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MOLECULAR CHARACTERIZATION OF DENITRIFYING BACTERIA AND THEIR POTENTIAL IN REDUCTION OF NITROGENOUS COMPOUNDS IN LAKE VICTORIA, KENYA

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DECLARATION

This thesis is my original work and has not been presented elsewhere for a degree or any other award.

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DEDICATION

I dedicate this work to my beloved family for the massive support, love and great inspiration during my research. Without your support and encouragement this journey would have been tough and unachievable.

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ACRONYMS AND ABBREVIATIONS

°C	Degree Celsius
OTUs	Operational Taxonomic Units
AOA	Ammonia-oxidizing Archaea
CFU	Colony forming unit
CO_2	Carbon dioxide
HNO_3	Nitric acid
LB	Luria-Bertani
N	Nitrogen
N_2O	Nitrous Oxide
NH_3	Ammonia
NH_4	Ammonium
NO	Nitric Oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NO_x	Nitrogen oxides
OD	Optical density
P	Phosphorus
PO_4^{3-}	Phosphate
Mg	Milligram
WWTPs	Waste Water Treatment Plants
SND	Simultaneous Nitrification and Denitrification
Long	Longitude
Lat	Latitude

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ABSTRACT

Nutrient loading with nitrogen and phosphorus in receiving waters is an increasing concern worldwide. In Lake Victoria basin, the substantial inflow of nutrients has led to extensive eutrophication which has resulted in the reiterative proliferation of invasive species of aquatic weeds like water hyacinth and cyanobacteria in the Lake Victoria basin hence causing mass mortality of fish and health risks. Nowadays, anthropogenic activities such as crop production, sewage discharge, fossil fuel combustion and fertilizer use have substantial intensified, therefore increasing the amount of nitrogen entering into terrestrial ecosystems. Heterotrophic nitrification and aerobic denitrification have been proposed as alternate strategies in nitrogen reduction in nitrogen-rich ecosystems. In this study, we examined the diversity of bacteria involved in nitrogen cycling and their potential application in bioremediation in Lake Victoria. Eight water samples were collected in triplicate using 500 ml sterile bottles and taken to SGS Kenya Limited Laboratory Services for physiochemical analysis. Total bacterial diversity in the collected samples was assessed using universal primers. Purified DNA (25 µl) was stabilized using DNAstable® (Biomatrica), air-dried, followed by Illumina sequencing. A culture dependent approach was used to recover aerobic denitrifiers using a medium with $(NH_4)_2SO_4$ as the main N source in Bromothymol blue medium (BTB). Serial dilution was done and spread plated onto the BTB media followed by incubation. Single isolates were selected from the plates and sub-cultured in a similar media to obtain pure cultures. Screening for Nitrogen removal was done by inoculating single colony into screening medium containing $(NH_4)_2SO_4$ as the main N source. Selected pure cultures were presumptively characterized based on molecular tests. Molecular characterization was achieved by extracting the DNA of positive isolate, amplifying 16S rRNA gene using universal primers (515F/806R), and sequencing. In order to determine how much nitrogen (N) was removed from effluent samples, 50 ml of sterilized wastewater was inoculated with a single colony and incubate at 30 °C for 120 hours and 150 rpm. Nitrogen removal by isolates was quantified using a spectrophotometer. Low absorbance indicated fewer molecules available to interact with the light, and vice versa. Physiochemical analysis showed there were elevated levels of total nitrogen and ammonia in both treated and untreated WWTPs as compared to lake and rivers samples. The sequence reads were grouped into 1,763 operational taxonomic units (OTUs). There OTUs were affiliated to 26 bacterial phyla with Proteobacteria being the most dominant phylum. Microbial diversity increased from WWTPs, rivers to lake. Isolated positive for nitrogen removal were recovered from the lake and WWTPs ecosystems and none from the river samples. The isolates were affiliated to genera *Pseudomonas*, *Klebsiella*, and *Enterobacter in* the phylum Proteobacteria. *Klebsiella quasivariicola* showed the highest utilization of nitrate and ammonium from the basal media as indicated by low absorbance values of 95.32% and 93.18%. The findings from this study show that bacteria play a very important role in removal of nitrogenous waste from waste water and some of them can be used in bioremediation efforts.

CHAPTER ONE

INTRODUCTION

1.1 Background of study

The global nitrogen cycle is crucial to the Earth's biogeochemistry (Fowler *et al*., 2013). Huge nitrogen (N) flows in terrestrial and aquatic environments are caused by biological nitrogen fixation, anthropogenic impacts, and a relatively small amount by lightning (Sutton *et al*., 2013). Microorganisms then convert the fixed nitrogen into amino acids as well as oxidized chemicals (Fowler *et al*., 2015). Products of chemical or biological processes can eventually be converted by microbes from sediments, soils, freshwater, and marine waters into molecular nitrogen and released back into the atmosphere (Galloway *et al*., 2004).

The current N biogeochemical cycle has been strongly accelerated through agricultural systems, and the trend is estimated to intensify further in the forthcoming decade (Bodirsky *et al*., 2014; Bouwman *et al*. 2017). N-based fertilizer production reached 150 million metric tonnes as of 2013 from 10 million metric tonnes in 1950 (Heffer and Prudhomme, 2016). Globally, humans introduce 120 million tons of N per year through synthetic fertilizer to supplement crop, and 50 million to 70 million tons of N is biologically fixed through irrigation (Sutton *et al*., 2013). However, only 28 million tons of N is available for food human consumption, whereas a huge portion of N is therefore lost to the ecosystem through atmospheric volatilization and direct runoff (Galloway *et al*., 2008).

Nitrogen and Phosphorous fertilizers have been useful in the improvement of global food production as well as other merits over the past several decades (Leach *et al*., 2012). However, widespread N cycle modification, along with surplus as well as cutbacks, has had a detrimental impact on the ecosystem as well as overall human health (Sutton *et al*., 2013). In particular, N has impacted multiple changes to the environment since it flows via the waters, soils, as well as atmosphere (Erisman *et al*., 2013). Release of excess nitrogen to the ecosystem impacts both freshwater and marine habitats through eutrophication (Juma *et al*., 2014). Nitrogen enrichment promotes the production of algal blooms and water hyacinth, which has a negative

impact on fisheries and human health (Glibert & Burford, 2017; Villamagna & Murphy, 2010). A significant percentage of people on the globe reside in metropolitan areas, and it has been projected that by 2050, approximately two-thirds of all people would move to urban areas, mostly along the coastlines (United Nations, 2014). Eutrophication occurs in many freshwater bodies as a result of rapid urbanization and the excess nitrogen discharged through agricultural, fuel combustion, municipal sewage, and other waste-water runoffs (Juma *et al*., 2014). For instance, Lake Victoria is a freshwater ecosystem affected by high nitrogen input from anthropogenic activities such as municipal sewage around Kisumu city and the surrounding agricultural farmlands (Lung'ayia *et al*., 2001).

Eutrophication destroys water quality by enhancing massive growth of water hyacinth, toxic algal blooms resulting in low oxygen levels has detrimental ramifications on the wetlands (Le Moal *et al*., 2019). Conventional nitrogen removal from wastewater can be achieved using either physicochemical or biological approaches. Physicochemical approaches are costly as well as inappropriate for in-situ usage. For this reason, biological treatment techniques have been suggested as an option considering that they are cheap and environmentally safe techniques of nitrogenous component reduction. (Medhi & Thakur, 2018).

Conventional denitrification systems typically aim to completely convert ammonia to dinitrogen gas depending on the denitrification and nitrification functions executed by two different microbial groups, known as heterotrophs as well as autotrophs, in two reactors, each operating within specific operational and also physiological conditions (Zhao *et al*. 2020). The variations in process parameters, like physiological performance to changes in oxygen concentration, need for various carbon sources, variation in treatment time and reactor configurations, among others, call for additional designing, space, as well as operational complexity, which raises the cost of the overall process (Rajta *et al*. 2020).

Unlike heterotrophic denitrifying bacteria, nitrite-oxidizing bacteria (NOB) as well as chemolithoautotrophic ammonia-oxidizing bacteria (AOB) execute the rate-limiting phase of nitrification in nitrogen cycling bacterial communities. This is due to the high sensitivity of AOB and NOB to parameters such dissolved oxygen (DO) concentration, C: N ratio, salinity, carbon supply, pH, temperature, ammonium and

nitrite concentrations (Wang *et al*. 2017). Due to these microbes notably slower growth rate, their inability to battle for oxygen as well as available space during the wastewater treatment process with a heavy loads of nitrogen and carbon results in a reduction in the efficacy of ammonium and nitrite oxidation. (Wang *et al*. 2017). High salt concentration (over 10 g NaCl) during treatment is also a factor that substantially inhibits the autotrophic AOB and NOB, making it difficult to sustain their rates in the aerobic reactor (Gonzalez-Silva *et al*. 2016). The above issues make conventional denitrification process more complicated generally and restrict its effectiveness when dominant heterotrophic microbes are available (Wang *et al*., 2020).

Simultaneous nitrification and denitrification (SND) process uses nitrate and oxygen as electron acceptors, and it's known to be more effective and efficient in removal of nitrogen as well as higher rate of carbon degradation. In SND, the level of oxygen is retained in a single reactor under constant controlled environments to complete the SND process (Dutta and Sarkar 2015). However, low levels of carbon may hinder nitrogen removal efficiency (Zhang *et al*., 2019). Biostimulation of aerobic denitrifiers with limiting nutrients such as carbon can strongly improve the bioremediation process (Adams *et al*., 2015).

Aerobic denitrifying bacteria, such as *Acinetobacter sp*. HA2 (Yao *et al*., 2013), *Vibrio diabolicus* SF16 (Duan *et al*., 2015), and *Paracoccus denitrificans* ISTOD1 (Medhi & Thakur, 2018), have been successfully cultured from contaminated ecosystems. However, very few studies have suggested the promising application of aerobic denitrifying bacteria in nitrate removal during wastewater treatment. Thus, the primary goal of this research was to evaluate the microbial population as well as relative abundance in the WWTPs and rivers that drain into Lake Victoria, Kenya. The recovered isolates were evaluated for their potential application in removal of nitrogenous compounds from wastewater.

