalysis. Coenzymes are low molecular weight compounds which are organic in nature and also produced from living cells (Gurung *et al.,* 2013). Coenzymes have also been identified to play a part in maintaining the structural integrity of enzymes that require coenzymes to function optimally. This is because, the absence of coenzymes for such enzymes might result in improper folding and instability of the enzyme, and therefore will be unable to enhance catalysis in any biochemical transformation (Broderick, 2001). At the initial stage of a biochemical conversion, coenzymes bind to the enzyme prior to the substrate binding, hence altering the shape of the enzyme. In any biochemical reaction, coenzymes act primarily as transporters of functional groups or electrons, and

unites these functional groups with the enzyme to assist biological reactions (Robinson, 2015).

In addition to coenzymes, some enzymes require additional compounds known as cofactors to facilitate catalysis. Co-factors are non-organic compounds which also assist in catalytically activating some enzymes. They include metal ions such as Magnesium, Zinc and Copper ions (Robinson, 2015). They facilitate catalysis by binding loosely to the enzyme, or firmly binding to the enzyme's active site. Metal ions generally delivers readily accessible oxidation states and high electrophilic centres that enables electron transfers and redox reactions in an enzymatically-catalysed reaction (Broderick, 2001). In situations where the enzyme requires a co-factor to catalytically function, but does not have a co-factor bound to it, is called an apoenzyme. This kind of enzyme is catalytically inactive. However, an enzyme which has a cofactor bound to it, is called a holoenzyme. Holoenzymes are catalytically functional as they contain the cofactor necessary to stimulate catalysis (Gurung *et al.,* 2013).

2.10 Bioprospecting for alpha-amylase from the natural environment

Isolation, identification, and screening of microorganisms for enzyme production in low-cost medium have been in existence for a long while and have been widely used due to their extensive applicability (De Oliveira *et al.*, 2015). Several studies have been conducted where culture-dependent bioprospecting methodology was applied to explore bacteria with potentials for alpha-amylase of unique characteristics in Nigeria. Some of such include studies by Oyeleke *et al*. (2011), Oziengbe and Onilude (2012), and Madika *et al.* (2017).

Alpha-amylase is one of the most sought-after enzyme in commercial industries, in addition to protease and cellulase enzymes. Alpha-amylase, plays a critical role in the conversion of starch (one of the largest polysaccharides in nature), by breaking down starch granules into oligosaccharides, comprising of glucose units (Souza and Magalhães, 2010). They are therefore essential in several major industries, that make use of starch-containing substrates as raw materials, to produce several industrially value-added products (De Oliveira *et al.,* 2015). They have gained widespread relevance in clinical, pharmaceutical, laundry, baking, brewing, pulp and paper, as well as in textile industries to generate products such as digestive tonics, medications containing

digestive enzymes, detergents, alcoholic beverages, pastries, laundry soaps, etc. (Mehta and Satyanarayana, 2016).

Even though amylases can be found in all life forms – plants, animals, and microorganisms, microbial amylases are by far the most widely used in industries, because they can be cultured in huge quantities within a relatively short time by fermentation. They are more stable and active than their counterpart from animals and plants, and they can be easily manipulated genetically due to their biochemical diversity (Elyasi *et al.*, 2020). Amylase producing organisms include Bacteria, Fungi and Achaea.

Producers of amylase in bacteria comprises of some genera such as those belonging to the phyla Firmicutes and Proteobacteria, amongst others. Among the phylum Firmicutes, are a sizable number of *Bacillus* species, selected species of *Ruminococcus* and certain species of *Staphylococcus* (Ze *et al.,* 2015). The phylum Proteobacteria include some species of *Pseudomonas, Halomonas* and some species of *Escherichia* (Ruginescu *et al.,* 2020). Fungi are also superb producers of amylase, which include widespread genera, such as *Rhizopus*, *Aspergillus* and *Penicillium* (Abdel-Azeem *et al.,* 2019a). In-like manner, members of Archaea have also been documented to produce alpha-amylase. Some of which are *Pyrococcus* sp., *Desulfurococcus* sp. and *Thermococcus* sp. (Lévêque *et al.,* 2000).

As cited earlier, alpha-amylase can be derieved from a variety of bacteria, but, the commercial production of amylase is predominantly sourced from the *Bacillus* genus (Souza and Magalhães, 2010). A large number of *Bacillus* species have been documented to produce alpha-amylase of various properties. Such as acid-tolerant alpha-amylase from *Bacillus acidicola* (Satyanarayana, 2017), halo-tolerant alpha-amylase from *Bacillus aquimaris* (Anupama and Jayaraman, 2011), cold-active alpha-amylase from *Bacillus cereus* GA6 (Kuddus and Ahmad, 2012), thermophilic and hyperthermophilic alpha-amylase from *Bacillus caldotenax* (Ginting *et al.,* 2021), mesostable alpha-amylase from *Bacillus marini* (Demirkan *et al.,* 2017) and alkaliphilic alpha-amylase from *Bacillus subtilis* (Al-Johani *et al.,* 2017). This genus has also been subjected to various genetic modification of the alpha-amylase gene (such as gene cloning and over expression strategies) for enhancement and overproduction of alpha-amylase have also been extensively applied (Elyasi *et al.*, 2020).

Alpha-amylases (E.C.3.2.1.1) are extracellular enzymes that catalyses the hydrolysis of the internal alpha-1,4-glycosidic bonds to products of low molecular weights such maltotriose, maltose and glucose units while retaining their alpha anomeric configuration (Elyasi *et al.*, 2020). Most alpha-amylase belong to the Glycoside Hydrolase family 13 (GH13), while a minority of them are found in GH57, GH119, and GH126. Amylases are the most widely used enzymes in industries, and as such, they constitute about 25 -33% of the enzyme market (Souza and Magalhães, 2010). Alpha-amylase production for industrial use is normally produced in submerged fermentation mode, employing various forms of microbes, such as Actinomycetes, bacteria, yeasts and fungi (Hiteshi, 2016).

Enzymes of industrial importance are chiefly produced by thermophilic microbes, which have been adapted to surviving in high temperature (Hiteshi and Gupta, 2014). They are able to live optimally within the temperature range of 40 to 80°C, secreting enzymes that are stable at high temperature. Such microorganisms can be found in environments such as Geothermal vents, coal refuse, compost and hot springs (Satyanarayana, 2017). Thermophilic bacteria that have been profiled for thermophilic and thermotolerant alpha-amylase production include *Bacillus subtilis*, *Bacillus licheniformis* *Thermococcus* sp., *Alicyclobacillus* sp. *Pyrococcus* sp. and *Alicyclobacillus* sp. (Hiteshi, 2016, Mehta and Satyanarayana, 2016, Al-Johani *et al.*, 2017).

2.11 Factors that affect the production of alpha-amylase

Alpha-amylase production is usually influenced by various nutritional and environmental factors. Among these factors are temperature, pH, aeration and agitation, period of incubation, nature of substrates used (nitrogen and carbon sources), inoculum size, moisture content, etc. (Elyasi *et al.*, 2020). However, to achieve maximum production of alpha-amylase and to prohibit the aggregation of protein during production, these factors must be controlled in order to achieve maximum production of alpha amylase (Elmansy *et al.*, 2018).

Temperature is one of the most important parameters that plays a significant role in the production of enzymes. The growth of an enzyme-producing organism is usually connected to the temperature at which it is subjected to (El-Fallal *et al.*, 2012). Normally, with an increase in temperature comes an increase in enzyme production, this is because

with an increased temperature comes increased kinetic energy and elevated rate of microbial metabolism, which results in increased enzyme production (Li *et al.*, 2015). However, when the temperature reaches a crescendo, the enzyme produced would peak, while further increment beyond the optimum growth of the enzyme-producer can result in enzyme inactivation and denaturation, hence a decrease in enzyme production and ultimately cell death (Elyasi *et al.*, 2020). However, the maximum temperature at which an enzyme is produced would also depend on if the enzyme-producer is psychrophilic, thermophilic or mesophilic in nature (El-Fallal *et al.*, 2012).

Hydrogen Ion concentration, also known as pH, also plays a strong role in the production of enzyme. Care must be taken to control the pH of an enzyme's production medium. This is because the shape of an enzyme's active site is usually affected by the hydrogen and hydroxyl ions (Demirkan *et al.*, 2017). At the optimum pH, enzyme production is at its maximum, but if the pH of the medium containing the enzyme-producer is changed above or below the optimal pH of the enzyme, a decline in the production of enzyme would set in, as the rate of reaction begins to decrease. The decrease in reaction rate is as a result of an increase in enzyme molecules whose shape of their active sites have been distorted, in such a way that their active sites are no longer complementary to the substrates to which they should normally bind (Demirkan *et al.*, 2017; Elmansy *et al.*, 2018). Also, extremely low or high pH values can have a debilitating effect on the membrane of enzyme-producing-microbes, by destabilising the transportation of solutes across their cell membrane, hence leading to a decline in enzyme production (Jin and Kirk, 2018).

Agitation and aeration of the culture medium where the microorganism is present is another determining factor. Agitation during enzyme production serves to aerate the production medium, break up cell aggregates into smaller pieces and constantly mix contents of the production media (Zhou *et al.*, 2018). Agitation can cause nutrients, oxygen and heat to completely mix in a fermentation broth, therefore causing them to be evenly transferred in the broth. It also aids in breaking up bubbles to establish better contact between the liquid and oxygen gas (Zhou *et al.*, 2018). It is paramount to also determine the best agitation speed for the enzyme being produced. This is because an extremely high or low agitation speed can also affect the production of enzymes. For example, an excessively high agitation speed can result in a change in enzyme formation, and can cause cell death of fragile cells. However, if the agitation speed is too low, the

production media can become viscous, and can lead to a decrease in the efficiency of transfers (El-Fallal *et al.,* 2012). Aeration, the process of supplying and circulating air into a media, such as a liquid or fermentation media. Aeration is imperative in the growth and metabolism of aerobic microbes, as it has an important effect on the synthesis of some metabolites (Zhou *et al.,* 2018). Not only is aeration responsible for circulating air into a media, it also gets rid of gas produced during the fermentation. In addition, it contributes to the homogenous mixture of the components of a growth or fermentation broth. However, if the rate of aeration supplied is too high, it can cause a loss in the volume of the liquid media and if excess oxygen is supplied to certain microorganisms, it can become deleterious to their survival (Zhou *et al.*, 2018, Elyasi *et al.,* 2020).

Generally, the production of enzymes, is directly connected to the consumption of substrate by the enzyme producer. However, the infusion of nitrogen source in the substrate is critical, as they provide the required vitamins and amino acids essential for growth and production (Deljou and Arezi, 2016). Nitrogen can be introduced into the medium as inorganic or organic forms. Organic forms of Nitrogen usually introduced into alpha amylase production media include tryptone, peptone, yeast extract and beef extract (Kumar *et al.,* 2014). While inorganic forms that have been extensively used in amylase studies include, ammonium sulphate, sodium nitrate and ammonium nitrate (Abel-Nabey and Farag, 2016). Studies such as those carried out by El-Fallal *et al.* (2012), Kumar *et al.* (2014) and Al-Johani *et al.* (2017), have however shown that organic forms of nitrogen best support maximum production of amylase from bacteria-producers.

Another important component of substrate which is likewise critical like Nitrogen is Carbon. Carbon sources can be in form of monosaccharides, disaccharides or polysaccharides (Abel-Nabey and Farag, 2016). They can also be in their natural forms (for example, food wastes and agro-industrial residues - rice straw, yam peels, cassava peels, etc.) or in their synthetic state (such as galactose, maltose, fructose, etc.) (Guimarães, 2012). Organic carbon sources seem richer than its counterpart, as it contains other minerals that may affect production of alpha amylase than its synthetic counterpart. Synthetic carbon sources do not contain other constituents, as they are purely synthesized and have a well-defined composition (Suribabu *et al.,* 2014). The incorporation of carbon sources into amylase production media acts as a source of energy and metabolic reactions, as they are an essential component required for

microbial cell growth. Enzyme production can be initiated in microorganism by the accurate addition of carbon source into the media (Guimarães, 2012).

2.12 Purification of alpha-amylase

Basically, enzymes are produced in their crude forms, and are normally subjected to purification to eliminate unwanted materials. Enzyme purification is the process of removing contaminants, unwanted proteins and cell debris from a medium containing the enzyme of interest (El-Fallal *et al.,* 2012). This process, is however achieved using several methodologies and steps, ranging from the less expensive method to the expensive techniques (Ramos and Malcata, 2011).

Purification of enzymes helps in understanding intrinsic details of the enzyme, such as its molecular and physiological characteristics, structural and functional attributes. It also facilitates the safe application of the enzyme particularly in food-related industries. (Sumrin *et al.,* 2011). Generally, enzymes produced from microorganisms require that it is separated from microbial debris and other cellular contents. For example, extracellular proteins/enzymes produced by microorganisms, would most importantly require separation of microbial cells from the supernatant containing the crude enzyme of interest (Ramos and Malcata, 2011). However, if the protein of interest is intracellularly produced by the microbe, then the first step, would be cell lysis of the host cell. Cell lysis is a technique used to rupture the cell membrane to release intercellular components of the host cell into the surrounding environment (Shehadul-Islam *et al.,* 2017). Purification of proteins (enzymes) commonly performed in a research laboratory and at a commercial/large-scale use involves techniques such as precipitation, centrifugation and filtration (Ramos and Malcata, 2011).

2.12.1 Purification Technique: Precipitation

This method is commonly used as part of the purification step of large macro molecules, especially, proteins. Precipitation can be employed in desalting and concentration of proteins (Duong-Ly and Gabelli, 2014). Protein precipitation involves the separation of protein molecules from liquid mixtures, by employing a reagent to modify the protein's solubility. Several reagents can be used in achieving precipitation such as organic solvents, neutral salts, hydrophilic polymers, polyvalent metal ions including Calcium, Magnesium, Manganese ions etc (Nickerson and Doucette, 2020).

Organic solvents such as acetone, dimethyl sulfoxide (DMSO) and several alcohols have been used in the recovery of proteins from solution, but not without some disadvantage. Generally, organic solvents have been documented to show low protein recovery yield, and also been recorded for their ability to interfere in secondary and tertiary structure of protein, which sometimes lead to protein denaturation (Nickerson and Doucette, 2020; Baghalabadi *et al*., 2021). Also, according to Tjernberg *et al.* (2006), their group showed that the inclusion of DMSO in protein purification can lead to protein degradation and denaturation. Hydrophilic polymers such as Polyethyleneglycol (PEG) is also described as a favourable reagent in the purification of proteins, due to its neutral chemical properties, in contrast to ethanol. The inclination of PEG to denature proteins is very minimal even when in high concentrations. The drawback of PEG in protein purification is the tendency to cause viscosity in solution (Cruz *et al.,* 2000).

The addition of neutral salts are also desirable reagents in protein purification. In 1887, Franz Hofmeister, a protein scientist was the first to describe the application of neutral salts in precipitation. He provided a catalogue of cations and anions in the order of which these ions could salt out proteins from aqueous solutions. The arrangement of these cations and anions is termed the Hofmeister series (Hofmeister,1888). In the series, anions known to encourage hydrophobic interactions are listed in order of decreasing strength: Phosphate ion > Sulphate ion > Acetate ion > Chloride ion > Bromide ion > Nitrate ion > Perchlorate ion > Iodide ion > Thiocyanate ion. While the order for cations in order of decreasing strength as described in the series are as follows; Ammonium ion > Rubidium ion > Potassium ion > Sodium ion > Caesium ion > Lithium ion > Magnesium ion > Calcium ion > Barium ion (Hofmeister,1888; Burgess, 2009).

The most popular neutral salt widely adopted and commonly used in protein precipitation is Ammonium sulphate. This is due to the salt comprising of cation and anion highly placed in the Hofmeister series, its water-soluble nature, low density and its relative ease to purchase. It is also a neutral salt of choice due to its ability to cause no detrimental effect on the structure and activity of proteins (Evans *et al.*, 2009; Duong-Ly and Gabelli, 2014). The use of neutral salt in the precipitation of proteins is commonly regarded as ‘salting-out’. Salting out can be defined as the phenomenon observed when there is a decrease in water solubility of organic compounds with an increase in the concentration of an ionic salt (Burgess, 2009). This occurs as a result of competition for water molecules between the salt and protein molecules. An increase in

the concentration of salt molecules results in the decrease of protein-water interactions, and an increase in the surface tension of water, thereby causing the proteins to fold firmly, triggering aggregation and consequently precipitation (Duong-Ly and Gabelli, 2014). Precipitation using ammonium sulphate is usually achieved by adding saturated amount of $(NH_4)_2SO_4$ solution gradually to an aqueous protein solution to a chosen concentration (Burgess, 2009). The only set-back associated with salting out in protein precipitation is the co-precipitation of contaminants alongside the protein of choice. It is therefore desirable that precipitated proteins be further purified following this procedure (Burgess, 2009).

2.12.2 Purification Technique: Dialysis

Dialysis is the process of separating a mixture of molecules based on size and diffusion principle. It is among the common technique used in removing compositions such as low-molecular-weight solutes, unwanted salts, contaminants from large molecules such as proteins (Evans *et al.*, 2009). The purification method follows the diffusion principle, in which movement of molecules occurs from regions of greater concentration to regions of lower concentration, until concentrations of both regions becomes equilibrium (Luo *et al.,* 2011). The molecules to be dialysed is placed with a semipermeable barrier, which acts to prevents the movement of large molecules across the provided membrane, while the smaller molecules are able to diffuse across the membrane through the membrane's pores (Scientific, 2009).

Ideally, the sample to be purified is placed in a dialysis bag and firmly clamped or tightly tied at both ends of the dialysis bag. The dialysis bag typically acts as the semipermeable membrane as it contains specific pore sizes, also known as Molecular Weight Cut Off (MWCO), it is these pore sizes that determines which molecules flow though the bag and which get retained (Drioli *et al.*, 2016). The bag is then lowered into a buffer solution (commonly referred to as dialysate) which is several times larger than the volume of the sample to be purified, to maintain concentration gradient. This way smaller molecules permeate through the dialysis bag through the pores, into the surrounding buffer, while the larger molecules, such as proteins are retained in the bag, due to restriction in movement provided by the pores of the dialysis bag (Evans *et al.*, 2009).

The time at which purification is achieved in dialysis is dependent on a number of factors

such as the MWCO of the dialysis bag, the concentration of the sample to be dialysed, the molecular weight of the sample, the availability of the surface area to sample molecules and the thickness of the membrane (dialysis bag), and the temperature at which the dialysis experiment was set-up (Scientific, 2009; Drioli *et al.*, 2016). Studies have shown that the more the concentration of the sample molecule, the higher the chances of these molecules being in contact with the membrane, hence increased rate of diffusion to the other environment. On other side, the larger the molecular weight of the sample, the lesser the chance of membrane-contact, therefore, the rate of diffusion is reduced (Luo *et al.*, 2011). The thicker the membrane bag, the lesser the diffusion rate. The flatter the surface area of the membrane, the better the process of dialysis, as this facilitates closer and immediate contact of ample molecules to the membrane. Hence, it is advised during dialysis to ensure the bag is sealed in a taut manner to encourage and facilitate better membrane-molecule contact (Walker, 2009). Another important factor that can facilitate increased rate of dialysis is stirring. Stirring the dialysis set-up, helps in breaking up a layer termed the Nernst diffusion layer. This layer is formed when the lower molecular weight components of the sample diffuse to the outer environment and concentrate around it dialysis bag. Non-stirring would result in the build-up of these components in high concentration, hence reducing the rate of diffusion from the bag to the buffer. Stirring thus aids in breaking up this pool of low molecular weight concentration, therefore driving the diffusion process faster (Scientific, 2009; Evans *et al.*, 2009).

2.12.3 Purification Technique: Chromatography

It is regarded as one of the most important purification techniques which has been widely adopted in analytical and biological science. This is because it allows for complex mixtures of compounds be separated, identified and purified into their respective compositions (Coskun, 2016). It is one of the most popular method used for resolving protein components, following the removal of crude extracts and contaminants (Kumari *et al.*, 2022). This method exploits the biological and chemical characteristics of proteins for their purification. Such as the proteins' binding ability with a static phase, total charge of the protein, size, shape, molecular weight and its hydrophobic groups available on its surface (Coskun, 2016).

Chromatography is based on the principle of physical separation of mixtures of molecules by applying the mixture to be separated onto two phases – the stationary and mobile phase. The stationary phase, also known as the solid phase is usually made up of a layer of fixed but porous bed of material. While the mobile phase is the fluid which moves over the solid phase under gravity (Kumari *et al.*, 2022). Over the years, several chromatographic variants have been adopted in the separation and purification of mixtures. They include: Column chromatography (Size Exclusion chromatography and Ion-exchange chromatography), affinity chromatography (Dye-Ligand chromatography, Hydrophobic Interaction Chromatography), Gas chromatography, Paper chromatography, Pseudo-affinity chromatography, Thin-layer chromatography and High Performance Liquid Chromatography (Coskun, 2016).

Colum chromatography can be in several variants: Size Exclusion Chromatography (SEC) and Ion-exchange chromatography (Hedhammah *et al.*, 2006). SEC is also known as Gel filtration, Gel-permeation chromatography, or molecular sieve/exclusion chromatography. SEC, Thin-layer chromatography, and paper-chromatography employs the partition principle in separating molecules on the basis of their shape and molecular sizes via sieving (Hagel, 1998).

In SEC, the immobile phase is usually made up of snugly packed but porous inert beads. Available spaces/channels between each bead allows for easy passage of large molecule, rather than them being impeded as in the case of Gel Electrophoresis (Hagel, 1998). These large molecules under gravity are pushed to travel through spaces outside these porous beads, and are quick to elute the chromatographic system first. The smaller molecules from the mixture however, if they are small in size enough to enter the pores of the inert beads, will be delayed, as they have to travel through these pores and then through the column, hence smaller molecular weight molecules elute later than large molecular weight molecules, resulting in separation of molecules based on their sizes (Hagel, 1998).

A commonly used material for stationary phase in SEC is a filtration bead commercially known as Sephadex. Sephadex is a highly porous bead which is made from dextrans (a polysaccharide), that has been highly cross linked with epichlorohydrin. Sephadex is chemically and physically stable due to these highly crosslinked particles (Coskun, 2016). Lastly, the diameter and height of the column used, size of beads, flow rate and

volume of protein mixture to be separated, all play a role in the resolution of proteins in gel filtration (Hagel, 1998).

In Ion-exchange chromatography, separation of protein mixtures is achieved by ionic interaction between the surface of a protein and the ionic charge coated on the surface of a provided solid support (an adsorbent) (Acikara, 2013). Ideally, the adsorbent- often called ion exchange adsorbent is coated with ions opposite to that of the protein to be separated. If the adsorbent is coated with negatively charged ions, it is called a cation exchanger, and it adsorb positively charged groups, but if the matrix or stationary phase is loaded with positively charged ions, it is referred to as an anion exchangers (Coskun, 2016), and adsorbs negatively charged group of proteins. To selectively release the adsorbed protein from the matrix, the mobile phase, usually a buffer is normally adjusted. The ionic strength of the buffer can be increased, such as supplementation of the buffer with sodium chloride, or by altering the pH of the buffer (Hedhammah *et al.*, 2006).

Affinity chromatography exploits the biological and highly specific interaction of proteins with other molecules in separating mixtures. For example, interactions between a ligand and a protein (such as cellulose, dextran, etc.), enzyme and its substrate, antibody and its antigen, hormone and its corresponding receptor, lectin and polysaccharides, vitamins and carrier proteins, etc. are typically used in this type of chromatography (Urh and Zhao, 2009). These pairs are highly specific in reaction and reversible, they can thus be used in separating and purifying macromolecules (Acikara, 2013). Separation is achieved by placing either of the pairs already mentioned, on to a matrix (stationary phase), and the desired molecule in the mobile phase. Most times, the stationary phase usually contains the ligands (Wilchek and Chaiken, 2000).

2.13 Structural and molecular characteristics of alpha-amylase

Both thermostable and mesostable enzymes, are made up of same 20 amino acids (Vieille *et al.*, 1996; Chakravarty and Varadarajan, 2000), the comparison of crystal structures, analysis of sequence alignments and comparison of amino acid contents suggests that thermophilic enzymes are very similar to their mesophilic counterparts (Vielle *at al.*, 1996). While the former statement is correct, it is paramount to note that the difference between thermostable and mesostable enzymes can be a function of subtle amino acid modifications, differences in the percentage of certain amino acid content,

as well as increase or decrease in certain types of amino acids (Chakravarty and Varadarajan, 2000). All alpha-amylase proteins irrespective of thermophilicity, are made up of polypeptide chains which folds into three distinct domains; Domains A, B and C (Hiteshi and Gupta, 2014).

Domain A contains the core catalytic domain in the form of a parallel (β/α)8-barrel, this barrel was first discovered in the structure of Taka-Amylase A, otherwise known as alpha-amylase from *Aspergillus oryzae* (Janecek, 2009). Because this sort of fold was first discovered in triose-phosphate isomerase (TIM), the barrel is frequently called the TIM-barrel (Farber and Petsko, 1990; Janecek, 2009). The TIM barrel (in domain A) consists of some highly important conserved regions (regions I - IV) which have been closely related to the active site present in all alpha-amylase (Kuriki *et al.*, 2005).

Domain B comprises of a long loop that extends between β strand 3 and α helix 3 with a β sheet configuration connected to domain A. Domain B has an uneven beta-sheet configuration, which varies significantly among various alpha-amylases. Domain B has allegedly been documented to be responsible for the difference observed among various alpha-amylase, with respect to their substrate specificity (Mehta and Satyanarayana, 2016). It is believed that the calcium binding-site for all recognised alpha-amylase (with very few exception) is located in between domains A and B, as the calcium binding-site contains some conserved amino acid residues. Also, in between domains A and B, is the active site cleft. These active sites, contains various amino acid residues which either directly undergo hydrogen bonding to the substrate or form hydrogen bonds using molecules of water (El-Fallal *et al.*, 2012). X- Ray crystallography and mutation studies showed that at the center of the active site were three indispensable amino acid residues which were critical and highly significant for catalysis to take place, namely; Glutamic acid (1 residue) and 2 residues of Aspartic acids (El-Fallal *et al.*, 2012).

Robert *et al.* (2003) showed that domain C, in alpha-amylase of barley, has a part to play in raw starch-binding, although it is not yet understood if this role is common across all alpha-amylases. Domain C consists of amino acid residues that have been folded on to an eight-stranded antiparallel barrel and is loosely connected with the other two domains. (Kraulis, 1991; Hiteshi and Gupta, 2014) as shown in Figure 2.4.

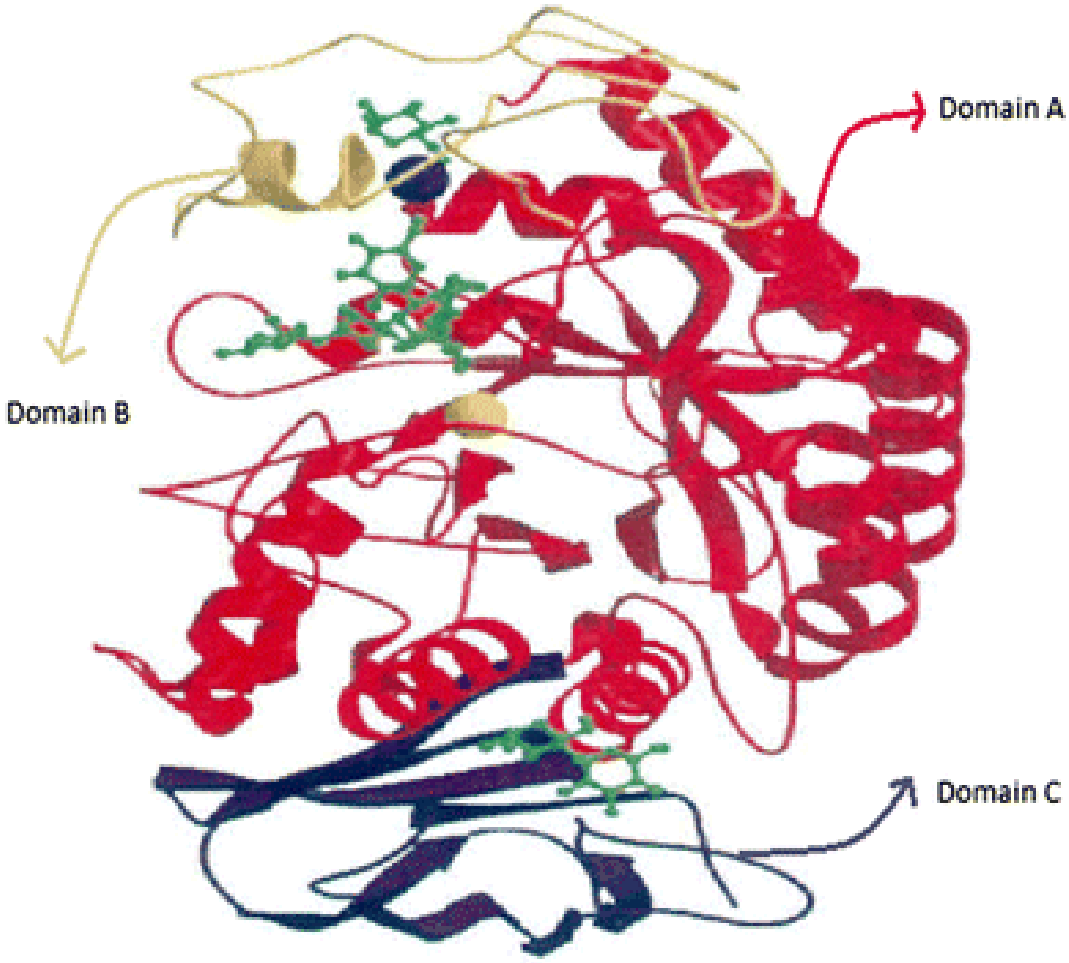


Figure 2.4: Alpha-amylase structure depicting domain A in red, domain B in yellow and domain C in purple. Calcium ion in the catalytic center is shown in the blue sphere.

Source: Kraulis, (1991).

2.14 Differences in signatures of thermophilic and mesophilic alpha-amylase

It is mostly assumed that charged amino acids are more present on the surface of thermophilic proteins. Charged amino acids such as Lysine, Arginine, Glutamic acid and Valine were implicated in the increment, and a reduction in the amount of uncharged amino acids such as Asparagine, Glutamine, Serine and Threonine in thermophiles (Chakravarty and Vardarajan 2000).

Ionic interactions such as Cation-pi interaction has also been labelled as another form of electrostatic contact that has been known for protein thermostability (Folch *et al.*, 2010). Cations are created by interaction which occurs among positively charged amino acids (Arginine and Lysine) and aromatic residues (Tryptophan, Phenylalanine and Tyrosine), a system of cations is preserved by Lysine in thermophiles while Arginine plays a main role in mesophilic proteins (Gromiha *et al.*, 2002). Disulfide bonds are structural components that are of significance with thermostable enzymes. They have been discovered to enhance stability within thermophilic proteins and prevents modification of the quaternary structure of proteins (Reed *et al.*, 2013). Amino acids at the protein's surface appear to seem flexible, and display free intra-protein interactions. These interactions in thermophilic microbes, augments the thermotolerance of their proteins. Example of such interactions include the formation of salt bridges as a result of large fraction of charged amino acids at the surface (Kumar *et al.*, 2000, Hiteshi and Gupta, 2014).

Other factors that have been linked to greater thermostability in proteins includes: Better packing, deletion or shortening of loops, greater hydrophobicity, amino acid substitutions within and outside the secondary structures, and decreased occurrence of thermolabile residues (Kumar *et al.*, 2000).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Samples Collection

Replicate samples of sediment and water were collected from 5 sample points on (2 separate occasions) of Ikogosi warm spring coded: SW (Source of the warm spring), MPW (Midpoint of the warm spring), C (Confluence), MPC (Midpoint of the cold spring) and SC (Source of the cold spring) in June, 2018 and also in December, 2018, resulting in a total of 20 composite samples during the course of this research. The location of the warm and cold spring was recorded as (7 º35'28.227''N, 4 º59'2.925'' E). The study area located in Ikogosi Ekiti, Ekiti State is shown in Figure 3.1.

Samples were collected in clean, sterile containers, placed in coolers with ice-packs and transported to the laboratory. Samples for physicochemical analysis were immediately transported to the Soil Microbiology Laboratory at the International Institute of Tropical Agriculture (IITA), Ibadan, while samples for microbial and metagenomic analysis were placed in the refrigerator prior to analyses.

3.2 Physicochemical analysis of water and sediment samples

Considered physicochemical parameters for water samples include Dissolved Oxygen, nitrates, sulphates, electrical conductivity, calcium, magnesium, manganese, potassium, sodium, copper, zinc, ferrous ions, Total Dissolved Solids, temperature and pH. Considered parameters for sediment samples were percentage nitrogen, available phosphorus, organic carbon, nitrates, sulphates, calcium, magnesium, potassium, sodium, manganese, copper, zinc and ferrous ions. Physicochemical parameters of all samples were investigated using the standard protocol stated in APHA (2005). Temperature and pH of the water body were analysed and recorded *in-situ.*

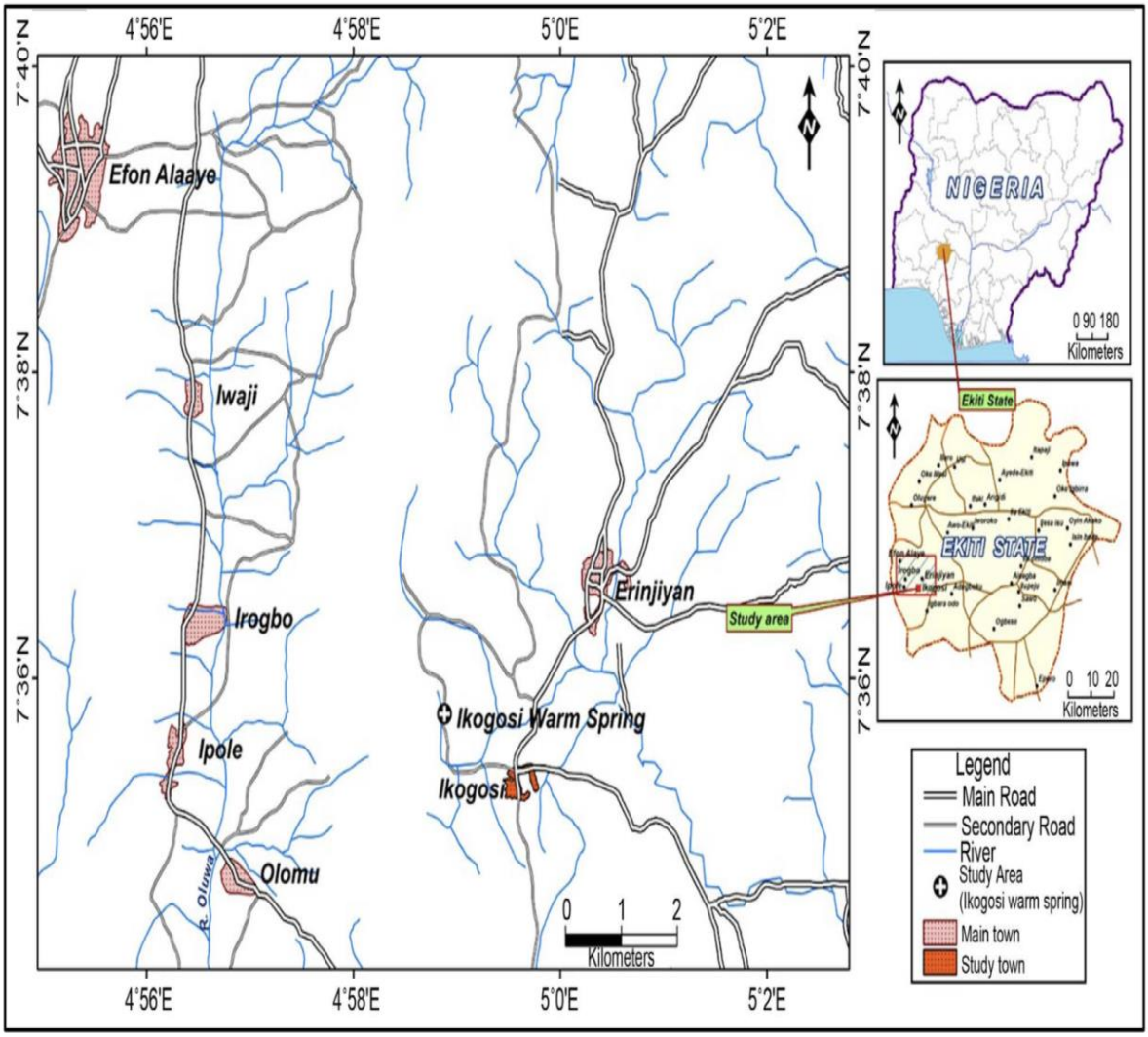


Figure 3.1: Map of Ikogosi warm spring and its neighbouring environment (Source, Fasesan *et al.*, 2020)

3.3 Selected physicochemical parameters performed on samples from Ikogosi Warm Springs

3.3.1 Sulphate determination from water samples of Ikogosi Warm Springs

Sulphate ions from water samples were analysed by the turbidimetric method by employing Barium chloride as a precipitant. Water samples were measured (100mL) into a 250 mL beaker, and then diluted with 300mL of distilled water. Concentrated hydrochloric acid (1 mL) was added to the solution and four droplets of an indicator was added (methyl orange). Following this, 20 mL of 10% $BaCl_2$ solution was added to the solution, boiled for few minutes and then left overnight. After this, Whatman's filter paper was used to filter the solution and then the filter was gently rinsed with distilled water to remove traces of chloride ions. Whatman filter paper was then dried in an oven at 80°C using the silica crucible in a muffle furnace for one hour. Following this procedure, the filter paper was cooled in a desiccator and weighed. The process of ignition, cooling and weighing was repetitively done to yield a constant value. Then, the sulphate ion of each sample was then computed based on APHA, (2005).

3.3.2 Electrical Conductivity

This parameter was measured using a conductivity meter (Fisher Scientific, USA). Before use, the conductivity device was calibrated by dipping the sensitive probe of the meter in standard solutions with pre-set conductivity values. The probe was cleaned gently with a lint-free tissue to remove all liquid from the standard solutions and was then dipped into the water sample to be analysed. The conductivity meter was dipped into water samples in such a way that the entire probe was immersed in the water. The probe was kept in the water sample, allowed to stabilise, and the electrical conductivity reading was taken.

3.3.3 Available Phosphorus

Sediment samples were extracted for phosphorus using Bray No. 1 solution as the extractant. Based on the reaction with ammonium molybdate and creation of the "Molybdenum Blue" hue, the extracted phosphorus was thereafter quantified using a spectrophotometer (Jenway, UK).

3.3.4 Percentage Nitrogen

Ten grams of sediment samples were placed in a Macro-Kjeldahl flask, 20 mL of distilled water was added and the flask was swirled for even distribution. Each flask containing the solution were allowed to stand for half an hour. Following this, 1g of K_2SO_4 mixture catalyst,10g of K_2SO_4 and 30mL of concentrated sulphuric acid were added using an automatic pipette. Each flask was gently heated on the digestion stand. After water was eliminated, and frothing had stopped, more heat was applied until the digest cleared. Thereafter, the mixture was boiled for 5 hours. Following this, the flasks were allowed to cool and 100mL of water was slowly added to the flask and the sand deposits were washed 4 times with distilled water. The indicator solution- H_3BO_3 in a flask, was placed under the condenser of the distillator, while another flask was attached to the distillation apparatus. Sodium hydroxide (10 M NaOH) was gently poured through the distillation flask. The distillate was then collected, after which NH4-N was determined by titration using 0.50M HCl. The change in colour at the end of the titration was from green to pink. The percentage of Nitrogen was thereafter calculated.

3.3.5 Determination of Sodium, Iron, Zinc, Copper, Manganese, Magnesium, Potassium and Calcium

Weighed amount of samples was placed in a digestion vessel, concentrated Hydrochloric acid and Hydrogen peroxide were added to the sample. For 10 minutes, the mixtures were heated in a microwave for digestion, after which samples were diluted with deionised water. Prepared samples were thereafter subjected to atomic absorption spectroscopy for quantitative determination of Sodium, Iron, Zinc, Copper, Manganese, Magnesium, Potassium and Calcium at ultra-low level (Albakaa *et al.*, 2021).

3.3.6 pH and temperature determination

The hydrogen ion concentration of water samples was taken by making use of a well calibrated pH meter. The pH meter was dipped into the water in a way that the probe of the pH meter was in contact with water for several seconds. The pH reading was thereafter recorded. The temperature of water samples was also done by making use of a mercury thermometer. The thermometer was dipped into the water for a few seconds and then the reading of the water was recorded in degree Celcius.

3.3.7 Total Disolved Solids

Water samples were shaken and poured into a filtering flask containing dried pre-weighed glass fibres (dried at 103°C). Water samples were then filtered through a pump, after which the filter in the filtering flask was recovered, and dried at 103°C, and allowed to cool, thereafter reweighed to establish the final weight of the filter. The total dissolved solid was then calculated as the difference in the weight of the glass fibre within the stipulated water samples.

3.4 Microbial analysis and culture methods

3.4.1 Isolation of bacteria and fungi from sediment and water samples

Isolation of bacteria and fungi from samples were carried out using the Serial Dilution Method (Harrigan and MacCance, 1966). Ten milliliters of water samples were measured and introduced into 90mL of sterile distilled water in an Erlenmeyer flask. The mixture was shaken thoroughly to ensure homogeneity and a series of ten-fold serial dilutions were prepared. For water samples, dilutions were made from 10^1-10^5. For sediment samples, 10g of sediment was weighed aseptically and introduced into 90mL of sterile distilled water, prepared in an Erlenmeyer flask. The mixture was vigorously shaken to ensure even distribution and series of ten-fold dilutions were prepared from 10^1-10^7. Sterile pipette was used to pipette 1mL from appropriate fold dilution into sterile Petri dishes, and sterile molten Nutrient agar was poured into each Petri dish for isolation of bacteria, while sterile molten Potato Dextrose Agar (supplemented with 100 µg/mL streptomycin) was used for fungal isolation. All Petri dishes containing sample and molten agar were swirled to ensure even distribution of samples. This technique was done in duplicates. Plates were allowed to cool, inverted and incubated (Memmert incubator) at 37 ^oC for 18 - 24hours (for bacterial growth) and for 3 – 5 days (for fungal growth) and observed for distinct colonies. Distinct bacterial colonies were then subcultured by proper streaking on similar medium in plates to obtain pure cultures. Grown colonies were then aseptically placed onto agar slants and 30% glycerol broths as stock cultures. Obtained pure cultures of fungal isolate were maintained on Potato Dextrose Agar slants and subcultured every three weeks to maintain their viability.

3.5 Morphological and biochemical identification of bacterial isolates

3.5.1 Gram's staining

A heat-fixed smear from an 18 - 24hour old culture was prepared on a clean glass slide with the aid of sterilised inoculating loop. The smear was stained with 2 -3 drops of Crystal Violet stain, the primary stain, for 60 seconds. Crystal Violet was poured off and rinsed with water. The slide was then flooded with Gram's iodine for 60 seconds and later rinsed with water, the slide was rinsed with 95% ethanol for 30 seconds followed by counterstaining with Safranin for 30-60 seconds. Eventually, the slide was washed with water, blotted dry and then examined under oil immersion objective lens (Olutiola *et al.*, 1991).

3.5.2 Catalase test

Freshly grown culture was emulsified with 3% freshly prepared Hydrogen peroxide solution on a clean slide. It was examined for efferversence, as the presence of efferversence indicates a catalase positive reaction while its absence indicates a catalase negative reaction (Olutiola *et al.*, 1991).

3.5.3 Methyl Red-Voges Proskauer (MRVP)

MRVP broth is composed of Dextrose (0.5g), KH_2PO_4 (0.5g) and Peptone (0.5g) in 100mL of distilled water. Ten milliliters of sterile MRVP broth was inoculated with the isolate and then incubated at 37ºC for 48 hours. Following incubation, the content of each tube was divided into two, one for methyl red test and the other Voges Prokauer test. For the Methyl Red test, five drops of methyl red solution was added and examined for immediate colour change. Red colouration indicates a positive reaction while yellow colouration indicates a negative reaction. For Voges Prokauer test, 0.5ml of 6% alpha naphthol and 0.5ml of KOH were added to 1ml of culture. The tubes were shaken and observed for change in colour. The development of a pink colour in the medium indicates a positive reaction (Olutiola *et al.*, 1991).

3.5.4 Utilisation of citrate

Simmons citrate Agar is made up of 0.10g of Ammonium dihydrogen phosphate, 0.10g of Potassium dihydrogen phosphate, 0.20g of Magnesium sulphate,0.20g of Sodium citrate, 0.5g of sodium chloride, 0.16mg of Bromothymol blue and 1.5g of agar in 100ml of distilled water. Sterile molten Simmon citrate agar was poured into sterile Petri dishes and allowed to set. Plates were then inoculated with 24 hour-old bacterial culture,

incubated for 48 hours and then examined for change in colour. A change in the indicator from green to blue indicates utilisation of citrate (Van Hofwegen *et al.*, 2016).

3.5.5 Hydrolysis of starch

The culture medium is composed of nutrient agar and 1% soluble starch. The medium was sterilised by autoclaving at 121^{o}C and 1.05kg cm^{-2} for 15 minutes. Twenty milliliters of molten agar were poured into Petri dishes and allowed to set. All test isolates were then spot inoculated on to the surface of the agar and incubated at 50 ^{o}C for 24 hours. After 24 hours, the surface of all plates were flooded with Gram's iodine and observed for colour change. Unhydrolysed starch formed a blue or blue black colour with iodine while hydrolysed starch appears as a clear zone (Olutiola *et al.*, 1991).

3.5.6 Oxidase test

Few drops of 1% aqueous solution of Tetramethyl-p-phenylenediamine hydrogen chloride was placed on a piece of Whatman No.1 filter paper in a Petri dish. With the inoculating loop, a 24 hour bacterial growth was smeared on to the impregnated filter paper and observed for purple colouration. A purple colouration produced within 5 to 10 seconds indicates a positive culture, while a delayed positive purple colouration is taken as negative culture (Olutiola *et al.*, 1991).

3.5.7 Motility Test

This was done using half strength nutrient agar. Test isolates were stab-inoculated into sterile tubes containing sterile nutrient agar and were incubated at 37^{o}C for 24 hours. The pattern of growth was then observed for motility. Motile isolates would be seen growing away from the line of stab, while non-motile would grow only at the line of stab.

3.5.8 Gelatin hydrolysis test

The culture medium which consisted of nutrient broth and 10 – 15% gelatin was sterilised in tubes and incubated with an 18 – 24hour old culture and an uninoculated nutrient gelatin tubes served as a negative control. All tubes were incubated at 37^{o}C for 3 days. Following incubation, tubes were refrigerated to solidify non-liquefied gelatin and were observed for liquefaction. When gelatin is broken down, it loses its gelling

qualities, hence a positive culture liquefies after refrigeration; while a negative culture to gelatin liquefaction retains its gelling qualities (Ekpenyong *et al.*, 2016).

3.5.9 Production of indole

Kovac's indole reagent consisting of 10g para-dimethyl amino benzaldehyde, 150mL of pure amyl alcohol and 50mL conc. pure hydrochloric acid was used to detect the presence of indole from tryptophane by test organisms. Sterile tryptone water in tubes were inoculated with the test organism, while some tubes were left uninoculated to serve as controls, and were all incubated for 48 hours 37^{o}C. Following incubation, 0.5mL Kovac's reagent was added to each tube, shaken gently and then allowed to stand. A deep red colour which separates out in the alcohol layer indicates the presence of indole which is caused by the breakdown of tryptophan by the test isolate (Prescott *et al.*, 2018).

3.5.10 Urease test

Christensen's urea was used for determining the ability of the test isolate to produce urease, agar medium (Qadri *et al.*, 1984). Tubes of the medium was prepared and used for each isolate. The tubes containing test isolates and uninoculated tubes (controls) were incubated at 37^{o}C for 72 hours. The production of urease was indicated by a change in the medium from yellow to pink. Uninoculated controls and negative test isolates showed no colour change.

3.5.11 Nitrogen reduction test

This test is used to test the ability of isolates if they are able to reduce nitrate compunds to nitrites and ammonia. Nitrate broth is composed of peptone water and 0.1% of $KNO_{3.}$ Sterile nitrate broth (10mL) containing inverted Durham tubes were inoculated with test isolates, while uninoculated tubes served as negative control and were incubated at 37^{o}C for 4 days. After incubation, drops of 0.5mL of 1% sulphanilic acid in 5N acetic acid were added to each tube, which was then followed by 0.5 mL of 0.6% N-N-dimethyl-p-diaminedihydrochloride in 5mL acetic acid. A deep red coloration and gas production indicated the production of nitrogen gas (Payne, 1973).

3.5.12 Spore staining test

Smears of 36 hour old cultures to be tested for endospore were heat fixed on clean slides and covered with a piece of absorbent paper and flooded with Malachite Green and exposed to steam for 10 minutes. As the paper began to dry, drops of malachite green was added to the adsorbent paper to keep it moist. After steaming, slides were rinsed thoroughly with water and was counterstained for 45seconds with 0.5% Safranin. The slides were then washed and examined for the presence of endospores. The vegetative cells appeared red while the spores appeared green (Olutiola *et al.,* 1991).

3.5.13 Growth on different media

Test isolates were grown on several differential media such as MacConkey, Cetrimide, Eosin Methylene blue and Mannitol Salt media in order to differentiate them to specific groups of bacteria. All media were prepared in accordance to the manufacturer's protocol.

3.5.14 Sugar fermentation test

One percent of different sugars (glucose, fructose, lactose, galactose, raffinose, mannitol, lactose, maltose, inositol, arabinose, inulin, sucrose and glycerol) were incorporated into freshly prepared media in tubes containing 1% peptone, 0.1% of NaCl and 0.01% of phenol red. This test is used to determine the ability of test isolates to utilise these sugars as their sole carbon source, thereby fermenting them. Fermentable sugars were then dispensed into each tube containing the prepared media as well as inverted Durham tubes and sterilised at 121ºC and 1.05kg cm^{-2} for 15 minutes. Test isolates were then inoculated into tubes for each sugar samples while some tubes were left uninoculated to serve as negative controls. All tubes were then incubated at 37ºC for 5 days. The test tubes were examined daily for change in colour and/or accumulation of gas. Acid production is shown by a change in colour of the indicator (phenol red) from red to yellow and gas production observed in the Durham tube (Olutiola *et al.*, 1991).

3.6 Cultural and morphological characterisation of fungal isolates

Fungal isolates were examined macroscopically on potato dextrose agar plates and microscopically, using a light microscope. Morphological characteristics observed included the spore, appearance, texture, colour and pigmentation (Robert *et al.*, 1995).

3.6.1 Microscopic characterisation of fungal isolates

A drop of Lactophenol cotton blue was placed on sterile microscope slides. With the aid of an inoculating needle, a portion of fungal mycelia was picked carefully, placed in lactophenol on slides, and were teased lightly using the inoculating needle and covered with a cover slip for clearer view under the microscope. Slides were viewed under a light microscope and photomicrographs taken. Identification of fungal isolates was done using the structural morphology, nature of mycelia, presence, shape and arrangement of spores, as well as the presence of septa. Observations were compared to standard manuals of Robert *et al.* (1995) and Barnett and Hunter (2006).

3.7 Molecular identification of bacterial isolates used for physiological studies

Two selected bacterial isolates used in the production of alpha-amylase were identified using 16S rRNA sequencing.

3.7.1 Genomic DNA extraction of bacterial isolates

Extraction of genomic DNA was done by picking a single colony of each bacteria, and grown in 10 mL of nutrient broth for 24 hours in a shaker incubator. Following incubation, cells were recovered by centrifugation of 2mL of cell culture at 10,000 x g for 3 minutes. The supernatant was discarded and pellet was suspended in 200µL of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0]). DNA was thereafter extracted using Quick-DNA™ Fungal/Bacterial MiniPrep Kit (Zymo Research, United States) according to the manufacturer's instructions and quantified using the NanoDrop Spectrophotometer 2000 (Thermo Scientific, USA). Briefly, 200µL of dissolved pellet in extraction buffer were aliquoted into a ZR bashing bead lysis tube together with 750 µL of bashing bead. The mixture was thoroughly vortexed for 5 minutes and a one-minute centrifugation was performed at 10,000 x g. Following this, 400 µL of the supernatant was aliquoted into Zymo-spin II-F Filter placed in a collection tube to collect the flow through. This set up was then centrifuged for 8,000 x g for 60 seconds. Genomic Lysis buffer (1200µL) was thereafter added to the flow through in the collection tube and was then transferred into a Zymo-Spin Column, placed in a collection tube. The set-up was again centrifuged for 1 minute at 10,000 x g. The flow through was discarded and 200 µL of DNA Pre-wash buffer was added to the seemingly empty spin column and centrifuged for 60 seconds at 10,000g. The spin column containing the DNA was treated to 500 µL of g-DNA Wash Buffer and centrifuged at 10,000 x g for 60 seconds. To elute the clean DNA, the spin

column was placed in a new collection tube, (containing 70 µL of elution buffer) spun in a centrifuge for 30 seconds at 10,000 x g. The eluted DNA was thereafter used as the template DNA for PCR amplification.

3.7.2 Determination of the purity, integrity and size of genomic DNA

Crude nucleic acid was quantified using Spectrophotometer 2000 (Thermo Scientific, USA). In addition to this, gDNA (genomic DNA) was also analysed by agarose gel electrophoresis, to determine the size of the amplified product and verify the purity. Amplified products were run on a 1.5% agarose gel, prepared in 1X TAE buffer containing ethidium bromide solution (10mg/mL). Amplified products were mixed with 6X gel loading dye (Genie, India), and 10µL of DNA samples were loaded into the wells of the agarose gel, alongside lambda DNA/HindIII molecular weight standard (Genie, India). Agarose gel electrophoresis was done at 100 V for 1hour (BioRad Power Pac Basic Electrophoresis systems, USA), and visualisation of the gDNA in gel was captured in the gel documentation system (Biorad Gel Documentation System, USA) under UV light.

3.7.3 Polymerase Chain Reaction from extracted DNA and sequencing reaction of the 16S rRNA Gene

The PCR cocktail mix consisted of 2.5µL of 10X PCR buffer, 1µL of 25mM of $MgCl_2$, 1µL of forward primer (27F - AGAGTTTGATCMTGGCTCAG), 1µL of reverse primer (1525R - AAGGAGGTGWTCCARCCGCA), 1µL of DMSO, 2µL of 2.5mM of DMSO (Dimethyl sulfoxide), 2µL of 2.5Mm DNTPs (Deoxynucleotide triphosphates), 0.1 µL of 5U/ µL Taq Polymerase and 3 µL of 10ng/ µL of template DNA. The mixture was made up to 25 µL by the addition of 13.4 µL of nuclease free water. PCR was accomplished in a VeritiTM 96-well thermal cycler (Applied Biosystems, USA) using the following PCR conditions: 94˚C for 5minutes for initial denaturation, followed by 36 cycles of denaturation at 94˚C for 30seconds, elongation step at 72˚C for 45seconds and a final elongation step at 72˚C for 7 minutes (Bharose *et al.,* 2017). Sequencing reactions (forward and reverse) were accomplished from these amplified PCR products using the ABI 3500 genetic analyser (Applied Biosystems, USA).

3.7.4 Molecular Identification and Phylogenetic Analysis of Amplified 16S rRNA Genes

The Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in NCBI (National Center for Biotechnology Information) was employed to identify the identity of bacterial isolates from their amplified 16S rRNA sequences. The nucleotide sequences of identified bacterial isolates were provided with accession numbers and deposited in the GenBank in NCBI. Molecular phylogenetic analysis was computed using MEGA 7 software (Kumar *et al.*, 2016), employing the Maximum Likelihood Method based on the Tamura-Nei model (Tamura and Nei, 1993) for the construction of phylogenetic trees. The bootstrap consensus trees were inferred from 1,000 replicates (Felsenstein, 1985).

3.8 Screening of bacterial isolates for bioprospecting thermostable alpha- amylase

All bacterial strains were subjected to starch hydrolysis test using Nutrient agar (Lab M, United Kingdom) fortified with 1% (w/v) of soluble starch (Trust Chemical Laboratories). All bacterial isolates were spot inoculated on starch agar plates and were incubated at 50°C for 24hours. Following incubation, all starch agar plates were flooded with Gram's iodine solution and observed for colour changes. The presence of blue black coloration surrounding colonies denotes a negative outcome for starch hydrolysis, while the presence of a clear halo around colonies were denoted as a positive result for starch hydrolysis. Positive isolates were selected as possible candidate for alpha-amylase production. With positive colonies, their zones of hydrolysis were measured using a clear transparent ruler (mm) and the isolate with the largest zone of hydrolysis were selected for further studies (Deljou and Arezi, 2016).

3.9 Production of thermostable alpha-amylase from selected bacterial isolate

3.9.1 Preparation of seed inoculum

Selected bacterial isolates were prepared for amylase production, by inoculating them into a growth medium (nutrient broth) for 18 hours (standardised to 0.5 MacFarland standard) and 1mL of bacterial broth was then introduced into the production medium (Msarah *et al.*, 2020).

3.9.2 Production of alpha-amylase

Production medium for Alpha-amylase consisted of a basal medium of the following composition dissolved sequentially and dispensed into aliquots of 100mL into a 250mL Erlenmeyer flask: Starch (10g/L), Yeast Extract (3g/L), Peptone (5g/L), NaCl (3g/L) and $MgSO_4.7H_2O$ (0.5g/L) (Deljou and Arezi, 2016). Into each flask was added 1% soluble starch (Trust Chemical Laboratories) which was then dissolved by mild heat accompanied by continuous stirring, over a Bunsen flame. Each flask was plugged with non-absorbent cotton wool and sterilized in an autoclave (Model YX-280A, Portable Autoclave, Zonkia, China) set at 1kgf/second for 15 minutes. All the flasks were allowed to cool to room temperature. Thereafter, 1mL of a standardised inoculum was introduced into each flask aseptically and incubated for 48 hours at 50°C under shaking conditions of 150 rpm using a controlled environment incubator shaker (New Brunswick Scientific Co, USA). After 48hours incubation, production medium was centrifuged using Sorvall RC 5C plus centrifuge at 10,000rpm for 15 minutes at 4˚C (Abel-Nabey and Farag, 2016). Cell debris were pelleted at the bottom, while the supernatant which contains the enzyme of interest were decanted and used for the estimation of alpha-amylase (Deljou and Arezi, 2016).

3.10 Enzyme assay using 3,5-Dinitrosalicylic Acid (DNS) method

One millilitre of crude enzyme obtained following centrifugation was added to 1 mL of 1% substrate (Soluble starch) in Citrate phosphate buffer (pH 6.9). The solution was then incubated in a water bath at 50°C for 15 minutes. This was then followed by the addition of 2mL of 3,5 – dinitrosalicylic acid, to halt the reaction. These tubes were thereafter boiled for 15 minutes and allowed to cool to room temperature and diluted with 5mL of distilled water. The absorbance of all cooled solutions were read at 540nm using a UV/Vis Spectrophotometer model 6305 (Jenway, UK). One Unit of alpha-amylase activity is expressed as one micromole of glucose liberated per mL per minute of enzyme (Miller, 1959). All experiments were done in replicates.

3.11 Optimisation of production media for optimum production of thermostable alpha-amylase

Several production conditions optimised for best production of enzyme were: varying temperature and pH, various carbon sources at various concentrations, several nitrogen sources at several concentrations, inorganic salts, varying inoculum sizes and different incubation hours. The One-Factor approach was utilised in the optimisation study of

production conditions. It involves varying a particular factor while keeping other factors constant. All optimization experiments were done in replicates.

3.11.1 Optimisation of temperature for alpha-amylase production

The effect of different temperature on the production medium was carried out to determine the best temperature suitable for better alpha-amylase production. This was determined by inoculating the 100mL of production medium prepared in a 250mL Erlenmeyer flask, with 1mL of a 0.5 McFarland standardised inoculum. The set-up was then incubated at different temperatures (40,45, 50, 55, 60, 65 and 70°C) for 48hours in a temperature controlled shaker incubator (New Brunswick Scientific Co, USA) at 120rpm. Thereafter, enzyme produced was then assayed as earlier described (Msarah *et al.,* 2020).

3.11.2 Optimisation of pH on production medium

In order to determine the optimal pH for the production of thermostable alpha-amylase, the production media were prepared using various buffer systems. Citrate phosphate buffer (5.0 and 6.0), Sodium phosphate buffer (7.0 and 8.0) and Glycine-NaOH buffer (9.0) with a concentration of 0.05M. An 18hour old seed culture (1 mL) was introduced into 100mL of the production medium prepared in a 250mL Erlenmeyer flask. The set-up was then incubated at both enzymes' optimum temperature for 48 hours in a temperature controlled shaker incubator (New Brunswick Scientific Co, USA) at 120rpm. Following completion of incubation, the enzyme was assayed using standard procedures as described.

3.11.3 Optimisation of various carbon sources

Several carbon sources (lactose, dextrose, sucrose, fructose, grounded cassava peels, blended cocoyam peels, Irish potato peels and yam peels) at 1% w/v were introduced into 100mL of the production medium as solo carbon source for alpha-amylase production. All production media were inoculated with 1mL of seed culture and incubated at 50°C for 48hours, at 120rpm. After 48 hours incubation, the enzyme produced was measured at 540nm using a spectrophotometer (Jenway, UK).

3.11.4 Optimisation of various nitrogen source

Different organic and inorganic sources of nitrogen were used to establish the best source of nitrogen for maximum production of alpha-amylase. Organic nitrogen sources used were peptone, tryptone and yeast extract, while inorganic nitrogen sources used were potassium nitrate (KNO_3), sodium nitrate ($NaNO_3$) and ammonium sulphate $(NH_4)_2SO_4$. (Abel-Nabey and Farag, 2016). Peptone and yeast extract was replaced in the basal medium with 8g/L of the above-named nitrogen sources. All production media (100mL) prepared in 250mL flasks were inoculated with 1mL of seed culture and incubated at 50°C for 48hours, at 120rpm. The crude enzyme was thereafter recovered and assayed as earlier described.

3.11.5 Optimisation of various concentrations using the best carbon source

Following the determination of the best carbon source, the best carbon source was varied as follows: 0.50%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0% (w/v) to determine the best concentration for maximum enzyme production (Msarah *et al.,* 2020). One millilitre of the bacterial inoculum was introduced into the production medium and the set up incubated for 48 hours at 50°C on a temperature controlled shaker incubator at 120rpm. Amylase assay was then performed after the incubation period.

3.11.6 Optimisation of various concentrations using the best nitrogen source

Following the determination of the best nitrogen source, the selected nitrogen source was varied from 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0% to determine the best concentration for optimum enzyme production (Deljou and Arezi, 2016). One millilitre of the freshly prepared bacterial inoculum was introduced into the production medium and the set up incubated for 48 hours at 50°C on a temperature controlled shaker incubator at 120rpm. Amylase assay was then performed after the incubation period.

3.11.7 Optimisation of inorganic salts

The effect of different metal ions on the production of alpha-amylase was studied. This was done by replacing $MgSO_4.7H_2O$ in the basal medium with other salts such as $NiSO_4.6H_2O$, $CuSO_4.5H_2O$, $CaCl_2.2H_2O$, $MnSO_4.H_2O$ and $FeCl_3.3H_2O$ at 0.5g/L. All production media (100mL) were prepared in a 250mL Erlenmeyer flask. They were inoculated with 1mL of freshly prepared culture and incubated at 50°C for 48hours, at

120rpm in a shaker incubator. The crude enzyme was thereafter recovered and assayed (Deljou and Arezi, 2016).

3.11.8 Optimisation of inoculum size

Selected bacteria for alpha-amylase production were grown in freshly prepared nutrient broth for 18hours. One millilitre of several concentrations of the 0.5 MacFarland standard broth was used. The concentrations were 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10% v/v. All basal media (100mL) containing different concentrations of the inoculum were incubated at 50°C and agitated in a shaker at 120 rpm for 48 hours. The crude enzyme was thereafter recovered and assayed for amylase activity at 540nm as earlier described (Indriati and Megahati, 2017).

3.11.9 Optimisation of various incubation time/hours

In order to determine the optimal incubation hour for best production of thermostable alpha-amylase, 100mL of basal medium was inoculated with 1mL of seed inoculum, incubated at 50°C and placed in a temperature-controlled shaker. Five millilitres of production media were withdrawn at 12-hour time intervals i.e. 12, 24, 36, 48, 60, 72, 84, 96 hours. The withdrawn media were centrifuged to obtain cell-free supernatant and thereafter assayed for alpha-amylase activity as previously described (Aygan *et al.*, 2014).

3.12 Characterisation of crude alpha-amylase from selected thermophilic bacteria, isolated from Ikogosi Warm Springs

3.12.1 Effect of temperature on the activity of the alpha-amylase and stability

Amylolytic activity was deduced by incubating crude enzymes with 1% w/v soluble starch as its substrate. This mixture was incubated at several temperatures (40, 45, 50, 55, 60, 65 and 70°C) for 15 minutes and then the enzyme activity was determined by reading the absorbance value at 540nm using the UV/Vis Spectrophotometer (Jenway). Thermal stability of crude enzymes were however determined by initially pre-incubating these enzymes at different temperatures close to the optimum temperature range (40, 45, 50 and 55°C) at 10 minutes and 30 minutes interval. Following pre-incubation, 1% w/v

of soluble starch was added as its substrate and its residual amylase activity was assayed. All characterisation studies were performed in replicates.

3.12.2 Effect of pH on alpha-amylase activity

Various buffer systems were prepared to determine the activity of alpha-amylase at the pH of 5, 6, 7, 8, 9 and 10. Buffers prepared were 0.05M of Citrate phosphate buffer (5.0 and 6.0), Sodium phosphate buffer (7.0 and 8.0) and Glycine-NaOH buffer (9.0). Soluble starch (1% w/v) was prepared with the above-named buffer systems. After this, 1mL of soluble starch and 1 mL of the crude enzyme were mixed together and incubated in a water bath at each enzyme's optimal temperature. The reaction was stopped by the addition of 3,5 – dinitrosalicylic acid, after 15 minutes of reaction, boiled for 15minutes, and the absorbance read at 540nm (Aygan *et al.,* 2014).

3.12.3 Effect of substrate concentration on alpha-amylase activity

Soluble starch dissolved in buffer system which indicated the enzyme's optimum activity was used for this procedure. Different concentration of soluble starch substrate was dissolved in 0.05M of appropriate buffer. The different concentration used were 0.25%, 0.5%, 0.75%, 1.0 %, 1.25%, 1.5%, 1.75% and 2.0% (w/v). Following incubation for 15 minutes at the enzyme's optimum temperature, the enzyme assay was carried out as described previously.

3.12.4 Kinetic analysis of crude alpha-amylase enzymes

The reaction rate of α-amylase was determined using different starch concentration (0.25, 0.5, 0.75,1.0, 1.25, 1.50, 1.75 and 2.0%) (w/v), 1mL variable soluble starch and 1 mL of the crude enzyme were mixed together and incubated in a water bath at each enzyme's optimal temperature. The reaction was stopped by the addition of 2mL of 3,5 – dinitrosalicylic acid, after 15 minutes of reaction, boiled for 15minutes, allowed to cool, and the absorbance read at 540nm. The enzyme velocity (enzyme activity per unit time) denoted as V were determined at each substrate concentration, and the inverse of the enzyme velocity (1/V) was plotted against the inverse of the substrate concentration (1/[S]) to determine the values of K_m and V_{max} by plotting the Lineweaver-Burk's double reciprocal plot (Lineweaver and Burk, 1934).

