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THE DIVERSITY AND SPATIAL VARIABILITY OF HETEROTROPHIC BACTERIA AND EVALUATION OF CULTURE MEDIA FOR ISOLATION OF BACTERIA FROM LAKE ZENGENA

MSc Thesis By Desta Alemu

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BAHIR DAR UNIVERSITY

COLLEGE OF SCIENCE

DEPARTMENT OF BIOLOGY

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A Thesis Submitted to the Department of Biology, College of Science Bahir Dar University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology (Applied Microbiology)

By

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Bahir Dar University, Ethiopia

APPROVAL SHEET

As a thesis research advisor, I certify that I have read and evaluated this thesis prepared, under my guidance by Mr Desta Alemu Abejehu entitled "The diversity and spatial variability of heterotrophic bacteria and evaluation of culture media for isolation of bacteria from lake zengena" and I recommended the paper to be submitted as fulfilling the requirement for the Degree of Master in Biology in the field of Applied Microbiology

…….. … …… ……

Name of advisor Signature Date

As members of the board examiners for the MSc thesis, open defense examination. We certify that we have read and evaluated the thesis prepared by Mr Desta Alemu Abejehu and examined the candidate. We recommend the thesis to be accepted as fulfilling requirement for the degree of MSc in Biology (Applied Microbiology).

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DECLARATION

I, Desta Alemu, hereby declare that this thesis is my original work and has not been previously published or presented for the award of a degree in any university. All the sources of the materials used for this thesis are accurately cited and all people and institutions who gave support for thesis work are faithfully acknowledged.

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LIST OF ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis of Variance
CFU	Colony Forming Units
MRVP	Methyl Red and Voges-Proskauer
OTUs	Operational Taxonomic Unit
SIM	Sulphide Indole Motility
SPSS	Statistical Package for Social Sciences
TSA	Tryptic soya agar
R2A	Reasoner's 2A agar
BCC	Bacterial community composition
TDS	Total dissolved solids
DO	Dissolved oxygen

ABSTRACT

Lake Zengena is one of the Crater Lake, which provides economic, social, and ecological benefits to the local community. The studies that were conducted in Lake Zangena concentrated mainly on Ecotourism. Information regarding the microbiology of the Lake Zengena is lacking. Thus, the aim of this study was determing the diversity and spatial variability of heterotrophic bacteria and evaluation of culture media for isolation of bacteria from lake zengena. Water samples were plated and incubated at 25^{o}C for 48-72hrs on (TSA) and at 25^{o}C for 5-7 days on R2A medium aerobically. Identification of was carried out according to Bergey's manual of determinative bacteriology. Diversity indices were used to measure the diversity across site and between Media. Analysis of variance was used to test mean difference of Operational Taxonomic Units (OTUs) abundance between sites and between media type. A total of 200 isolates were picked from the two sites, purified, and grouped in to 76 OTUs based on morphological and biochemical characteristics. Eight OTUS were the most abundant and constitute about 22.5% of the total number of isolates and common to all sites and media types. Twenty-six OTUs (52 %) isolates were common to all media and 34 OTUs (57.7%) isolates were common to all sites. There were 27 unique OTUs in R2A and 24 unique OTUs in TSA medium. In case of sites, site 1 had 22 unique OTUs while site 2 had 20 unique OTUs. Variability in terms of media type was higher than spatial (sites). There was statistically significant difference in OTUs abundance between media type and sites, ($p=0.001$) R2A Site I ($H=3.652$) and R2A site II ($H=3.569$) exhibited higher diversity than TSA site I and TSA site II. Nine bacterial genera were identified, of which seven genera were present in all sites and media types were most dominant. Eight of identified genera were naturally occurring freshwater genera. The result of this study signifies conducting further research to identify each phenotype at species level to understand the ecological and economical role of the bacterial community.

Key words: *Bacteria, Diversity, Ecology, Isolates, Lake Zengena, spatial variability.*

1. INTRODUCTION

1.1. BACKGROUND OF THE STUDY

Freshwater lakes are important habitats that shelter highly complex and diverse microbial communities. They provide a unique habitat for microbes and they differ from larger oceanic and moving water systems in physical, chemical, and biological characteristics. Many freshwater lakes have low salt content than marine and ocean ecosystems, which is usually less than 1% (Pernthaler, 2013). Freshwater lake habitats vary with different environmental variables and bacterial community composition (Yang *et al.,* 2016). Bacteria are the dominant and major biotic component of the freshwater ecosystem, which comprise an important part of the lake microbial flora. Analysis of the bacterial diversity and distribution patterns in freshwater lakes has received growing attention in the last decades (Roguet *et al*., 2015). Freshwater ecosystems are the most endangered habitats in the world and have an alarming rate of species extinction, anthropogenic stressors including excess input of nutrients, hydro-morphological alterations, and continuous pollution increases extinction rates of freshwater species (Bernhardt *et al*., 2017).

Bacteria are very diverse and ubiquitous in their distribution, being able to survive under extreme conditions such as high or low temperatures, acidic conditions, and saline habitats (Edbeib *et al*., 2016). The number and type of bacteria in which ecosystems diverge and greatly are influenced by the ecosystem processes (Lindstrom *et al*., 2005). The relative analysis revealed that the abundance of bacteria in the eutrophic lake was higher than that in the oligotrophic lake, but the bacterial diversity in the oligotrophic lake was higher than that in the eutrophic lake (Feng *et al*., 2019). Phylum *Proteobacteria,* phylum *Actinobacteria,* phylum *Cyanobacteria,* and phylum *Bacteroidetes* are common bacteria in freshwater lakes (Khalil *et al*., 2015).

Bacteria perform numerous ecological roles in lake ecosystems. Freshwater bacteria take part in different biogeochemical cycles which play a role in remineralizing, restoring, and biodegradation of nutrients and contaminants (Josh *et al*., 2016). Bacteria also important sources of food and nutrients for other organisms in the aquatic ecosystems and sustain ecosystem balance (Joshi *et al*., 2016). Bacteria activate many of the environmental processes that sustain life existing in the lake (Madson, 2015; Bouskill *et al*., 2010). Bacteria contain high aquatic biomass and have fast growth rates, can respond to all environmental changes,

which has been used as indicators of environmental changes (Ager *et al*., 2010). Besides ecological importance, bacteria have been greatly applied in the search for medicine, agriculture, and industry (Prakash *et al*., 2012). Additionally, now a day bacterium is gaining attention for their extracellular enzyme and antibiotic production. Industrially, lake bacteria are a rich source of hydrolytic enzymes such as amylases, lipases, proteases, and catalases (Lutz *et al*., 2012).

An analysis of bacterial diversity, there is no general agreement on which diversity indices the best to use. However, the uses of Shannon Weaver and Simpson diversity indices have been recommended strongly to measure bacterial diversity (Haegeman *et al*., 2013). A more evenly distributed community is more diverse than a community is with few dominant species (Magurran, 2004). Most aquatic bacteria are gram-negative, with relatively few gram-positive representatives. Phylum proteobacteria are the dominant groups of bacteria. From this phylum, the genus *Pseudomonas* is most widespread and routinely found in freshwater lake samples (Lindstorm *et al*., 2005; Perscott, 2005).

Identification of bacterial into species involves laboratory culturing, isolation, and characterization to genera and species based on a wide range of phenotypic characteristics and biochemical attributes following procedures in Bergey's manual of determinative bacteriology (Holt *et al*., 1994). Identification of bacteria has a fundamental importance in the field of microbial systematics as well as in searching for potential bacteria for industrial, agricultural, environmental, and food production purposes (Claire *et al*., 2003; Rastogi, 2011). Advances in molecular biology have opened new possibilities for microbial identification and characterization. As a result, bacterial identification remains a changing and expanding field (Logue and Lindstrom, 2008).

In bacterial identification techniques, the phenotypic technique is more classical and is based on an organism's morphology and metabolic attributes. Whereas the genotypic technique is based on profiling an organism's genetic material, primarily it's DNA (Elbouri *et al*., 2012). Phenotypic identification can yield more direct and functional information. However, it has limitations time consuming and variability of culture due to different environmental conditions (Ruiz *et al*., 2000; Bosshard *et al*., 2004).

Information regarding diversity and abundance of bacteria are available in different freshwater lake ecosystem around the world. A lot of studies have been carried out on the bacterial community composition of freshwater lakes such as Lake Baikal, Russia (Khakhinov *et al*.,

2012). Lake Tanganyika, Africa (De Wever *et al*., 2005), Dianchi Lake, China (Bai *et al*., 2012), and Manasbal Lake, India (Shafi *et al*., 2017). Ethiopia possesses several natural freshwater lakes; Lake Tana, Lake Gummare, Lake Hawasa, Lake Chamo, Lake Hayq, Lake Langano, Lake Abaya, Lake Zway, Lake Zengena and others (Tenalem Ayenew, 2009). However, the bacterial diversity and abundance in these freshwater lakes were not yet intensely researched.

Lake Zengena was less researched in all aspects. Information regarding the diversity and abundance of the bacteria and totally the microbiology of the lake has not been studied. Thus, this study is intended to determine the diversity and spatial variability of heterotrophic bacteria and the evaluation of culture media for the isolation of bacteria from Lake Zengena.

1.2. Statement of the problem

Most studies on bacterial communities of freshwater were tilted to their impact on human and animal health other than diversity and their economic importance. Until recently, not much attention has been given to the study of their diversity and their importance in industries and ecological applications (Flandroy *et al*., 2018. It is known that bacteria that occupy Lake ecology play a critical role in ecological processes and have the potential for industrial and environmental applications (Newton *et al*., 2011).

Ethiopia has many freshwater lakes resources though the microbiology of lakes resources was not adequately studied. This is true for Lake Zengaena too which has not been well addressed in all aspects of the lake including microbiology. The studies that were conducted in Lake Zangena concentrated mainly on Ecotourism, some others on the Floristic diversity of woody plant species, and the physicochemical characteristics of the lake. The diversity of the bacteria and totally the microbiology of the lake has not been studied yet. Thus, the aim of this study was intended to investigate the diversity and spatial variability of heterotrophic bacteria and evaluation of culture media for isolation of bacteria from Lake zengena..

1.3. Objectives of the study

1.3.1. General objective

The aim of this study is to investigate the diversity and spatial variability of heterotrophic bacteria and evaluation of culture media for isolation of bacteria from Lake zengena.

1.3.2. Specific Objectives

- To investigate the diversity of heterotrophic bacteria in Lake Zengena using culture based techniques.
- To evaluate the efficacy of two culture media TSA and R2A on the isolation of bacteria isolated from the Lake Zengena.
- To determine spatial variability in bacterial diversity of Lake Zengena
- To determines physiochemical characteristics of the lake.