1.2 Statement of the problem

Despite several research conducted during the last five decades, eutrophication has remained a major global concern worldwide (Maure *et al*., 2021). The municipal wastewater from Kisumu still holds a significant amount of total nitrogen, which is

directly deposited into Lake Victoria owing to the limitations associated with the conventional sewage treatment system. The lake's freshwater quality is impacted by eutrophication when excessive nitrogen gets released into the surrounding waters. Eutrophication has led to extensive proliferation of harmful algal blooms that have introduced hypoxic zones, which could be hazardous to both human health as well as aquatic life.

Over time, Lake Victoria's water quality has deteriorated due to substantial release of nutrient and this possess a major threat to the ecological functions offered by the lake ecosystem (Villamagna & Murphy, 2010). Several studies done elsewhere have suggested how aerobic denitrifying bacteria can be employed to remove nitrogenous substances from wastewaters (Hao *et al*., 2022). Therefore, we proposed to study the microbial relative abundance and use selected aerobic denitrifiers as a bioremediation strategy for removal of nitrogenous compounds from wastewater being released into the lake.

1.3 Justification

One of the primary goals of treating wastewater is to remove nutrients from contaminated water, particularly nitrogen and phosphorus, as they are the cause of eutrophication. The available physicochemical techniques for nitrogenous compounds removal such as adsorption, ion exchange, catalysis, and electrodialysis are expensive, unsuitable for *in-situ* applications, and forms by-products after treatment. Biological denitrification is an alternative process for nitrogenous compounds removal since it’s efficient, eco-friendly, and cost effective (Medhi & Thakur, 2018).The recent discovery of simultaneous nitrification and denitrification has been proposed as an alternative technique in the reduction of nitrogen in a nitrogen-rich ecosystem. With the recent discovery of heterotrophic nitrification and aerobic denitrification, interest in unconventional nitrogen removal technique has surged. The heterotrophic nitrifiers and aerobic denitrifiers can simultaneously exploit nitrate and oxygen as electron acceptors, therefore overcoming the limitations of the traditional process of alternating aerobic and anaerobic treatment (Zhang *et al*., 2019). Aerobic denitrifiers have the potential to transform NH4-N to atmospheric nitrogen gas aerobically through simultaneous nitrification and denitrification process (Khanichaidecha *et al*., 2019). Understanding the significance and

diversity of heterotrophic nitrifiers and aerobic denitrifiers in managing nitrogen pollution upon different environmental settings can assist in establishing an empirical knowledge for the SND process to deal with the issues encountered by conventional techniques of anaerobic heterotrophic denitrification and aerobic autotrophic nitrification (Gupta *et al*., 2022). This study explored the total microbial diversity in waste water as well as fresh water using a molecular approach as well as isolation of pure cultures capable of nitrogen removal fromnitrogen rich environments.

1.4 Hypothesis

Aerobic denitrifying bacteria can reduce nitrogenous compounds in wastewater to acceptable levels.

1.5 Objective

1.5.1 General objective

To assess the potential of aerobic denitrifying bacteria in the removal of nitrogenous compounds from wastewater entering Lake Victoria.

1.5.2 Specific objectives

1. To assess the bacterial diversity within wastewater discharged into Lake Victoria with a view of identifying the proportion of aerobic denitrifiers.
2. To isolate and characterize aerobic denitrifying bacteria from the fresh water and wastewater using cultural and molecular approaches.
3. To evaluate the nitrogenous compounds removal potential of different aerobic denitrifying bacteria.

1.6 Significance of the study

Pollution within Lake Victoria has been increasing at an alarming rate. The lake is severely impacted by nutrient loading from agricultural practices, municipal sewage, and other wastewater runoffs,

Understanding the role of microorganisms in waste degradation can help develop intervention measures towards pollution management. The treatment of contaminated water using the heterotrophic nitrification and aerobic denitrification processes can be a reliable and cost-effective approach. This will aid in lowering nitrogen loading

in Lake Victoria, thereby enhancing the lake water quality, minimizing fish mortality and eventual supporting the economy and the welfare of the Lake Victoria region. The findings of this study will serves as the basis for bioremediation-based using highly efficient pollutant-degrading bacteria (Lv *et al*., 2017).

CHAPTER TWO

LITERATURE REVIEW

2.1 Water pollution

All natural resources possess water as a basic component. It is fundamental to the existence of all lifeforms, especially humans, as well as for economic development as well as food production. Many countries throughout the world are currently experiencing a severe water scarcity, and the majority of industrial operations relies on water, and about forty percent of the food produced worldwide also depend on irrigation. Anthropogenic activities have a significant impact on water quality, which is decreasing as a result of increased industrial output, environmental degradation, urbanization, and population growth, among other concerns. Pollution of water systems is a global concern that has greatly affected both developed and developing nations, compromising economic activities and people's health worldwide. The percentage of freshwater reservoirs is about 0.5% all over the world with a volume of 2.84x105 Km^3. Waterbodies such as rives possesses an insignificant quantity (0.1%) of the land surface). Only a portion of 0.01% of the waters in river channels occur in the earth. Even though there are minimal amounts, running waters are of great importance (Wetzel, 2001).

As a result of the recent rapid growth in both the population as well as the industrial sector, water usage has increased escalated significantly (Ramakrishnaiah *et al*., 2009). In various regions of the world, anthropogenic activities including agriculture practices, population increase, and widespread urbanization have led to a decline in quality of water (Khatri *et al.,* 2015). The pollution in the rivers has become a very crucial challenge because of insufficient surface water quality protection strategies as well as sanitation. Rivers, stream and lagoons have become sinks for disposing wastes.

Pollutants are frequently released into receiving waters with minimal or no regard to their assimilative potential (Ladu *et al*., 2018). Critical challenges with the potential dilution of rivers and lagoons in metropolitan areas include the discharge of raw waste, sewage, as well as oil spills. The natural purification process of contaminated waters takes more time, and huge amount of pollutants may require a long duration

before an acceptable levels of purification is reached (McGauhey, 1968). Water pollution risks food production, human health as well as the environment conditions (Ahmed & Rahman, 2000). Scientists and the government are concentrating more of their studies on the problem of contamination in water systems. Due to the detrimental impacts of water contamination and the limited availability of water resources, safeguarding water quality demands prompt attention.

2.2 Sources of water pollution

Water pollution remains one of the critical environmental concern, and it's among the most vulnerable natural resources in terms of pollution. Generally, there are three major causes of water pollution: Surface runoff from crop production practices such as fertilizer, manures, insecticides and pesticides use. Sewage released directly to rivers. Release of untreated industrial effluents into rivers. All these pollutants makes water from rivers unsafe for bathing and drinking as well. A study reported approximately 1500 pollutants which are available in freshwater environments and the list of the pollutants includes the following: organic toxic pollutants (phenols, formaldehyde), nutrients (nitrates, phosphates) heat, metals (mercury, lead, zinc, cadmium), residential wastewater comprising pathogenic microorganisms, metals, minerals, detergents, farm manure, polychlorinated biphenyls and radionuclides, acids, anions and alkalis such as sulphide, cyanide, sulphite, oil and oil dispersants as well as other substances (Tripathi *et al*., 1990).

Nowadays, several factors have been applied to study about water pollution. Nitrates and Silicon pollution in freshwater was captured by House *et al*. (2001). Biological features in terms of physiochemical parameters in ponds was studies by Dwivedi (2017). Chen and Twillery (1999) explained about changes that occurs in biology of polluted water. Water reservoirs; oceans, rivers, and are receiving large quantities of domestic sewage, industries wastes and agricultural runoff. These pollutants have been marked as the major water pollution sources in various studies. Similar studies investigated the effects of sewage on the quality of rivers (Ray and David, 1966; Singh and Bhowmik, 1985). The chemistry of municipal runoff water was reported by Lee and Bang (2000). A study was done on heavy metals present in sewage sludge (Oake, 1985). Impacts of sewage release in water bodies was studied by Cooke (1994). The biology of sewage was reported by Sutton and Ornes (1977).

Agriculture is recognized as an essential source of water pollution. A study detected pesticides and herbicides pollutants in river water (Blanchard and Lerch, 2000; (Galiulin *et al*., 2001). The studies above indicated the presence of large quantity of heavy metals and pesticides in milk, fruits, grains and vegetables. These pollutants accumulated directly or indirectly through biomagnification. Studies also shows that Nitrogen and Phosphorous fertilizers have been useful in the improvement of food production globally as well as other merits over the last century (Leach *et al*., 2012). However, widespread modification to the N cycle, with surpluses as well as deficits, have negatively impacted the ecosystem and human health at large (Sutton *et al*., 2013). Other agricultural wastes such as manures from cows, pigs, as well as chicken are considered to be water contaminants. Since animal feces has the risk of contaminating both surface and groundwater, it is a pollutant that both the general public and regulatory authorities are becoming more concerned about (Gerba & Pepper, 2009).

Industries contribute huge amounts of contaminants which ultimately finds its way to freshwater ecosystem. The discharge from industries contains harmful substances which puts the aquatic ecosystem under great risk. Several studies have reported channels in which industries contribute to water pollution. A study was done on the effects of pharmaceutical industries to the microbial population (Ajmal, 1980). Azad *et al*. (1982) studied industrial units which were releasing heavy metals to rivers. Another study also detected some trace elements in river water (Paul and Pillai, 1983). Experts and the government are devoting more of their attention on the problem of water contamination. Protecting quality of the water therefore needs quick attention due to the negative effects caused by water pollution as well as scarcity of water resources.