3.12.5 Effect of metal ions on alpha-amylase activity

The effect of various divalent cations on the activity of each crude enzyme was determined at 1mM and 5mM for each metal ion. This was performed by pre-incubating both enzymes with $CaCl.2H_2O$, $FeSO_4.7H_2O$, $MnSO_4.H_2O$, $MgSO_4.6H_2O$, $ZnSO_4.7H_2O$ and $CuSO_4.5H_2O$ respectively for 15 minutes at the enzyme's optimum temperature and pH. Following this, the enzyme assay was then performed, as described ealier.

3.12.6 Effect of inhibitors on alpha-amylase activity

The modified method of Tiwari *et al.* (2014) was used in determining the effect of various inhibitors (1% v/v) on the activity of both enzymes. The effect was determined by pre-incubating enzymes with inhibitors for 15 minutes, at the enzymes' optimum pH and temperature. Thereafter, the residual activity was investigated by testing the pre-incubated enzyme under standard assay conditions. The various inhibitors used includes SDS (Sodium Dodecyl Sulphate), EDTA (Ethylenediaminetetraacetic acid) and Urea.

3.13 Partial purification of alpha-amylase

Crude amylase was partially purified using ammonium sulphate precipitation, dialysis and column chromatography using sephadex G-100 (Indriati and Megahati, 2017).

3.13.1 Ammonium sulphate precipitation

Isolates for amylase production were grown in 100 mL of the optimised production medium. The filtrate containing the crude enzyme was centrifuged at 12,000rpm for 15 minutes at 4°C using a high speed refrigerated floor centrifuge (Sorvall Rc 5c Plus, USA) to separate cells from crude extracts. Both crude extracts obtained were treated three times with appropriate quantity of solid ammonium sulphate to achieve 0 - 20%, 20 - 60% and from 60 - 80% saturation as described by Dixon and Webbs (1962). The crude extract was first treated with 11.3g of solid ammonium sulphate to achieve 0 - 20% saturation. This procedure was done with the set-up on a stirrer, with a magnetic stirrer inside the cell-free crude extract continuously stirring the mixture (the magnetic stirrer was properly controlled to avoid rigorous stirring and bubbles) and was maintained overnight at 4°C in a refrigerator. The treated supernatant was centrifuged at 12,00rpm for 15minutes at 4°C using the same cold centrifuge as described above and the supernatant was used for the next salting out treatment. The second and third

application of ammonium sulphate was done using 26g and 14g of the salt. The procedure was again repeated by continuous stirring, maintaining the set-up at 4°C overnight and centrifuging to pelletise precipitated proteins. At the end, the precipitate from each batch were pooled and re-suspended in 0.05M sodium phosphate buffer pH 7.0 and filled to 100mL (Sumrin *et al.*, 2011).

3.13.2 Dialysis

Dialysis tubing was tied at one end with a clean twine rope. Ten millilitres of the re-dissolved precipitated alpha-amylase from both isolates in Sodium phosphate buffer (pH 7.0) were placed in separate dialysis tubing. The other end of the tubing was also tied and the tubing made taut. Tubings containing re-dissolved alpha-amylase were dialysed against the same buffer in a beaker. Dialysis was allowed to proceed for 48 hours with continuous buffer changing (three times) and stirring of the buffer, at 4 ºC. The partly purified alpha-amylase were transferred into sterile tubes and kept at 4 ºC (Aygan *et al.*, 2014).

3.13.3 Column chromatography

3.13.3.1 Preparation of sephadex beads and loading the column

Ten (10) grams of Sephadex G-100 was suspended in 100 mL of sterile distilled water to form a paste during which it was stirred gently. The mixture was allowed to swell for 3 days (at 4 ºC) in 0.05M sodium phosphate buffer (pH 7.0) to form a suspension. Sodium azide (NaN_3) at a concentration of 0.1% (w/v) was added to the suspension to prevent microbial contamination. The gel suspension was then poured into a column to bring the liquid to the top of the glass tube, simultaneously allowing the buffer to flow through the growing bed of gel. The addition of the gel was continued until a reasonable height of bead bed in the column was attained, the packed column was allowed to stand for 3days to allow for proper gel compacting by gravity (Modified method of Olutiola *et al.*, 1981).

3.13.3.2 Sample application and elution

Five millilitres of the dialysed enzyme concentrate were applied carefully at the top of the gel of the column and allowed to pass into the gel by running the column, a flow rate

of 0.3mL/min was maintained. Forty (40) fractions were collected in 5mL tubes and total protein as well as enzyme assay procedures were carried out on each fraction.

3.13.3.3 Estimation of total protein content

The total protein content of the partially purified alpha-amylase from selected bacteria was determined using Lowry's method with Bovine Serum Albumin (BSA) used as a standard (Lowry *et al.*, 1951). Three solutions denoted as solution A, B, C and 2N of Folin and Ciocalteu's Phenol reagent were used in this analysis. The composition of solution A consisted of 5.7196g/L of NaOH and 28.6168g/L of Na_2CO_3, solution B consisted of 1.4232g of $CuSO_4.5H_2O$ (for 100mL) and solution C consisted of 2.85299g of Na_2 Tartrate.$2H_2O$. All reagents used were freshly prepared. All solutions were mixed in ratio of 100:1:1 to form the Lowry solution, while Folin's reagent was diluted in water in ratio 1:1 (solution D). The standard curve was computed by first preparing 100mg/L of BSA as stock solution and then aliquoted in different volumes, 0.0, 2.0, 4.0, 6.0, 8.0 and 10.0 mL in to tubes and topped up with distilled water to make a total volume of 10mL. This resulted in a final concentration of 0, 20, 40, 60, 80 and 100 mg/L. After this, 0.5mL of all prepared standard solution were aliquoted into separately labelled Eppendorf tubes and 0.7mL of Lowry solution was aliquoted into each solutions in the labelled tubes. The mixture was vortexed briefly and incubated in the dark at room temperature for 20 minutes. After incubation, 0.1 mL of solution D was added and vortexed for proper mixing. The mixture was again incubated for 30 minutes at room temperature in the dark. Upon completion of incubation, the absorbance was read at 600nm using a UV/Vis spectrophotometer. The same procedure was used for all protein samples and their absorbance measured and recorded.

3.13.4 Statistical analysis

Generated results were subjected to analysis of variance, while all means were separated using the Duncan's Multiple Range Test at 5% level of significance, using the SPSS version 25 statistical software.

3.14 Metagenomic DNA analysis

3.14.1 Total genomic DNA extraction and quantification for 16S and ITS metagenomic analysis

Sediment samples were air-dried for 7 days, and grinded into fine powder using a mortar and pestle. Following this, total genomic DNA was thereafter extracted using kit-based DNA extraction protocol of Quick DNA Fecal/Soil Microbe Miniprep kit (Zymo Research, USA).

About 0.25g of sediment sample were placed in a tube containing bashing bead, and 750µL of lysis buffer was aliquoted into the tube and vortex thoroughly for about 5 minutes. Following this, the tube was centrifuged at 10,000 x g for a minute using a microcentrifuge. The supernatant (400 µL) was aliquoted in to a clean spin filter placed in a clean collection tube. The set-up was then centrifuged at 8000 x g for one minute. The filtrate in the collection tube was then treated with 1,200 µL of genomic lysis buffer and mixed using a pipetteman. A part of the mixture (800 µL) was placed in a column and centrifuged at 10,000 x g, the process was repeated again for the rest of the mixture, then the filtrate was discarded and the column containing trapped nucleic acid was treated with 200 µL of DNA pre-wash buffer and subject to centrifugation at 10,000 x g for 1 minute, the tube was further treated to g-DNA wash buffer and centrifuged as described previously. The column containing the "cleaned" DNA was placed in a clean collection tube and 70 µL of DNA elution buffer was added to the centre of the column carefully and left to stand for 5 minutes and then centrifuged at 10,000 xg for 30 seconds. Additional step of transferring the eluted DNA in to a treated Zymo-spin III-HRC Filter (treated with 600 µL prep solution and centrifuged at 8,000 x g for three minutes) and centrifuged at 16,000 xg for 3 minutes was included to remove humic acid associated with soil and sediment particles. All extracted DNA were stored in -20°C freezer at the International Institute of Tropical Agriculture (IITA), Ibadan prior to sequencing.

Water samples were passed through a membrane filter with pore size 0.2micron (using a vacuum pump) to trap all forms of microbial cells. Further to this, these membrane filters were then subjected to total DNA extraction using kit-based DNA extraction protocol of Quick DNA Fecal/Soil Microbe Miniprep kit (Zymo Research, USA) as described earlier. All extracted DNA were also stored in -20°C freezer at the International Institute of Tropical Agriculture (IITA), Ibadan prior to sequencing.

3.14.2 DNA quantification, PCR amplification, library construction and sequencing

The paired index Hi-Seq protocols were employed to sequence targeted regions of interest from samples from Ikogosi Warm Spring. DNA samples were quantified using a NanoDrop Spectrophotometer 2000 (Thermo Scientific, USA) to evaluate the quality and purity of all extracted DNA. All DNA samples were also analysed on an ethidium bromide-stained 1% agarose gel, and visualised in the gel documentation system (Biorad Gel Documentation System, USA) under UV light. Having confirmed the integrity of all DNA samples, they were shipped to the sequencing facility - BGI tech Solutions, Hong Kong. Upon arrival of DNA samples in the sequencing facility, all samples were subjected to further evaluation and quality control checks such as re-evaluating the quality and integrity of the DNA.

High quality DNA template (30ng) was used for Polymerase Chain Reaction (PCR). PCR amplification was performed using two primer sets: one primer set for amplification of one of the regions (V1 –V3) of the bacterial 16S ribosomal RNA (rRNA), and a primer set for amplification of a portion of the nuclear ribosomal Internal Transcribed Spacer (ITS) region (ITS1) in fungi. V1-V3 primer sets used were 8F(5'-AGAGTTTGATYMTGGCTCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3'), while ITS1 primers employed were ITS1(5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3').

PCR products were thereafter purified using Agencourt AMPure XP beads (Beckman Coulter, USA), and further tagged for library construction using an illumina adapter (i7) -AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC. The library size and the concentration of the library were determined by Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Paired end sequencing was performed on one flow cell for 301 cycles using a HiSeq 2500 platform for V1-V3 dataset and on one flow cell for 250 cycles for ITS dataset on a HiSeq 2500 platform (Illumina Inc., USA).

3.14.3 Sequence analysis/bioinformatics workflow

3.14.3.1 Sequence pre-processing

Quality checks were performed on all raw datasets generated from the HiSeq Illumina platform, using FastQC (version 0.11.2) (Andrews, 2010), a Java-based quality control

tool for high throughput sequence data. Raw reads generated from both amplicons (V1-V3 and ITS1) were then quality filtered to remove primers, adapters, low-quality/poor reads and reads with ambiguous base calls, using cutadapt (version 2.6) (Martin, 2011) and DADA2 Bioconductor workflow (version 1.18) (Callahan *et al.,* 2019) in R (version 4.1.1) (R Core Team, 2020). After sequences were quality filtered and cleaned, FastQC was performed again to check the quality of the cleaned reads and check for ambiguity.

The forward and reverse reads of the bacterial dataset were merged using DADA2 (version 1.18) in R to yield a consensus sequence of a longer read length. However, due to varying lengths of ITS1 in different populations of microorganisms, only forward reads of the fungal dataset were quality filtered and trimmed, to eliminate biases of certain taxa with longer ITS lengths being lost during joining as they would be unable to overlap into a consensus sequence (Pauvert *et al.,* 2019). A mapping file was created in a text editor and validated in R. The mapping file contained metadata (information that links samples generated sequences to experimental variables, such as, name of sample, period of sample collection, location of samples, type of samples, etc.) of each dataset. Each experimental variable was written on separate columns in the mapping file (Mbareche *et al.,* 2020).

3.14.3.2 Upstream analysis

Both fungal and bacterial datasets were processed in R studio (Rstudio Team 2015; R core Team, 2020). Upstream processing such as denoising, detection and removal of chimera, read merging (for bacterial dataset only), generation of Amplicon Sequence Variants (ASVs), and assignment of taxonomy to generated ASVs were computed in DADA2 bioconductor workflow package in R, using training set FASTA files (Training set 18, release 11.5) from the Ribosomal Database Project (RDP) that have been formatted for analysis in DADA2 workflow - for bacterial dataset; (Callahan *et al.* 2019; Callahan, 2020; R core Team, 2020) and the UNITE reference database (Nilsson *et al.,* 2019) for fungal dataset using the following commands: “filterAndTrim”, “mergePairs”, “removeBimeraDenovo” and “assigntaxonomy”.

However, the DADA2 workflow does not include steps in making a phylogenetic tree for each generated ASVs. In order to analyse phylogenetic relatedness among these ASVs, a phylogenetic tree is needed. This was done by initially performing multiple

sequence alignment of all generated ASVs using the DECIPHER package (version 2.20) (Wright, 2016) in R. Aligned sequences were thereafter analysed to construct a phylogenetic tree for downstream processing using the phangorn (version 2.7.1) package in R (Schliep, 2011).

Generated ASV table, taxonomy table, phylogenetic tree and metadata file were merged as a single phyloseq object for downstream analysis, using the phyloseq package (version 1.36.0) (McMurdie and Holmes, 2013), available as a Bioconductor package in R.

3.14.3.3 Downstream analysis

For comparable results, both datasets were standardised to handle outliers and erroneous relative abundances. Thereafter, diversity analysis (alpha and beta analysis), taxonomic compositional plots, core microbiota, Venn diagrams, differential abundance and statistical analysis were computed in R studio using several packages.

Alpha diversity metrics computed include observed species, Shannon, Simpson and Faith's - PD. Alpha diversity box plots were created in R using the following packages: microbiomeutilities (version 1.00.16) (Lahti *et al.*, 2017), a package for data and microbiome analysis, reshape (version1.4.4) a package for restructuring and manipulating data (Wickham, 2007); picante (version 1.8.2) a software tool for integrating and analysing ecological data (Kembel *et al.*, 2010) and ggplot2 (version 3.3.5), for creating publishable graphics (Wickham, 2007). Significant differences in computed alpha diversity metrics were tested using Kruskal-Wallis's non-parametric statistical test (McKight and Najab, 2010) from base packages available in R software (Li *et al.*, 2017).

Beta diversity for bacterial and fungal datasets were computed in R using the Phyloseq package (McMurdie and Holmes, 2013). Bacterial beta diversity was determined using Bray-Curtis dissimilarity distances, weighted and unweighted unifrac distances (Lozupone *et al.*, 2011). Beta diversity metrics for fungal data was computed using weighted Unifrac and Bray-Curtis dissimilarity index (Baselga, 2013). Beta diversity of the bacterial communities were visualised using non-metric multidimensional scaling (NMDS) in phyloseq package (McMurdie and Holmes, 2013), while statistical analysis was computed using ADONIS (permutation-based Analysis of Variance, PerMANOVA) with 999 permutations in Vegan package (Oksanen *et al.*, 2018)

computed in R to determine significant differences between bacterial communities among several groups.

Taxonomic compositional plots were generated to identify the population of various taxa (with their respective relative abundance) identified from each sample and from groups of samples. Samples were group on the mapping file's column by "Spring location", "Sample Types", "Month of Collection" and "Sample types and Collection period". These bar plots were created using phyloseq (version 1.36.0) and microbiome (version 1.14.0) packages in R (McMurdie and Holmes, 2013, Lahti *et al.,* 2017).

Core microbiota was computed to determine prevalent core microbes among groups of samples, and from specific locations within the spring. Core members in this study was defined as shared taxa present in 90% of all samples (90% core threshold). This was computed using the microbiome package (version1.14.0) in R (Lahti *et al.*, 2017; Estendorfer *et al.,* 2020). High quality Venn diagrams were generated in R using microbiome and Eulerr packages (Chen and Boutros, 2011; Lahti *et al.*, 2017), to indicate the number of shared and distinct taxa among groups of samples grouped as: Spring location"- Source of the warm spring, source of the cold spring, midpoint of the warm spring, midpoint of the warm spring and confluence of the spring, "Sample Types"- sediment and water, "Month of Collection"- December and June, and "Sample types and Collection period.

Differential abundance testing was computed for both datasets to identify significantly differentially abundant taxa between two groups of samples. To compute this analysis, taxa were initially merged at the lowest obtainable taxonomic by making use of a customised script in R (Lennard *et al.*, 2017). Thereafter, taxa were considered significant if they showed a fold change of ≥ 1.50, had an adjusted p-value of ≤ 0.05 and if at least one of the two groups under comparison had $\geq 50\%$ of samples with the taxa. These statistics were analysed in R using MetagenomeSeq package (version 1.34.0) (Paulson *et al.,* 2013), GridExtra (version 2.3) and NMF (0.30.4) (Gaujoux and Seoighe, 2020).

CHAPTER FOUR

RESULTS

4.1. Physicochemical properties of sediment and water samples from different sampling points during different seasons

The physicochemical parameters of the Ikogosi warm spring sediment and water samples are shown in Table 4.1A and 4.1B. Considering sediment samples, there was a significant difference ($P < 0.05$) between the organic carbon content of the spring between June and December, where higher organic carbon (%) was recorded in the SW (source of the warm spring) sample in June. There was also a significant difference ($P < 0.05$) in the organic carbon content of all the 5 different sample sites in the wet and dry seasons, highest organic carbon content was found in the wet season for SW and C (confluence of the spring), and in the dry season it was highest in the SC and MPC (midpoint of the cold spring) samples. Available Phosphorus across all 5 sample sites of the spring showed a significantly high content in SW during the wet season, and a significantly high content in SC samples during the dry season. Across both seasons, highest available Phosphorus was recorded in SW samples during the wet season.

The Nitrogen (%) showed that there was a significant difference ($P < 0.05$) observed from samples collected in June and December, and the highest % Nitrogen was found in MPC site during the dry season. During the wet season, however, the site with the highest significant record of percentage Nitrogen was recorded at the Confluence (C). Potassium (K) was significantly the highest at the MPW (midpoint of the warm spring) site during the wet season, while it was highest at C during the dry season. There was a significant difference in the sodium content between the dry and wet season, where highest sodium content was found at the confluence (C) in December/Dry samples. Also,

significantly low sodium content was found across sample sites in June samples, in comparison to all sample sites in December/dry season. Iron (Fe^{2+}) across both months of sampling (June and December samples, recorded high values. However, the SW sample site in the wet season (June) showed the highest amount of this element. In the dry season, the SC site was found with the highest value in the dry (December) season.

Copper was highest in the MPW dry season sample, while it was highest in the SW sample during the wet season. Zinc, was the highest in the C sample site in the dry season, while it was the highest in SW during the wet sample. Comparing both seasons for this element, significantly high values were recorded across all 5 sample sites during the dry season in comparison to all 5 sample sites recorded in the wet season. Manganese, was found to be highest in the SC sample during the wet season, while it showed the highest value in the C sample during the dry season. The sample site that showed a significantly high value comparing both seasons, was the C site during the dry season. Significant differences ($P < 0.05$) were observed among values observed in the Magnesium content during the wet season and during the dry season. During the wet season, the highest value of this element was found in MPW and C site, while the highest value was found in C sample site during the dry season. However, higher values were recorded across all the 5 sample sites during the dry season, in comparison to all the 5 sample sites observed during the wet season.

There was a significant difference ($P < 0.05$) in Calcium discovered across all sample sites, at both seasons. During the wet seasons, the highest value of Calcium was recorded in C sample sites, while during the dry season, the highest value of this element was recorded at MPW and C sample sites. Sulphate concentration across 5 sample sites were significantly different, during the wet and dry season. In June, the sample site with the highest sulphate concentration was recorded at SC, while in December, the highest sulphate concentration was recorded at MPW and SC. Nitrates had the highest value during the wet season at 2 sample sites, namely: SW and SC, while during the dry season, sample site with the highest value was at MPC and SC.

Table 4.1A Mean physicochemical values from sediment samples obtained at respective sampling points from Ikogosi Warm Springs in June and December,2018

	WET SEASON (JUNE)					DRY SEASON (DECEMBER)				
	SAMPLE SITES					SAMPLE SITES				
Parameter	SW	MPW	C	MPC	SC	SW	MPW	C	MPC	SC
Organic C (%)	0.233^{e}	0.015^{c}	0.300^{a}	0.007^{d}	0.024^{b}	0.006^{c}	0.038^{b}	0.023^{b}	0.047^{b}	1.207^{a}
Available P(mg/L)	7.404^{a}	2.953^{c}	1.902^{d}	3.170^{b}	1.105^{e}	4.787^{a}	1.426^{b}	1.771^{b}	2.377^{b}	6.009^{a}
% Nitrogen	0.003^{b}	0.002^{b}	0.018^{a}	0.002^{b}	0.002^{b}	0.004^{b}	0.005^{b}	0.006^{a}	0.063^{a}	0.017^{b}
K(Cmol/kg)	0.113^{e}	1.182^{a}	0.226^{c}	0.275^{b}	0.134^{d}	2.040^{b}	5.400^{c}	27.260^{a}	2.040^{b}	2.040^{b}
Na (Cmol/kg)	0.355^{c}	0.209^{e}	0.281^{d}	1.264^{a}	0.430^{b}	82.370^{d}	96.903^{c}	125.220^{a}	104.630^{b}	85.890^{d}
Fe(ppm)	505.000^{a}	132.500^{b}	82.500^{e}	120.000^{c}	97.500^{d}	468.860^{d}	605.830^{b}	615.570^{b}	551.060^{c}	654.440^{a}
Cu (ppm)	0.226^{a}	0.049^{c}	0.049^{c}	0.049^{c}	0.108^{b}	22.750^{b}	30.540^{a}	14.940^{c}	22.750^{b}	17.550^{c}
Zn(ppm)	0.721^{a}	0.145^{b}	0.088^{c}	0.074^{d}	0.074^{c}	17.080^{d}	18.360^{d}	154.770^{a}	127.000^{b}	26.030^{c}
Mn (ppm)	12.805^{b}	4.672^{e}	5.058^{d}	9.698^{c}	15.144^{a}	7.920^{c}	18.540^{b}	83.760^{a}	18.553^{b}	13.220^{b}
Mg (Cmol/kg)	0.129^{a}	0.045^{b}	0.033^{c}	0.027^{d}	0.015^{e}	6.990^{b}	6.990^{b}	28.030^{a}	6.990^{b}	4.590^{c}
Ca (Cmol/kg)	0.128^{b}	0.085^{c}	0.173^{a}	0.042^{d}	0.085^{c}	66.970^{b}	74.280^{a}	75.740^{a}	37.543^{c}	26.160^{d}
SO_4(ppm)	4.677^{d}	5.411^{c}	2.456^{e}	6.888^{b}	8.569^{a}	5.866^{c}	23.730^{a}	15.733^{b}	4.533^{c}	22.660^{a}
NO_3(ppm)	13.088^{a}	5.290^{c}	6.590^{b}	5.940^{d}	13.080^{a}	0.272^{b}	1.611^{a}	0.332^{b}	2.293^{a}	2.293^{a}

Key: SW – Source of warm spring, MPW – Midpoint of warm spring, C – Confluence of the warm and cold spring, SC – source of cold spring, MPC – Midpoint of cold spring. Organic C. - Organic Carbon, Available P- Available Phosphorus, K – Potassium, Na – Sodium, Fe – Iron, Cu – Copper, Zn – Zinc, Mn –Manganese, Mg – Magnesium, Ca – Calcium, SO_4 – Sulphates, NO_3 – Nitrates. Mean values with similar letter(s) across rows are significantly different at ≤ 5 % level of probability by Duncan Multiple Range Test (DMRT).

Table 4.1B. Mean physicochemical values from water samples obtained at respective sampling points from Ikogosi Warm Springs in June and December, 2018.

	WET SEASON					DRY SEASON				
	SAMPLE SITES					SAMPLE SITES				
Parameter	SW	MPW	C	MPC	SC	SW	MPW	C	MPC	SC
Temp.(°C)	38.000^{a}	37.330^{a}	28.330^{b}	29.000^{b}	28.330^{b}	38.330^{a}	37.330^{a}	29.670^{b}	25.670^{c}	26.670^{c}
pH	6.360^{d}	7.930^{a}	7.430^{b}	7.170^{c}	7.070^{c}	5.800^{b}	7.400^{a}	7.600^{a}	7.400^{a}	7.600^{a}
DO (ppm)	7.060^{b}	7.000^{c}	7.220^{a}	6.540^{d}	6.250^{e}	6.840^{b}	8.020^{a}	6.450^{b}	7.250^{ab}	7.150^{ab}
TDS (ppm)	42.013^{a}	36.000^{b}	22.000^{c}	20.967^{c}	18.000^{d}	40.330	42.330	32.330	42.330	36.330
K(ppm)	0.449^{a}	0.427^{b}	0.326^{c}	0.312^{d}	0.283^{e}	0.840^{a}	0.840^{a}	0.840^{a}	0.580^{b}	0.580^{b}
Na (Cmol/kg)	28.022^{b}	30.111^{a}	20.011^{d}	21.962^{c}	9.027^{e}	0.040	0.070	0.040	0.070	0.070
Fe(ppm)	1.750^{a}	1.750^{a}	1.500^{b}	1.001^{c}	1.000^{c}	0.320^{c}	0.560^{ab}	0.320^{c}	0.480^{b}	0.640^{a}
Cu (ppm)	0.005	0.005	0.005	0.005	0.344	0.060	0.060	0.040	0.040	0.040
Zn(ppm)	0.003^{b}	0.005^{a}	0.003^{b}	0.003^{b}	0.003^{b}	0.090^{a}	0.040^{c}	0.040^{c}	0.080^{ab}	0.050^{bc}
Mn (ppm)	0.121^{a}	0.830^{b}	0.044^{c}	0.044^{c}	0.086^{b}	0.060^{b}	0.150^{ab}	0.060^{b}	0.160^{a}	0.063^{b}
Mg (Cmol/kg)	1.023^{b}	1.133^{a}	0.586^{c}	0.476^{d}	0.295^{e}	3.410^{b}	8.040^{a}	3.020^{b}	3.140^{b}	3.140^{b}
Ca (Cmol/kg)	18.152^{a}	15.807^{b}	10.304^{c}	6.272^{d}	6.272^{d}	11.000^{b}	10.210^{b}	9.010^{b}	11.000^{b}	16.170^{a}
SO_4(ppm)	3.806^{a}	3.174^{b}	2.574^{e}	2.658^{c}	2.674^{c}	2.130^{ab}	1.680^{bc}	1.070^{c}	1.240^{c}	2.670^{a}
NO_3(ppm)	4.040^{c}	3.540^{a}	3.350^{d}	3.350^{d}	5.940^{b}	2.750^{d}	2.550^{d}	3.140^{c}	3.850^{b}	4.440^{a}
Electrical Conductivity(S/m)	85.037^{a}	72.000^{b}	45.133^{c}	44.000^{c}	38.000^{d}	82.330^{a}	84.670^{a}	64.330^{b}	83.670^{a}	74.000^{c}

WHO LIMITS: Temperature (22.00-32.00), pH (6.50-8.50), DO (<5.00), TDS (500), K (30.00),Na (200.00), Fe(0.50 - 50.00),Cu (2.00), Mg (200.00), Ca (200.00), SO_4 (100.00),NO3 (<50.00), Electrical conductivity (<1000.00).

Key: SW – Source of warm spring, MPW – Midpoint of warm spring, C – Confluence of the warm and cold spring, SC – source of cold spring, MPC – Midpoint of cold spring. Temp. – Temperature, DO – Dissolved Oxygen, TDS – Total Dissolved Solids, K – Potassium, Na – Sodium, Fe – Iron, Cu – Copper, Zn – Zinc, Mn –Manganese, Mg – Magnesium, Ca – Calcium, SO_4 – Sulphates, NO_3 – Nitrates. Mean values with similar letter(s) across rows are not significantly different at 5 % level of probability by Duncan Multiple Range Test (DMRT).

Considering water samples from the spring (Table 4.1B), the temperature of the SW and MPW were not significantly different at $P < 0.05$, during both seasons. Both SW and MPW recorded the highest value in comparison to other sites of the spring. The pH of the spring showed that there was no significant difference in the values recorded across 4 sample sites (MPW, C, MPC and SC) during the dry season (December). During the wet season, there was no significant difference in the pH of the spring at MPC and C site. During both seasons/months, the lowest statistically significant pH values were recorded in the SW site only.

The amount of Dissolved Oxygen (DO) across all the 5 sample sites during the wet season showed that the highest amount of DO was found in C sample site, which was significantly different from other sample sites. The dry season however, showed that DO values at MPW was significantly different from SW and C, but was not significantly different from MPC and SC. Total Dissolved Solids (TDS) showed significant differences at $P < 0.05$, across all sample sites computed during the wet season, with SW site showing the highest significant value. For the dry season, all values recorded across all the 5 sample sites were not significantly different from one another.

For Potassium in the spring during June sampling, there was a significant difference in the values derived across all the 5 sample sites, with SW recording the highest value in Potassium. However, in December samples, Potassium had a uniform value of 0.840ppm at SW, MPW and C sites and a uniform value of 0.580ppm at MPC and SC sites. There was a significant difference across all sample sites for Sodium values, with MPW site showing the highest value during the wet season. During the dry season, all values derieved from all the 5 sample sites were not significantly different from each other. Iron content in the spring showed that in June (wet season) the highest significant value was observed at SW and MPW (1.750ppm), while sites with the lowest value was observed at MPC and SC (1.001ppm and 1.000ppm) respectively. During the dry season, it was observed that there was no significant difference observed between MPW, MPC and SC sites, while similar values with no significant difference was observed between SW and C sites (0.320ppm). In addition, it was observed that across all sample sites, higher values were derived during the dry season, than in the wet season.

Copper ions during the wet season showed that there was no significant difference at $P < 0.05$, across all 5 sampled sites. A constant value of 0.005ppm was observed from SW, MPW, C and MPC, while 0.344 was observed only at SC point. During the dry season, a similar trend was observed with no significance observed across all sampled sites, a uniform value of 0.060ppm was observed at SW and MPW, while 0.040ppm was maintained at C, MPC and SC sites. Zinc, had the highest significant value in MPW, while other sites (SW, C, MPC and SC) showed no significant difference in their values during the wet season. While during the dry season, the highest value was observed at SW and MPC sites. Comparing both seasons, SW value during the dry season showed a significantly higher value than in SW site during the wet season.

Manganese showed significant differences at $P < 0.05$ across sample sites during the wet season, with SW site showing the highest significant value, while during the dry season, MPW showed the highest value albeit with no significant difference across other sample sites. Furthermore, comparing both seasons, higher significant values were observed at SW and MPC sites only during the wet season than in the dry season. Magnesium ions in the spring showed that there was a significant difference observed across all 5 sample during the wet season, with the highest value recorded at MPW site. Similar observation was observed in the dry season, with the highest significant value observed at MPW likewise, while the rest of the sample sites showed no significant difference from each other. Across both seasons, the highest significant value was observed at MPW site during the dry season.

During the wet season, calcium was observed as the highest significant value at SW site only, while during the dry season, the highest significant values were observed in SW and MPC sites. Across both sampling seasons, calcium had the highest significant value at SW during the wet season. Sulphates were observed across all samples sites, within the range of 2.674 – 3.804ppm during the wet season. Across all sample sites, SW sites recorded the highest significant mean value at 3.804ppm. while the lowest mean value was recorded at C site with a mean value of 2.574 ppm. During the dry season, however, the highest value of sulphates was observed at SW site, but it was not statistically significant from values observed from other sample sites.

Nitrates, during both seasons was significantly higher at SC sites only, than other sample sites. During the wet season, electrical conductivity was observed as the highest at SW site, while during the dry season, the highest value was observed at MPW, however, the value obtained at MPW was not significantly different from those obtained from SW and MPC sites as well.

4.2. Bacterial load of the sediment and water samples collected at different seasons

The mean CFU/mL of bacteria and fungi isolated from 5 sample sites of the spring during the dry (December) and wet (June) season is shown in Table 4.2. Across both seasons (wet and dry seasons), highest bacterial count from water samples was recorded in the confluence of the warm and cold spring (C) during the dry season with a value of 14.5 x 10^1 CFU/mL. Considering sediment samples, the highest bacterial load was recorded at the confluence of the spring with a value of 14.0 x 10^4 CFU/mL, while the lowest bacterial load was recorded at the midpoint of the cold spring(MPC) having 6.5 x 10^4 CFU/mL.

The overall number of bacteria and fungi that were isolated from 5 sample collection points of Ikogosi warm spring during the course of this study were 174 and 151 respectively. Considering sediment samples, the total number of characterised bacteria from sediments in June were 47 (Table 4.3A), while bacterial isolates that were characterised from sediment samples in December were 49 (Table 4.3B). Considering water samples, June samples (wet season) recorded a total number of characterised bacterial isolates as 29 (Table 4.3C), while 49 bacterial isolates were identified from water samples in December (Table 4.3D).

In Tables 4.3A-D, shows the biochemical characteristics of the 174 bacteria which grouped them into the genera *Bacillus, Staphylococcus, Micrococcus, Sporosarcina, Acinetobacter, Pseudomonas, Enterobacter, Citrobacter, Streptococcus, Enterococcus, Alcaligenes and Aeromonas, Escherichia* and *Klebsiella*.

Table 4.2 Mean CFU/mL values from water and sediment samples obtained at respective sampling points from Ikogosi Warm Springs in June and December, 2018.

		Mean CFU/mL on Nutrient Agar					Mean TFU/mL on Potato Dextrose Agar				
		SAMPLE SITES					SAMPLE SITES				
	Month	SW	MPW	C	MPC	SC	SW	MPW	C	MPC	SC
Water	June	6.5×10^1	7.5×10^1	5.0×10^1	5.5×10^1	7.0×10^1	7.0×10^1	6.5×10^1	5.5×10^1	5.0×10^1	10.5×10^1
	December	7.5×10^1	1.25×10^2	1.45×10^2	6.0×10^1	9.5×10^1	4.5×10^1	7.5×10^1	1.50×10^3	1.15×10^2	1.95×10^2
Sediment	June	13.5×10^4	9.5×10^4	8.0×10^4	6.5×10^4	1.15×10^5	5.5×10^4	7.5×10^4	6.5×10^4	4.0×10^4	6.5×10^4
	December	8.5×10^4	1.35×10^5	1.4×10^5	7.5×10^4	9.0×10^4	9.0×10^4	11.5×10^4	14.0×10^4	7.5×10^4	1.05×10^5

Key: SW – Source of warm spring, MPW – Midpoint of warm spring, C – Confluence of the warm and cold spring, SC – source of cold spring, MPC – Midpoint of the cold spring. CFU –Colony Forming Unit, TFU - Total Fungal Unit.

Table 4.3A: Biochemical characteristics of bacteria isolated from sediment samples at different sampling points of Ikogosi Warm Springs (June -Wet Season)

ISOLATE C ODE	CHROMOGENES IS ON NA	GRAM'S REACTION	SHAPE	CATALASE TEST	OXIDASE TEST	CITRATE TEST	STARCH HYDRO.	GROWTH ON CAB	MR	VP	GELATIN HYDRO.	MOTILITY	INDOLE'S TEST	UREA HYDRO.	NITRATE REDUCTION TEST	SPORE TEST	FRUCTOSE	GALACTOSE	RAFFINOSE	MANNITOL	LACTOSE	MALTOSE	INOSITOL	ARABINOSE	INULIN	SUCROSE	GLYCEROL	PROBABLE IDENTITY
SW3F	none	+	rod	+	+	-	+	-	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	*Bacillus sp.*
SW24B	none	+	rod	+	-	+	+	-	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	+	*Bacillus licheniformis*
SW24C	none	+	rod	+	-	+	+	-	-	+	-	+	-	+	+	+	+	-	-	-	+	+	-	-	-	+	+	*Bacillus licheniformis*
SW25C	none	+	rod	+	-	-	+	-	+	+	+	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	-	*Bacillus coagulans*
SW24D	none	+	rod	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-	+	+	-	+	+	-	+	+	-	*Bacillus subtilis*
SW3C	none	+	rod	+	-	+	+	-	-	+	-	+	-	+	+	+	+	-	-	-	+	+	-	-	-	+	+	*Bacillus cereus*
SW25B	none	+	rod	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-	+	+	-	+	+	-	+	+	-	*Bacillus subtilis*
SW25A	none	+	rod	+	+	-	+	-	+	-	+	+	-	-	+	+	-	+	-	-	-	+	-	-	-	-	+	*Bacillus sp.*
SW4D	none	+	cocci	-	+	+	+	-	-	+	+	+	-	-	+	+	+	-	+	+	-	+	+	-	+	+	-	*Bacillus subtilis*
SW15A	yellow	+	cocci	+	+	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-	*Micrococcus luteus*
SW14B	yellow	+	cocci	+	+	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-	*Micrococcus luteus*
SW35A	none	+	rod	+	-	+	+	-	-	+	-	+	-	+	+	+	+	-	-	-	+	+	-	-	-	+	+	*Bacillus cereus*
SW24A	none	+	rod	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-	+	+	-	+	+	-	+	+	-	*Bacillus subtilis*
MPW44	none	+	cocci	+	-	+	-	-	+	+	+	-	-	+	-	-	+	-	-	-	+	+	-	-	-	+	-	*Staphylococcus sp.*
MPW47	none	+	cocci	+	-	+	-	-	+	+	+	-	-	+	-	-	+	-	-	-	+	+	-	-	-	+	-	*Staphylococcus sp.*
MPW1A	yellow	+	cocci	+	+	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-	*Micrococcus luteus*
MPW46	yellow	+	cocci	+	+	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-	*Micrococcus luteus*
MPW49	orange	-	rod	+	+	+	-	-	-	-	+	+	-	-	-	+	+	+	-	-	+	+	-	-	-	+	+	*Sporosarcina luteola*
MPW30	orange	-	rod	+	+	+	-	-	-	-	+	+	-	-	-	+	+	+	-	-	+	+	-	-	-	+	+	*Sporosarcina luteola*
MPW48	orange	-	rod	+	+	+	-	-	-	-	+	+	-	-	-	+	+	+	-	-	+	+	-	-	-	+	+	*Sporosarcina luteola*
MPW50	yellow	+	cocci	+	+	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-	*Micrococcus luteus*

MPW31	orange	+	rod	+	+	+	-	-	-	-	+	+	-	-	-	+	+	+	-	-	+	+	-	-	-	+	+	*Sporosarcina luteola*
C3B	none	+	rod	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-	+	+	-	+	+	-	+	+	-	*Bacillus subtilis*
C2A	none	-	rod	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-	+	+	-	+	+	-	+	+	-	*Bacillus subtilis*
CID	none	-	rod	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	*Acinetobacter baumanii*
C3A	none	+	rod	+	-	+	+	-	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	+	*Bacillus licheniformis*
C15A	orange	-	rod	+	+	+	-	-	-	-	+	+	-	-	-	+	+	+	-	-	+	+	-	-	-	+	+	*Sporosarcina luteola*
C4B	none	+	rod	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-	+	+	-	+	+	-	+	+	-	*Bacillus subtilis*
C4A	none	+	rod	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-	+	+	-	+	+	-	+	+	-	*Bacillus subtilis*
CIC	light green	-	rod	+	+	+	-	+	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	*Pseudomonas aeruginosa*
MPC21	none	+	cocci	+	-	+	-	-	+	+	+	-	-	+	-	-	+	-	-	-	+	+	-	-	-	+	-	*Staphylococcus sp.*
MPC41	yellow	+	cocci	+	+	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-	*Micrococcus luteus*
MPC33	none	+	cocci	+	-	+	-	-	+	+	+	-	-	+	-	-	+	-	-	-	+	+	-	-	-	+	-	*Staphylococcus sp.*
MPC1D	none	+	rod	+	-	+	+	-	-	+	-	+	-	+	+	+	+	-	-	-	+	+	-	-	-	+	+	*Bacillus cereus*
MPC1A	none	+	rod	+	+	-	-	-	+	-	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	*Bacillus brevis*
MPC39	light green	-	rod	+	+	+	-	+	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	*Pseudomonas aeruginosa*
MPC1B	none	+	rod	+	+	-	+	-	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	*Bacillus sp.*
SCIC	none	-	rod	+	-	+	-	-	-	+	-	+	-	-	+	-	+	+	+	+	+	+	+	+	-	+	+	*Enterobacter sp.*
SCIE	none	-	rod	+	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+	+	+	+	-	-	*Acinetobacter sp.*
SCIB	none	+	cocci	+	-	+	-	-	+	+	+	-	-	+	-	-	+	-	-	-	+	+	-	-	-	+	-	*Staphylococcus sp.*
SCIA	none	-	rod	+	-	+	-	-	-	+	-	+	-	-	+	-	+	+	+	+	+	+	+	+	-	+	+	*Enterobacter sp.*
SC41	none	+	rod	+	+	-	+	-	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	*Bacillus sp.*
SC2C	none	-	rod	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	*Acinetobacter sp.*
SC45	none	+	cocci	+	-	+	-	-	+	+	+	-	-	+	+	-	+	+	-	+	+	+	-	-	-	+	-	*Staphylococcus aureus*
SC1D	none	-	rod	+	-	+	-	-	-	+	-	+	-	-	+	-	+	+	+	+	+	+	+	+	-	+	+	*Enterobacter sp.*
SC44	light green	-	rod	+	+	+	-	+	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	*Pseudomonas aeruginosa*
SC2A	light green	-	rod	+	+	+	-	+	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	*Pseudomonas aeruginosa*

Key: SW – Source of warm spring, MPW – Midpoint of warm spring, C – Confluence of the warm and cold spring, SC – source of cold spring, MPC – Midpoint of cold spring, Hydro –hydrolysis. NA-Nutrient Agar, CAB – Centrimide Agar Base, MR – Methyl Red, VP – Voges-Proskauer.

Table 4.3B: Biochemical characteristics of bacteria isolated from sediment samples at different sampling points of Ikogosi Warm Springs (December – Dry season)

ISOLATE CODE	GRAM'S REACTION	SHAPE	CATALASE TEST	OXIDASE TEST	CITRATE TEST	STARCH HYDRO.	GROWTH ON CAB	MR	VP	GELATIN HYDRO.	MOTILITY	INDOLE'S TEST	UREA HYDRO.	NITRATE REDUCTION	SPORE TEST	FRUCTOSE	GALACTOSE	RAFFINOSE	MANNITOL	LACTOSE	MALTOSE	INOSITOL	ARABINOSE	INULIN	SUCROSE	GLYCEROL	PROBABLE IDENTITY
SW4B	+	cocci	+	-	+	-	-	+	+	+	-	-	+	+	-	+	+	-	+	+	+	-	-	-	+	-	*Staphylococcus aureus*
SW8	-	rod	+	-	+	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	*Acinetobacter moffi*
SW3B	+	rod	+	-	-	+	-	+	+	+	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	-	*Bacillus coagulans*
SW4D	-	rod	+	-	+	-	-	+	-	-	+	-	+	+	-	-	-	-	+	+	+	-	+	-	+	+	*Citrobacter sp.*
SW1	+	cocci	-	-	-	-	-	+	-	+	-	-	-	-	-	+	+	+	-	+	+	-	+	+	+	+	*Streptococcus sp.*
SW3C	+	rod	+	+	-	+	-	+	+		+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	*Bacillus sp.*
SW14	-	rod	+	-	-	-	-	+	-	-	+	+	-	+	-	-	-	-	+	+	+	-	+	-	-	-	*E.coli*
SW16	+	cocci	+	-	+	-	-	+	+	+	-	-	+	+	-	+	+	-	+	+	+	+	-	-	+	-	*Staphylococcus aureus*
MPW5	-	rod	+	+	+	-	+	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	*Pseudomonas aeruginosa*
MPW6	-	rod	+	+	+	-	-	+	+	+	+	+	-	+	-	+	+	-	+	-	+	+	-	+	+	+	*Aeromonas hydrophila*
MPW2B	-	rod	+	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	*Alcaligenes sp.*
MPW2C	-	rod	+	-	-	-	-	+	-	-	+	+	-	+	-	-	-	-	+	+	+	-	+	-	-	-	*E.coli*
MPW29	+	cocci	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	-	-	-	+	-	-	+	+	-	*Staphylococcus sp.*
MPW10	+	cocci	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	*Micrococcus sp.*
MPW25	+	rod	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+	-	-	-	+	+	-	*Bacillus mycoides*
MPW9	+	cocci	+	+	-	-	-	-	+	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	+	-	*Micrococcus sp.*
MPW32	+	cocci	+	-	+	-	-	+	+	+	-	-	+	+	-	+	+	-	+	+	+	-	-	-	+	-	*Staphylococcus aureus*
MPW7	+	rod	+	+	-	+	-	+	+	+	+	-	-	+	+	+	+	-	+	+	-	-	-	+	+	-	*Bacillus mycoides*
MPW3E	+	rod	+	-	+	+	-	-	+	-	+	-	+	+	+	+	-	-	-	+	+	-	-	-	+	+	*Bacillus cereus*
MPW8	+	cocci	+	-	+	-	-	+	+	+	-	-	+	-	-	+	-	-	-	+	+	-	-	-	+	-	*Staphylococcus sp.*

C14	+	rod	+	+	-	-	-	+	-	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	*Bacillus brevis*
CB	+	cocci	+	-	+	-	-	+	-	+	-	-	+	-	-	+	+	-	-	-	+	-	-	-	-	-	*Staphylococcus sp.*
C25	-	rod	+	-	-	-	-	+	-	-	+	+	-	+	-	-	-	-	+	+	+	-	+	-	-	-	*Escherichia coli*
C2A	+	rod	+	-	+	+	-	-	+	-	+	-	+	+	+	+	-	-	-	+	+	-	-	-	+	+	*Bacillus cereus*
C15	+	rod	+	+	-	-	-	+	-	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	*Bacillus brevis*
C26	-	rod	+	+	+	-	+	-	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	*Pseudomonas aeruginosa*
C4C	-	rod	+	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+	+	+	+	-	-	*Acinetobacter sp.*
C1	-	rod	+	-	+	-	-	-	+	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	*Enterobacter aerogenes*
C3	-	rod	+	-	+	-	-	-	+	-	+	-	-	+	-	+	+	+	+	+	+	+	+	-	+	+	*Enterobacter sp.*
C2B	+	cocci	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	+	+	+	-	+	+	+	*Streptococcus pyogenes*
C2C	-	rod	+	+	+	-	+	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	*Pseudomonas aeruginosa*
C2F	-	rod	+	-	-	-	-	+	-	-	+	+	-	+	-	-	-	-	+	+	+	-	+	-	-	-	*Escherichia coli*
C4A	+	rod	+	+	-	+	-	+	-	+	+	-	-	+	+	-	+	-	-	-	+	-	-	-	-	+	*Bacillus sp.*
C20	+	rod	+	-	+	+	-	-	+	+	+	-	-	+	+	+	-	+	+	-	+	+	-	+	+	-	*Bacillus subtilis*
MPC21	+	rod	+	+	-	+	-	+	-	-	+	-	-	+	+	+	+	-	-	-	-	-	-	+	-	-	*Bacillus sp.*
MPC18	+	rod	+	+	+	-	+	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	*Pseudomonas aeruginosa*
MPC20	+	rod	+	+	+	-	-	-	+	+	+	-	-	+	+	+	-	+	+	-	+	+	-	+	+	-	*Bacillus subtilis*
MPC7	-	rod	+	+	+	-	-	-	+	-	-	+	+	+	-	-	-	+	+	+	+	-	+	-	+	-	*Klebsiella sp.*
MPC10	+	cocci	+	-	-	-	-	-	+	-	-	-	+	+	-	+	+	-		+	+	-	-	-	+	-	*Staphylococcus epidermidis*

MPC16	+	rod	+	-	-	+	-	-	-	+	+	-	-	+	+	-	-	-	+	+	-	-	+	-	+	-	*Bacillus sp.*
SC4	-	rod	+	+	+	-	+	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	*Pseudomonas aeruginosa*
SCB	+	cocci	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-	+	+	*Streptococcus sp.*
SCC	+	cocci	+	-	+	-	-	+	+	+	-	-	+	+	-	+	+	-	+	+	+	-	-	-	+	-	*Staphylococcus aureus*
SC9	+	cocci	+	-	+	-	-	+	+	+	-	-	+	+	-	+	+	-	+	+	+	-	-	-	+	-	*Staphylococcus aureus*
SC10	+	cocci	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	+	+	+	-	-	+	-	*Enterococcus sp.*
SC11	+	cocci	-	-	-	-	-	-	+	+	-	-	-	+	-	+	+	-	+	+	+	-	-	-	+	-	*Enterococcus faecalis*
SC12	-	rod	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	*Acinetobacter sp.*
SCA	+	cocci	+	-	-	-	-	-	+	-	-	-	+	+	-	+	+	-	-	+	+	-	-	-	+	-	*Staphylococcus epidermidis*
SCB4	+	cocci	+	-	+	-	-	+	+	+	-	-	+	+	-	+	+	-	+	+	+	-	-	-	+	-	*Staphylococcus aureus*

Key: SW – Source of warm spring, MPW – Midpoint of warm spring, C – Confluence of the warm and cold spring, SC – source of cold spring, MPC – Midpoint of cold spring, Hydro –hydrolysis. NA-Nutrient Agar, CAB – Centrimide Agar Base, MR-Methyl Red, VP- Voges-Prokauer.

Table 4.3C: Biochemical characteristics of bacteria isolated from water samples at different sampling points of Ikogosi Warm Springs (June –Wet Season)

ISOLATE CODE	GRAM'S REACTION	SHAPE	CHROMOGENESIS ON NA	CATALASE TEST	OXIDASE TEST	CITRATE TEST	STARCH HYDRO.	GROWTH ON CAB	MR	VP	GELATIN HYDRO.	MOTILITY	INDOLE'S TEST	UREA HYDRO.	NITRATE REDUCTION TEST	SPORE TEST	FRUCTOSE	GALACTOSE	RAFFINOSE	MANNITOL	LACTOSE	MALTOSE	INOSITOL	ARABINOSE	INULIN	SUCROSE	GLYCEROL	PROBABLE IDENTITY
SW21W	+	rod	None	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+	-	-	-	+	+	-	*Bacillus mycoides*
SW3W	-	rod	None	+	-	+	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	*Acinetobacter moffi*
SW10W	+	cocci	None	+	-	+	-	-	+	+	+	-	-	+	-	-	+	-	-	-	+	+	-	-	-	+	-	*Staphylococcus sp.*
SW6W	+	rod	None	+	-	-	+	-	+	+	+	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	-	*Bacillus coagulans*
SW8W	+	rod	None	+	-	+	+	-	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	+	*Bacillus licheniformis*
SW1W	-	rod	None	+	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	*Alcaligenes sp.*
MPW22W	+	cocci	Yellow	+	+	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-	*Micrococcus luteus*
MPW41W	-	rod	Light green	+	+	+	-	+	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	*Pseudomonas aeruginosa*
MPW44W	-	rod	Orange	+	+	+	-	-	-	-	+	+	-	-	-	+	+	+	-	-	+	+	-	-	-	+	+	Sporosarcina luteola
MPW14W	+	cocci	Yellow	+	+	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-	Micrococcus luteus
MPW11W	+	cocci	None	+	-	+	-	-	+	+	+	-	-	+	+	-	+	+	-	+	+	+	-	-	-	+	-	*Staphylococcus aureus*
MPW16W	+	rod	None	+	+	+	-	-	-	+	+	+	-	-	+	+	+	-	+	+	-	+	+	-	+	+	-	*Bacillus subtilis*
MPW35W	-	rod	None	+	-	-	-	-	+	-	-	+	+	-	+	-	-	-	-	+	+	+	-	+	-	-	-	*Escherichia coli*
MPW411W	+	cocci	Light pink	+	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	*Micrococcus roseus*
C36W	+	cocci	None	+	-	-	-	-	-	+	-	-	-	+	+	-	+	+	-	-	+	+	-	-	-	+	-	*Staphylococcus epidermidis*
C12W	+	cocci	None	+	-	-	-	-	-	+	-	-	-	+	+	-	+	+	-	-	+	+	-	-	-	+	-	*Staphylococcus epidermidis*
C34W	-	rod	Orange	+	+	+	-	-	-	-	+	-	-	-	-	+	+	+	-	-	+	+	-	-	-	+	+	*Sporosarcina luteola*
C11W	+	Rod	None	+	+	-	-	-	+	-	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	*Bacillus brevis*
C20W	-	Rod	None	+	-	+	-	-	-	+	-	+	-	-	+	-	+	+	+	+	+	+	+	+	-	+	+	*Enterobacter sp.*

MPC12W	+	cocci	Light pink	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	*Micrococcus roseus*
MPC22W	+	cocci	None	+	-	+	-	-	+	+	+	-	-	+	+	-	+	+	-	+	+	+	-	-	-	+	-	*Staphylococcus aureus*
MPC23W	-	rod	Light green	+	+	+	-	+	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	*Pseudomonas aeruginosa*
MPC26W	+	cocci	Yellow	+	+	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-	*Micrococcus luteus*
MPC24W	+	rod	None	+	+	-	-	-	+	-	+	+	-	-	+	+	-	+	-	-	-	+	-	-	-	-	+	*Bacillus sp.*
SC42W	-	rod	Light green	+	+	+	-	+	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	*Pseudomonas aeruginosa*
SC35W	+	cocci	None	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-	+	+	*Streptococcus sp.*
SC33W	-	rod	None	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	*Acinetobacter sp.*
SC41W	+	cocci	None	+	-	-	-	-	-	+	-	-	-	+	+	-	+	+	-	-	+	+	-	-	-	+	-	*Staphylococcus epidermidis*
SC12W	+	rod	None	+	+	-	-	-	+	-	+	+	-	-	+	+	-	+	-	-	-	+	-	-	-	-	+	*Bacillus sp.*

Key: SW – Source of warm spring, MPW – Midpoint of warm spring, C – Confluence of the warm and cold spring, SC – source of cold spring, MPC – Midpoint of cold spring, NA-Nutrient Agar, CAB – Centrimide Agar Base, Hydro-hydrolysis, MR-Methyl Red, VP-Voges-Proskauer.

Table 4.3D: Biochemical characteristics of bacteria isolated from water samples at different sampling points of Ikogosi Warm Springs (December –Dry season)

ISOLATE CODE	GRAM'S REACTION	SHAPE	CATALASE TEST	OXIDASE TEST	CITRATE TEST	STARCH HYDRO.	GROWTH ON CAB	MR	VP	GELATIN HYDRO.	MOTILITY	INDOLE'S TEST	UREA HYDRO.	NITRATE REDUCTION	SPORE TEST	FRUCTOSE	GALACTOSE	RAFFINOSE	MANNITOL	LACTOSE	MALTOSE	INOSITOL	ARABINOSE	INULIN	SUCROSE	GLYCEROL	PROBABLE IDENTITY
SW4B	+	cocci	+	-	+	-	-	+	+	+	-	-	+	+	-	+	+	-	+	+	+	-	-	-	+	-	*Staphylococcus aureus*
SW8	-	rod	+	-	+	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	*Acinetobacter moffi*
SW3B	+	rod	+	-	-	+	-	+	+	+	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	-	*Bacillus coagulans*
SW4D	-	rod	+	-	+	-	-	+	-	-	+	-	+	+	-	-	-	-	+	+	+	-	+	-	+	+	*Citrobacter sp.*
SW1	+	cocci	-	-	-	-	-	+	-	+	-	-	-	-	-	+	+	+	-	+	+	-	+	+	+	+	*Streptococcus sp.*
SW3C	+	rod	+	+	-	+	-	+	+		+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	*Bacillus sp.*
SW14	-	rod	+	-	-	-	-	+	-	-	+	+	-	+	-	-	-	-	+	+	+	-	+	-	-	-	*E.coli*
SW16	+	cocci	+	-	+	-	-	+	+	+	-	-	+	+	-	+	+	-	+	+	+	+	-	-	+	-	*Staphylococcus aureus*
MPW5	-	rod	+	+	+	-	+	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	*Pseudomonas aeruginosa*
MPW6	-	rod	+	+	+	-	-	+	+	+	+	+	-	+	-	+	+	-	+	-	+	+	-	+	+	+	*Aeromonas hydrophila*
MPW2B	-	rod	+	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	*Alcaligenes sp.*
MPW2C	-	rod	+	-	-	-	-	+	-	-	+	+	-	+	-	-	-	-	+	+	+	-	+	-	-	-	*E.coli*
MPW29	+	cocci	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	-	-	-	+	-	-	+	+	-	*Staphylococcus sp.*
MPW10	+	cocci	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	*Micrococcus sp.*
MPW25	+	rod	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+	-	-	-	+	+	-	*Bacillus mycoides*
MPW9	+	cocci	+	+	-	-	-	-	+	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	+	-	*Micrococcus sp.*
MPW32	+	cocci	+	-	+	-	-	+	+	+	-	-	+	+	-	+	+	-	+	+	+	-	-	-	+	-	*Staphylococcus aureus*
MPW7	+	rod	+	+	-	+	-	+	+	+	+	-	-	+	+	+	+	-	+	+	-	-	-	+	+	-	*Bacillus mycoides*
MPW27	+	rod	+	-	+	+	-	-	+	-	+	-	+	+	+	+	-	-	-	+	+	-	-	-	+	+	*Bacillus cereus*
MPW8	+	cocci	+	-	+	-	-	+	+	+	-	-	+	-	-	+	-	-	-	+	+	-	-	-	+	-	*Staphylococcus sp.*

C14	+	rod	+	+	-	-	-	+	-	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	*Bacillus brevis*
CB	+	cocci	+	-	+	-	-	+	-	+	-	-	+	-	-	+	+	-	-	-	+	-	-	-	-	-	*Staphylococcus sp.*
C25	-	rod	+	-	-	-	-	+	-	-	+	+	-	+	-	-	-	-	+	+	+	-	+	-	-	-	*Escherichia coli*
C2A	+	rod	+	-	+	+	-	-	+	-	+	-	+	+	+	+	-	-	-	+	+	-	-	-	+	+	*Bacillus cereus*
C15	+	rod	+	+	-	-	-	+	-	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	*Bacillus brevis*
C26	-	rod	+	+	+	-	+	-	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	*Pseudomonas aeruginosa*
C4C	-	rod	+	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+	+	+	+	-	-	*Acinetobacter sp.*
C1	-	rod	+	-	+	-	-	-	+	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	*Enterobacter aerogenes*
C3	-	rod	+	-	+	-	-	-	+	-	+	-	-	+	-	+	+	+	+	+	+	+	+	-	+	+	*Enterobacter sp.*
C2B	+	cocci	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	+	+	+	-	+	+	+	*Streptococcus pyogenes*
C2C	-	rod	+	+	+	-	+	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	*Pseudomonas aeruginosa*
C2F	-	rod	+	-	-	-	-	+	-	-	+	+	-	+	-	-	-	-	+	+	+	-	+	-	-	-	*Escherichia coli*
C4A	+	rod	+	+	-	+	-	+	-	+	+	-	-	+	+	-	+	-	-	-	+	-	-	-	-	+	*Bacillus sp.*
C20	+	rod	+	-	+	+	-	-	+	+	+	-	-	+	+	+	-	+	+	-	+	+	-	+	+	-	*Bacillus subtilis*
MPC21	+	rod	+	+	-	+	-	+	-	-	+	-	-	+	+	+	+	-	-	-	-	-	-	+	-	-	*Bacillus sp.*
MPC18	+	rod	+	+	+	-	+	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	*Pseudomonas aeruginosa*
MPC20	+	rod	+	+	+	-	-	-	+	+	+	-	-	+	+	+	-	+	+	-	+	+	-	+	+	-	*Bacillus subtilis*
MPC7	-	rod	+	+	+	-	-	-	+	-	-	+	+	+	-	-	-	+	+	+	+	-	+	-	+	-	*Klebsiella sp.*
MPC10	+	cocci	+	-	-	-	-	-	+	-	-	-	+	+	-	+	+	-		+	+	-	-	-	+	-	*Staphylococcus epidermidis*

MPC16	+	rod	+	-	-	+	-	-	-	+	+	-	-	+	+	-	-	-	+	+	-	-	+	-	+	-	*Bacillus sp.*
SC4	-	rod	+	+	+	-	+	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	*Pseudomonas aeruginosa*
SCB	+	cocci	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-	+	+	*Streptococcus sp.*
SCC	+	cocci	+	-	+	-	-	+	+	+	-	-	+	+	-	+	+	-	+	+	+	-	-	-	+	-	*Staphylococcus aureus*
SC9	+	cocci	+	-	+	-	-	+	+	+	-	-	+	+	-	+	+	-	+	+	+	-	-	-	+	-	*Staphylococcus aureus*
SC10	+	cocci	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	+	+	+	-	-	+	-	*Enterococcus sp.*
SC11	+	cocci	-	-	-	-	-	-	+	+	-	-	-	+	-	+	+	-	+	+	+	-	-	-	+	-	*Enterococcus faecalis*
SC12	-	rod	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	*Acinetobacter sp.*
SCA	+	cocci	+	-	-	-	-	-	+	-	-	-	+	+	-	+	+	-	-	+	+	-	-	-	+	-	*Staphylococcus epidermidis*
SCB4	+	cocci	+	-	+	-	-	+	+	+	-	-	+	+	-	+	+	-	+	+	+	-	-	-	+	-	*Staphylococcus aureus*

Key: SW – Source of warm spring, MPW – Midpoint of warm spring, C – Confluence of the warm and cold spring, SC – source of cold spring, MPC – Midpoint of cold spring, Hydro –hydrolysis. NA-Nutrient Agar, CAB – Centrimide Agar Base, MR –Methyl Red, VP – Voges Proskauer.

4.3. Fungal load of the sediment and water samples collected at different seasons

Highest total fungal counts from water samples (considering both wet and dry seasons) was recorded at the source of the cold spring (SC) with a mean value of 19.5 x 10^1 CFU/mL during the dry season, while the lowest fungal load was recorded at the source fof the warm spring (SW)during the dry season too, with a mean value of 4.5 x 10^1TFU/mL. Considering sediment samples across different seasons, the highest bacterial load was recorded at the confluence of the spring (C) with a mean value of 14.0 x 10^4 CFU/mL during the dry season (December), while the lowest bacterial load was recorded at the midpoint of the cold spring (MPC) with a mean value of 4.0 x 10^4 CFU/mL observed during the wet season (June). (Table 4.2)

In the dry and wet season, the same array of fungi was recorded as shown in Table 4.4A-D. The predominant fungi were *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp. and *Trichoderma* sp. A total number of fungal isolates identified from 5 sample collection points of Ikogosi warm spring were 151.

4.4. DNA Concentration and Purity of Extracted DNA from Sediment and Water Samples from Ikogosi Warm Springs

From sediment samples, the highest DNA concentration was 567.200 ng/µl, while the least concentration of DNA was 71.400 ng/µl. From water samples, the highest DNA concentration was recorded as 554.500 ng/µl, and the lowest DNA extract was 14.700 ng/µl. Table 4.5 reveals the concentration and purity of DNA extracted during the course of this research work.

Table 4.4A Morphological and microscopic characteristics of fungi from sediment samples at different sampling points of Ikogosi Warm Springs (June – Wet Season)

Isolate code	Morphological characteristics			Microscopic observation	Probable name
	Chromogenesis on PDA	Elevation	Surface		
SW12S	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
SW23S	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate condidiophores with brush-like phialides	*Penicillium* sp.
SW14S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
SW10S	Deep Green velvet-like colonies with white edges.	Raised	Smooth	Septate condidiophores with brush-like phialides	*Penicillium* sp.
SW4S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
MPW1S	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
MPW2S	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
MPW11S	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate condidiophores with brush-like phialides	*Penicillium* sp.
MPW3S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.

MPW4S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
MPW15S	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
C01S	Light brown velvet-like colony with folded white edges	Raised	Rough	Septate conidiophore with tree-like branches with chains of conidia	*Cladosporium* sp.
C03S	Light green colonies with white edges and colonies, which over time turns to an abundance of green mycelium on plate	Flat	Rough	Long unbranched conidiophores with vesicles at the tip which carries the conidia.	*Aspergillus flavus*
C4S	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
C9S	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate condidiophores with brush-like phialides	*Penicillium* sp.
MPC5S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
MPC13S	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
MPC12S	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate condidiophores with brush-like phialides	*Penicillium* sp.
MPC13S	Light green colonies with white edges and colonies, which over time turns to an abundance of green mycelium on plate	Flat	Rough	Long unbranched conidiophores with vesicles at the tip which carries the conidia.	*Aspergillus flavus*
SC4S	Creamish colony which produces a pink pigment around its colony	Raised	Rough	Septate, smooth-walled conidiophore with distinctively long phialides	*Talaromyces* sp.

SC7S	Light brown velvet-like colony with folded white edges	Raised	Rough	Septate conidiophore with tree-like branches with chains of conidia	*Cladosporium* sp.
SC3S	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate condidiophores with brush-like phialides	*Penicillium* sp.
SC10S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
SC11S	Light green colonies with white edges and colonies, which over time turns to an abundance of green mycelium on plate	Flat	Rough	Long unbranched conidiophores with vesicles at the tip which carries the conidia.	*Aspergillus flavus*
SC14S	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.

Key: SW – Source of warm spring, MPW – Midpoint of warm spring, C – Confluence of the warm and cold spring, SC – source of cold spring, MPC – Midpoint of cold spring, PDA-Potato Dextrose Agar.

Table 4.4B Morphological and microscopic characteristics of fungi from sediment samples at different sampling points of Ikogosi Warm Springs (December –Dry Season)

Isolate code	Morphological characteristics			Microscopic observation	Probable name
	Chromogenesis on PDA	Elevation	Surface		
SW3S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
SW5S	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
SW6S	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
SW9S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
SW13S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
SW19S	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
SW20S	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
SW21S	Light green colonies with white edges and colonies, which over time turns to an abundance of green mycelium on plate	Flat	Rough	Long unbranched conidiophores with vesicles at the tip which carries the conidia.	*Aspergillus flavus*
MPW1S	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*

MPW2S	Light green colonies with white edges and colonies, which over time turns to an abundance of green mycelium on plate	Flat	Rough	Long unbranched conidiophores with vesicles at the tip which carries the conidia.	*Aspergillus flavus*
MPW4S	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
MPW7S	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
MPW8S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
MPW11S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
MPW14S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
MPW15S	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
MPW27S	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
C 4S	Light green colonies with white edges and colonies, which over time turns to an abundance of green mycelium on plate	Flat	Rough	Long unbranched conidiophores with vesicles at the tip which carries the conidia.	*Aspergillus flavus*
C1S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
C20S	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.

C22S	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
C23S	Creamish colony which produces a pink pigment around its colony	Raised	Rough	Septate, smooth-walled conidiophore with distinctively long phialides	*Talaromyces* sp.
C10S	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
C14S	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
C13S	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
MPC21S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
MPC2S	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
MPC1S	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
MPC7S	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
MPC10S	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.

SC1S	Light green colonies with white edges and colonies, which over time turns to an abundance of green mycelium on plate	Flat	Rough	Long unbranched conidiophores with vesicles at the tip which carries the conidia.	*Aspergillus flavus*
SC8S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
SC4S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
SC22S	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
SC23S	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
SC13S	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
SC10S	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched condiophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
SC3S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
SC30S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
SC33S	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.

Key: SW – Source of warm spring, MPW – Midpoint of warm spring, C – Confluence of the warm and cold spring, SC – source of cold spring, MPC – Midpoint of cold spring, PDA-Potato Dextrose Agar.

Table 4.4C Morphological and microscopic characteristics of fungi from water samples at different sampling points of Ikogosi Warm Springs (June – Wet Season)

Isolate code	Morphological characteristics			Microscopic observation	Probable name
	Chromogenesis on PDA	Elevation	Surface		
SW33	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
SW13	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
SW12	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
SW32	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
SW3	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
SW12	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
SW12B	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
MPW12	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
MPW22	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
MPW33	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.