1.4. Significance of the study

Determining the diversity and spatial variability of heterotrophic bacteria and evaluation of culture media for isolation of bacteria from Lake has paramount importance to the scientific community, the local government and community in particular. The study shows the diversity and spatial variability of heterotrophic bacteria and evaluation of culture media for isolation of bacteria from Lake Zengena.

Thus, the results of the study will serve as first hand information to the community who will benefit directly or indirectly from the resources of the lake. The result also gives information about the physico chemical characteristics of the lake. The outcome of this study also increases implementation of the lake resource for further economic use. Furthermore, it will serve as information for researchers who will have interest to conduct similar study in other areas in the country.

1.5. Scope and limitations of the study

This study mainly encounters and encompasses the diversity and spatial variability of heterotrophic bacteria and evaluation of culture media for isolation of bacteria from two different sample sites of lake by using two different media types and the study also determines the physicochemical characteristics of the lake. The study did not include anaerobic cultures and identification of bacterial isolates to the species level by using molecular techniques. In addition, physiological characterization (pH, temperature, salt and concentration) was not done on the isolates.

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2. LITERATURE REVIEW

Microorganisms are the richest index of living diversity on earth, constituting about 60% of the total biomass and all basic ecosystem processes are reliant on them (Wooley *et al*., 2010) although 99.99% of them are not yet discovered (Jurasinski and Koch, 2011). Microbes are key players and major components of the biosphere, compared to macro-organisms. However, little is known about their diversity and ecology in their natural environment (Martiny *et al*., 2006). Studies have shown that microbes are not only very abundant and ecologically important but are also highly diverse and have a multitude of ecological and technological applications (Madigan, 2012).

Microbial diversity is the key to human survival and economic security as it provides a vast variety and pool of resources, which can be utilized by humans for their benefits (Anizzon *et al*., 2012). Microorganisms are useful for innovative applications to humankind because of their vital biogeochemical cycling starved for all life on earth would cease (Gilbert and Neufeld, 2014). Man has long been manipulating the microbial wealth for food production and preservation, production of antibiotics, controlling of pests, pathogens, wastewater treatment, examining chemicals and as tools for medical research (Sharma, 2014).

The study of microbial diversity is also vital to answering new and emerging challenges like diseases (Ferreira *et al*., 2010). Despite the recognized values of microbes, our knowledge of their diversity, and many of their key roles in sustaining the global life support system is still very scarce. Continued exploration, evaluation, and exploitation of microorganisms for their diversity are essential for scientific, industrial, and social development. Thus, due attention on research to describe and characterize unexplored resources and protecting, preservation of natural ecosystems and the future benefit of mankind is important (Torsvik and Ovreas, 2002). In the last century, there has been an intensive study on microorganisms, and their applications expanded in different sectors of human activity (Ovreas and Curtis, 2011).

This huge microbial resource is undertreated due to mainly anthropogenic pressures, especially in the aquatic ecosystems where there is a high degree of human interferences (Ovreas and Curtis, 2011). With the increasing human population, such impacts on the environment are intensifying at an alarming rate and may ultimately lead to the loss of vital microbial resources. It is also clear that from studies done so far, only had a very limited picture of microbial diversity as compared to the theoretically estimated diversity believed to exist in natural

environments (Fierer, 2008). Continued efforts on the study of the microbial community in space and time are thus needed to increase our understanding of microbial diversity.

2.1. Diversity of Heterotrophic bacteria in Freshwater Lake

Freshwater ecosystems are vital to all life. Only 3% of the world's water is fresh. Of this, 99% locked in glaciers or underground water (Wetzel, 2001). Apart from several niches present on earth, freshwater lakes are ideal habitats for diverse bacterial populations, both beneficial as well as harmful. Freshwater bacteria are a very diverse group of prokaryotes, differing in their morphology, physiology, and ecological preferences (Sigee, 2005). They mediate many of the environmental processes that sustain life on the earth. The two main microbial processes that occur in freshwater lake habitats are the nitrogen and carbon cycle, both of these cycles affect the lives of the macro flora and fauna which share this habitat. Besides, their diversity is greatly applied in the alleviation of environmental problems and search for novel enzymes and antibiotics used in medicine, agriculture, and industry (Claire *et al*., 2003). The term heterotrophic bacteria are microorganisms that require organic carbon for growth. These organisms are found throughout the environment in both natural and treated water. The presence of heterotrophic bacteria in water has implications for public health, especially pathogenic organisms (Allen *et al*., 2004).

Freshwater lake bacteria varied in function and taxonomy (Ye, 2009; Sugumar and Anandharaj, 2016). The dominant phylum in the lake ecosystem is phylum proteobacteria. Along proteobacteria, different studies revealed, phylum Actinobacteria and phylum Bacteroidetes as main phyla in the freshwater Lake ecosystem (Zwart *et al*., 2002; Newton *et al*., 2011).

2.1.1. Phylum Actinobacteria

Historically soils are considered as the prime environment of habitation and optimal activity for the Actinobacteria. The introduction of molecular-based studies of aquatic systems has changed this opinion. Initial 16S rRNA gene and fish-based studies revealed that members of the Actinobacteria are common and often a numerically important component in a variety of freshwater habitats (Gomez *et al*., 2007; Zwart *et al*., 2002).

The phylum *Actinobacteria* constitutes of Gram-positive bacteria, small, rod, coccus, or solenoid shaped. They are free-living and found at the surface water where they contribute over 50% of the bacteria in the surface water. In energy generation, they are phototrophic and heterotrophic (Ventura *et al*., 2007). Actinobacteria in freshwater lake actively synthesize DNA

and proteins, which delivers evidence that the phylum is a native resident of freshwater. Apart from this, they also present in the bottom waters of the lake their number often lowers with decreasing oxygen level (Newton *et al*., 2011).

2.1.2. Phylum Bacteroidetes

The members of this phylum are mostly found in soil, in aquatic environments, or as symbionts of plants, animals, and humans. They play a significant role in the degrading of organic compounds in saline and freshwater environments (Krieg *et al*., 2010). Many representatives of the Bacteroidete are known to have close relationships with animal and human hosts, where they can be either synergists or antagonists. The comparative phylogenetic investigation provided a strong provision for the phylum Bacteroidetes that it shares numerous phenotypic characteristics and a common ancestor with the phyla Fibrobacteres and Chlorobi (Guts, 2004). The phylum consists of bacteria like Gram-negative, bacillus bacteria, and exhibits enormous phenotypic, and metabolic diversity (Newton *et al*. 2011). In Lake Epilimnia, biopolymers degradation can occur due to these bacteria. Lake Bacteroidetes abundance increases following cyanobacteria blooms and accounts for more than 40% of the total bacterial biomass in a lake, as measured by fluorescence in situ hybridization probes (Zwart *et al*., 2002).

2.1.3. Phylum Firmicutes

These are a phylum of bacteria, most of which have a Gram-positive cell wall structure. But a few, such as *Megasphaera*, *Pectinatus*, *Selenomonas,* and *Zymophilus*, have a spongy pseudo outer membrane that causes them to stain Gram-negative. Scientists classified Firmicutes to comprise all Gram-positive bacteria but recently defined them to be of the main group of related forms called the low G+C group, in contrast to the Actinobacteria. Firmicutes play an essential role in beer, wine, and cider decomposition. The group is divided into the *Clostridia*, which are anaerobic, the *Bacilli*, which are obligate or facultative aerobes (Wolf *et al*., 2004).

2.1.4. Phylum Proteobacteria

Generally, Members of proteobacteria are the dominant prokaryotes in aquatic systems and they are the most heterogeneous in terms of physiology, distribution, and taxonomy. They found in both oxic and anoxic environments and predominately in saline, alkaline, and freshwater systems (Brenner *et al*., 2005; Glbert and Lenive, 2017).

The phylum Proteobacteria is a group of Gram-negative bacteria encompassing the majority of recognized agriculturally, industrially, medically relevant organisms, and the most studied

bacterial phyla. Six classes of Proteobacteria are currently recognized as Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, Gammaproteobacteria, and Zetaproteo bacteria (Yannarell and Kent, 2009).

2.1.5. Phylum Verrucomicrobia

Members of the Verrucomicrobia were identified and cultured early as genus *Prosthecobacter* but the phylum, which took its name from its first aerobic freshwater isolates *Verrucomicro biaspinosum*, phylum Verrucomicrobia were identified in lakes, soil, oceans, and human feces and even as ecto and endosymbionts of eukaryotes (Wanger and Horn, 2006). Recently, Methane oxidizing representatives of the phylum Verrucomicrobia have been isolated from extremely low pH, thermophilic environments (Semrau *et al*., 2008). The phylum Verrucomicrobia has an elevated degree of phylogenetic relationship with the phylum Planctomycetes. Some representatives of those groups have "compartmentalized intracellular structures" (Wanger and Horn, 2006).

2.1.6. Flora of the lake Zengena

Lake Zengena is surrounded by fragmented and small-sized remnant forest patches. The forest consists of woody plant species including *Allophylusa abyssinica*, *Terminalia brownii*, *Prunus africana, Apodytes dimidiata*, and *Schefflera abyssinica*, which were common 50 woody plant species belonging to 31 families were recorded. Zengena forest is characterized by a higher number of woody species but a smaller number of the woody plant compared with other Afromontane forests in Ethiopia (Desalegn Tadele *et al.,* 2014). Zengena forest is not such exposed to disturbance by humans and livestock. The forest, especially close to the shore of the lake, is not easily accessible for use by humans and livestock due to its steepness. Low disturbance in the forest may contribute to the maintaining volume and quality of the lake (Desalegn Tadele *et al.,* 2014).

2.1.7. Fauna of the lake Zengena

The faunal diversity of the lake has not been studied well. The lake was not rich in faunal diversity because of the closed crater and other geological and hydrological nature of the lake. There were some bird species include *Inter alia*, *Alopochenae gyptiaca* (Dakiye), *Aquila rapax* (Chilifit), *Wattled Ibis (*Bale-Enitiltil Gagano). There are some species of fish, *T.zilli* and *O.nilticus* species are native and *C.carpio* which is exotic to the lake (Yared Tigabu, 2010).

2.2. Spatial variability and Bacterial abundance in Freshwater lakes.

The structure of bacterial communities is maintained by variable environmental conditions across space and time that represent habitat heterogeneity (Shade *et al.*, 2008). Ecological niche separation of coexisting microbial taxa might be triggered by bottom-up (resource availability) and top-down control (mortality factors), leading to distinct spatial (longitudinal and vertical) and temporal patterns of distribution of different microbes within lakes (Salcher., 2014). Among the factors that regulate prokaryotic assemblages, some of the most important are temperature, ultraviolet radiation, quality and quantity of dissolved organic matter, nutrient concentrations, and grazing pressure (Newton *et al*., 2011). Which can vary with the geographic position, watershed, and surrounding landscape of the lakes.