2.3 Components of wastewater

Sewage, commercial and industrial waste, agricultural leftovers, and other sources are only a few of the sources of wastewater, which is made up of a vast array of harmful compounds. These compounds can be distinguished based on their physical characteristics, chemical composition, and multiplicity of microbes. (Ritter *et al*., 2002). Typically, effluent is produced by normal biological functions, or it can be defined as any water that has been contaminated by anthropogenic activities

(Rajasekhar *et al*., 2018). Agricultural waste, industrial waste, commercial waste, and home wastewater are the primary sources of effluents (Ahmad *et al*., 2016). Large water bodies get filthy as a result of the significant amount of polluted and contaminated water that is produced and flows into them. Nearly all significant sources require high-quality water, especially industries, however in exchange, vast quantities of filthy water are produced and discharged into huge waterways, making them contaminated. (Jassby *et al*., 2018; Deshpande *et al*., 2020).

The complex matrix that makes up wastewater consists of 99.9% water, particulate matter with a chemical oxygen demand of 250–1000 mg/L, a variety of microorganisms (up to 109 number/m), organic waste (such as faeces, toilet paper, and food waste), dissolved biodegradable organics (such as proteins, carbohydrates, and lipids), inorganic solids (such as sediment, soil, salts, and metals), and 0.1% suspended solids (350–1200 mg/L), nutrients as well as micro-pollutants (Jain *et al*., 2021).

Microbial population

Wastewater is an ideal environment for both infectious and non-infectious microbes. Pathogens include viruses, protozoa, parasitic worms, as well as their eggs are all enteric organisms. (Abdel-Raouf *et al*., 2012). The bulk of human pathogens found in wastewater come from feces, which make up a significant portion of domestic sewage (Symonds & Breitbart, 2014). Pathogenic microbes can also be found in industrial waste from the food industry, especially waste from animal processing.

Heavy Metals

The ecology has recently been exposed to a sizable volume of wastewater containing heavy metals (O'Connell *et al*., 2008). Toxic heavy metals like copper, chromium, cadmium, nickel, and zinc are mostly introduced into wastewater by industrial wastes (Barakat, 2011). Through the assistance of the food supply chain carcinogenic and teratogenic heavy metals may pose critical health risks by accumulating in the body systems (Lee *et al*., 2014). Thus, it is essential to recover wastewater that has been contaminated with heavy metals.

Nutrient content:

Excessive amounts of certain chemical nutrients, including nitrogen and phosphorus, are highly toxic to fish, people's health, and other lifeforms. They may also encourage eutrophication in receiving streams, which may lead to massive growth of algal blooms that pose significant risks to both people's health and environment as well (Templeton & Butler, 2011). Wastewater left untreated will deteriorate and turn toxic, polluting and endangering the environment. Furthermore, urban effluents have become a major nutrient source that can be directly reclaimed or provide additional benefits if water can be reused again for crop production (Greenway, 2005).

DO, BOD and COD

The total concentration of oxygen dissolved in a litre of water is referred to as dissolved oxygen. The biochemical oxygen demand is the quantity of dissolved oxygen required by aerobic bacteria to break down organic materials. Chemical Oxygen Demand is the amount of dissolved oxygen that is needed to oxidize chemical organic materials. Elevated biochemical oxygen demand may reduce the concentration of dissolved oxygen, resulting in an anoxic condition that may have a substantial impact on microbial abundance in WWTPs (Xu *et al*., 2019).

pH

pH is the negative log of H+ concentration available in a sample. Organisms requires a specific pH for them to thrive well in an ecosystem. pH affects the activity of enzymes, and this may affect the elemental mobilization indirectly. A study investigated the shifting of phytoplanktonic composition with respect to pH lowering (Berge *et al*., 2010)

Temperature

Most industries causes thermal pollution by discharging hot water directly in reservoirs which cause destruction to the aquatic ecosystem. (Dodds *et al*., 2010) reported reduction of bacterial density due to thermal pollution. Temperature interferes with electrical conductivity of water and this is an important factor in regulation and control of biodiversity (Talbot *et al*., 1990). High temperature affects oxygen content available in the water. Increasing temperature minimizes the level of

oxygen available in the water (Alisawi *et al*., 2020). Thermal pollution disrupts the aquatic environment hence destroying the natural food web.

Organic matter

Water consumption introduces a variety of organic, inorganic, as well as synthetic substances to wastewater, including dirt, grit, oil, micronutrients, chemicals, metals, and animals and plants waste (Abdel-Raouf *et al*., 2012). Inorganic particulates in effluent comprises surface particles and salts, metals (Templeton & Butler, 2011). In general, organic substances are biodegradable and include human and food wastes which bacteria can metabolize, reducing the oxygen accessible for other life forms (Templeton & Butler, 2011). Therefore, by evaluating BOD as well as COD, organics in wastewater may be evaluated (Henze *et al*., 2008).

Turbidity

Water turbidity is impacted by suspended particulate materials present in water, which constitute approximately 0.1 percent of the total of all wastewater. (Middleton, 1977). The light penetrance as well as temperature of a surface water can be altered by suspended matter, which will destabilize benthic vegetation and obstruction of streams (Bilotta and Brazier, 2008, Templeton and Butler, 2011). Turbidity is a particulate pollution indicator that may be employed to assess and regulate effluent quality, which is especially critical for wastewater discharge considering suspended material can have negative effects on the ecosystem (Hannouche *et al*., 2011). Bacteria, Giardia cysts, and Cryptosporidium oocyst concentrations have all been linked to rising wastewater turbidity (Crittenden *et al*., 2012). Turbidity affects water chemically and also regulate the photosynthetic process of water body which delays the dissolved oxygen hence causing suffocation to the aquatic life.

2.4 Major recalcitrant chemical (RCs) and threats to aquatic ecosystem

Diverse pollutants from urban, agricultural, as well as industrial wastewater sources are regularly released into rivers, lakes, and seas. Compounds which are biodegradable are often degraded by different chemical, biological or physical processes once discharged into the ecosystem. However, persistent and widely dispersed recalcitrant contaminants (RCs) and/or their transformation products

continue to exist in the ecosystem (Englande *et al*., 2015). Because of their adverse effects on the ecosystem as well as public health, their tenacity, constant discharge over time, concentrations of these pollutants have been identified in various pertinent water sources and have become a widespread problem even at minimal concentrations (Muller *et al*., 2020). Even though some of the detrimental consequences of RCs are recognized, still, there is a great deal of ambiguity surrounding their impacts as exposure as well as concentrations rise, or the potential changes in toxicity that might arise from possible side effects of recalcitrant compounds.

Studies on marine species has revealed that the existence of nanomaterials in an environment promotes physiological alterations, behavioral disorders, and an elevated risk of death (Roberts *et al*., 2007). Aquatic species are reported to ingest microplastics while consuming their food, with clear evidence of various species consuming microplastics owing to their size resemblance with their food (Walkinshaw *et al*., 2020). Some of the typical pollutants found on microplastics are polycyclic aromatic hydrocarbons, DDT, PCB, organo halogenated pesticides, chlorinated benzenes and hexachloro cyclohexanes, (O'Donovan *et al*. 2018). Microplastics causes physiological impacts linked with consumption. Severe effects caused by the release of dangerous substances, ingredients employed into the fabrication of plastic, such as plasticizers, antioxidants, flame retardants, pigments, and so forth, may seep into human tissues and cause induced alterations or bioaccumulation (Pedersen *et al*., 2020). Numerous POPs have been identified as hormone disruptors, which can impair the endocrine and reproductive systems in both animals and people (Sweetman *et al*., 2005). As a result, there is an urgent effort needed to address, identify, and conduct research in these areas in relation to the comprehension of RCs in the water habitats and their remediation and removal techniques.

2.5 Treatment technologies for emerging contaminants (ECs) in wastewater

In the past couple of decades, the buildup of numerous known chemicals of human or natural source in waterways has emerged as a major phenomenon of growing environmental concerns. These pollutants, according to Rodriguez-Narvaez *et al*. (2017), are typically organic in form and frequently detected in minute levels at

concentrations between parts per trillion (ppt) and parts per billion (ppb), or g/L. Emerging contaminants (ECs) is a well-known term that has been used by several research institutions to describe these compounds (Philip *et al*., 2018).

It is likely that some ecosystems will go extinct because of the ECs' environmental resilience, whether they exist alone or in mixture (de Oliveira *et al*., 2019). Thus, it is necessary to establish higher EC release limitations and implement enhanced technologies for removing ECs from samples. There are numerous EC removal technologies that can be widely divided into conventional, natural attenuation as well as sophisticated techniques. Natural attenuation mechanisms such as photolysis, biodegradation, volatilization, sorption, dilution, and others are all low-cost as well as effective. Nonetheless, some techniques for instance volatilization, are less productive, suspended particles prevent photocatalysis, EC buildup are boosted during the sorption process as well (Barbosa *et al*., 2016).

As a result, treatment systems serve as critical barriers to the proliferation of ECs, bolstering the way that conventional Treatment systems can further minimize EC discharge into the environment. EC retention, physicochemical properties, treatment mechanism involved, and operational/environmental conditions all have a significant impact on EC sorption capacity. In general, primary, secondary, and perhaps tertiary treatment phases are used by WWTPs. Even though the first treatment processes are meant to remove colloidal as well as suspended materials, emerging contaminants are also somewhat eradicated, primarily by sorption onto the primary sludge (Luo *et al*., 2014; Tran *et al*., 2018).

The secondary treatment phase's objective is to eliminate organics or nutrients through biological degradation processes. The ECs are put through a range of processes, including sorption, dispersion, biodegradation, photodegradation, dilution as well as volatilization, although the major techniques of EC elimination are sorption, biotransformation and biodegradation (Luo *et al*., 2014; Barbosa *et al*., 2016). Likewise, tertiary treatment procedures for removing nutrients, suspended particles, as well as pathogens have been shown to have significant EC removal efficiency, notably for recalcitrant ECs removed through ozonation-like standard oxidation techniques (Rout *et al*., 2016; Ahmed *et al*., 2017). According to studies, the removal efficiency of EC following primary treatment ranges between 20-50

percent, while secondary treatment techniques yield a significantly greater removal effectiveness in the range of 30-70 percent (Tiwari *et al*., 2017).