MPW4	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
MPW43	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
C12	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
C24	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
C34	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
C3	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
C2	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
MPC2	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
MPC3A	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
MPC2B	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
MPC13	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*

MPC33	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
SC23	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
SC2B	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
SC30	Creamish colony which produces a pink pigment around its colony	Raised	Rough	Septate, smooth-walled conidiophore with distinctively long phialides	*Talaromyces* sp.
SC32	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
SC2	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
SC13	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
SC12	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
SC42	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
SC02	Creamish colony which produces a pink pigment around its colony	Raised	Rough	Septate, smooth-walled conidiophore with distinctively long phialides	*Talaromyces* sp.
SC9	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.

Key: SW – Source of warm spring, MPW – Midpoint of warm spring, C – Confluence of the warm and cold spring, SC – source of cold spring, MPC – Midpoint of cold spring, PDA- Potato Dextrose Agar

Table 4.4D Morphological and Microscopic Characteristics of Fungi from Water Samples at Different Sampling Points of Ikogosi Warm Springs (December – Dry Season)

Isolate code	Morphological characteristics			Microscopic observation	Probable name
	Chromogenesis on PDA	Elevation	Surface		
SW11	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
SW31	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
SW12	Light brown velvet-like colony with folded white edges	Raised	Rough	Septate conidiophore with tree-like branches with chains of conidia	*Cladosporium* sp.
SW30	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
MPW2	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
MPW3	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
MPW7	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
MPW10	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
MPW11	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.

MPW23	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
C2	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
C23	Light green colonies with white edges and colonies, which over time turns to an abundance of green mycelium on plate	Flat	Rough	Long unbranched conidiophores with vesicles at the tip which carries the conidia.	*Aspergillus flavus*
C20	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
C13	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
C14	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
C18	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
C10	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
C15	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
C3	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
C33	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
C12	Large, white ringed-like colony with a greenish colour at the middle	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.

	of the colony, and woolly in appearance.				
C11	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
C4	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
C5	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
C16	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
MPC23	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
MPC11	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
MPC10	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
MPC4	Light green colonies with white edges and colonies, which over time turns to an abundance of green mycelium on plate	Flat	Rough	Long unbranched conidiophores with vesicles at the tip which carries the conidia.	*Aspergillus flavus*
MPC2	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
MPC8	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
MPC3	Light brown velvet-like colony with folded white edges	Raised	Rough	Septate conidiophore with tree-like branches with chains of conidia	*Cladosporium* sp.

MPC14	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
MPC15	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
MPC12	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
MPC18	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
SC17	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
SC10	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
SC10B	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
SC22	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
SC11	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia	*Aspergillus niger*
SC4	Light brown velvet-like colony with folded white edges	Raised	Rough	Septate conidiophore with tree-like branches with chains of conidia	*Cladosporium* sp.
SC22	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.

SC3	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
SC5	Light brown velvet-like colony with folded white edges	Raised	Rough	Septate conidiophore with tree-like branches with chains of conidia	*Cladosporium* sp.
SC17	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.
SC13	Light green colonies with white edges and colonies, which over time turns to an abundance of green mycelium on plate	Flat	Rough	Long unbranched conidiophores with vesicles at the tip which carries the conidia.	*Aspergillus flavus*
SC12A	Light brown velvet-like colony with folded white edges	Raised	Rough	Septate conidiophore with tree-like branches with chains of conidia	*Cladosporium* sp.
SC1	Dense layer of black conidia with creamy edges surrounding the colonies.	Flat	Rough	Long unbranched coniodophore with an enlarged vesicle at the tip, bearing the conidia..	*Aspergillus niger*
SC02	Light brown velvet-like colony with folded white edges	Raised	Rough	Septate conidiophore with tree-like branches with chains of conidia	*Cladosporium* sp.
SC06	Large, white ringed-like colony with a greenish colour at the middle of the colony, and woolly in appearance.	Flat	Rough	Short conidiophores with conidia growing at the ends of the phialides	*Trichoderma* sp.
SC18	Light green colonies with white edges and colonies, which over time turns to an abundance of green mycelium on plate	Flat	Rough	Long unbranched conidiophores with vesicles at the tip which carries the conidia.	*Aspergillus flavus*
SC21	Deep green velvet-like colonies with white edges.	Raised	Smooth	Septate conidiophores with brush-like phialides	*Penicillium* sp.
SC42	Large cotton-like colony with dense mycelium	Flat	Rough	Septate hyphae with short and single phialides	*Fusarium* sp.

Key: SW – Source of warm spring, MPW – Midpoint of warm spring, C – Confluence of the warm and cold spring, SC – source of cold spring, MPC – Midpoint of cold spring, PDA-Potato Dextrose Agar

4.5 Screening bacterial isolates from Ikogosi Warm Springs for alpha-amylase activity

Out of 174 bacterial isolates, obtained from sediment and water samples of Ikogosi warm springs, 46 isolates were deemed thermophilic, as they were able to thrive at 50°C and elicited amylolytic zones of hydrolysis ranging between 4.00mm and 12.00mm on the screening medium. All the positive thermophilic amylolytic bacterial isolates were identified to belong to the genus *Bacillus* (Table 4.6).

The isolates exhibited clear zones on 1% starch agar plates and 27.05% of total isolates were amylolytic and 72.94% non-amylolytic. However, among amylolytic isolates, some clearings were more pronounced and wider than others. The widest zone of clearing for amylase activity was observed with respect to *Bacillus cereus* MPW3E with a diameter size of 12.00 mm and *Bacillus siamensis* SW3F with a diameter of 10.00mm. These two isolates were thus selected for further studies.

The phylogenetic relatedness of selected Bacilli, is depicted in Figure 4.1 A and B for *Bacillus siamensis* SW3F and *Bacillus cereus* MPW3E, respectively. Bootstrap values inferred from 1000 replicates showed significant bootstrapping (Felsenstein, 1985).

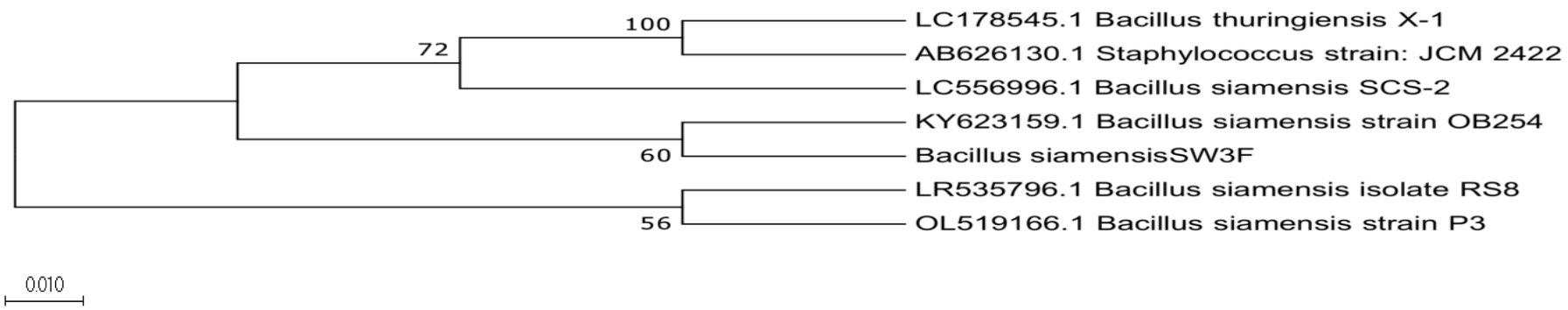
Table 4.5 DNA concentration of water and sediment samples from Ikogosi Warm Springs sampled in 8th June, 2018 and 18th December, 2018

Period of collection	Sample Type	Sample ID	Nucleic Acid Conc. (ng/µl)	A260(abs)	A280(abs)	A260/280	A260/230
8th June, 2018	Water	SW	307.900	6.159	3.235	1.900	1.830
	Water	MPW	131.000	2.621	1.433	1.830	1.920
	Water	C	170.900	3.418	1.828	1.870	2.020
	Water	MPC	356.000	7.120	3.785	1.880	1.770
	Water	SC	71.400	1.427	0.800	1.780	1.070
18th December, 2018.	Water	SW	567.200	11.344	5.987	1.890	1.160
	Water	MPW	243.500	4.871	2.638	1.850	0.280
	Water	C	412.300	8.247	4.309	1.910	1.160
	Water	MPC	191.000	3.819	1.967	1.940	0.660
	Water	SC	175.400	3.508	1.815	1.930	0.960
8th June, 2018	Sediment	SW	14.700	0.294	0.162	1.820	0.310
	Sediment	MPW	15.800	0.317	0.158	2.000	0.310
	Sediment	C	67.700	1.354	0.718	1.890	1.230
	Sediment	MPC	38.600	0.772	0.425	1.820	0.470
	Sediment	SC	78.600	1.573	0.884	1.780	0.210
18th December, 2018	Sediment	SW	259.800	5.196	2.710	1.920	1.920
	Sediment	MPW	554.500	11.089	5.850	1.900	2.070
	Sediment	C	251.400	5.027	2.634	1.910	1.930
	Sediment	MPC	225.200	4.505	2.357	1.910	2.120
	Sediment	SC	324.600	6.493	3.381	1.920	1.900

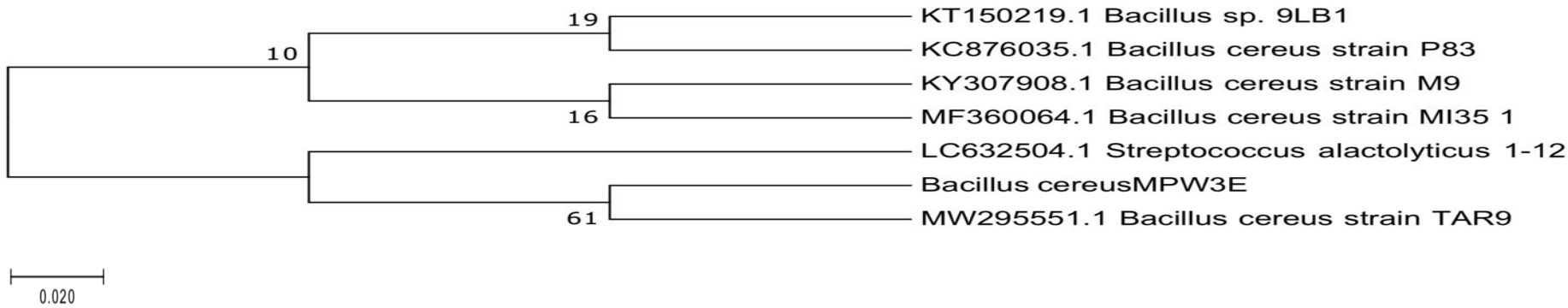
Table 4.6: Hydrolytic zones of bacterial isolates from Ikogosi Warm Springs

Isolate Code	Source and Date	Zone of Hydrolysis (mm)
SW3C	Source of Warm Spring, sediment, (June, 2018).	6.00
SW24B	Source of Warm Spring, sediment (June, 2018).	6.00
SW24C	Source of Warm Spring, sediment (June, 2018).	4.00
SW25C	Source of Warm Spring, sediment. (June, 2018).	8.00
SW3F	Source of Warm Spring, sediment. (June, 2018).	12.00
SW24D	Source of Warm Spring, sediment. (June, 2018).	9.00
SW25B	Source of Warm Spring, sediment (June, 2018).	7.00
SW25A	Source of Warm Spring, sediment (June, 2018).	5.00
SW4D	Source of Warm Spring, sediment (June, 2018).	5.00
SW35A	Source of Warm Spring, sediment (June, 2018).	9.00
SW24A	Source of Warm Spring, sediment (June, 2018).	9.00
C3B	Confluence of both springs, sediment (June, 2018).	9.00
C2A	Confluence of both springs, sediment (June, 2018)	9.00
C4B	Confluence of both springs, sediment (June, 2018).	9.00
C4A	Confluence of both springs, sediment (June, 2018).	6.00
MPC1D	Midpoint of cold spring, sediment (June, 2018).	6.50
MPC1A	Midpoint of cold spring, sediment. (June, 2018).	7.00
MPC1B	Midpoint of cold spring, sediment. (December, 2018).	7.00
SC41	Source of cold spring, sediment (December, 2018).	4.00
SW3B	Source of Warm Spring, sediment (December, 2018).	5.50
SW3B	Source of Warm Spring, sediment (December, 2018).	6.50
SW3C	Source of Warm Spring, sediment (December, 2018).	8.00
MPC25	Midpoint of Cold Spring, sediment (December,2018)	8.00
MPW3E	Midpoint of Warm Spring, sediment (December,2018)	10.00
C14	Confluence of both springs, sediment (December,2018)	9.50

C2A	Confluence of both springs, sediment (December,2018)	9.00
C4A	Confluence of both springs, sediment (December,2018)	6.00
C20	Confluence of both springs, sediment (December,2018)	6.00
MPC20	Midpoint of Cold Spring, sediment (December,2018)	5.50
MPC16	Midpoint of Cold Spring, sediment. (December,2018)	5.00
SW6W	Source of Warm Spring, Water (June, 2018).	4.00
SW8W	Source of Warm Spring, Water (June, 2018).	6.00
MPW16W	Midpoint of Warm Spring, water. (June, 2018)	5.00
C11W	Confluence, Water (June,2018)	4.00
MPC24W	Midpoint of Cold Spring, water (June, 2018)	4.50
SC12W	Source of Cold Spring, water (June, 2018)	4.50
SW3B	Source of Warm Spring, water (December, 2018)	8.50
MPW7	Midpoint of Warm Spring, water (December,2018)	8.50
MPW27	Midpoint of Warm Spring, water (December,2018)	9.00
C14	Confluence of both springs, Water (December,2018)	8.00
C2A	Confluence of both springs, Water (December,2018)	7.00
C25	Confluence of both springs, Water (December,2018)	6.50
C4A	Confluence of both springs, Water (December,2018)	6.00
C20	Confluence of both springs, Water (December,2018)	4.00
MPC21	Midpoint of Cold Spring, Water (December 2018)	6.00
MPC16	Midpoint of Cold Spring, Water (December 2018)	6.50



(A)



(B)

Figure 4.1: Molecular Phylogenetic analysis of (A) *Bacillus siamensis* SW3F and (B) *Bacillus cereus* MPW3E using Maximum Likelihood method computed in MEGA 7 (Kumar *et al.*, 2016)

4.6 Alpha-amylase production from selected thermophilic bacteria, isolated from Ikogosi Warm Springs sediments

The best culture conditions for the production of alpha-amylase by *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F are shown in Figures 4.2 – 4.6.

The effect of incubation temperature was illustrated in Figure 4.2. Analysis revealed the best optimisation temperature for best production of thermostable alpha-amylase was at 50°C for both bacteria. The *Bacillus cereus* MPW3E production medium showed an enzyme activity of 14.690 U/mL, while the medium containing *Bacillus siamensis* SW3F recorded a value of 15.220 U/mL. Findings showed that production media incubated above 50°C adversely depleted the production of amylase, with the least value (2.100 U/mL) for *Bacillus cereus* MPW3E and 1.539 U/mL for *Bacillus siamensis* SW3F recorded at 70°C.

Figure 4.3 demonstrated that the production media whose pH was 7 showed the highest production of alpha amylase from both *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F with values of 11.206 U/mL and 23.942 U/mL respectively. The production media which demonstrated the least support for production of alpha amylase using both bacteria, were production media which had been prepared in buffers of pH 5, they showed values of 5.026 U/mL and 5.403 U/mL for *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F respectively.

In Figure 4.4, the effect of various carbon sources on the production of thermostable alpha amylase is shown. Cassava peel-supplemented medium, best supported the production of thermostable alpha amylase for both *Bacillus cereus* MPW3E (21.223 U/mL) and *Bacillus siamensis* SW3F (22.105 U/mL), followed by yam-peel supplemented media, which also supported thermostable alpha amylase production, recording 18.574U/mL and 20.340 U/mL for *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F respectively. However, sucrose supplemented media showed the least support for the production of the amylolytic enzyme, with an activity of 2.507 U/mL for *Bacillus cereus* MPW3E and 3.743 U/mL for *Bacillus siamensis* SW3F.

In Figure 4.5, the effect of several nitrogen sources in the production medium is shown. Investigations revealed that the production medium supplemented with tryptone was the

most favourable (24.048 U/mL) for the production of the enzyme using *Bacillus siamensis* SW3F as an inoculum, while the inclusion of yeast extract to the production medium containing *Bacillus cereus* MPW3E as inoculum, showed the highest (19.281 U/mL) production of thermophilic amylolytic enzyme. The production medium which showed the least support of the production of the enzyme of interest was the medium supplemented with KNO_3 with a value of 4.626U/mL, for *Bacillus siamensis* SW3F media, as well as $NH_4(SO_4)_2$ which showed poor production of amylolytic enzyme with a record value of 1.448 U/mL for the production media containing *Bacillus cereus* MPW3E as inoculum.

Further studies were conducted to investigate the optimum concentration (for maximum thermostable alpha amylase production) from the best carbon sources for both selected bacteria. Figure 4.6. showed that for *Bacillus cereus* MPW3E, the production medium supplemented with 2.0% cassava peel best supported production of amylase, (23.631U/mL), while for *Bacillus siamensis* SW3F, the production medium supplemented with 1.0% of cassava peel best supported the production of alpha amylase (25.461U/mL). Further increase in the concentration of carbon sources resulted in the decrease of thermostable alpha amylase from both selected Bacilli. A concentration of 3.0% showed the least value for alpha amylase production, with values of 6.498U/mL and 7.098U/mL for *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F respectively.

Figure 4.7 showed the varying concentration of the best nitrogen sources for both Bacilli used in this study. It showed that when the concentration of yeast extract (for *Bacillus cereus* MPW3E) and tryptone (for *Bacillus siamensis* SW3F) was at 1.50%, the production of thermostable alpha amylase was optimum, with values of 21.688U/mL and 26.992 U/mL respectively. However, as the concentration of the nitrogen source was increased in both production media, there was a significant decrease in the production of alpha amylase using *Bacillus cereus* MPW3E, with the least enzyme production recorded at 3.0% (9.746 U/mL), whereas, for *Bacillus siamensis* SW3F had the least amylase production at 0.5% of tryptone (8.687 U/mL).

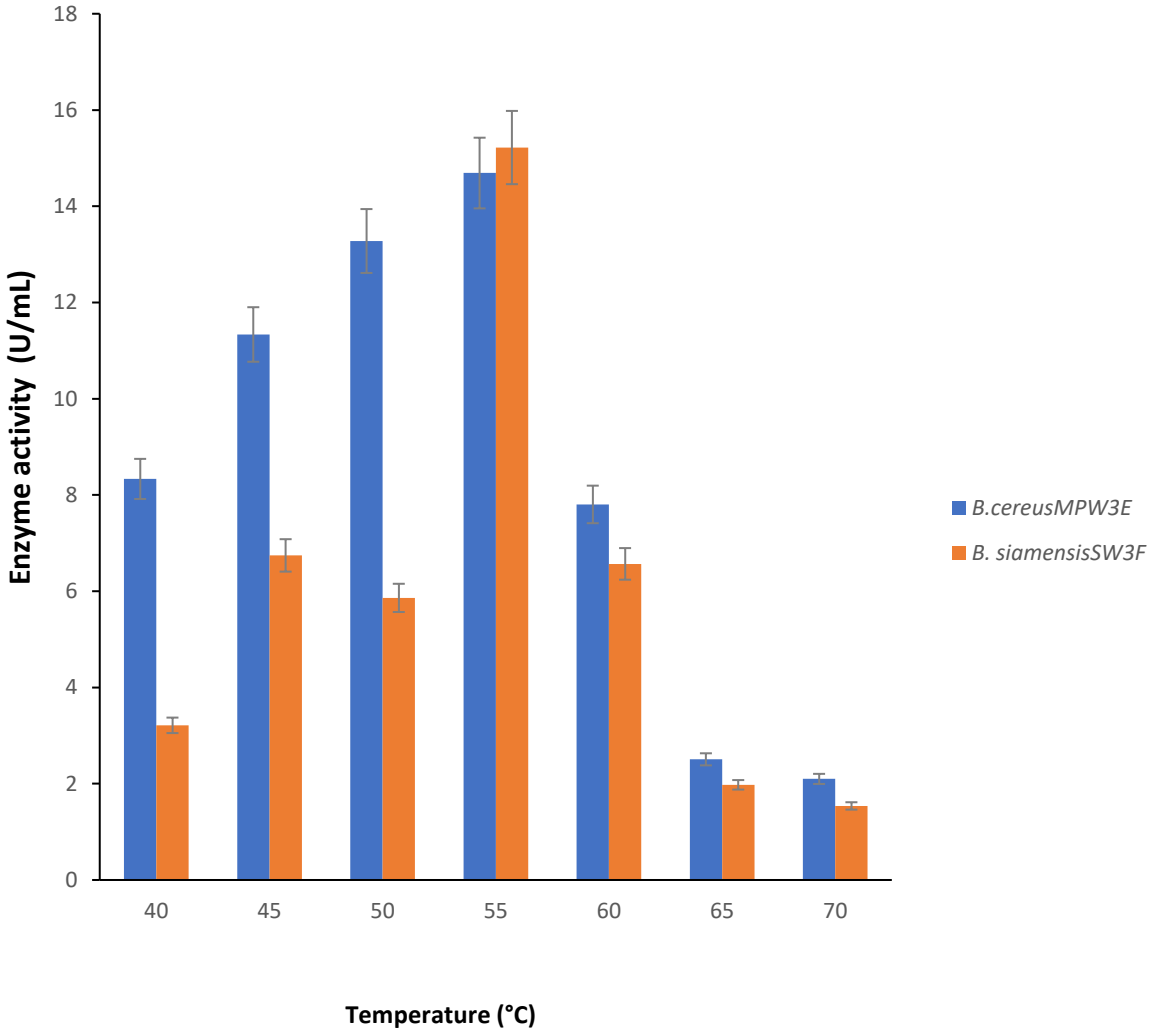


Figure 4.2: The effect of varying temperature on the production of thermostable alpha amylase using *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F

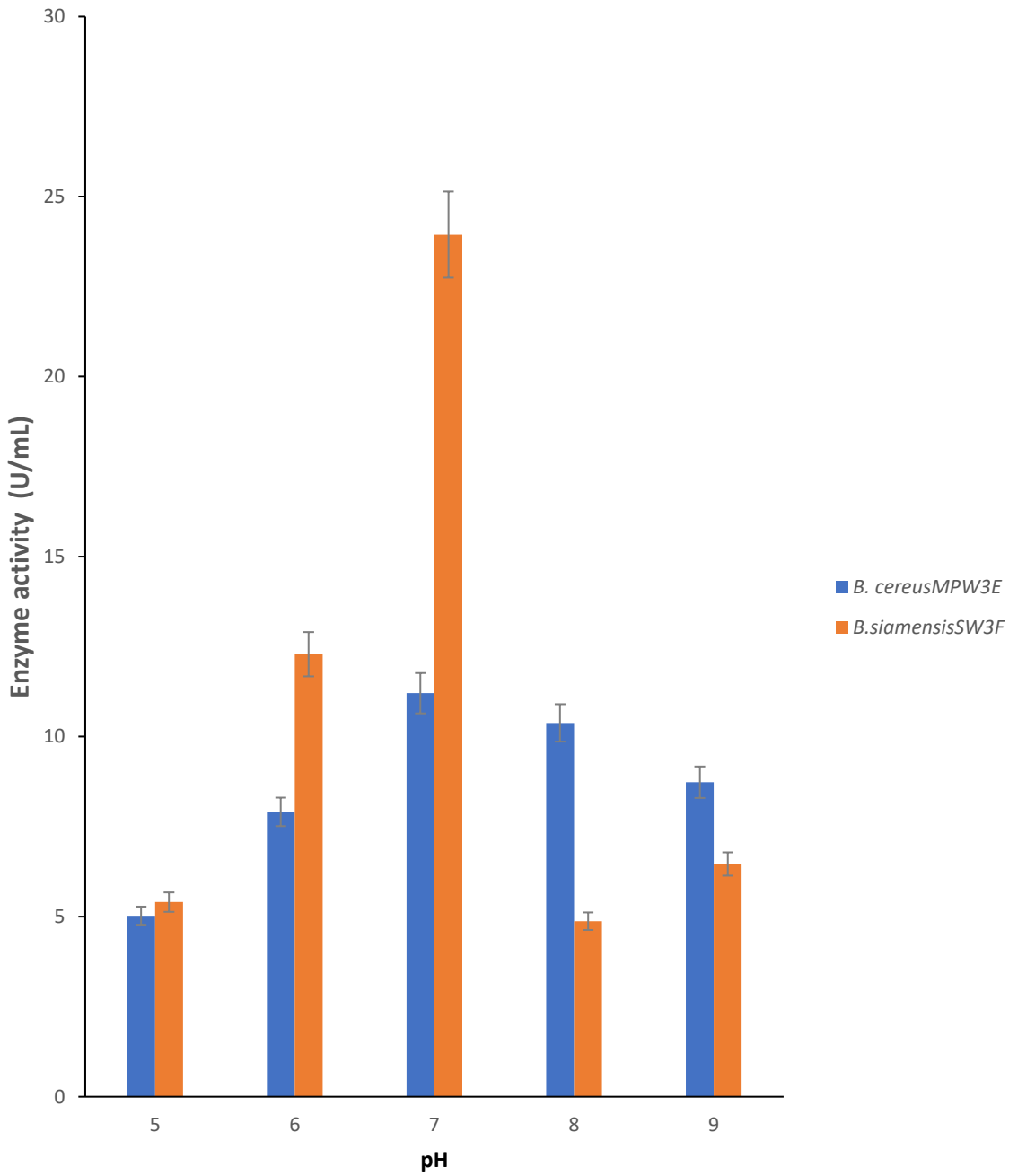


Figure 4.3: The effect of varying pH on the production of thermostable alpha amylase using *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F

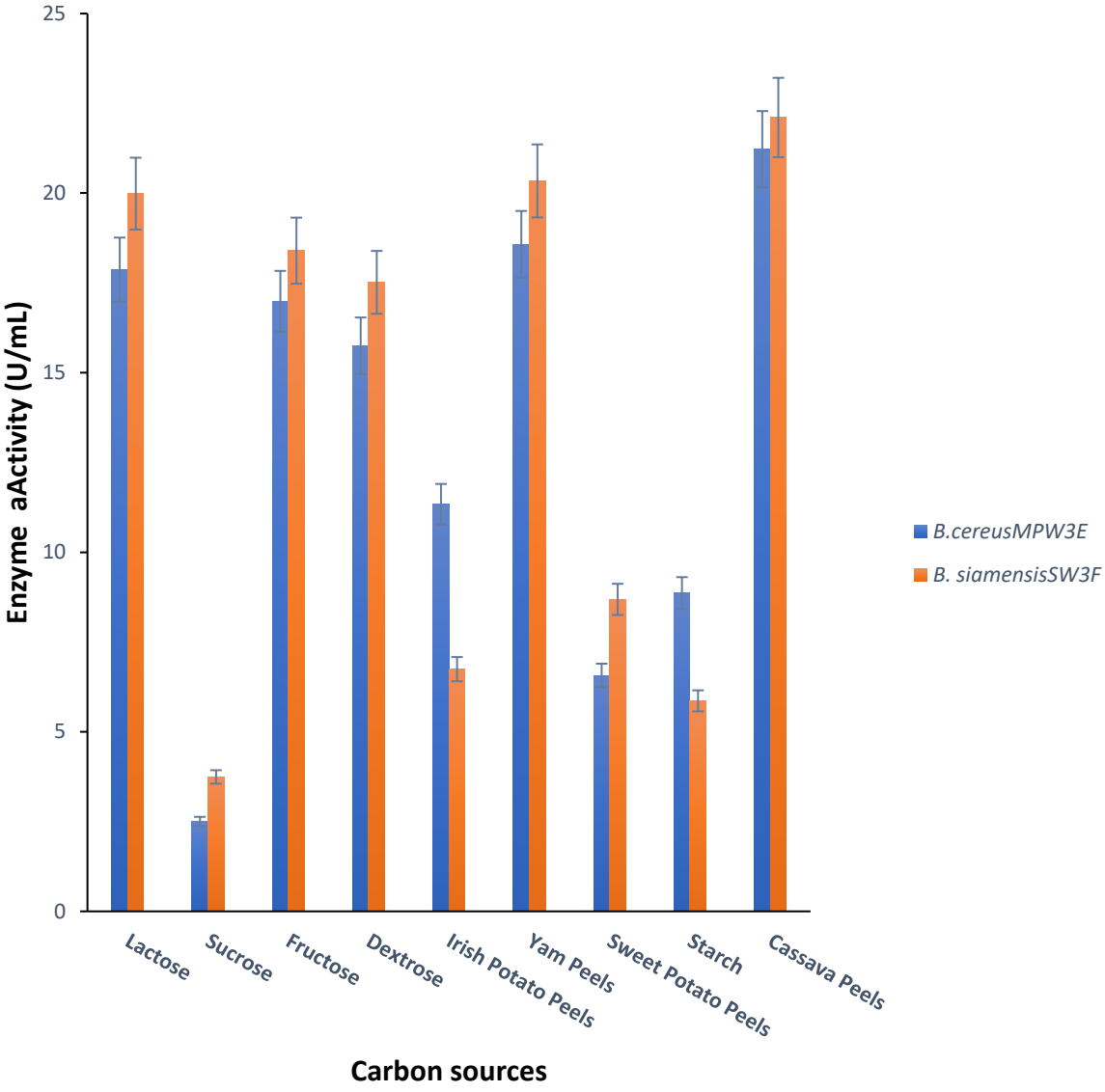


Figure 4.4: The effect of various carbon sources on the production of thermostable alpha amylase using *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F

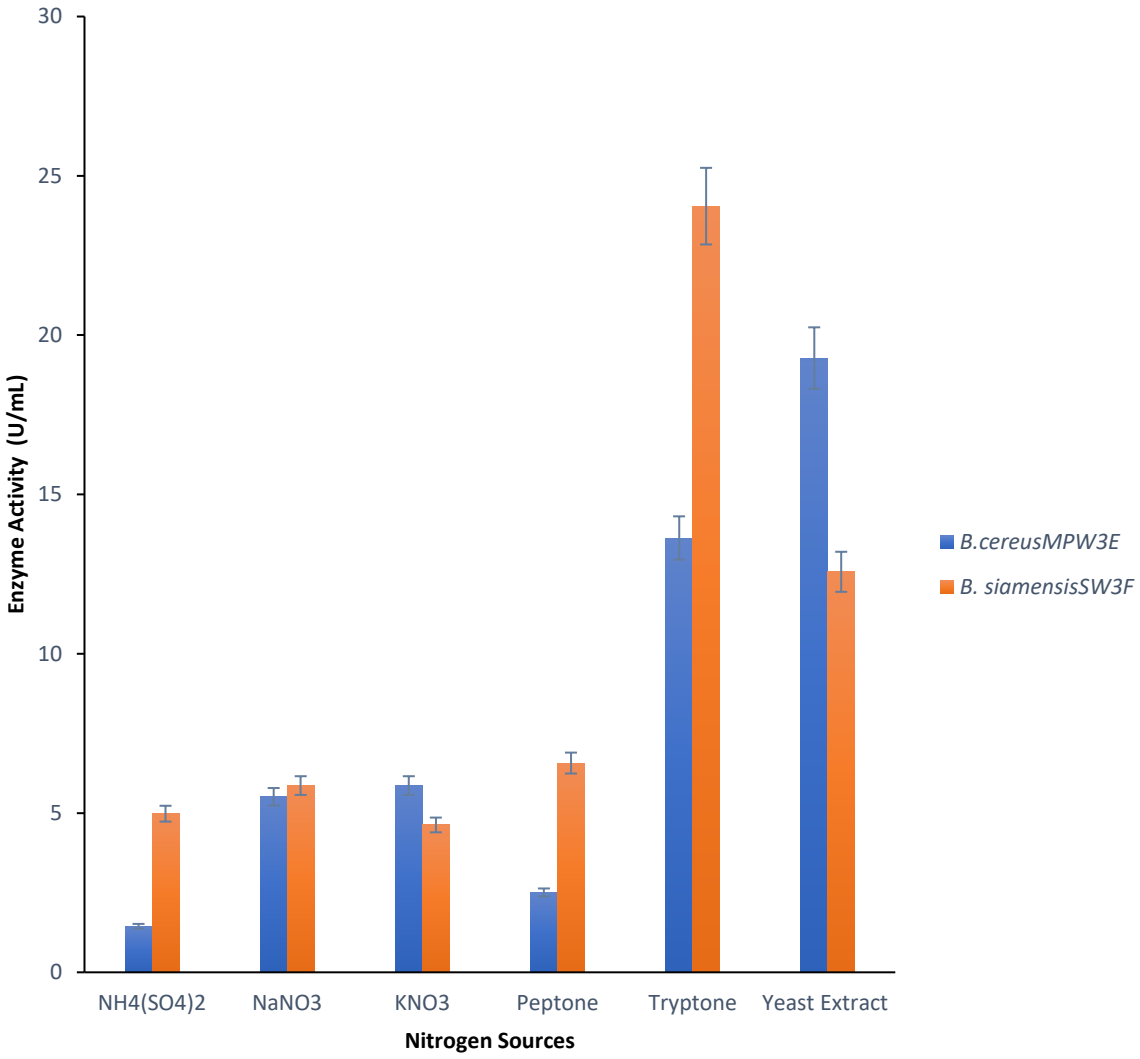


Figure 4.5. Effect of various nitrogenous sources on the production of thermostable amylolytic enzyme using *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F

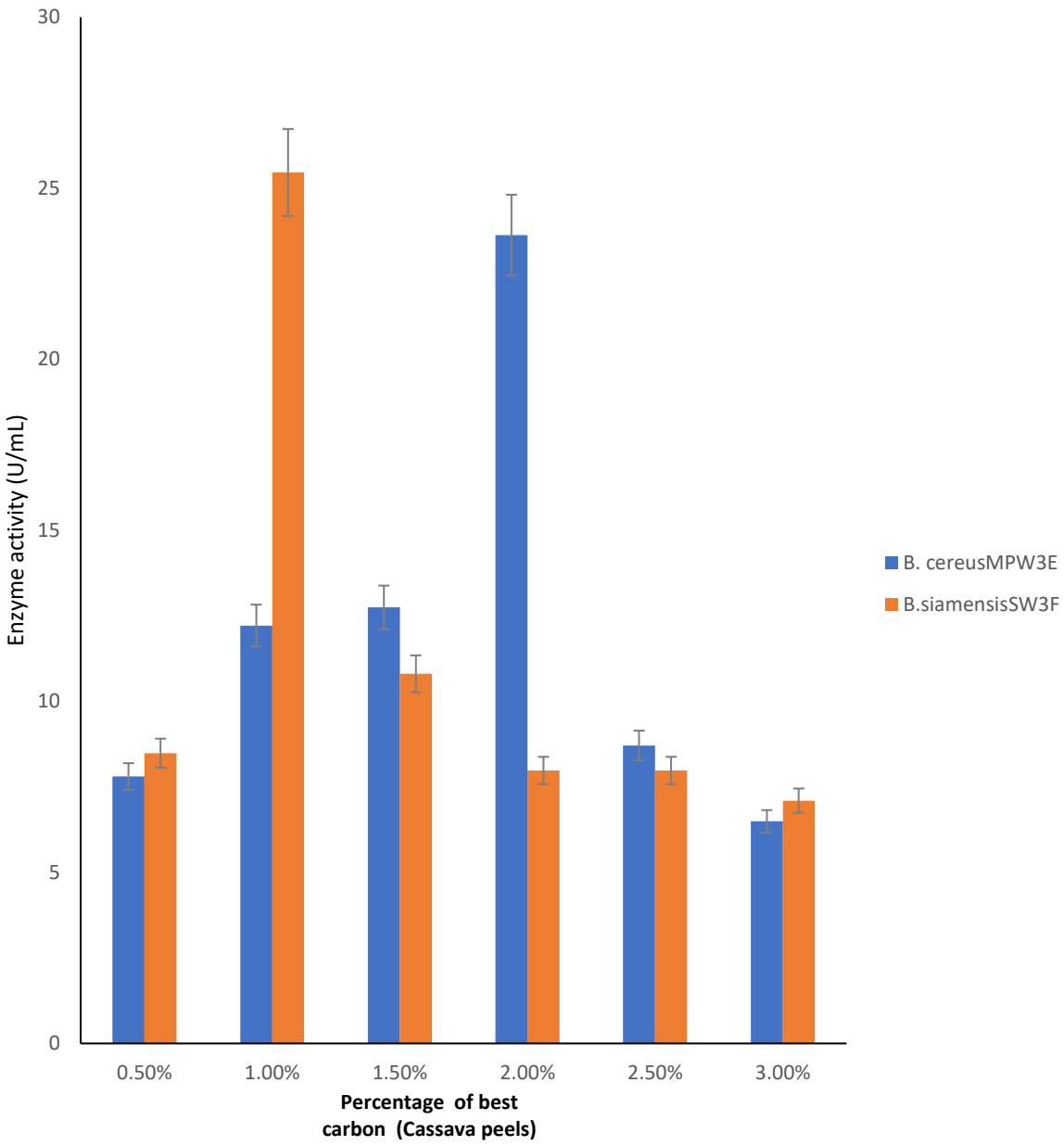


Figure 4.6: The effect of varying concentration of the best carbon source on the production of thermostable alpha amylase using *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F

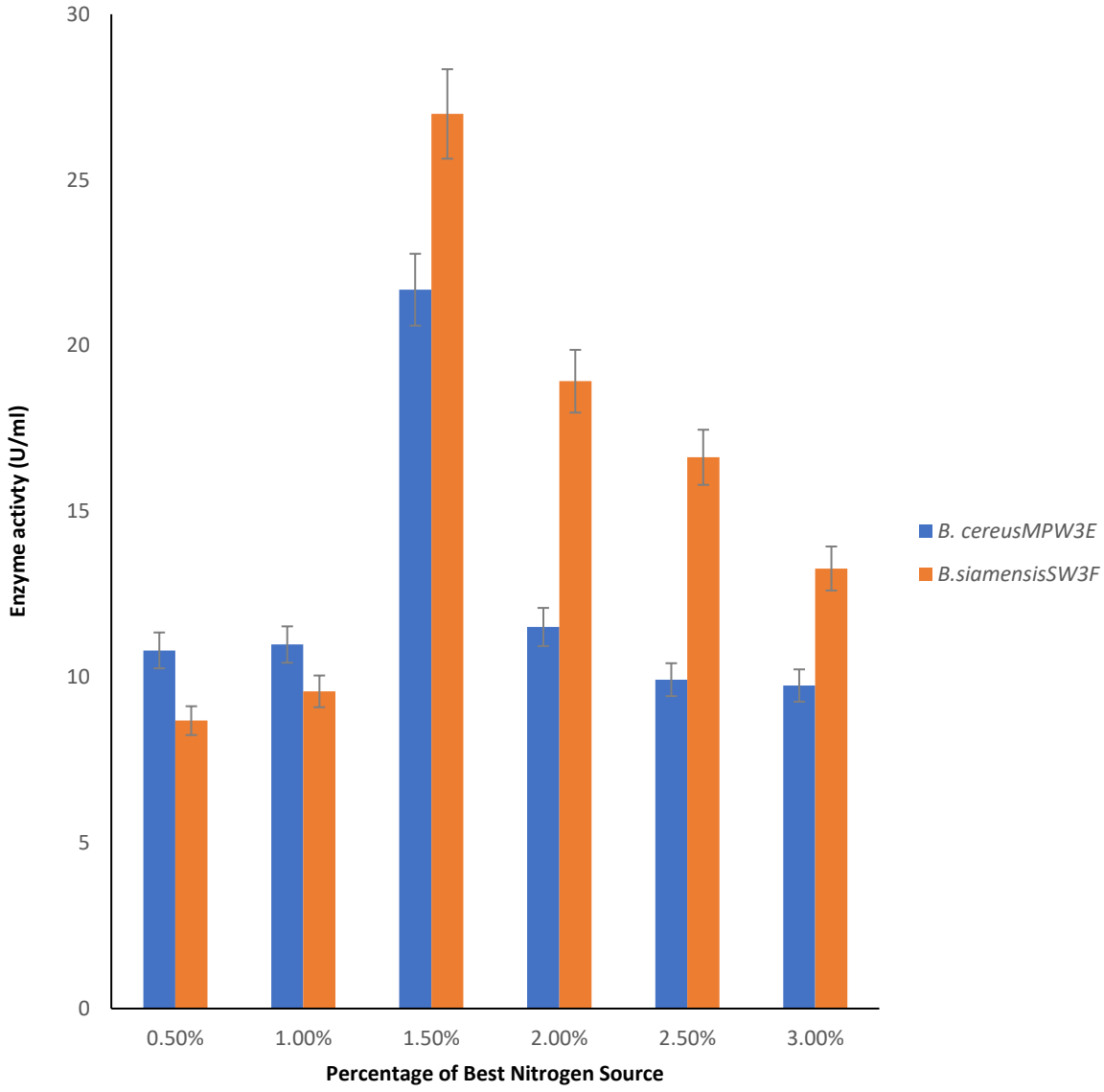


Figure 4.7 Effect of various percentage of best nitrogenous sources on the production of thermostable amylolytic enzyme using *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F

The effect of various metal ions (0.01%) on the production of thermostable alpha amylase from *Bacillus cereus*MPW3E and *Bacillus siamensis* SW3F was shown in Figure 4.8. Production medium of both bacteria, containing divalent metal ions of Nickel, Copper and Manganese suppressed the production of amylase, as well as the trivalent ferrous ion. But the inclusion of Calcium and Magnesium ions in both production media, improved the production of alpha amylase (Calcium and Magnesium for *Bacillus cereus*MPW3E and for *Bacillus siamensis* SW3F respectively.

Figure 4.9 showed the effect of varying inoculum size for each production media, using *Bacillus cereus*MPW3E and *Bacillus siamensis* SW3F in each medium. The highest production of thermostable alpha amylase was produced when 1% inoculum size was maintained in the medium of production for both bacteria. Using *Bacillus cereus*MPW3E, 1% inoculum size, yielded 30.81U/mL while *Bacillus siamensis* SW3F yielded 32.28 U/mL.

In Figure 4.10, the effect of several incubation time (hours) was depicted to optimise for the production of thermostable alpha amylase using *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F. A 48-hour incubation time best supported the production of alpha amylase from both Bacilli. However, *Bacillus siamensis* SW3F produced more alpha amylase (42.488 U/mL) than its counterpart *Bacillus cereus* MPW3E (37.184 U/mL). Production of alpha-amylase began to depreciate as the incubation hour increased as observed in the Figure.

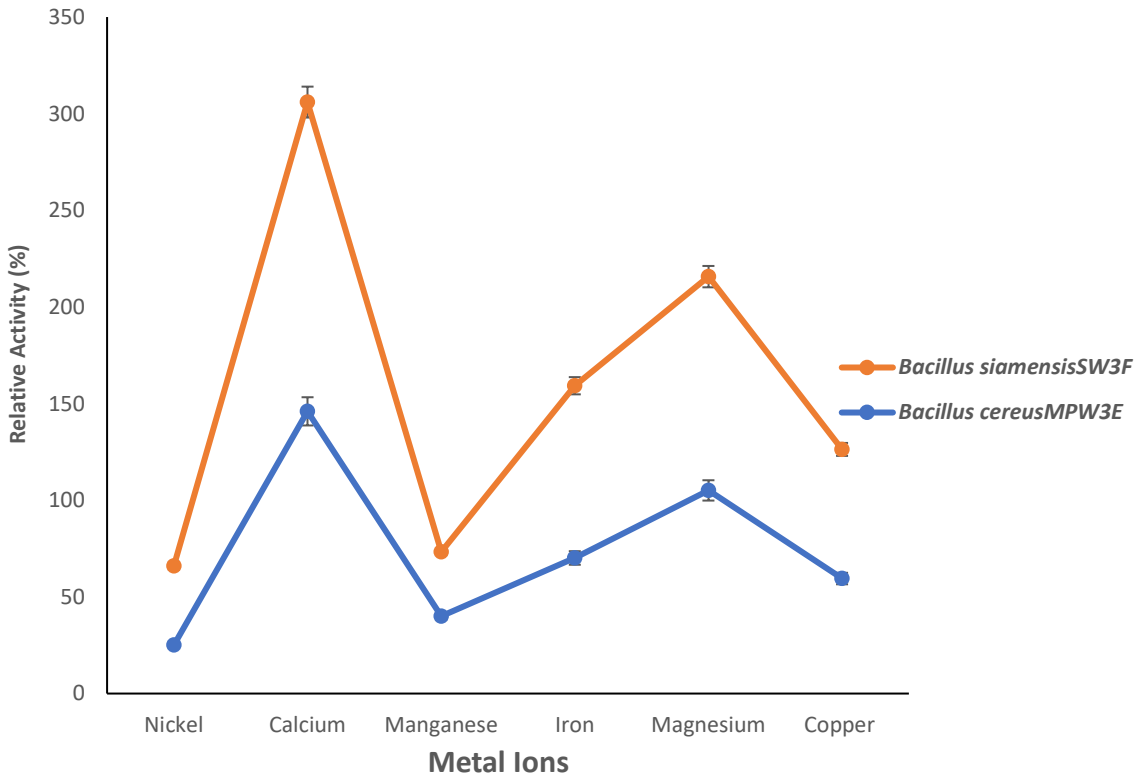


Figure 4.8: Effect of various inorganic salts (0.01%) on the production of thermostable amylolytic enzyme using *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F

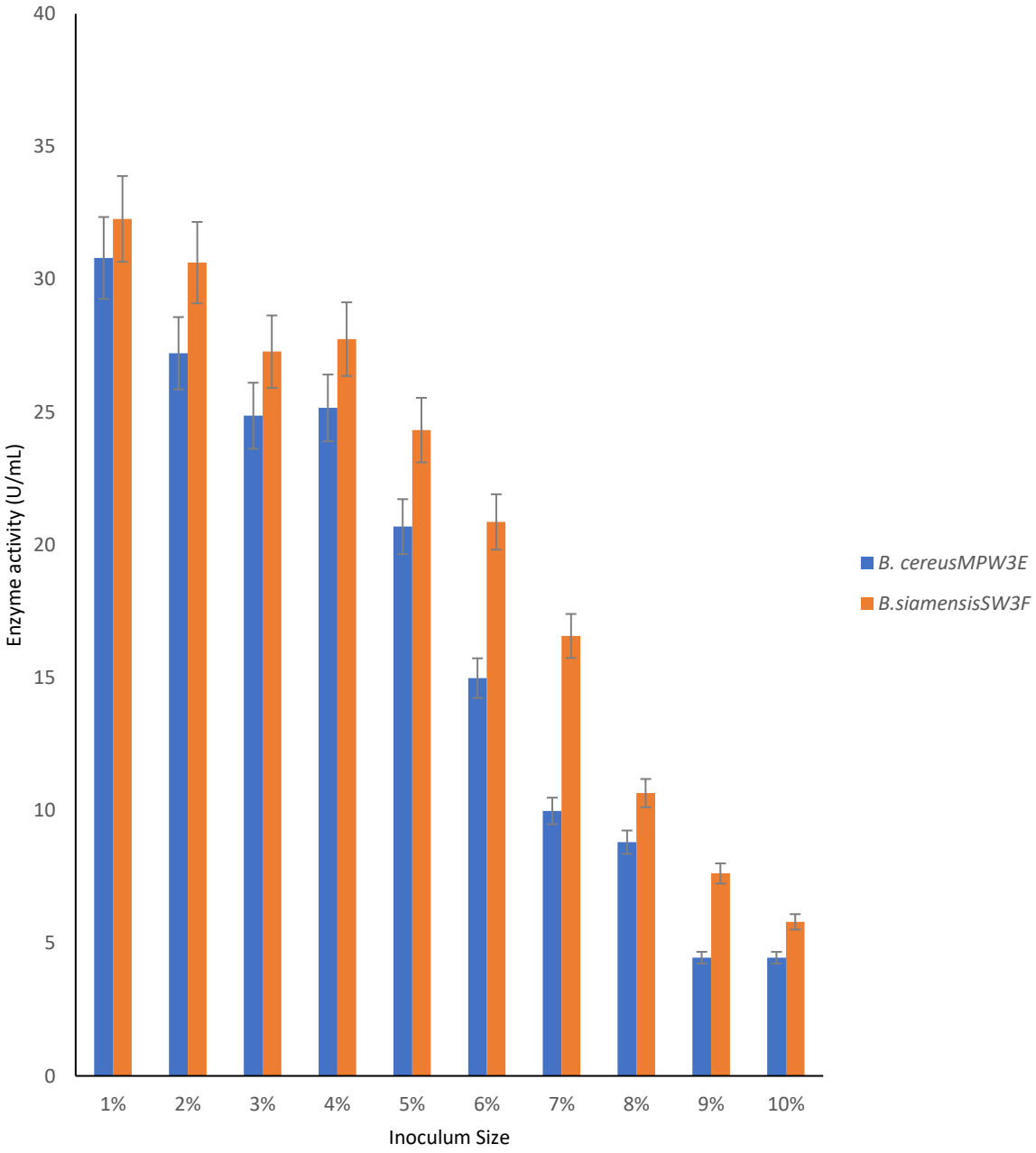


Figure 4.9 The effect of varying inoculum sizes on thermostable alpha amylase production using *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F

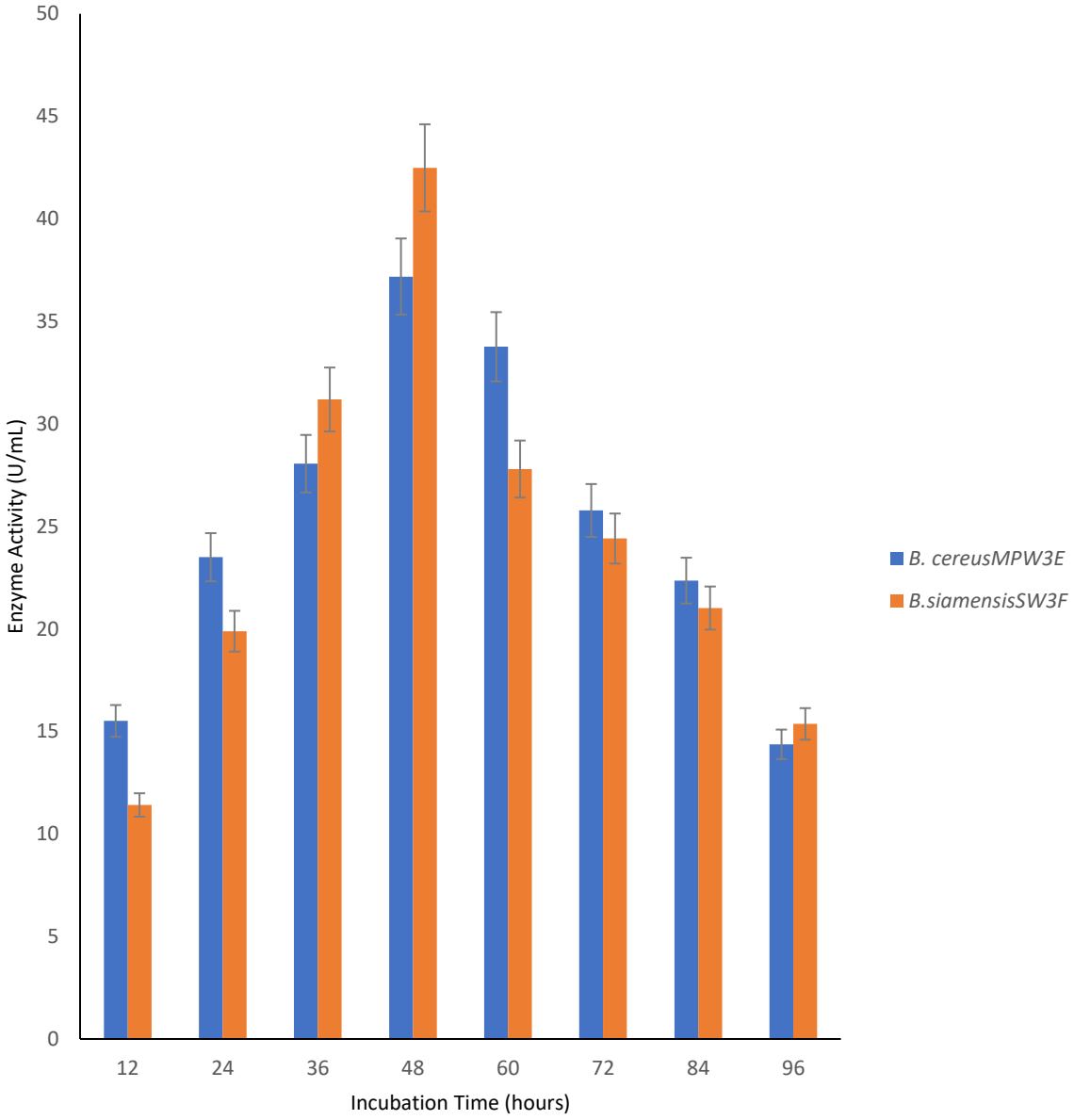


Figure 4.10: The effect of varying incubation time on thermostable alpha amylase production using *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F

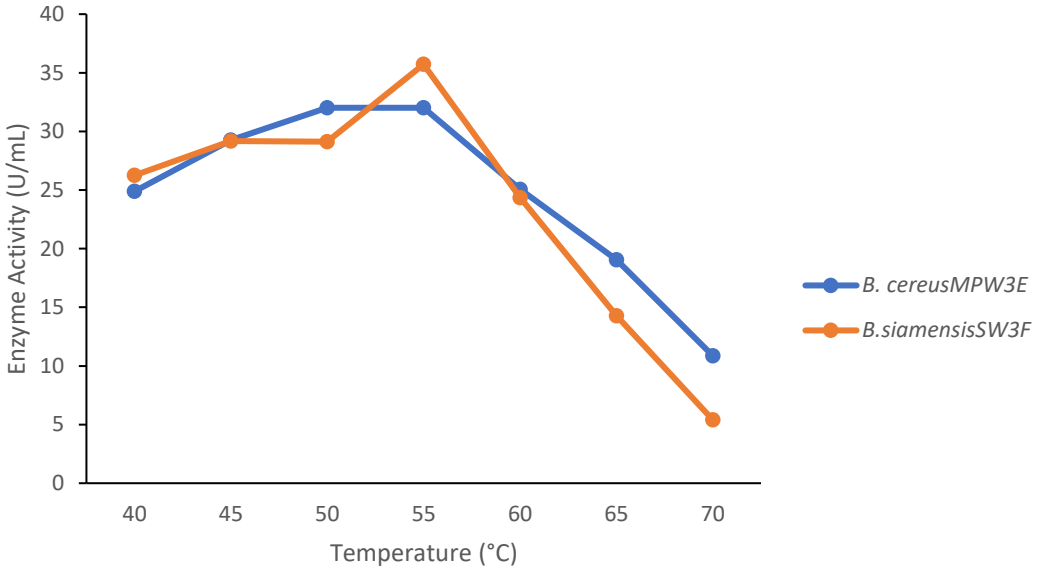
4.7 Characteristics of alpha-amylase produced from selected thermophilic bacteria, isolated from Ikogosi Warm Springs sediments

The effect of varying temperature on the crude enzyme's activity from both bacteria is shown in Figure 4.11A. It revealed that 55°C supported the best activity of amylase produced from *Bacillus siamensis* SW3F (35.718 U/mL), while both 50 and 55°C exerted no significant difference (32.018 U/mL) on the alpha amylase activity of *Bacillus cereus* MPW3E. Beyond 55°C, there was a sharp reduction in alpha amylase activity in both organisms.

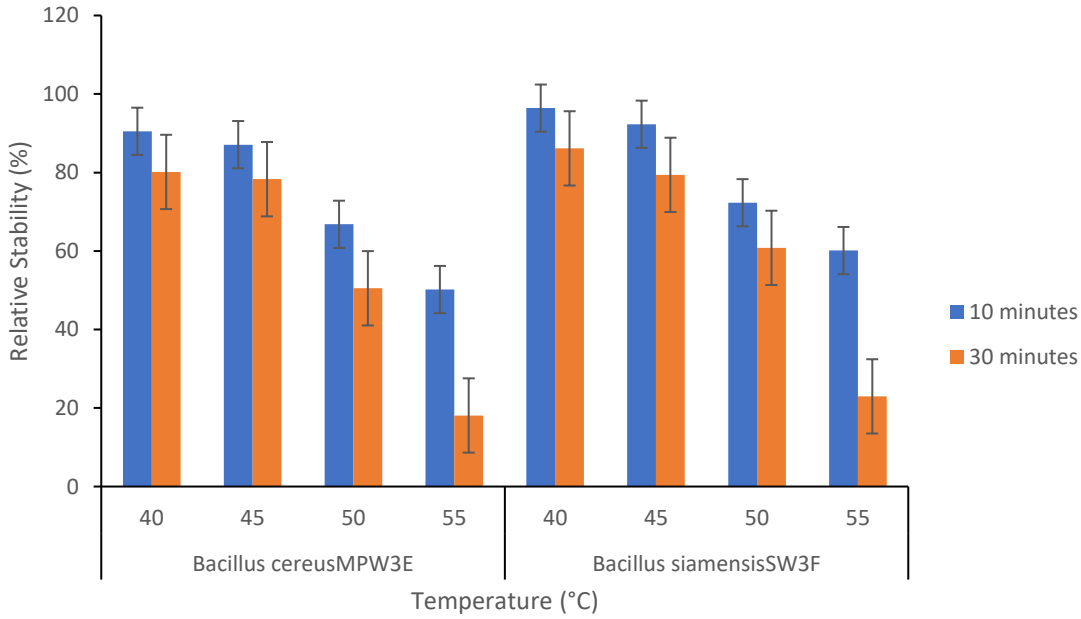
Furthermore, the thermal stability of the alpha amylase of both bacteria reduced at 30 minutes of incubation as shown in Figure 4.11B. After 10 minutes of exposure of both amylase from *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F to temperature regimes between 40 and 55°C, the enzyme activity reduced with a relative stability recorded at 90.50 and 96.40% respectively. By 55°C after 30 minutes of exposure, only 50.19 and 22.97% of relative stability was recorded for both bacteria respectively.

Figure 4.12 depicts the effect of pH on α-amylase activity of *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F. The pH 6.0 was optimal pH for optimal enzyme activity (23.748 U/mL) from *Bacillus cereus* MPW3E, while pH 7.0 supported maximum enzyme activity (26.008 U/mL) from *Bacillus siamensis* SW3F and was seen as a peak on the line graph. The lowest pH activity was recorded at pH 9 for enzymes from both bacteria with enzyme activity values of 9.285 U/mL and 12.710 U/mL for *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F respectively.

The effect of various starch concentration on the activity of alpha amylase from *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F were determined and shown in Figure 4.13. The activity of crude enzymes from both test isolates gradually increased from 0.25% to peak at 1.00%. The optimum enzyme activity was recorded at the substrate concentration of 1.0%, depicting that reaction mixture containing 1.0% of starch supported the maximum activity of enzymes produced from both isolates. The highest value of the enzyme activity for *Bacillus cereus* MPW3E was 28.602 U/mL, while enzyme activity from *Bacillus siamensis* SW3F was 30.510 U/mL. Thereafter, the activity of crude enzymes showed a steady decline as the substrate concentration increased.



(A)



(B)

Figure 4.11: The effect of varying temperature on (A) the activity and (B) stability of crude enzyme produced from *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F

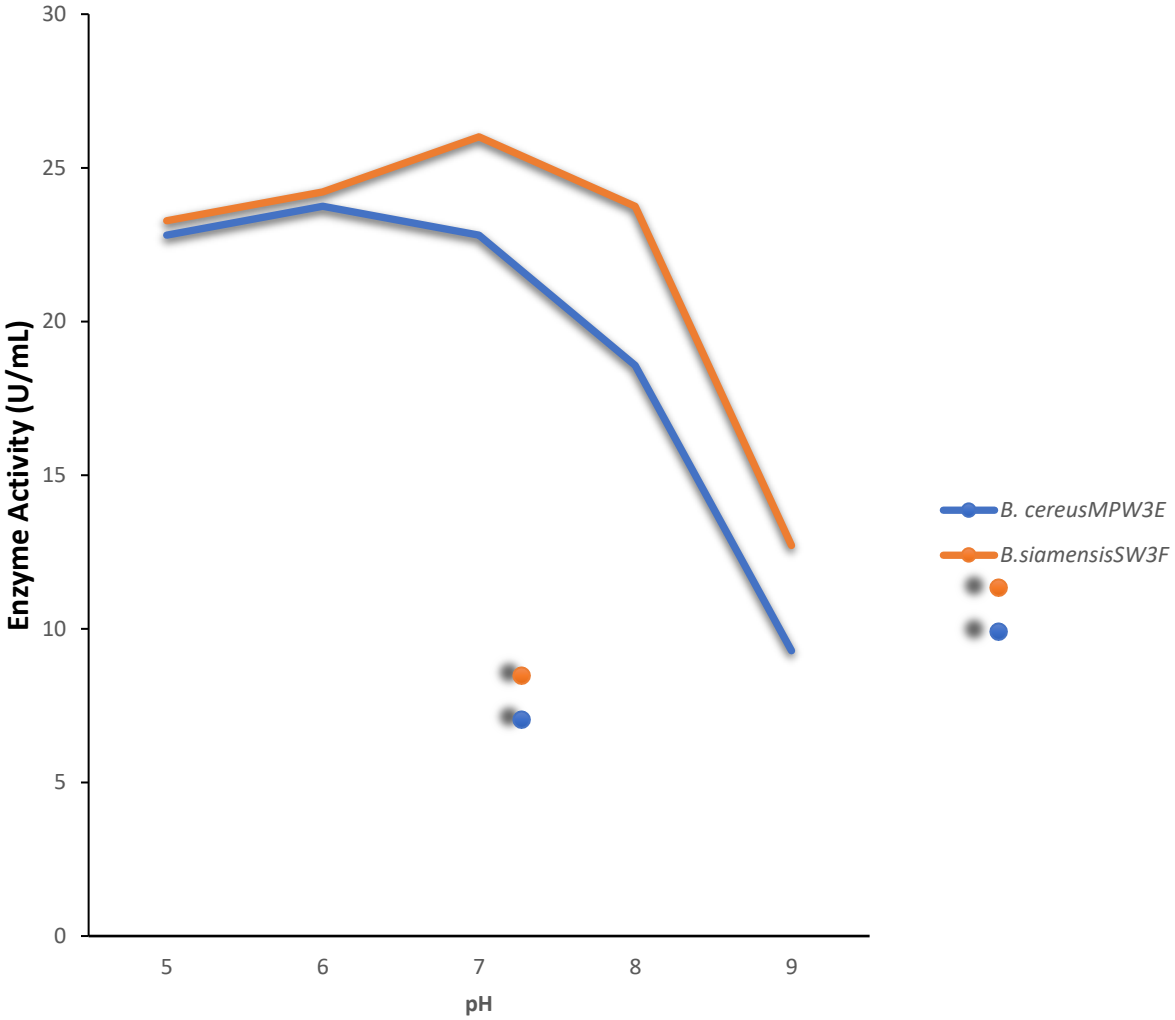


Figure 4.12: The effect of pH on the activity of crude alpha amylolytic enzyme produced from *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F

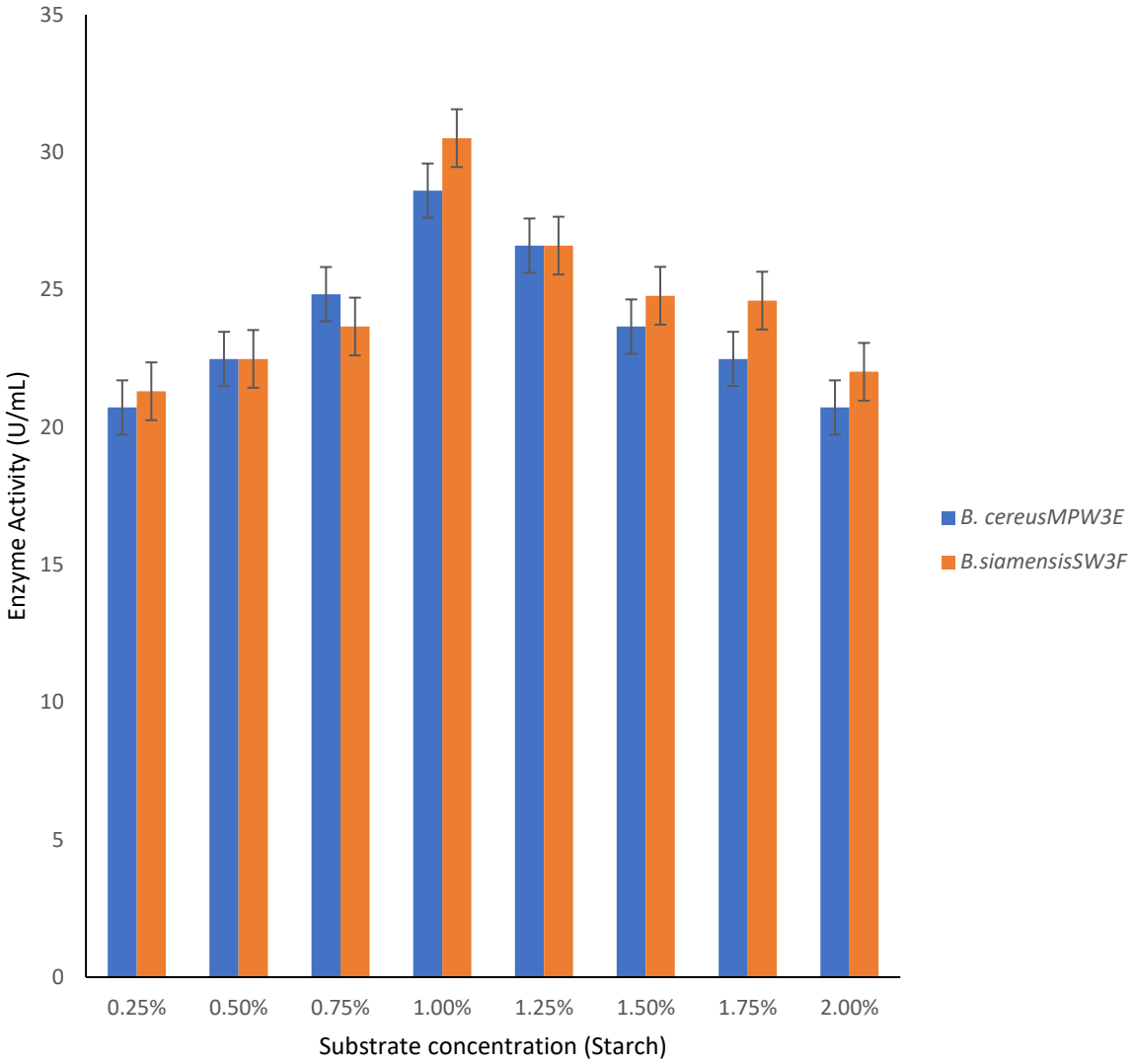
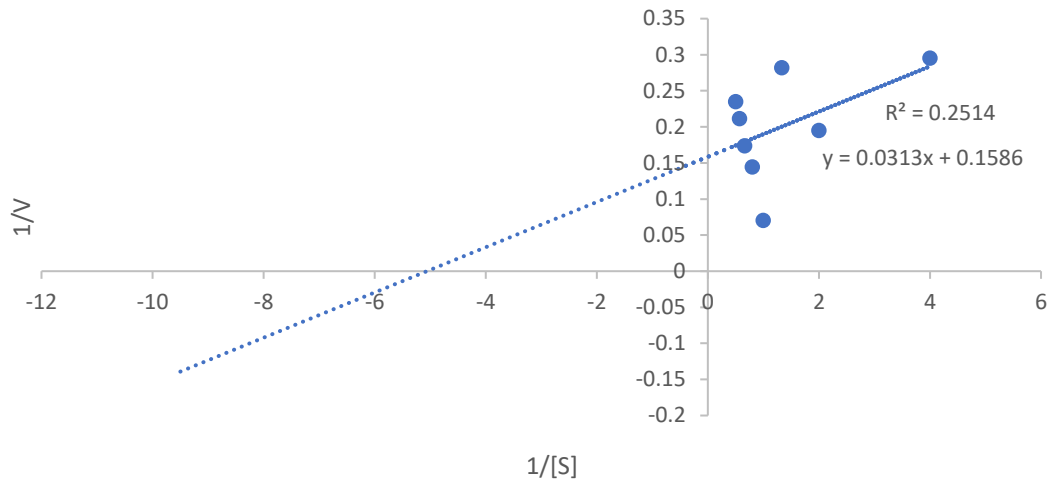


Figure 4.13: Effect of varying starch concentration on the activity of crude alpha amylolytic enzyme produced from *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F

The Lineweaver-Burke double reciprocal plot of the kinetic parameters, Km (Michaelis-Menten constant) and Vmax of crude extracellular amylase of *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F, using starch as substrate in concentration ranging from 0.25 – 2.0% is shown in Figure 4.14A. The Vmax was 6.3052 µmol/min for *Bacillus cereus* MPW3E and its Km was 0.1973mM, while that for *Bacillus siamensis* SW3F is shown in Figure 4.14B. The Km was 0.0519mM and its Vmax was 25.9740 µmol/min.