In aquatic ecosystems, hydrologic regimes quickly move water parcels thus transporting bacteria and other planktonic organisms with the water mass. A pyrosequencing study compared the composition of bacterial community at three different depths throughout the water column at multiple stations in the North Atlantic Ocean and reported that bacterial community composition was most similar within the same water mass, defined by depth, regardless of horizontal space (Suman, & Saxena, 2015). Long-term observation of aquatic ecosystems has provided the data mandatory for showing that, along with the spatial scale, bacterial communities also vary over time which also affects the interpretation of the relative contribution of the underlying processes of bacterial community composition(Gilbert et al., 2012).

2.3 Roles of culture media in isolation of heterotrophic bacteria

Different growth medias have to be used to since all bacetria will not grow in single growth medium. It is important to compare the growth efficacy of routinely used media in microbiology laboratories. Depending on medium for bacteria isolation, it is problem for bacteriology laboratory, individual enrichment, and plating media (Ifeanyi *et al*., 2014). One of the challenges is in studying bacterial diversity is unculturability. Only 1% of bacteria are cultivable. Unculturability is probably caused by growth states of cells in nature, exceptionally high concentration of nutrients, and complex organic compounds that present in the laboratory media Mostly laboratory conditions are poorly mimic than natural environmental conditions. As a result, improved culture media that is more closely mimic natural conditions is needed to study and identify new roles and functions of microorganisms (Vartoukian *et al*.,2010).

Tryptic Soy Agar (TSA) is used as a general growth medium for the isolation and cultivation of microorganisms. It is recommended for transportation, cultivation, storage, , and maintenance

of cultures of microorganisms. New culture media today mimic the natural environment of bacteria by adding different elements in culture medium to cultivate previously uncultivated bacteria (Heylen *et al*.,2006).

R2A Agar is a low nutrient medium that was developed by Reasoner and Geldreich for the bacteriological plate counts of treated drinking water. This application demands a medium that allows stressed bacteria to recover without being overgrown by faster-growing organisms in the meanwhile. The low nutrient R-2A Agar in combination with long incubation times and optional low incubation temperatures meets these requirements and gives distinctly higher recovery rates for debilitated microorganisms (Christesen *et al*., 2012).

Casein hydrolysate and peptone provide nitrogen, vitamins, amino acids, carbon, and minerals. Yeast extract is a source of vitamins and essential trace elements. Soluble starch has a positive impact on the recovery of injured organisms due to its ability to absorb toxic metabolic by-products. Sodium pyruvate also aids with the recovery of stressed cells. Magnesium sulphate provides divalent cations and sulphate. Potassium phosphate is added to stabilize the pH and as a phosphate source agar acts as a solidifying agent Appendix(II). appropriate standard procedures should be considered for sampling and storage. Generally, it is recommended to test water samples as soon as possible and store them refrigerated to minimize changes. R-2A Agar can be applied for the spread or pour plate technique. Dilution of 30-300 colonies per plate is Chosen to compute CFU (Colony Forming Units) per volume considering your specific dilution. Plates should be counted after 5-7 days of incubation (Imazaki and Kobori, 2010).

2.4. Physicochemical characteristics and effect on the isolation of bacteria

In aquatic ecosystems the diversity of microorganisms is influenced by physical and chemical factors which will interact or oppose each other. The interactions of physicochemical parameters of water play a significant role in the species composition of the primary organisms (Sahato et al., 2004). It is found that the bacterial community variance is strongly correlated to water temperature, conductivity, pH, and dissolved oxygen (DO) content in freshwater, intertidal wetland, and marine sediments (Wang et al., 2010). In lakes, multivariate analysis showed that several factors such as temperature, nutrient concentrations, water flow, and biomass of other plankton groups vary with bacterioplankton fingerprints. Such associations suggested that these environmental factors largely affect the abundances and taxonomic composition and distribution of bacterial communities (Allison and Martiny, 2008).

Different studies have confirmed that out of various factors temperature affecting the aquatic bacterial diversity, significance, as it regulates various abiotic and biotic activities of an aquatic system, has a decisive influence on bacterial community composition. Fluctuations in temperature influencing various phenomena such as stratification, the solubility of gases, pH, conductivity, and distribution of organisms in aquatic ecosystems (Radhika et al.,2004). Change in pH values beyond the optimum range adversely affects the microbial physiology. Desirable pH for freshwater ranges between 6.5 and 9.0 and any fluctuation in this range affects natural aquatic life by controlling the solubility of metal ion (Hassanin, 2006).

Measurement of total dissolved solids (TDS) helps to determine the combined of all organic and inorganic substances contained in a liquid in molecular, ionized, and suspended form. It is vital parameter for describing chemical constituents of water and edaphic relations that contribute to productivity within the water bodies. In aquatic ecosystems TDS comprises mainly of bicarbonates, nitrates, carbonates, phosphates, magnesium, potassium, calcium, chlorides, , sodium, organic matter, manganese, salt, and other particles (Mahananda, 2010). Dissolved oxygen (DO) supports life in water and affects the solubility and availability of many gases and nutrients. DO, the most significant parameter affecting the productivity of aquatic systems. Numerous studies have been carried out to ascertain the association of BCC with the dissolved oxygen content in lake ecosystems (Wang *et al*., 2010).

2.4.1. Physical and chemical parameters of Lake Zengena

The mean values of lake temperature, 22oC, dissolved oxygen 7.3mg!, Turbidity 5NTU, pH 7.7, conductivity 52JISem, and total dissolved solute of the lake is 31.4 mg!. No significant seasonal changes in conductivity, turbidity, total dissolved solids, and pH were observed in Lake. As compared to other open lakes and reservoirs. This is the indication of the absence of mixing of the water column from rivers, agricultural and municipal effluents from the catchments to the lake. Associated with these, general levels of nutrients, turbidity, conductivity, and total dissolved solids significantly lower in high land Crater Lake Zengena as compared to other open lakes and reservoirs (Goraw Goshu, 2007).

2.5. The potential application of bacteria from the lake

2.5.1. Ecological importance of freshwater lake bacteria

Bacteria have ecological a swell as economic importance. Understanding ecological processes that result in the structure and function of the microbial community in the environment is a field that elevated some greater interest in current years. This is because of the vital role of microbial in health, ecosystem, and biotechnology (Kastman *et al*., 2016; Widder *et al*., 2016). The lake is a site of tremendous bacterial activity, which plays an important role in nutritional chains as well as maintaining the biological balance. Freshwater bacteria have an important role in geochemical transitions within the aquatic environment and are important in the cycling of metabolically vital elements; nitrogen, carbon, and Sulphur (Madsen, 2015). Microorganisms are important sources of food and nutrients for organisms present in the aquatic ecosystem such as protozoa. In the absence of aquatic microorganisms, the system of food chain might be disturbed leading to ecosystem imbalance and ultimately affect the existence of biotic and abiotic systems associated with it (Madsen, 2015).

2.5.2 Industrial importance of freshwater lake bacteria

In addition to ecological importance in these ecosystems, bacteria have currently is gained attention for their extracellular enzymatic and antibiotic production. Most of the aquatic bacteria are a rich source of hydrolytic enzymes such as amylases, lipases, proteases, phospholipase, catalases, and other important industrial enzymes. Hydrolases are by far the most widely used class of enzymes in industry (Busto *et al*., 2010). Enzyme proteases produced by microorganism has industrial potential due to its wide biochemical applications in food industries, medicinal formulations, detergents, waste treatment and remediation of pollutants from contaminated environments (Saurabh, 2007). Bacterial hydrolases are classes of enzymes that are capable of degrading numerous pollutants containing recalcitrant plastic polymers. An extracellular esterase is involved in the degradation of polyester polyurethanes (Wei and Zimmermann, 2017).

Bacteria also have great importance in the production of antibiotics. Antibiotics are chemical compounds natural or artificial that inhibits the growth of or kills microbes and are used primarily to treat or prevent infections (Reybitz, 2015). Antibiotic production is a feature of several kinds of bacteria. The order Actinomycetales became renowned as a source of antibiotics (Clardy *et al*., 2006). Members of Actinomycetales accounted for almost 70% of the world so occurring antibiotics used in humans (Lazzarini *et al*., 2001) while non-actinomycetes

bacteria account for 12% of known secondary metabolites (Baltz, 2019). The phyla Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, and Proteobacteria are known to have species that produce bioactive compounds (Keller, 2004).

The current global major challenge concerning human health is the spreading and appearance of antibiotic resistance among the pathogens (Martinez *et al*., 2008). Screening of microorganisms from their natural habitat is an important step for the isolation of therapeutic compounds. As a result, researchers are trying to look for new organisms that could have the potential to produce antibiotics from unexplored habitats (Oskay *et al*., 2004). Aquatic microorganisms are of special interest, as they have not been exploited extensively compared to terrestrial microbes (Zhang *et al*., 2005).

2.5.3. Economic importance of lake Zengena

Lake Zengena and the surrounding area is rich in abundance of tourism and natural resources. A good and aesthetic environment with breathtaking walkways and camping ground. It possesses a luxuriant look and beautiful landscape with a highland environment. The lake was not given due attention and due to this very little conservation activity was done by local guides and scouts. It is visited by both domestic and foreign tourists although they are very few. The sale of local handicrafts could have a big potential to help, develop, and promoting community business enterprises in Lake Zengena and its environs. The environs of Lake Zengena are rich with the bamboo tree (Kerkeha). The most common handicrafts in the surrounding include those prepared from Bamboo trees, Horsetails (Chira) souvenirs, and weaved goods (Zemenu Bires, 2017).

2.6. Methods of microbial diversity analysis in freshwater

Microbial ecology methods include systemic sampling from the environment, culturing and/or of isolation, detection of microorganisms in the environment (in situ), determination of microbial number and structures, and microbial activity measurement in-situ or in-vivo. These methods are conventionally classified as culture-dependent and culture-independent techniques, both methods have their strengths and weakness that should be at least minimized or compromised. Due to the inherent limitation in either approach using a combination of both techniques offers a better understanding of microbial ecology (Martiny *et al*., 2006).

Both methods require careful sampling, sample handling, and processing. There are several important factors considered in sample collection. The major factors are amounts of samples, representatives of the ecosystem, activities of microorganisms, contamination-free, and correct

storage conditions. Furthermore, microbial communities are dynamic in time and space (Martiny *et al*., 2006). More importantly, immediate processing of samples or processing on-site is highly recommendable to minimize the negative effects during sample handling (Maruta *et al*., 2012).

2.6.1. Counting bacterial population

The population ecology of freshwater bacteria is characterized by high cell counts and the capacity for the rapid rate of reproduction. Counts of viable (metabolically active) heterotrophic bacteria can be readily carried out by plating bacteria on agar plates and counting the number of colonies that develop an isolated colony. Bacterial counts expressed as colony forming units (CFU) records organisms that can grow and multiply on a nutrient medium. This approach gives information on the total number of metabolically active heterotrophic bacteria (total heterotrophic viable count) present in the sample. There are problems in these methods; all plating media are highly selective and many viable organisms with the complex nutrient requirement will be excluded, Bacteria require specific Physico-chemical parameters (particularly pH or oxygen) may also exclude from the count (Mwirchio *et al*., 2010).