2.6 Global Nitrogen Cycle and its anthropogenic perturbation

The culmination of the globe cycling of nitrogen (N) in a broad range of biochemical forms between atmospheric, terrestrial, and ocean reservoirs is the biotic and abiotic transformation of N, its physical conveyance via rivers as well as the atmosphere. Human activities have been reported with substantial influence on the globe nitrogen flow since the initial decades of the 20th century. These activities include the Haber Bosch process (NH_3 production), growth of nitrogen-fixing crops, and combustion. Anthropogenic Nitrogen Cycling modification is beneficial through food supply to humans and the stimulation of global carbon dioxide sequestration by marine and terrestrial environments (Sutton *et al*., 2013; Zaehle, 2013). Anthropogenic N has value for people, but there are also significant adverse implications on both the ecosystem and people's health.

The phrase "nitrogen cascade" was used by Galloway *et al*., (2003) to emphasize how a single anthropogenic nitrogen atom can have a range of different environmental implications before it is eliminated from the fixed N inventory through denitrification. These implications include a decrease in ecosystem biodiversity driven by eutrophication as well as acidification, a rise in greenhouse gases as well as ozone depletion, and a surge in disorders associated with air pollution. It also contributes negative effects on the ecosystem through irradiative constraints of climate, human health damage due to ozone and aerosols production, and biodiversity reduction at regional scales in terrestrial environments (Erisman *et al*., 2013). The today's world nitrogen cycle study is primarily concerned with the amount of current fluxes, the influence of human activities on the mechanisms, and the repercussions on environments, climate, as well as public health. (Fowler *et al*., 2013).

2.7 Eutrophication and effects on biodiversity

Eutrophication of freshwater and coastal water bodies has been widely facilitated by excessive N and P nutrient additions via atmospheric deposition and fertilizer runoff. For the past five decades, human global population, food productivity, and energy

consumption have increased with around five-folds (FAO, 2017). The alteration of global nutrient cycles results in both negative and positive impacts on the ecosystem. Nutrient loading in rivers and soils supports the formation of a biocenosis that connects the ecosystems of land and ocean (Jickells, 1998). Over past decades, human nitrogen release from various activities such as combustion, leguminous productivity, and fertilizers application have more than intensified the rate at which biologically available nutrients penetrates the ecosystem (Galloway *et al*., 2004).

Through the exploitation of ground as well as river waters, humans have impacted the global hydrologic systems (Zeng *et al*., 2017). For instance, dams have been reported with unintentional amounts of nutrients, which are transported by rivers to receiving waters and irrigation practices, which have been noted to cause nitrogen leaching (Woli *et al*., 2016). All those modifications in the world's nutrient and hydrological flows result in the proliferation of toxic algal blooms, the establishment of hypoxic zones after aerobic bacteria minimize O_2 thresholds while consuming bloom biomass, fish mortalities among other damages associated with the biodiversity, acid deposition caused by discharge of CO_2 through active decomposition, and other effects (Bouwman *et al*., 2009). Significant build-up of these nutrients within the ecosystem leads to contamination of marine ecosystems. This has eventually resulted to multiple health problems and ecosystem effects such as pollution of groundwater, acidification of the ecosystem, loss of biodiversity, eutrophication, accumulation of hazardous blooms, fish-killing (Diaz & Rosenberg, 2008 ; Paerl & Scott, 2010; Erisman *et al*., 2013; Isbell *et al*., 2013). The build-up of toxic algal blooms deteriorates the quality of drinking water, odor nuisance, and human health problems for those living within the vicinity of the lake (Glibert *et al*., 2005).

There are currently numerous of these seasonally or episodically occurring hypoxic zones around the planet. The yearly occurrence of a hypoxic sections in the Gulf of Mexico is one well-known instance, which is induced by nitrogen and phosphorus inflow from farmland practices and urban activities in the Mississippi River Basin (Rabalais *et al*., 2006). Another study in Lake Victoria reported that eutrophication has enhanced intensive growth of weeds such as water hyacinth and toxic algal blooms which has negative effects on the economic, aesthetic, and ecological functions steered by the lake ecosystem (Villamagna & Murphy, 2010). As the

Chesapeake Bay amply demonstrates, minimizing both nitrogen and phosphorus seems to be crucial in reducing marine as well as coastal eutrophication (Paerl *et al*., 2009; Lefcheck *et al*., 2018).

2.8 Heterotrophic nitrifiers and aerobic denitrifiers in removal of nitrogenous compounds

Researchers aiming to eliminate nitrogen from effluents with a high nitrogen content have a great challenge in choosing and optimizing bioprocesses to identify the optimum treatment recommendations. Over the past decades, nitrogen removal in conventional treatment technologies has been accomplished either through alternating aerobic and anaerobic processes in a single reactor or through various phases (modifying O_2 concentrations) (Winkler and Straka 2019). However, attempts are being made to develop novel methods of wastewater treatment that use bacteria species which can perform both nitrification as well as denitrification aerobically in the same reactor within the same conditions (He *et al*., 2016).

Conventional denitrification systems typically aim to completely convert ammonia to dinitrogen gas depending on the denitrification and nitrification mechanism performed in confined vessels by two distinct types of microbes known as autotrophs as well as heterotrophs, both functioning in a different physiological as well as operational settings. (Zhao *et al*., 2020). Nitrite is generated aerobically from ammonia during a process known as nitritation and nitrite is then further oxidized to nitrate during nitratation. In order for heterotrophic denitrifying bacteria to further convert nitrate to dinitrogen gas under anaerobic environment, organic carbon must be present as an electron donor (Kuypers *et al*., 2018).

The variations in process parameters, like physiological performance to changes in oxygen concentration, need for various carbon sources, varying treatment intervals as well as reactor designs, among others, call for additional designing, space, as well as operational complexity, which raises the cost of the overall process (Rajta *et al*., 2020). Contrary to heterotrophic denitrifying bacteria, the rate-limiting stage of nitrification is facilitated by chemolithoautotrophic ammonia-oxidizing bacteria (AOB) as well as nitrite-oxidizing bacteria (NOB) in nitrogen cycling bacterial communities. This is due to the extreme sensitivity of AOB and NOB to factors such as salinity, carbon source, pH, temperature, temperature, C:N ratio,

ammonium, nitrite and dissolved oxygen. (Wang *et al*., 2017). This is linked with their significantly slower rate of growth that makes these microbes unable to battle for oxygen as well as space during the wastewater treatment process with a high load of carbon/nitrogen, affecting the performance of ammonium or nitrite oxidation (Wang *et al*., 2017, 2020).

Due to the aforementioned concerns with autotrophic nitrification and heterotrophic denitrification, interest has transitioned to simultaneous nitrification and denitrification (SND), that is referred to as a "nonconventional" system since oxygen concentration is maintained under constant restricted environment in a single reactor to accomplish the SND processes (Dutta and Sarkar 2015).

The benefits of heterotrophic nitrifiers over autotrophic nitrifiers are numerous: Greater resistance to minimal temperatures, utilization of a range of substances, improved growth and preservation of active biomass all through the treatment process, and general protocol simplicity resulting in higher ammonium and organics reduction in less timeframe (Yao *et al*., 2013). These microbes also have lower neutralizing abilities as a result of the alkalinity produced through denitrification that substantially compensates for the alkalinity utilized during nitrification. This helps to solve the acclimation issue as well as minimizing the additional cost associated with pH adjustment. Moreover, the heterotrophic nitrification/aerobic denitrification (HN/AD) process intrigues investigators since nitrogenous compounds like nitrite/nitrate are undetected in the influx of aerobic chambers due to their synchronous denitrification in the treatment unit. (Yao *et al*. 2013).

Current studies have shifted to bacteria that are capable to perform coupled HN/AD process. These microbes have been investigated and have shown to be promising in biological nitrogen removal systems. Understanding aerobic denitrifiers has become of great importance to environmental microbiology. Several genera of microorganism have been reported to conduct the process successfully. The following microbes have been previously identified with a potential of modulating nitrogen level from contaminated ecosystem; *Bacillus sp* YX-6 (Song *et al*., 2011) *Acinetobacter sp* HA2 (Yao *et al*., 2013), *Paracoccus denitrificans* ISTOD1 (Medhi & Thakur, 2017) and, *Paracoccus denitrificans* ISTOD1 (Medhi & Thakur, 2018).

2.9 Molecular techniques for characterization of denitrifying bacteria

The nitrogen cycle is powered by symbiotic as well as free-living microbes which have nitrogenase activity. They transform dinitrogen gas to bioavailable ammonia whereby ammonia is fully oxidized, nitrite and nitrate through nitrite oxidoreductase and ammonia monooxygenase enzymes in a two-step aerobic pathway. Finally, denitrifying microbes close the nitrogen cycle by reducing nitrate to dinitrogen gas (Correa-Galeote *et al*., 2013).

Nitrification

Molecular analysis of nitrogen-fixing bacteria is often achieved through the *nifH* gene, which is used as a marker gene (Morales *et al*., 2010). During nitrification, two different groups of microbes are required for this process; nitrite-oxidizers and chemolithotrophic ammonia-oxidizers. The process starts when NH_3 is converted to hydroxylamine, nitrite, and nitrate, which is accomplished by hetero-trimeric Cu enzyme ammonia monooxygenase (AMO) (Levy-Booth *et al*., 2014). The *amoA* (marker gene) that encodes the α-subunit of the AMO enzyme, is used in nitrification research and molecular analysis of the AOA and AOB communities.