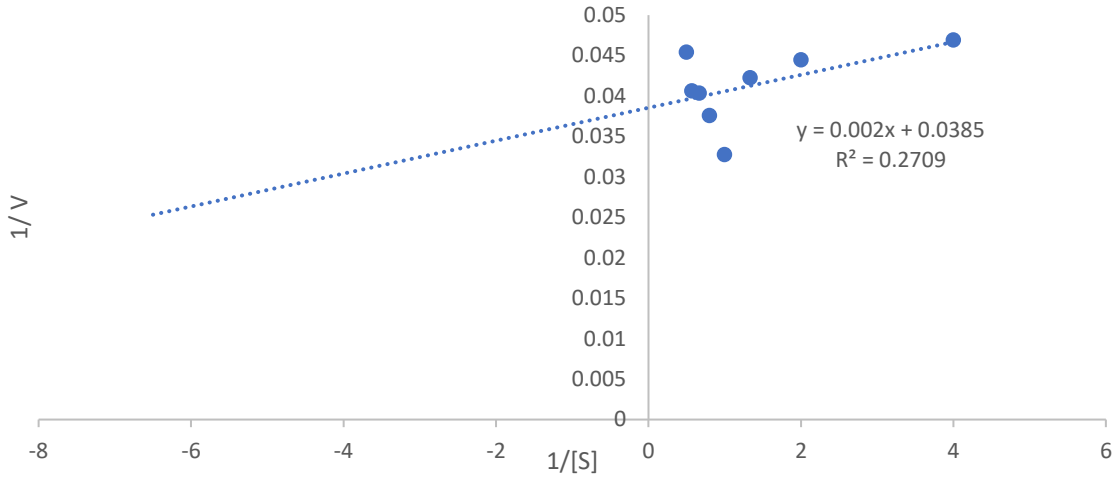
Table 4.7 shows the effect of selected metal ions (Ca^{2+}, Fe^{3+}, Mg^{2+}, Cu^{2+}, Mn^{2+} and Ni^{2+}) at 1mM and 5mM on the activity of crude alpha amylase from *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F. High percentage relative activity was recorded in the presence of Ca^{2+} and Mg^{2+} at 1mM for both bacteria. Values of 120% and 108% were recorded from the crude enzyme of *Bacillus cereus*MPW3E from Ca^{2} and Mg^{2+} respectively, likewise values of 150% and 110% were observed from the crude enzyme of *Bacillus siamensis* SW3F from Ca^{2} and Mg^{2+} respectively. The metal ion which showed the least support for the activity of the enzyme was Ni^{2+} for both isolates at 1mM and 5mM concentration.

The percentage relative activity of inhibitors at 0.1% and 1% on alpha amylase activity is shown in Table 4.8. All inhibitors used in this study (Sodium Dodecyl Sulphate (SDS), Ethylene diamine tetra acetic acid (EDTA), Urea and Tween-20) showed inhibitory reaction to both crude enzymes tested. The strongest inhibition reaction on the activity of alpha amylase from both *Bacillus* species was observed in the presence of SDS at both tested concentrations, with *Bacillus cereus* MPW3E retaining 35.00 % and 23.07% (at 0.1% and 1.0%) of its activity. While *Bacillus siamensis* SW3F retained only 25.06% and 11.00% of its activity in the presence of 0.1% and 1.0% of SDS.



Vmax = 6.3052 µmol/min, Km = 0.1973mM

(A)



Vmax = 25.9740 µmol/min , Km = 0.0519mM

(B)

Figure 4.14: Lineweaver-Burke double reciprocal plots for the determination of Km and Vmax value of crude extracellular alpha amylase from (A) *Bacillus cereus* MPW3E and (B) *Bacillus siamensis* SW3F

Table 4.7: Percentage relative activity of metal ions (1mM and 5mM) on the activity of alpha-amylase from *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F

	Relative activity (%)			
	*Bacillus cereus*MPW3E		*Bacillus siamensis* SW3F	
	1mM	5mM	1mM	5mM
Control	100	100	100	100
Metal ions				
Ni^{2+}	20.00	5.00	13.00	09.09
Ca^{2+}	120.01	89.12	150.1	77.00
Mn^{2+}	40.09	34.11	50.56	33.16
Fe^{3+}	40.31	30.45	56.03	48.11
Mg^{2+}	108.11	90.81	110.00	89.34
Cu^{2+}	81.23	10.00	92.23	43.05

Table 4.8: Percentage relative activity of different inhibitors on alpha-amylase from *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F

	Relative activity (%)			
	Bacillus cereus MPW3E		*Bacillus siamensis* SW3F	
	0.1%	1%	0.1%	1%
Control	100	100	100	100
Inhibitors				
SDS	35.00	23.07	25.06	11.00
EDTA	77.71	34.34	81.12	70.01
Urea	58.21	40.00	61.45	08.07
Tween 20	50.02	44.12	55.78	32.55

4.8 Alpha-amylase purification from *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F

Partial purification of alpha-amylase from *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F isolated from the sediment of Ikogosi warm spring is shown in Table 4.9. Using ammonium sulphate precipitation, the specific activity of alpha-amylase of *Bacillus cereus* MPW3E was 0.211 U/mg in comparison to the crude enzyme. The dialysed precipitated alpha-amylase resulted in an increase in the specific activity (0.405 U/mg) of the partly purified amylase with a purification fold of 1.361. Column chromatographic purification of the enzyme resulted in a further increase in the specific activity (0.792 U/mg) and purification fold (5.111) of the enzyme. The percentage yield of the amylase following the three-step purification procedure produced 51.366% yield.

The level of purification for crude alpha-amylase of *Bacillus siamensis* SW3F showed that the initial precipitation of crude alpha- amylase using ammonium sulphate resulted in a specific activity of 0.298 U/mg and a purification fold of 1.743, while increased specific activity of 0.416 U/mg and purification fold of 2.430 was recorded following dialysis of the precipitated enzyme. A purification fold of 4.701 and 63.028% yield was recorded following sephadexG100 purification step.

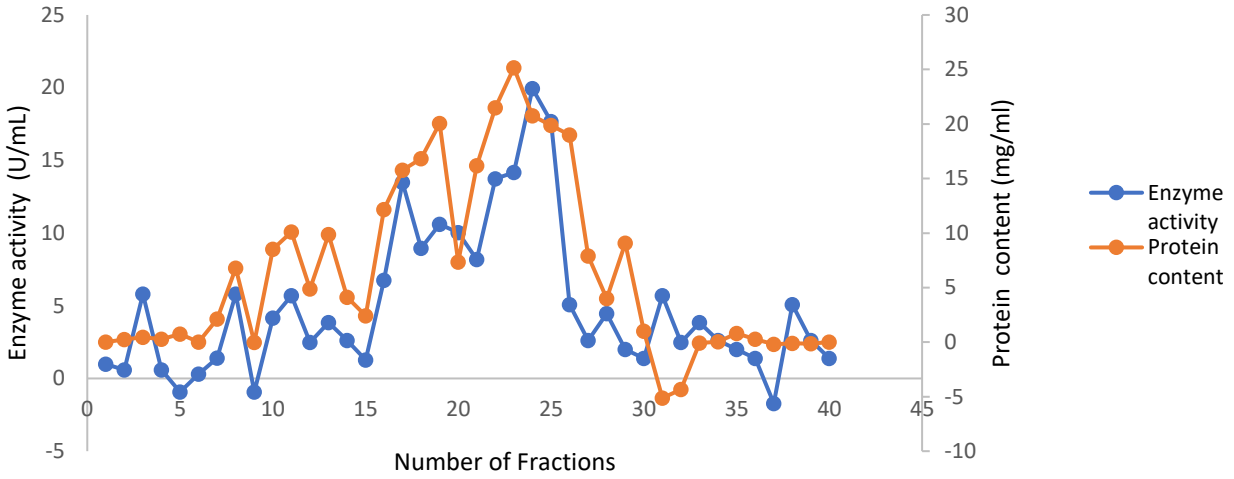
4.9 Column chromatographic purification of extracellular alpha-amylase from *Bacillus* cereus MPW3E and *Bacillus siamensis* SW3F

Various peaks of enzyme activity and corresponding protein content were detected from 40 fractions collected following column chromatography purification step. The different amylolytic activity peaks and the protein contents produced by *Bacillus cereus* MPW3E are shown in Figure 4.15A. Peaks of enzyme activity and protein content were found in fractions 17 and 23 - 25. However, highest peaks of amylase and protein were detected in fractions 23 - 25, with enzyme activity of 14.176 U/mL, 19.935 U/mL, and 17.665 U/mL, respectively and their respective protein content were 20.746 mg/mL, 25.160 mg/mL, and 19.889 mg/mL.Figure 4.15B depicts enzyme activity and protein content detected from column purified alpha amylolytic enzyme produced from *Bacillus siamensis* SW3F. From 40 fractions collected, peaks of enzyme activity and protein content were detected in fractions 10 and 17 - 19. Although the highest enzyme activity and protein content were recorded from fractions 17 – 19. Enzyme activity for each were

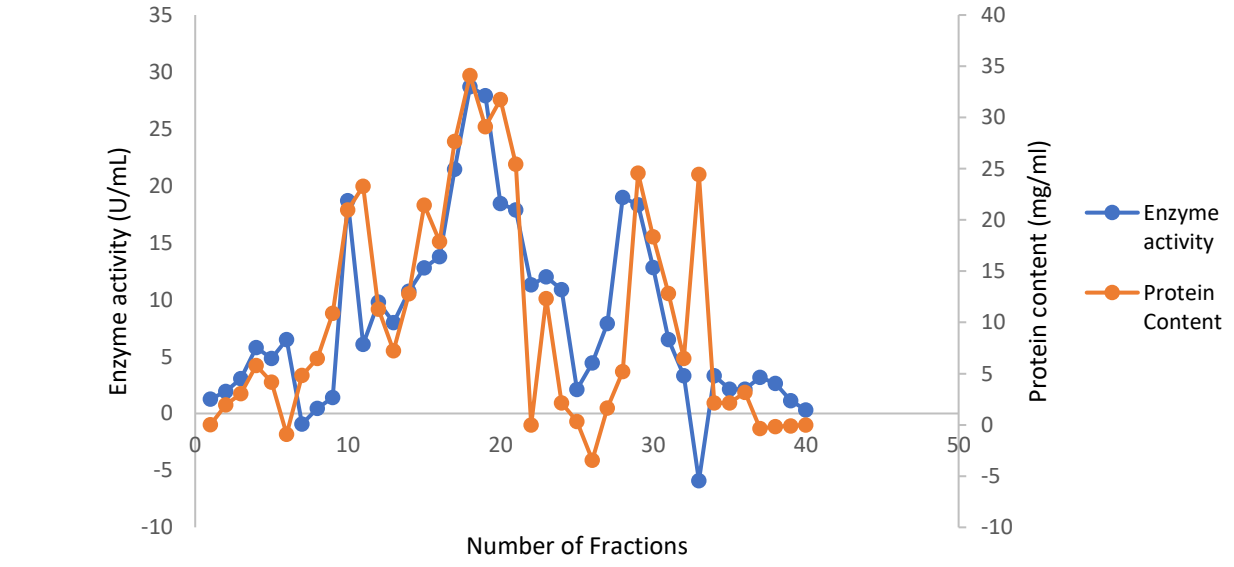
21.454 U/mL, 28.701 U/mL and 27.909 U/mL respectively, while their corresponding protein content were 27.648mg/mL, 34.109mg/mL, and 29.100mg/mL.

Table 4.9: Level of purification of alpha-amylase from *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F from the sediment of Ikogosi Warm Springs

***Bacillus cereus* MPW3E**	Total volume (mL)	Total enzyme activity (U/mL)	Total protein content (mg/mL)	Specific activity (U/mg)	Purification fold	Percentage yield
Crude Enzyme	100	38.881	250.01	0.155	1	100
$(NH_4)_2SO_4$	50	31.887	151.35	0.211	1.361	82.012
Dialysed	10	28.718	70.917	0.405	2.613	73.861
SephadexG100	5	19.935	25.160	0.792	5.111	51.366
***Bacillus siamensis* SW3F**	Total volume (mL)	Total enzyme activity (U/mL)	Total protein content (mg/mL)	Specific activity (U/mg)	Purification fold	Percentage yield
Crude Enzyme	100	45.537	266.320	0.171	1	100
$(NH_4)_2SO_4$	50	39.125	131.351	0.298	1.743	85.919
Dialysed	10	33.556	80.752	0.416	2.430	73.690
SephadexG100	5	28.701	34.109	0.804	4.701	63.028



(A)



(B)

Figure 4.15: Column chromatography fractions of purified extracellular alpha amylase produced from (A) *Bacillus cereus* MPW3E and (B) *Bacillus siamensis* SW3F

4.10 Targeted metagenomic analysis of the bacterial diversity of Ikogosi Warm Springs

The targeted metagenomic analysis of the bacterial diversity of the sediment and water samples through the amplification of V1-V3 of the 16S bacterial rRNA showed a decline in the number of the demultiplexed reads pre-processed for V1 –V3 dataset (Table 4.10). Quality-filtered and denoised forward and reverse reads merged to give a consensus high-quality read suitable for downstream processing showed that a total of 693,916 raw reads were processed and 308,601 were certified quality filtered and fit for downstream processing. From these quality-filtered reads, a total of 14,499 Amplicon Sequence Variants (ASV) were generated. Further filtering to remove unwanted taxa such as "chloroplasts, mitochondria, etc." removed 2,286 unwanted ASVs while 12,213 ASVs were retained, generating 12,213 taxa. Their 12,213 taxa were taxonomically ranked into: "Kingdom – Bacteria", and their "Phylum, "Class", "Order", "Family", "Genus", "Species" were as revealed in Table 4.11.

Comparing water and sediment samples, at the phyla level, 8 phyla with 119 genera was discovered from water samples while 6 phyla classified into 112 genera was identified from sediment samples. Comparing the months of collection, June samples recorded higher number of phyla (6), in contrast to December samples which had 7 phyla. With respect to the genera, June samples showed a higher number of identified genera (125) in comparison to December samples which showed that only 113 genera were identified. Comparing different locations within the spring, the midpoint of the warm spring recorded the lowest number of phyla (4), while the source of the cold spring was recorded as the location with the largest number of phyla (8), similarly, the spring location with the lowest number of genera was identified as the midpoint of the warm spring with 73 genera identified, which was closely followed by the source of the warm spring which showed that 74 genera was identified. The spring location with the highest bacterial genera was observed at the confluence of the cold and warm spring recording its total number of genera as 109 in number.

Table 4.10: Summary of the number of reads before and after quality preprocessing for each demultiplexed reads using DADA2 pipeline

Sample name	Raw reads	Filtered reads	Denoised F.	Denoised R.	Merged reads	Non-chimeric reads
SW-water-dry	34504	30735	30155	30313	19246	14411
SW-water-wet	34893	29525	29024	29194	18209	13054
SW-soil-dry	34495	31079	30672	30793	28636	25596
SW-soil-wet	35039	28897	28467	28581	11263	9376
MPW-water-dry	34615	30339	29870	29921	19239	15769
MPW-water-wet	34725	29588	28944	29156	23966	16634
MPW-soil-dry	34626	29742	29415	29554	6816	6181
MPW-soil-wet	34956	29735	29360	29514	4767	4505
C-water-dry	34629	30424	29618	29712	22087	16481
C-water-wet	34729	29618	29041	29138	19704	14536
C-soil-dry	34473	31281	30395	30736	24923	19595
C-soil-wet	34887	30034	29464	29574	20258	18134
MPC-water-dry	34723	30392	29375	29575	22557	18584
MPC-water-wet	34786	30061	29240	29264	24546	17304
MPC-soil-dry	34611	30726	30148	30334	26820	18672
MPC-soil-wet	34404	30922	30029	30324	24819	18897
SC-water-dry	34830	29952	29141	29234	22830	17131
SC-water-wet	34785	30300	29447	29637	24605	17156
SC-soil-dry	34759	30157	29008	29497	19059	14766
SC-soil-wet	34447	30642	30031	30278	14270	11819
	693,916	**573,757**	**598,844**	**565,095**	**398,620**	**308,601**

Key: SW – Source of warm spring, MPW – Midpoint of warm spring, C – Confluence of the warm and cold spring, SC – source of cold spring, MPC – Midpoint of cold spring, Denoised F. – Forward denoised reads, Denoised R. – Reverse denoised reads.

Table 4.11: Summary of bacterial taxonomic classification identified using RDP database for V1 –V3 dataset

Sample groupings		BACTERIAL TAXONOMIC CLASSIFICATION					
		Phylum	Class	Order	Family	Genus	Species
Sample Types	Water	8	18	28	53	119	79
	Sediment	6	14	25	56	112	74
Month of Collection	June	7	16	26	58	125	76
	December	6	15	26	52	113	77
Spring Location	Source of warm spring	6	13	22	43	74	45
	Midpoint of warm spring	4	10	21	39	73	43
	Confluence	5	15	24	49	109	69
	Midpoint of cold spring	7	16	24	49	105	60
	Source of cold spring	8	17	27	51	109	57
Sample types and Month of collection	June Sediment	5	11	19	47	89	53
	June Water	7	15	23	43	96	52
	December Sediment	6	13	22	44	87	60
	December Water	5	14	23	42	93	65

4.11. Taxonomic composition and relative abundance of bacterial taxa present in sediment and water samples of Ikogosi Warm Springs

Samples from the spring were grouped into four categories namely: Sampling location, month of collection, the sample types, and the collection period of the sample types as shown below.

(A) Sampling location of the spring: categorised as source of warm spring (SW), midpoint of warm spring (MPW), confluence of warm and cold spring(C), source of cold spring (SC) and, midpoint of cold spring (MPC).

(B) Month of collection: Grouped as June and December.

(C) Sample types: Grouped as sediment and water

(D) Sample type and collection period: categorised in to Sediment_June, Sediment_Dec, Water_June and Water_Dec.

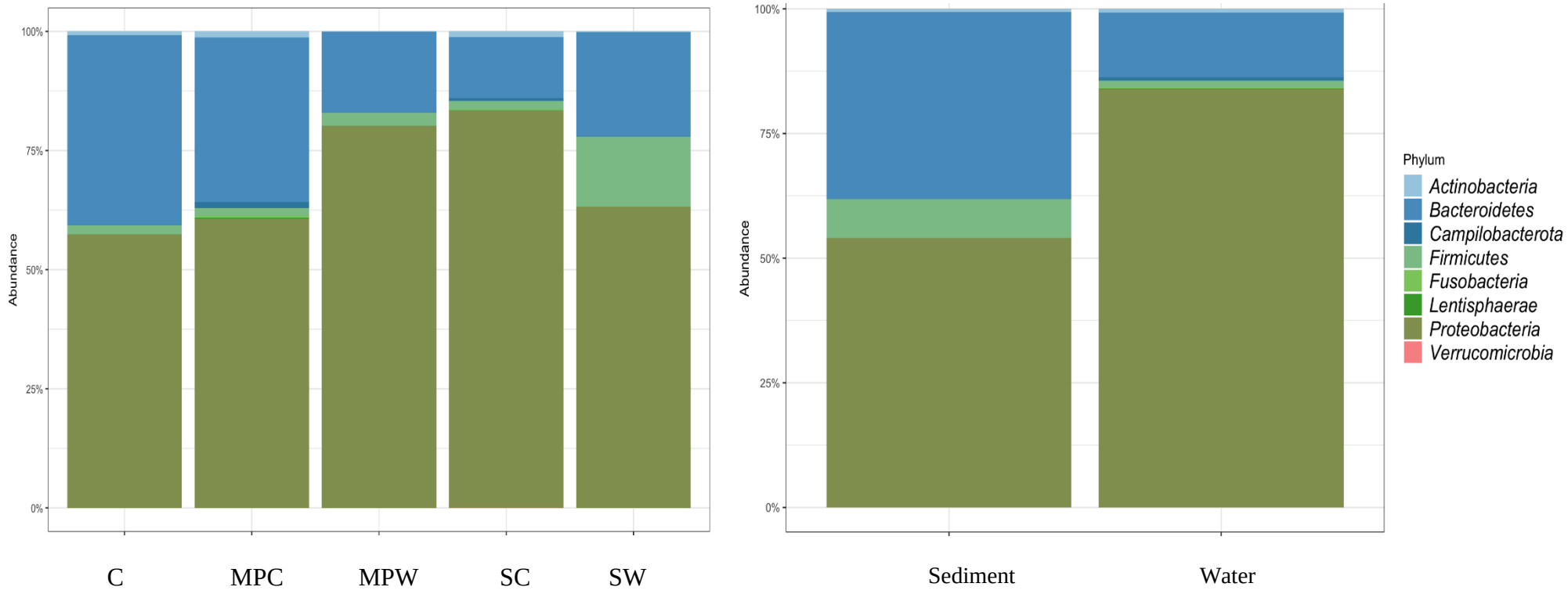
Figure 4.16A – 4.16D shows the relative abundance of phylum in four groups, namely: Sampling location of the spring, sample types, Month of collection and Sample type&collection period.

Figure 4.16A depicts that the source of cold spring has the highest relative abundance of Proteobacteria (83.33%), while the confluence of the spring is observed as the site with the least relative abundance of Proteobacteria (57.35%). Figure 4.16B shows the phyla observed in water and sediments of Ikogosi warm springs. It was observed that Proteobacteria was the most dominant phyla, and showed highest relative abundance (83.97%) in water samples, while sediment samples had a high prevalence of Bacteroidetes (37.47%) and Firmicutes (7.76%) than in water samples. From sampled months (June and December), three phyla showed dominance - Bacteroidetes, Firmicutes and Proteobacteria, in samples collected in June with a relative abundance of 73.51%, 15.78% and 8.72% respectively. December samples however showed the dominance of 2 phyla – Bacteroidetes (34.73%) and Proteobacteria (64.47%) (Figure 4.16C). Among water and sediment samples collected at different months, only sediment collected in June showed high prevalence of Firmicutes (15.01%), with the lowest abundance of Firmicutes recorded in water samples collected in December (0.07%). Also, the highest abundance of Bacteriodetes were seen in sediment samples collected in December (53.53%), while the least relative abundance of Bacteroidetes was recorded from water samples collected in June (10.07%) (Figure 4.16D).

Figure 4.17A – 4.17D shows the taxonomic bar plots of top 19 most abundant bacterial genera detected according to the following groupings:Sampling location of the spring (grouped as source of warm spring (SW), midpoint of warm spring(MPW), confluence of warm and cold spring(C), source of cold spring (SC) and, midpoint of cold spring (MPC)), sample type (grouped as sediment and water), month of collection (grouped as June and December), and Sampletype_collection period (categorised in to Sediment_June, Sediment_Dec, Water_June and Water_Dec) respectively.

Figure 4.17A depicts the dominance of *Empedobacter, Acinetobacter, Pseudomonas,* and *Paraclostridium* from samples collected at the source of the warm spring with a prevalent abundance of 21.52%, 20.47%,13.67% and 13.57% respectively, while at the source of the cold spring, *Comamonas* (30.40%), *Brevundimonas* (21.31%), *Flavobacterium* (6.51%) and *Providencia* (6.29%) were seen in larger abundance in comparison to other present genera. In Figure 4.17B, among the top 20 genera, sediment samples showed high relative abundance of *Vogesella* (9.74%), *Aeromonas* (3.47%) and *Sphingobacterium* (5.57%) than its corresponding water samples. However, some genera such as *Providencia* and *Empedobacter* were not discovered in water samples. Figure 4.17C shows the relative abundance plot from samples collected at different months, it was observed that June samples showed increased presence of *Vogesella* (10.35%), *Pseudomonas* (8.20%), *Providencia* (4.05%), *Delftia* (3.00%) and *Acromobacter* (2.61%) than December samples. In December samples, *Comamonas* (20.85%) and *Acinetobacter* (16.96%) were discovered in higher abundance than from samples collected in June. However, *Sphingobacterium* and *Empedobacter* were discovered only from samples collected in December.

Grouping according to sample types and collection period (Figure 4.17D) showed the dominance of *Vogesella* (18.76%) in sediment collected in June only, while *Sphingobacterium* had a high relative abundance (11.00%) in sediment collected in December. The dominance of *Pseudomonas* (16.04%) was pronounced in water samples collected in June, while *Acinetobacter* (26.31%) was the most dominant in water samples collected in December.



Key: SW – Source of warm spring, MPW – Midpoint of warm spring, C – Confluence of the warm and cold spring, SC – source of cold spring, MPC – Midpoint of cold springs

Figure 4.16. Taxonomic compositional bar plot of bacterial phyla detected (A) at different sampling points (B) from sediment and water samples of Ikogosi warm springs

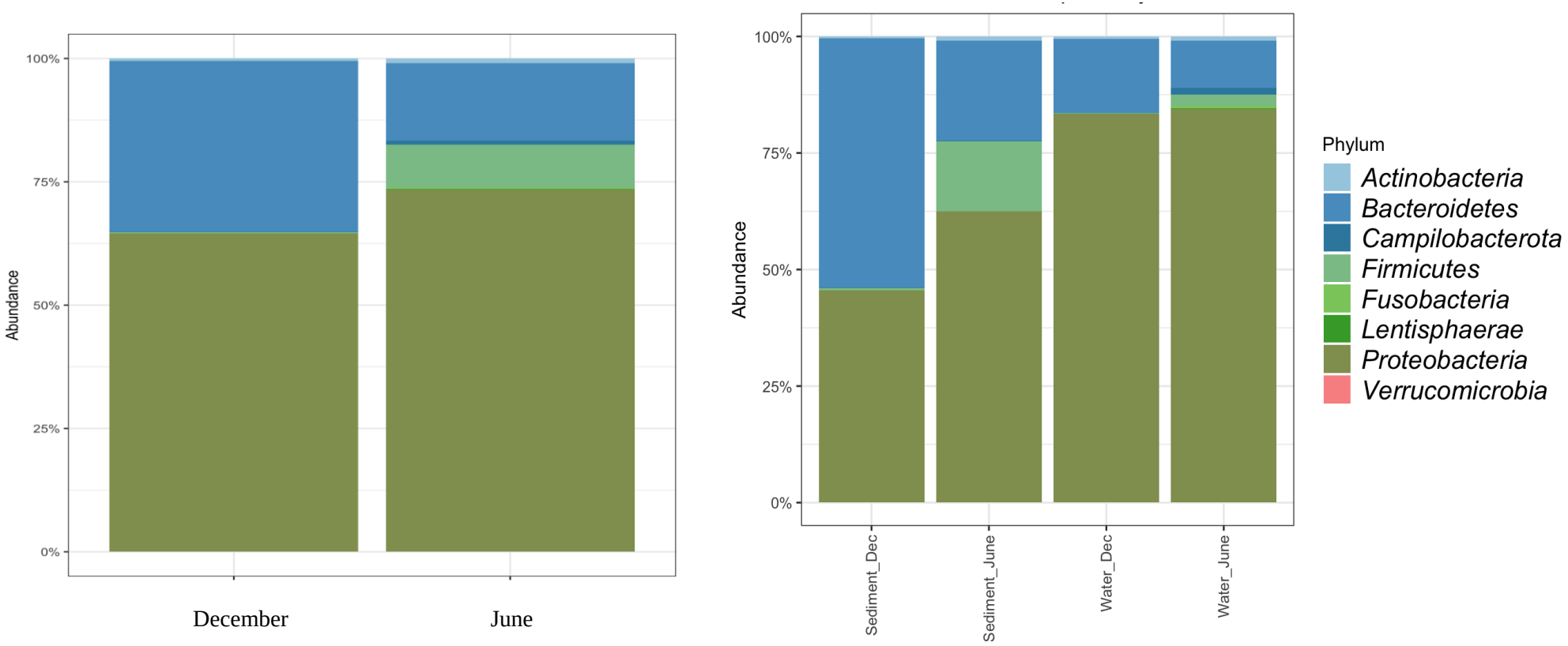
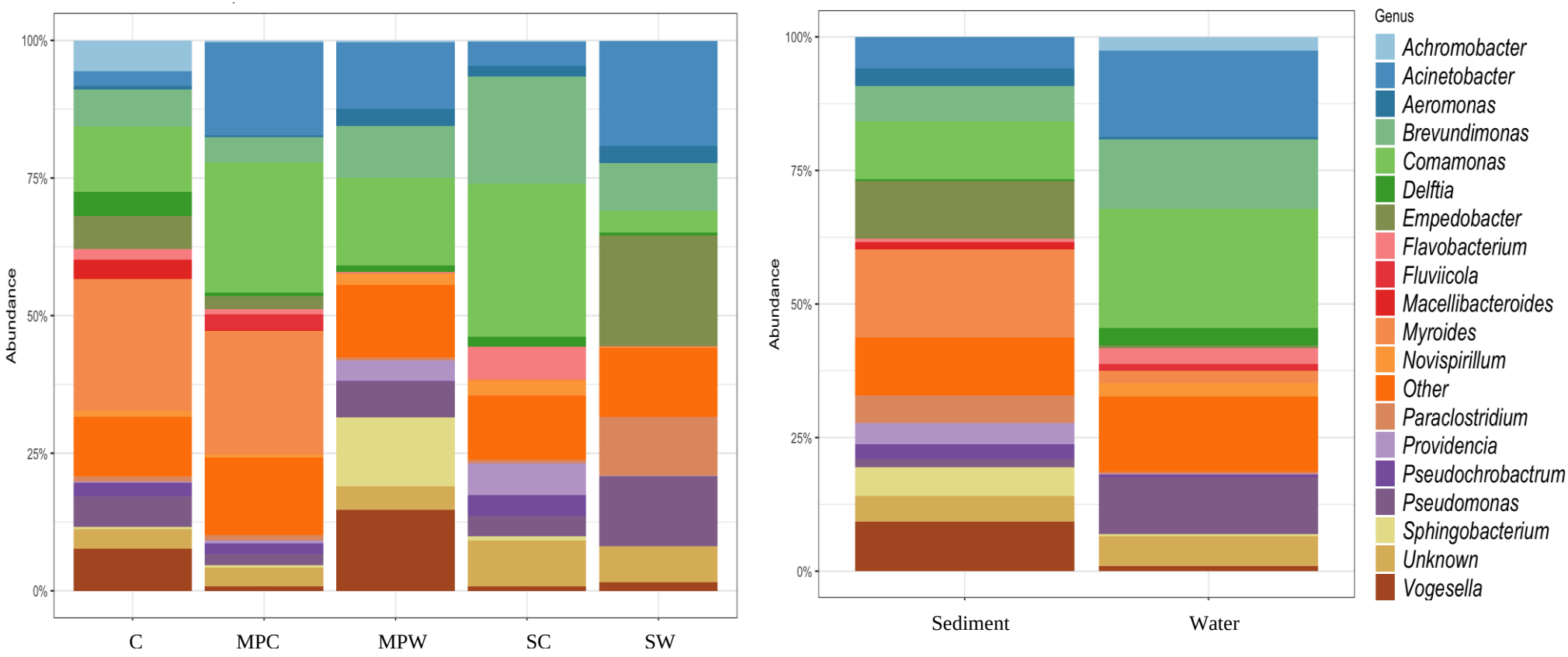


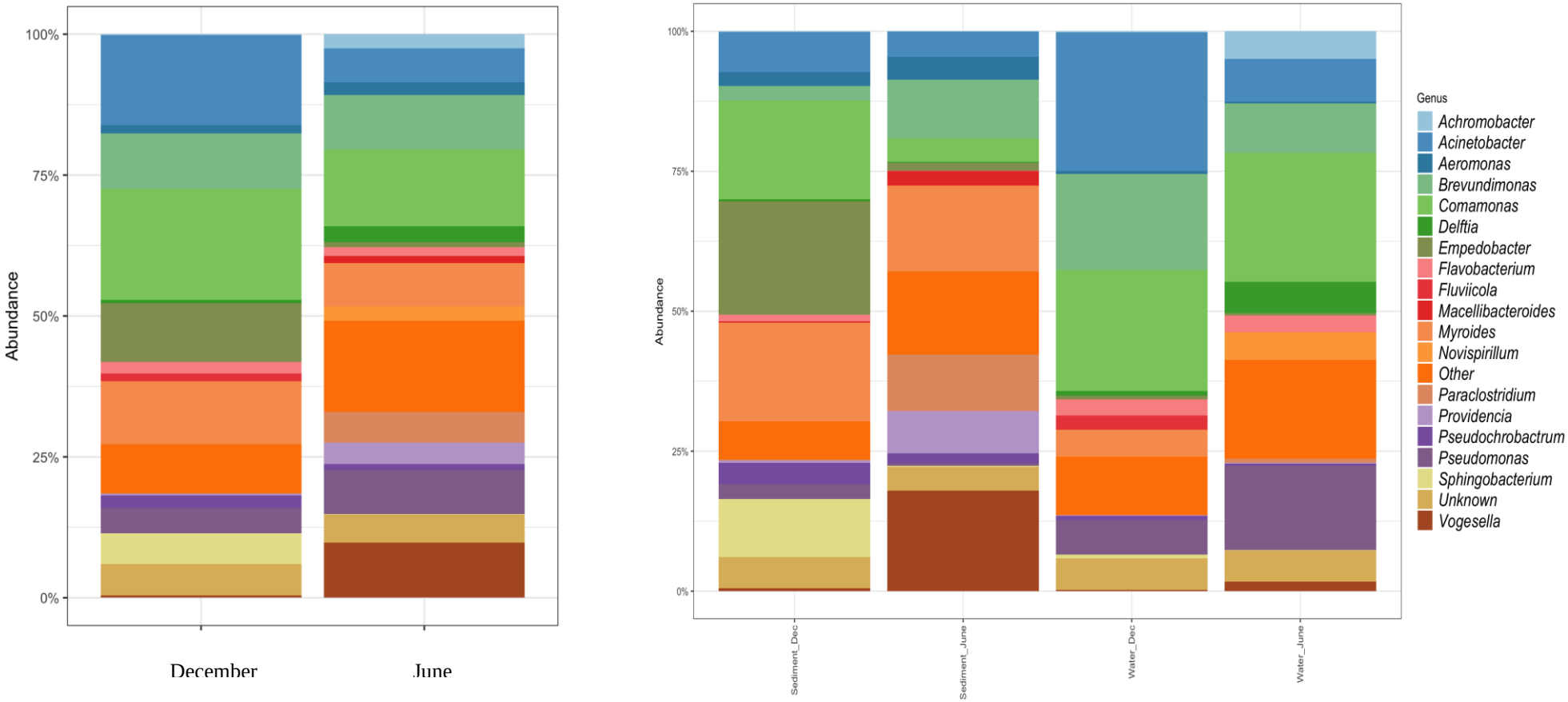
Figure 4.16. Taxonomic compositional bar plot of bacterial phyla detected at (C) different sampling months (D) different months from sediment and water samples from Ikogosi warm springs



(A) **(B)**

Key: SW – Source of warm spring, MPW – Midpoint of warm spring, C – Confluence of the warm and cold spring, SC – source of cold spring, MPC – Midpoint of cold springs

Figure 4.17 Taxonomic composition bar plot of top19 most abundant genera detected at (A) different sampling points and (B) from sediment and water samples of Ikogosi warm springs



(C) **(D)**

Figure 4.17.C Taxonomic compositional bar plot of top 19 bacterial genus detected during (C) different sampling months and (D) at different months from sediment and water samples of Ikogosi warm springs

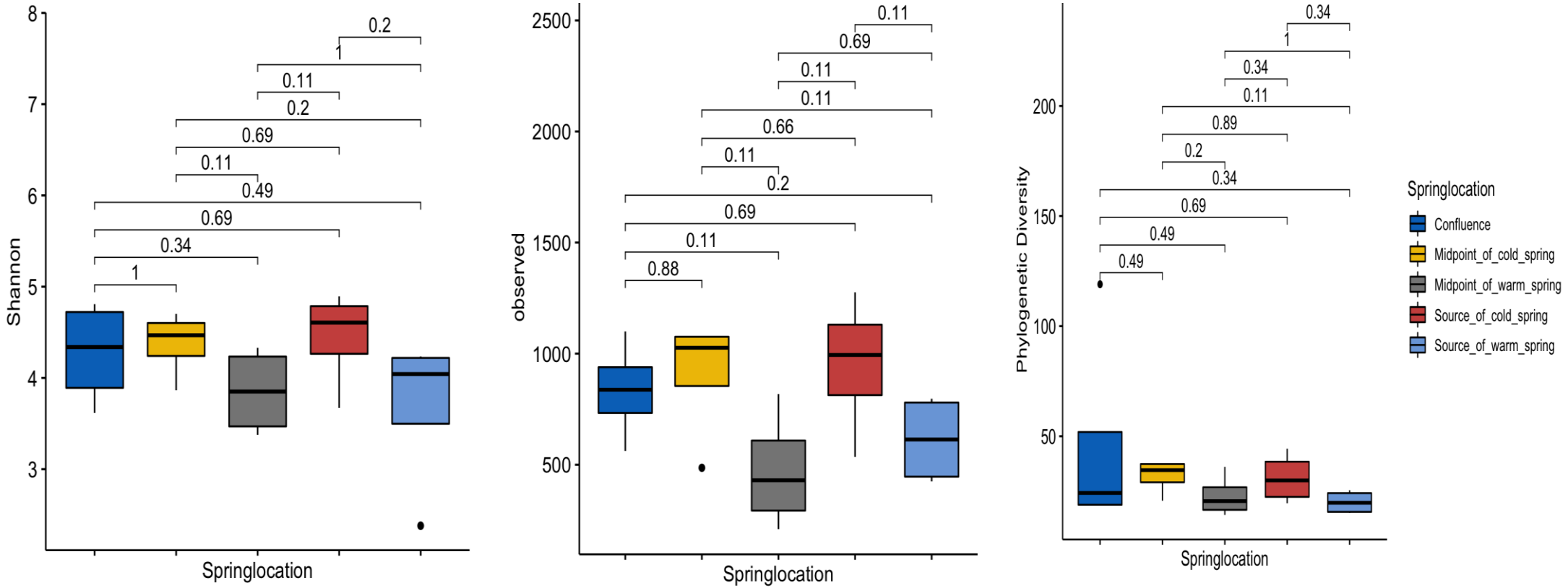
4.12 Boxplots of alpha diversity from samples of Ikogosi Warm Springs

The use of alpha diversity metrics (Shannon, Phylogenetic Diversity and Observed Species) depicted values which were either significantly or not significantly different. The alpha diversity boxplots of all named metrics from the sediment and the water samples are shown in Figures 4.18- 4.21.

The Shannon diversity index (Figure 4.18A) showed the richness and evenness of the species in the samples. There was no significant difference ($P<0.05$) from the samples from different points of the spring. The observed species which measured the number of species or number of Operational Taxonomic Unit showed that the number of species from all sampled locations of the spring were not significantly different (Figure 4.18B). Likewise, the Phylogenetic Diversity (PD) which measured the biodiversity based on phylogenetic distances in each sample, showed no significant difference at $P <0.05$ (Figure 4.18C).

In all sediment and water samples, water samples had numerically higher richness and evenness value than sediment samples, however, the variations were not significantly different at $P < 0.05$ (Figure 4.19A). In the sediment and water samples using Phylogenetic Diversity and observed species index, higher diversity was depicted in water samples than in sediment samples and thus was statistically significant at $P <0.05$ (Figure 4.19B and Figure 4.19C).

Using the three alpha diversity index, there were no significant differences recorded from samples collected at different months of the year as shown in Figure 4.20A – 4.20C. Furthermore, sediment and water samples collected at different months did not depict any statistical difference at $P <0.05$, using Shannon and Observed species and PD metrics (Figure 4.21A – Figure 4.21C).



(A) **(B)** **(C)**

Figure 4.18: Bacterial alpha diversity box plots of samples collected at different sampling points of Ikogosi warm springs, using (A) Shannon (B) Observed species (C) Phylogenetic Diversity Index (P <0.05)

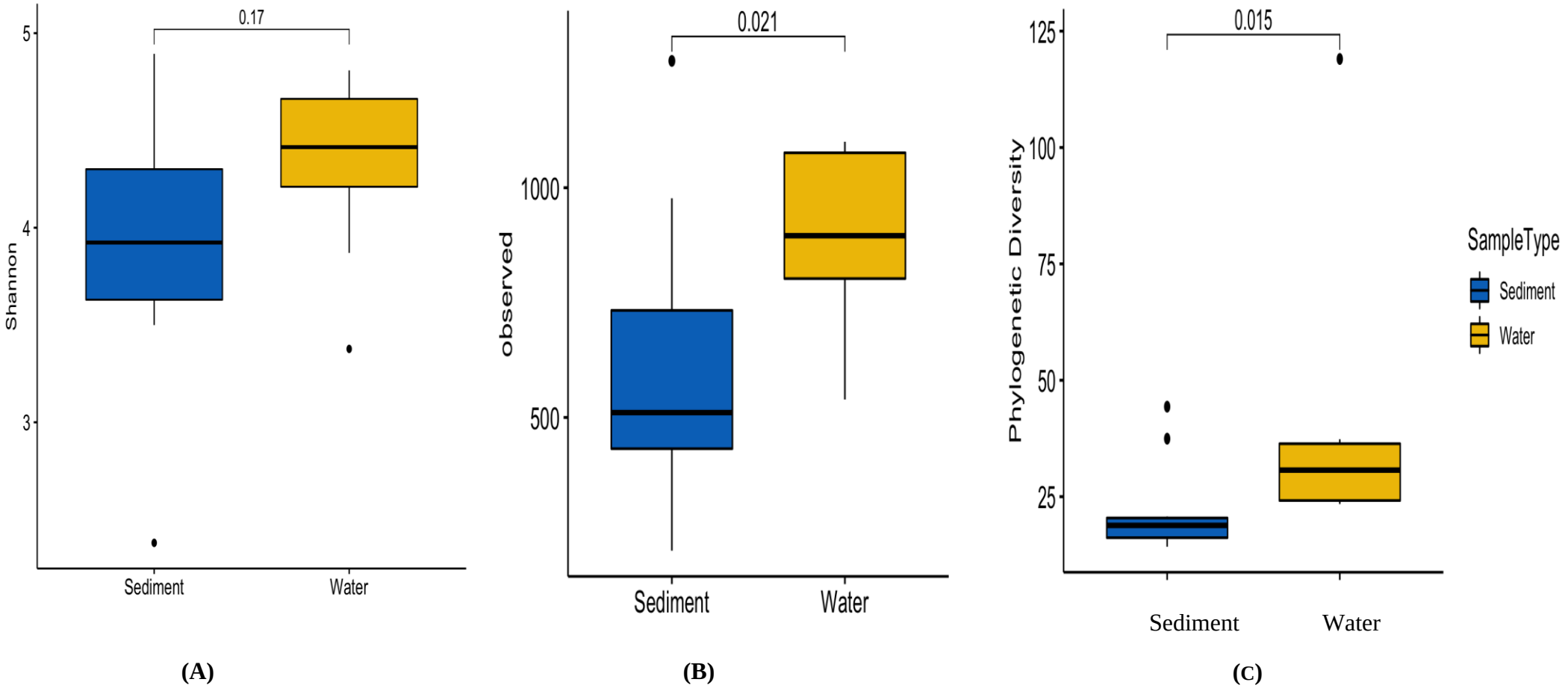
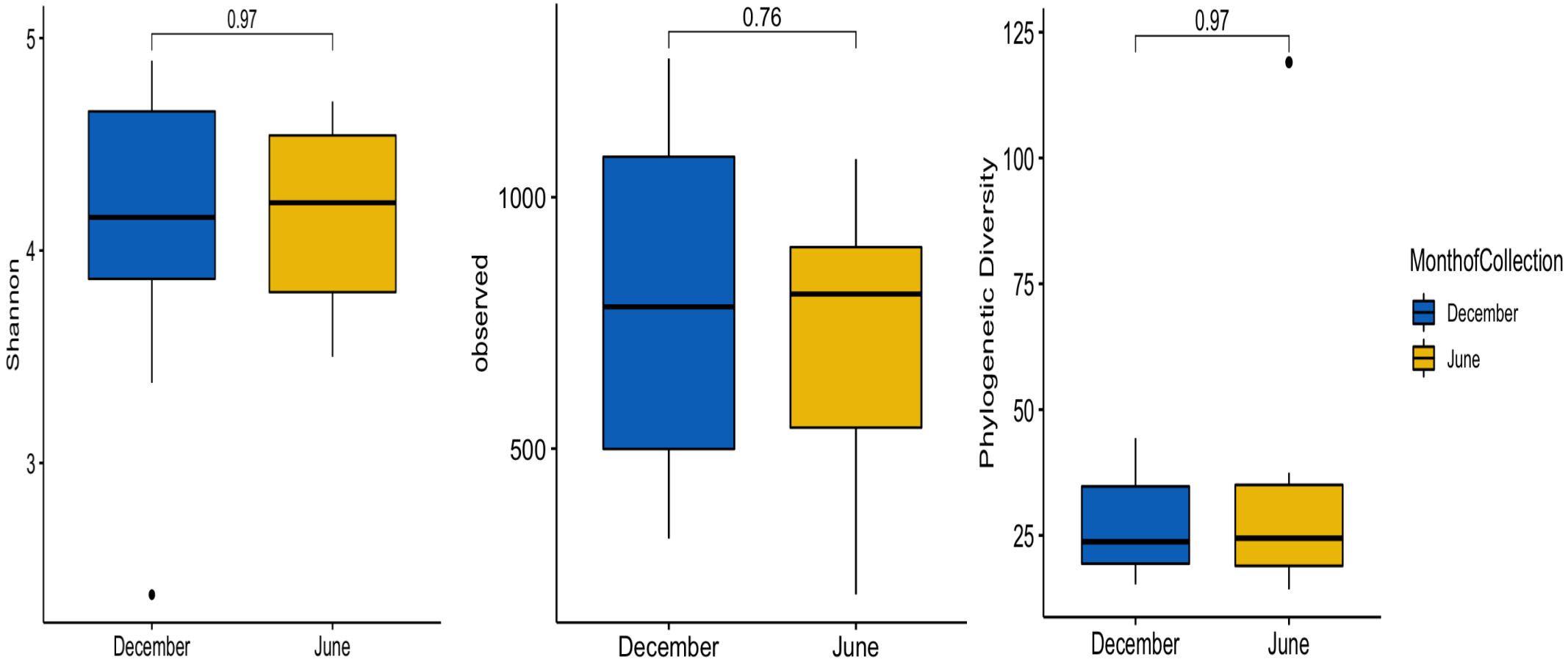


Figure 4.19. Bacterial alpha diversity box plots of sediment and water samples of Ikogosi warm springs, using (A) Shannon, (B) Observed species and (C) Phylogenetic index (P <0.05)



(A) **(B)** **(C)**

Figure 4.20 Bacterial alpha diversity box plots of samples collected at different sampling months from Ikogosi warm springs, using (A)Shannon, (B) Observed species and (C) Phylogenetic Diversity index (P <0.05)

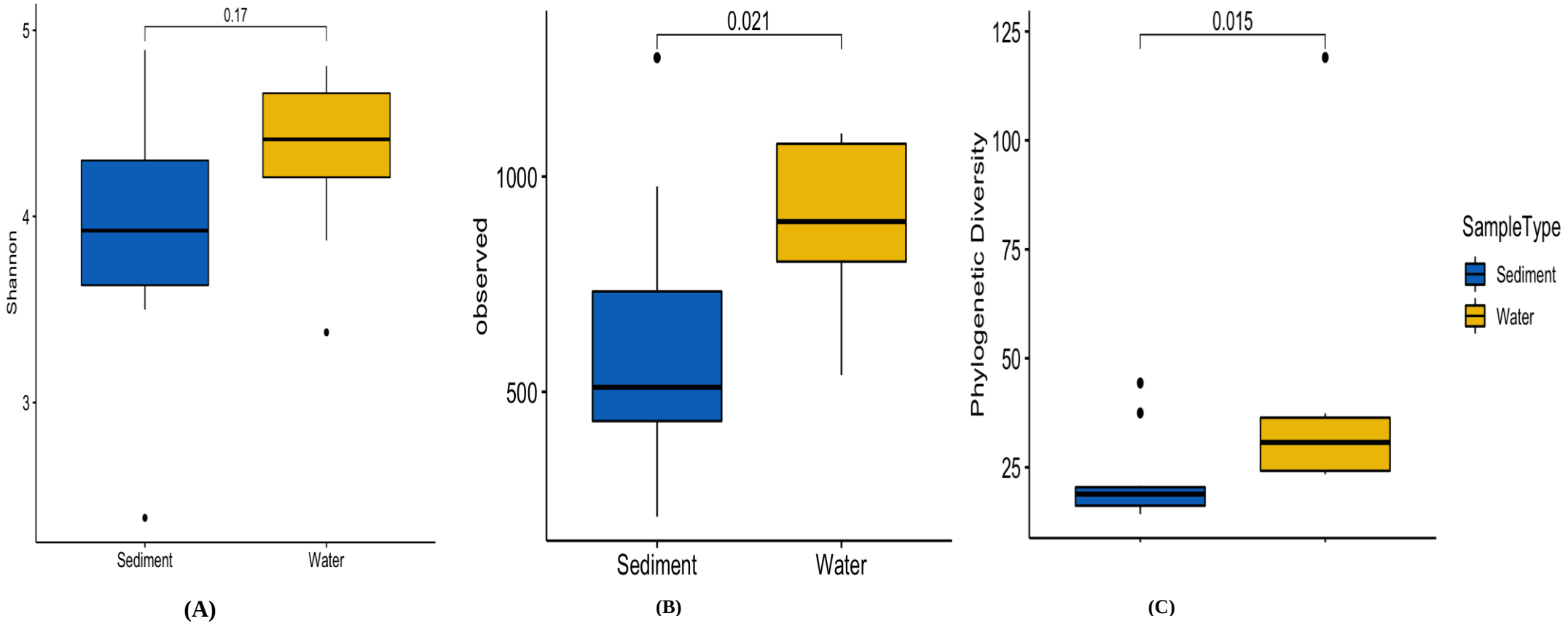


Figure 4.21. Bacterial alpha diversity boxplots of sediment and water samples collected at different sampling months from Ikogosi warm springs using (A) Shannon, (B) Observed species and (C) PD index of water and sediment samples collected in December and June ($P<0.05$)

4.13 Scattered plots of beta diversity of from samples of Ikogosi Warm Springs

Beta diversity plots of Bray-Curtis distance, weighted and unweighted distances of all samples collected from the five sampling parts of the spring are shown in Figure 4.22A – 4.22C. All metrics indicated no statistical dissimilarity among bacterial communities from samples collected from the five designated location of the spring. Beta diversity plots of all named distance methods for different sample types (sediment and water) are depicted in Figure 4.19.

In sediment and water samples (Figure 4.23), Bray-Curtis metrics which is based on the relative abundance from each site, showed significant separation of the bacterial structure present in water samples away from sediment samples, with minimal overlap. ($P= 0.001$, $P <0.05$) (Figure 4.23A). Interestingly, weighted Unifrac metrics indicated no distinct clustering and no significant difference in the bacterial communities present in water and sediment samples ($P= 0.202$, $P <0.05$) (Figure 4.23B). However, Unweighted Unifrac metrics, indicated a significant difference between bacterial population in sediment and water samples ($P = 0.016$), hence a clustering of sediment samples, with minimal overlap between water and sediment samples at $P <0.05$ (Figure 4.23C).

Beta diversity plots of above named distances of samples collected at different months of the year are shown in Figure 4.24. Figure 4.24A depicts bacterial structure present in samples collected in June and December, using Bray-Curtis dissimilarity distance, $P = 0.065$, hence no statistical difference in bacterial communities present from both group of samples ($P<0.05$). Figure 4.24B shows the relationship between samples of June and December, using the weighted Unifrac method. There was a significant difference between the group of sample at $P = 0.021$. There was also an observed separation of December samples, with minimal inclusion from June samples. Figure 4.24C shows the unweighted distance between December and June samples, however, there was no clustering nor significant difference observed at $P<0.05$.

Figure 4.25 shows the beta diversity plots of sediment and water samples collected in separate months. Bray-Curtis approach in comparing these group of samples showed clear separation of water samples collected in June, and a clear separation of sediment samples collected in June. These clusters were highly significant with $P = 0.044$,

between samples of water collected in December and sediments collected in December, and P seen as 0.0034 between samples of sediment collected in June and water samples collected in December (Figure 4.25A).

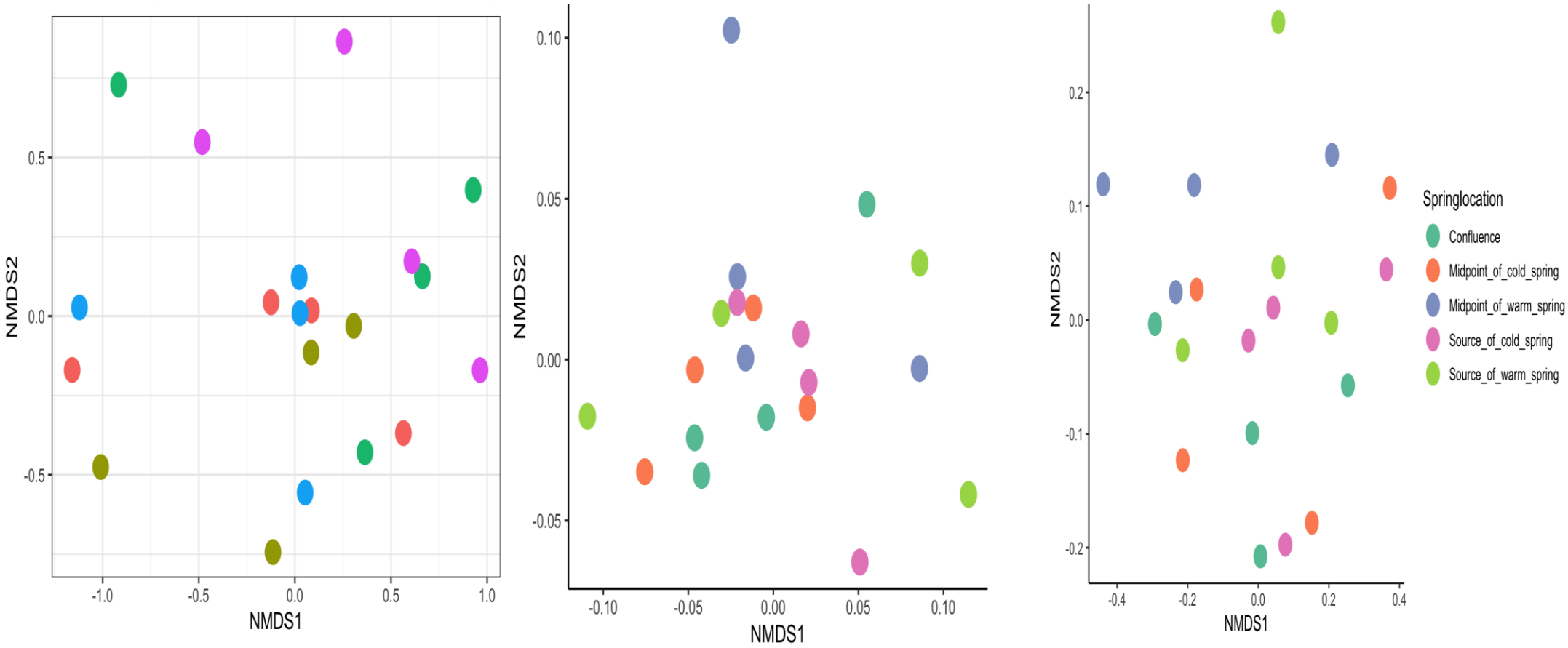
Figure 4.25B depicts beta diversity of water and sediment samples collected at different months of the year, using weighted unifrac metrics. However, there was no indication of bacterial community differences among these samples (P = 0.08). Figure 4.25C shows the beta diversity among samples using unweighted unifrac metrics. Statistical results indicated no difference in the bacterial structure present among these group of samples.

4.14 Core bacterial members from samples collected from Ikogosi Warm Springs

In this study, core members were defined as microbial Amplicon Sequence Variants (ASVs) found across 90% of grouped samples (90% core threshold). The Venn diagram depicting unique and common core members revealed shared taxa/ASVs/ core members among four groups were (sample type, month of sample collection, spring location, and sample types collected at different months of the year) represented as overlapping areas in the circles of the Venn diagram. Unique core ASVs of each group were represented as non-overlapping regions of the Venn diagram as shown in Figure 4.26A-D respectively.

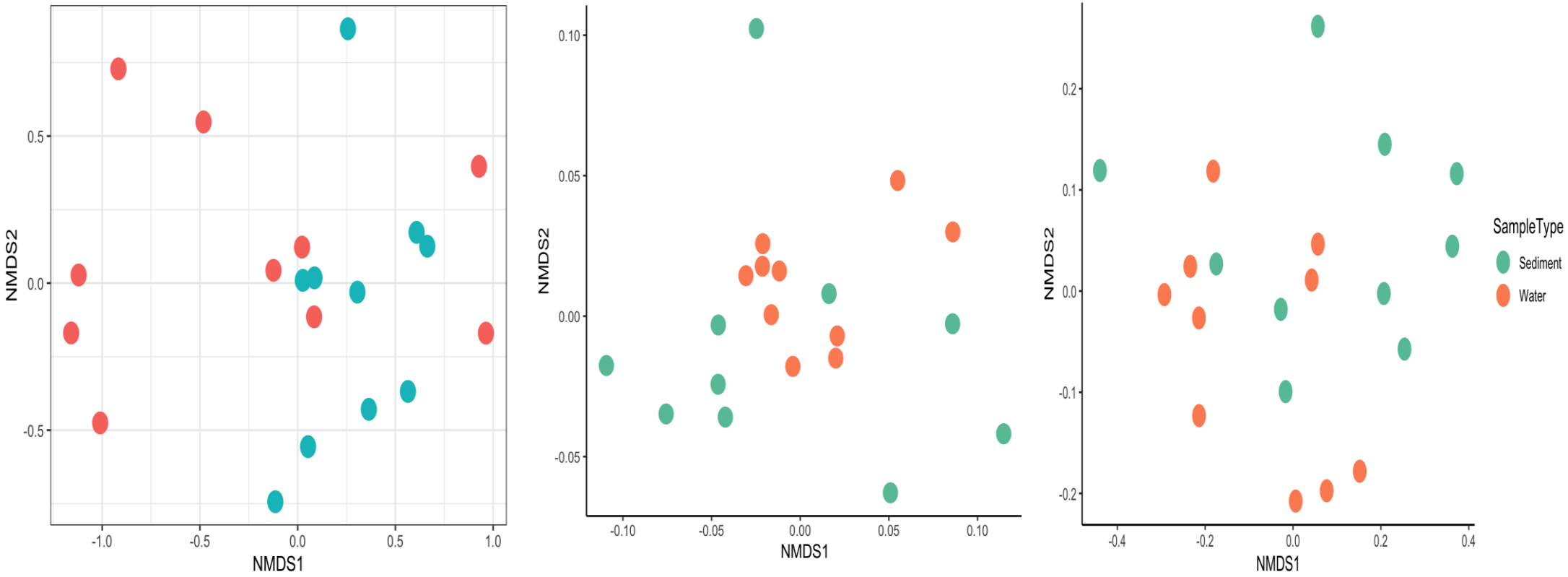
Figure 4.26A showed that 2 ASVs were shared among water and sediment samples, occupying 18.18% of all ASVs discovered at 90% core threshold, while Figure 4.26B showed two taxa (28.57%) as shared members discovered among December and June samples. In Figure 4.26C, 2 taxa (3.92%) were identified to share members among samples collected from different points of the spring. Like in previous groups, 2 bacterial taxa were also identified to be present in all samples (shared members) collected from sediment and water samples collected in the months of December and June (Figure 4.26D). These shared taxa/members represented 4.76% of total core members of these groups.

Bacterial names of unique and shared core taxa present among these groups are tabulated in Tables 4.12A-D respectively.



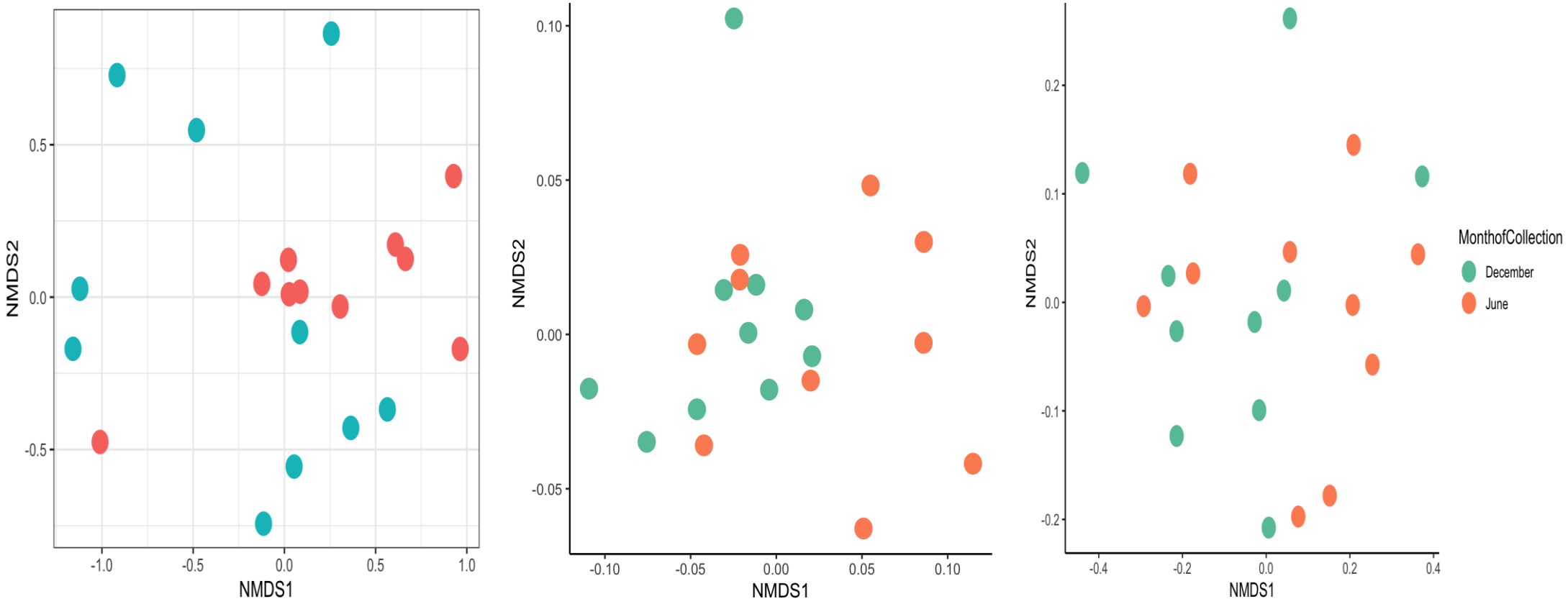
(A) **(B)** **(C)**

Figure 4.22. Bacterial beta diversity plots of samples collected at different sampling of Ikogois warm springs, using (A)Bray-Curtis (B) weighted Unifrac and (C) unweighted unifrac distance metrics at P<0.05, visualised via NMDS



(A) **(B)** **(C)**

Figure 4.23. Bacterial beta diversity plots of sediment and water samples of Ikogosi warm springs, using (A)Bray-Curtis, (B) weighted and (C) unweighted Unifrac distance metrics at P<0.05, visualised via NMDS



(A) **(B)** **(C)**

Figure 4.24. Bacterial beta diversity plots of samples collected at different sampling months from Ikogosi warm springs, using (A)Bray-Curtis, (B)weighted and (C)unweighted Unifrac distance metrics at P<0.05, visualised via NMDS

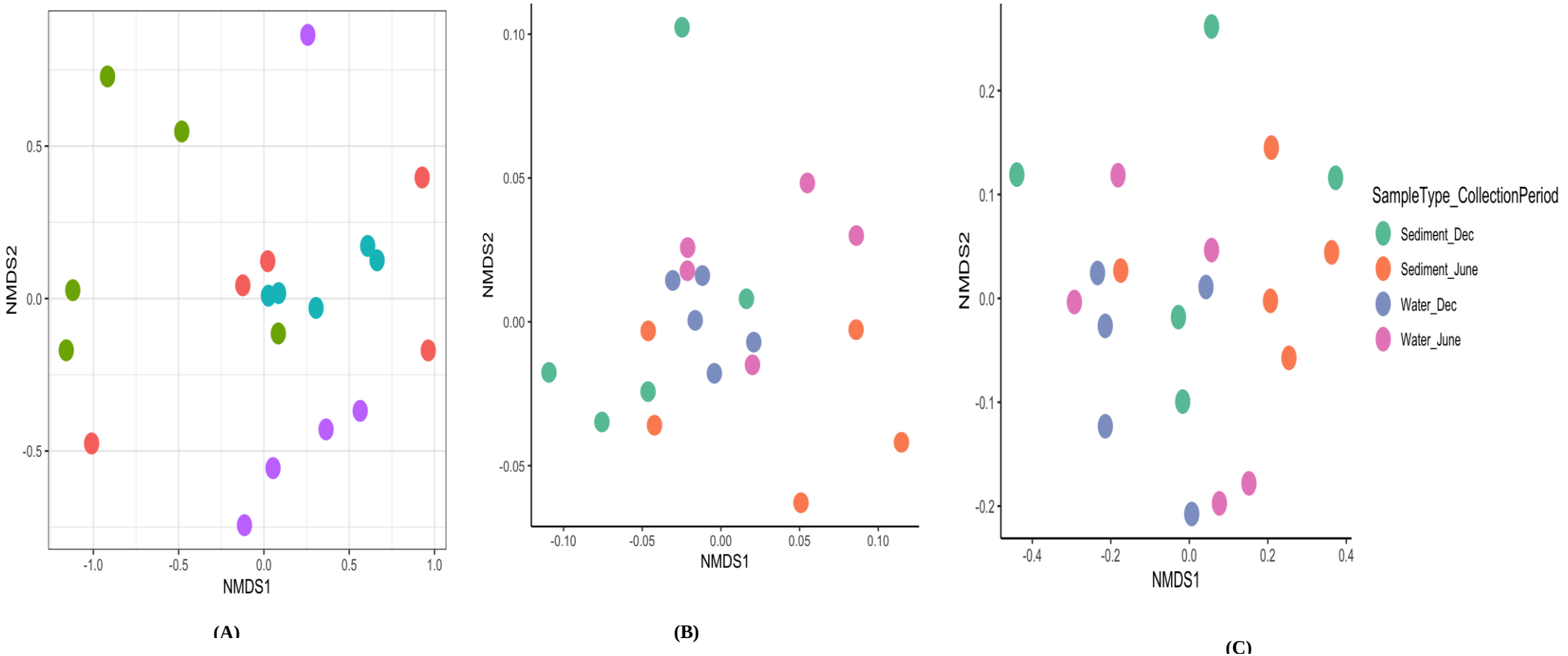
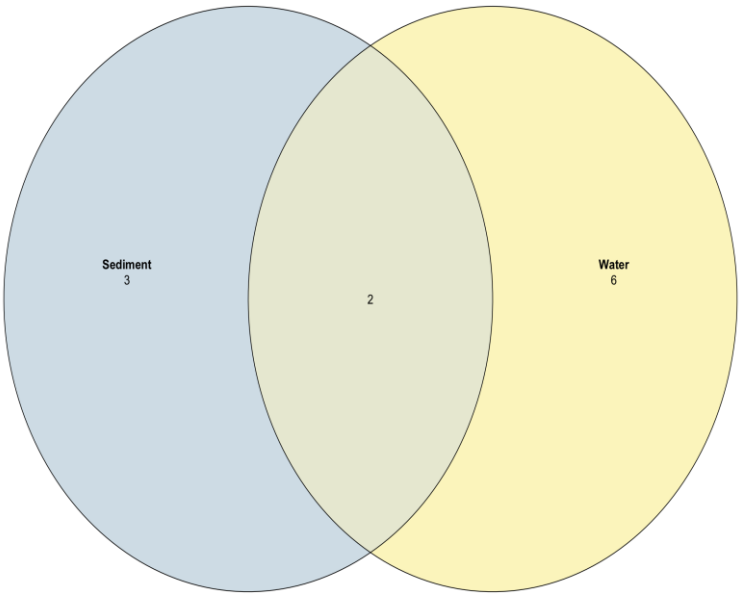
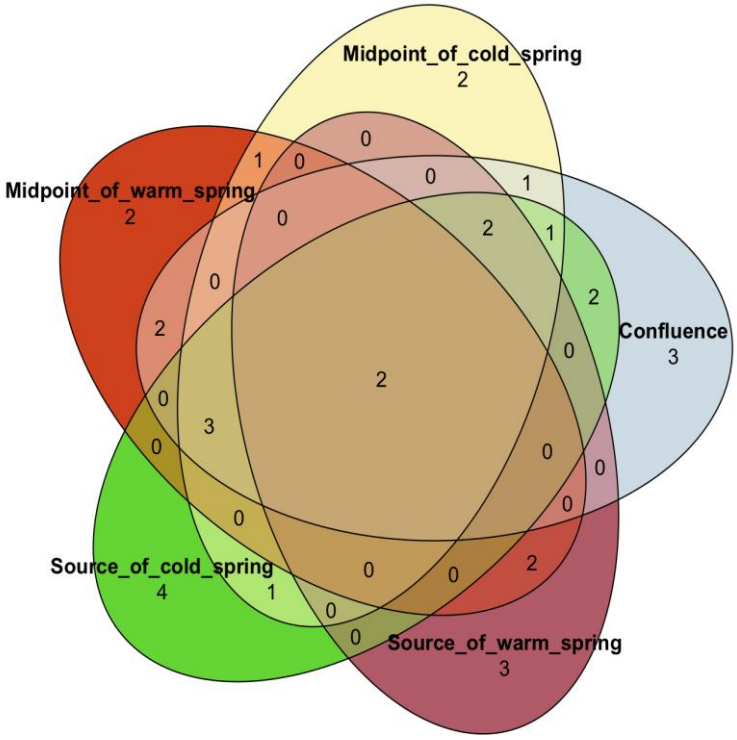


Figure 4.25. Bacterial beta diversity plots of sediment and water samples collected at different sampling months from Ikogosi warm springs, using (A)Bray-Curtis, (B)weighted and (C)unweighted Unifrac distance metrics at P<0.05, visualised via NMDS

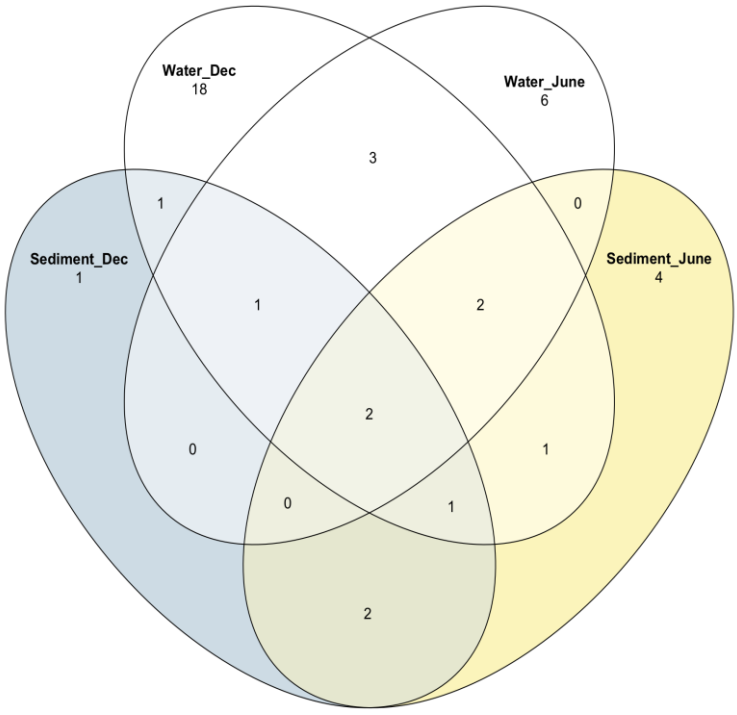


(A)

(B)



(C)



(D)

Figure 4.26. Venn diagram depicting number of shared taxa/Amplicon Sequence Variants (ASVs) and unique taxa/ASVs among (A) sediment and water samples (B) samples collected at different sampling months (C) samples collectd at different sampling points (D) sediment and water samples collected at different sampling months, from Ikogosi warm springs

Table 4.12A. Unique and shared core members from sediment and water samples of Ikogosi Warm Springs

SEDIMENT	
ASV- ID	**Unique Taxa**
ASV 3	*Comamonas aquatica*
ASV 14	*Comamonas* sp.
ASV 439	*Chromobacterium* sp.
WATER	
Unique Taxa	
ASV71	*Pseudomonas* sp.
ASV72	*Aeromonas* sp.
ASV272	*Stenotrophomonas*
ASV 177	*Comamonadaceae*
ASV 184	*Enterobacteriaceae* sp.
ASV 36	*Pseudomonadaceae*
SHARED TAXA	
ASV 22	*Vogesella* sp.
ASV 31	*Acinetobacter* sp.