2.6.2. Isolation and identification of bacteria

Culture-based techniques have been used for a century and helped to characterize a large number of microbial species that can be grown under conventional laboratory conditions. However, only 1% of bacteria grow under culture-based laboratory techniques. In recent years, Molecular techniques used to overcome the limitation of the cultivation approach and helped in revealing many hitherto uncultured microorganisms (Konne *et al*., 2005).

The identification of individual bacteria species involves laboratory culture, isolation, and characterization. Identification of bacteria into genera and species based on a wide range of phenotypic characteristics and biochemical attributes (Holt *et al*., 1994).

2.6.2.1. Limitations and strengths of phenotypic identification

Phenotypic identification can yield more direct and functional information that revels what metabolic activities are taking place to aid the survival and growth of the bacteria. If implemented properly phenotypic methods are accurate and reliable. However, it has limitations. This technique is solely applicable to cultivable organisms. Time-consuming and variability of culture due to different environmental conditions may lead to an ambiguous result and subjective interpretation (Ruiz *et al*., 2000; Bosshard *et al*., 2004).

2.6.3. Bacterial diversity measures

The diversity of microbes will be determined by the number of species or different operational Taxonomic Units (OTUs) of microbes living in a certain environment, as well as the evenness of the species distribution. The number of species present and their numerical composition characterize important features of the bacterial community in a certain niche. To relate the bacterial diversity a range of bioinformatics tools has developed. Shannon Weiner and Simpson diversity indices commonly used in bacteria diversity measurement based on operational taxonomic units OTUs (Scholss and Handelsman, 2006). OTUs are in ferried to exist based on sorting out data and can be defined at different levels of resolution (phylum, class, order, family, genus, and species) (Chano and Bunge, 2002; Scholss and Handelsman, 2006).

2.6.4. Regulation of bacterial populations and biomass

Bacterial biomass in freshwater systems is affected by environmental factors including predation protozoa and rotifers, parasitic viral attack, inorganic nutrients and dissolved organic carbon availability. In addition to the food web factors oxygen depletion affect bacterial abundances by changing the environment (Ricciardi and Rigault, 2000).

3. MATERIALS AND METHODS

3.1. Study area

The study was conducted on a diversity of heterotrophic bacteria in Lake Zengena. Lake Zenegena is high land closed Crater Lake located at 10°54′50″N 36°58′00″E in the Awi Zone Banja woreda Kessa-Chewsa Kebele, Amhara Region in Ethiopia. It is located at an elevation of 2523 m above sea level (Figure 1). The diameter of the lake is 930 m from North to South and 970 m from East to West and area of 0.54 square kilometers with a maximum depth of 166 m (Endalew *et al.*, 2004). The area is, seasonally dominated by the dry season, the main rainy season extends from mid-June to mid-October with maximum rainfall occurring between July and August, with mean annual rainfall ranging between 1,300 mm up to 1,800 mm and the mean annual temperature ranges between 16^{o} C and 20^{o}C (Banja woreda statical office,2020).

Lake Zengena believed to be formed by volcanic explosion and collapse. Its rim is made of unconsolidated ash deposits. The Lake is a volcanic crater that is a field with water (Tenalem Ayenew, 2009). It is circular and surrounded by a cliff covered with vegetation without any indentation. It is a closed basin with no inlet and outlet. There are no rivers which tribute to the lake and no outlet. Surrounded by both indigenous and non-indigenous plants with a higher number of woody species and trees that are evergreen (Desalegn Tadele *et al*., 2014). The lake provides economic, social, and ecological benefits (Zemenu Bires, 2017).

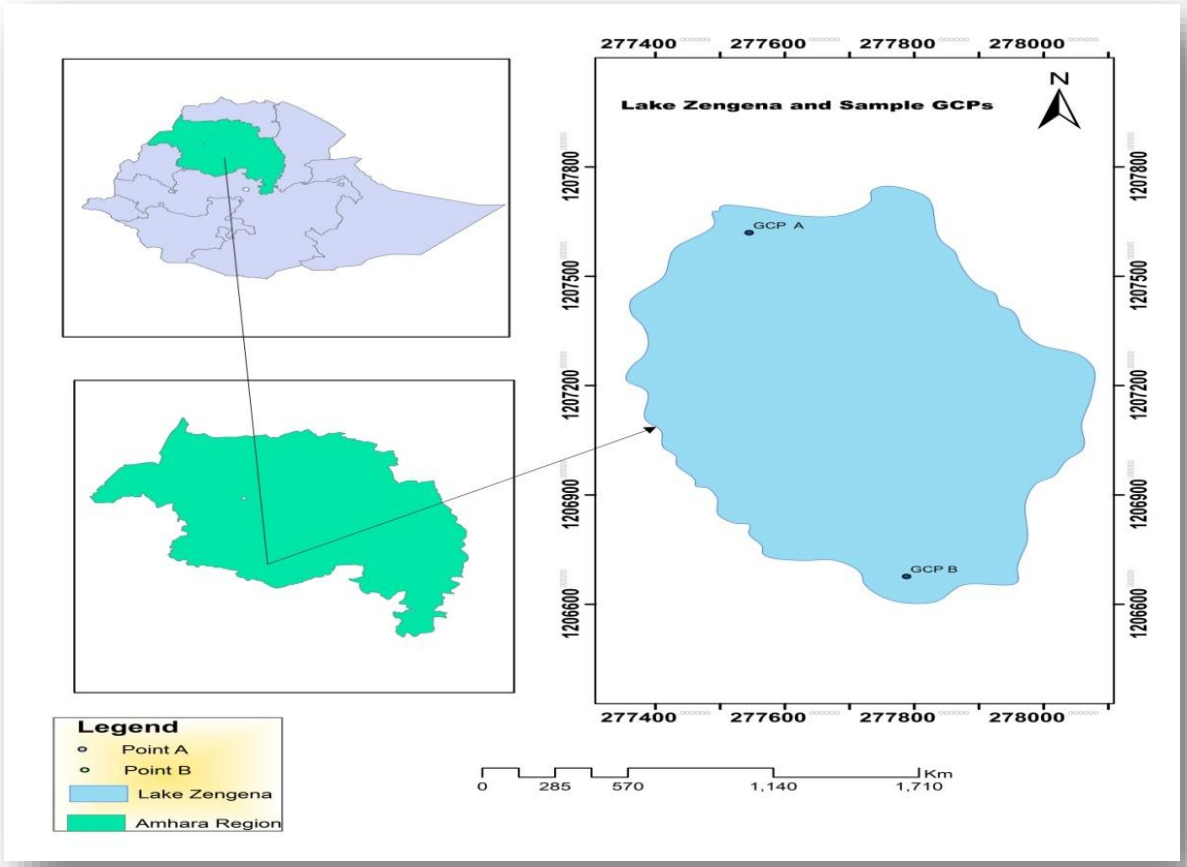

Figure 1: The map of the study area showing the two study sites of lake zengena.

3.2. Study design and period

Expermental study design was conducted aimed to investigate the diversity and spatial variability of heterotrophic bacteria and evaluation of culture media for isolation of bacteria from Lake Zengena along with major physiochemical characteristics of the lake. The study was conducted from water samples of Lake Zengena by considering two sample sites and two culture media types. Water sample was taken from two different sites of lake and cultured on two different media. Bacterial isolates was characterized and identified based on their bases of morphological features, biochemical characteristics, and physiological parameters. The study was conducted from December 2019 to June, 2020).

3.3. Sample site and sample collection

Water sample was collected from two different sites of the lake located 36.96 ° 44 ′ 66 N″ and 10.91° 59 ′ 02″ E north of the lake and 36.96° 44′ 66″ N and 10.91° 15′ 36″ E to the south of the lake at specific coordinates. At each sample site, one liter of water samples was collected

in two liter autoclaved glass bottle container aseptically, kept in icebox, and transported to the microbiology laboratory of Bahir Dar University within 3 hour after collection.

3.4. Measurement of physicochemical parameters of lake water

Water temperature (using mercuric thermometer), dissolved Oxygen (DO) (using Bante 820, China), pH, specific electrical conductivity and total dissolved solid (TDS) (using Bante901P, china). Were measured in situ.

3.5. Isolation of bacteria

Water samples obtained from each site were serially diluted up to 10^{-10} using physiological saline water. This was done by transferring 1 ml sample to the 9 ml of dilution water. Then from serially diluted samples. 0.1 ml of water was spread on plates containing; Trptone soy agar (TSA) and low nutrient media (R2A). Then Trptone soy agar (TSA) plates were incubated at 25^{o}C for 48-72hrs and R2A medium a were incubated at 25^{o}C for 5-7 days. After incubation, 50 colonies from each culture type were picked up from plate containing colonies. Isolates were then sub cultured for purity check and finally preserved on nutrient agar slants for further processes.

3.6. Identification of the isolates

During the identification, 50 colonies (from each culture type) were picked up from a plate containing 30-300 colonies. Total of 200 bacterial colonies were chosen by considering their shape, size, margin, color, opacity, elevation, surface, texture of colonies and arrangement of the cells, size and form to conduct identification. Bacterial isolates were characterized and identified based on their morphological features, Gram's test, motility test, endspore formation and biochemical characteristics. Bacterial isolates were characterized and identified to genus level following procedures in Bergey's manual of determinative bacteriology (Holt and Bergey, 1994).

3.6.1. Morphological characteristics

Colony morphologies of the isolates were described using standard microbiological criteria and macroscopic techniques with special emphasis on shape, size, margin, color, opacity, elevation, surface, texture, arrangement of the cells, size, and form were recorded for each colony. Bacterial isolates were characterized morphologically by; gram's test, motility test, and endospore formation test (Cappuccino and Sherman, 2002).

3.6.2. Biochemical tests

Identification of isolate was carried out by subjecting the isolated bacterial isolates various biochemical tests depending on their metabolic activities and changes in products. Bacterial isolates were tested biochemically by; catalase test, triple sugar iron test (glucose fermentation, lactose fermentation, sucrose fermentation, H_2S, and acid formation). Citrate test, Indole production test, Methyl Red test, Voges-Proskauer test, and Mannitol fermentation test (Cappuccino and Sherman, 2002).

3.7. Data analysis

The computer-based Statistical Package for the Social Sciences (SPSS version 23 for windows) was used for data analysis. Rarefaction curve was calculated to determine the sample sufficiency. Analysis of variance was used to analyze differences in OTUs abundance between media and sites. The species diversity (richness and evenness of each culture type) in terms of OTUs were analyses using the Shannon Weiner Diversity Index and Simpson's index. P-values less than 0.05 were used to compare the results.