PCR primers for amplification of amo operon sequences across various contexts have been devised aiming at *amoA* gene, which encodes the subunit that contains the enzyme's active site [48-50]. The part of the genome expressing the C-terminus of *amoA* suggests being an appropriate target site for primers as well as probes to distinguish between AOB of the β and γ subclasses of the *Proteobacteria*, and also between methane and ammonia oxidizers within the γ subclass (Limburg *et al*., 2000).

A study confirms that *Nitrosomonas spp*. were the main nitrifiers in the lower section of the waters of the Elbe, based on data from DNA hybridization, immunofluorescence microscopy, as well as high MPN dilutions analysis of 16S rRNA partial gene sequences. Fluorescent in situ hybridization (FISH) was applied to detect and quantify *Proteobacteria* of the β subclass in samples taken from a collection of permanently ice-covered wetlands in the Taylor Valley of Antarctica (Voytek *et al*., 1999). Thirty oligonucleotides with documented specificity for AOB of the β-*Proteobacteria* were proposed from the 16S rRNA gene sequences

available up to 1997 for hybridizations or PCR with isolated DNA/RNA or entire cells. These oligonucleotides have been exploited to learn virtually everything there is to understand the wide range of AOB communities found in natural settings. Although published (Utaker *et al*., 1998).

Denitrification

Denitrification is achieved by reducing the nitrate to dinitrogen gas through sequential enzymatic activities; nitrate, nitrite, nitric oxide, and nitrous oxide reductase encoded by the *nar/nap, nirK/nirS, c-nor/q-nor*, and *nos* genes respectively (Levy *et al*., 2014). A periplasmic nitrate reductase or a membrane-bound nitrate reductase enzyme catalyzes the conversion of nitrate to nitrite, which is the first step of denitrification (Van *et al*., 2005). Nar enzyme consists of three subunits NarGHI, NarG and NarH which are situated in cytoplasm, link with Narl, where its N-terminus meets the periplasm.

A study confirmed the PCR primer sequences utilized for identifying denitrifying bacteria from various habitats, and the results displayed specific bands of *narG, napA, nirS,* and *nosZ* which were detected more frequently in hyporheic zone water samples. The PCR primer sequences from this study can be utilized to analyze how denitrifying bacteria respond to seasonal as well as regional variations during denitrification process (Kim, 2020). The occurrence of the cd1 NIR and Cu NIR among various bacteria has primarily been investigated using immunological approaches (Korner *et al*., 1987).

Target DNA can be amplified by PCR, cloned. Large sample sets may be screened for the comparison of local and seasonal variations using methods like DGGE, TGGE, or T-RFLP (Bothe *et al*., 2000).

2.10 Methodologies for nitrogenous compounds removal from nitrogen-rich ecosystem using microbes

Simultaneous Nitrification and Denitrification with Activated Sludge

Simultaneous nitrification and denitrification (SND) is a biological technique for removing nitrogenous substances from wastewater. This process achieves nearly complete nitrogen removal by combining the functions of nitrifying and denitrifying

bacteria in a single reactor. SND offers a number of benefits over conventional nitrification and denitrification regarding the removal of nitrogen: It removes the requirement for two different tanks to be controlled in serial or intermittent aeration in a single vessel, allowing for constant wastewater circulation with a lower footprint (Li *et al*., 2008). This process employs 22-40% fewer carbon sources and minimizes effluent yield by thirty percent. SND consumes less energy due to the reduced aeration requirement, and a neutral pH level with a lower demand for alkalinity can be achieved in the reactor since alkalinity is utilized during nitrification but produced during denitrification. (Yoo *et al*., 1999; Hibiya *et al*., 2003; Third *et al*., 2005).

Microalgal Growth

Over the growth season, microalgae consume an immense amount of trace metals and nutrients in natural aquatic environments (Gangstad, 1979). Microalgae can additionally take in inorganic nitrogen sources for instance ammonium, nitrite, and nitrate. Ammonium was selected among those because microalgae can metabolize it with less effort compared with nitrite and nitrate. Microalgae have benefits in several functions: they grow fast, and generate a large number of bio-valuable waste substances. The byproducts of microalgae residues can be utilized as agricultural fertilizer and animal feed, and they require less area for waste disposal (Cai *et al*., 2013). This system is an excellent choice for wastewater treatment because of the aforementioned benefits.

Anaerobic ammonium oxidation (anammox)

Anaerobic ammonium oxidation (anammox) is an innovative method for removing ammonia from industrial and municipal wastewater and decreasing nitrogen in effluent that is ammonium-rich (Anjali and Sabumon, 2014). Anammox is now gaining prominence due to its low cost and energy requirements. In comparison to the traditional nitrification/denitrification techniques, anammox relies on the anoxic oxidation of ammonia with nitrite as the preferred electron acceptor; utilizes 50% less oxygen, 100% less organic carbon, and conserves 90% of the operational expenses in sludge disposal. As a result, firms that produce effluent with elevated levels of ammonium are becoming more interested in the anammox technique (Anjali and Sabumon, 2014).

Bioelectrochemical Systems (BESs)

Bio-electrochemical systems (BESs) involve microbial processes that generate power from organic wastes while producing some valuable compounds like acetate, medium-chain fatty acids, alcohols, and butyrate (Kumar *et al*., 2017). The principal output of MFC technology has been bioelectricity production, and one of the most important uses of BES is wastewater treatment. The use of bioelectrochemical systems (BESs), a newly developed environmentally friendly invention for both the generation of energy and sewage treatment, has significantly increased during the previous ten years. Additionally, BESs have distinct prospects for the effective and safe microbial production of valuable compounds and biofuels (Bajracharya *et al*., 2016).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Description of the study site

The largest freshwater reservoir in Africa is Lake Victoria, occupying an area of 69,484 Km^2 with an average depth of approximately 40 meters (**Figure 1**). The lake extends between latitudes and longitudes (0˚30' N and 3˚12' S 31° 37′ E and 34° 53′ E) (Muyodi *et al*., 2010). The reservoir is found in Kenya, Tanzania, and Uganda, while the catchment basin area in Rwanda and Burundi (Juma *et al*., 2014).

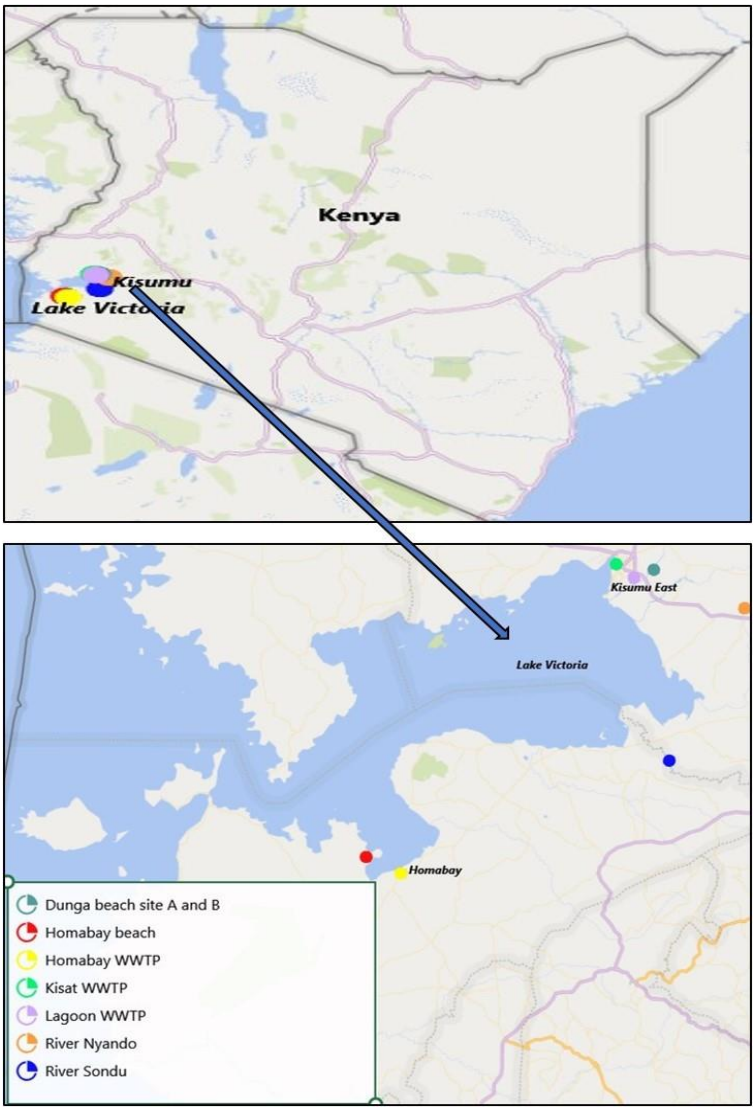

Figure 1: The map indicating the study sections and sample collection areas in the vicinity of Lake Victoria

3.2 Sample collection

A total of eight samples comprising water were collected randomly using 500 ml sterile bottles from the shores of the lake, rivers, and WWTPs that drains to Lake Victoria including Dunga beach site A and B, Homabay beach, River Sondu, River Nyando, Kisat treated WWTP, Lagoon untreated WWTP, and Homabay untreated WWTP. The bottles were properly sealed, kept in ice-cooled cases, and conveyed to the University of Embu microbiological lab for further investigations.

3.3 Chemical analysis of the samples

After sample collection, the samples were taken to SGS Kenya Limited Laboratory Services for the physiochemical parameters analysis. The parameters to be analyzed included the following: pH, total nitrogen, chloride, biochemical oxygen demand, nitrate, ammonium, total dissolved solids, and nitrite. Nitrate was analyzed via APHA-4500-NO3 method, nitrite was analyzed via APHA-4500-NO2, and ammonia as nitrogen was analyzed through APHA-4500- NH3 F method (APHA, 1992).