Table 4.12B. Unique and shared core members depicted at different sampling months (June and December) from Ikogosi Warm Springs

JUNE	
ASV- ID	**Unique Taxa**
ASV 72	*Aeromonas* sp.
ASV 184	Enterobacteriaceae
DECEMBER	
	Unique Taxa
ASV 14	*Comamonas* sp.
ASV 118	Moraxellaceae
ASV 117	Comamonadaceae
SHARED TAXA	
ASV 22	*Vogesella* sp.
ASV 31	*Acinetobacter* sp.

Table 4.12C. Unique and shared core members depicted at different sampling points from Ikogosi Warm Springs

SOURCE OF WARM SPRING	
Unique Taxa	
ASV 199	*Enterobacter* sp.
ASV 272	*Stenotrophomonas* sp.
ASV 364	Pseudomonadaceae
ASV 26	*Delftia acidovorans/lacustris/ tsuruhatensis*
ASV 439	*Chromobacterium* sp.
CONFLUENCE	
Unique Taxa	
ASV 4	*Brevundimonas diminuta*
ASV 8	*Brevundimonas naejangsanensis*
ASV 2671	*Alicycliphilus*
MIDPOINT OF COLD SPRING	
Unique Taxa	
ASV 150	*Pseudochrobactrum* sp.
ASV 291	*Dysgonomonas* sp.
SOURCE OF COLD SPRING	
Unique Taxa	
ASV 187	*Kluyvera ascorbata*
ASV 6	*Brevundimonas* sp.
ASV 521	*Yokenella s*p.
ASV 1779	*Erwinia*
SHARED TAXA	
ASV 22	*Vogesella*
ASV 31	*Acinetobacter* sp.

Table 4.12D. Unique and shared core members depicted in sediment and water samples collected at different sampling months from Ikogosi Warm Springs

JUNE SEDIMENT SAMPLES	
Unique Taxa	
ASV15	*Clostridium* sp.
ASV 194	*Clostridium-sensu-stricto*
ASV 323	*Klebsiella* sp.
ASV1568	*Pseudogulbenkiania* sp.
ASV 19	*Pseudomonas aeruginosa/fulva/monteilii*
ASV 25	*Novispirillum* sp.
ASV 43	*Pseudomonas otitidis*
ASV 82	*Acetobacteroides* sp.
ASV 94	*Bacteroides* sp.
ASV 1085	*Sporomusaceae*
DECEMBER SEDIMENT SAMPLES	
Unique Taxa	
ASV 8	*Brevundimonas naejangsanensis*
DECEMBER WATER SAMPLES	
Unique Taxa	
ASV 1	*Acinetobacter calcoaceticus*
ASV 291	*Brevundimonas diminuta*
ASV 21	*Pseudomonas aeruginosa/entomophila*
ASV 24	*Pseudomonas putida/mosselii*
ASV 6	*Brevundimonas* sp.
ASV 59	*Kinneretia* sp.
ASV 62	*Chryseobacterium* sp.
ASV 80	*Delftia* sp.
ASV 199	*Enterobacter* sp.
ASV 213	*Agrobacterium* sp.
ASV 265	*Brucella* sp.
ASV 872	*Acidovorax* sp.
ASV 886	*Lelliottia* sp.
ASV 914	*Mitsuaria* sp.
ASV 2323	*Tianweitania* sp.
ASV 2792	*Pseudocitrobacter* sp.
ASV 347	*Alcaligenaceae*
ASV 390	*Aeromonadaceae*
SHARED TAXA	
ASV 22	*Vogesella*
ASV 31	*Acinetobacter* sp.

4.15 Differential abundance testing of taxa between two groups of samples from Ikogosi Warm Springs

Figure 4.27A - Figure 4.27C depicts various taxa using metagenomeSeq to identify bacterial taxa that were differently abundant among the compared groups of sediment versus water samples, the June versus December samples and source of warm spring versus source of cold spring samples, respectively.

Figure 4.27A revealed that the relative abundances of 49 bacterial taxa significantly differed between sediment and water samples. Seven bacterial taxa that were more abundant in sediment samples included those belonging to the families *Planococcaceae, Morganellaceae* (*Providencia rettgeri,), Morganellaceae (Morganella), Neisseriaceae* (*Pseudoglubenkiania), Peptostreptococcaceae,* and *Clostridiaceae (Clostridiaceae_1*). However, among water samples, 19 bacterial taxa were more significantly abundant, of the following families: Rhizobiaceae (*Agrobacterium, Ensifer*), *Pseudomonadaceae* (*Pseudomonas cf/mosselli/putida*), *Comamonadaceae* (*Kinneretia, Mitsuaria, Delftia acidovorans/lacustris/tsuruhatensis), Xanthomonadaceae, Enterobacteriaceae* (*Metakosakonia*), *Rhodanobacteraceae, Sphingobacteriaceae* (*Pedobacter, Nubsella), Alcaligenaceae* (*Achromobacter, Alcaligenaceae, Achromobacter aegrifaciens/insuavis/xylosoxidans*), *Dysgonomonadaceae* (*Dysgonomonas wimpennyi*), *Rhodospirillaceae (Novispirillum), Rikenellaceae* (*Acetobacteroides*) and, *Moraxellaceae (Acinetobacter baumanii, Acinetobacter calcoaceticus/pittii).*

Figure 4.27B illustrated that 19 bacterial taxa had relative abundances that were significantly different among samples collected in June and December. June samples revealed that 10 bacterial taxa were more abundant than its counterpart December samples. These abundant taxa *are Romboutsia, R. lituseburensis, Paraclostridium, Chromobacteriaceae, Aquitalea, Clostridium_sensu_stricto, Vogesella perlucida, Novispirillum, Acetobacteroides* and *Terrisporobacter,*while from December samples, 2 bacterial taxa (*Kinneretia* and *Acinetobacter calcoaceticus/pittii*) were identified as the most abundant bacteria in comparison to June samples. Twelve taxa were identified to be significantly present among samples collected from the designated source of warm spring and the designated source of the cold spring (Figure 4.27C). Among the source of the warm spring, bacterial taxa with the most significant abundance were *Pseudomonadaceae* (*Pseudomonas otitidis*), *Moraxellaceae* (*Acinetobacter baumannii,*

and *Acinetobacter nosocomialis*). However, bacteria taxa which were discovered as the most abundant from samples collected from the source of the warm spring include *Planococcaceae, Morganellaceae (Providencia rettgeri, Morganella), Caulobacteraceae (Brevundimonas naejangsanensis*) and *Bacteroidaceae (Bacteroides).*

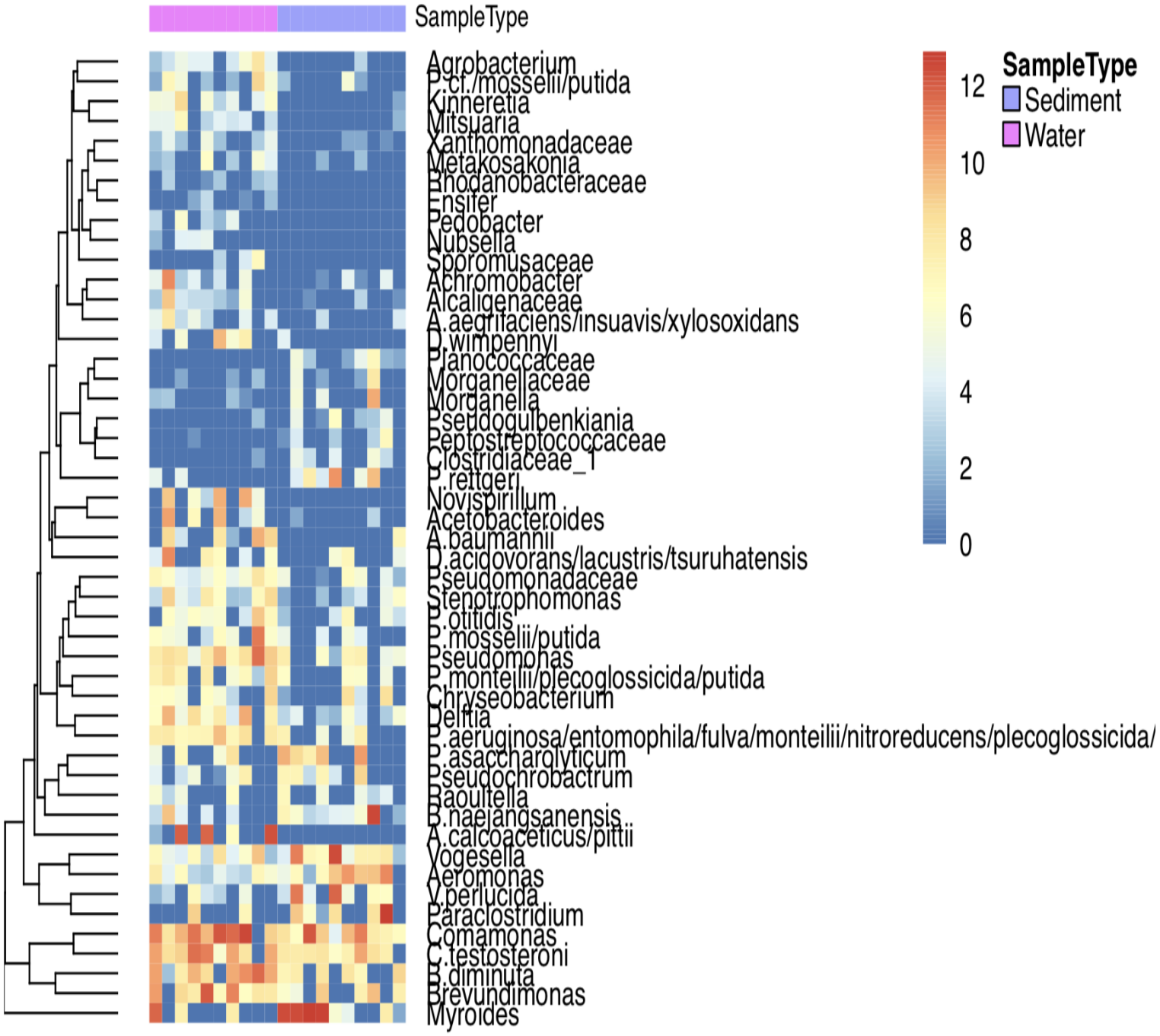


Figure 4.27A Bacterial taxa significantly abundant among sediment and water samples from Ikogosi warm springs, using metagenomeSeq (Adjusted P value ≤0.05)

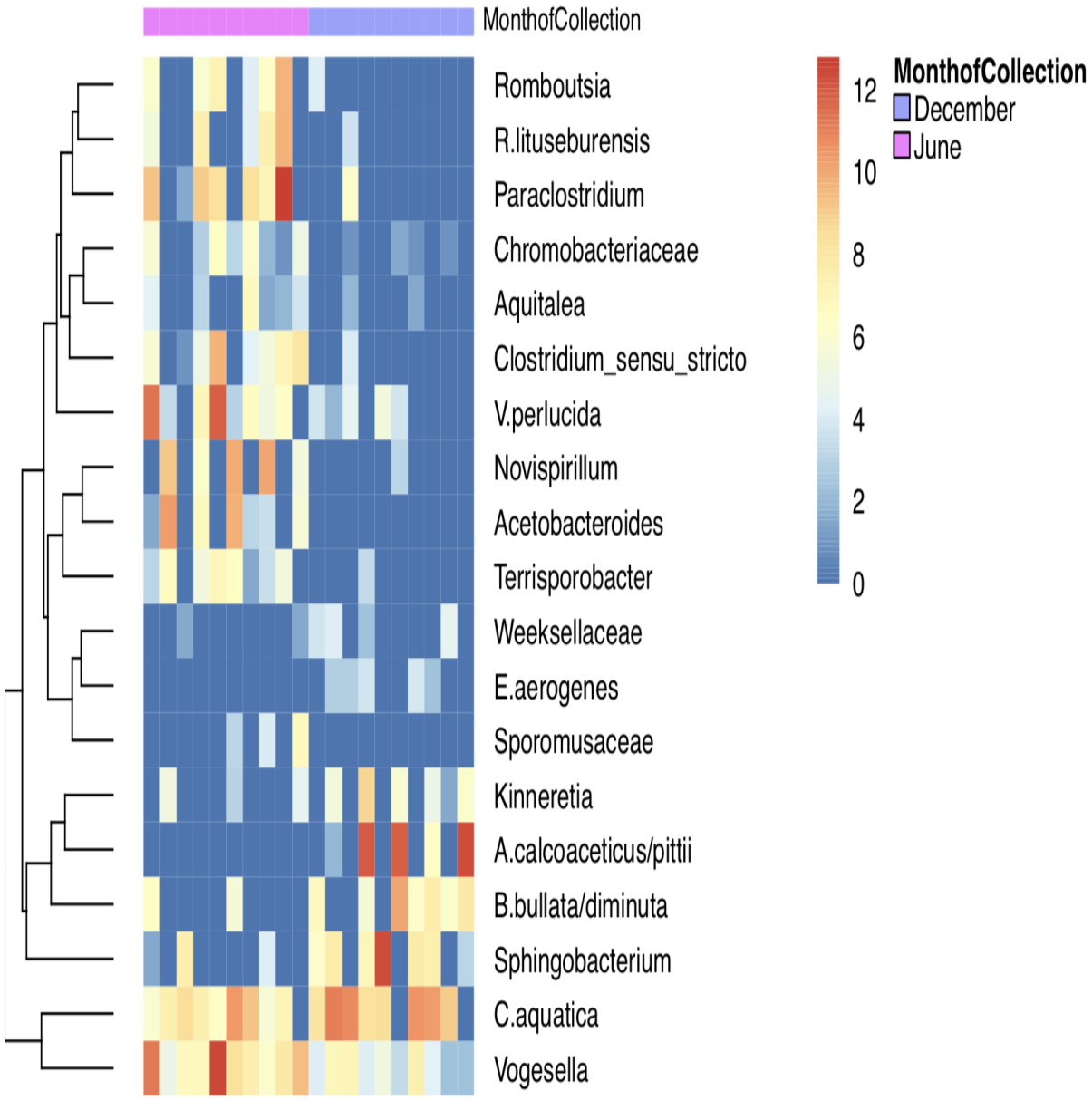


Figure 4.27B Bacterial taxa significantly abundant among samples collected at different sampling months from Ikogosi warm springs, using metagenomeSeq (Adjusted P value ≤0.05)

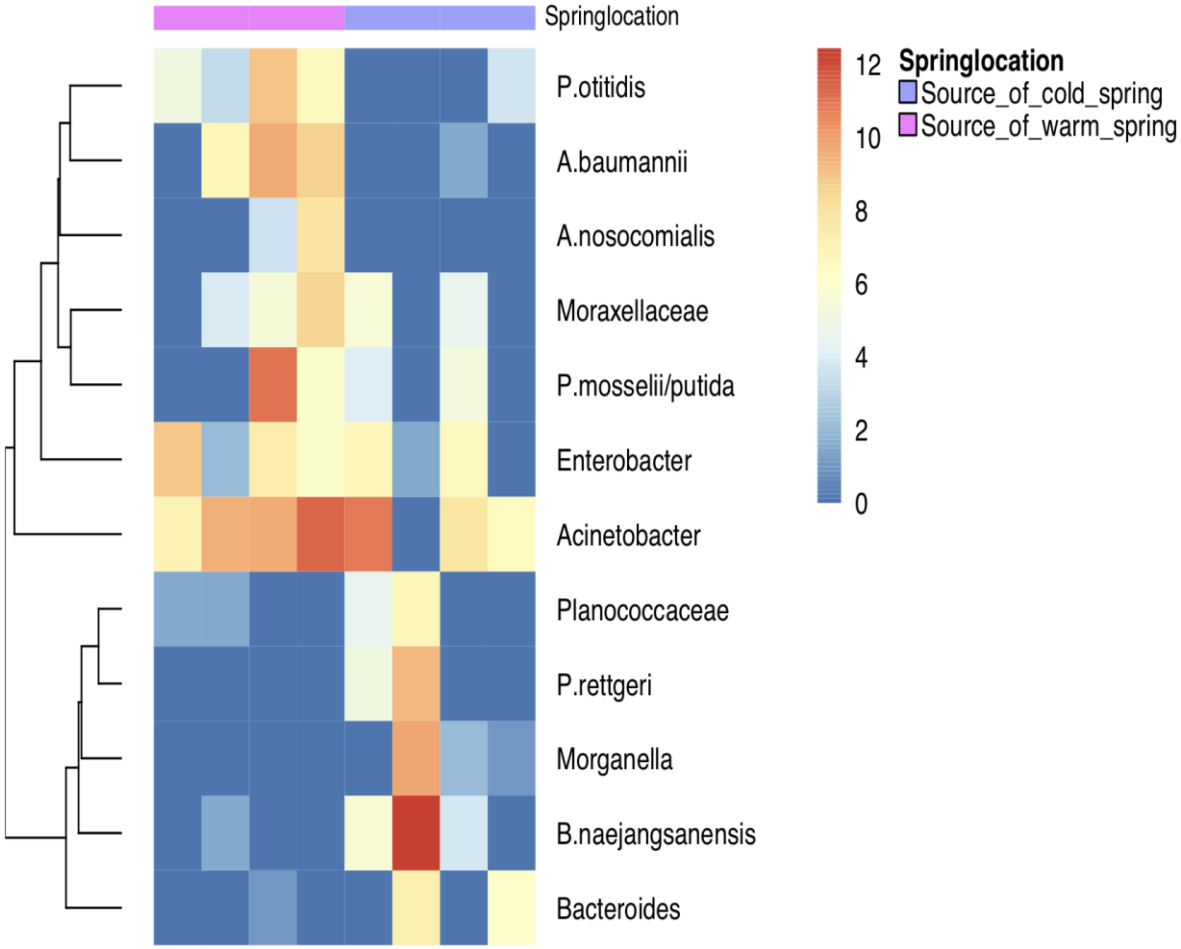


Figure 4.27C. Bacterial taxa significantly abundant among samples collected from the source of the warm spring and source of the cold spring, using metagenomeSeq (Adjusted P value ≤0.05)

4.16. Targeted metagenomic analysis of the fungal diversity of Ikogosi Warm Springs

The targeted metagenomic analysis of the fungal diversity of the sediment and water samples through the amplification of ITS1 of the fungal genome showed a decline in the number of the demultiplexed reads pre-processed for ITS1 dataset (Table 4.13). Quality-filtered forward reads suitable for downstream processing showed that a total of 796,572 raw reads were processed and 790,091were certified quality-filtered and fit for downstream processing. Table 4.15. shows the decline in the number of demultiplexed reads preprocessed for ITS1 dataset. After removal of primers and adapters from all reads, they were quality filtered and denoised (by applying error correcting models). Thereafter, denoised forward reads were processed to remove chimeric reads to yield high quality data suitable for downstream processing. From these quality-filtered reads, a total of 4,327 Amplicon Sequence Variants (ASVs) were derieved. Further filtering to remove unwanted taxa such as chloroplasts and mitochondria resulted in the removal of 1841 ASVs, and retaining 2,4,86 ASV which produced 2,486 taxa which were taxonomically ranked into Kingdom –Fungi, and their "Phylum, "Class", "Order", "Family", "Genus", "Species" as shown in Table 4.14.

With respect to sediment and water groupings, the genera discovered in water was more diverse (306) than those found in the sediment (275), the phylum of both sediment and water were highly similar, with sediment recording 4 phyla and 5 phyla in water samples. Comparing months of collection, December samples recorded 6 fungal phyla as present, unlike June samples that recorded 4 phyla. In addition, higher fungal diversity in terms of fungal genera was recorded in December samples having recorded 327 genera, while June samples revealed lower fungal genera (177). In terms of spring location, the highest phyla were discovered in two locations namely: midpoint of the cold spring (MPW) and the source of the cold spring (SC), having same number of phyla (6). The location with lowest fungal genera was at the midpoint of the warm spring recording 3 fungal phyla only. In terms of genera, the spring location with the lowest number of fungal genera was at the midpoint of the warm spring recording total genera of 73, which was closely followed by the source of the warm spring with total genera of 78. The highest fungal genera were recorded at the midpoint of the cold spring (307).

Table 4.13. Summary of ITS1 reads before and after quality preprocessing for each demultiplexed reads using DADA2 pipeline

Sample name	Raw reads	Filtered reads	Denoised reads	Non-chimeric reads
SW-water-dry	38955	38813	38811	38735
SW-water-wet	47109	47089	47085	47085
SW-soil-dry	38153	37952	37938	37938
SW-soil-wet	38430	38163	38089	38018
MPW-water-dry	38510	38369	38290	38290
MPW-water-wet	39546	39229	39228	39228
MPW-soil-dry	37856	37607	37605	37605
MPW-soil-wet	38321	38020	37995	37882
C-water-dry	39484	39365	39318	39318
C-water-wet	36565	36106	35952	35952
C-soil-dry	41335	41114	41054	40939
C-soil-wet	38390	38079	38072	38032
MPC-water-dry	39317	39127	38974	38974
MPC-water-wet	39780	39557	39490	39365
MPC-soil-dry	39273	39002	38988	38803
MPC-soil-wet	41145	40949	40920	40920
SC-water-dry	39071	38840	38445	38370
SC-water-wet	38986	38717	38713	38713
SC-soil-dry	41127	40892	40870	40870
SC-soil-wet	45319	45066	45054	45054
	796,672	**792,056**	**790,891**	**790,091**

Key: SW – Source of warm spring, MPW – Midpoint of warm spring, C – Confluence of the warm and cold spring, SC – source of cold spring, MPC – Midpoint of cold spring, Denoised F. – Forward denoised reads, Denoised R. – Reverse denoised reads

Table 4.14 Summary of Fungal Taxonomic Classification Identified Using UNITE Database for ITS1 Dataset

Sample groupings		FUNGAL TAXONOMIC CLASSIFICATION					
		Phylum	Class	Order	Family	Genus	Species
Sample Types	Water	5	24	66	161	306	370
	Sediment	4	14	33	58	275	91
Month of Collection	June	4	19	52	117	177	191
	December	6	25	70	171	327	400
Spring Location	Source of warm spring	4	14	33	58	78	91
	Midpoint of warm spring	3	14	26	58	73	81
	Confluence	5	18	50	125	219	237
	Midpoint of cold spring	6	23	62	148	270	307
	Source of cold spring	6	22	63	146	268	304
Sample types and Month of collection	June Sediment	4	19	52	117	177	191
	June Water	3	16	41	103	152	167
	December Sediment	6	20	52	127	217	248
	December Water	5	23	64	149	275	317

4.17 Taxonomic composition and relative abundance of fungal taxa present in sediment and water samples of Ikogosi Warm Springs

Samples from the spring were grouped into four categories namely: Sampling location, month of collection, the sample types, and the collection period of the sample types as shown below.

(A) Sampling location of the spring: categorised as source of warm spring (SW), midpoint of warm spring(MPW), confluence of warm and cold spring(C), source of cold spring (SC) and, midpoint of cold spring (MPC).

(B) Month of collection: Grouped as June and December.

(C) Sample types: Grouped as sediment and water

(D) Sample type andcollection period: categorised in to Sediment_June, Sediment_Dec, Water_June and Water_Dec.

Figure 4.28A – 4.28D shows the relative abundance of phylum across the four groups, namely: spring location, sample type, period of collection and sample type&collection period.

Figure 4.28A illustrated that the midpoint of the warm spring was mostly dominated by Ascomycota, and also recorded the highest relative abundance (83.71%) of the phylum with respect to other sample sites, the least relative abundance of Ascomycota (55.43%) was recorded at the source of the warm spring. However, the source of the warm spring had the largest relative abundance of Basidiomycota (44.51%) while the midpoint of the warm spring had the least abundance of Basidiomycota recorded as 16.28%. Neither Basidiobolomycota nor Monoblepharomycota were discovered from all sampled sites.

Figure 4.28B. shows the phyla identified in water and sediments of Ikogosi warm springs. It was observed that Ascomycota was the most dominant phyla, and showed highest relative abundance (74.64%) in water samples, while sediment samples had a high prevalence of Basidiomycota (34.27%) than in water samples. From sampled months (June and December), Ascomycota and Basidiomycota were the major phyla identified. Samples collected in June had relative abundance of 63.45% and 36.41% of Ascomycota and Basidiomycota respectively. While December samples recorded the dominance of Ascomycota as 79.28% and Basidiomycota as 20.01% (Figure 4.28C). Among water and sediment samples collected at different months, water samples

collected in June showed highest prevalence of Ascomycota (89.02%), with the lowest abundance of Ascomycota recorded in water samples collected in June (62.01%). However, other phyla such as Mortierellomycota, Chytridiomycota and Monoblepharomycota had extremely low relative abundance across all group of samples (Figure 4.28D).

Figure 4.29A – 4.29D shows the taxonomic bar plots of top 20 most abundant bacterial genera detected according to the following groupings:Sampling location of the spring (grouped as source of warm spring (SW), midpoint of warm spring(MPW), confluence of warm and cold spring(C), source of cold spring (SC) and, midpoint of cold spring (MPC)), month of collection (grouped as June and December), sample type (grouped as sediment and water) and Sampletype_collection period (categorised in to Sediment_June, Sediment_Dec, Water_June and Water_Dec).

Figure 4.29.A depicts the dominance of *Trichosporon, Meyerozyma* and *Condenascus* from samples collected at the source of the warm spring with a prevalent abundance of 39.27%, 25.38% and 22.79% respectively, while at the source of the cold spring, *Aspergillus* (18.33%), *Penicillium* (21.31%) and *Malassezia* (7.56%) were seen in large abundance. In Figure 4.29B, among the top 19 genera, sediment samples showed high relative abundance of *Meyerozyma* (27.47%), *Trichosporon* (15.22%) and *Aspergillus* (14.04%) than its corresponding water samples. However, some genera such as *Cladosporium* (5.34%), *Condenascus* (11.53%), and *Apiotrichum* (5.90%) were discovered in water samples in high prevalence than in sediment samples, where these phyla were present in less than 2.00%. Figure 4.29C shows the relative abundance plot from samples collected at different months, it was observed that June samples showed increased presence of *Aspergillus* (20.56%), *Trichosporon* (15.64%), *Condenascus* (8.73%), *Malassezia* (6.88%) and *Penicillium* (5.56%) than from December samples. In December samples, *Meyerozyma* (34.87%) and *Cladosporium* (4.16%) were discovered in higher abundance than from samples collected in June. Grouping according to sample types and collection period (Figure 4.29D) showed the dominance of *Meyerozyma* (56.40%) in water samples collected in December, while *Trichosporon* had the highest bacterial relative abundance (28.03%) in water collected in June. The dominance of *Malassezia* (11.83%) was pronounced in sediment samples collected in December, while *Condenascus* (19.42%) was the most dominant in sediment samples collected in June.

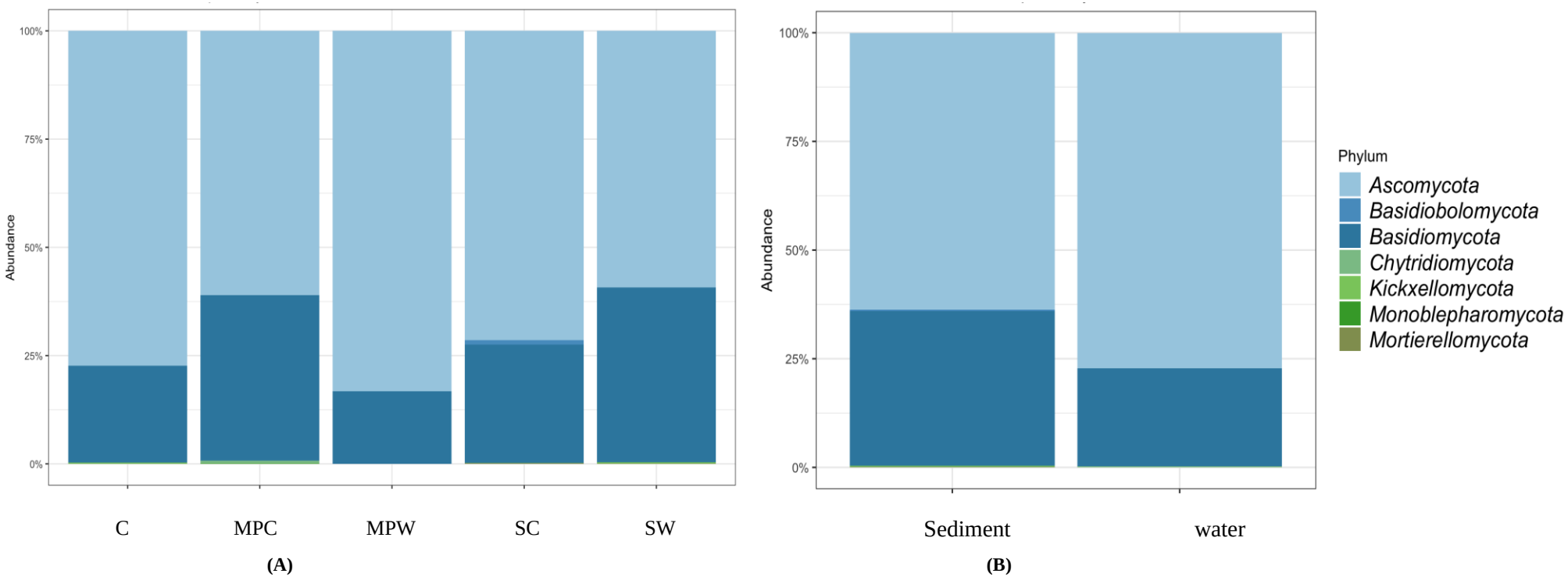
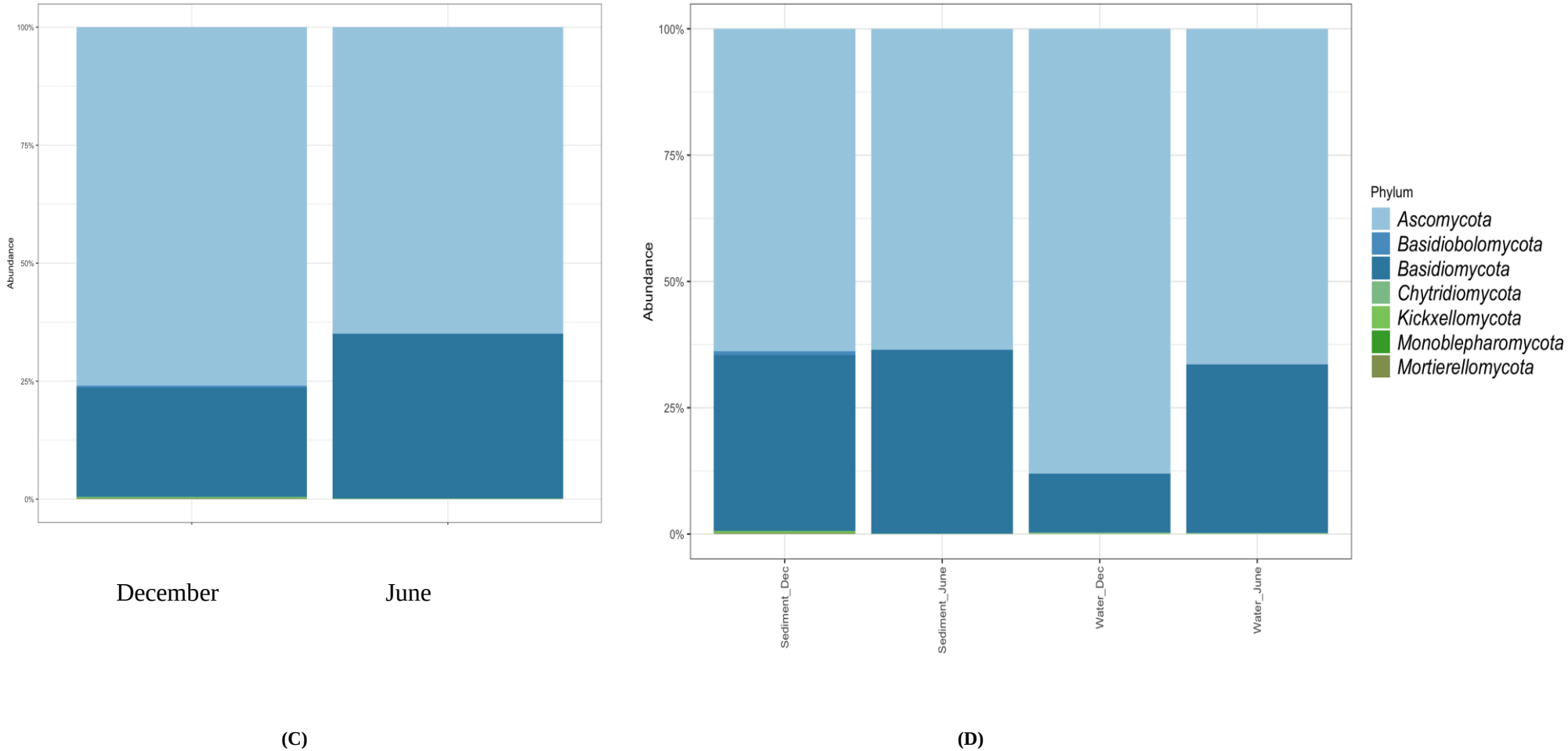


Figure 4.28. Taxonomic compositional bar plot of fungal phyla detected (A) at different sampling points (B) from sediment and water samples of Ikogosi warm springs



(C) **(D)**

Figure 4.28. Taxonomic compositional bar plot of fungal phyla detected at (C) different sampling months (D) at different months from sediment and water samples from Ikogosi warm springs

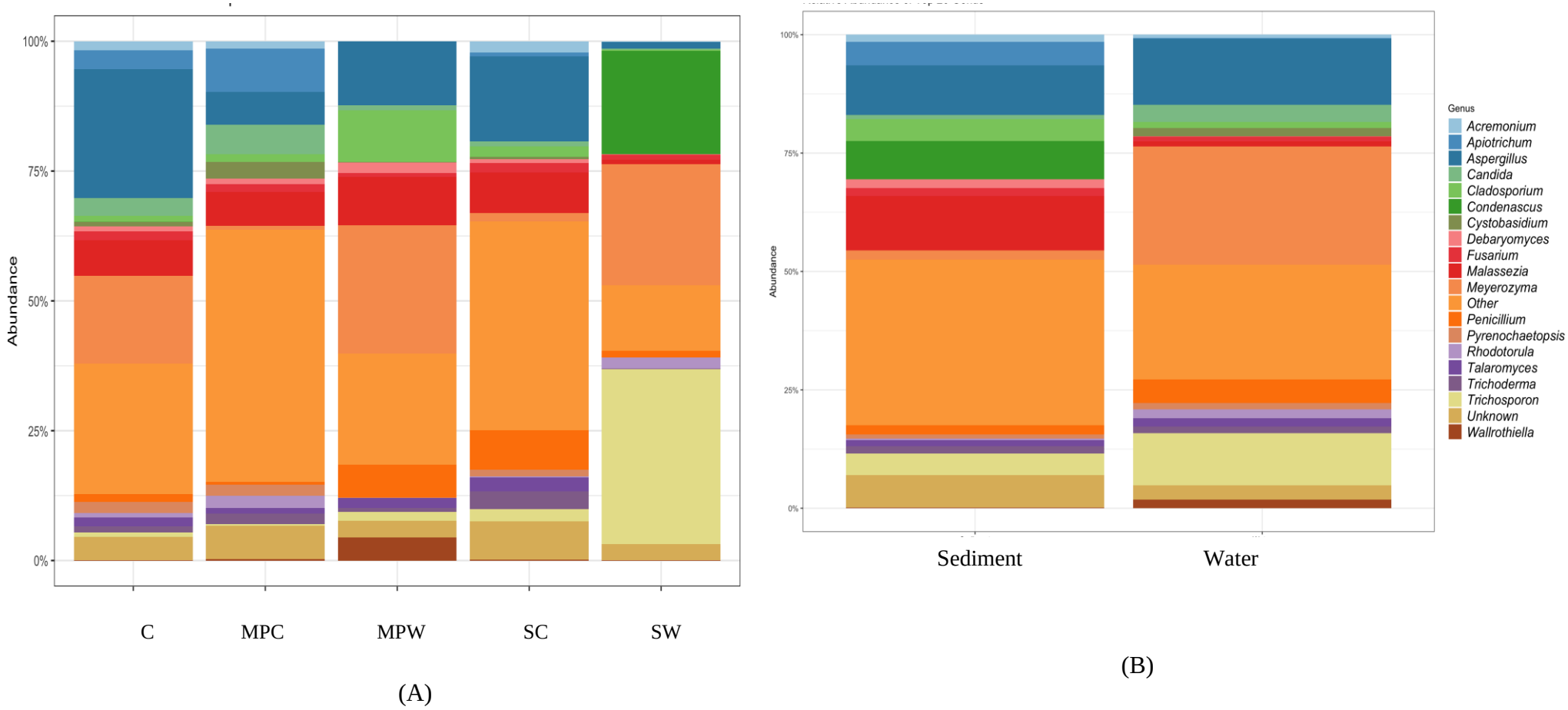
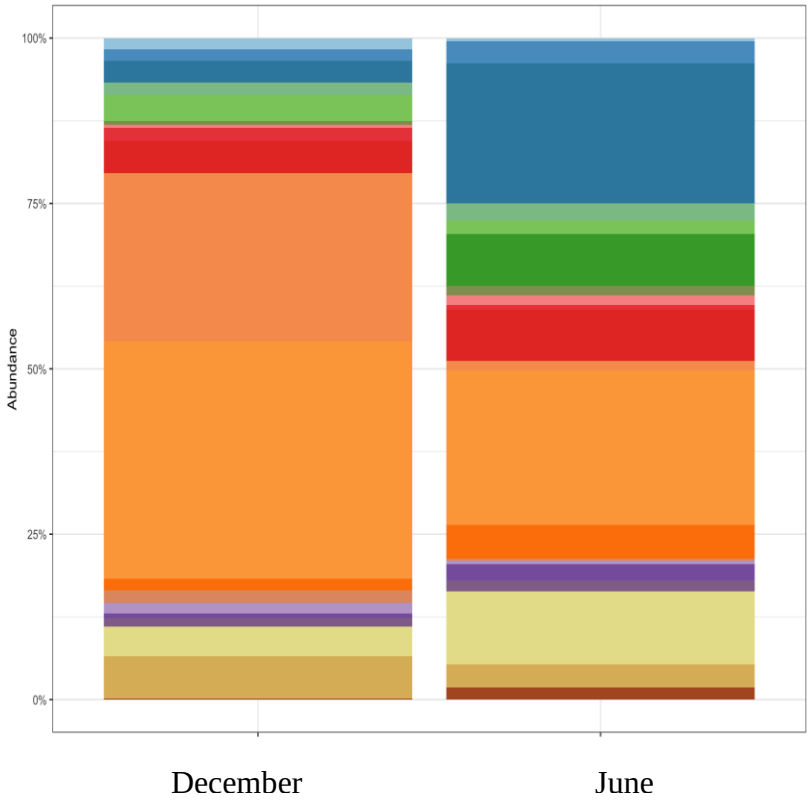
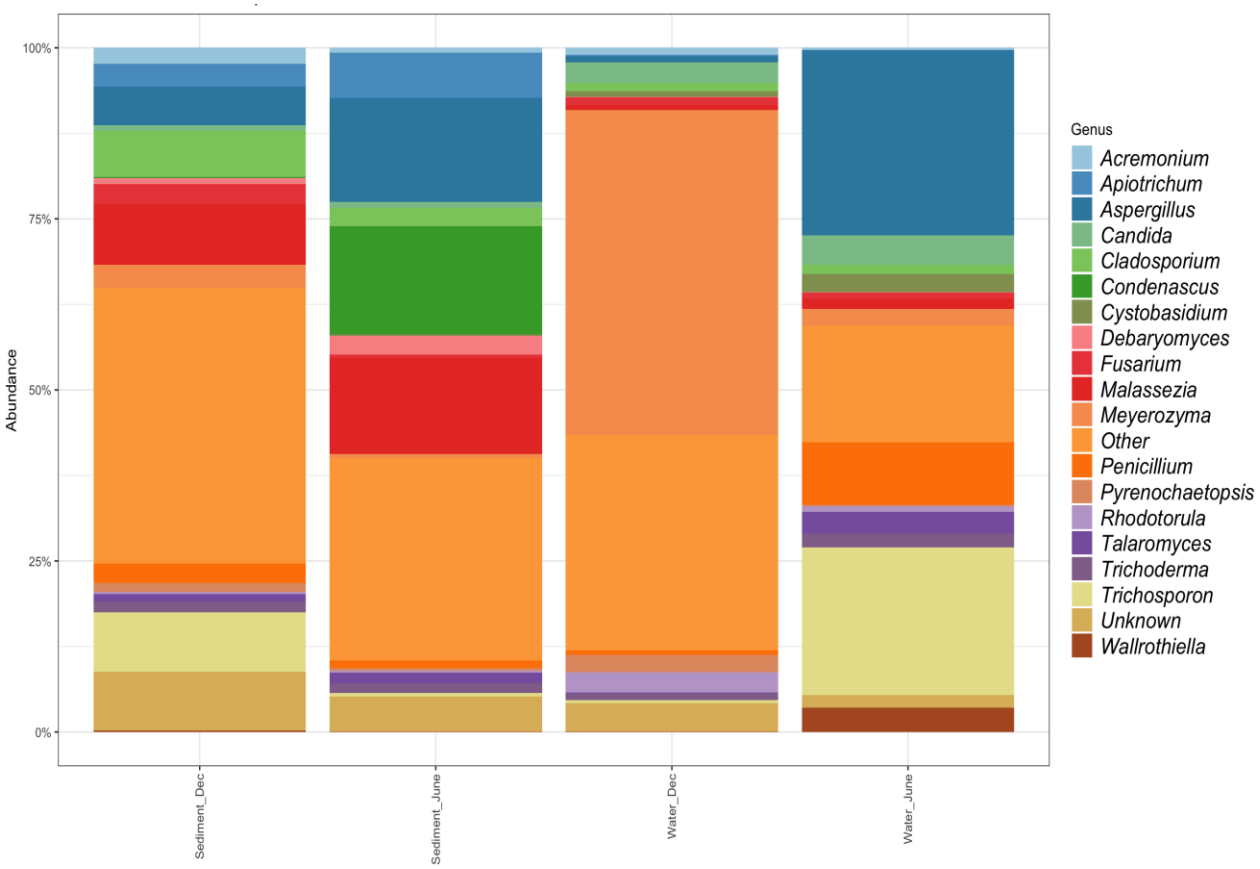


Figure 4.29 Taxonomic composition bar plot of 19 most abundant fungal genera detected (A)at different sampling points and (B) from sediment and water samples from Ikogosi warm springs



(C)



(D)

Figure 4.29 Taxonomic compositional bar plot of 19 most abundant fungal phyla detected (C) at different sampling months (D) at different months from sediment and water samples from Ikogosi warm springs

4.18 Boxplots of alpha diversity from samples of Ikogosi Warm Springs

The use of alpha diversity metrics (Shannon, Phylogenetic Diversity and Observed Species) depicted values which were either significantly or not significantly different. The alpha diversity boxplots of all named metrics from samples collected from different locations of the spring are shown in Figure 4.30 - 4.33. Shannon diversity revealed that richness of species from samples collected from the midpoint of the cold spring was significantly different from samples midpoint of the warm spring at $P<0.05$, (Figure 4.30A), similar deduction was made with a significant difference also observed from samples collected at the midpoint of the cold spring and from samples collected at the source of the warm spring. Observed specie metrics showed that there was a significant difference in the Operational Taxonomy Units discovered from samples collected from the midpoint of the cold spring than from those observed from samples collected from the midpoint of the warm spring. Similar significant difference was observed as well from samples collected from the midpoint of the warm spring from samples collected from the source of the cold spring (Figure 4.30B). Phylogenetic Diversity (PD) metrics, also recorded that there was a significant difference among the fungal population from samples collected from the midpoint of the cold spring and the midpoint of the warm spring (Figure 4.30C).

Alpha diversity boxplots of all metrics from different sample types (sediment and water) are shown in Figure 4.31A – 4.31C. Neither Shannon, Observed species nor phylogenetic Diversity metrics showed any dissimilarity in the evenness, richness and phylogenetic variation of the fungal population at $P<0.05$. Alpha diversity analysis from samples collected in December and June are shown in Figure 4.32A - 4.32C. Shannon diversity showed that similar evenness and richness of the same fungal population was present in both group of samples (Figure 4.32A). Observed species, depicted that there were similar taxa identified at both months of the year, with no significant difference (Figure 4.32B). Lastly, phylogenetic diversity metrics indicated that the fungal population observed in December was also the same observed in June and there was no phylogenetic difference among them (Figure4.32C). Boxplots of the alpha diversity of all named metrics from sediment and water samples collected at different months of the year are depicted in Figure 4.33A – 4.33C. None of the alpha diversity metrics depicted any statistically significant differences from all grouped samples.

4.19. Scattered plots of beta diversity from samples of Ikogosi Warm Springs

Beta diversity plots of the Bray-Curtis distance, weighted and unweighted distances of samples collected from five sampling parts of the spring are shown in Figure 4.34A – 4.34C. Bray-Curtis scattered plots showed that there were no clustering or dissimilarity found among group of samples collected from different points of the spring at $P<0.05$, where $P = 0.338$ (Figure 4.34A). Weighted Unifrac based on the relative abundance of species and phylogeny indicated that fungal communities present in sample from the midpoint of the cold spring were significantly different from the fungal community existing in samples collected from the source of the cold spring at $P < 0.05$, where $P = 0.022$ (Figure 4.34B). Unweighted Unifrac which calculated distances between samples by considering only the presence or absence of taxa also showed no difference in fungal community among samples collected from different locations of the springs ($P = 00538$, $P<0.05$) (Figure 4.34C).

In sediment and water samples (Figure4.35A-C), Bray-Curtis metrics, showed significant distinction of the fungal community observed in water samples and sediment samples, hence clustering of sediment sample away from water samples. ($P= 0.004$, $P <0.05$) (Figure 4.35A). Interestingly, Weighted Unifrac metrics indicated no distinct clustering and no significant difference in the fungal communities present in water and sediment samples ($P= 0.108$, $P <0.05$) (Figure 4.35B). Likewise, Unweighted Unifrac metrics, indicated no significant difference in the fungal structure of sediment and water samples ($P = 0.141$), at $P <0.05$(Figure 4.35C).

Figure 4.36A-C depicts fungal structure present in samples collected in June and December, using Bray-Curtis dissimilarity distance, $P = 0.057$, hence no statistical difference in their fungal communities of samples collected from both months ($P<0.05$) (Figure 4.36A). Figure 4.36B shows the relationship between samples of June and December, using the weighted Unifrac method. There was no significant difference between these group of samples at $P = 0.219$, while Unweighted unifrac metrics also showed non-significant fungal communities ($P = 0.109$) from both group of samples at $P<0.05$. (Figure 4.36C).

Sediment and water samples collected at different months are shown in Figure 4.37A-C (December and June). Figure 4.37A shows the Bray-Curtis approach in checking for

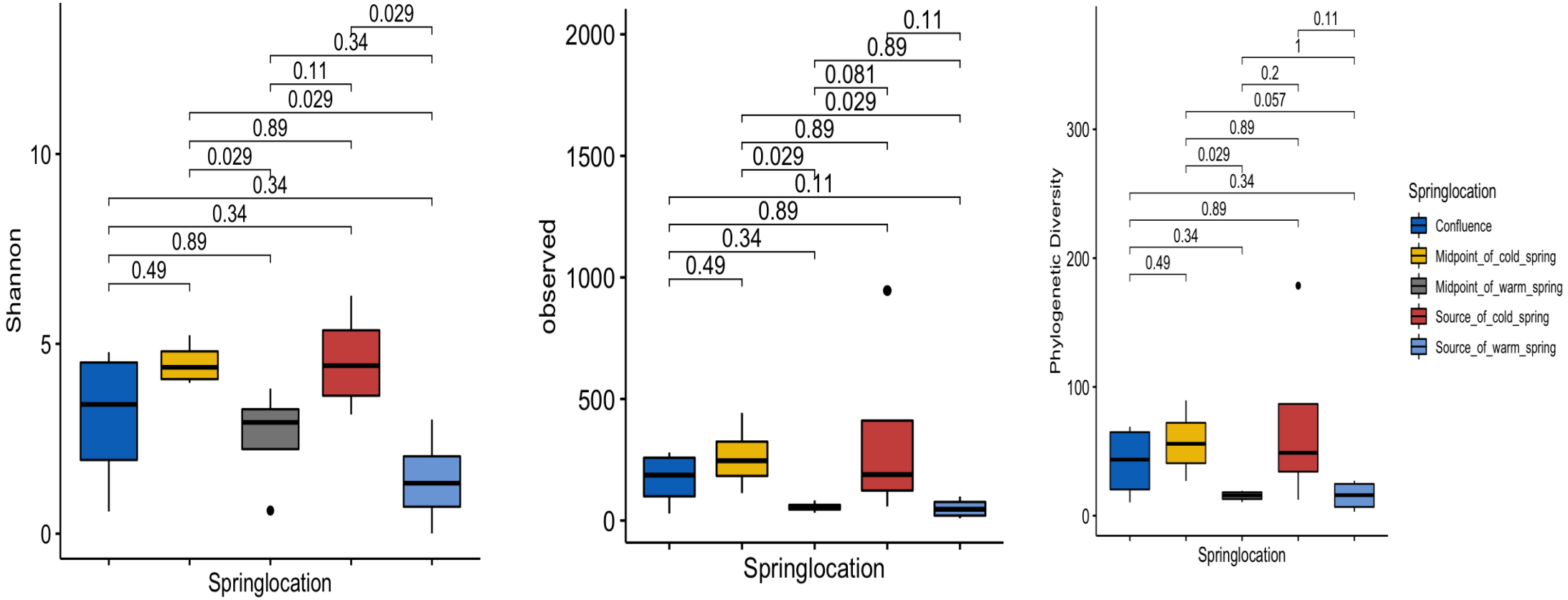
fungal community among these group of samples. There was a clear separation of water samples collected in June from other group of samples. This separation was highly significant with P = 0.002. However, there was no indication of fungal community difference among these samples using weighted Unifrac (P = 0.208) (Figure 4.37B). While Figure 4.37C also depicts the beta diversity among these group of samples using unweighted unifrac metrics, with statistical results indicating no difference in the fungal structure present among these samples, P = 0.142 at P<0.05.

4.20 Core fungal members from samples collected from Ikogosi Warm Springs

In this study, core members were defined as microbial ASVs found across 80% of grouped samples (80% core threshold). The Venn diagram depicting unique and common core members revealed shared taxa/ASVs/ core members among four groups were (sample type, month of sample collection, spring location, and sample types collected at different months of the year) represented as overlapping areas in the circles of the Venn diagram. Unique core ASVs of each group were represented as non-overlapping regions of the Venn diagram as shown in Figure 4.38A-D respectively.

Figure 4.38A showed that 2 fungal ASVs were shared among water and sediment samples, occupying 50% of all ASVs discovered at 80% core threshold. Figure 4.38B also depicted two taxa (66.67%) as shared members discovered among December and June samples, however, December samples had no identified unique taxa at 80% threshold core. Figure 4.38C revealed that no taxa was identified as core member (shared member) for all sampled sites of the spring, setting the core microbiome threshold at 80%. From sediment and water samples collected in the months of December and June, no fungal taxa were identified to be present in 80% of all samples, as shared members. However, 2 fungal taxa were identified as core members among sediments collected in December, sediments collected in June and water sampled in December which accounts for 22.22% of all core members of the group. In addition, one fungal taxa was identified as a core member of sediment collected in June and water sampled in December (Figure 4.38D).

Fungal names of unique and shared core taxa present among these groups are tabulated in Tables 4.15A – D respectively.