4. RESULTS AND DISCUSSION

4.1. Physicochemical characteristics of lake water

Major physicochemical characteristics of lake water were measured during sampling time and the mean value was listed (Table: 1).

Table 1: Physicochemical characteristics of Lake Water (pH, Temperature, conductivity, dissolved oxygen and TDS) site one measured on Junuary4 /2020 and site two measured February 12/2020).

Physicochemical characteristics of lake	Site	
	Site 1	Site 2
Temperature	19˚C	20˚C
pH	7.4	7.6
Conductivity	47.9 µ/cm	48.3 µ/cm
Dissolved oxygen	5.2 mg/L	5.3 mg/L
TDS	23.1mg/L	24.4mg/L

Bacterial community difference is strongly correlated to water temperature, conductivity, pH, and dissolved oxygen (DO) content in freshwater (Wang *et al*., 2010). In lakes, multivariate analysis revealed that several factors such as temperature, pH, nutrient concentrations, water flow, and biomass of other plankton groups mainly affect the abundances and taxonomic composition and distribution of bacterial communities (Allison and Martiny, 2008).

The temperature of lake water, which was measured 19℃ in site one and 20℃ in site two, was comparable to most freshwater lakes temperature, which is favorable and available for most dominant freshwater bacterial tax (Greenberger *et al*, 2015). Water temperature is environmental parameters affecting the aquatic bacterial diversity, which has enormous significance as it regulates various abiotic and biotic activities of an aquatic system (Radhika *et al*., 2004). As studies have shown that out of different factors temperature has a significant influence on bacterial community composition. *Pseudomonas* and *Aeromonas* were more frequently exist and

abundant in freshwater at 22◦C (Greenberger *et al*, 2015) which coincide with the mean temperature of the lake water.

The pH of the lake measured 7.4 and 7.6, which was correlates with favorable pH for freshwater ranges between 6.5 and 9.09. Change in pH values beyond the optimum range adversely affects the microbial physiology (Hassanin *et al*., 2006). The pH of lake water is considered to be more favorable for microbial growth.

Total dissolved solids were measured 23mg/L and 24.4mg/L which were in the range of most oligotrophic freshwater lakes and favorable for common freshwater bacteria (Goher, 2002). Total dissolved solids strongly correlated with the coliform counts (Hassanin *et al.*, 2006).

Dissolved oxygen (DO) was measured 5.2 mg/L and 5.3 mg/L**.** This is less measured as compared to many freshwater lakes because of the absence of mixing of the water column from rivers, agricultural and municipal effluents from the catchments to the lake. Dissolved oxygen in aquatic ecosystems supports life in water and affects the solubility and availability of many gases and nutrients which is the most significant parameter affecting the productivity of aquatic systems. A study has been shown a strong correlation between dissolved oxygen and Bacterial community composition (Naik *et al*., 2012).

Absence of mixing of water column from rivers, agricultural and municipal effluents from the catchments to the lake, makes generally levels of nutrients, turbidity, conductivity, and total dissolved solids significantly lower in high land Crater Lake Zengena as compared to other open lakes and reservoirs (Goraw Goshu, 2007)

4.2. Heterotrophic bacterial counts from Lake Zengena on the two media count.

The mean heterotrophic plate counts (HPC) of bacteria ranges from 1.78×10^8 in site one rich media (S1TSA) to 2.1×10^8 CFU/ml in site two low nutrient medium (S2R2A). Bacterial divesty in the low nutrient media was greater than rich media in all sites. However, bacterial counts in site two were more than site one (Figure 2). The result correlates with the measured physicochemical characteristics of site two, better to fit the range of most freshwater lakes than site one. Generally mean heterotrophic plate counts (HPC) indicates that low nutrient media has greater bacterial count than rich media in all sites. This indicates that bacterial biomass was influenced by a rich of media. The type of media found in the study and the result obtained is more conformed. with the study result done on improving the cultivability of

freshwater bacteria using FW70 a low nutrient solid medium modified with sodium private (Imazaki and Kobori, 2010).

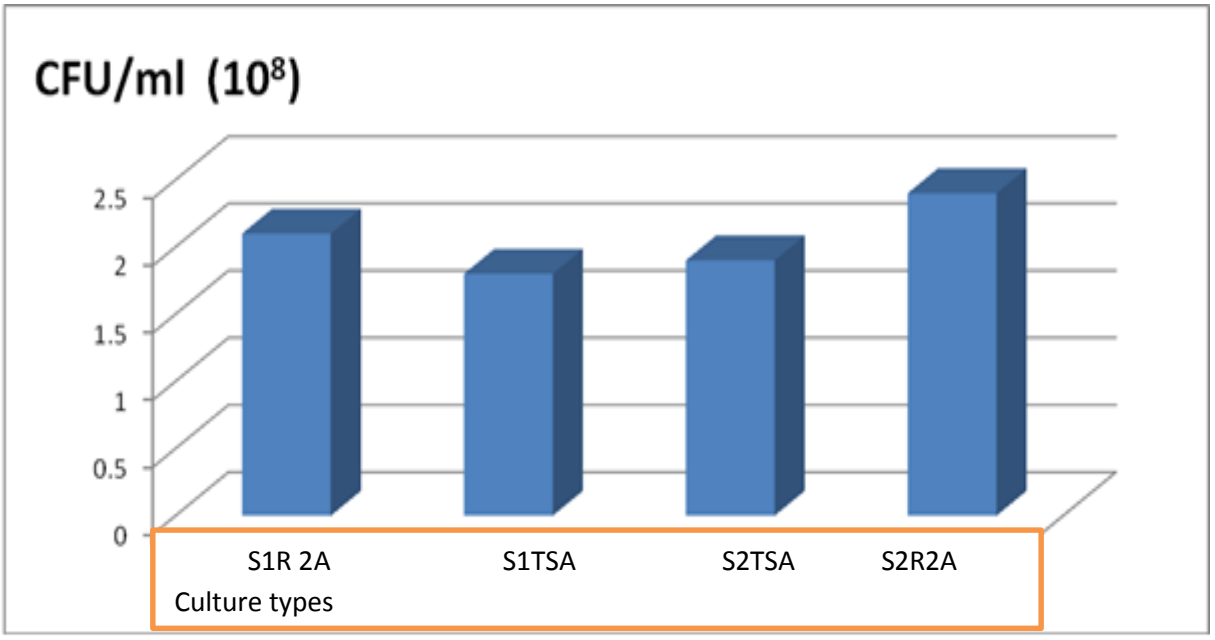

Figure 2: CFU/ml of water sample in different culture media

Where; S1: Site one, S2: Site two, R2A: Media type, TSA: Media type media

4.3. Heterotrophic bacterial diversity

Based on colony characteristic and biochemical tests considered the 200 isolates were grouped into 76 distinct isolates (Operational Taxonomic Unit, OTU) OTUs were then used to determine the diversity of the isolates and comparisons between media types and sites. Bacterial diversity was thus assessed in terms of unique operational taxonomic units (OTUs). There was a total of 76 OTUs, which demonstrate unique morphological and biochemical features and can be a proxy for at least a species.

The sample rarefaction curve indicates that 200 isolates were sufficient to explain the diversity provided by the given methods. As more individuals are sampled, the total number of OTUs recorded in the sample increases initially and species accumulation curve was generated (Figure 3). The graph shows analyzing isolates more than 200 will not reveal much new bacterial OTUs.

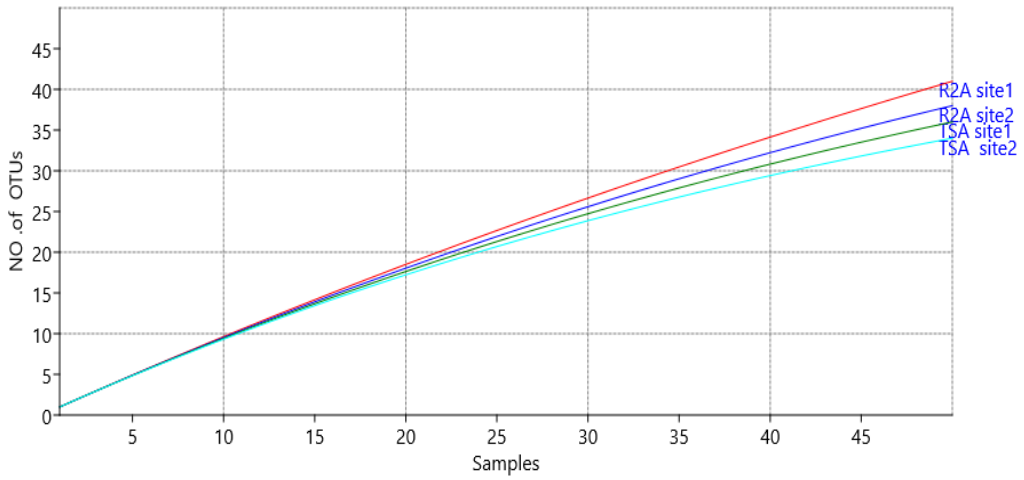

Figure 3: Rarefaction curve of each culture type

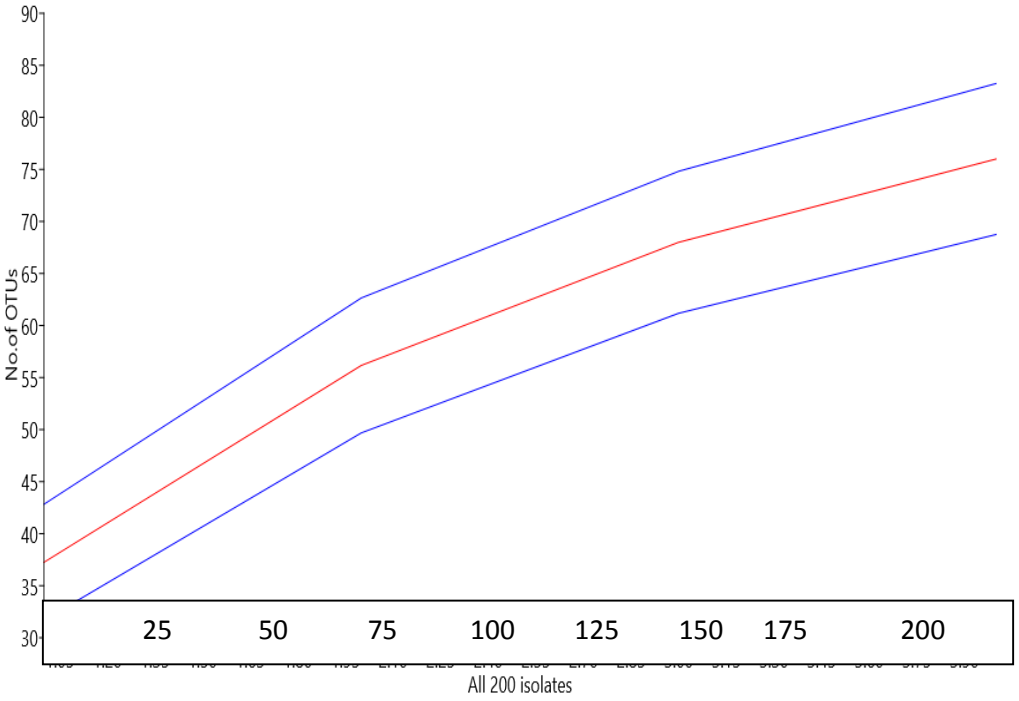

Figure 4: Rarefaction curve of all 200 isolates.