3.4 Molecular diversity of the bacteria within the sample

3.4.1 Total DNA isolation, amplicon library preparation and sequencing

The phenol-chloroform methodology was applied to extract the total community DNA (Sambrook *et al.,* 1989). DNA extraction solution contained the following reagents: Stock solution: 1 M Tris-HCL, 0.5 M EDTA, 5 M NaCl. Solution A: 2 ml- 1 M Tris HCl pH 8.5, 4 ml- 0.5 M EDTA pH 8.0, 10 g. Solution B (lysis buffer): 20 ml- 400 mM Tris HCl pH 8.5, 6 ml- 60 mM EDTA pH 8.0, 1.5 ml- 5 M NaCl, 0.5 g- SDS.

Cells were harvested by centrifuging 10 ml of the water sample. After being air dried, the obtained DNA pellets were resuspended in 30.0 µl of PCR water. The extracted DNA's quality was examined by separating 4 µl on 1% gel at 115V for 40 minutes. Purified DNA (25 µl) was stabilized using DNAstable® (Biomatrica), air-dried, and shipped to MR DNA Research Lab, United State, where sequencing was performed. Using the barcoded primers 515F (GTGCCAGCMGCCGGTAA), and 806R (GGACTACHVGGGTWTCTAAT), the 16S rRNA gene V4-V7 region was amplified from the isolated DNA. The PCR products were amplified in 30 cycles via

HotStarTaq Plus Master Mix Kit from Qiagen (USA). The products were denatured at 94 °C for 3 minutes, 30 cycles at 94 °C for 30 seconds, at two different annealing temperatures of 53 °C for 40 seconds and 72 °C for 1 minute, and finally elongated at 72 °C for five minutes. All PCR products were inspected in a 2% agarose gel after amplification to assess whether the amplification process was successful and the relative intensities of bands. Based on the molecular weight and DNA quantity from the gel pictures, barcoded samples were merged in equimolar ratios. Calibrated Ampure XP beads were used to purify pooled samples for use in library preparation. The pooled and purified PCR products were used to generate the DNA library in accordance with the Illumina TruSeq protocol. At Molecular Research DNA, sequencing was completed on a MiSeq 2x300bp Version 3 in compliance with the manufacturer guidelines (www.mrdnalab.com, Shallowater, TX, USA).

3.4.2 Analysis of sequences data and taxonomic classification

The Q25 sequencing data were processed using the MR DNA ribosomal as well as functional gene analysis pipeline that was obtained during the sequencing procedure (www. mrdna lab. com, MR DNA, Shallowater, TX). Sequences depleted of primers, reads less than 250 base pairs, as well as sequences with ambiguous base calls were all discarded. The obtained readings were quality screened using a maximum anticipated error threshold of 1.0. Uchime was employed to denoise the dereplicated sequences and eliminate any chimeric sequences (Edgar *et al*., 2011). Operational taxonomic units (OTUs) were picked at 3% divergence, which is equivalent to 97% similarity. BLASTn was used to match each OTU's taxonomic classification against a curated database provided from NCBI (www. ncbi. nlm. nih. gov). Analysis of sequence data was done on R version 3.6.2 software and Excel to generate plots. Richness, Shannon, and Simpson indices were estimated from the resultant OTUs via microbiomeSeq (Ssekagiri *et al*., 2017), phyloseq (McMurdie *et al*., 2013) and vegan (Oksanen *et al*., 2015) R packages. Heatmaps were generated using gplots package in R (Warnes *et al*., 2016). Principle component analysis (PCA) was done to explore the impacts of environmental element on the microbial community using AmpViz2 package in R (Andersen *et al*., 2018). Under the accession number, PRJNA824944, the assembled sequence reads were submitted in the SRA.

3.5 Isolation and culturing of aerobic denitrifying bacteria

Aerobic denitrifiers were isolated from the water samples using screening media and solid bromothymol blue media (Guo *et al*., 2013). Screening media (pH 7.2) contained one liter each of these ingredients: sodium succinate (2.84g), $NaNO_3$ (10 mM), ; KH_2PO4, 1.36g; $(NH_4)_2SO_4$, 0.27g; Yeast extract, 1g; $MgSO_4.7H_2O$, 0.19g; TE (trace element) solution, 1 ml. Solid BTB (pH 7.0 - 7.3) included the following reagents per liter: agar, 20g; KNO_3, 1g; KH_2PO4, 1g; $FeCl_2.6H_2O$, 0.5g;, $CaCl_2.7H_2O$, 0.2g; $MgSO_4.7H_2O$, 1 g; sodium succinate, 8.5g and BTB reagent (1% in alcohol) 1ml.

The effluent samples (50 ml) and 100 ml of screening media were added to sterile 500 ml Erlenmeyer flasks with cotton plugs in order to enrich denitrifying bacteria. These flasks were then rotary shaken at 150 rpm for three days at 30 °C. The experiment was performed in triplicates for each sample. The enriched samples were then serially diluted to 10 folds, whereby 100 µl of 10^8, 10^9, and 10^{10} of each sample was spread plated on solid BTB plates and incubated for 48 hours. Pure bacterial colonies were selected and purified using multiple streaking on the same fresh media.

3.6 Evaluation of nitrogenous compounds removal

A single colony of each bacteria was inoculated into 100 ml of screening media. under aerobic environment and maintained at 30 °C for 24 hours to evaluate the capacity of each bacteria for the removal of nitrogenous compounds (Ma *et al*., 2016). Media without the inoculum served as the negative control. The Phenate technique was used to determine the ammonium utilization by each bacteria isolate. Phenate method involve addition of phenol solution in a mixture of nitroprusside catalyst and hypochlorite. Indophenol, which is a bright blue compound, will react with ammonia if it is present. The color absorbs 640 nm-wavelength light (Park *et al*., 2009). N-(1-naphthyl)-ethylene diamine photometry was used to measure nitrite oxidation. Nitrite and sulphanilamide combine to form a diazo compound in an acidic solution, and this combination reacts with NEDA (N-(1-naphthyl)-ethylenediamine-dihydrochloride) to generate a red azo dye. The color absorbs light with a 543 nm wavelength (Oliveira *et al*., 2004). The Phenol Disulphonic Acid (PDA) technique was used to quantify nitrate reduction. Nitrate reacts with PDA to

forma nitro derivative, which forms a yellow color in alkaline solution. The development of yellow colour is attributed to rearrangement of nitro derivative sturucture. The absorbance is measured at 410 nm wavelength (Taras, 1950). There were three replicas of each procedure. The percentage of NH_4^+, NO_2^-, and NO_3^- reduction was calculated following the formula listed below:

(Ci-Cf)/Ci x 100

Whereby Ci = initial concentration, Cf =final concentration of N. One-way ANOVA was used to analyze the results at a statistical significance threshold of $p \leq 0.05$. All the statistical analysis in this work was analyzed using R version 3.6.2 software.

3.7 Molecular characterization of the isolates

3.7.1 DNA extraction from pure bacteria isolates

All pure cultures selected were grown in 1 ml of LB broth in Eppendorf tubes and incubated at 30˚C for 96 hours. The growing cells were extracted by centrifuging at 8,000 rpm. DNA extraction solution contained the following reagents: Stock solution: 1 M Tris-HCL, 0.5 M EDTA, 5 M NaCl. Solution A: 2 ml- 1 M Tris HCl pH 8.5, 4 ml- 0.5 M EDTA pH 8.0, 10 g. Solution B (lysis buffer): 20 ml- 400 mM Tris HCl pH 8.5, 6 ml- 60 mM EDTA pH 8.0, 1.5 ml- 5 M NaCl, 0.5 g- SDS.

The cells were re-suspended in 100 µl of Solution A (100 mM Tris-HCI (pH 8.0), 5 µl of Lysozyme added (from a 20 mg/m1 solution) and incubated at 37˚C for 15 minutes in a water bath. 400 µl of Lysis buffer (400 mM Tris-HCI [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaC1, 1% sodium dodecyl sulfate) was added and the tube was left at room temperature for 10 min. Proteinase K (10 µl, 20 mg/m1) was added, mixed gently to digest any proteins and nucleases That may degrade the DNA. The solution was then incubated at 65˚C for 15 minutes in a water bath. An equal volume of chloroform (515 µl) was added and centrifuged at 13, 200 rpm for 5 minutes at 4˚C. The supernatant was transferred into a new tube and the volume of the supernatant was noted. Sodium acetate (150 µl) and 665 µl of isopropyl alcohol were added, mixed by inversion and spun at 13,200 rpm for 10 min to pellet the DNA. The supernatant was carefully pipetted out and the resultant DNA pellet was washed in 300 µl of 70% ethanol. The DNA pellet was air-dried and dissolved in 30

µl of PCR water. Gel electrophoresis was done to confirm the quality of DNA by separating 4 µl on 1% gel at 115 V for 40 min. Purified DNA (1 µl) was used as a template for PCR amplification.

3.7.2 DNA amplification

Using the primer pairs 8F forward (5'-AG(A/G) GTTTGATCCTGGCT-3' and 1492R reverse (5'-CGGCTACCTTGTTACGACTT), the bacterial 16S rRNA gene was amplified successfully (Embley & Stackebrandt, 1994; Lane, 1991). Amplification of the 16S rRNA gene was achieved via the Sure Cycler 8800 (Agilent Technologies). The PCR mixture was comprised up of 30.0 µl of molecular biology grade water, 10.0 µl of polymerase buffer, 1.0 µl of dNTPs, 1.0 µl of MgCl2, 1.5 µl of dimethyl sulfoxide (DMSO), and 0.3 µl Taq polymerase in a total reaction quantity of 50 µl. The PCR reactions were carried out under the following parameters: 95 °C for 5 minutes, afterward 35 cycles (1 minutes of denaturing at 94 °C, 1 minutes of annealing at 53 °C, 1 minutes of extension at 72 °C), and finally, a final extension phase at 72 °C for 5 minutes. After 35 cycles, the amplified products were run and visualized for size and quantity on a 1% agarose gel in 1× TBE buffer for 35 minutes. Staining with fluorescent dye enhanced visualization under UV box (Thurston & Saffer, 1989)

3.7.3 Purification of PCR products, sequencing and data analysis

The QIAquick PCR purification kit methodology (Qiagen) was employed to purify the PCR products prior shipping them to Inqaba Biotech in South Africa for sequencing. Cleaning the amplified fragments was accomplished by combining 12.5 µl of PCR product with 2.5 µl of ExoSAP-IT™ (Thermo Fisher Scientific), incubating at 37 °C for 30 minutes, accompanied by heat inactivation at 95 °C for 5 minutes to terminate the experiment. Similar universal primers 8F and 1492 R were used to sequence PCR products at Inqaba biotech in South Africa. The sequenced amplicons were checked for quality and edited using Chromas Lite 2.0.1 (https://technelysium.com.au/wp/chromas). Sequence similarity level to existing type strains was done on EzBioCloud server (Yoon *et al*., 2017.