(A) **(B)** **(C)**

Figure 4.30: Fungal alpha diversity box plots of samples collected at different sampling points of Ikogosi warm springs, using (A) Shannon (B) Observed species and (C) Phylogenetic Diversity index (P <0.05)

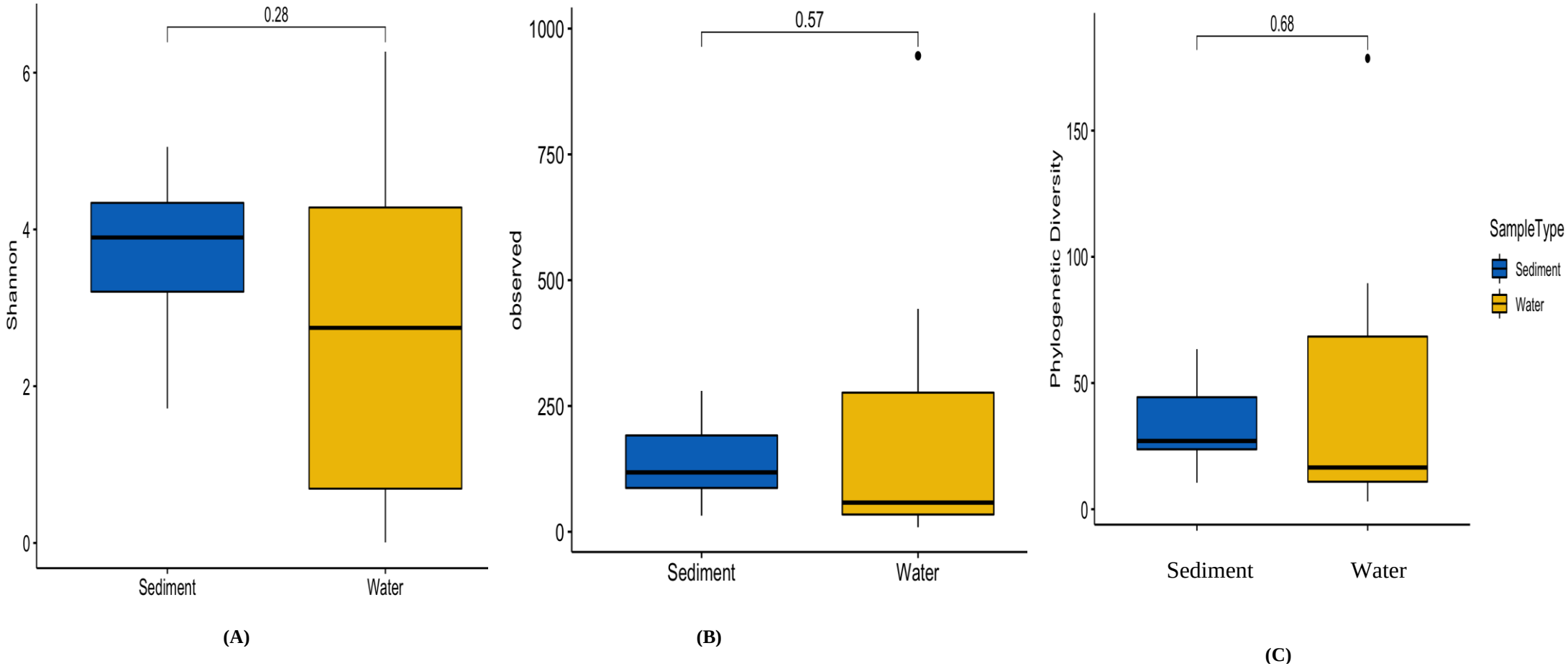


Figure 4.31: Fungal alpha diversity boxplots of sediment and water samples of Ikogosi warm springs using (A) Shannon's (B) Observed species and (C) Phylogenetic Diversity index (P<0.05)

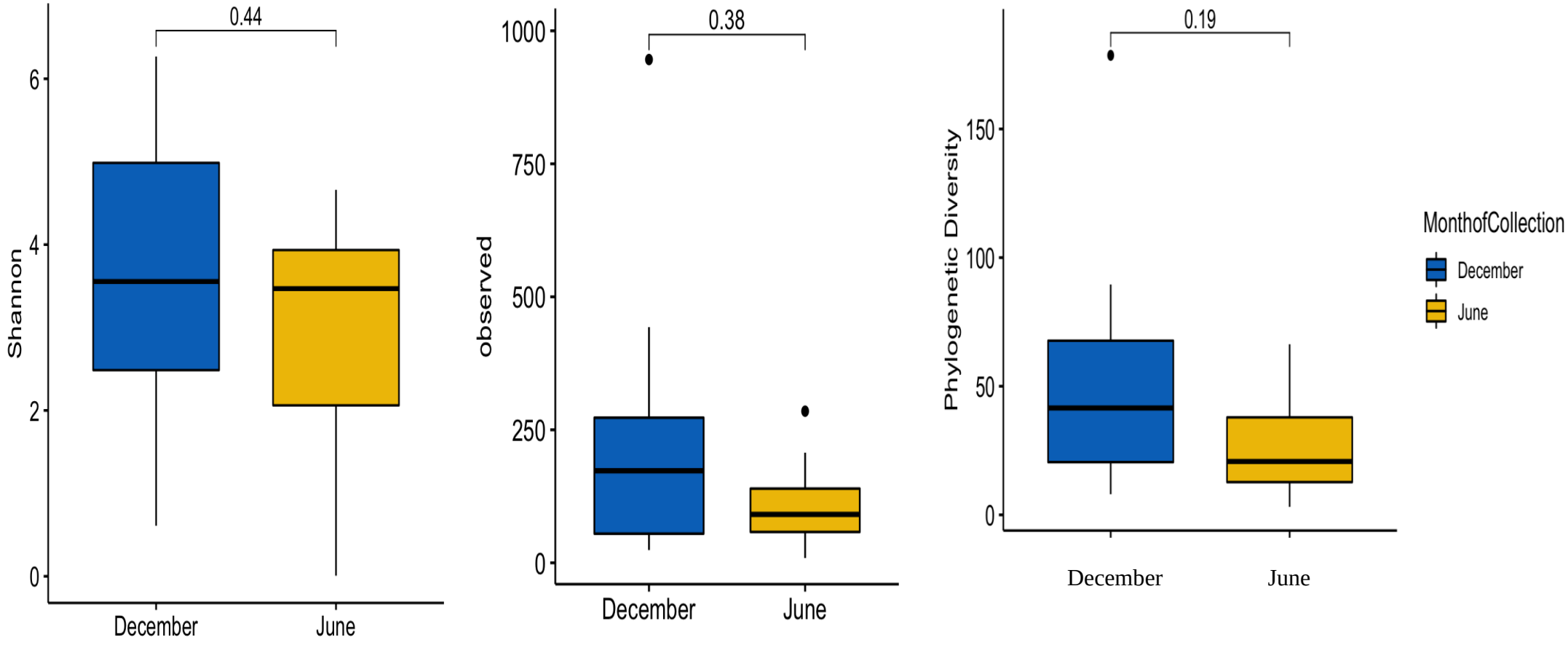


Figure 4.32. Fungal alpha diversity boxplots of samples collected at different sampling months from Ikogosi warm springs using (A) Shannon (B) Observed species and (C) Phylogenetic index (P<0.05)

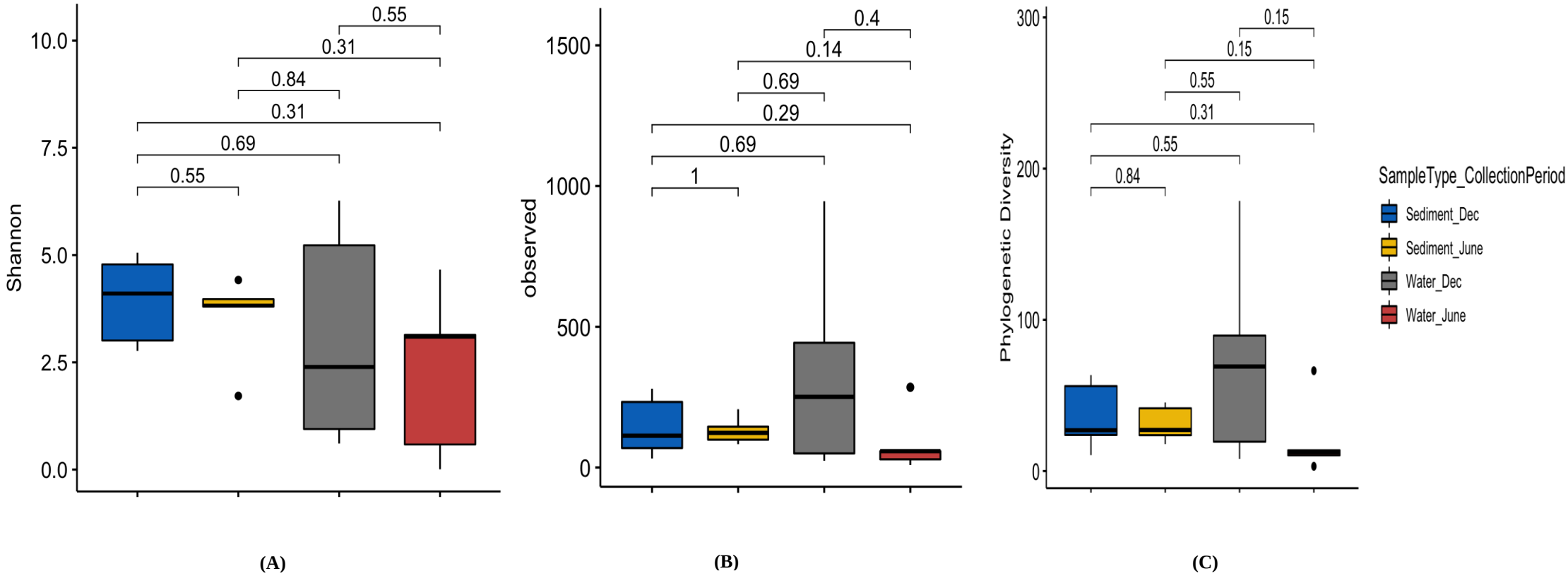
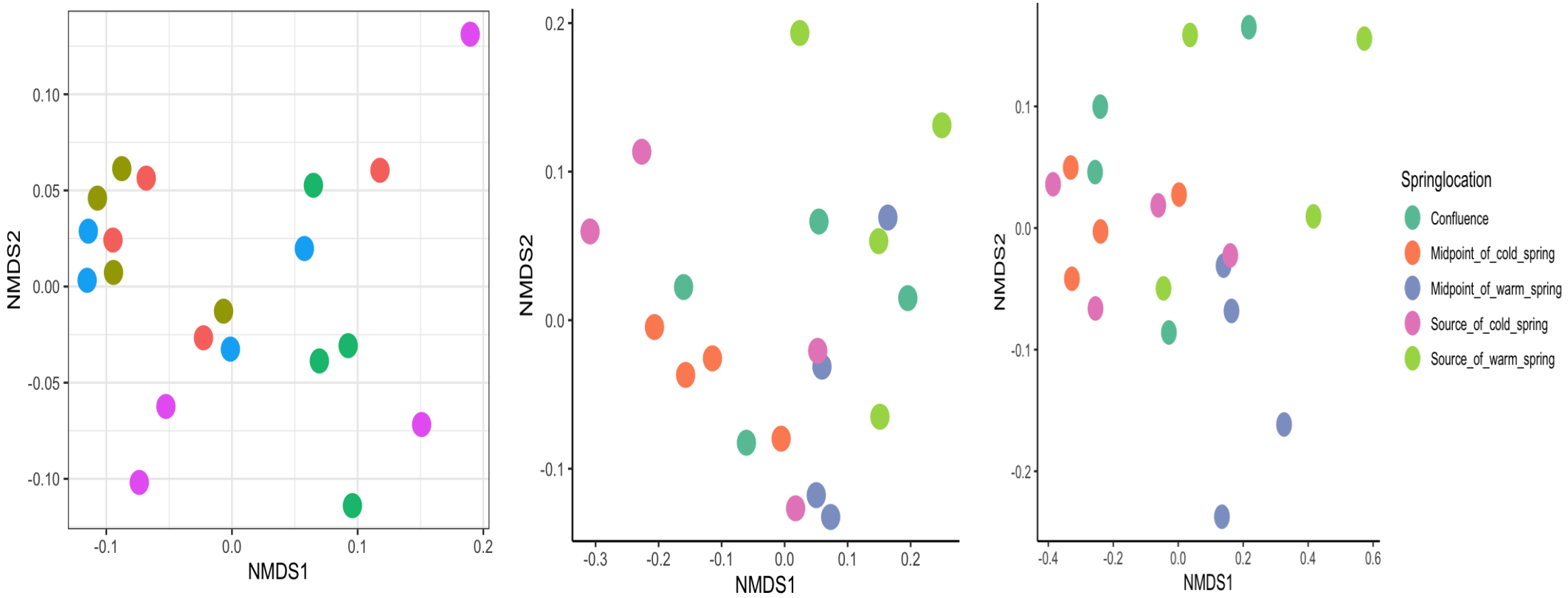
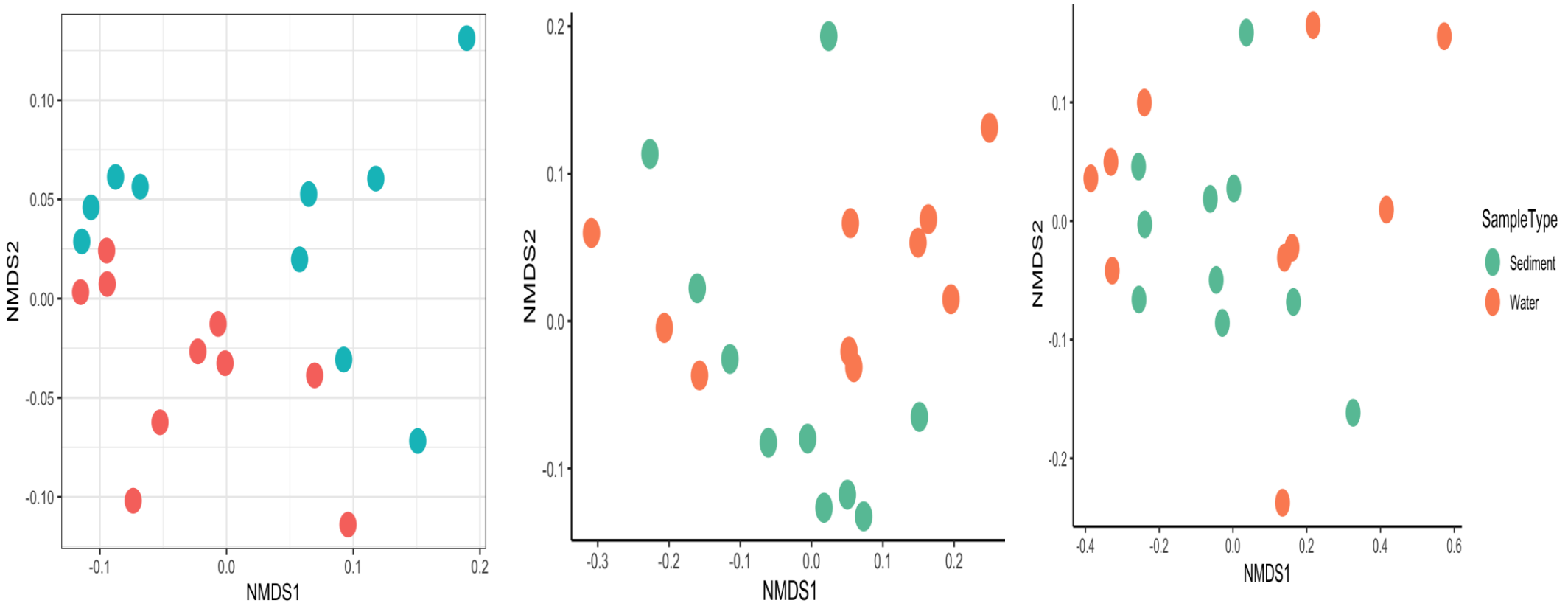


Figure 4.33 Fungal alpha diversity boxplots of sediment and water samples collected at different sampling months from Ikogosi warm springs using (A) Shannon, (B) Observed species and (C) Phylogenetic Diversity index (P<0.05)



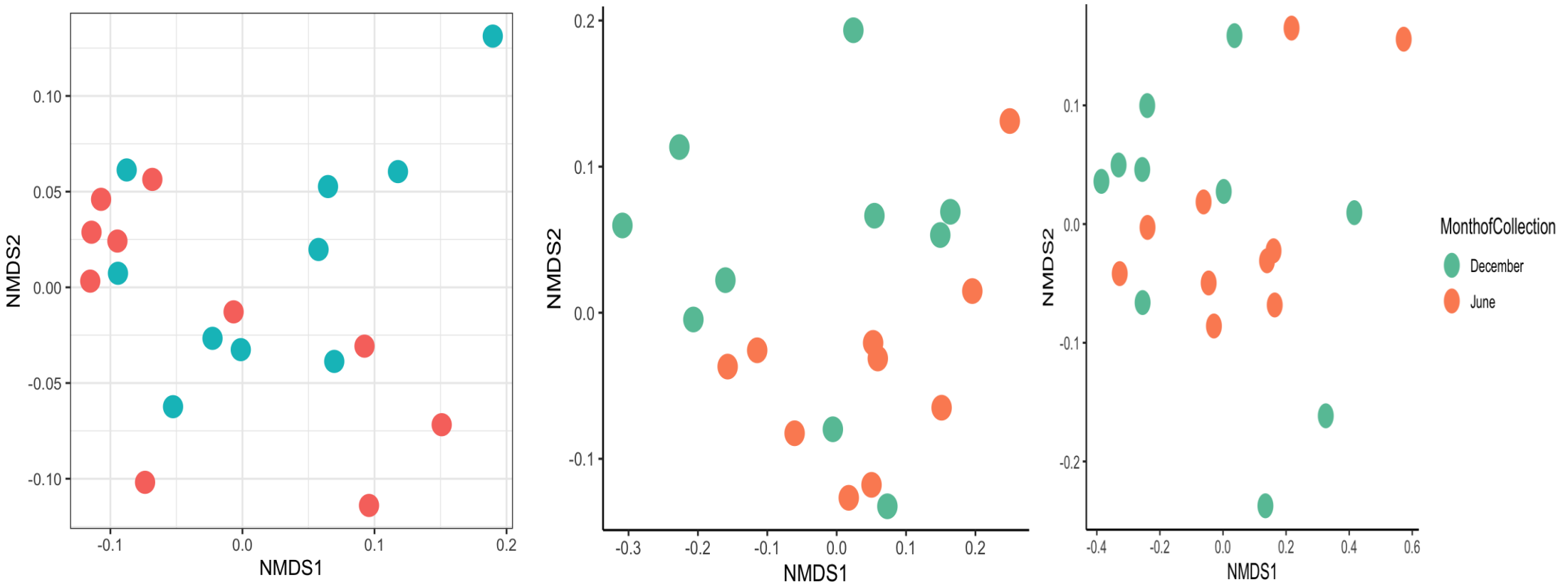
(A) **(B)** **(C)**

Figure 4.34. Fungal beta diversity plots of samples collected at different sampling points of Ikogosi warm springs using (A) Bray-Curtis (B) weighted and (C) unweighted unifrac distance metrics at P<0.05, visualised via NMDS



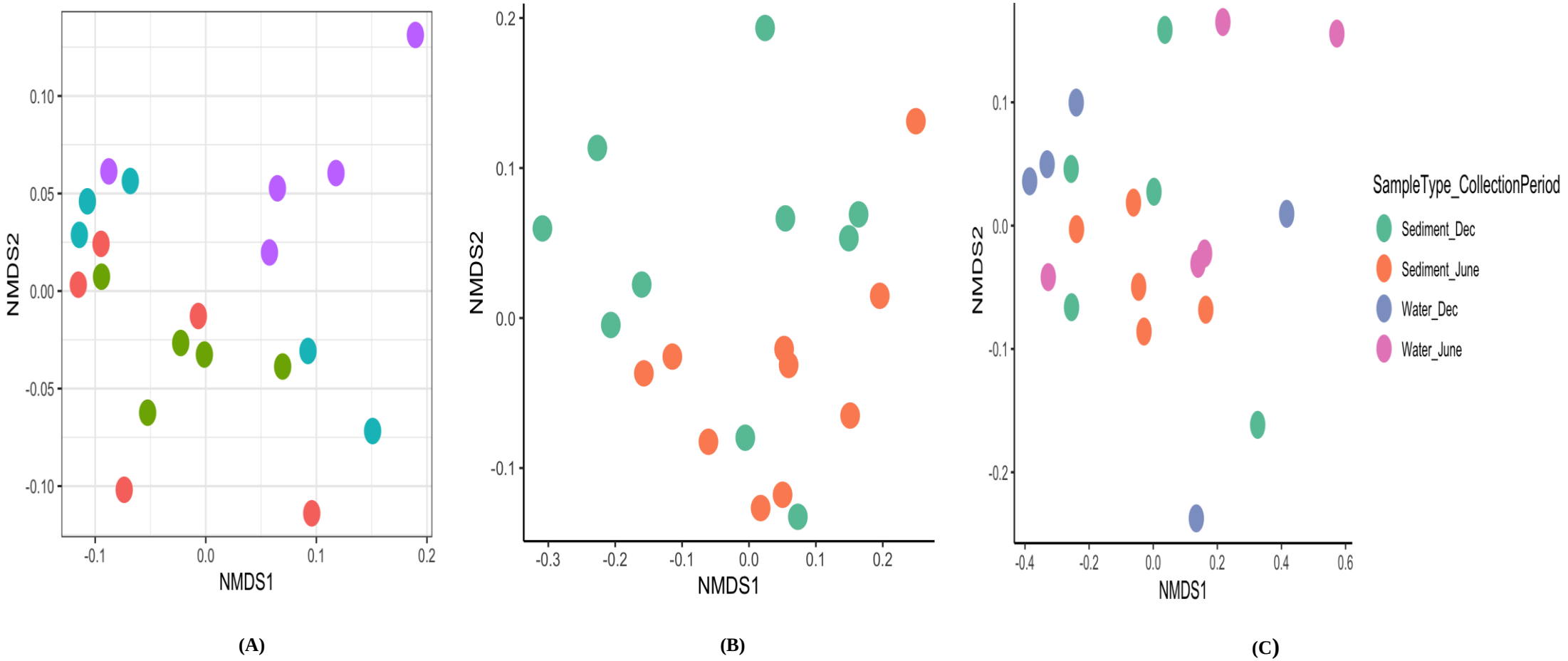
(A) **(B)** **(C)**

Figure 4.35. Fungal beta diversity plots of sediment and water samples of Ikogosi warm springs , using (A) Bray-Curtis (B) weighted and (C) unweighted unifrac distance metrics at P <0.05, visualised via NMDS



(A) **(B)** **(C)**

Figure 4.36. Fungal beta diversity plots of samples collected at different sampling months from Ikogosi warm springs, using (A) Bray-Curtis (B) weighted and (C) unweighted unifrac distance metrics at P < 0.05, visualised via NMDS



(A) **(B)** **(C)**

Figure 4.37 Fungal beta diversity plots of sediment and water samples collected at different sampling months from Ikogosi warm springs, using (A) Bray-Curtis (B) weighted and (C) unweighted unifrac distance metrics at P < 0.05, visualised via NMDS

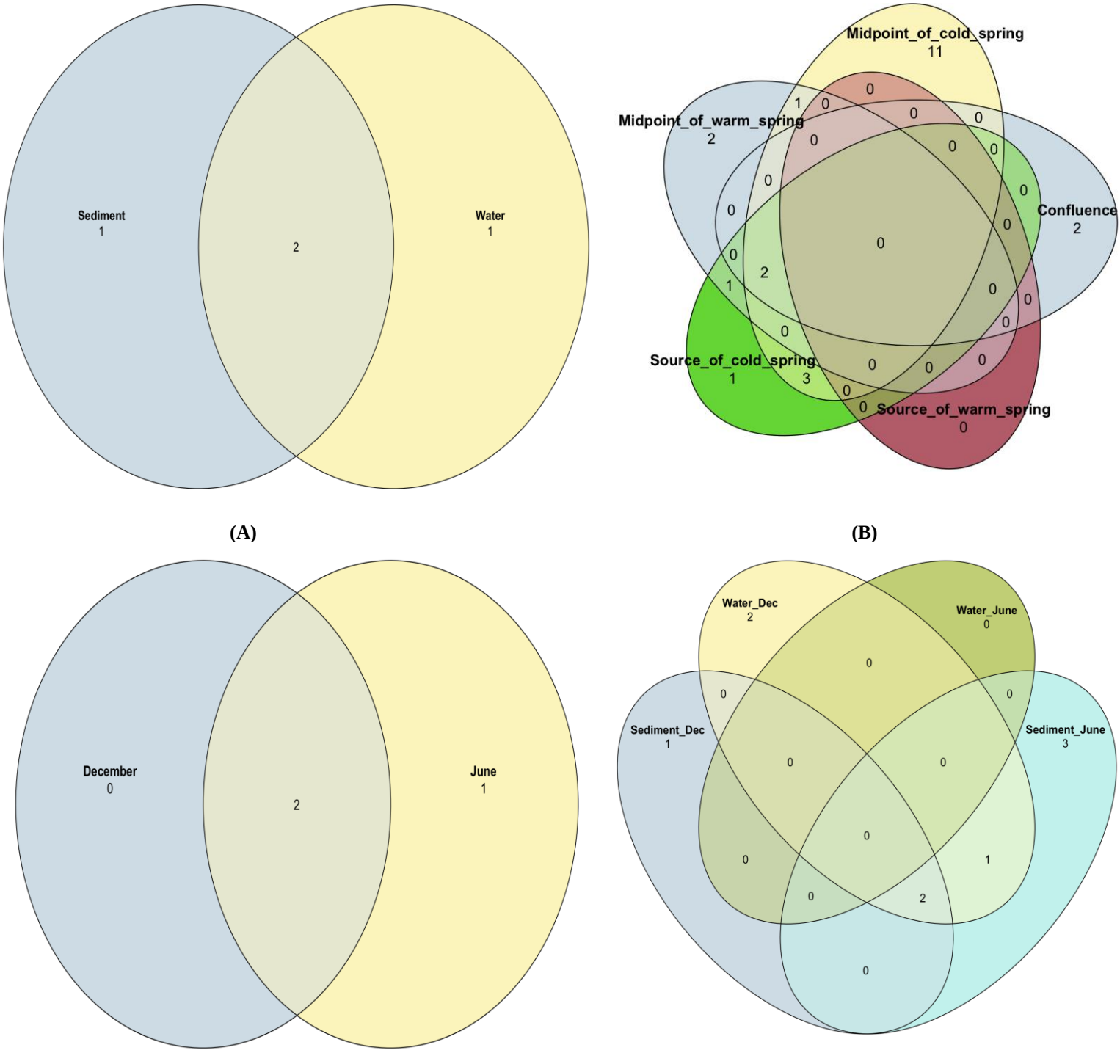


Figure 4.38. Venn diagram depicting number of shared fungal taxa/ASV and unique taxa/ASVs among (A) sediment and water samples (B) samples collected at different sampling months (C) samples collected at different sampling months (D) sediment and water samples collected at different sampling months from Ikogosi warm springs

Table 4.15A. Unique and shared core members from sediment and water samples of Ikogosi Warm Springs

SEDIMENT	
ASV- ID	**Unique Taxa**
ASV 158	*Plectospharellaceae*
WATER	
Unique Taxa	
ASV 4	*Penicillium hetheringtonii*
SHARED TAXA	
ASV 16	*Aspergillus penicillioides*
ASV 65	*Malassezia restricta*

Table 4.15B. Unique and shared core members depicted at different sampling months from Ikogosi Warm Springs

	JUNE	
S/NO	ASV- ID	**Unique Taxa**
1	ASV 3	*Aspergillus* sp.
	DECEMBER	
	Unique Taxa	
	None	
	SHARED TAXA	
1	ASV 16	*Aspergillus penicillioides*
2	ASV 65	*Malassezia restricta*

Table 4.15C. Unique and shared core members depicted at different sampling points from Ikogosi Warm Springs

SOURCE OF WARM SPRING		
S/NO	**Unique Taxa**	
1	None	
MIDPOINT OF WARM SPRING		
	Unique Taxa	
1	ASV 3	*Aspergillus* sp.
2	ASV 50	*Malassezia* sp.
CONFLUENCE		
	Unique Taxa	
1	ASV 13	*Stachybotryaceae*
2	ASV 678	*Malassezia globosa*
MIDPOINT OF COLD SPRING		
	Unique Taxa	
1	ASV 15	*Wallrothiella gmelinae*
2	ASV 36	*Nectriaceae*
3	ASV 57	*Cladosporium* sp.
4	ASV 62	*Rhodotorula mucilaginosa*
5	ASV 72	*Cladosporium halotolerans*
6	ASV 115	*Trichoderma* sp.
7	ASV 118	*Acremonium*
8	ASV 155	*Coprinellus disseminatus*
9	ASV 158	*Plectosphaerellaceae*
10	ASV 270	*Paramicrothyrium*
11	ASV 333	*Alloconiothyrium aptrootii*
SOURCE OF COLD SPRING		
	Unique Taxa	
1	ASV 116	*Nigrospora oryzae*
SHARED TAXA		
1	ASV 4	*Penicillium hetheringtonii*
2	ASV 9	*Cladosporium delicatum*
3	ASV 16	*Aspergillus penicillioides*
4	ASV 26	*Pyrenochaetopsis tabarestanensis*
5	ASV 35	*Fusarium* sp.
6	ASV 65	*Malassezia restricta*
7	ASV 260	*Ganoderma*

Table 4.15D. unique and shared core members depicted in sediment and water samples collected at different sampling months from Ikogosi Warm Springs

JUNE SEDIMENT SAMPLES		
S/NO	**Unique Taxa**	
1	ASV24	*Aspergillus gracilis*
2	ASV 72	*Cladosporium halotolerans*
JUNE WATER SAMPLES		
	Unique Taxa	
2	ASV 25	*Trichoderma scalesiae*
DECEMBER SEDIMENT SAMPLES		
	Unique Taxa	
1	ASV 158	*Plectosphaerellaceae*
DECEMBER WATER SAMPLES		
	Unique Taxa	
1	ASV 1	*Meyerozyma guilliermondi*
SHARED TAXA		
1	ASV 3	*Aspergillus* sp.
2	ASV 4	*Penicillium hetheringtonii*
3	ASV 9	*Cladosporium delicatum*
4	ASV 16	*Aspergillus penicillioides*
5	ASV 65	*Malassezia restricta*

4.21 Differential abundance testing of fungal taxa between two groups of samples from Ikogosi Warm Springs

Samples were grouped into two, namely: sediment versus water samples, June versus December samples and source of warm spring versus source of cold spring samples, in order to identify differentially abundant bacterial taxa. Figure 4.39A-D depicts various taxa using metagenomeSeq to identify bacterial taxa that were differently abundant among compared groups of sediment versus water samples, June and December samples and source of warm spring versus source of cold spring samples respectively.

Figure 4.39A revealed that the relative abundances of 30 fungal taxa significantly differed between sediment and water samples. Twelve fungal taxa that were more abundant in sediment samples included those belonging to the families; *Malasseziaceae (Malassezia obtusa, Malassezia slooffiae, Malassezia cuniculi), Trichocomaceae* (*Aspergillus halophilicus, Aspergillus gracilis*), *Mycosphaerellaceae (Ramularia), Clavicipitaceae* (*Metarhizium majus*), *Malasseziaceae, Nectriaceae (Fusarium keratoplasticum), Ophiocordycipitaceae (Purpureocillium lilacinum), Wallemiaceae* (*Wallemia tropicalis*), and *Nectriaceae* (*Gibberella intricans*). However, among water samples, 10 fungal taxa were more significantly abundant, namely: *Paraconiothyrium, Torula, Cerrena unicolor, Cystobasidium, Colletotrichum, Fusarium, Candida hyderabadensis, Trichoderma scalesiae, Nigrospora oryzae and Rhodotorula paludigena.*

Figure 4.39B illustrated that out of 34 significantly abundant fungal taxa, 20 of them were more significantly abundant in December samples than in June samples, namely: *R. solani, F. waltergamsii, Sporocadaceae, Xylariaceae, Acrocalymma, Herpotrichiellaceae, Roussoella mukdahanensis, Acremonium polychromum, A.fucatum, Pleurotheciaceae, Roussoella neopustulans, Prunus scoparia, Stachybotrys aurantia, Spiromyces aspiralis, Stilbocrea walteri, Roussoella, Cladosporium sphaerospermum* and *Ceriporia lacerata.*

Only three taxa were identified to be significantly present among samples collected from the designated source of warm spring and the designated source of the cold spring (Figure 4.39C). However, only *Malassezia cuniculi* and *Aspergillus gracilis* were more significantly abundant at the source of the cold spring.

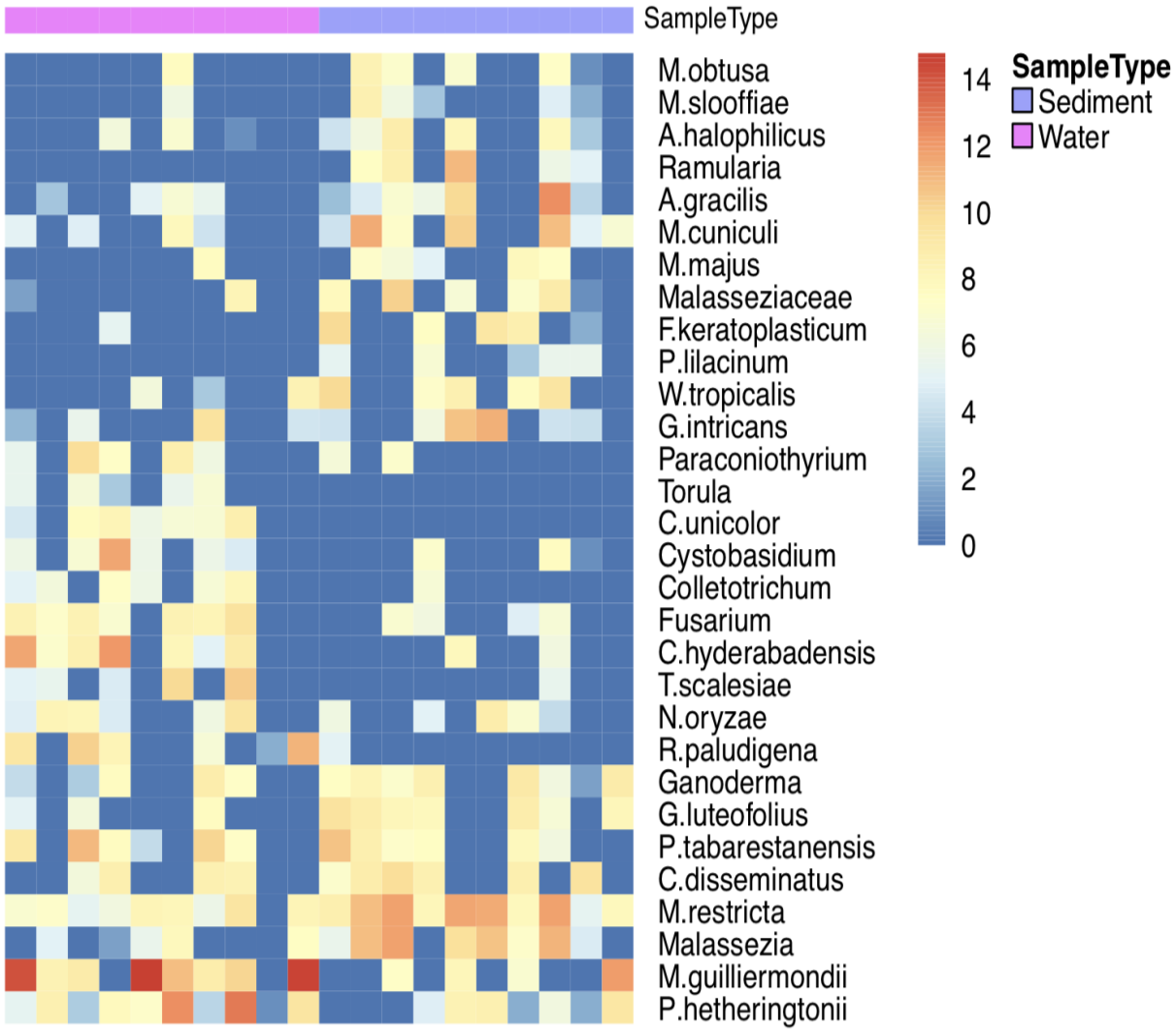


Figure 4.39A. Fungal taxa significantly abundant among sediment and water samples from Ikogosi warm springs, using metagenomeSeq (Adjusted P value ≤0.05)

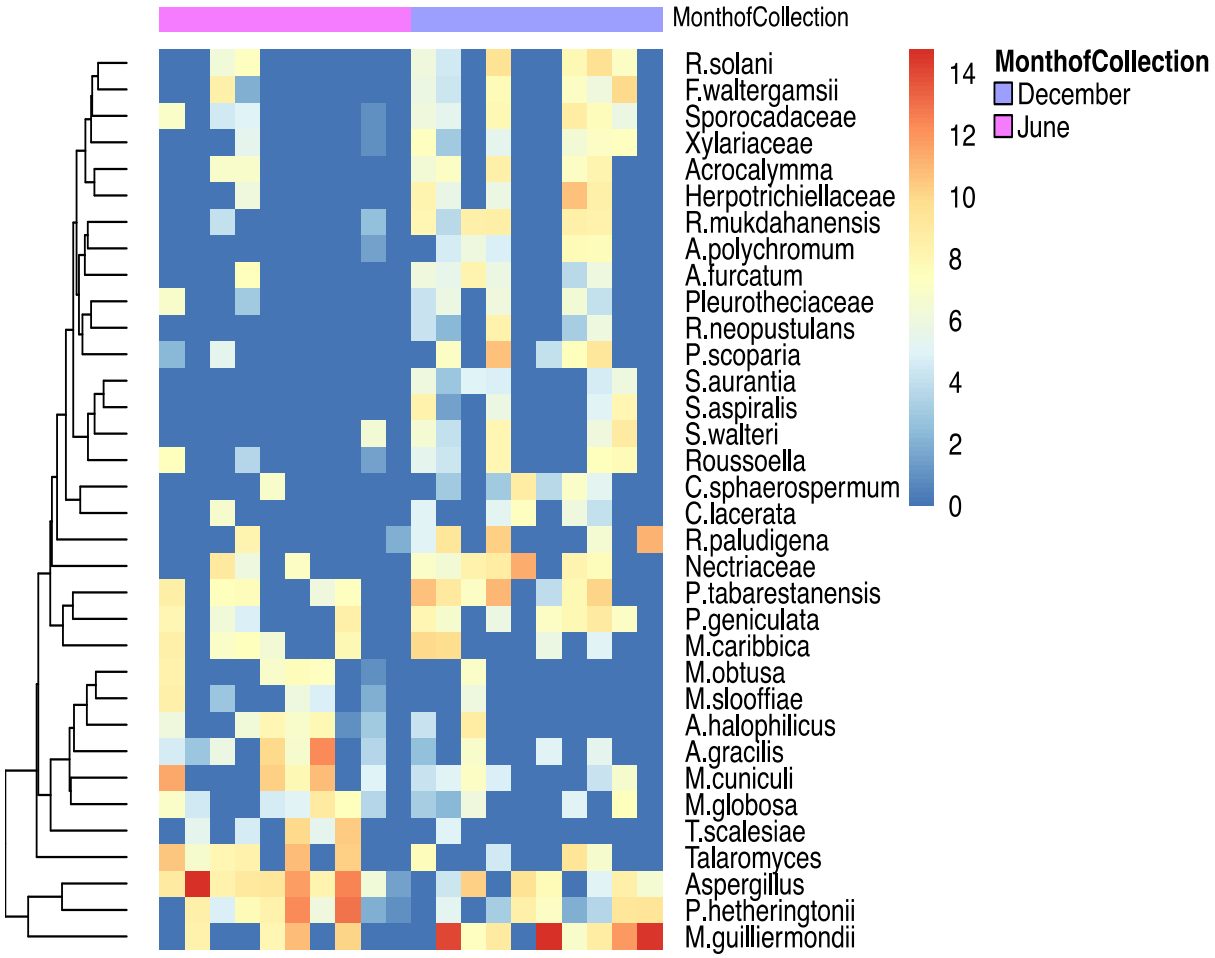


Figure 4.39B Fungal taxa significantly abundant among samples collected at different sampling months from Ikogosi warm springs, using metagenomeSeq (Adjusted P value ≤0.05)

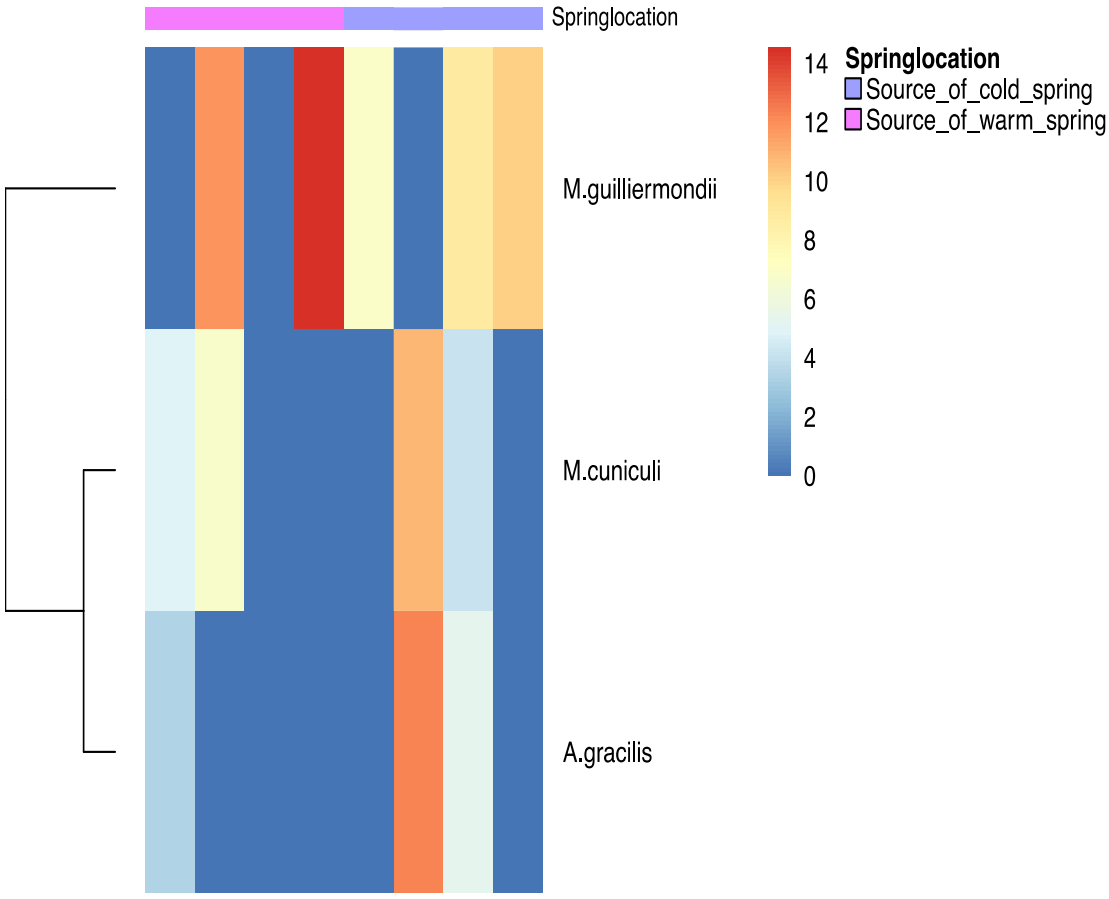


Figure 4.39C. Fungal taxa significantly abundant among samples collected from the source of warm spring and source of the cold spring, using metagenomeSeq (Adjusted P value ≤0.05)

CHAPTER FIVE

DISCUSSION

5.1 Physicochemical properties of sediment and water samples from different sampling points during different seasons

Water samples collected in this study at both sampling seasons showed that the physical parameters of the spring were similar. For example, the Hydrogen Ion Concentration (pH) recorded at the source of the warm spring for both seasons showed that the water was slightly acidic with values of 6.360 and 5.800 for December and June samples. Likewise, all other sampling points of the springs had neutral pH values irrespective of the season of collection.

All the pH of water samples fell within the range of 7.170 – 7.900, with the exception of samples from the oozing point of the warm spring. This above-mentioned range were well within the acceptable standard set by WHO (2014). However, the mean pH range in the seasons differed (5.800 – 7.400 during the dry season and 6.36 – 7.930 during the wet season). This slightly different pH range might be as a result of the dilution of the spring water by constant rain fall usually observed during the wet season. The slightly acidic pH observed at the source of the warm spring is in agreement with the findings of Zarikas *et al.* (2014) who recorded a slightly acidic pH from a Thermopylae Natural Hot Water Springs in India. Furthermore, the finding is also in agreement with a previous study carried out on the warm source of the Ikogosi warm spring in 2005 by Oladipo and co-workers (Oladipo *et al.,* 2005), who recorded similar acidic pH. This acidic value may be attributed to the type of sediment and the geological formation of the location of the source of the warm spring which Talabi (2013) reported to best be described as quartzite sediments made up of loose weathered rocks, and often described as poor soil for agricultural purposes. Quartzite soils undergo weathering, especially when constantly immersed in water, as is the case at Ikogosi, (as water and oxygen are among the major sources of chemical weathering) and over time in the process can leach

their components into the waterbody thus increasing the pH value of the water body (Crundwell, 2017).

Temperature is one of the key environmental factors that shapes the microbial community of waterbodies. It is also a strong determinant of several activities in waterbodies such as solubility of gases and rate of chemical reactions (Adedire *et al.,* 2021). The highest temperature recorded from water samples collected from the source of the warm spring was 38.33°C, which is in tandem with the findings of Ikudayisi *et al.* (2015) who recorded the temperature of the warm spring to be within the same range. A gradual decrease in temperature was observed as the distance between the warm part of the spring and other sampling locations widened. However, the temperature range observed between the confluence and the source of the cold spring during both seasons was within the acceptable temperature range (25.67 - 29.67°C) as recommended by WHO (2014). This temperature range was also observed in other waterbodies such as those recorded by Adesakin *et al.* (2020) who observed similar results from natural underground waters - boreholes and wells. The reported temperature in this study is in agreement with the works of Ojo and Agbede (2014) who reported similar temperature of water samples collected from the same warm spring as 37.50°C while assessing the water quality of the spring.

Dissolved Oxygen (DO) is the measure of oxygen that is accessible to aquatic life in waterbodies. It can also be described as the amount of oxygen dissolved in an aqueous system (Adedire *et al.,* 2021). This chemical parameter plays a significant role in determining the metabolic activities, nutrient availability and activities of microbes in waterbodies (Schindler *et al.,* 2017). All DO (6.25 -8.02 ppm) observed in this study were slightly above the standard guide (<5.00) provided by WHO (2014). This might be attributed to the canopies created by tall green trees shrouding the Spring, which forms a shelter for trooping tourists. Since photosynthesis takes place in all green leafy plants to release sugar and oxygen (Johnson, 2016), this might be a contributing factor to excess DO observed across all points of the Spring that were sampled during both seasons. However, Derso *et al.* (2015) reported lower DO values from water samples collected from Hot Springs in Eastern Amhara Region, Ethiopia, which is at variance to results observed in this study.

Significant differences in chemical parameters of water sampleswas observed among all

five sampled sites. Example of such chemicals with significant differences included Potassium, Iron, Manganese, Magnesium, Copper, Sulphates and Nitrates. For instance, more ions (cations and anions) were present in the warmer regions than in the mixed and colder regions of the Spring. This significant variation might be as a result of the influence of several features such as temperature, water retention time and water mixture as it flows towards the colder region of the Spring (Talabi, 2013).

The presence of Potassium ion is key to the survivability of microbial cells in solutions in which they contribute to osmotic balance in cells, by aiding their intracellular ionic strength (Etesami *et al.*, 2017). High concentrations of Potassium ions can have a debilitating effect on bacterial proteins, thus affecting protein synthesis (Etesami *et al.*, 2017). During the dry season, there was an increase in the concentration of potassium ions when compared to the wet season. This might be as a result of reduced water mixture during the dry season, than in the wet season where adequate water mixing and faster water migration would occur due to rainfall. In addition, all potassium ion from all water samples (0.427 – 0.840 ppm) from the spring were well within the range stipulated by WHO (2014). Higher potassium ion concentration has been reported by Farhat *et al.* (2021) who observed Potassium ions in Chutrun thermal springs in Pakistan, was within 3.8– 4.1 ppm. Copper (a known heavy metal of water bodies) was also considered for observation during both seasons and across all sample sites. Studies have shown that the release of Copper ions into bacteria cells can cause intracellular and irreparable damage and ultimately cell death (Mattews *et al.*, 2013). The range of copper ions recorded in the research were well within the permissible range as stipulated by WHO (2014) and were all near zero in values (within the narrow range of 0.005 – 0.344). This observation is not surprising as the spring is devoid of industrial and chemical contamination. Similar low levels were observed in Limpopo Hot Springs, South Africa with 0.0 ppm in Copper ions (Tekere *et al.*, 2011).

Aqueous concentrations of other cations and anions such Sodium, Iron, Zinc, Manganese, Magnesium, Calcium and Sulphates showed variations during both seasons. Interestingly, all were within the required standard set by the WHO (2014). Findings by Amanial (2015) who analysed Calcium, Magnesium and Sulphates from a spring water in Ethiopia showed similar values, without exceeding the threshold set in place by the World Health Organisation. Findings in this study is in agreement with the findings of Ikudayisi and his colleagues, who in 2015 assessed the chemical and physical

parameters of the same Spring and documented similar values of Magnesium, Iron, Zinc and Manganese as near zero values as well.

Sediments collected from the different parts of the Spring were found to comprise of both organic and inorganic constituents. Their properties are mainly detected using their chemical and mineralogical composition. The inorganic composition of sediments could be grouped as non-crystalline, clay and non-clay minerals and amorphous materials (Bradford and Horowitz,1988). Carbon, phosphorus and nitrogen cycles in sediments are all known to play a role in the determination of species and microbial population of sediments (Wang and Wang, 2020).

Organic carbon analysis across all sample sites depicted a low amount of organic carbon in the sediments of all sample sites within the range of 0.007 – 0.233 from sediment samples collected in June (Wet season), while a similar narrow and low organic carbon was detected in sediments collected from all sample sites in December (0.006 – 1.207). These low values may be attributed to an extremely low primary productivity in the spring with concomitantly low rate of microbial decomposition and organic matter/content with minimal deposition into sediments (Bianchini *et al.,* 2006). For example, from the source of the warm spring in December, the sample recorded the lowest organic content which may not be surprising as the sediment is mainly made up of rocks, stones and granite with an absent aquatic life. In addition, this Spring is not prone to contamination by fishing, agricultural wastes and pesticides, which might be another reason for such low organic carbon observed across all sampled sites during both seasons. Mushtaq *et al.* (2016) in their reports on the physicochemical analysis of sediment samples from Dal Lake Srinagar, Kashmir, India observed a similarly low organic carbon range attributable to the nature of the sediment.

Phosphorus is a major nutrient involved in regulating primary production in shallow ecosystems. Sediments serve as a source or a sink for phosphorus and it is strongly related to organic carbon in freshwater sediments (Mok *et al.,* 2021). Phosphorus can be released into sediments from weathered phosphate minerals, from the degradation of plants, animals or microbes (Mok *et al.*, 2021). Microorganisms are involved in the regulation of phosphorus through their biomass degradation which can make up about 25 - 50% of the total Phosphorus (Wright *et al.,* 2001). Available Phosphorus can be defined as a part of total Phosphorus that is freely accessible for absorption and

utilization by living organisms (Johan *et al.*, 2021). The available phosphorus observed across all sampled sites, from both seasons were within a narrow range of 1.771 – 7.404 ppm, which is relatively low. This result is in concordance with the findings of Singare *et al.* (2011) who also observed low values for available phosphorus in the sediments of Vasai Creek in Mumbai, India. However, the finding in this study is in variance with the result of a study conducted by Wang and Liang, (2015) on the sediment of a lake in China with an elevated increase in Phosphorus. The low values observed in this study is not surprising, as studies have shown Phosphorus to be limiting in aquatic freshwater systems (Watson *et al.*, 2018). In addition, Kalkhoff (2016) stated that Phosphorus is less attached to large sand particles than smaller clay and silt-like sized particle. The physical attributes of the sediments in the warm part of Ikogosi Springs are large-sized particle as compared to sediments in the cold spring of the water body (clay-like size), which might be another reason for low levels of Phosphorus detected.

In particular, Iron was detected in high values across all 5 sample sites, irrespective of the period of sampling. The highest value of Iron was recorded at the source of the warm spring (505.000ppm) during the wet season, while the highest value was recorded at the source of the cold spring (654.440ppm) during the dry season. One of the physical properties of the sediment at the source of the cold spring is its clay-like texture, which is in contrast to the sediment observed at the source of the warm spring that is rocky, glasslike and granite-like. The texture of this sediment, which is similar to coarse-looking, metamorphic miniature rocks may be a contributing factor in the high amounts of Iron detected from both sources (Talabi, 2013). For example, studies have shown that claylike sediments could contain Iron and Manganese nodules (Zhang *et al.*,2020). This might probably be a factor which could be responsible for the high concentration of Iron detected. Furthermore, John *et al.* (1995) stated that the process of rock and stone crushing has been strongly associated with the release of Iron. The high spikes of Iron recorded for sediments collected from the warm spring might have been as a result of crushing the stony, rocky sediment to powder in the analytical laboratory. Iron concentrations of 82.50 – 654.440 ppm in sediments of Ikogosi Warm Springs from both sampling periods, were lower than those observed by Başyiğit and Tekin-Özan (2013) from the sediment of a freshwater lake in Turkey and Akan *et al.* (2012) from sediment samples in Lake Chad.

5.2 Phenotypic characteristics of bacteria and fungi from sediment and water samples of Ikogosi Warm Springs at different seasons

Both culture-based and culture-independent techniques aided the study of the bacterial and fungal diversity of the spring. Culture-based method still remains an important aspect in Microbiology as it is still being used in understanding the metabolic and physiological attributes of single microbes (Tshikhudo *et al.,* 2013).

Characterising microbial communities in their natural habitat could aid in providing more insights concerning prospective metabolic interaction among each other, and between microbial population and environmental variables (López-López *et al.,* 2015). It could also be used to discover and harness microbes that can be valuable in biotechnology process. For example, the discovery of the metabolite - Bryostatin 1, from extracts of the marine *Bugula neritina.* This metabolite have been of interest in medical biotechnology, as it had been discovered to contain antioxidants effective against several cancer cells (Trindade *et al.,* 2015).

A large number of bacteria and fungi were biochemically characterised in this study. However, based on sample location, the location with the least bacterial and fungal diversity was from the source of the warm spring even at different period of collection. This might be as a result of the thermal identity and sparse nutrient availability characterising the location. With a constant average temperature of 38.00 - 38.33°C, the warm spring, although not as heated as in the case of hot springs like Siloam Hot Water Springs, South Africa (Tekere *et al.,* 2011), Hot springs of Galacia, Spain (López-López *et al.,* 2015) and Xiamen Hotsprings, in China (Zhao *et al.,* 2017) was somewhat discouraging to the growth of some non-spore formers and some species of fungi when characterised biochemically. For instance, the genus *Bacillus* was more dominant than other genera identified.

It is also interesting to note that the temperature of the spring began to drop as the spring water made its way to the confluence, during which the number of identified microbial flora increased. They ranged from *Bacillus* sp., *Acinetobacter* sp., *Staphylococcus* sp., *Micrococcus* sp., *Pseudomonas* sp., *Enterobacter* sp. to *E. coli*. The increase in the diversity might not be unconnected to a reduction in the temperature of the spring at the confluence. Furthermore, the surge in the bacterial diversity may also be as a result of

regular recreational activity occurring at the site. A number of tourist and community dwellers often dip varieties of cans, plastics and bowls to fetch water for several reasons at the confluence of the spring. The findings of Olajuyigbe and Ajele (2005) is in agreement with this report, these researchers also identified *Micrococcus* sp., *Enterobacter* sp., *Bacillus* sp., *Pseudomonas* sp. and *Staphylococcus* sp. from the spring while in search of a prospective microbe capable of producing protease at elevated temperature. Likewise, the works of Amadi *et al.* (2020) who performed bacterial diversity studies on water samples from Krakrama River in Rivers State, Nigeria, also isolated *Enterobacter* sp., *Bacillus* sp., *Pseudomonas* sp. and *Staphylococcus* sp. observed in the River with a recorded temperature of 26.00°C. More so, some species of *Bacillus* isolated from the source of the warm spring have been recorded to produce proteolytic enzymes at an elevated temperature of 65°C (Olajuyigbe and Ajele, 2005).

Fungal diversity of the spring using culture-based methodology, identified all characterised fungi as belonging to only two phyla- Ascomycetes and Deuteromycetes. The representative of Ascomycota recovered from the sediment and water samples of the spring were *Aspergillus, Penicillium, Fusarium, Cladosporium,* and *Talaromyces*, while the phylum Deuteromycetes was only represented by one member of the genus *Trichoderma* belonging to the family Hypocreaceae. Irrespective of the temperature difference observed across sampled points, *Aspergillus, Penicillium* and *Fusarium* spp. were isolated and identified across all sites from sediment samples irrespective of the period of collection. This observation is not surprising as several studies have proven the ubiquity of *Aspergillus* and *Penicillium* in aquatic environments (Abdel-Azeem *et al.,* 2019a; Heo *et al.,* 2019). Abdullah and Abbas (2008) also studied the fungal diversity of surface sediments present in Shatt Al-Arab River in Iraq, using the conventional approach, and reported the most frequent fungal genera in the sediment as *Aspergillus, Fusarium* and *Penicillium.* Another study conducted by Salano *et al.* (2018) also reported the high frequency of *Aspergillus* and *Penicillium* sp. isolated from the sediment of a hot spring in Kenya. In this work, the recovery of *Talaromyces* sp. and *Cladosporium* sp. was predominantly high at the source of the cold spring and at the confluence of the spring and in very low abundance during both sample collection period. Orwa *et al.* (2020) also isolated *Talaromyces* sp. and *Cladosporium* sp. from water samples collected from the halophilic Lake Magadi in Kenya.

The pH of the warm spring was recorded as being slightly acidic only at the source of the warm spring (5.80 – 6.36), but other parts of the springs were observed as neutral in pH. Just like temperature, hydrogen ion concentration is also one of the major variables that have been documented to induce significant responses of metabolic activities of microbes in the natural environment (Ye *et al.,* 2012). pH has been shown to have a strong correlation with the structure of microbial population across different ranges of biogeochemical environments (Thompson *et al.,* 2017). All isolated bacterial and fungal forms from all five points of the spring appeared to be neutrophiles. This may be connected to the near-neutral pH of the spring which encourages the growth and dominance of neutrophiles. All recovered bacterial isolates from the sediment of the spring including *Bacillus, Micrococcus, Sporosarcina, Staphylococcus, Acinetobacter, Pseudomonas* and *Enterobacter* during the wet season and an inclusion of *Escherichia coli, Streptococcus, Aeromonas, Alcaligenes* and *Citrobacter* during the dry season seemed to be neutrophilic in nature. The isolation of *Pseudomonas* and *Bacillus* species in the sediment of Ikogosi warm springs is in agreement with the report of Ajayi and Ige (2019) who reported the isolation and identification of these microbes using Standard Plate method from the sediment of River Ose, with a pH of 6.92. In addition, these heterotrophic Proteobacteria were also discovered in high abundance from alkaline sediments of a Hot Spring in Northeast China (Wang and Pecoraro, 2021). The presence of *Sporosarcina* at the midpoint of the warm spring and at the confluence of the spring was scanty, making up only 11.11% of the bacteria genera discovered. *Sporosarcina*, according to Tominaga *et al.* (2009) is a Gram variable, endospore-forming rods whose spores are terminally positioned. This interesting bacterial isolate has been reportedly isolated in the warm locations of Sikkim Himalaya Hot Springs (Sharma *et al.,* 2020) and has also been reportedly identified in Geothermal Springs of Armenia and Nagorna-Karabakh (Saghatelyan *et al.,* 2021). According to Vos *et al.* (2011), *Sporosarcina* is an heterotroph with various species of variable physiological attributes and metabolism - Psychrophilic, neutrophilic, alkaliphilic and halophilic in nature. However, Tominaga *et al.* (2009) cited their temperature range as 5 – 40°C with a pH range of 6.5 – 8.8, which is well within the range of Ikogosi warm springs.

In the sediment of the warm spring particularly at the midpoint and at the confluence, *Aeromonas* and *Alcaligenes* were recovered, albeit only from December sediment samples. In addition to this, *Acinetobacter* sp. was isolated in both seasons, at the

confluence and at the source of both cold and warm spring. *Acinetobacter* is an aerobic bacterium which thrives and grow within a wide pH range (5 - 8) (Hrenovic *et al.*, 2014). They are abundant in diverse natural environments such as oceans, soil, sediments, rivers, springs, lakes, and pesticide-contaminated sites (Hrenovic *et al.*, 2014; Jung and Park, 2015). They play a critical part in nutrient cycling, and are involved in a wide array of biodegradation of chemicals. *Acinetobacter* sp. are aerobic in nature and can be either pathogenic or non-pathogenic (de Berardinis *et al.*, 2009). Usually, the non-pathogenic ones can be dominant in places like the aforementioned, while the pathogenic ones, can be contracted in hospital environments and infected apparatus (Jung and Park, 2015). Therefore, the presence of this genera in the sediment of the spring across both sampling sites is not unexpected as the pH of the spring is adequate enough to facilitate their growth and ubiquity. It is also in agreement with the study of Saxena *et al.,* (2017) who likewise noticed the ubiquity of *Acinetobacter* in the hot springs of Tattapani in India.

The identification of *Talaromyces* sp. in freshwater sediment such as that of Ikogosi warm springs has not been commonly reported. According to Gao *et al.* (2021), *Talaromyces* are acid-tolerant and hardly survive at pH above 7. However, several studies including Barbosa *et al.* (2018) and Rajeshkumar *et al.* (2019) did not report the acidophilic nature of the fungus. Therefore, it appears that the species of *Talaromyces* isolated at the site of the cold spring might be species of the fungi that is neutrophilic in nature, in contrast to reports of Gao *et al.* (2021). The abundance of *Fusarium* and *Trichoderma* were evident from both water and sediment samples of the Spring as they were recovered from all sample sites. The abundance and constant presence of *Fusarium* sp. might be as a result of its widespread dissemination of spores, and also due to *Fusarium's* ability to thrive on an extensive variety of substrates of variable pH (Abdel-Azeem *et al.*, 2019b). For instance, Panwar *et al.*, (2016) studied the effect of temperature and pH on the growth of *Fusarium* sp. responsible for Fusarium Head Blight in wheat. Their study reported that the fungal isolate sporulated and thrived at a pH range from 4.0 – 8.0. Another investigation conducted by Oritsejafor (1986), deduced the pH range for optimum growth of the fungus as 5.0 – 7.0, whereas Panwar *et al.* (2016) placed the sporulation and growth of *Fusarium solani* at 5.5 – 7.0. Findings in this study is supported with results of Panwar (2016). It therefore appears that different *Fusarium* sp. adapt according to the physicochemical condition of their environmental habitats.

Microorganisms can generally be classified according to the temperature range at which they can grow. They include psychrophiles/psychrophilic microbes, mesophiles/mesophilic microbes, thermophiles/thermophilic microbes and hyperthermophiles/hyperthermophilic microbes (Coleman and Smith, 2007). However, the term "thermo-tolerant" is not alien in temperature studies. According to Matsushita *et al.* (2016), thermo-tolerant microbes are mesophiles that typically have their optimum growth temperature slightly above the 35 - 45°C growth temperature range. They are usually of the same mesophilic genus or species, but have their optimum growth temperature range about 5 – 10°C higher than the regular mesophilic optimum temperature range (Matsushita *et al.*, 2016). They are also found in warm or hot habitats and are capable of producing thermotolerant/ thermophilic enzymes (Ghilamicael *et al.*, 2018).

5.3 Screening bacterial isolates from Ikogosi Warm Springs for alpha-amylase activity

In this study, when 174 bacterial isolates were screened for alpha-amylase enzymes at a temperature of 50°C, only 27.05% were alpha-amylolytic at the subjected high temperature and all of these positive isolates were of the genus *Bacillus.* This is expected as *Bacillus* species possesses mechanisms which it uses to resist heat and other deleterious environmental hardship. A good instance is the possession of their high-stress resistant spores which makes them survive in extreme environments (Li *et al.*, 2021). Among those positive to alpha-amylase at high temperature, the two *Bacillus* spp. - *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F (obtained from the midpoint of the warm spring (sediment) and from the source of the warm spring (sediment)) and selected as best amylase producers were used for physiological studies. Their selection was as a result of the diameter of their hydrolytic zones, as they had the widest diameter upon measurement of their zones of hydrolysis. Lal and Cheeptham. (2012) had stated that the more the amylase produced by a bacterium, the larger the zone of hydrolysis would be, hence the selection of *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F.

This result is similar to the works of Abel-Nabey and Farag, (2016); Indriati and Megahati, (2017) and Msarah *et al.*, (2020), who likewise identified *Bacillus* sp. as their choice inoculum after subjecting various bacterial isolates to screening for alpha-amylase at elevated temperatures. More specifically, Priyadarshini *et al.* (2020) and

Cotta *et al.* (2021) also characterised thermostable alpha-amylase from *Bacillus cereus* isolated from Chilika lake in India and from a compost respectively.

Bacillus siamensis is a member of the Operational Group *Bacillus amyloliquefaciens* (OGBa), which are taxonomically classified under the *Bacillus subtilis* species complex. They are known to be widely distributed in natural habitats such as soil, water, plants and sediments (Fan *et al.*, 2017) and have enormous biotechnological applications in industries. They have also been reported to produce several important enzymes such as alpha-amylase, lacasse, protease and pectinase (Ngalimat *et al.*, 2021). The findings in this study is also in line with studies of Seo *et al.*, (2019), who isolated several bacteria from bentonite samples in Korea and identified *Bacillus siamensis* as the bacteria with the highest alpha amylase activity.

5.4 Optimisation of alpha-amylase production from selected bacteria, isolated from sediments of Ikogosi Warm Springs

Optimisation of production media is usually performed to boost yields in metabolites often produced by the bacterium being studied. It is one of the most important phenomenon that is generally performed before production on a large-scale is carried out (Singh *et al.*, 2017). In this study, the OFAT approach was used in determining the best conditions for optimum production of alpha-amylase from selected isolates of *Bacillus cer*eus MPW3E and *Bacillus siamensis* SW3F. It was observed that at 55°C, yield of 14.690U/mL and 15.220U/mL were respectively obtained from the two organisms. The production of alpha-amylase diminished by approximately 50% when the temperature was increased to 60°C. Both organisms seemed to prefer temperature within 40 - 55°C temperature range. This might be as a result of the warm environment from which both *Bacillus* spp. were isolated and which could have contributed to the temperature preference of the enzyme produced. Similar studies involving *Bacillus cereus* isolated from a Nigerian soil, showed a similar pattern by its gradual increase in alpha-amylase production until 55°C was reached and further production resulted in lesser amylase yield (Bello *et al.*, 2021). Sumpavapol *et al.* (2010) described *Bacillus siamensis* as a mesophilic *Bacillus* with a broad growth temperature range of 4 - 55°C. This observation appears to group both organisms as thermo-tolerant microbes.

With respect to pH optimisation of the culture medium, maximum yield of alpha-amylase by *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F were 11.206 U/mL and 23.942 U/mL respectively when the production media was maintained at 7.0. Saha and Mazumdar (2019) reported similar results that which showed that at pH 7.0, maximum alpha-amylase was produced by *Bacillus cereus* AMY3 isolated from a garden soil in India, using the OFAT approach. Additionally, Sivakumar *et al.* (2012) also described *Bacillus cereus* with maximum alpha-amylase production at pH 7.0 and this was isolated from a vermi-compost site in India. However, according to Sumpavapol *et al.* (2010), *Bacillus siamensis* is an endospore-forming halophilic Bacilli with a broad pH range of 4.5 – 9.0 although there is a dearth of information regarding optimisation of pH for maximum alpha-amylase production from *Bacillus siamensis.* It is apparent that alpha-amylase production by both *Bacillus* spp. are better at the neutral range of pH which might be as a result of the pH range of the warm spring where both *Bacillus* spp. were isolated.

The production of alpha-amylase by microorganisms is also dependent on the composition of the medium. Carbon is an important component in bacterial fermentation media. It is a source of energy to the inoculum introduced into the medium, as it is actively involved in cell growth and positively influences the production of metabolites by the inoculum (Singh *et al.*, 2017). In this study, cassava peel was found to be the best stimulator for alpha-amylase production for both *Bacillus* sp. producing 21.223U/mL of alpha-amylase from *Bacillus cereus* MPW3E and 22.105 U/mL from *Bacillus siamensis* SW3F. Cassava peel at a concentration of 2% (w/v) for *Bacillus cereus* MPW3E and 1% (w/v) of Cassava peel for *Bacillus siamensis* SW3F supported maximum production of the enzyme, after which further increment in cassava peels concentration resulted in lower yields. This phenomenon can be likened to carbon catabolite repression in which microbial regulatory system coordinates its regulatory genes to efficiently make use of its preferred carbon source, while inhibiting the stimulation of redundant catabolic pathway in a bid to preserve energy (Deutscher, 2008). Furthermore, lower yield in the alpha-amylolytic enzyme following an increment in the concentration of cassava peels might be as a result of increased viscosity of the production medium at elevated concentrations (of cassava peels) that might reduce the amount of dissolved oxygen available to the microorganism necessary for its growth. Thus, cassava peels may be useful as a cheaper substitute and natural carbon sources for the production of alpha-

amylase from *B. cereus* and *B. siamensis.*

The type of nitrogenous sources is also an important factor to consider in the production of alpha-amylases. According to Elibol (2004), nitrogen sources play a significant role in the biosynthesis of metabolites as they are known to be actively involved in the synthesis of amino acids and proteins. Investigations in this study revealed that maximum alpha-amylase (24.048 U/mL) was produced from *Bacillus siamensis* SW3F when the production medium was supplied with Tryptone as the sole Nitrogen source, while maximum alpha-amylase (19.281 U/mL) was produced when the production medium containing *Bacillus cereus* MPW3E was provided with yeast extract as its source of Nitrogen. In addition, typtone and yeast extract at a concentration of 1.50% (w/v) best supported maximum production of alpha-amylase using *Bacillus siamensis* SW3F and *Bacillus cereus* MPW3E respectively. Several investigations such as Dash *et al.* (2015) and Lal *et al.* (2016) have reported that organic sources of Nitrogen like yeast extract, peptone and tryptone usually act as enhancers for the production of alpha-amylases in several *Bacillus* species. Findings in this study is in agreement with these previous investigations. This might be because organic Nitrogen sources, also contain additional nutrients such as vitamins, which provides tremendous growth conditions to microorganisms, unlike their inorganic counterparts. However, studies revealed that as the concentration of the best nitrogen source increased above the optimum nitrogen concentration required (1.5%, w/v), a decline in the production of the enzyme was observed. The decrease in the production of the amylolytic enzyme might be due to a change in the pH of the medium due to increased acidity which might play a part in repressing amylase production.

The results of the influence of metal ions on bacterial amylases have been well documented, and has been established that majority of alpha-amylases are metal ion-dependent (Dutta *et al.,* 2016). The increase in the production of alpha-amylase following the addition of some specific metal ions maybe as a result of these ions' ability to interact with negatively charged amino acid residues of the enzyme (Dutta *et al.,* 2016). In this study, the highest production of enzyme was observed when the production medium was supplemented with Calcium ions (0.01%). In addition to this, Magnesium ions were also found to have a positive effect on alpha-amylase production using both *Bacillus* species. Similarly, Halder *et al.* (2014) and Rehman *et al.* (2019) also reported the positive influence of calcium ions on the production of alpha-amylase

from *Bacillus cereus*.

In submerged fermentation, inoculum size plays an essential role in enzyme production (Dash *et al.*, 2015). In this analysis, enzyme production was optimal at 1% (v/v) using *Bacillus cereus* MPW3E (30.81U/mL) and *Bacillus siamensis* SW3F (32.28U/mL). Further increment in the concentration of inoculum resulted in a gradual depletion of alpha amylase production. It is important to note that there is no fixed bacterial inoculum volume appropriate for the production of alpha-amylase (Elmansy *et al.*, 2018), but generally, its production is usually affected by large sizes of inoculum which can be attributed to nutrient depletion as a result of rapid biomass growth which generally bring about reduction in metabolic activities, including enzyme production (Malhotra *et al.*, 2000).

The findings in this study is similar to that reported by Bandal *et al.* (2021) who reported that 1.0% of inoculum concentration was best for the production of alpha-amylase for a halophilic *Bacillus* sp. Optimisation of incubation time is also important in the production of alpha-amylase. Several studies have shown that as the fermentation period increased, the production of enzyme increased until it reached the optimum incubation period. Additional increment in the incubation period then resulted in a decrease in enzyme production (Dash *et al.*, 2015; Bandal *et al.*, 2021). In this investigation, a 48-hour incubation time resulted in the highest production of alpha-amylase from both Bacilli, with *Bacillus siamensis* SW3F producing alpha amylase of 42.488U/mL than its counterpart *Bacillus cereus* MPW3E (37.184U/mL). Further increment in incubation time resulted in a drastic drop in enzyme production, as stated by previous investigators (Deb *et al.*, 2013; Elmansy *et al.*, 2018). This could be due to several factors including nutrient exhaustion, deleterious accumulation of by-product in the production medium and microbial cell death (Elmansy *et al.*, 2018; Rehman *et al.* 2019). These findings agree with the study described for *Bacillus amyloliquefaciens* P-001 in the production of alpha-amylase, who also recorded 48hour incubation time as the best incubation period (Deb *et al.*, 2013). Result of this investigation is in contrast to the findings of Rehman *et al.* (2019) who reported that the efficient incubation time for maximum alpha-amylase production produced by *Bacillus cereus* AS2 was 72hours.

5.5 Characteristics of alpha-amylase produced from selected thermophilic bacteria, isolated from Ikogosi Warm Springs sediment

Characterisation of enzymes is an important aspect in Enzymology studies. This is because characterisation aids the researcher in understanding the properties of the enzyme of interest and also serves as a guide to match enzymes to industrial applications (Souza and Magalhães, 2010). In this study, the effect of temperature on the activity and stability of crude alpha-amylase was determined. Temperature is a fundamental biochemical element which governs enzymatic activities (Gurung *et al.,* 2013). It was observed that the activity of alpha-amylase produced from *Bacillus cereus* MPW3E showed its optimal temperature at 50°C which plateaued to 55°C (32.018U/mL) while alpha-amylase produced from *Bacillus siamensis* SW3F showed its maximum enzyme activity at 55°C (35.718 U/mL). This finding is in agreement with that of Anto *et al.* (2006) and Mahdavi *et al.* (2010) both of who reported maximum crude alpha-amylase activity from *Bacillus cereus* MK and *Bacillus cereus* MTCC 1305 strains at 50 and 55°C respectively.

Before an optimum temperature is reached (depicted as peaks), an increase in temperature produces an increase in the rate of molecular movements, which results in a corresponding increase in the rate of reaction. However, as the temperature climbs beyond the optimum, denaturation of enzymatic protein begins to occur, hence decreasing and even halting the activity of enzymes (Robinson, 2015). Thermal stability studies from both *Bacillus* sp. revealed that alpha-amylolytic activity was not highly stable at 55°C after 10 and 30 minutes. The optimum temperature and results of thermal stability observed in this analysis might be as a result of the thermo-tolerant nature of both *Bacillus* sp. used in this assay, hence the activity of the enzyme began to decline rapidly as the temperature climbed well into the thermophilic temperature range, and the crude alpha-amylase activity could not be sustained for a lengthy period of time.

In addition to temperature, pH is also a part of environmental factors capable of affecting the rate of enzyme-catalysed reaction (Robinson, 2015). Both enzymes assayed in this study were highly active at pH 5.0 – 7.0 with optimum pH 6.0 for amylase produced from *Bacillus cereus* MPW3E (23.748 U/mL) and an optimum pH 7.0 for alpha-amylase produced from *Bacillus siamensis* SW3F (26.008 U/mL). This study suggests that alpha-amylase from both *Bacillus* spp. seem to prefer slightly acidic to neutral pH for

maximum activity. Previous studies with similar pH conditions (pH 6.0 and 7.0) for alpha-amylase produced from *Bacillus* sp. include the work of Mahdavi *et al.* (2010) and Msarah *et al.* (2020). As the pH increased above the optimum, a decrease in amylolytic activity was recorded. This decrease might have resulted from a change in the ionic state of the enzyme's active site, which may alter catalytic reactions resulting in a decline in enzyme activity.

The activity of crude alpha-amylase was studied with increases in various substrate concentrations. The amylolytic activity was observed to increase as the concentration of substrate increased from 0.25% until 1.0% (28.602U/mL for *Bacillus cereus* MPW3E and 30.510U/mL for *Bacillus siamensis* SW3F), after which a decrease in activity of both enzymes was observed. Optimum activity at moderately low substrate concentration is a desired quality of enzymes used in industries (Adedire *et al.,* 2013). This means that such enzymes would already have been optimally active in the presence of low amounts of substrate, which would inherently result in rapid completion of the desired reaction. Similar substrate concentration for amylolytic activity from *Bacillus* sp. was observed in studies conducted by Adedire *et al.* (2013) and Alonazi *et al.* (2021).