OTUs were represented by one or more isolates and used for the subsequent analysis. Of all the76 OTUs, eight OTUs (OUT 12, OUT 13, OUT 15, OUT 20, OUT 38, OUT 40, OUT 44 and OUT 60) were found in all sites and culture types, 4 OUTs (OUT47, OUT 52, OUT 68, and OUT 76) were found in all conditions except in site two in TSA media, two OUTs (OUT 33and OUT 46) were found in all conditions except in site two in R2A media, two OTUs (OUT 19 and OUT 31) was found in all conditions except in site two in TSA media and OUT 7 was

present in all conditions except in site one in R2A media. These 16 OTUS were abundant and constitute about 37.5% of the total number of isolates. Two OTUs (OUT 12 and OUT 15) each has 6 isolates, 7 OTUs (OUT 13 OUT, OUT 20, OUT38, OUT40, OUT31, OUT 33 and OUT 52) has 5 isolates, seven OTUs (OUT 7, OUT 19, OUT 44, OUT 46, OUT 47, OUT 60 and OUT 76) each has 4 isolates. (Appendix 9). This indicates that these OTUs thrive in all culture conditions are probably stable and permanent microbial communities.

Moreover, 26 OTUs were common to all sites and represented by 104(52 %) isolates and 34 OTUs were common to all media represented by 115(57.75%) isolates. Regarding unique OTUs media, R2A had 27 unique OTUs and TSA had 23 unique OTUs. In the case of sites, the site I had 22unique OTUs while site2 had 20 unique OTUs. Therefore, variability in terms of media type was higher than sites. In which R2A has higher 27 unique OTUs in site one. This could imply that R2A media can improve the culturability of bacteria more than TSA (Table 2). This result indicated the use of different culture media and different sites increased bacteria cultivability as supported by the presence of unique isolates obtained on each medium and each site.

Table 2: Unique and common OTUs in the different culture and sites.

OTUs	R2Asite I	R2Asite II	TSA site I	TSA site II
Total	41	38	36	34
Unique	27	23	22	20
Common	26		34	

OTUs structure (diversity and abundance) showed a statistically significant difference between media and sites ($p<0.05$). Within media ($p=0.010$) and in sites ($p=0.02$) (Figure 5). A difference in media is large and more significant than sites. Low nutrient media R2A improves the culturability of bacteria and increases the abundance and diversity of bacteria. oligotrophic nature of the lake and it's less exposed to anthropogenic activities makes the lake supports diverse bacterial Taxas and responsible for variation in phylotype diversity and this idea is supported by many studies (Gilbert *et al*., 2012)

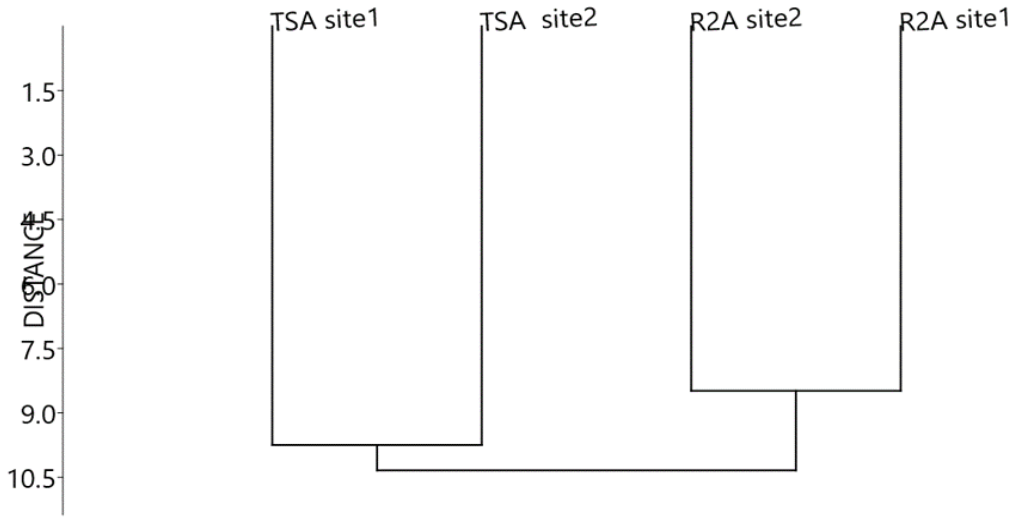

Figure 5: Dendrogram showing bacterial community clustering based on the different culture types.

4.4. Bacterial diversity indices

According to the results of Shannon-Weiner Index, R2A site one (H=3.652; Hmax= 3.652and D=0.028) was more diverse than TSA site one (H=3.503; Hmax= 0.9226and D=0.0328) and R2A site two (H=3.569, Hmax=0.9336and D=0.0304) was also diverse than TSA site two (H=3.437, Hmax=0.9145 and D=0.0352). As the analysis showed, low nutrient R2A media-generated higher diversity in each site than TSA rich media (Appendix 1). This result thus supports the fact that the type of media influences the diversity of bacteria.

4.5. Identified genera from water samples of Lake Zengena

All the 200 isolates from the water samples of Lake Zengena were identified to 9 genera and belongs to 4 Phyla. The dominant Phylum was *Proteobacteria t*hat consists of 5 genera, while 2 genera belong to Phylum *Actinobacteria*, 1 genus to phylum *Fermicute*s, and 1 genus to phylum *Bacteriodetes* (Appendix 8). Based on the results of this study phylum proteobacteria were the dominant (60%) phyla. This is not a surprise in that proteobacteria are dominant groups in all-natural environments (Tamaki *et al*., 2005). Thus, the results of this study revealed that the water of Lake Zengena had a varied group of bacteria genera.

The most dominant genera were *Pseudomonas* 48 (24%) and *Aeromonas* 33 (16.5%). This result was similar to the study reported by (Greenberger *et al*.,2015), conducted on the effect of different heterotrophic plate count methods on the estimation of the composition of the

cultivable microbial community. *Pseudomonas* and *Aeromonas* more frequently existed and abundant at 22 ◦C. confirmed by the OTU category significance test, which showed significant associations with 22 ◦C for the OTU's assigned to the *Aeromonas* ($p < 0.01$) and to *Pseudomonas* ($p < 0.05$) (Greenberger *et al*., 2015) which coincide to the lake temperature of zengena.

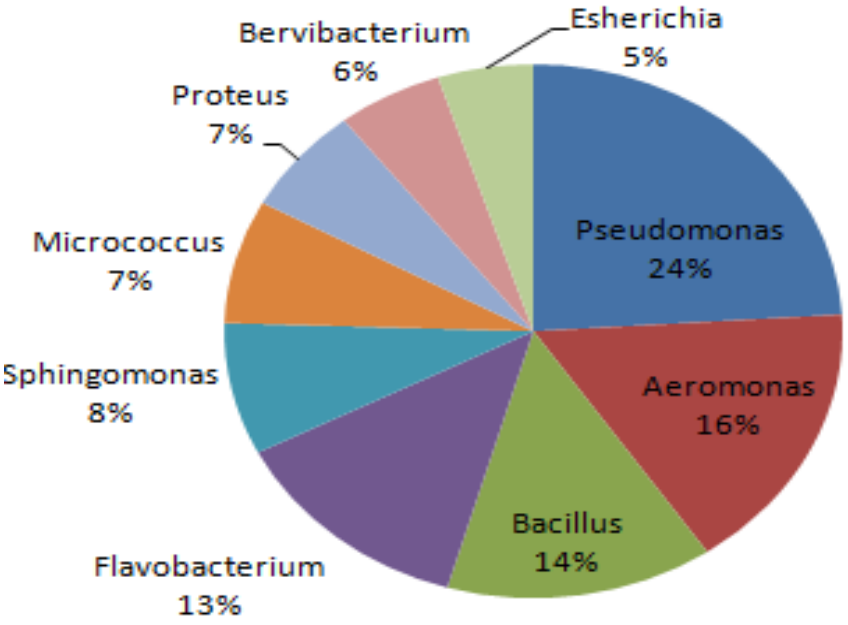

Figure 6: Relative percentage of each genus in the water samples of Lake Zengena.

4.6. Distribution of the identified genera among the different sites and media type

Seven genera were found in all sites and media types showing their stable presence in the lake water (Figure 7). Besides, most of these common genera were abundant. However, two genera, namely, *Sphignomonas* and *Bervibacterium* were not found in some media types. *Sphignomonas* was not found in TSA media in site two cultures and *Bervibacterium* was not found in site one TSA media. *Pseudomonas* and *Aeromonas* were found at the highest percentage at site two (26%) and (18%) respectively. *Flavobacterium* was highly improved and supported by R2A media than TSA; this study is supported by similar studies done on *Flavobacterium* isolated from a freshwater river (Lee and Jeon, 2018) and *Sphignomonas* were grown with the highest percentage (18% and 12%) respectively at R2A media (Figure 7). This indicates low nutrient media R2A supported fastidious and slow-growing genera more than TSA. this result was confirmed with the study result on a proposal for a method to estimate nutrient shock effects in bacteria by comparing the growth of *Sphignomonas* on R2A and TSA medium in which Sphignomonas grows more on in R2A than TSA medium (Azevedo *et al*., 2012).