Phylogenetic analysis was done using Molecular Evolutionary Genetics Analysis (MEGA) version X (Kumar *et al*., 2018). Molecular Evolutionary Genetics Study

(MEGA) version X was employed for the phylogenetic analysis (Kumar *et al*., 2018). The evolutionary history was inferred via the Neighbor-Joining procedure. The optimal tree is displayed. The branches were accompanied with the proportion of replicate trees in which the relevant taxa clustered during the bootstrap test (500 replicates). Using with *Bacillus subtilis* IAM12118 as the outgroup, the evolutionary distances were evaluated via the Maximum Composite Likelihood technique. The isolate sequences have been submitted to NCBI under the accession numbers ON227417 to ON227434.

3.8 Assessment of nitrogenous compounds removal activity in the wastewater

The ability of aerobic denitrifiers to remove nitrogenous substances from wastewater was investigated using bacterial isolates that were efficient at removing nitrogen from the basal media. Pure isolates were grown on a basal media which included the following reagents per liter: agar, 20g; KNO_3, 1g; KH_2PO4, 1g; $FeCl_2.6H_2O$, 0.5g;, $CaCl_2.7H_2O$, 0.2g; $MgSO_4.7H_2O$, 1 g; sodium succinate, 8.5g and BTB reagent (1% in alcohol) 1ml, and incubated at 30˚C for 24 hours. A single colony of each bacteria was added to 50 ml of sterilized lake wastewater in a 250 ml conical flask, and the amount of nitrogen removed was measured after 120 hours of incubation at 30 °C and 150 rpm. Ammonium oxidation was evaluated by the Phenate technique. The colour absorbs light of 640 nm wavelength (Park *et al*., 2009). N-(1-naphthyl)-ethylene diamine photometry was applied to measure nitrite utilization. The colour absorbs light at a wavelength of 543 nm (Oliveira *et al*., 2004). The Phenol Disulphonic Acid (PDA) methodology was employed to detect nitrate present. The absorbance is measured at 410 nm wavelength (Taras, 1950). Each treatment was carried out in triplicates. The percentage reduction of NH_4^+, NO_2^-, and NO_3^- was determined using the formula below: (Ci-Cf)/Ci x 100

Ci, initial concentration, Cf, final concentration of N. One-way ANOVA was used to analyze the results at a statistical significance threshold of $p \leq 0.05$. All the statistical analysis in this work was analyzed using R version 3.6.2 software.

CHAPTER FOUR

RESULTS

4.1 Physico-chemical parameters at the sampling site

Eight samples in total were collected and examined. The samples were collected in the following areas; Dunga beach, sites A and B and one sample from Homabay beach, River Sondu, River Nyando, Kisat WWTP, Lagoon WWTP, and Homabay WWTP. All the physico-chemical parameters of the sampling sites are highlighted in **Table 1**. The pH of all samples was found to range between 6.5 - 8.5 except for River Sondu (6.25) and Dunga B (6.46) which were slightly lower. High levels of ammonia were reported in WWTPs (Kisat WWTP, 19.49 mg/l, Homabay WWTP, 38.69 mg/l, Lagoon WWTP, 10.77 mg/l). Total nitrogen was high in both treated and untreated WWTPs (Kisat WWTPs, 24.64 mg/l, Homabay WWTP, 45.43 mg/l, Lagoon WWTP 18 mg/l). However, results shows that lakes and river samples had relatively low levels of total nitrogen and ammonia compared to WWTPs. Both WWTP and lake water had elevated levels of biochemical oxygen demand compared to the rivers. For instance, Kisat had 153 mg/l, Homabay WWTP with 126 mg/l, while Dunga B had 183 mg/l. A study reveals that water system is deemed extremely contaminated with organic waste at BOD concentrations of 100 mg/l or even greater (Zaid *et al*., (2019). The level of chloride was high in WWTPs compared to rivers and lake samples. **Table 1** indicates the raw figures of the physico-chemical parameters at the sampling points of Rivers, WWTP and Lake Victoria during sampling.

Table 1: Sample collection points and physicochemical analysis of water samples from rivers, treated and untreated WWTPs, and Lake environments.

Sample origin			**Rivers**		**Treated WWTP**	**Untreated WWTPs**		**Lake**		
Parameters	**Method**	**Unit**	**(J1) River Sondu**	**(J8) River Nyando**	**(J7) Kisat Treated WWTP**	**(J2) Lagoon Untreated WWTP**	**(J4) Homabay Untreated WWTP**	**(J3) Homabay B**	**(J5) Dunga A**	**(J6) Dunga B**
Sampling points	GPS	Lat	0° 22' 46"	0° 0' 57"	0° 4' 57"	0° 28' 35"	0° 31' 27"	0° 30' 41"	0° 8' 43"	0° 8' 20"
		Long	34° 49' 21"	35° 16' 32"	34° 44' 58"	34° 30' 42"	34° 26' 53"	34° 28' 34"	34°44' 13.2"	34°44' 11"
pH	APHA 4500 H+		6.27	6.78	6.76	6.71	7.46	7.42	7	6.44
Total Nitrogen	Calculation	mg/l	1.23	1.34	24.64	18	45.43	1.57	3.36	3.92
TDS	APHA 2540 C	mg/l	45	144	661	553	1308	264	276	242
Nitrite-N	APHA-4500-NO2	mg/l	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Chloride	APHA-4500-CL B	mg/l	7.88	12.32	62.09	60.12	146.35	10.35	9.36	11.83
Ammonia-N	APHA-4500- NH3 F	mg/l	<0.02	<0.02	19.49	10.77	38.69	<0.02	2.84	2.55
Nitrate-N	APHA-4500-NO3	mg/l	0.11	0.22	<0.01	0.08	0.07	0.45	<0.01	<0.01
BOD@20ºC	APHA 5210	mg/l	45	30	153	60	126	63	73	183

4.2 Microbial community diversity

Based on 16S rRNA high throughput sequencing, the number of OTUs obtained ranged from 710 to 12541. The number of sequences ranged from 18,205 to 29,531 across all samples. A total of 26 bacterial phyla were observed across the eight samples (**Figure 2**). The results revealed that 60% of the sequences belonged to phylum *Proteobacteria*, 19% to *Bacteriodetes*, 3.9% to *Firmicutes*, 2.4% to *Cyanobacteria*, and 1.5% to *Verrucomicrobia.*

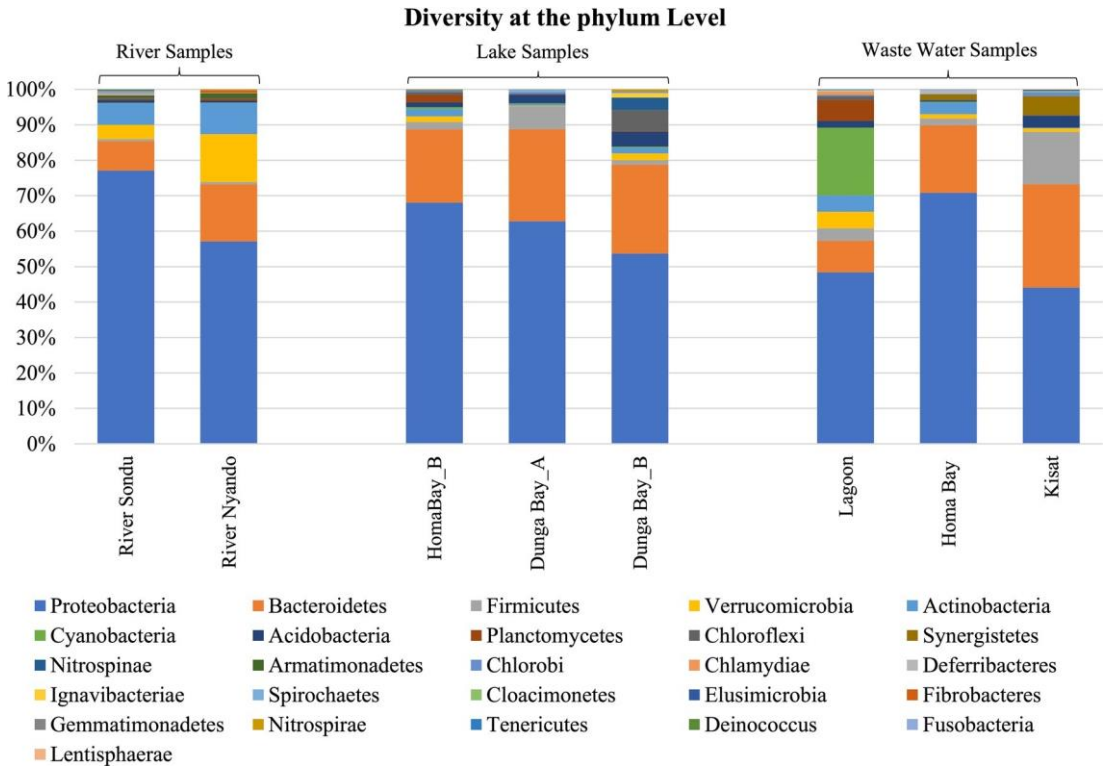

Figure 2: Illumina high throughput sequencing of 16S rRNA amplicons shows the relative abundance of different phylum. A total of 26 bacterial phyla were observed with the number of operational taxonomic units (OTUs) ranging from 710 –1,255 in all samples.