In the consideration of the effect of some metal ions on the activity of crude alpha-amylase, both enzyme activity showed an increase in their activity with Calcium (*Bacillus cereus* MPW3E: 120% and *Bacillus siamensis* SW3F: 150.1%) and Magnesium ions (*Bacillus cereus* MPW3E: 108.11% and *Bacillus siamensis* SW3F: v110.0%) at 1Mm. The increase in the enzymatic activity observed during the addition of Ca^{2+} and Mg^{2+} is not surprising as several studies have shown that most alpha-amylase of microbial origin are calcium-dependent metalloenzymes. These ions have been discovered to have a stabilizing effect on the enzyme's structure (Mehta and Satyanarayana, 2016; Gómez-Villegas *et al.,* 2021). However, in this study, when the concentration of these stimulating metal ions was increased, a dramatic decline in the enzymatic activity was observed. Studies have shown that metals in sufficiently high concentrations inhibit enzyme activity, by binding strongly to sulfhydryl groups of proteins of the enzyme. The structure of the enzyme is usually altered and enzyme activities inhibited due to the binding of these sulfhydryl groups (Mizrahi and Achituv,1989). Moreover, in this study, metal ions including Fe^{3+}, Ni^{2+}, Cu^{2+} and Mn^{2+}were observed to be detrimental to the activity of both enzymes at both concentrations used. According to Mizrahi and Achituv (1989) heavy metals such as

Copper, Zinc, Nickel and Lead when present, can interrupt the enzyme's activity by altering the enzyme's active site making it non-conformable to substrates, hence reducing enzyme-substrate reactions. Similar results; Alonazi *et al.* (2021) and Hu and Liu (2021) confirmed calcium and magnesium ions as activators of alpha-amylase, as observed also in this research work.

Results of the present study showed that the activity of crude enzymes from both *Bacillus* spp. were diminished in the presence of chemical agents such as Sodium dodecyl sulfate (SDS), Ethylenediamine tetraacetic acid (EDTA), Urea and Tween 20 at concentrations of 0.1% and 1.0%. The decline of amylase enzymes by chelating agent EDTA suggests that these enzymes are metalloenzymes which can be damaged by heavy metals via their strong binding to the enzymes amino acid (Adedire *et al.*, 2013). This study is in consonance with studies of Alonazi *et al.* (2021) and Hu and Liu, (2021) who also described the chelating effect of EDTA on alpha-amylase isolated from *Bacillus* sp.

The maximum enzyme velocity, (V_{max}) and Affinity constant (K_m) are kinetic constants that describes the kinetic properties of an enzyme, with respect to substrate concentration. These constants are important in understanding the mechanism by which enzymes uses in controlling metabolism (Benjamin *et al.*, 2013). It was observed in this study that values of K_m for *Bacillus cereus* MPW3E and *Bacillus siamensis* SW3F were low (0.1973mM and 0.0519mM respectively) at various substrate concentrations. If an enzyme possesses low K_m, it is an admired kinetic parameter in industrial enzymes, because it is an indication that such enzyme would form an enzyme-substrate complex rapidly with its substrate, reach V_{max} faster hence, better enzyme activity (Chandra and Madakka, 2019). Irshad *et al.* (2012) and Abootalebi *et al.* (2020) also reported a low value of K_m for alpha-amylase derived from *Bacillus* spp. and stated that these enzymes possessed adequate degradation rate which led to enhanced transformation of substrate.

5.6 Targeted metagenomics analysis of the bacterial diversity of Ikogosi Warm Springs observed during two seasons of the year

Targeted metagenomics is a high-throughput approach that aims at amplifying, sequencing and analysing a particular region of the genome, (that is commonly shared across several microbes) for the purpose of describing their taxonomic contents (Siegwald *et al.*, 2017). This research work targeted the V1- V3 of the bacterial 16S

rRNA gene, in an effort to characterise, understand and gain insights into the microbial consortia inhabiting Ikogosi warm springs. In this work, bacterial assemblages in water samples recorded more taxonomic group of bacteria than in sediment samples. Similar studies, Espín *et al.* (2021) also recorded similar scenario where the bacterial community structure in water samples were more diverse than in samples collected from sediments of a saline Lake in Spain. Furthermore, with respect to phyla discovered, Proteobacteria, Bacteriodetes and Firmicutes were the most abundant phyla discovered from both habitats. The presence of Proteobacteria in relatively large abundance in sediment and water samples is not surprising as several studies have shown their dominance in diverse ecosystems such as in sediments, aquatic waterbodies, plants and animals (Fang *et al.*, 2022). The occurrence of Proteobacteria in water and sediment can vary considerably due to the nature of mineral and organic matter present. The physicochemical evaluation of the Ikogosi warm spring as conducted in this study, indicated that the sediment contained relatively low organic content. However, the presence of a large percentage of Proteobacteria and Firmicutes in the spring is not unusual, as they have been known to be microbes with diverse physiological adaptabilities (Vaz-Moreira *et al.* 2017).

Saprophytes, primary producers and unidentified microbes were present in the spring's water (119) and sediment (112) using culture-independent approach. It was observed that water samples reflected overlapping bacterial clades, albeit with differences in their abundances. For instance, *Comamonas* sp. recorded higher relative abundance in water samples than in sediment samples. The same can be said of *Acinetobacter*, *Brevundimonas*, *Flavobacterium, Pseudomonas* and *Delftia* sp. In a similar study by Zhang *et al.* (2021), using high-throughput sequencing approach, the presence of *Acinetobacter* and *Pseudomonas* sp. was also observed in high abundance from water samples collected from a flowing river in China. However, sediment samples contained higher abundance values of *Vogesella, Sphingobacterium, Novispirillum* and *Paraclostridium. Empedobacter* sp. was captured among the top 19 in sediment samples only, with a relative abundance of 11.40%. These microbes were not captured in culture-based analysis, thus indicating the sensitivity of culture-independent approaches to "difficult-to-grow" microbes, and their advantage of being able to identify previously unknown microorganisms (Kumar *et al.*, 2015).

Sequence analysis conducted on December and June samples revealed the presence of numerous genera - 125 and 113 bacterial genera for June and December samples

respectively. An overlap within the top 20 bacterial genera was also noticed, although the pattern of distribution of the 20 bacterial genera was quite different. For instance, a larger percentage of *Acinetobacter* (16.96%), *Comamonas* (20.85%) and *Empedobacter* (11.09%) were discovered among samples collected in December, being the dry season unlike the samples collected during the wet period of the year (June). Several conditions might have influenced such microbial abundances, such as water mixing and exchange during the rainy/wet season or different physicochemical attributes of the spring at different seasons which could have had an impact on the bacterial population (Talabi, 2013).

Alpha diversity analysis is used to determine the structure of an ecological community. The structure can be determined in terms of the community's richness (which is defined by the number of taxonomic groups), the community's evenness (which is characterised by the distribution of abundances of microbial groups) or the community's phylogenetic relatedness (Willis, 2019). In microbial ecology studies using amplicon sequencing, alpha diversity analysis is one of the first approach used in understanding the structure of the microbial consortia and also used in evaluating differences between environments (Vendrell *et al.,* 2022). When analysing the differences in community structure present in sediment and water samples, the Shannon index indicated that there was no statistically significant difference in their evenness and richness in both communities. However, in this study, the observed species metrics (which takes into consideration the number of taxonomic groups present in samples), illustrated that there was a significant difference between the communities of water and sediment samples, with water samples containing a richer community of microbes than those of sediment samples. More so, the phylogenetic diversity of water samples indicated a significantly higher mean alpha-diversity than that of sediment samples. This finding is in contrast to reports of Fang *et al.* (2022) who likewise conducted a microbiome study on the water and sediment in Laoshan Bay in China and reported a richer community in sediment sample than river samples. The significant increase in the phylogenetic diversity and number of taxonomic groups present in water samples than in sediment samples might be that the microbiota in water are more vulnerable to anthropogenic disturbances than microbial communities present in the sediments of the spring. In addition, alpha diversity analysis conducted among samples collected in December (Dry) and June (Wet), even though the mean alpha-diversity obtained in December samples had a higher mean value than June

samples, yet, there was no statistical difference recorded in terms of richness, evenness and phylogenetic diversity in the microbial communities present in both group of samples.

Beta diversity analysis is another critical evaluation which is commonly used in microbiome analysis. It is used to quantify similarities/dissimilarities/distances between two or more pairs of microbiome (Su, 2021). A number of approaches can be used in this analysis. In this study, Bray-Curtis dissimilarity distance and Unifrac metrics were used, supported with statistical analysis. Bray-Curtis dissimilarity metrics clusters/groups microbial communities away from each other based on abundance of taxa/abundance of Operational Taxonomic Units between samples (OTUs) (Li *et al.*, 2018). Whereas Unifrac metrics (unweighted and weighted Unifrac) is also used in separating groups in microbiome analysis but requires the inclusion of a phylogenetic tree. This is because weighted unifrac makes use of a tree to infer evolutionary distances as a means of measuring similarity/dissimilarity between samples, while unweighted unifrac simply clusters/groups microbial communities based on the presence or absence of taxa (Wong *et al.*,2016).

According to the cluster results depicted using Bray-Curtis dissimilarity metrics. There were notably different microbial community found in water and sediment samples, hence separate clusters of sediment and water samples were observed. However, this was not the case using weighted unifrac distance metrics, as clusters depicted showed that microbial communities from sediment and water samples were not phylogenetically different. This might be as a result of highly similar bacterial phyla discovered among both group of samples. For instance, both group of samples were dominated by the phylum Proteobacteria and Bacteroidetes, albeit with different relative abundances. Using unweighted unifrac metrics, clusters depicted indicated that separation of microbial community by clusters were significant and therefore the unweighted unweighted unifrac detected significant microbial richness between both samples. That is, the microbial species varied considerably among the samples. For instance, the number of genera and species detected in water samples were higher than those generated in samples from sediments.

Considering samples collected at different months – June and December, using beta diversity analysis with the above-named metrics, according to Bray-Curtis metrics, it

showed that there was no difference in the taxonomic composition of the microbes harvested during both periods, hence similar microbial community. Using weighted unifrac metrics, it however stated that microbial communities observed at different months were phylogenetically different, therefore it assumes that June samples possessed taxa that were distinct from December samples. Finally, unweighted unifrac showed indistinguishable clusters among these two group of samples, indicating that there was an insignificant microbial richness. For examples, the number of species detected in June was 76, while those detected in December was 77, slightly different in number from each other.

The core microbiome was analysed to identify shared OTUs (Operational Taxonomic Units) at 90% core threshold, across groups of samples. The presence of a core microbiome is used to refer to organisms, functions or genes that are common by all or most samples in a given habitat (usually set at a particular percentage of the total sample, known as core threshold) (Turnbaugh *et al.*, 2007). Among sediment and water samples, two bacterial species were identified as common to these group of samples: namely *Vogesella* sp. and *Acinetobacter* sp. Both microbes belong to the phylum Proteobacteria, which is not surprising, as the phylum recorded the largest abundance from both sediment and water samples. *Acinetobacter* sp. is also known for its ubiquity in both habitats (Adewoyin and Okoh, 2018). Strains of *Vogesella* sp. have been documented to be capable of denitrification in their natural environment. They also adapt to changes in the concentration of oxygen available in their surrounding (Duan *et al.*, 2020). *Vogesella* sp. have been isolated from freshwater habitats such as those reported by Sheu *et al.* (2016). Likewise, from samples collected at separate months, the common microbiome observed were *Vogesella* sp. and *Acinetobacter* sp. as the core taxonomic composition of microbiomes from June and December samples. It can be inferred that their constant presence (*Acinetobacter* and *Vogesella* sp.) might play vital roles in the function and stability of the spring's microbiota irrespective of sampling location, type of samples collected and period of collection. However, the presence of *Acinetobacter* species might call for concern, as some species of this microbe have been grouped as part of the common multidrug-resistant pathogen such as *Acinetobacter baumannii.*

Differential abundance testing was also performed on several groups of samples. Differential abundance testing is another means of statistically testing the abundance of the taxonomic composition of microbes between defined groups of samples (Wallen,

2021). Statistical testing done between sediment versus water samples clearly indicated that among several significant taxa *Acinetobacter baumannii, Novispirillum* and *Acetobacteroides* showed a significantly increased abundance in water samples. Although in culture-based analysis, the presence of *A. baumannii,* was detected, but not that of *Novispirillum* and *Acetobacteroides.* Furthermore, significantly prevalent genera in sediment samples included *Pseudoglubenkiania, Morganella* and *Providencia rettgeri.* However, *Brevundimonas diminuta, Comamonas testosteroni, Vogesella* and *Aeromonas* among others, were unaffected by the physicochemical parameters of both water and sediment of the spring, as their abundance was significantly high in both group of samples. It is interesting to note that *Comamonas* sp. are versatile in their catabolic capabilities, and can adapt to a number of different environments.

Studies have suggested that *Comamonas* might play an important role in nutrient cycling, particularly in the nitrogen biogeochemical cycle. This is because this group of genera contain several genes for denitrification as well as ammonification in their genome (Wu *et al.,* 2018). Therefore, its significant presence in both samples is an indication that it also plays a significant role in nutrient transformation, food-web, and maintaining the biogeochemical cycle of Ikogosi warm spring environment. Differential abundance testing also revealed that only *Comamonas aquatic* and *Vogesella* were significantly detected in the months of December and June. Probably, the seasonal fluctuations, such as water exchange due to rainfall, changes in the physicochemical parameters, anthropogenic disturbances and in-situ hydrological changes of the spring might have limited impact on these microbes, with respect to their abundance in the freshwater habitat under study.

5.7 Targeted metagenomics analysis of the fungal diversity of Ikogosi Warm Springs observed during two seasons of the year

Fungal biodiversity studies in aquatic habitats have not been thoroughly investigated using high-throughput sequencing techniques, unlike its prokaryotic counterpart. This bias might be as a result of the assumption that they would most likely be present in low abundances in such region (Lepère *et al.,* 2019). However, recent studies have begun to reveal larger abundance and higher taxonomic richness in various freshwater habitats (Ishii *et al.,* 2015). In this study, several genera and species were discovered, hence buttressing the opinion that fungal species are also important in waterbodies. They are

famous as decomposers of debris in aquatic ecosystems and also as zoo- and phytoplankton's parasites in freshwater habitats (El-Elimat *et al.,* 2021).

To completely access the fungal biodiversity of Ikogosi warm spring, and make comparative analysis, fungal taxonomic composition of the spring was analysed as well as their diversity patterns and fungal structure occupying the spring. In illustrating the various fungal phylotypes identified from samples collected from the sediment of the spring, as well as samples collected from the ever-flowing water body, it was observed that only two phylotypes occupied the entire niche of water and sediment samples. With phylum Ascomycota being the most abundant and Basidiomycota. This result is in concordance with the report of Kambura *et al.* (2016) and Lepère *et al.* (2019), who also reported the dominance of Ascomycota, which was closely followed by Basidiomycota in samples of water and sediments from hot springs, lakes, ponds and rivers. However, a similar pattern of Ascomycota dominating more than half of the fungal community was also observed for both periods, and followed by Basidiomycota.

Ascomycota is the largest phylum of fungi with an approximate species of about 64,000. They are ubiquitous and can be found in a wide range of habitats – from terrestrial habitats such as in temperate and tropical forests, to aquatic habitat such as freshwater and marine habitats (Schoch *et al.*, 2009) hence, their dominating presence in this study is therefore expected. This was further reflected in the top 19 most abundant fungal genera detected in sediment and water samples. An average of 65% of top 19 fungal genera belonged to the large phylum Ascomycota while the rest of the identified genera belonged to the Basidiomycota phylum (25%). It is noteworthy to state that there were sequences grouped as "unknown", as their taxonomic classification as of the time of this analysis was not identified in the fungal database.

In water samples, *Meyerozyma*, *Trichosporon* and *Aspergillus* sp. topped the chart as the most abundant genera, all belonging to the large Ascomycota phylotype. The same scenario can be deduced from samples collected from sediment, as *Malassezia*, *Aspergillus*, *Condenascus* sp. were all sac-fungi with exception to *Malassezia* belonging to the phylum Basidiomycota. The presence of *Malassezia*, *Aspergillus* and *Condenascus* discovered in the sediment of this spring is anticipated, as microbial ecology studies have confirmed their presence in sediments, soil and freshwater habitats (Schoch *et al.*, 2009, Kambura *et al.* 2016). With minimal nutrient availability in the

waters of Ikogosi springs, these fungal taxa were able to survive and probably proliferate, which might be possibly due to their ability to produce spores, enabling them to endure unfavourable conditions. Also, it might be that their spores are a contributing factor to their ubiquity in the waterbody. The discovery of these microbes in water samples of the Spring is in agreement with studies of Okpako *et al.* (2009) and Lepère *et al.* (2019). The December and June samples were also analysed to have a fungal profile of the most dominant fungal genera. During the dry season (December), *Meyerozyma* and *Malassezia* sp. were the most dominant but these are yeasts in nature, unlike what was reflected during the wet season, whose 2 most dominant genera were a filamentous fungus (*Aspergillus* sp.) and a yeast-like fungus (*Trichosporon* sp).

Alpha diversity analysis was also conducted to understand the underlying fungal community inhabiting the spring. Shannon metrics indicated that even though the mean alpha-diversity for sediment was higher than in water samples, the differences observed were not statistically significant. This might be as a result of highly overlapping presence of similar fungi belonging majorly to two phyla – Ascomycota and Basidiomycota. Specifically, the Spring was heavily dominated by members of the Ascomycota family leaving minimal taxa to phyla such as Chytridiomycota, Kickxellomycota and Monoblepharomycota, hence, indicating their richness and evenness as insignificant. Other metrics used in the analysis: Observed species and Phylogenetic Diversity both recorded their fungal communities as highly similar even though their mean alpha diversity values were different, which might be as a result of highly similar fungi observed in both samples. For example, the ubiquitous *Aspergillus* detected among the most dominating fungi in both sediment and water samples. Considering samples collected at various months, the alpha diversity statistics also conducted to check for differences in the fungal community occupying the spring at specific periods of the year revealed that none of these metrics indicated any statistical significance, even though the mean alpha-diversity of samples collected in December were higher than its June counterpart samples. It can be inferred that the fungal diversity/fungal microbiome of the spring seems limited unlike its prokaryotic counterpart analysed from the spring.

To further evaluate dissimilarities between the fungal communities of Ikogosi warm springs using sediment and water samples, Bray-Curtis deduced that there was a significant dissimilarity between the fungal communities of sediment and water from

the spring, therefore the cluster segregated sediment samples from water samples. This deduction might have been as a result of the difference observed in the number of detected genera and species in the sediment and water samples. Nonetheless, weighted and unweighted unifrac metrics depicted otherwise, stating that fungal communities of the sediment and water samples were not dissimilar form each other, but indeed similar to one another. Since weighted unifrac considers phylogenetic diversity, it seems to infer that both fungal communities are not divergent enough, but might rather share a common phylogenetic ancestor. Evaluating fungal population of December and June samples, none of the above-named metrics yielded a statistically dissimilar population. In terms of microbial abundance or phylogenetically. It might be safe to infer that the OTUs observed in this diversity study were majorly represented by Ascomycota and Basidiomycota, which might not be diverse enough to yield a robust fungal diversity.

In a bid to identify fungal taxa that are constantly present/stable within the Spring, core microbiome analysis was performed. This methodology enables the researcher to recognise those taxa that are characteristics of an environment. Another purpose of performing this analysis is to be able to identify microbes that are essential for the environment's biotic and abiotic functions (Risely, 2020; Neu *et al.*, 2021). Two fungal phylotypes were identified as the core microbiome existing in the sediment and water of Ikogosi warm springs. These two phylotypes – *Aspergillus penicillioides* and *Malassezia restricta* are members of the dominant Ascomycota, therefore their persistence in the sediment and water of the spring is not unexpected. The presence of these persistent fungal microbes was also noticed among samples collected in December and June. It can be inferred that it is likely these microbes are an essential component of the Spring. For instance, *Aspergillus* sp. have been recognised for their indispensable role in nutrient cycling, such as Phosphorus, Carbon, Sulphur, and Nitrogen by organic matters such as plant debris and litters, and also through the production of metabolites (Heo *et al.*, 2019). They are also excellent solubilisers and mobilisers of insoluble phosphates making soluble phosphate compounds available for plant-use (Nahas, 2015). A number of *Aspergillus* species that have been recognised as phosphate solubilisers include *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus terreus* and *Aspergillus avus* (Nayak *et al.*, 2020). The second core fungal species - *Malassezia restricta*, has been associated with the human skin as an opportunistic yeast-like fungus involved in seborrheic dermatitis. It has also been detected in marine habitats using culture-

independent techniques (Amend, 2014).

Differentially abundance testing was conducted between two group of samples, to identify significantly abundant taxa. However, a number of fungal taxa were significantly abundant in both sediment and water samples. They include *Malassezia restricta* and *Malassezia sp.* which emphasises the high traffic of anthropogenic activities that might be entrenched in human traditional belief systems which supports the washing of head into a flowing stream. Genera such as *Torula* and *Cerrena unicolor* were exclusively found in water samples of the spring only. *Torula* is a yeast-like fungus associated with dry leaves and twigs of shrubs known as *Felia microphylla*. *Torula* sp. are saprophytic in nature, and can be found in both aquatic and terrestrial environments (Oh *et al.*,2001). The spring is shrouded with heavy vegetation and the absence of leaves, shrubs and debris dropping in the water body in any part of its environment is almost impossible. It is likely that these microbes might have been introduced into the water of the Spring by its surrounding dense vegetation.

Testing for abundance in December sample vs June samples, it seems a number of fungal species were significantly abundant in December samples, than in June samples. Also, worthy of note is the presence of *Aspergillus* and *Malassezia globosa* whose abundance were significant in both group of samples, hence reiterating the fact that these genera could be of environmental importance in Ikogosi warms spring environment. Fungi generally are known to be important source of food to phytoplanktons and zooplanktons and occupy an important niche in the food web (El-Elimat *et al.,* 2021).

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

Culture-dependent and culture-independent bacterial and fungal diversity studies were conducted from sediment and water samples collected from Ikogosi warm springs, Ekiti state, Nigeria. Inclusive was physicochemical analysis of the spring during the dry and wet season. Physicochemical studies revealed the slightly acidic nature of the spring at the source of the warm spring, while other parts of the spring were neutral to alkaline in pH. The temperature of the warm spring at the source of the warm spring was significantly different from the temperature of cold spring even at different seasons. This study also showed that all physicochemical parameters tested for water samples during both seasons were well within WHO standard. In culture-dependent bacterial analysis, members of the phyla Firmicutes, Bacteroidetes and Proteobacteria were predominantly identified from all samples collected at different sampling points of the warm spring, which is in tandem with analysis from culture-independent bacterial analysis which also reported same phyla from the spring.

Sediment and water samples showed that there was an overlapping community of bacteria and fungi present in water and sediment samples of the spring using both culture and culture-independent methodology. However, in culture-dependent studies, using Phylogenetic Diversity (PD) metrics in alpha diversity computation, it revealed that there were phylogenetically different community of bacteria present in water and sediment samples of the spring. Beta diversity metrics using Bray-Curtis dissimilarity depicted that there were significantly different abundances of microbes in water samples than in sediment samples, hence stating that the communities were dissimilar. The core microbiome analysis stated that there were two significantly present bacteria in more than 90% of samples analysed namely: *Acinetobacter* and *Vogesella* both of them belonging to the large phylum of Proteobacteria. Differentially abundant testing, which informs researchers about the significance of taxonomic abundance revealed that bacterial taxa abundances differed between sediment and water samples. However,

Brevundimonas diminuta, *Comamonas testosteroni*, *Vogesella* and *Aeromonas* were significantly abundant in both sediment and water samples.

Comparing samples collected at different months (December and June), the most abundant phylum observed from both samples were Proteobacteria and Bacteroidetes. However, major dinstinctions among these samples collected in separate months include, a large relative abundance of Firmicutes were observed in June samples, which was more than thirty times higher in abundance than those observed in December samples. In addition, beta diversity reports using weighted unifrac, showed that December and June samples had phylogenetically different community of bacteria. Core microbiome analysis revealed the *Acinetobacter* and *VogeseIla* as core microbiota present in both months, nonetheless this analysis revealed *Aeromonas* sp. as the only unique taxa discovered from samples collected in June, and *Comamonas* sp. as the only unique taxa discovered in December samples. Differential abundance testing illustrated that 19 bacterial taxa had relative abundances that were significantly different among samples collected in June and December. However, the most prevalent bacteria occupying more than 90% samples collected during both months were *Comamonas aquatic* and *Vogesella* sp. Bacterial metagenome sequences were deposited into the NCBI SRA Archive with the study ID PRJNA783904, with accession numbers: SAMN23457566 - SAMN23457585.

In fungal microbiome studies, the most prevalent phyla were Ascomycota and Basidiomycota in groups of sediment and water samples as well as groups of December and June samples. At the genera level, a larger percentage of *Meyerozyma*, *Trichosporon* and *Aspergillus* were observed in water samples than in sediment samples, while sediment samples contained higher abundance of *Debaryomyces*. December samples contained *Meyerozyma* sp. which was more than twenty times larger in abundance than in samples collected in June. However, June samples recorded a larger abundance of *Aspergillus* sp. and *Penicillium* sp. which were more than five times greater in abundance than those found in December samples. Alpha diversity analysis using Shannon, observed species and phylogenetic Diversity (PD) revealed no statistical differences in their population in water versus sediment samples and in December versus June samples. However, using Bray-Curtis dissimilarity index in beta diversity analysis, it depicted that the abundances of fungal microbes in the sediment versus water samples

and in December versus June samples were not similar, hence they were statistically different from each other. Core microbiome analysis revealed the dominance of *Aspergillus penicillioides* and *Malassezia restricta* as their core fungal microbiome in sediment and water samples, as well as in December and June samples. Differential abundance testing showed that the abundance of *Malassezia restricta* was significantly abundant in both sediment and water samples from the spring. This analysis also revealed the dominating presence of *Penicillium hetheringtonii* and *Meyezyroma guilliermondii* from both sediment and water samples collected at different months of the year. Fungal metagenome sequences generated in this study have been archived in NCBI under the SRA study ID PRJNA779450 with accession numbers: SAMN23038675 - SAMN23038694.

In culture-dependent studies, two best alpha-amylase-producing bacteria were chosen for alpha-amylase production. Both isolates were members of the genus *Bacillus* namely: *Bacillus cereus* MPW3E (accession number OL639188) and *Bacillus siamensis* SW3F (accession number OP554284). Using OFAT design, the best optimisation condition for the production of alpha-amylase using *Bacillus cereus* MPW3E was at 55°C (14.690 U/mL), pH 7.0 (15.220 U/mL), using 2% cassava peel as its carbon source (23.631U/mL), and 1.5% yeast extract as nitrogen source (21.688U/mL), supplemented with 0.01% calcium ions with a 1% seed inoculum size (v/v) in the production medium (30.81U/mL) and a 48-hour incubation time (37.184U/mL). The produced alpha-amylase had characteristics similar to a relatively weak thermotolerant alpha-amylase enzyme with an optimum temperature of 50°C and at pH 6.0 and at a substrate concentration of 1%. The enzyme activity was disrupted with the inclusion of denaturants and chelators (SDS, Urea, EDTA and Tween-20). However, the enzyme activity increased in the presence of calcium and magnesium salts. Kinetic parameters of the crude enzyme were 0.1973mM as its K_m and 6.3052µmol/min as its $V_{max.}$ An evidence that the enzyme would attain maximum velocity rapidly, hence transformation of substrate to products would be rapid. The crude enzyme was purified using a 3-step approach: Dialysis, salting out and column chromatography. Each purification approach resulted in an increase in its specific activity (within the range of 0.155 – 0.792) and purification fold (within a of 1.000 – 5.111).

Using *Bacillus siamensis*SW3F in the production of alpha-amylase, physiological conditions that supported the best production of alpha-amylase was at a temperature of

55°C (15.220 U/mL), pH 7.0 (23.942 U/mL), using 1% cassava peel as its carbon source (25.461U/mL), and 1.5% tryptone as nitrogen source (26.992 U/mL), supplemented with 0.01% calcium ions, using a 1% seed inoculum size (v/v) in the production medium (32.28U/mL) and a 48-hour incubation time (42.488U/mL). The physiological characteristics of the produced enzyme was as follows: A optimum pH and temperature of 7.0 (35.718 U/mL) and 55°C (26.008 U/mL) and maximum enzyme activity at 1% substrate concentration (30.510U/mL). The enzyme activity was disrupted when denaturants and chelators such as SDS, Urea, EDTA and Tween-20 were introduced into the medium. However, the enzyme activity increased in the presence of calcium and magnesium salts as observed in enzymes produced from *Bacillus cereus* MPW3E. Kinetic parameters of the crude enzyme were 0.0519mM as its K_m and 25.9740 µmol/min as its $V_{max.}$ Purification approach used were dialysis, salting out and column chromatography. Each purification approach resulted in an increase in its specific activity (within the range of 0.171 – 0.804) and purification fold (within a range of 1.000 – 4.701).

6.2 Conclusion

This research work has demonstrated the importance of Ikogosi warm springs, as an indigenous reservoir of microbes that are of industrial relevance. Some of these microbes also showed the capability to produce metabolites which can withstand high temperature. In addition, both culture and culture-independent approaches showed the presence of both bacteria and fungi, however, culture-independent analysis was able to establish microbial structures, core microorganisms and unidentified microbes inhabiting various locations of the spring.

6.3 Recommendations

Taxonomic composition and abundance of microorganisms in the spring varied with location and sample types (sediment and water). This should be adopted to source for specific phyla of bacteria and fungi with diverse potentials especially in industrial applications.

Using targeted metagenomics studies, Ikogosi warm spring is a potential source of relevant genera of microorganism which are tolerant to seasonal changes in environmental conditions.

Having shown the industrial importance of Ikogosi warm springs, particularly with respect to microbes inhabiting the spring and their ability to tolerate elevated temperature, genetic studies can be done to increase their metabolites' thermal and pH stability, and mass producing them for local purchase. This would give indigenous enzyme production companies a competitive edge in the global enzyme market.

The identified non-culturable organisms in Ikogosi warm springs should be further characterised through functional metagenomics analysis to establish their potential for high-temperature, enzyme-linked industrial applications.

6.4 Contributions to Knowledge

I. This is the first report that brings to the fore, differences occuring within the microbial community structure of Ikogosi warm springs, using both culture-dependent and metagenomic methodologies.
II. The study reports the total diversity and abundance of microbial taxa in the Ikogosi warm springs, thereby establishing the spring as a reservoir of unique microbial source to solve environmental and industrial-related challenges.
III. This research study has shown that there are seasonal differences in the physicochemical parameters of the spring water and sediment, as well as in their microbial community.
IV. Culture-independent analysis revealed the presence of some unidentified microbes as well as "difficult-to-grow" microbial strains such as *Malassezia restricta* and *Fluviicola* in the warm spring.

REFERENCES

Abdel-Azeem, A.M., Abdel-Azeem, M.A., Abdul-Hadi, S.Y. and Darwish, A.G. 2019a (eds) *Aspergillus*: Biodiversity, Ecological Significances, and Industrial Applications. In *Recent Advancement in White Biotechnology through Fungi*. Yadav, A., Mishra, S., Singh, S., Gupta, A. (eds) Springer Cham. Switzerland. 121-179.

Abdel-Azeem, A.M., Abdel-Azeem, M.A., Darwish, A.G., Nafady, N.A. and Ibrahim, N.A., 2019b. *Fusarium*: Biodiversity, Ecological Significances, and Industrial Applications. In *Recent Advancement in White Biotechnology through Fungi*. Yadav, A., Mishra, S., Singh, S., Gupta, A. (eds) Springer, Cham. 201-261.

Abdeljabbar, H., Cayol, J. L., Hania, W. B., Boudabous, A., Sadfi, N. and Fardeau, M. L. 2013. *Halanaerobium sehlinense* sp. nov., an Extremely Halophilic, Fermentative, Strictly Anaerobic Bacterium from Sediments of The Hypersaline Lake Sehline Sebkha. *International Journal of Systematic and Evolutionary Microbiology* 63.6:2069-2074.

Abdullah, S.K. and Abbas, B.A. 2008. Fungi inhabiting surface sediments of Shatt Al-Arab River and its Creeks at Basrah, Iraq. *Basrah Journal of Science* 26.1:68-81.

Abel-Nabey, H.M. and Farag, A.M. 2016. Production, Optimization and Characterization of Extracellular Amylase from Halophilic *Bacillus licheniformis* AH214. *African Journal of Biotechnology* 15.17: 670-683.

Abootalebi, S.N., Saeed, A., Gholami, A., Mohkam, M., Kazemi, A., Nezafat, N., Mousavi, S.M., Hashemi, S.A. and Shorafa, E. 2020. Screening, Characterization and Production of Thermostable Alpha-Amylase Produced By a Novel Thermophilic *Bacillus megaterium* Isolated from Pediatraic Intensive Care Unit. *Journal of Environmental Treatment Techniques* 8.3:952 - 960.

Acikara, Ö. B. 2013. Ion Exchange Chromatography and Its Applications. *Column Chromatography 10*: 1- 6.

Adedire, D.E., Jimoh, A.O., Kashim-Bello, Z., Shuaibu, B.A.W., Popoola, O.A., Pate, K.I., Uzor, O.S., Etingwa, E., Joda, J.F., Opaleye, O.O. and Adeniran, K.R., 2021. Assessment of the Physicochemical Qualities and Microbiological Profile of Idah

River, Kogi State, Nigeria. *African Journal of Microbiology Research* 15.7:377-387.

Adedire, D.E., Jimoh, A.O., Kashim-Bello, Z., Shuaibu, B.A.W., Popoola, O.A., Pate, K.I., Uzor, O.S., Etingwa, E., Joda, J.F., Opaleye, O.O. and Ogunlowo, V.A., 2022. Microbiome Diversity Analysis of the Bacterial Community in Idah River, Kogi State, Nigeria. *Advances in Microbiology* 12.5:343-362.

Adedire, O., Ogundipe, W. and Farinu, A. 2013. Studies on Crude Thermostable Amylase Produced by *Bacillus* Spp. Isolated from Starch Waste. *International Journal of Science and Research* 2.4: 396 – 400.

Adesakin, T.A., Oyewale, A.T., Bayero, U., Mohammed, A.N., Aduwo, I.A., Ahmed, P.Z., Abubakar, N.D. and Barje, I.B. 2020. Assessment of Bacteriological Quality and Physico-Chemical Parameters of Domestic Water Sources in Samaru Community, Zaria, Northwest Nigeria. *Heliyon* 6.8:1 – 13.

Adewoyin, M.A. and Okoh, A.I. 2018. The Natural Environment as a Reservoir of Pathogenic and Non-Pathogenic *Acinetobacter* species. *Reviews on Environmental Health* 33.3: 265 - 272.

Agrawal, P. K., Agrawal, S. and Shrivastava, R. 2015. Modern Molecular Approaches for Analyzing Microbial Diversity from Mushroom Compost Ecosystem. *3 Biotechnology* 5.6: 853-866.

Ajayi, A.O. and Ige, O.F. 2019. Microbes from Water and Fish of Ose River in Ondo State, Nigeria. *Science Research Annals* 10: 101 -107.

Akan, J.C., Abbagambo, M.T., Chellube, Z.M. and Abdulrahman, F.I. 2012. Assessment of Pollutants in Water and Sediment Samples in Lake Chad, Baga, North Eastern Nigeria. *Journal of Environmental Protection* 2012:1428 – 1441.

Albakaa, A. R. M., Ameen, D. S. M., Abed, N. K., Jabbar, Z. A., and Musaa, L. A. 2021. Quantification of Ca, K, Mg, Zn and Fe Elements in Grape Leaves from Different Regions of Iraq By Atomic Absorption Spectroscopy. In *Journal of Physics: Conference Series* 1853.1:12018 - 12023

Alexopoulos, A., Plessas, S., and Bezirtzoglou, E. 2009. Water Microbial Ecology –An Overview. *Encyclopedia of Life Science* 1-24.

Al-Johani, N.B., Al-Seeni, M.N. and Ahmed, Y.M., 2017. Optimization of Alkaline α-Amylase Production by Thermophilic *Bacillus subtilis*. *African Journal of Traditional, Complementary and Alternative Medicines* 14.1:88-301.

Alonazi, M., Karray, A., Badjah-Hadj-Ahmed, A.Y. and Ben Bacha, A. 2021. Alpha Amylase from *Bacillus pacificus* Associated with Brown Algae *Turbinaria ornata*: Cultural Conditions, Purification, and Biochemical Characterization. *Processes* 9.1:16 – 21.

Amadi, L.O., Berembo, B.T. and Wemedo, S.A. 2020. Microbiological and Physicochemical Properties of Krakrama Brackish Water in Rivers State, Niger Delta, Nigeria. *Acta Scientific Microbiology* 3.5:123-132.

Amanial, H.R, 2015. Assessment of Physicochemical Quality of Spring Water in Arbaminch, Ethiopia. *Journal of Environmental Analytical Chemistry* 2.157:2380 - 2391.

Amend, A. 2014. From Dandruff to Deep-Sea Vents: Malassezia-Like Fungi are Ecologically Hyper-Diverse. *PLoS Pathogens* 10.8:1 - 7.

Andrews S. 2010. FastQC: A Quality Control Tool for High Throughput Sequence Data. Available Online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc. Retrieved June 27, 2022.

Andronov, E.E., Petrova, S.N., Pinaev, A.G., Pershina, E.V., Rakhimgalieva, S.Z., Akhmedenov, K.M., Gorobets, A.V. and Sergaliev, N.K. 2012. Analysis of the Structure of Microbial Community in Soils with Different Degrees of Salinization using T-RFLP and Real-Time PCR Techniques. *Eurasian Soil Science* 45.2:147-156.

Anto, H., Trivedi, U. and Patel, K. 2006. α-Amylase Production by *Bacillus cereus* MTCC 1305 Using Solid-state Fermentation. *Food Technology and Biotechnology* 44.2:241-245.

Anupama, A. and Jayaraman, G. 2011. Detergent Stable, Halotolerant α-Amylase from *Bacillus aquimaris* vitp4 Exhibits Reversible Unfolding. *International Journal of Applied Biology and Pharmaceutical Technology* 2:366-76.

APHA, 2005. Standard Methods for the Estimation of Water and Waste Water, 21th edn. American Waste Water Association and Water Pollution Control Federation, Washington.

Aragno, M. 1981. Responses of Microorganisms to Temperature. In *Physiological Plant Ecology I*. Encyclopedia of Plant Physiology. Lange, O.L., Nobel, P.S., Osmond, C.B., Ziegler, H. (eds) Springer, Berlin, Heidelberg 339-369.

Ari, Ş. and Arikan, M. 2016. Next-Generation Sequencing: Advantages, Disadvantages, and Future. In Plant omics: *Trends and Applications*. Hakeem, K., Tombuloğlu, H., Tombuloğlu, G. (eds) Springer Cham, Switzerland 109-135.

Arya, M., Shergill, I. S., Williamson, M., Gommersall, L., Arya, N. and Patel, H. R. 2005. Basic Principles of Real-Time Quantitative PCR. *Expert Review of Molecular Diagnostics* 5.2:209-219.

Aryal, S., Karki, G. and Pandey, S. 2015. Microbial Diversity in Freshwater and Marine Environment. *Nepal Journal of Biotechnology* 3.1: 68-70.

Aygan, A., Sariturk, S., Kostekci, S. and Tanis, H. 2014. Production and Characterization of Alkaliphilic Alpha-amylase from *Bacillus subtilis* A10 Isolated from Soils of Kahramanmaras, Turkey. *African Journal of Microbiology Research* 8.21:2168 - 2173.

Baghalabadi, V., Razmi, H. and Doucette, A. 2021. Salt-Mediated Organic Solvent Precipitation for Enhanced Recovery of Peptides Generated by Pepsin Digestion. *Proteomes* 9.4:1 – 14.

Baeza, M., Barahona, S., Alcaíno, J. and Cifuentes, V. 2017. Amplicon-metagenomic Analysis of Fungi from Antarctic Terrestrial Habitats. *Frontiers in Microbiology*, 8:2235 – 2245.

Balvočiūtė, M., Huson, D.H., SILVA, R. and Greengenes, N.C.B.I., 2017. OTT—How Do These Taxonomies Compare. *BMC Genomics* 18.2:114.

Bandal, J.N., Tile, V.A., Sayyed, R.Z., Jadhav, H.P., Azelee, N.I., Danish, S. and Datta, R. 2021. Statistical Based Bioprocess Design for Improved Production of Amylase from Halophilic *Bacillus* sp. H7 Isolated from Marine Water. *Molecules* 26.10:2833

Barbosa, R.N., Bezerra, J.D., Souza-Motta, C.M., Frisvad, J.C., Samson, R.A., Oliveira, N.T. and Houbraken, J., 2018. New *Penicillium* and *Talaromyces* species from Honey, Pollen and Nests of Stingless Bees. *Antonie van Leeuwenhoek* 111.10:1883-1912.

Barnett, H.L. and Hunter, B.B. 2006. *Illustrated Genera of Imperfecti Fungi*. 4th Ed. Rockville, Maryland. APS Press 92 -94.

Baselga, A. 2013. Separating the Two Components of Abundance-based Dissimilarity: Balanced Changes in Abundance vs. Abundance Gradients. *Methods in Ecology and Evolution* 4.6: 552-557.

Bausum, H. T. and Matney, T. S. 1965. Boundary between Bacterial Mesophilism and Thermophilism. *Journal of Bacteriology* 90.1:50-53.

Başyiğit, B. and Tekin-Özan S. 2013. Concentrations of Some Heavy Metals in Water, Sediment, and Tissues of Pikeperch Sander lucioperca from Karataş Lake Related to Physico-Chemical Parameters, Fish Size, and Seasons. *Polish Journal of Environmental Studies* 22.3:1 – 13.

Bradford, W.L. and Horowitz, A.J. (eds). 1988. The Role of Sediments in the Chemistry of Aquatic Systems*: Proceedings of the Sediment Chemistry Workshop* 969: 8-12.

Bellemain, E., Carlsen, T., Brochmann, C., Coissac, E., Taberlet, P. and Kauserud, H. 2010. ITS as an Environmental DNA Barcode for Fungi: An In-silico Approach Reveals Potential PCR biases. *BMC Microbiology* 10.1:189 – 200.

Bello, A.Y., Abdulkadir, N., Abubakar, S. and Lawal, A. 2021. Studies on Screening and Optimization of Amylase Enzyme Production using Bacteria Isolated from Soil. *Journal of Microbiology and Experimentation* 9.6: 196 – 200.

Benjamin, S., Smitha, R.B., Jisha, V.N., Pradeep, S., Sajith, S., Sreedevi, S., Priji, P., Unni, K.N. and Josh, M.S. 2013. A Monograph on Amylases from *Bacillus* sp. *Advances in Bioscience and Biotechnology* 4.2: 1 - 15.

Bergkessel, M. and Guthrie, C. 2013. Colony PCR. In *Methods in Enzymology*. Lorsch, J.(eds) Academic Press. USA 529: 299 - 309.

Besser, J., Carleton, H. A., Gerner-Smidt, P., Lindsey, R. L. and Trees, E. 2018. Next-Generation Sequencing Technologies and their Application to the Study and Control of Bacterial Infections. *Clinical Microbiology and Infection* 24.4: 335-341.

Bharose, A.A., Gajera, H.P., Hirpara, D.G., Kachhadia, V.H. and Golakiya, B.A., 2017. Molecular Identification and Characterization of *Bacillus* Antagonist to Inhibit aflatoxigenic *Aspergillus flavus*. *International Journal of Current Microbiology and Applied Sciences* 6.3:2466-2484.

Bhatia, S. 2018 (eds). Introduction to Enzymes and their Applications. In *Introduction to Pharmaceutical Biotechnology;* IOP Publishing Ltd. Bristol, UK. 1 -29

Bianchini Jr, I., Peret, A.M. and Cunha-Santino, M.B., 2006. A Mesocosm study of Aerobic Mineralization of Seven Aquatic Macrophytes. *Aquatic Botany* 85.2:163-167.

Birtel, J., Walser, J. C., Pichon, S., Bürgmann, H. and Matthews, B. 2015. Estimating Bacterial Diversity for Ecological Studies: Methods, Metrics, and Assumptions. *PloS One* 10.4:1 -23.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F. and Bai, Y. 2019. Reproducible, Interactive, Scalable and extensible Microbiome Data Science using QIIME 2. *Nature Biotechnology* 37.8:852-857.

Branton, D., Deamer, D.W., Marziali, A., Bayley, H., Benner, S.A., Butler, T., Di Ventra, M., Garaj, S., Hibbs, A., Huang, X., Jovanovich, S.B. *et al*. 2008. The Potential and Challenges of Nanopore Sequencing. *Nature Biotechnology* 26.10: 1146-1153.

Brock, T. D., Brock, K. M., Belly, R. T. and Weiss, R. L. 1972. *Sulfolobus*: A New Genus of sulfur-oxidizing bacteria living at low pH and high temperature. *Archives of Microbiology* 84.1:54 - 68.

Broderick, J.B. (eds) 2001. *Coenzymes and cofactors.* In Encyclopedia Of Life Sciences *Nature*.United Kingdom 1 - 11.

Bromberg, J. S., Fricke, W. F., Brinkman, C. C., Simon, T. and Mongodin, E. F. 2015. Microbiota—Implications for Immunity and Transplantation. *Nature Reviews Nephrology* 11.6:342 – 352.

Brown, J. L., Ross, T., McMeekin, T. A. and Nichols, P. D. 1997. Acid Habituation of *Escherichia coli* and the Potential Role of Cyclopropane Fatty Acids in low pH tolerance. *International Journal of Food Microbiology* 37.2-3:163-173.

Burgess, R.R. 2009. Protein Precipitation Techniques. In *Methods in Enzymology*. Burgess, R.R., Deutscher, M.P (eds). Academic Press, USA 463: 331–342

Buzzini, P., Branda, E., Goretti, M.and Turchetti, B. 2012. Psychrophilic Yeasts from Worldwide Glacial Habitats: Diversity, Adaptation Strategies and Biotechnological Potential. *FEMS Microbiology Ecology* 82.2: 217-241.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A. and Holmes, S.P. 2016. DADA2: High-resolution Sample Inference from Illumina Amplicon Data. *Nature Methods* 13.7:581-583.

Callahan, B.J., McMurdie, P.J. and Holmes, S.P. 2017. Exact Sequence Variants Should Replace Operational Taxonomic Units in Marker-Gene Data Analysis. *The ISME Journal* 11.12: 2639-2643.

Callahan, B.J., Wong, J., Heiner, C., Oh, S., Theriot, C.M., Gulati, A.S., McGill, S.K. and Dougherty, M.K. 2019. High-Throughput Amplicon Sequencing of the Full-Length 16S rRna Gene with Single-Nucleotide Resolution. *Nucleic Acids Research* 47.18:103 – 110.

Callahan, B. 2020. RDP Taxonomic Training Data Formatted for DADA2 .RDP Trainset 18/release 11.5 [Data set]. Zenodo. Accessed 21/09/2021

Cambillau, C. and Claverie, J. M. 2000. Structural and Genomic Correlates of Hyperthermostability. *Journal of Biological Chemistry* 275.42: 32383-32386.

Cañedo-Argüelles, M., Kefford, B. and Schäfer, R. 2018. Salt in Freshwaters: Causes, Effects and Prospects - Introduction to the Theme Issue. *Philosophical Transactions of the Royal Society B: Biological Sciences* 374:1–6.

Canion, A., Prakash, O., Green, S. J., Jahnke, L., Kuypers, M. M. and Kostka, J. E. 2013. Isolation and Physiological Characterization of Psychrophilic Denitrifying Bacteria from Permanently Cold Arctic Fjord Sediments Svalbard, Norway. *Environmental Microbiology* 15.5: 1606-1618.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I. and Huttley, G.A. 2010. QIIME Allows Analysis of High-Throughput Community Sequencing Data. *Nature Methods* 7.5:335 - 347.

Caruso, V., Song, X., Asquith, M., and Karstens, L. 2019. Performance of Microbiome Sequence Inference Methods in Environments with Varying Biomass. *mSystems 4*.1: e00163-18.

Case, R. J., Boucher, Y., Dahllöf, I., Holmström, C., Doolittle, W. F. and Kjelleberg, S. 2007. Use of 16S rRNA and rpoB Genes as Molecular Markers for Microbial Ecology Studies. *Applied and Environmental Microbiology* 73.1: 278-288.

Castelle, C. J. and Banfield, J. F. 2018. Major New Microbial Groups Expand Diversity and Alter our Understanding of the Tree of Life. *Cell* 172.6: 1181-1197.

Cavicchioli, R., Curmi, P. M., Siddiqui, K. S. and Thomas, T. 2006. 17 Proteins from Psychrophiles. In *Methods in Microbiology* 35:395-436.

Chakravarty, S. and Varadarajan, R. 2000. Elucidation of Determinants of Protein Stability through Genome Sequence Analysis. *Febs Letters* 470.1:65-69.

Chandra, M.R.G.S. and Madakka, M. 2019. Comparative Biochemistry and Kinetics of Microbial Lignocellulolytic Enzymes. In *Recent Developments in Applied*

Microbiology and Biochemistry. Buddolla, V. (eds) Academic Press USA 147-159.

Chaudhary, N., Sharma, A.K., Agarwal, P., Gupta, A. and Sharma, V.K. 2015. 16S Classifier: A Tool for Fast and Accurate Taxonomic Classification of 16S rRNA Hypervariable Regions in Metagenomic Datasets. *PloS One*, 10.2:e0116106.

Chen, H. and Boutros, P.C. 2011. VennDiagram: A Package for the Generation of Highly-Customizable Venn and Eulerr Diagrams in R. *BMC Bioinformatics* 12.1:1-7.

Chen, B., Landry, M. R., Huang, B. and Liu, H. 2012. Does Warming Enhance the Effect of Microzooplankton Grazing on Marine Phytoplankton in The Ocean? *Limnology and Oceanography* 57.2: 519-526.

Chen, C. Y., Chen, P. C., Weng, F. C. H., Shaw, G. T. W. and Wang, D. 2017. Habitat and Indigenous Gut Microbes Contribute to the Plasticity of Gut Microbiome in Oriental River Prawn During Rapid Environmental Change. *PloS One* 12.7*:* 1-17.

Cochrane, G., Karsch-Mizrachi, I., Nakamura, Y. and International Nucleotide Sequence Database Collaboration 2011. The International Nucleotide Sequence Database Collaboration. *Nucleic Acids Research* 39:15–18.

Cochrane, G., Alako, B., Amid, C., Bower, L., Cerdeño-Tárraga, A., Cleland, I., Gibson, R., Goodgame, N., Jang, M., Kay, S. and Leinonen, R., 2012. Facing growth in the European Nucleotide Archive. *Nucleic Acids Research* 41.1:30 - 35.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M. and Tiedje, J.M. 2009. The Ribosomal Database Project: Improved Alignments and New Tools for rRNA Analysis. *Nucleic Acids Research*, 37:141-145.

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A., Kuske, C.R. and Tiedje, J.M. 2014. Ribosomal Database Project: Data and Tools for High Throughput rRNA Analysis. *Nucleic Acids Research* 42: 633-642.

Coleman, J.P. and Smith, C.J. 2007. Microbial classification. In: *XPharm: The Comprehensive Pharmacology Reference.* Elsevier, Amsterdam, 1–4

Coskun, O. 2016. Separation Techniques: Chromatography. *Northern Clinics of Istanbul* 3.2:156-160.

Cotta, S.P.M., Marins, M.S., Marriel, I.E., Lana, U.D.P., Gomes, E.A., Figueiredo, J.E.F. and Oliveira-Paiva, C.A. 2021. Thermo-resistant Enzyme-producing Microorganisms Isolated from Composting. *Brazilian Journal of Biology* 83 - 89.

Crundwell, F.K. 2017. On the Mechanism of the Dissolution of Quartz and Silica in Aqueous Solutions. *ACS Omega* 2.3:1116-1127.

Cruz, M.S., Chumpitaz, L.D., Alves, J.G.L. and Meirelles, A.J. 2000. Kinematic viscosities of polyethylene glycols. *Journal of Chemical and Engineering Data* 45.1:61-63.

DasSarma S and Arora P. (eds). 2002. Halophiles. In: *Encyclopedia of Life Sciences*. 2002. Nature London 458-466.

Dash, B.K., Rahman, M.M. and Sarker, P.K. 2015. Molecular Identification of a Newly Isolated *Bacillus subtilis* BI19 and Optimization of Production Conditions for Enhanced Production of Extracellular Amylase. *BioMed Research International* 2015:1 - 9.

de Berardinis, V., Durot, M., Weissenbach, J. and Salanoubat, M. 2009. *Acinetobacter baylyi* ADP1 as a Model for Metabolic System Biology. *Current Opinion in Microbiology* 12.5:568-576.

Deb, P., Talukdar, S.A., Mohsina, K., Sarker, P.K. and Sayem, S.M. 2013. Production and Partial Characterization of Extracellular Amylase Enzyme from *Bacillus amyloliquefaciens* P-001. *SpringerPlus* 2.1:1-12.

De Mandal, S., Panda, A. K., Bisht, S. S. and Kumar, N. S. 2015. Microbial Ecology in the Era of Next Generation Sequencing. *Next Generation Sequencing and Applications*1:1 – 6.

De Oliveira, A.P.A., Silvestre, M.A., Alves-Prado, H.F., Rodrigues, A., da Paz, M.F., Fonseca, G.G. and Leite, R.S.R. 2015. Bioprospecting of Yeasts for Amylase Production in Solid State Fermentation and Evaluation of the Catalytic Properties of Enzymatic Extracts. *African Journal of Biotechnology* 14.14:1215-1223.

Derso, S., Beyene, A., Getachew, M. and Ambelu, A., 2015. Assessment of Ecological Quality of Hot Springs in the Eastern Amhara Region. *Environmental Systems Research* 4.1:1-13.

Deljou, A. and Arezi, I. 2016. Production of Thermostable Extracellular A-Amylase By a Moderate Thermophilic *Bacillus licheniformis* Isolated from Qinarje Hot Spring (Ardebil prov. of Iran). *Periodicum biologorum 118*.4.

DeLong E (ed). 2013. Methods in Enzymology. In Microbial Metagenomics, Metatranscriptomics, and Metaproteomics, 1st edn. Academic Press, San Diego 12 -45

Demirkan, E., Sevgi, T. and Başkurt, M. 2017. Optimization of Physical Factors Affecting the Production of the Alpha-amylase from a Newly Isolated *Bacillus* sp. M10 Strain. *Karaelmas Science and Engineering Journal 7*.1.

Deutscher, J. 2008. The Mechanisms of Carbon Catabolite Repression in Bacteria. *Current Opinion in Microbiology* 11.2: 87 - 93.

Dixon, M. and Webb, E.C., 1962. Enzyme fractionation By Salting-Out: A Theoretical Note. In: *Advances in Protein Chemistry*. Anfinsen, C.B., Anson, M.L., K. Bailey, Edsall, J.T (eds). Academic Press USA 16:197-219.

Dodds, W. and Whiles, M. 2010 (eds). Freshwater Ecology. In Concepts and Environmental Applications of Limnology. 2nd Edition, Elsevier, Amsterdam, 330-333.

Drioli E., Quist-Jensen C., Giorno L. 2016. Molecular Weight Cutoff. In: *Encyclopedia of Membranes*.Drioli E., Giorno L. (eds) Springer, Heidelberg. 47 -72.

Duan, J.L., Sun, J.W., Ji, M.M., Ma, Y., Cui, Z.T., Tian, R.K., Xu, P.C., Sun, W.L. and Yuan, X.Z. 2020. Indicatory Bacteria and Chemical Composition Related to Sulfur Distribution in the River-Lake Systems. *Microbiological Research* 236:1-9.

Ducklow, H. 2008. Microbial Services: Challenges for Microbial Ecologists in a Changing World. *Aquatic Microbial Ecology* 53.1: 13-19.

Dunkley, E. A., Guffanti, A. A., Clejan, S. and Krulwich, T. A. 1991. Facultative Alkaliphiles Lack Fatty Acid Desaturase Activity and Lose the Ability to Grow at Near-neutral pH when Supplemented with an Unsaturated Fatty Acid. *Journal of Bacteriology* 173.3: 1331-1334.

Duong-Ly, K.C. and Gabelli, S.B. 2014. Salting-out of Proteins Using Ammonium Sulfate Precipitation. *Methods in Enzymology* 541:5-94.

Dutta, P., Deb, A. and Majumdar, S. 2016. Optimization of the Medium for the Production of Extracellular Amylase by the *Pseudomonas stutzeri* ISL B5 Isolated from Municipal Solid Waste. *International Journal of Microbiology* 2016:1 – 7.

Edbeib, M. F., Wahab, R. A. and Huyop, F. 2016. Halophiles: Biology, Adaptation, and their Role in Decontamination of Hypersaline Environments. *World Journal of Microbiology and Biotechnology* 32.8: 135 – 146.

Ekkers, D. M., Cretoiu, M. S., Kielak, A. M. and van Elsas, J. D. 2012. The Great Screen Anomaly—A New Frontier in Product Discovery Through Functional Metagenomics. *Applied Microbiology and Biotechnology* 93.3:1005-1020.

Ekpenyong, M., Asitok, A., Odey, A. and Antai, S. 2016. Production and Activity Kinetics of Gelatinase by *Serratia* sp. SLO3. *Nigerian Journal of Biopesticides* 1:70-82.

El-Elimat, T., Raja, H.A., Figueroa, M., Al Sharie, A.H., Bunch, R.L. and Oberlies, N.H. 2021. Freshwater Fungi as a Source of Chemical Diversity: A Review. *Journal of Natural Products* 84.3:898 -916.

Elibol, M. 2004. Optimization of Medium Composition for Actinorhodin Production by *Streptomyces coelicolor* A3 (2) with Response Surface Methodology. *Process Biochemistry* 39.9:1057-1062.

El-Fallal, A., Dobara, M.A., El-Sayed, A. and Omar, N.(eds) 2012. Starch and Microbial Alpha-Amylases. In: From Concepts to Biotechnological Applications. *Carbohydrates – Comprehensive Studies on Glycobiology and Glycotechnology*, IntechOpen Limited, London 459-488.

Elmansy, E.A., Asker, M.S., El-Kady, E.M., Hassanein, S.M. and Fawkia, M. 2018. Production and Optimization of Alpha-Amylase from Thermo-Halophilic Bacteria Isolated from Different Local Marine Environments. *Bulletin of the National Research Centre* 42.1:1-9.

Elyasi Far, B., Ahmadi, Y., Yari Khosroshahi, A. and Dilmaghani, A. 2020. Microbial Alpha-Amylase Production: Progress, Challenges and Perspectives. *Advanced Pharmaceutical Bulletin* 10.3: 350–358.

Escobar-Zepeda, A., Vera-Ponce de Leon, A. and Sanchez-Flores, A. 2015. The Road to Metagenomics: From Microbiology to DNA Sequencing Technologies and Bioinformatics. *Frontiers in Genetics 6:* 348 – 356.

Espín, Y., Menchén, A., Moreno, J.L., Sanz, D., Álvarez-Ortí, M., Fernández, J.A. and Gómez-Alday, J.J. 2021. Water and Sediment Bacterial Communities in a Small Mediterranean, Oxygen-Stratified, Saline Lake (Lake Alboraj, SE Spain). *Applied Sciences* 11.14: 1 -11.

Estendorfer, J., Stempfhuber, B., Vestergaard, G., Schulz, S., Rillig, M.C., Joshi, J., Schröder, P. and Schloter, M. 2020. Definition of Core Bacterial Taxa In Different Root Compartments of *Dactylis glomerata*, Grown in Soil under Different Levels of Land Use Intensity. *Diversity* 12.10:1 -18.

Etesami, H., Emami, S. and Alikhani, H.A. 2017. Potassium Solubilizing Bacteria (KSB): Mechanisms, Promotion of Plant Growth, and Future Prospects. A Review. *Journal of Soil Science and Plant Nutrition* 17.4: 897 - 911.

Evans, D.R., Romero, J.K. and Westoby, M. 2009. Concentration of Proteins and Removal of Solutes. *Methods in Enzymology* 463:97-120.

Fakruddin, M. and Mannan, K. 2013. Methods for Analyzing Diversity of Microbial Communities in Natural Environments. *Ceylon Journal of Science (Biological Sciences)* 42.1: 19-33.

Fan, B., Blom, J., Klenk, H.P. and Borriss, R., 2017. *Bacillus amyloliquefaciens, Bacillus velezensis,* and *Bacillus siamensis* form an "operational group *B. amyloliquefaciens*" within the *B. subtilis* species complex. *Frontiers in Microbiology* 8:22 – 28.

Fan, Z. Y., Li, X. R., Mao, D. P., Zhu, G. F., Wang, S. Y. and Quan, Z. X. 2009. Could Nested PCR be Applicable for the Study of Microbial Diversity? *World Journal of Microbiology and Biotechnology* 25.8: 1447-1452.

Fang, G., Yu, H., Sheng, H., Chen, C., Tang, Y. and Liang, Z. 2022. Seasonal Variations and Co-Occurrence Networks of Bacterial Communities in the Water and Sediment of Artificial Habitat in Laoshan Bay, China. *PeerJ 9*: 1 - 8.

Farber, G.K. and Petsko, G.A. 1990. The Evolution of α/β barrel enzymes. *Trends in Biochemical Sciences* 15.6:228-234.

Farhat, N., Hussain, S., Faisal, F., Batool, I. and Noreen, M. 2021. Physico-chemical Characteristics and Therapeutic Potential of Chutrun Thermal Springs in Shigar Valley, Gilgit-Baltistan (Pakistan). *Applied Water Science* 11.2:1 - 8.

Fasesan, D., Dawkins, K., Ramirez, R., Rasheed-Jada, H., Onilude, A., Nash, O., and Esiobu, N. 2020. Analysis of a Tropical Warm Spring Microbiota Using 16S rRNA Metabarcoding. *Advances in Microbiology* 10.4:145 – 155.

Felder, R.A. 1998. Fully Automated, Production-Scale DNA Analyzer Débuts: PE Biosystems Introduces ABI PRISM® 3700 DNA Analyzer for Genomics and other Large-Scale DNA Analysis Applications. *Journal of the association for Laboratory Automation* 3.5: 22-24

Felsenstein J. 1985. Confidence Limits on Phylogenies: An Approach Using the Bootstrap. *Evolution* 39:783-791.

Ferrera, I. and Reysenbach, A.L.(eds) 2007. Thermophiles In: *Encyclopedia of Life Sciences*.John Wiley and Sons, New Jersey. 300 – 305.

Ferroni, G. D. and Kaminski, J. S. 1980. Psychrophiles, Psychrotrophs, and Mesophiles in an Environment which Experiences Seasonal Temperature Fluctuations. *Canadian Journal of Microbiology* 26.10:1184-1191.

Fichot, E. B. and Norman, R. S. 2013. Microbial Phylogenetic Profiling with the Pacific Biosciences Sequencing Platform. *Microbiome* 1.1:10 – 25.

Fischer. E. 1894. "Einfluss der configuration auf die wirkung der enzyme," *Berichte der Deutschen Chemischen Gesellschaft* 27.3:2985–2993.

Folch, B., Dehouck, Y. and Rooman, M., 2010. Thermo- and Mesostabilizing Protein Interactions Identified by Temperature-dependent Statistical Potentials. *Biophysical Journal* 98.4:667-677.

Fricker, A. M., Podlesny, D. and Fricke, W. F. 2019. What is New and Relevant for Sequencing-Based Microbiome Research? A Mini Review. *Journal of Advanced Research* 19:105–112.

Fröhlich-Nowoisky, J., Pickersgill, D. A., Després, V. R. and Pöschl, U. 2009. High Diversity of Fungi in Air Particulate Matter. *Proceedings of the National Academy of Sciences* 106.31:12814-12819.

Fukuchi, S., Yoshimune, K., Wakayama, M., Moriguchi, M., and Nishikawa, K. 2003. Unique Amino Acid Composition of Proteins in Halophilic Bacteria. *Journal of Molecular Biology* 327.2: 347-357.

Gao, H., Wang, Y., Luo, Q., Yang, L., He, X., Wu, J., Kachanuban, K., Wilaipun, P., Zhu, W. and Wang, Y. 2021. Bioactive Metabolites from Acid-Tolerant Fungi in a Thai Mangrove Sediment. *Frontiers in Microbiology* 11:3587 -3592.

García-López, R., Cornejo-Granados, F., Lopez-Zavala, A.A., Sánchez-López, F., Cota-Huízar, A., Sotelo-Mundo, R.R., Guerrero, A., Mendoza-Vargas, A., Gómez-Gil, B. and Ochoa-Leyva, A. 2020. Doing More with Less: A Comparison of 16S Hypervariable Regions in Search of Defining the Shrimp Microbiota. *Microorganisms* 8.1:134 -144.

Garland, J. L. 1996. Patterns of potential C Source Utilization by Rhizosphere Communities. *Soil Biology and Biochemistry* 28.2: 223-230.

Garland, J. L. and Mills, A. L. 1991. Classification and Characterization of Heterotrophic Microbial Communities on the Basis of Patterns of Community-Level Sole-Carbon-Source Utilization. *Applied and Environmental Microbiology* 57.8: 2351-2359.

Gaucher, E. A., Kratzer, J. T. and Randall, R. N. 2010. Deep Phylogeny--How a Tree Can Help Characterize Early Life on Earth. *Cold Spring Harbor Perspectives in Biology* 2.1: 1 – 15.

Gaujoux R, Seoighe, C and Gaujoux, M.R. 2020. *The package NMF: Manual Pages*. Accessed on 21/09/2021.

Genilloud, O., González, I., Salazar, O., Martín, J., Tormo, J. R. and Vicente, F. 2011. Current approaches to exploit actinomycetes as a source of novel natural products. *Journal of Industrial Microbiology and Biotechnology* 38.3:375-389.

Ghilamicael, A. M., Budambula, N. L. M., Anami, S. E., Mehari, T., and Boga, H. I. 2018. Thermotolerant bacteria of biotechnological potential from hot springs in Eritrea. *African Journal of Microbiology Research* 12.22:512-524.

Ghosh, A., Mehta, A., Khan, A.M. 2019. Metagenomic Analysis and its Applications. In: *Encyclopedia of Bioinformatics and Computational Biology*. Ranganathan S, Gribskov, M., Nakai, K., Schönbach., C (eds). Academic Press. USA 184–193.

Ghosh, W and Dam, B. 2009. Biochemistry and Molecular Biology of Lithotrophic Sulfur Oxidation by Taxonomically and Ecologically Diverse Bacteria and Archaea. *FEMS Microbiology Reviews* 33.6: 999 -1043.

Ginting, E.L., Wantania, L., Moko, E.M., Tumbol, R., Siby, M. and Wullur, S. 2021. Isolation and Identification of Thermophilic Amylolytic Bacteria from Likupang Marine Hydrothermal, North Sulawesi, Indonesia. *Biodiversitas Journal of Biological Diversity* 22.6:3326 -3332.

Glenn, A. R. and Dilworth, M. J. 1991. Soil Acidity and the Microbial Population: Survival and Growth of Bacteria in Low pH. In: *Plant-soil interactions at low pH.* Wright, R.J., Baligar, V.C., Murrmann, R.P. (eds) Springer, Dordrecht. 567-579.

Glöckner, F.O., Yilmaz, P., Quast, C., Gerken, J., Beccati, A., Ciuprina, A., Bruns, G., Yarza, P., Peplies, J., Westram, R. and Ludwig, W. 2017. 25 Years of Serving the Community with Ribosomal RNA Gene Reference Databases and Tools. *Journal of Biotechnology* 261:169-176.

Gómez, F.(eds) 2011. Acidophile. In: *Encyclopedia* of *Astrobiology*. Springer, Heidelberg. 100 -130.