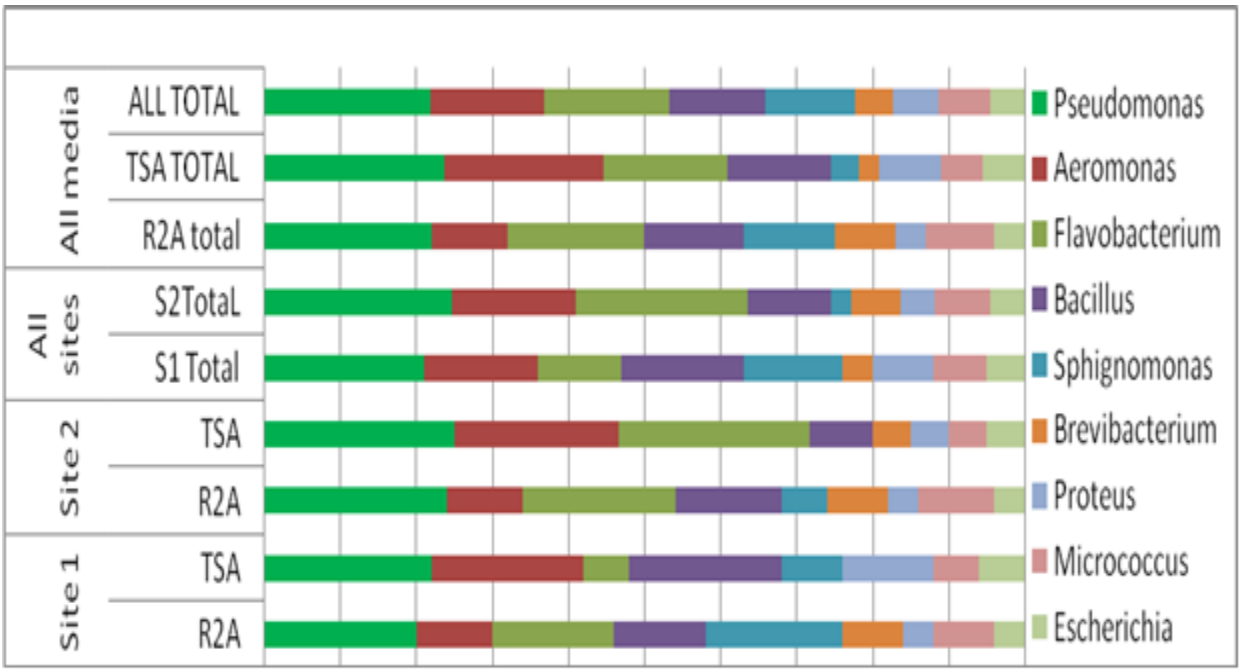

Figure 7: Relative abundance of the identified genera in the water sample of Lake Zengena at the different culture types.

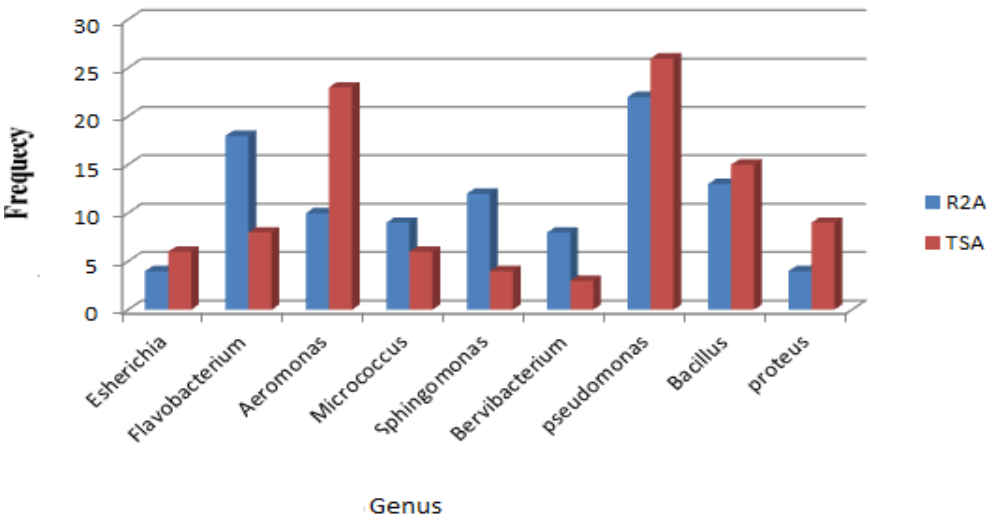

Figure 8: Relative abundance of each genus at media type

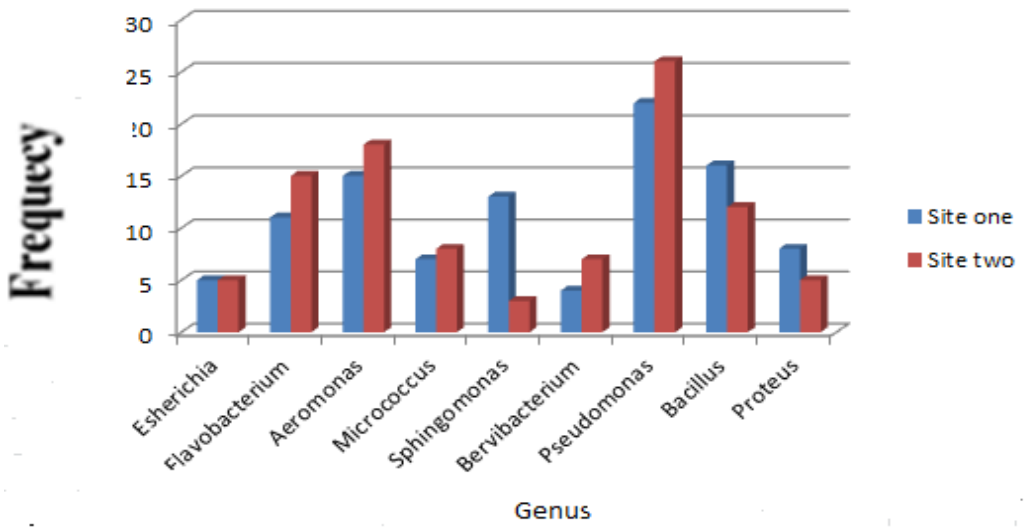

Figure 9: Relative abundance of each genus at sites.

In this study, most of the genera identified have naturally occurring bacteria, which indicates the lake is ecologically segregated and less exposed to anthropogenic activities, deposition of municipal wastes and fecal matters, *Aeromonas* and *Flavorbacterium* are common fish pathogens which are found naturally in freshwater (Newton *et al*., 2011) (Table 3).

Table 3: Taxonomic rank of nine identified genera from a water sample of LakeZengena

Phylum	*Class*	*Order*	*Family*	*Genus*
Proteobacteria	*Gamaproteobacteria*	*Pseodumoales*	*Pseodomonaliscea*	*Pseudomonas*
Proteobacteria	*Gamaproteobacteria*	*Aeromonadales*	*Aeromonadaceae*	*Aeromonas*
Firmicutes	*Bacilli*	*Basillales*	*Bacilliaceae*	*Bacillus*
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*
Proteobacteria	*Gamaproteobacteria*	*Enteralesobacteriales*	*Enterobacteriaceae*	*Proteus*
Actinobacteria	*Actinobacteria*	*Micrococcales*	*Brevibacteriaceae*	*Brevibacterium*
Actinobacteria	*Actinobacteria*	*Micrococcales*	*Micrococcaceae*	*Micrococcus*
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingomonas*
Proteobacteria	*Gamaproteobacteria*	*Enteralesobacteriales*	*Enterobacteriaceae*	*Escherichia*

5. CONCLUSION AND RECOMMENDATIONS

5.1. CONCLUSION

Lake Zengena is likely supporting diverse bacterial phonotypes. 76 OTUs were identified into nine genera and 4 Phyla, the dominant Phylum *Proobacteria* consists 5 genera, Phylum *Actinobacteria* 2 genera, phylum *Firmicute*s 1 and Plum *Bacteriodetes*1 genera. OTUs diversity differ significantly between media ($p=0.001$) and between sites ($p=0.002$). As result of this study, it can be concluded that the lake water was dominant by naturally occurring bacteria than enteric group. This might be due to the hydrology, geological and absence of mixing of the water column from rivers, agricultural and municipal effluents from the catchments to the lake and the lake to be more of Oligotrophic and favorable to maintain diverse bacterial species. In addition to this, the lake is ecologically segregated and less exposed to anthropogenic activities. Result of this the natural diversity were not disturbed and their stability is maintained. These results in existence and concentration of naturally occurring bacterial in the lake.

The diversity indices showed differences in Media and sites. Species diversity and richness higher in R2A media than TSA and Site one was higher in species diversity than site two. Low nutrient (R2A) media supports more species diversity. Oligotrophic nature of lake and other physicochemical factors in the study sites might be responsible for variation in phylotye diversity.

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5.2. RECOMMENDATIONS

Based on the results of this study, the following points are recommended

- The identified genera need to be further characterized for their ecological role and identified to species level.
- Isolates further characterized and tested in activities of enzyme and antibiotic production and industrial applications.
- Isolates further sequenced and molecularly characterized.

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APPENDICES\

Appendix I: Composition of Tryptic soy agar (TSA)

Compound	g/Liter
pancreatic digest of casein	15 g
papanic digest of soy bean meal	5g
sodium chloride	5g
Agar	15 g
pH at 25oC	7.3 ± 0.2

Appendix II: Composition of low nutrient (R2A) Agar medium

Compound	g/Liter
Yeast extracts	0.5
Peptone	0.5
Casamino acid	0.5
Dextrose	0.5
Soluble starch	0.5
Sodum puruvate	0.3
Agar	15
Dipotassium phosphate	0.3
Magnesium sulphate	0.05
pH at 25oC	7±0.2

Appendix III: OTUs diversity indices

	R2A site1	R2A site2	TSA site1	TSA site2
Taxa_S	41	38	36	34
Individuals	50	50	50	50
Dominance_D	0.028	0.0304	0.0328	0.0352
Simpson_1-D	0.972	0.9696	0.9672	0.9648
Shannon_H	3.652	3.569	3.503	3.437
Evenness_e^H/S	0.9403	0.9336	0.9226	0.9145

Appendix IV: Distribution of each genus with in media and sites in each culture type

Genera	R2Asite1	R2Asite2	TSAsite 1	TSAsite 2	Total	%
Pseudomonas	10	12	11	15	48	24 %
Flavobacterium	8	10	3	5	26	13 %
Aeromonas	5	5	10	13	33	16.5 %
Bacillus	6	7	10	5	28	14 %
Sphingomonas	9	3	4	0	16	8 %
Bervibacterium	4	4	0	3	11	5 %
Proteus	2	2	6	3	13	6.5 %
Micrococcus	4	5	3	3	15	7.5 %
Esherichia	2	2	3	3	10	5 %
Total	50	50	50	50	200	

Appendix V: Percentage occurrence of each genus in different media type

Genera	R2A	%	TSA	%	Grand total	%
Esherichia	4	4%	6	6%	10	5%
Flavobacterium	18	18%	8	8%	26	13%
Aeromonas	10	10%	23	23%	33	16.5%
Micrococcus	9	9%	6	6%	15	7.5%
Sphingomonas	12	12%	4	4%	16	8%
Bervibacterium	8	8%	3	3%	11	5.5%
pseudomonas	22	22%	26	26%	48	24%
Bacillus	13	13%	15	15%	28	14%
proteus	4	4%	9	9%	13	6.5%
Total	100		100		50	

Appendix VI: Percentage occurrence of each genus at different sites

Genera	Site one	%	Site two	%	Grand total	%
Esherichia	5	5%	5	5%	10	5%
Flavobacterium	11	11%	15	15%	26	13%
Aeromonas	15	15%	18	18%	33	16.5%
Micrococcus	7	7%	8	8%	15	7.5%
Sphingomonas	13	13%	3	3%	16	8%
Bervibacterium	4	4%	7	7%	11	5.5%
Pseudomonas	22	22%	26	26%	48	24%
Bacillus	16	16%	12	12%	28	14%
Proteus	8	8%	5	5%	13	6.5%
Total	100		100		200	

Appendix VII: ANOVA Table

1: Within sites

Descriptives

Genera

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
site 1	100	3.8300	2.38707	.23871	-3.3564	4.3036	1.00	9.00
site2	100	3.5800	2.51533	.25153	-3.0809	4.0791	1.00	9.00
Total	200	3.7050	2.44907	.17318	-3.3635	4.0465	1.00	9.00

ANOVA

Genera

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.125	1	3.125	.520	0.02
Within Groups	1190.470	198	6.012		
Total	1193.595	199			

2: Within media

Descriptives

Genera

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
R2A	100	3.9500	2.38419	.23842	-3.4769	4.4231	1.00	9.00
TSA	100	3.4600	2.50018	.25002	-2.9639	3.9561	1.00	9.00
Total	200	3.7050	2.44907	.17318	-3.3635	4.0465	1.00	9.00

ANOVA

Genera

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12.005	1	12.005	2.012	0.01
Within Groups	1181.590	198	5.968		
Total	1193.595	199			

Appendix VIII: Individual isolates with their corresponding OTUs based on similar morphological and biochemical characteristics.

OTUs	Isolates
OUT 1	W S1R2A 37
OUT 2	WS1TSA83, WS1TSA 79, WS2TSA151.WS2TSA157,
OUT3	WS2R2A108
OUT4	WS1R2A3, WS1TSA53, WS1TSA55,WS1TSA93
OUT5	WS1R2A7, WS1TSA88, WS2TSA184
OUT 6	WS1TSA 94, WS2TSA152, WS2TSA191
OUT 7	WS1TSA 99, WS2R2A147, WS2TSA170
OUT 8	WS1R2A 43, WS1TSA85,WS2R2A117
OUT 9	WS2R2A109
OUT 10	WS1TSA90, WS2R2A125
OUT11	WS1R2A1,WS2R2A122
OUT12	WS1R2A16,WS1TSA69, WS2R2A126, WS2TSA173,WS2TSA200, WS1TSA154
OUT13	WS1R2A31,WS1TSA62, WS1TSA71, WS2R2A145, WS2TSA162
OUT14	WS1TSA87, WS2TSA163, WS2TSA164
OUT15	WS1R2A12,WS1TSA78, WS1TSA81, WS2R2A119, WS2R2A132, WS2TSA167
OUT16	WS2TSA177, WS2TSA186
OUT17	WS1R2A 30, WS1R2A39
OUT18	WS1R2A45
OUT19	WS1R2A25, WS1TSA52,WS2R2A123, WS2R2A139
OUT20	WS1R2A5,WS1TSA64, WS1TSA97, WS2R2A121,WS2TSA 197
OUT21	WS1TSA63, WS1TSA98,.WS2TSA192
OUT22	WS1R2A6, WS2R2A115
OUT23	WS2R2A106,WS2TSA172,WS2TSA183

OUT24	WS1R2A46, WS1TSA92, WS2R2A143
OUT 25	WS1R2A18, WS2TSA148
OUT26	WS2R2A130
OUT27	WS1R2A19, WS1R2A48
OUT28	WS1R2A40, WS1TSA77,WS2TSA199
OUT29	WS2R2A101, WS2R2A146
OUT30	WS1R2A27, WS1R2A36
OUT31	WS1R2A21, W1STSA51, WS1R2A47, WS1TSA96, WS2R2A140
OUT32	WS2R2A113, WS2TSA189
OUT33	WS1R2A28, WS1TSA61, WS1TSA76,WS2TSA165, WS2TSA195
OUT34	WS1R2A41, WS2R2A124
OUT35	WS2TSA107,WS2TSA111
OUT36	WS1TSA57
OUT37	, WS2TSA166
OUT38	WS1R2A2,WS1TSA56, WS1TSA59, WS2R2A116, WS2TSA194
OUT39	WS1R2A4, WS2R2A135, WS2R2A150
OUT40	WS1R2A8, WS1R2A24, WS1TSA82, WS2TSA120,WS2TSA190
OUT41	WS1R2A20
OUT42	WS1R2A42, WS2TSA153
OUT43	WS1TSA65, WS1TSA91
OUT44	WS1R2A34,WS1TSA75, WS2TSA175,WS2R2A129
OUT45	WS1R2A26
OUT46	WS1R2A29, WS1TSA72, WS1TSA156,WS2TSA193
OUT47	WS1R2A32,WS1R2A128, WS2TSA178, WS2TSA198
OUT48	WS1R2A155
OUT49	WS2R2A112, WS2R2A133
OUT50	WS1R2A86
OUT51	WS1R2A73,WS2TSA196

OUT52	WS1R2A50,WS2R2A103, WS2R2A158,WS2TSA185,WS2R2A188
OUT53	WS2TSA160, WS2TSA174
OUT54	WS1TSA66
OUT55	WS1TSA68, WS1TSA64, WS2TSA161
OUT56	WS1R2A17, WS1R2A44
OUT57	WS1TSA74, WS2TSA169,WS2TSA180
OUT58	WS1R2A100
OUT59	WS1R2A10, WS2R2A102,WS2R2A141
OUT 60	WS1R2A9, WS1TSA58, WS2R2A134, WS2TSA168
OUT61	WS2TSA187
OUT62	WS1R2A49, WS2R2A104
OUT63	WS1TSA60, WS1TSA67,WS1TSA95
OUT64	WS1R2A33, WS2TSA127, WS2R2A136, WS2R2A142
OUT65	WS1TSA80
OUT66	WS2R2A105, WS2R2A110
OUT67	WS1R2A11, WS1R2A23, WS1R2A35
OUT68	WS1R2A38, WS2R2A114,WS2R2A144, WS2TSA159, WS2TSA179, WS2TSA181
OUT69	WS2TSA176
OUT70	WS1TSA70
OUT71	WS1TSA54, WS2TSA182
OUT72	WS1TSA89
OUT73	WS1R2A14, WS2R2A131, WS2R2A149
OUT74	WS1R2A22, WS2R2A137
OUT 75	WS2R2A138
OTU76	WS1R2A13, WS1R2A15, WS2R2A118, WS2TSA171

Appendix IX: The 9 identified genera based on morphological and biochemical test.

Shape	Size	Margin	Color	Opacity	Elevation	Surface	Texture	Gs	C.shpe	Ca	Edo	Mo	GL	Lac	Su	Gas	H_2S	Ci	In	MR	VP	MnF	Genus
Circular	small	undulte	Yellow	Transparent	Raised	smooth	Moist	-	Shortrod	+		+	+	-	-	-	+		-	-	-		Pseudomonas
Circular	Medium	Entire	Yellow	Transparent	Raised	smooth	Moist	-	palisade	+		+	-	+	+	-	+	-	-	-	-		Flavobacterium
Circular	Medium	Entire	White	Transparent	Convex	smooth	Moist	-	streptobacillus	+		+	+	-	+	+	-		-	+	+		proteus
Circular	Small	Entire	Yellow	Transparent	Convex	smooth	Moist	+	streptobacillus	+	+	+	+	-	-	+	+						Bacillus
Irregular	Medium	Entire	Yellow	Opaque	Convex	smooth	Moist	+	Shortrod	+	-	-	+	-	+	-	+						Bervibacterium
Irregular	small	Entire	Yellow	Opaque	Convex	smooth	Moist	+	staphylococci	+		-	+	-	-	-	+					-	Micrococcus
Circular	Medium	Entire	White	Transparent	Raised	smooth	Moist	-	Shortrod	+		+	+	-	-	+	+		-	+	-		Aeromonas
Circular	small	Entire	Yellow	Transparent	Convex	Rough	Dry	-	Shortrod	+		+	-	+	+	+	+	-	-	-	-		Sphingomonas
Circular	Medium	Entire	White	Transparent	Raised	smooth	Moist	-	Shortrod	+		+	+	+	-	+	+	-	-	+	-		Escherichia

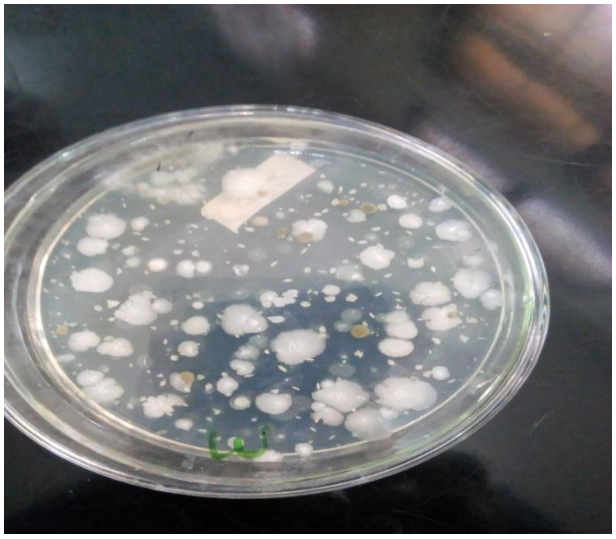
Where GS: Gram Stain; Mo: Motility; I: indole, Ca: catalase, MR: methyl red, VP: Voges Proskauer's, Mnf: mannitol fermentation ,Edo: endospore formation, C: Citrate utilization, H_2S: Hydrogen Sulphide production, L: Lactose, Gl: giucose fermentation, Su; sucrose fermentation, Gs: gas production.

Appendix X: Refreshing culture for biochemical test

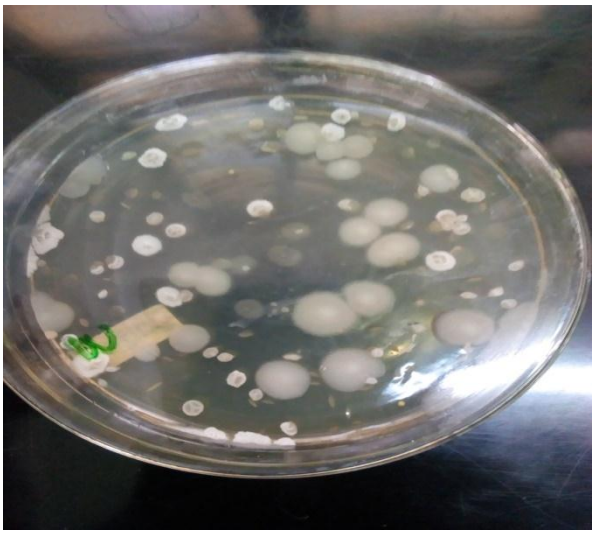
Appendix XI: morphological observation by microscope.


B-150
OPTIKA

Appendix XII: Isolation on solid media



A: R2A agar media



B: TSA agar media