Gómez-Villegas, P., Vigara, J., Romero, L., Gotor, C., Raposo, S., Gonçalves, B. and Léon, R. 2021. Biochemical Characterization of the Amylase Activity from the New Haloarchaeal Strain *Haloarcula* sp. Hs Isolated in the Odiel Marshlands. *Biology* 10.4: 337 -353.

Gooch J.W. 2011. Facultative Psychrophile. In: *Encyclopedic Dictionary of Polymers*. Gooch J.W. (eds) Springer, New York. 1 – 10.

Gosavi, M.C 2016. Applications of DNA Barcoding in Molecular Systematics of Fungi: A Review. *International Journal of Life Sciences* 7:111-115.

Gromiha, M. M., Thomas, S. and Santhosh, C. 2002. Role of Cation-π Interactions to the Stability of Thermophilic Proteins. *Preparative Biochemistry and Biotechnology* 32.4: 355-362.

Grant, S., Sorokin, D. Y., Grant, W. D., Jones, B. E., and Heaphy, S. 2004. A Phylogenetic Analysis of Wadi el Natrun Soda Lake Cellulase Enrichment Cultures and Identification of Cellulase Genes from these Cultures. *Extremophiles* 8.5: 421-429.

Guimarães, L.H.S.(eds), 2012. Carbohydrates from biomass: Sources and Transformation by Microbial Enzymes. In: *Carbohydrates-Comprehensive Studies on Glycobiology and Glycotechnology; Intech: London*.441-456.

Gurung, N., Ray, S., Bose, S. and Rai, V. 2013. A Broader View: Microbial Enzymes and their Relevance in Industries, Medicine, And Beyond. *BioMed Research International* 1 -18.

Gunde-Cimerman, N., Plemenitaš, A. and Oren, A. 2018. Strategies of Adaptation of Microorganisms of the Three Domains of Life to High Salt Concentrations. *FEMS Microbiology Reviews* 42.3:353-375.

Hagel, L. 1998. Gel-filtration Chromatography. *Current Protocols in Molecular Biology* 44.1: 10-19.

Hairul, N.B.I., Ojo, K.A., Kasimu, M.A., Gafar, O.Y., Okoloba, V. and Mohammed, S.A. 2013. Ikogosi Warm Water Resorts: What You Don't Know? *Interdisciplinary Journal of Contemporary Research in Business* 4: 280-303.

Halder, D., Biswas, E. and Basu, M. 2014. Amylase Production by *Bacillus cereus* strain BRSC-S-A26MB Under Optimal Laboratory Condition. *International Journal of Current Microbiology and Applied Sciences* 3.6:1035- 1047.

Harrigan, W.F. and McCance, M.E. (eds) 1976. *Laboratory Methods in Food and Dairy Microbiology*. Academic Press London.

Heo, I., Hong, K., Yang, H., Lee, H.B., Choi, Y.J. and Hong, S.B. 2019. Diversity of *Aspergillus*, *Penicillium*, and *Talaromyces* species Isolated from Freshwater Environments in Korea. *Mycobiology* 47.1:12-19.

Hedhammar, M., Karlström, A.E. and Hober, S. 2006. Chromatographic Methods For Protein Purification. *Stockholm: Royal Institute of Technology* 1-31.

Helke, E. and Weyland, H. 2004. Psychrophilic versus Psychrotolerant Bacteria-Occurrence and Significance in Polar and Temperate Marine Habitats. *Cellular and Molecular Biology* 50.5:553-561.

Heo, I., Hong, K., Yang, H., Lee, H.B., Choi, Y.J. and Hong, S.B. 2019. Diversity of *Aspergillus*, *Penicillium*, and *Talaromyces* species Isolated from Freshwater Environments in Korea. *Mycobiology* 47.1:12 - 19.

Hill, G.T., Mitkowski, N.A., Aldrich-Wolfe, L., Emele, L.R., Jurkonie, D.D., Ficke, A., Maldonado-Ramirez, S., Lynch, S.T. and Nelson, E.B. 2000. Methods for Assessing the Composition and Diversity of Soil Microbial Communities. *Applied Soil Ecology* 15.1:25-36.

Hiteshi, K. 2016. Production Optimization of Alpha-amylase from *Bacillus licheniformis*. *Journal of Advance Research in Pharmacy & Biological Science 2*.5:01-14.

Hiteshi, K and Gupta, R. 2014. Thermal Adaptation of α-amylases: A Review. *Extremophiles* 18.6:937-944.

Hofmeister, F. 1888. About the Science of The Effects of Salts: About the Water Withdrawing Effect of the Salts. *Archive for Experimentelle Pathologie und Pharmakologie* 24:247-260.

Hood, L. and Rowen, L. 2013. The Human Genome Project: Big Science Transforms Biology and Medicine. *Genome Medicine* 5.9: 79 – 88.

Horikoshi, K. 1999. Alkaliphiles: Some Applications of their Products for Biotechnology. *Microbiology and Molecular Biology Reviews* 63.4:735-750.

Hu, Q. and Liu, J. 2021. Production of α-Amylase by *Bacillus subtilis* QM3 and its Enzymatic Properties. *Open Access Library Journal* 8 .3:1 - 8.

Hug, L.A., Baker, B.J., Anantharaman, K., Brown, C.T., Probst, A.J., Castelle, C.J., Butterfield, C.N., Hernsdorf, A.W., Amano, Y., Ise, K. and Suzuki, Y., 2016. A New View of the Tree of Life. *Nature Microbiology* 1.5:16048 – 16064.

Huttenhower, C., Gevers, D., Knight, R., Abubucker, S., Badger, J. H., Chinwalla, A. T., Creasy, H.H., Earl, A.M., FitzGerald, M.G., Fulton, R.S. and Giglio, M. G. 2012. Structure, Function and Diversity of the Healthy Human Microbiome. *Nature* 486.7402: 207 – 217.

Hrenovic, J., Durn, G., Goic-Barisic, I. and Kovacic, A. 2014. Occurrence of an Environmental *Acinetobacter baumannii* strain Similar to a Clinical Isolate in Paleosol from Croatia. *Applied and Environmental Microbiology* 80.9:2860 - 2866.

Ikudayisi, A., Adeyemo, F. and Adeyemo, J. 2015. Chemical and Hydro-Geologic Analysis of Ikogosi Warm Spring Water in Nigeria. *International Journal of Environmental, Chemical, Ecological, Geological and Geophysical Engineering* 9.9:1126-1130.

Illumina Inc. 2014. HiSeq 2500 Applications. Publication No. 770-2012-003. Retrieved from https://www.illumina.com/content/dam/illumina-marketing/documents/products/brochures/brochure_hiseq_applications.pdf. Accessed on July 29,2023.

Indriati, G. and Megahati, R.R.P. 2017. Optimization Medium of Amylase Production by *Bacillus licheniformis* Strain Mgi Originated from Pariangan Hot Spring, West Sumatera, Indonesia. *International Journal of Advanced Research* 5.11:660-664.

Irshad, M., Anwar, Z., Gulfraz, M., Butt, H.I., Ejaz, A. and Nawaz, H. 2012. Purification and Characterization of α-amylase from *Ganoderma tsuage* growing in Waste Bread Medium. *African Journal of Biotechnology* 11.33:8288 - 8294.

Ishii N, Ishida S, Kagami M. 2015. PCR Primers for Assessing Community Structure of Aquatic Fungi Including Chytridiomycota and Cryptomycota *Fungal Ecology* 13:33– 43.

Itoh, N., Kazama, M., Takeuchi, N., Isotani, K. and Kurokawa, J. 2016. Gene-specific Amplicons from Metagenomes as an Alternative to Directed Evolution for Enzyme Screening: A Case Study Using Phenylacetaldehyde Reductases. *FEBS Open Bio* 6.6: 566-575.

Jain, P. and Sinha, S. 2009. Neutrophiles: Acid Challenge and Comparison with Acidophiles. *Internet Journal of Microbiol*ogy 7.1:1-9.

Jamal, M., Sharma, S. P., Chung, H. J., Kim, H. J., Hong, S. T. and Lee, S. 2017. Ultra-High Efficient Colony PCR for High Throughput Screening of Bacterial Genes. *Indian Journal of Microbiology* 57.3:365–369.

Janeček, S., 2009. Amylolytic enzymes-focus on the Alpha-amylases from Archaea and Plants. *Nova Biotechnologica et Chimica* 9.1:5-25.

Janto, B., Ahmed, A., Ito, M., Liu, J., Hicks, D.B., Pagni, S., Fackelmayer, O.J., Smith, T.A., Earl, J., Elbourne, L.D. and Hassan, K. 2011. Genome of Alkaliphilic

Bacillus Pseudofirmus OF4 Reveals Adaptations that Support the Ability to Grow in an External pH Range from 7.5 to 11.4. *Environmental Microbiology* 13.12: 3289-3309.

Jian, C., Luukkonen, P., Yki-Järvinen, H., Salonen, A. and Korpela, K. 2020. Quantitative PCR Provides a Simple and Accessible Method for Quantitative Microbiota Profiling. *PloS One* 15.1: 1- 23.

Jin, H., Mo, L., Pan, L., Hou, Q., Li, C., Darima, I. and Yu, J. 2018. Using PacBio Sequencing to Investigate the Bacterial Microbiota of Traditional Buryatian Cottage Cheese and Comparison with Italian and Kazakhstan Artisanal Cheeses. *Journal of Dairy Science* 101.8:6885-6896.

Jin, Q. and Kirk, M. F. 2018. pH as a Primary Control in Environmental Microbiology: 1. Thermodynamic Perspective. *Frontiers in Environmental Science* 6: 21- 35.

Johan, P.D., Ahmed, O.H., Omar, L. and Hasbullah, N.A., 2021. Phosphorus transformation in Soils Following Co-Application of Charcoal and Wood Ash. *Agronomy* 11.10: 2010 – 2018.

John, R.G., Heidi, C.H., David, A.R., Ronald, C.A., Terry, I.B. and Howard, E.T. 1995. Heavy Metals in the Mississippi River. *US Geological Survey Circular*, 1133.

Johnson, D. B. 1998. Biodiversity and Ecology of Acidophilic Microorganisms. *FEMS Microbiology Ecology* 27.4: 307-317.

Johnson, J.S., Spakowicz, D.J., Hong, B.Y., Petersen, L.M., Demkowicz, P., Chen, L., Leopold, S.R., Hanson, B.M., Agresta, H.O., Gerstein, M. and Sodergren, E. 2019. Evaluation of 16S rRNA Gene Sequencing for Species and Strain-Level Microbiome Analysis. *Nature Communications* 10.1:1-11.

Johnson, M.P. 2016. An overview of Photosynthesis. *Essays in Biochemistry* 60.3:255 - 273.

Johny, T.K and Bhat, S.G. 2017. PCR in Metagenomics. In: *Methods in Molecular Biology*. Domingues L. (eds) New York. 30 -150.

Joshi, P., Pande, V. and Joshi, P. 2016. Microbial Diversity of Aquatic Ecosystem and its Industrial Potential. *Journal of Bacteriology & Mycology: Open Access* 3.1: 48 -54.

Jung, J. and Park, W. 2015. *Acinetobacter* species as Model Microorganisms In Environmental Microbiology: Current State and Perspectives. *Applied Microbiology and Biotechnology* 99.6: 2533 - 2548.

Junge, K., Cameron, K. and Nunn, B. 2019. Diversity of Psychrophilic Bacteria in Sea and Glacier Ice Environments—Insights Through Genomics, Metagenomics, and Proteomics Approaches. In *Microbial Diversity in the Genomic Era.* Das, S. and Dash, H.R. Academic Press. USA. 197-216.

Kalkhoff, S.J., 2016. *Phosphorus in sediment in the Kent Park Lake watershed,* Johnson County, Iowa, 2014–15. No. 1001. US Geological Survey. Retrieved from https://pubs.er.usgs.gov/publication/ds1001 Accessed 21/09/2022

Kambura, A.K., Mwirichia, R.K., Kasili, R.W., Karanja, E.N., Makonde, H.M. and Boga, H.I. 2016. Diversity of Fungi in Sediments and Water Sampled from the Hot Springs of Lake Magadi and Little Magadi in Kenya. *African Journal of Microbiology Research* 10 .10:330 - 338.

Katayama, T., Yoshioka, H., Mochimaru, H., Meng, X.Y., Muramoto, Y., Usami, J., Ikeda, H., Kamagata, Y. and Sakata, S. 2014. *Methanohalophilus levihalophilus* sp. nov., A Slightly Halophilic, Methylotrophic Methanogen Isolated from Natural Gas-Bearing Deep Aquifers, and Emended Description of the Genus *Methanohalophilus*. *International Journal of Systematic and Evolutionary Microbiology* 64.6:2089-2093.

Kchouk, M., Gibrat, J. F. and Elloumi, M. 2017. Generations of Sequencing Technologies: From First to Next Generation. *Biology and Medicine* 9.3:1 -8.

Kembel, S.W., Cowan, P.D., Helmus, M.R., Cornwell, W.K., Morlon, H., Ackerly, D.D., Blomberg, S.P. and Webb, C.O. 2010. Picante: R tools for Integrating Phylogenies and Ecology. *Bioinformatics* 26 .11:1463-1464.

Kim, M., Lee, K. H., Yoon, S. W., Kim, B. S., Chun, J. and Yi, H. 2013. Analytical Tools and Databases for Metagenomics in the Next-Generation Sequencing Era. *Genomics and Informatics* 11.3:102- 113.

Kim, S. J., Lee, J. C., Han, S. I. and Whang, K. S. 2015. *Halobacillus sediminis* sp. nov., a Moderately Halophilic Bacterium Isolated from a Solar Saltern Sediment. *International Journal of Systematic and Evolutionary Microbiology* 65.12: 4434 - 4440.

Kishore, D., Kundu, S. and Kayastha, A.M., 2012. Thermal, Chemical and pH Induced Denaturation of a Multimeric β-galactosidase Reveals Multiple Unfolding Pathways. *PloS One* 7.11:1 -12.

Kittelmann, S., Seedorf, H., Walters, W. A., Clemente, J. C., Knight, R., Gordon, J. I. and Janssen, P. H. 2013. Simultaneous Amplicon Sequencing to Explore Co-

Occurrence Patterns of Bacterial, Archaeal and Eukaryotic Microorganisms In Rumen Microbial Communities. *PloS One* 8.2: 1 – 17.

Klein, M., Ralya, T., Pollak, B., Obenza, R. and Harbour, M. G. (eds) 2012. *A Practitioner's Handbook for Real-Time Analysis: Guide to Rate Monotonic Analysis for Real-Time Systems*. Springer Science & Business Media. 1 – 10.

Konermann, L. (eds.) 2012. Protein Unfolding and Denaturants. *Encyclopaedia of Life Sciences.* John Wiley & Sons, London. 1–7.

Kostylev, M., Otwell, A. E., Richardson, R. E. and Suzuki, Y. 2015. Cloning Should be Simple: *Escherichia coli* DH5α-mediated Assembly of Multiple DNA Fragments with Short End Homologies. *PLoS One* 10.9: 1 – 24.

Kralik, P. and Ricchi, M. 2017. A Basic Guide to Real Time PCR In Microbial Diagnostics: Definitions, Parameters, and Everything. *Frontiers in Microbiology* 8:108 – 123.

Kraulis, P.J., 1991. MOLSCRIPT: A Program to Produce Both Detailed and Schematic Plots of Protein Structures. *Journal of Applied Crystallography* 24.5:946-950.

Kresic, N. 2010. Types and classifications of springs. In *Groundwater Hydrology of Springs.* Kresic, N., Stevanovic, Z. (eds). Butterworth-Heinemann London. 31-85.

Kuddus, M. and Ahmad, I.Z. 2012. Cold-active Extracellular α-Amylase Production from Novel Bacteria *Microbacterium foliorum* GA2 and *Bacillus cereus* GA6 Isolated from Gangotri Glacier, Western Himalaya. *Journal of Genetic Engineering and Biotechnology* 10.1:151-159.

Kumar, S., Krishnani, K. K., Bhushan, B. and Brahmane, M. P. 2015. Metagenomics: Retrospect and Prospects in High Throughput Age. *Biotechnology Research International* 1 -12.

Kumar, Y., Singh, P. K., Singh, A. K., Masih, H., Peter, K. J., Benjamin, J. C. and Rath, S. 2014. Production Optimization of Alpha-amylase from *Bacillus altitudinis*. *The International Journal of Scientific Research and Engineering Technology* 3.4: 654-673.

Kumar, S., Stecher G., and Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for Bigger Datasets. *Molecular Biology and Evolution.* 33:1870-1874.

Kumar, S., Tsai, C. J. and Nussinov, R. 2000. Factors Enhancing Protein Thermostability. *Protein Engineering* 13.3: 179-191.

Kumari, V.C., Patil, S.M., Ramu, R., Shirahatti, P.S., Kumar, N., Sowmya, B.P., Egbuna, C., Uche, C.Z. and Patrick-Iwuanyanwu, K.C. 2022. Chromatographic techniques: types, principles, and applications. In *Analytical Techniques in Biosciences*, Egbuna, C., Patrick-Iwuanyanwu, K.C., Shah, M.A., Ifemeje, J. C., Rasul, A. (eds) Academic Press. USA 73-101.

Kuriki, T., Hondoh, H. and Matsuura, Y. 2005. The Conclusive Proof that Supports the Concept of the Alpha-Amylase Family: Structural Similarity and Common Catalytic Mechanism. *Biologia Bratislava* 60:13-16.

Ladoukakis, E., Kolisis, F.N. and Chatziioannou, A.A. 2014. Integrative Workflows for Metagenomic Analysis. *Frontiers in Cell and Developmental Biology* 2:70-78.

Lahti, L., Shetty, S., Blake, T. and Salojarvi, J. 2017. Microbiome R package.

Lal, A. and Cheeptham, N. 2012. Starch Agar Protocol. *American Society for Microbiology* 1-9.

Lal, N., Jyoti, J. and Sachan, P. 2016. Optimization of Nitrogen Sources for the Growth and Amylase Production from *Bacillus licheniformis* JAR-26 Under Submerged Fermentation. *Indian Journal of Biology* 3.2:1 - 9.

Lange, M., Eisenhauer, N., Sierra, C.A., Bessler, H., Engels, C., Griffiths, R.I., Mellado-Vázquez, P.G., Malik, A.A., Roy, J., Scheu, S. and Steinbeiss, S. 2015. Plant Diversity Increases Soil Microbial Activity and Soil Carbon Storage. *Nature Communications* 6.1:1-8.

Lasa, I. and Berenguer, J. 1993. Thermophilic Enzymes and their Biotechnological Potential. *Microbiologia* 9.2: 77-89.

Le, T-V. 2023. Securing Group Patient Communication in 6G-Aided Dynamic Ubiquitous Healthcare with Real-Time Mobile DNA Sequencing. *Bioengineering*. 10.7:839.

Lennard, K., Dabee, S., Barnabas, S.L., Havyarimana, E., Blakney, A., Jaumdally, S.Z., Botha, G., Mkhize, N.N., Bekker, L.G., Lewis, D.A. and Gray, G. 2018. Microbial Composition Predicts Genital Tract Inflammation and Persistent Bacterial Vaginosis in South African Adolescent Females. *Infection and Immunity* 86.1: 410 – 417.

Lepère, C., Domaizon, I., Humbert, J.F., Jardillier, L., Hugoni, M. and Debroas, D. 2019. Diversity, Spatial Distribution and Activity of Fungi in Freshwater Ecosystems. *PeerJ 7*:1 - 22.

Lévêque, E., Janeček, Š., Haye, B. and Belarbi, A. 2000. Thermophilic Archaeal Amylolytic Enzymes. *Enzyme and Microbial Technology* 26.1:3-14.

Li, H., Li, T., Li, X., Wang, G., Lin, Q. and Qu, J. 2018. Gut microbiota in Tibetan Herdsmen Reflects the Degree of Urbanization. *Frontiers in Microbiology* 9:1745 – 1752.

Li, H., Yang, Q., Li, J., Gao, H., Li, P. and Zhou, H. 2015. The Impact of Temperature on Microbial Diversity and AOA Activity in the Tengchong Geothermal Field, China. *Scientific Reports* 5.1:1-12.

Li, J., Sun, Y., Chen, F., Hu, X. and Dong, L. 2021. Pressure and Temperature Combined with Microbial Supernatant Effectively Inactivate *Bacillus subtilis* Spores. *Frontiers in Microbiology* 12:834 – 840.

Li, K., Yan, E. and Feng, Y. 2017. How is R cited in research outputs? Structure, Impacts, and Citation Standard. *Journal of Informetrics* 11.4: 989-1002.

Lineweaver, H. and Burk, D. 1934. The Determination of Enzyme Dissociating Constants. *Journal of American Chemical Society* 56:658–666.

López-García, A., Pineda-Quiroga, C., Atxaerandio, R., Pérez, A., Hernández, I., García-Rodríguez, A. and González-Recio, O. 2018. Comparison of Mothur and QIIME for the Analysis of Rumen Microbiota Composition Based on 16S rRNA Amplicon Sequences. *Frontiers in Microbiology* 9: 3010 - 3018.

López-López, O., Knapik, K., Cerdán, M. E. and González-Siso, M. I. 2015. Metagenomics of an Alkaline Hot Spring in Galicia (Spain): Microbial Diversity Analysis and Screening for Novel Lipolytic Enzymes. *Frontiers in Microbiology 6*: 1291- 1300.

Lovett, S., Price, P. and Edgar, B. (eds) 2007. Salt, Nutrient, Sediment and Interactions: Findings from the National River Contaminants Program, Land and Water Australia 1-141.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein Measurement with the Folin Phenol Reagent. *Journal of Biological Chemistry* 193:265-275.

Lozupone, C., Lladser, M.E., Knights, D., Stombaugh, J. and Knight, R. 2011. UniFrac: An Effective Distance Metric for Microbial Community Comparison. *The ISME Journal 5*.2:169-172.

Lu, H., Giordano, F. and Ning, Z. 2016. Oxford Nanopore MinION Sequencing and Genome Assembly. *Genomics, Proteomics and Bioinformatics* 14.5: 265-279.

Luo, J., Wu, C., Xu, T. and Wu, Y. 2011. Diffusion Dialysis-Concept, Principle and Applications. *Journal of Membrane Science* 366.1-2:1-16.

Madhavan, A., Sindhu, R., Parameswaran, B., Sukumaran, R. K. and Pandey, A. 2017. Metagenome Analysis: A Powerful Tool for Enzyme Bioprospecting. *Applied Biochemistry and Biotechnology* 183.2:636-651.

Madigan, M. and Martinko, J. 2005. Brock Biology of Microorganisms, *International Microbiology* 8:149-152

Madika, A., Ameh, J., and Machido, D. 2017. Production of Alpha Amylase by *Bacillus* subtilis Using Maize Husk as Substrate. *Journal of Advances in Microbiology*, 6.2: 1-9.

Maester, T.C., Pereira, M.R., Sierra, E.M., Balan, A. and de Macedo Lemos, E.G. 2016. Characterization of EST3: A Metagenome-Derived Esterase with Suitable Properties for Biotechnological Applications. *Applied Microbiology and Biotechnology* 100.13: 5815-5827.

Mahdavi, A., Sajedi, R.H., Rasa, M. and Jafarian, V. 2010. Characterization of an α-Amylase with Broad Temperature Activity from An Acid-Neutralizing *Bacillus cereus* strain. *Iranian Journal of Biotechnology* 8.2:103 – 111.

Malhotra, R., Noorwez, S.M. and Satyanarayana, T. 2000. Production and Partial Characterization of Thermostable and Calcium-Independent Alpha-Amylase of an Extreme Thermophile *Bacillus thermooleovorans* NP54. *Letters in Applied Microbiology* 31.5: 378-384.

Malla, M. A., Dubey, A., Kumar, A., Yadav, S., Hashem, A. and Abdallah, E. F. 2019. Exploring the Human Microbiome: The Potential Future Role of Next-Generation Sequencing in Disease Diagnosis and Treatment. *Frontiers in Immunology* 9: 2868 – 2881.

Martin, M. 2011. Cutadapt Removes Adapter Sequences from High-Throughput Sequencing Reads. *EMBnet Journal* 17.1:10-12.

Matsushita, K., Azuma, Y., Kosaka, T., Yakushi, T., Hoshida, H., Akada, R. and Yamada, M. 2016. Genomic Analyses of Thermotolerant Microorganisms used for High-Temperature Fermentations. *Bioscience, Biotechnology, and Biochemistry* 80.4:655-668.

Mathews, S., Hans, M., Mücklich, F. and Solioz, M. 2013. Contact Killing of Bacteria on Copper is Suppressed if Bacterial-Metal Contact is Prevented and is Induced

on Iron by Copper Ions. *Applied and Environmental Microbiology* 79.8:2605 - 2611.

Mbareche, H., Dumont-Leblond, N., Bilodeau, G. J. and Duchaine, C. 2020. An Overview of Bioinformatics Tools for DNA Meta-Barcoding Analysis of Microbial Communities of Bioaerosols: Digest for Microbiologists. *Life* 10 .185:1 - 20.

Mckight, P.E. And Najab, J. (Eds) 2010. Kruskal-Wallis Test. In: *The Corsini Encyclopedia Of Psychology*.1-10.

McMurdie, P. J. and Holmes, S. 2013. Phyloseq: An R package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PloS One* 8.4: 1 – 19.

Mehta, D. and Satyanarayana, T. 2016. Bacterial and Archaeal Alpha-Amylases: Diversity and Amelioration of the Desirable Characteristics for Industrial Applications. *Frontiers in Microbiology* 7:1129 – 1135.

Mendes, R., Kruijt, M., De Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J. H., Piceno, Y.M., DeSantis, T.Z., Andersen, G.L., Bakker, P.A. and Raaijmakers, J. M. 2011. Deciphering the Rhizosphere Microbiome for Disease-Suppressive Bacteria. *Science* 332.6033: 1097-1100.

Metpally, R. P. R. and Reddy, B. V. B. 2009. Comparative Proteome Analysis of Psychrophilic Versus Mesophilic Bacterial Species: Insights Into the Molecular Basis of Cold Adaptation of Proteins. *BMC Genomics* 10.1:11 – 31.

Miller, G.L. 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry* 31:426-429.

Mizrahi, L. and Achituv, Y. 1989. Effect of Heavy Metals Ions on Enzyme Activity in the Mediterranean Mussel, *Donax trunculus*. *Bulletin of Environmental Contamination and Toxicology* 42.6: 1-5.

Mok, J.S., Choi, A., Kim, B., An, S.U., Lee, W.C., Kim, H.C., Kim, J., Yoon, C. and Hyun, J.H. 2021. Phosphorus Dynamics Associated with Organic Carbon Mineralization by Reduction of Sulfate and Iron in Sediment Exposed to Fish Farming. *Frontiers in Marine Science* 8: 1 -13.

Msarah, M.J., Ibrahim, I., Hamid, A.A. and Aqma, W.S. 2020. Optimisation and Production of Alpha-Amylase from Thermophilic *Bacillus* Spp. and its Application in Food Waste Biodegradation. *Heliyon* 6.6: e04183.

Murray, I.A., Clark, T.A., Morgan, R.D., Boitano, M., Anton, B.P., Luong, K., Fomenkov, A., Turner, S.W., Korlach, J. and Roberts, R.J. 2012. The Methylomes of Six Bacteria. *Nucleic Acids Research* 40.22:11450-11462.

Mushtaq, B., Raina, R., Yousuf, A.R., Wanganeo, A. and Jehangir, A. 2016. Exploring the Various Physico-Chemical Parameters of Surface Water and Sediments of Dal Lake, Srinagar, Kashmir. *Journal of Fisheries and Aquatic Science* 11:391-401.

Nahas, E. 2015. Control of Acid Phosphatases Expression from *Aspergillus Niger* By Soil Characteristics. *Brazilian Archives of Biology and Technology* 58.5: 658 - 666.

Nakamura, Y., International Nucleotide Sequence Database Collaboration, Cochrane, G., International Nucleotide Sequence Database Collaboration, Karsch-Mizrachi, I. and International Nucleotide Sequence Database Collaboration. 2012. The International Nucleotide Sequence Database Collaboration. *Nucleic Acids Research* 41.1:21-24.

Nayak, S., Samanta, S. and Mukherjee, A.K. 2020. Beneficial Role of *Aspergillus* sp. in Agricultural Soil and Environment. *Frontiers in Soil and Environmental Microbiology* 2020: 17 - 36.

Nedwell, D. B. 1999. Effect of Low Temperature on Microbial Growth: Lowered Affinity for Substrates Limits Growth at Low Temperature. *FEMS Microbiology Ecology* 30.2:101-111.

Neu, A.T., Allen, E.E. and Roy, K. 2021. Defining and Quantifying the Core Microbiome: Challenges and Prospects. *Proceedings of the National Academy of Sciences* 118.51: 1 – 15.

Ngalimat, M.S., Yahaya, R.S.R., Baharudin, M.M.A.A., Yaminudin, S.M., Karim, M., Ahmad, S.A. and Sabri, S. 2021. A Review on the Biotechnological Applications of the Operational Group *Bacillus amyloliquefaciens*. *Microorganisms* 9.3:614.

Ngara, T. R. and Zhang, H. 2018. Recent Advances in Function-Based Metagenomic Screening. *Genomics, Proteomics and Bioinformatics* 16.6: 405-415.

Nickerson, J.L. and Doucette, A.A. 2020. Rapid and Quantitative Protein Precipitation for Proteome Analysis by Mass Spectrometry. *Journal of Proteome Research* 19.5:2035-2042.

Nilsson, R. H., Kristiansson, E., Ryberg, M., Hallenberg, N. and Larsson, K. H. 2008. Intraspecific ITS Variability in the Kingdom Fungi as Expressed in The

International Sequence Databases and its Implications for Molecular Species Identification. *Evolutionary Bioinformatics 4.4*:193-201.

Nilsson, R. H., Ryberg, M., Abarenkov, K., Sjökvist, E. and Kristiansson, E. 2009. The ITS Region as a Target for Characterization of Fungal Communities Using Emerging Sequencing Technologies. *FEMS Microbiology Letters* 296.1:97-101.

Nilsson, R.H., Larsson, K.H., Taylor, A.F.S., Bengtsson-Palme, J., Jeppesen, T.S., Schigel, D., Kennedy, P., Picard, K., Glöckner, F.O., Tedersoo, L. and Saar, I. 2019. The UNITE Database for Molecular Identification of Fungi: Handling Dark Taxa and Parallel Taxonomic Classifications. *Nucleic Acids Research* 47:259-264.

Noll, K. M. (eds) 2001. Thermophilic Bacteria. In *Encyclopedia of Genetics,* Elsevier. 1961–1963.

Nuryshev, M. Z., Stoyanova, L. G., and Netrusov, A. I. 2016. New Probiotic Culture of *Lactococcus lactis* sp. lactis: Effective Opportunities and Prospects. *Journal of Microbial and Biochemical Technology* 8.4:290-295.

Nguyen, N.P., Warnow, T., Pop, M. and White, B. 2016. A Perspective on 16S rRNA Operational Taxonomic Unit Clustering Using Sequence Similarity. *NPJ Biofilms and Microbiomes 2*.1:1-8.

Oh, D.K., Cho, C.H., Lee, J.K. and Kim, S.Y. 2001. Increased Erythritol Production in Fed-Batch Cultures of *Torula* Sp. by Controlling Glucose Concentration. *Journal of Industrial Microbiology and Biotechnology* 26.4: 248 - 252.

Ojo, O. M. and Agbede, O. A. 2014. Assessment of Ikogosi Warm Spring Water Quality in Ekiti State, South Western Nigeria. *Sci-Afric Journal of Scientific Issues, Research and Essays* 2.3:116-123.

Okpako, E. C., Osuagwu, A. N., Duke, A. E. and Ntui, V. O. 2009. Prevalence and Significance of Fungi In Sachet Water and Borehole Drinking in Calabar, Nigeria. *African Journal of Microbiology Research* 3.2: 056 – 061.

Oksanen, J., Friendly, F.G.B., Kindt, M., Legendre, R., McGlinn, P., Minchin, D., O'Hara, P.R., Simpson, R.B., Solymos, G.L., Stevens, M.H.H. and Szoecs, E. 2018. Vegan: Community Ecology Package. *R package version*, 2(6).

Oladipo, A.A., Oluyemi, E.A., Tubosun, I.A., Fasisi, M.K. and Ibitoye, F.I, 2005. Chemical Examination of Ikogosi Warm Spring in South Western Nigeria. *Journal of Applied Sciences* 5.1:75-79.

Olajuyigbe, F. M. and Ajele, J. O. 2005. Production Dynamics of Extracellular Protease from *Bacillus* species. *African Journal of Biotechnology* 4.8:776-779.

Olutiola, P.O., 1981. Extracellular Maltase from *Aspergillus flavus*. *Mycologia* 73.6:1130-1142.

Olutiola, P.O., Famurewa, O. and Sonntag, H.G. 1991. *An introduction to General Microbiology. A practical Approach*, 1st Edition, 157-180.

Oren, A. 2013. *Salinibacter*: An Extremely Halophilic Bacterium with Archaeal Properties. *FEMS Microbiology Letters* 342.1: 1-9.

Oritsejafor, J.J. 1986. Influence of Moisture and pH on Growth and Survival of *Fusarium oxysporum* F. sp. *elaeidis* in soil. *Transactions of the British Mycological Society* 87.4: 511-517.

Orwa, P., Mugambi, G., Wekesa, V. and Mwirichia, R. 2020. Isolation of Haloalkaliphilic Fungi from Lake Magadi in Kenya. *Heliyon* 6.1: 1- 10.

Otlewska, A., Adamiak, J. and Gutarowska, B. 2014. Application of Molecular Techniques for the Assessment of Microorganism Diversity on Cultural Heritage Objects. *Acta Biochimica Polonica* 61.2: 1 – 13.

Oyeleke, S. B., Ibrahim, A. D., Manga, S. B., Rabah, A. B., Auta, H., and Ladan, F. 2011. Production of bacterial amylase by *Bacillus* species isolated from rice husk dumpsites in Sokoto metropolis, Nigeria. *International Journal of Biological and Chemical Sciences*, 5.1:1 – 7.

Oziengbe, E. O. and Onilude, A. A. 2012. Production of a Thermostable α-amylase and its Assay Using *Bacillus licheniformis* Isolated from Excavated Land Sites in Ibadan, Nigeria. *Bayero Journal of Pure and Applied Sciences*, 5.1: 132-138.

Pankhurst, C.E., Yu, S., Hawke, B.G. and Harch, B.D. 2001. Capacity of Fatty Acid Profiles and Substrate Utilization Patterns to Describe Differences in Soil Microbial Communities Associated with Increased Salinity or Alkalinity at Three Locations in South Australia. *Biology and Fertility of Soils* 33.3:204-217.

Panwar, V., Aggarwal, A., Paul, S., Singh, V., Singh, P.K., Sharma, D. and Shaharan, M.S. 2016. Effect of Temperature and pH on the Growth of *Fusarium* spp. causing *Fusarium* Head Blight (FHB) in Wheat. *South Asian Journal of Experimental Biology* 6.5:186-193.

Park, S. C. and Won, S. 2018. Evaluation of 16S rRNA Databases for Taxonomic Assignments Using a Mock Community. *Genomics and Informatics*16.4: 1 – 4.

Paulson, J.N., Stine, O.C., Bravo, H.C. and Pop, M. 2013. Differential Abundance Analysis for Microbial Marker-Gene Surveys. *Nature Methods* 10.12:1200-1202.

Pauvert, C., Buée, M., Laval, V., Edel-Hermann, V., Fauchery, L., Gautier, A., Lesur, I., Vallance, J. and Vacher, C. 2019. Bioinformatics Matters: The Accuracy of Plant and Soil Fungal Community Data is Highly Dependent on the Metabarcoding Pipeline. *Fungal Ecology* 41:23-33.

Payne, W.J. 1973. Reduction of Nitrogenous Oxides by Microorganisms. *Bacteriology Reviews* 37:409-452.

Pedron, R., Esposito, A., Bianconi, I., Pasolli, E., Tett, A., Asnicar, F., Cristofolini, M., Segata, N. and Jousson, O. 2019. Genomic and Metagenomic Insights Into the Microbial Community of a Thermal Spring. *Microbiome*,7.1:8- 23.

Pereira, F., Amorim, A. and Van Asch, B. 2013. Genetic and DNA-based Techniques. In *Comprehensive Analytical Chemistry* 60:195-220.

Petrosino, J. F., Highlander, S., Luna, R. A., Gibbs, R. A. and Versalovic, J. 2009. Metagenomic Pyrosequencing and Microbial Identification. *Clinical Chemistry* 55.5: 856–866.

Prakash, P. Y., Irinyi, L., Halliday, C., Chen, S., Robert, V. and Meyer, W. 2017. Online Databases for Taxonomy and Identification of Pathogenic Fungi and Proposal for a Cloud-Based Dynamic Data Network Platform. *Journal of Clinical Microbiology* 55.4: 1011-1024.

Prescott, L.M., Willey, J.M., Sherwood, L.M. and Woolverton C.J. 2018. *Microbiology* 11th Ed. McGraw Hill New York. 33-286.

Priyadarshini, S., Pradhan, S.K. and Ray, P. 2020. Production, Characterization And Application of Thermostable, Alkaline α-amylase (AA11) from *Bacillus cereus* strain SP-CH11 Isolated From Chilika Lake. *International Journal of Biological Macromolecules* 145:804-812.

Purves, W.K., Sadava, D., Orians, G.H. and Heller, H.C.(eds) 2004. Life. In: *The Science of Biology: volume III: Plants and Animals*. Macmillan. London 1601 – 1700.

Qadri, S. H., Zubairi, S., Hawley, H. P., Mazlaghani, H. H. and Ramirez, E. G. 1984. Rapid Test for Determination of Urea Hydrolysis. *Antonie van Leeuwenhoek* 50.4:417-423.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. and Glöckner, F.O. 2012. The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools. *Nucleic Acids Research* 41:590-596.

Rahman, M. T., Uddin, M. S., Sultana, R., Moue, A. and Setu, M. 2013. Polymerase Chain Reaction (PCR): A Short Review. *Anwer Khan Modern Medical College Journal* 4.1:30-36.

Rajan, S. K., Lindqvist, M., Brummer, R. J., Schoultz, I. and Repsilber, D. 2019. Phylogenetic Microbiota Profiling in Fecal Samples Depends on Combination of Sequencing Depth and Choice of NGS Analysis Method. *PloS One* 14.9: 1- 23.

Ramos, O.S. and Malcata, F.X. 2011. 3.48 Food-Grade Enzymes. Pergamon, *Comprehensive Biotechnology*.555-569.

R Core Team 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. Retrieved from https://www.R-project.org/. Accessed on 20/09/2021

R Studio Team. RStudio: Integrated Development for R. Boston, Massachusetts. 2015.

Vos, P., Garrity, G., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.H. and Whitman, W.B. (eds)., 2011. *Bergey's manual of systematic bacteriology: Volume 3: The Firmicutes.* Springer Science & Business Media.1 - 1405

Rajeshkumar, K. C., Yilmaz, N., Marathe, S. D., and Seifert, K. A. 2019. Morphology and Multigene Phylogeny of *Talaromyces amyrossmaniae*, A New Synnematous Species Belonging to the Section Trachyspermi From India. *Mycokeys* 45:41 – 56.

Reed, C. J., Lewis, H., Trejo, E., Winston, V. and Evilia, C. 2013. Protein Adaptations in Archaeal Extremophiles. *Archaea* 2013:1 - 5.

Reid, A. and Buckley, M., 2011. Washington, DC: American Academy of Microbiology. The Rare Biosphere:In *A Report from the American Academy of Microbiology*. 1 -18.

Rehman, A., Saeed, A., Asad, W., Kiran, T., Baloch, M.N. and Eijaz, S. 2019. Optimization of physicochemical parameters for maximum amylase production by indigenously isolated *Bacillus cereus* AS2 strain. *Pakistan Journal of Pharmaceutical Sciences* 32.2:889-894.

Ribosomal Database Project - Release 11. (August, 2020). Centre for Microbial Ecology, Michigan State University. Retrieved from https://rdp.cme.msu.edu. Accessed on 21/09/2021.

Risely, A. 2020. Applying the core microbiome to understand host–microbe systems. *Journal of Animal Ecology* 89.7:1549 -1558.

Robert, A., Samson, E.S., Hoekstra, J., Frisvad, C. and Filterberg, O (eds). 1995. *Introduction to Food-borne Fungi*. 4th Ed. Atlas. United Kingdom. 54 -261.

Robert, X., Haser, R., Gottschalk, T.E., Ratajczak, F., Driguez, H., Svensson, B. and Aghajari, N. 2003. The Structure of Barley Alpha-Amylase Isozyme 1 Reveals a Novel Role of Domain C in Substrate Recognition and Binding: A Pair of Sugar Tongs. *Structure* 11.8:973-984.

Robinson, P.K., 2015. Enzymes: Principles and Biotechnological Applications. *Essays in Biochemistry* 59 :1 – 41.

Roncarati, D. and Scarlato, V. 2017. Regulation of Heat-Shock Genes in Bacteria: From Signal Sensing to Gene Expression Output. *FEMS Microbiology Reviews* 41.4:549-574.

Rosselli, R., Romoli, O., Vitulo, N., Vezzi, A., Campanaro, S., de Pascale, F., Schiavon, R., Tiarca, M., Poletto, F., Concheri, G., Valle, G. and Squartini, A. 2016. Direct 16S rRNA-seq from bacterial communities: A PCR-independent Approach to Simultaneously Assess Microbial Diversity and Functional Activity Potential of Each Taxon. *Scientific Reports* 6:32165 – 32175.

Ruginescu, R., Gomoiu, I., Popescu, O., Cojoc, R., Neagu, S., Lucaci, I., Batrinescu-Moteau, C. and Enache, M. 2020. Bioprospecting for Novel Halophilic and Halotolerant Sources of Hydrolytic Enzymes in Brackish, Saline And Hypersaline Lakes of Romania. *Microorganisms* 8.12:1- 16.

Ruzaike, A., Muizniece-Brasava, S. and Kovalenko, K. 2015. Facultative Thermophilic Microorganisms in Potato Products in Retort Packaging. *Chemical Technology* 66.1: 19-23.

Saghatelyan, A., Margaryan, A., Panosyan, H., & Birkeland, N. K. 2021. Microbial Diversity of Terrestrial Geothermal Springs in Armenia and Nagorno-Karabakh: A Review. *Microorganisms* 9.7:1473 - 1480

Saha, S.P. and Mazumdar, D. 2019. Optimization of Process Parameter for Alpha-Amylase Produced By *Bacillus Cereus* Amy3 Using One Factor At a Time (OFAT) And Central Composite Rotatable (CCRD) Design Based Response Surface Methodology (RSM). *Biocatalysis and Agricultural Biotechnology* 19:101168 -101173.

Salano, O.A., Makonde, H.M., Kasili, R.W. and Boga, H.I., 2018. Isolation and Characterization of Fungi from a Hot-Spring on the Shores of Lake Bogoria, Kenya. *Journal of Yeast and Fungal Research* 9.1:1-13.

Satyanarayana, T. 2017. Acidic α-amylase of *Bacillus acidicola*: Attempts in Ameliorating Catalytic Efficiency and Production. *Advances in Biotechnology and Microbiology* 3.4:1-5.

Sanger, F., Nicklen, S. and Coulson, A. R. 1977. DNA Sequencing with Chain-Terminating Inhibitors. *Proceedings of the National Academy of Sciences* 74.12: 5463-5467.

Santamaria, M., Fosso, B., Consiglio, A., De Caro, G., Grillo, G., Licciulli, F., Liuni, S., Marzano, M., Alonso-Alemany, D., Valiente, G. and Pesole, G. 2012. Reference Databases for Taxonomic Assignment in Metagenomics. *Briefings in Bioinformatics*13.6: 682-695.

Santamaria, M., Fosso, B., Licciulli, F., Balech, B., Larini, I., Grillo, G., De Caro, G., Liuni, S. and Pesole, G., 2018. ITSoneDB: A Comprehensive Collection of Eukaryotic Ribosomal RNA Internal Transcribed Spacer 1 (ITS1) sequences. *Nucleic Acids Research* 46:127-132.

Saxena, R., Dhakan, D.B., Mittal, P., Waiker, P., Chowdhury, A., Ghatak, A. and Sharma, V.K. 2017. Metagenomic Analysis of Hot Springs in Central India Reveals Hydrocarbon Degrading Thermophiles and Pathways Essential for Survival in Extreme Environments. *Frontiers in Microbiology* 7:2123 - 2129

Schindler, D.E., Jankowski, K., A'mar, Z.T. and Holtgrieve, G.W. 2017. Two-stage Metabolism Inferred from Diel Oxygen Dynamics in Aquatic Ecosystems. *Ecosphere* 8.6:1- 10.

Schliep K. 2011. "Phangorn: Phylogenetic Analysis in R." *Bioinformatics* 27.4: 592–593.

Schloss, P. D and Handelsman J. 2005. Metagenomics For Studying Unculturable Microorganisms: Cutting the Gordian Knot. *Genome Biology* 6.8:229 – 235.

Schoch, C.L., Sung, G.H., López-Giráldez, F., Townsend, J.P., Miadlikowska, J., Hofstetter, V., Robbertse, B., Matheny, P.B., Kauff, F., Wang, Z. and Gueidan, C. 2009. The Ascomycota Tree of Life: A Phylum-Wide Phylogeny Clarifies the Origin and Evolution of Fundamental Reproductive and Ecological Traits. *Systematic Biology* 58.2: 224 - 239.

Scientific, T.F., 2009. *Thermo Scientific Pierce Protein Assay Technical Handbook*. Thermo Scientific. NewYork.1- 5.

Seo, D.H., Cho, E.S., Hwang, C.Y., Yoon, D.J., Chun, J., Jang, Y., Nam, Y.D., Park, S.L., Lim, S.I., Kim, J.H. and Seo, M.J. 2019. Cultivable Microbial Diversity in Domestic Bentonites and Their Hydrolytic Enzyme Production. *Microbiology and Biotechnology Letters* 47.1:125-131.

Shajani, Z., Sykes, M. T. and Williamson, J. R. 2011. Assembly of Bacterial Ribosomes. *Annual Review of Biochemistry* 80:501-526.

Sharma, A., Parashar, D. and Satyanarayana, T. 2016. Acidophilic Microbes: Biology and Applications. In *Biotechnology of Extremophiles.* Springer, USA 215-241.

Sharma, N., Kumar, J., Abedin, M., Sahoo, D., Pandey, A., Rai, A.K. and Singh, S.P. 2020. Metagenomics Revealing Molecular Profiling of Community Structure and Metabolic Pathways in Natural Hot Springs of the Sikkim Himalaya. *BMC Microbiology* 20.1:1-17.

Sharma, P. and Giri, A. 2018. Productivity Evaluation of Lotic and Lentic Water Body in Himachal Pradesh, India. *MOJ Ecology and Environmental Sciences* 3.5: 311-317.

Sharpton, T. J. 2014. An Introduction to the Analysis of Shotgun Metagenomic Data. *Frontiers in Plant Science* 5: 209 – 219.

Shehadul-Islam, M., Aryasomayajula, A. and Selvaganapathy, P.R. .2017. A Review on Macroscale and Microscale Cell Lysis Methods. *Micromachines* 8.3:83 - 110.

Sheu, S.Y., Chen, Y.L., Young, C.C. and Chen, W.M. 2016. *Vogesella facilis* sp. nov., Isolated from a Freshwater River, and Emended Description of the Genus *Vogesella*. *International Journal of Systematic and Evolutionary Microbiology* 66.2: 817 - 823.

Shuikan, A., Alharbi, S. A., Alkhalifah, D. H. M. and Hozzein, W. N.(eds) 2019. High-Throughput Sequencing and Metagenomic Data Analysis. In *Metagenomics-Basics, Methods and Applications*. IntechOpen United Kingdom 223 – 256.

Siegwald, L., Caboche, S., Even, G., Viscogliosi, E., Audebert, C. and Chabé, M. 2019. The Impact of Bioinformatics Pipelines on Microbiota Studies: Does the Analytical "Microscope" Affect the Biological Interpretation? *Microorganisms* 7.10:393 – 402.

Siegwald, L., Touzet, H., Lemoine, Y., Hot, D., Audebert, C. and Caboche, S. 2017. Assessment of Common and Emerging Bioinformatics Pipelines For Targeted Metagenomics. *PLoS One* 12.1: 1 -11.

Sigee, D.C.(eds) 2005. Freshwater Microbiology; Biodiversity and Dynamic Interactions of Microorganisms in the Aquatic Environment. John Wiley and Sons, UK. 212 – 244.

Sillanpää, S., Kramna, L., Oikarinen, S., Sipilä, M., Rautiainen, M., Aittoniemi, J., Laranne, J., Hyöty, H. and Cinek, O. 2017. Next-Generation Sequencing Combined with Specific PCR Assays to Determine the Bacterial 16S rRNA Gene Profiles of Middle Ear Fluid Collected from Children with Acute Otitis Media. *mSphere* 2.2: 1 – 23.

SILVA 2019. High quality ribosomal databases. Retrieved from https://www.arb-silva.de/documentation/release-138/ assessed on 23/05/2020.

Simon, C. and Daniel, R. 2011. Metagenomic Analyses: Past and Future Trends. *Applied and Environmental Microbiology* 77.4: 1153-1161.

Singare, P.U., Trivedi, M.P. and Mishra, R.M., 2011. Assessing the Physico-Chemical Parameters of Sediment Ecosystem of Vasai Creek at Mumbai, India. *Marine Science*1.1: 22-29.

Singh, J. S., Singh, S. P. and Gupta, S. R. 2014a (eds). *Ecology, Environmental Science and Conservation*. S. Chand Publishing. New Dehli 45 – 78.

Singh, J., Birbian, N., Sinha, S. and Goswami, A. 2014b. A Critical Review on PCR, its Types and Applications. *International Journal of Advanced Research in Biological Sciences* 1.7: 65-80.

Singh, V. and Das, D. 2019. Potential of Hydrogen Production from Biomass. In: *Science and Engineering of Hydrogen-Based Energy Technologies.* De Miranda P.E.V (eds) Academic Press. USA 123-164.

Singh, V., Haque, S., Niwas, R., Srivastava, A., Pasupuleti, M. and Tripathi, C., 2017. Strategies for fermentation medium optimization: An In-Depth Review. *Frontiers in Microbiology 7*: 2087- 2092.

Sivakumar, T., Shankar, T., Vijayabaskar, P., Muthukumar, J. and Nagendrakannan, E. 2012. Amylase Production Using *Bacillus cereus* Isolated from a Vermicompost Site. *International Journal of Microbiology Research* 3.2:117-123.

Smith, P. B., Tomfohrde, K. M., Rhoden, D. L. and Balows, A. 1972. API System: A Multitube Micromethod for Identification of Enterobacteriaceae. *Applied Microbiology* 24.3:449 – 454.

Souza, P.M.D. and Magalhães, P.D.O. 2010. Application of Microbial α-amylase in Industry-A Review. *Brazilian Journal of Microbiology* 41:850-861.

Stein, J. L., Marsh, T. L., Wu, K. Y., Shizuya, H. and DeLong, E. F. 1996. Characterization of Uncultivated Prokaryotes: Isolation and Analysis of a 40-Kilobase-Pair Genome Fragment from a Planktonic Marine Archaeon. *Journal of Bacteriology* 178.3: 591-599.

Su, X., 2021. Elucidating the Beta-Diversity of the Microbiome: From Global Alignment to Local Alignment. *Msystems* 6.4:3 - 21.

Suribabu, K., Govardhan, T.L. and Hemalatha, K.P.J., 2014. Influence of Carbon Sources on α-amylase Production by *Brevibacillus* sp. Under Submerged Fermentation. *Cellulose* 3.2:292 - 299.

Sumpavapol, P., Tongyonk, L., Tanasupawat, S., Chokesajjawatee, N., Luxananil, P. and Visessanguan, W. 2010. *Bacillus siamensis* sp. nov., isolated from Salted Crab (poo-khem) in Thailand. *International Journal of Systematic and Evolutionary Microbiology* 60.10: 2364-2370.

Sumrin, A., Ahmad, W., Ijaz, B., Sarwar, M.T., Gull, S., Kausar, H., Shahid, I., Jahan, S., Asad, S., Hussain, M. and Riazuddin, S. 2011. Purification and Medium Optimization of α-Amylase from *Bacillus subtilis* 168. *African Journal of Biotechnology* 10.11: 2119-2129.

Suutari, M. and Laakso, S. 1994. Microbial Fatty Acids and Thermal Adaptation. *Critical Reviews in Microbiology* 20.4: 285-328.

Talabi, A. O. 2013. Hydrogeochemistry and Stable Isotopes (δ18O and δ2H) Assessment of Ikogosi Spring Waters. *American Journal of Water Resources* 1.3: 25 - 33.

Tamás, M.J., Sharma, S.K., Ibstedt, S., Jacobson, T. and Christen, P. 2014. Heavy Metals and Metalloids as a Cause for Protein Misfolding and Aggregation. *Biomolecules* 4.1: 252-267.

Tamura K. and Nei M. 1993. Estimation of the Number of Nucleotide Substitutions in the Control Region of Mitochondrial DNA in Humans and Chimpanzees. *Molecular Biology and Evolution* 10: 512-526.

Tekere, M., Lötter, A., Olivier, J., Jonker, N. and Venter, S. 2011. Metagenomic Analysis of Bacterial Diversity of Siloam Hot Water Spring, Limpopo, South Africa. *African Journal of Biotechnology* 10.78: 18005-18012.

Thomas, T., Gilbert, J. and Meyer, F., 2012. Metagenomics-A Guide from Sampling to Data Analysis. *Microbial Informatics and Experimentation* 2.1-12.

Thompson, L.R., Sanders, J.G., McDonald, D., Amir, A., Ladau, J., Locey, K.J., Prill, R.J., Tripathi, A., Gibbons, S.M., Ackermann, G. and Navas-Molina, J.A. 2017. A Communal Catalogue Reveals Earth's Multiscale Microbial Diversity. *Nature* 551.7681: 457-463.

Tiwari, S., Shukla, N., Mishra, P. and Gaur, R. 2014. Enhanced Production and Characterization of a Solvent Stable Amylase from Solvent Tolerant *Bacillus tequilensis* RG-01: Thermostable and Surfactant Resistant. *The Scientific World Journal*, 2014:1- 9.

Tjernberg, A., Markova, N., Griffiths, W.J. and Hallén, D. 2006. DMSO-related Effects in Protein Characterization. *Journal of Biomolecular Screening* 11.2: 131-137.

Tominaga, T., An, S.Y., Oyaizu, H. and Yokota, A. 2009. *Sporosarcina luteola* sp. nov. Isolated from Soy Sauce Production Equipment in Japan. *The Journal of General and Applied Microbiology* 55.3: 217-223.

Torsvik, V., Øvreås, L. and Thingstad, T. F. 2002. Prokaryotic Diversity--Magnitude, Dynamics, and Controlling Factors. *Science* 296 .5570:1064-1066.

Trevors, J. T. 1998. Bacterial Biodiversity in Soil with an Emphasis on Chemically-Contaminated Soils. *Water, Air, and Soil Pollution* 101.1-4:45-67.

Trindade, M., Van Zyl, L.J., Navarro-Fernández, J. and Abd Elrazak, A. 2015. Targeted Metagenomics as a Tool to Tap Into Marine Natural Product Diversity for the Discovery and Production of Drug Candidates. *Frontiers in Microbiology* 6:890 – 899.

Tshikhudo, P., Nnzeru, R., Ntushelo, K., & Mudau, F. 2013. Bacterial Species Identification Getting Easier. *African Journal of Biotechnology* 12.41: 5975-5982.

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., and Gordon, J. I. 2007. The Human Microbiome Project: Exploring the Microbial Part of Ourselves in a Changing World. *Nature* 449:804–810.

Tuson, H. H. and Weibel, D. B. 2013. Bacteria-surface Interactions. *Soft Matter* 9.18: 4368–4380.

Urh M, Simpson D, Zhao K. 2009. Affinity Chromatography: General methods. *Methods in Enzymology* 463:417-438.

Ursell, L.K., Robbins-Pianka, A., Scott, N., Gonzalez, A., Knights, D., Rideout, J.R., Kshatriya, A., Caporaso, J.G. and Knight, R. 2015. Using QIIME to Evaluate the Microbial Communities within Hydrocarbon Environments. McGenity, T., Timmis, K., Nogales Fernández, B. (eds) *Hydrocarbon and Lipid Microbiology Protocols*. Springer, Heidelberg, 89-113.

Van Hofwegen, D. J., Hovde, C. J. and Minnich, S. A. 2016. Rapid Evolution of Citrate Utilization by *Escherichia coli* by Direct Selection Requires citT and dctA. *Journal of Bacteriology* 198.7:1022-1034.

Vaz-Moreira, I., Nunes, O.C. and Manaia, C.M. 2017. Ubiquitous and Persistent Proteobacteria And Other Gram-Negative Bacteria in Drinking Water. *Science of the Total Environment* 586:1141-1149.

Venter, J.C., Remington, K., Heidelberg, J.F., Halpern, A.L., Rusch, D., Eisen, J.A., Wu, D., Paulsen, I., Nelson, K.E., Nelson, W. and Fouts, D.E. 2004. Environmental Genome Shotgun Sequencing of the Sargasso Sea. *Science* 304.5667:66-74.

Ventosa, A., Nieto, J. J. and Oren, A. 1998. Biology of Moderately Halophilic Aerobic Bacteria. *Microbiology and Molecular Biology Reviews* 62.2:504–544.

Vendrell, J.A., Henry, S., Cabello-Aguilar, S., Heckendorn, E., Godreuil, S. and Solassol, J. 2022. Determination of the Optimal Bacterial DNA Extraction Method to Explore the Urinary Microbiota. *International Journal of Molecular Sciences* 23.3:1336 – 1342.

Vester, J.K., Glaring, M.A. and Stougaard, P. 2015. Improved Cultivation and Metagenomics as New Tools for Bioprospecting in Cold Environments. *Extremophiles* 19.1:17-29.

Vieille, C., Burdette, D. S. and Zeikus, J. G. 1996. Thermozymes. *Biotechnology Annual Review* 2:1-83.

Vieille, C. and Zeikus, G. J. 2001. Hyperthermophilic Enzymes: Sources, Uses, and Molecular Mechanisms for Thermostability. *Microbiology and Molecular Biology Reviews* 65.1:1–43.

Vitorino, L. C. and Bessa, L. A. 2018. Microbial Diversity: The Gap Between the Estimated and the Known. *Diversity* 10.2:46 – 56.

Walker, J.M.(eds) (2009). The Bicinchoninic Acid (BCA) Assay for Protein Quantitation. In: The Protein Protocols Handbook. *Springer Protocols Handbooks* New Jersey 11-15.

Wallen, Z.D. 2021. Comparison Study of Differential Abundance Testing Methods Using Two Large Parkinson Disease Gut Microbiome Datasets Derived From 16S Amplicon Sequencing. *BMC Bioinformatics* 22.1:1 - 29.

Watson, S.J., Cade-Menun, B.J., Needoba, J.A. and Peterson, T.D. 2018. Phosphorus Forms in Sediments of a River-Dominated Estuary. *Frontiers in Marine Science* 5:302 – 310.

Wang, L. and Liang, T. 2015. Distribution Characteristics of Phosphorus in the Sediments and Overlying Water of Poyang Lake. *PloS One*, 10.5: e0125859.

Wang, X. and Pecoraro, L. 2021. Diversity and Co-Occurrence Patterns of Fungal and Bacterial Communities from Alkaline Sediments and Water of Julong High-Altitude Hot Springs at Tianchi Volcano, Northeast China. *Biology* 10.9:894 -890.

Wang, X., Jordan, I. K. and Mayer, L. W. 2015a. A Phylogenetic Perspective on Molecular Epidemiology. In *Molecular Medical Microbiology*, 2nd Edition. Tang, Y., Sussman, M., Liu, D., Poxton, I., Schwartzman, J. (eds) Academic Press.USA 517-536.

Wang, X.C., Liu, C., Huang, L., Bengtsson-Palme, J., Chen, H., Zhang, J.H., Cai, D. and Li, J.Q. 2015b. ITS 1: A DNA Barcode Better Than ITS 2 In Eukaryotes? *Molecular Ecology Resources* 15.3:573-586.

Wang, Y. and Wang, C. 2020. Comparative Study on Archaeal Diversity in the Sediments of Two Urban Landscape Water Bodies. *PlosOne* 15.2:1 – 9.

Wang, Z., Brown, J. H., Tang, Z. and Fang, J. 2009. Temperature Dependence, Spatial Scale, and Tree Species Diversity in Eastern Asia and North America. *Proceedings of the National Academy of Sciences* 106.32: 13388-13392.

Weinisch, L., Kühner, S., Roth, R., Grimm, M., Roth, T., Netz, D., Pierik, A. J. and Filker, S. 2018. Identification of Osmoadaptive Strategies in the Halophile, Heterotrophic Ciliate *Schmidingerothrix salinarum*. *PLoS Biology* 16.1:1 -15.

Wickham, H., 2007. Reshaping Data with the Reshape Package. *Journal of Statistical Software* 21.12:1-20.

Wiegel, J. 1990. Temperature Spans for Growth: Hypothesis and Discussion. *FEMS Microbiology Reviews* 6.2-3:155-169.

Wilchek M and Chaiken I. 2000. An Overview of Affinity Chromatography in Affinity Chromatography–Methods and Protocols. *Humana Press* 1–6.

Willis, A.D. 2019. Rarefaction, Alpha Diversity, and Statistics. *Frontiers in Microbiology* 10: 2407- 2414.

Wright, E. S. 2016. Using DECIPHER v2. 0 to Analyze Big Biological Sequence Data in R. *R Journal* 8.1: 352-359.

Wright, R.B., Lockaby, B.G. and Walbridge, M.R. 2001. Phosphorus Availability in an Artificially Flooded Southeastern Floodplain Forest Soil. *Soil Science Society of America Journal* 65.4: 1293-1302.

Woese, C. R. 1987. Bacterial Evolution. *Microbiological Reviews* 51.2: 221 - 231.

Woese, C. R., Kandler, O. and Wheelis, M. L. 1990. Towards a Natural System of Organisms: Proposal for the Domains Archaea, Bacteria, and Eucarya. *Proceedings of the National Academy of Sciences* 87.12: 4576-4579.

Wong, R.G., Wu, J.R. and Gloor, G.B. 2016. Expanding the UniFrac toolbox. *PloS One* 11.9:1 -9.

World Health Organization (WHO) 2014. The United Nations International Children's Emergency Fund (UNICEF). Joint Monitoring Programme for Water Supply and Sanitation. In Progress on Drinking Water and Sanitation: 2014 Update; UNICEF: New York, NY, USA.

Wu, Y., Zaiden, N. and Cao, B. 2018. The Core-and Pan-Genomic Analyses of the Genus *Comamonas*: From Environmental Adaptation to Potential Virulence. *Frontiers in Microbiology* 9:3096 - 3101.

Yang, B., Wang, Y. and Qian, P. Y. 2016a. Sensitivity and Correlation of Hypervariable Regions in 16S rRNA Genes in Phylogenetic Analysis. *BMC Bioinformatics* 17.1:135 – 145.

Yang, C., Xia, Y., Qu, H., Li, A., Liu, R., Wang, Y., Zhang, T. 2016b. Discovery of New Cellulases from the Metagenome by a Metagenomics-Guided Strategy. *Biotechnology for Biofuels* 9:138 – 149.

Ye, R., Jin, Q., Bohannan, B., Keller, J. K., McAllister, S. A. and Bridgham, S. D. 2012. pH Controls Over Anaerobic Carbon Mineralization, the Efficiency of Methane Production, and Methanogenic Pathways in Peatlands Across an Ombrotrophic–Minerotrophic Gradient. *Soil Biology and Biochemistry* 54: 36-47.

Yu, G., Fadrosh, D., Goedert, J. J., Ravel, J. and Goldstein, A. M. 2015. Nested PCR Biases in Interpreting Microbial Community Structure in 16S rRNA Gene Sequence Datasets. *PloS One* 10.7: 1 – 14.

Yuk, H. G. and Marshall, D. L. 2004. Adaptation of *Escherichia coli* O157: H7 to pH Alters Membrane Lipid Composition, Verotoxin Secretion, and Resistance to Simulated Gastric Fluid Acid. *Applied and Environmental Microbiology*, 70.6: 3500-3505.

Zahn, L. M. 2017. Archaeal Diversity and Evolution. *Science* 357.6351: 560-562

Zarikas, V., Anagnostou, K.E., Avlakiotis, P., Kotsopoulos, S., Liolios, C., Latsos, T., Perantzakis, G., Lygdis, A., Lykourgiotis, A. and Antoniou, D. 2014. Measurement and Analysis of Physicochemical Parameters Concerning Thermopylae Natural Hot Spring Waters. *Journal of Applied Sciences* 14.19:2331-2340.

Ze, X., Ben David, Y., Laverde-Gomez, J.A., Dassa, B., Sheridan, P.O., Duncan, S.H., Louis, P., Henrissat, B., Juge, N., Koropatkin, N.M. and Bayer, E.A. 2015. Unique Organization of Extracellular Amylases Into Amylosomes in the Resistant Starch-Utilizing Human Colonic Firmicutes Bacterium *Ruminococcus bromii*. *MBio 6*.5: e01058-15.

Zhang, Q., Wu, Z., Zhao, J., Wang, G., Hao, J., Wang, S., Lin, Y., Guan, H., Zhang, J., Jian, S. and Li, A. 2021. Composition and Functional Characteristics and Influencing Factors of Bacterioplankton Community in the Huangshui River, China. *Microorganisms* 9.11: 2260 – 2267.

Zhang, Z., Xiao, C., Adeyeye, O., Yang, W. and Liang, X. 2020. Source and Mobilization Mechanism of Iron, Manganese and Arsenic in Groundwater of Shuangliao City, Northeast China. *Water* 12.2: 534 – 540.

Zhao, C., Chu, Y., Li, Y., Yang, C., Chen, Y., Wang, X. and Liu, B. 2017. High-Throughput Pyrosequencing Used for the Discovery of a Novel Cellulase from a Thermophilic Cellulose-Degrading Microbial Consortium. *Biotechnology Letters* 39.1: 123-131.

Zhao, L., Ma, T., Gao, M., Gao, P., Cao, M., Zhu, X. and Li, G. 2012. Characterization of Microbial Diversity and Community in Water Flooding Oil Reservoirs in China. *World Journal of Microbiology and Biotechnology* 28 .10: 3039-3052.

Zhou, J., Deng, Y., Shen, L., Wen, C., Yan, Q., Ning, D., Qin, Y., Xue, K., Wu, L., He, Z., Voordeckers, J. W., Nostrand, J. D., Buzzard, V., Michaletz, S. T., Enquist, B.

J., Weiser, M. D., Kaspari, M., Waide, R., Yang, Y. and Brown, J. H. 2016. Temperature Mediates Continental-Scale Diversity of Microbes in Forest Soils. *Nature Communications* 7: 12083 – 12095.

Zhou, Y., Han, L.R., He, H.W., Sang, B., Yu, D.L., Feng, J.T. and Zhang, X. 2018. Effects of Agitation, Aeration and Temperature on Production of a Novel Glycoprotein GP-1 by *Streptomyces kanasenisi* ZX01 and Scale-Up Based on Volumetric Oxygen Transfer Coefficient. *Molecules* 23.1:125 – 130.

Zhou, Y., Zhang, D., Peatman, E., Rhodes, M. A., Liu, J. and Davis, D. A. 2017. Effects of Various Levels of Dietary Copper Supplementation with Copper Sulfate And Copper Hydroxychloride On Pacific White Shrimp *Litopenaeus vannamei* Performance and Microbial Communities. *Aquaculture* 476: 94-105.