The most abundant phylum at each of the eight sampling sites was the *Proteobacteria*, which constituted approximately 76%, 48%, 68%, 69%, 62%, 52%, 44%, and 56% of samples J1, J2, J3, J4, J5, J6, J7, and J8, accordingly. (Figure 2). Phylum *Lentisphaerae*, *Deinococcus_Thermus* and *Fusobacteria* were less dominant in all samples. Phylum *Nitrospirae* was detected with a relative abundance of 0.1% across all samples. Within the phylum *Proteobacteria*, the most abundant classes were *Betaproteobacteria* (31%) and *Gammaproteobacteria* (10%), whereas

Bacteroidia (8.8%) was the most abundant class within the phylum *Bacteriodetes* followed by *Sphingobacteria* with 5.5% (**Figure 3**).

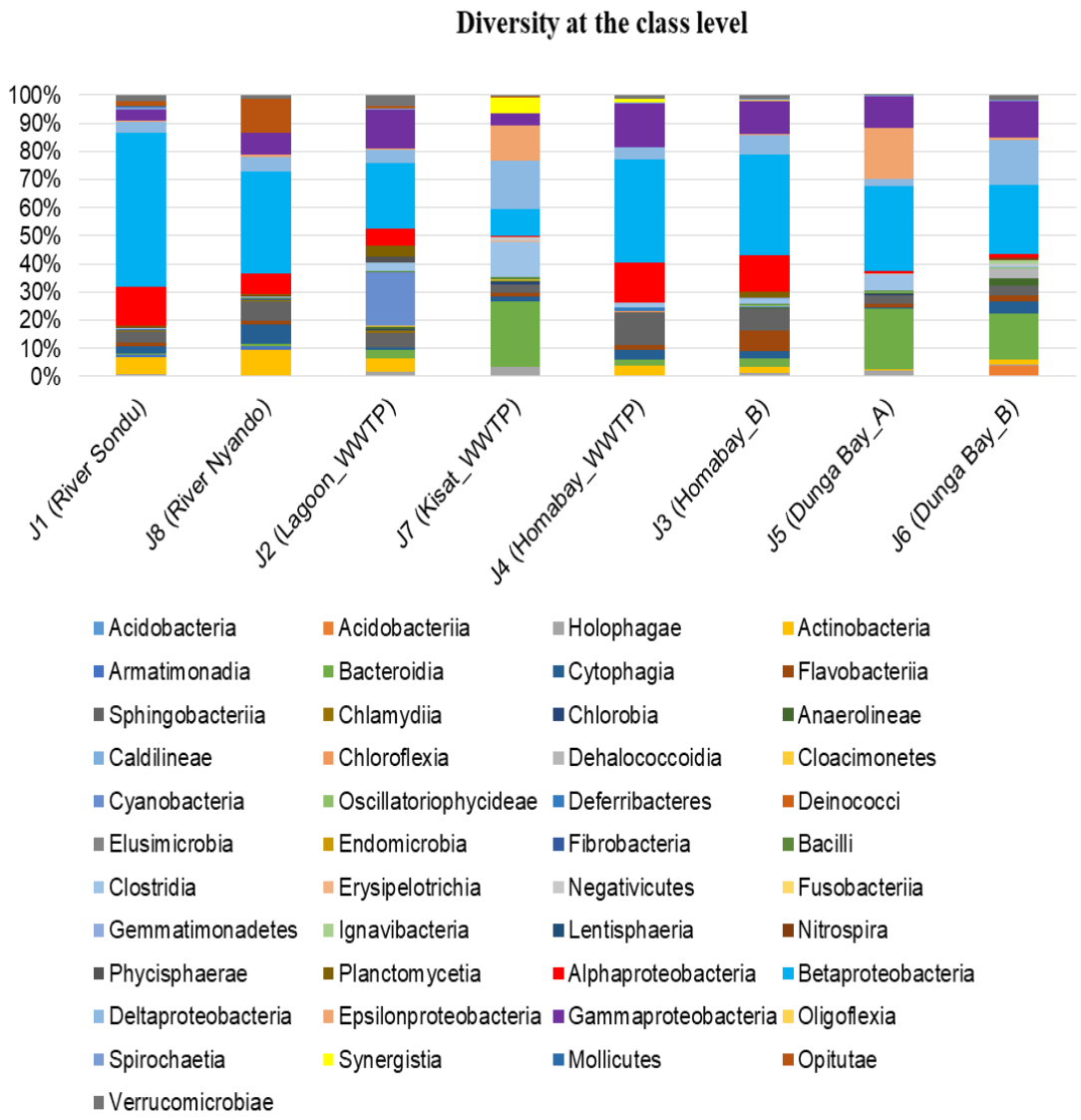

Figure 3: Illumina high throughput sequencing of 16S rRNA amplicons shows the relative abundance of different class. A total of 45 bacterial class were observed with the number of operational taxonomic units (OTUs) ranging from 710–1,255 in all samples.

At the genus level, the major *Proteobacteria* genera were *Dechloromonas* and *Hydrogenophaga*, and some of the species in these two genera are known for their ability to perform denitrification (Jewel *et al*., 2017) (**Figure 3**). *Dechloromonas* represented the most dominant group with 5.1%, followed by *Hydrogenophaga*,

4.0%. *Bacteriodetes* group was the second most abundant phylum which accounted for 19% of the bacterial sequences. The major genus belonging to *Bacteriodetes* was *Bacteroides* which constituted 1.8%, and it's well documented with nitrates reduction capabilities (Jewell *et al*., 2017). The group of *Firmicutes* constituting 3.9% were largely represented by obligate anaerobes, *Clostridium*, accounting for 2.1% of all bacterial sequences. A study reported *Clostridium* with the potential to convert benzaldehyde to benzoate and benzyl alcohol (Wexler *et al*., 2005). Distinctive representative genus *Planktothrix agardhii* constituted 2.4% and it was rich in sample J2 (Lagoon WWTP) accounting for 18.9% (**Figure 4**) and it wasn't detected in sample J7 (Kisat treated WWTP). *Planktothrix agardhii* is a toxin-producing strain of *Cyanobacteria* that possesses a potential health risk to human populations, fish and other aquatic organisms (Marie *et al*., 2012).

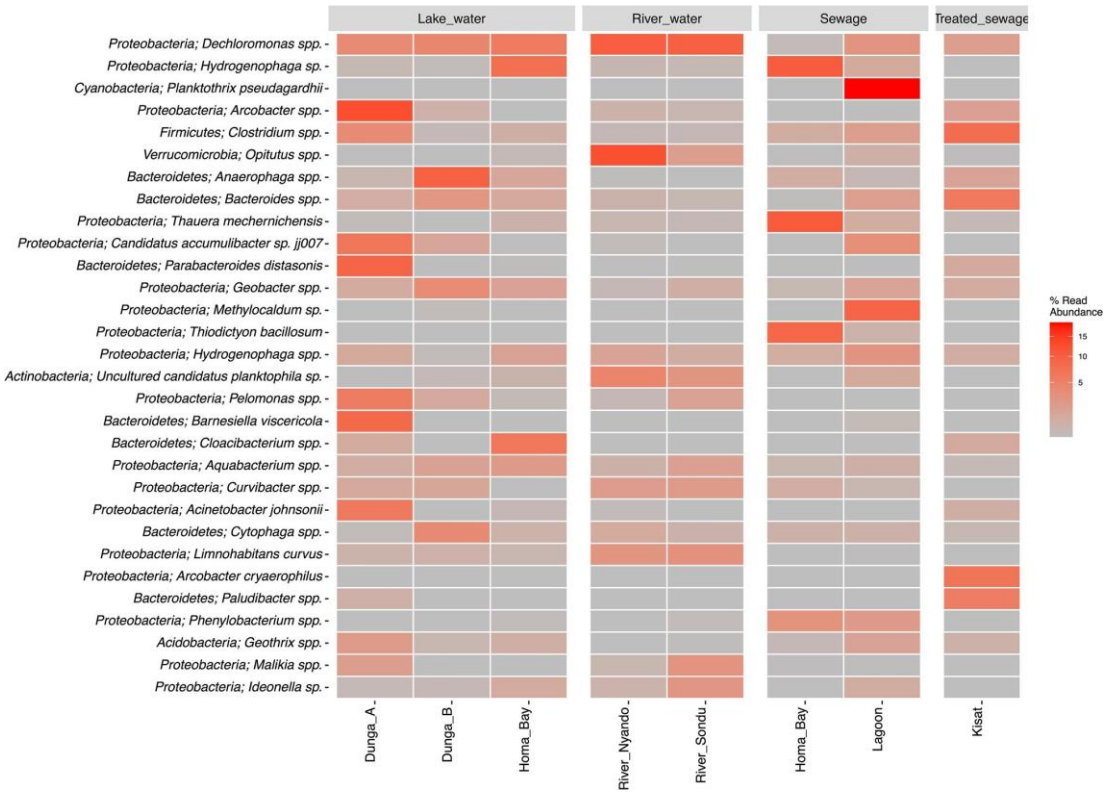

Figure 4: Heat map indicating the relative abundance of the major genera based on Illumina high throughput sequencing of bacterial 16S rRNA amplicons. The relative abundance for each sample is depicted by the colour intensity of each panel.

4.3 Diversity index analysis

The Sequences, OTUs and α-diversity indices of bacterial species obtained from different samples are presented in Table 2. A rich microbial diversity is represented by high values of the diversity indices. Simpson index value ranged from 0.96-0.98, whereas Shannon diversity ranged from 5.9–7.9. Based on the diversity indices, the diversity showed an increasing trend from WWTP, rivers to lake (**Figure 5**).

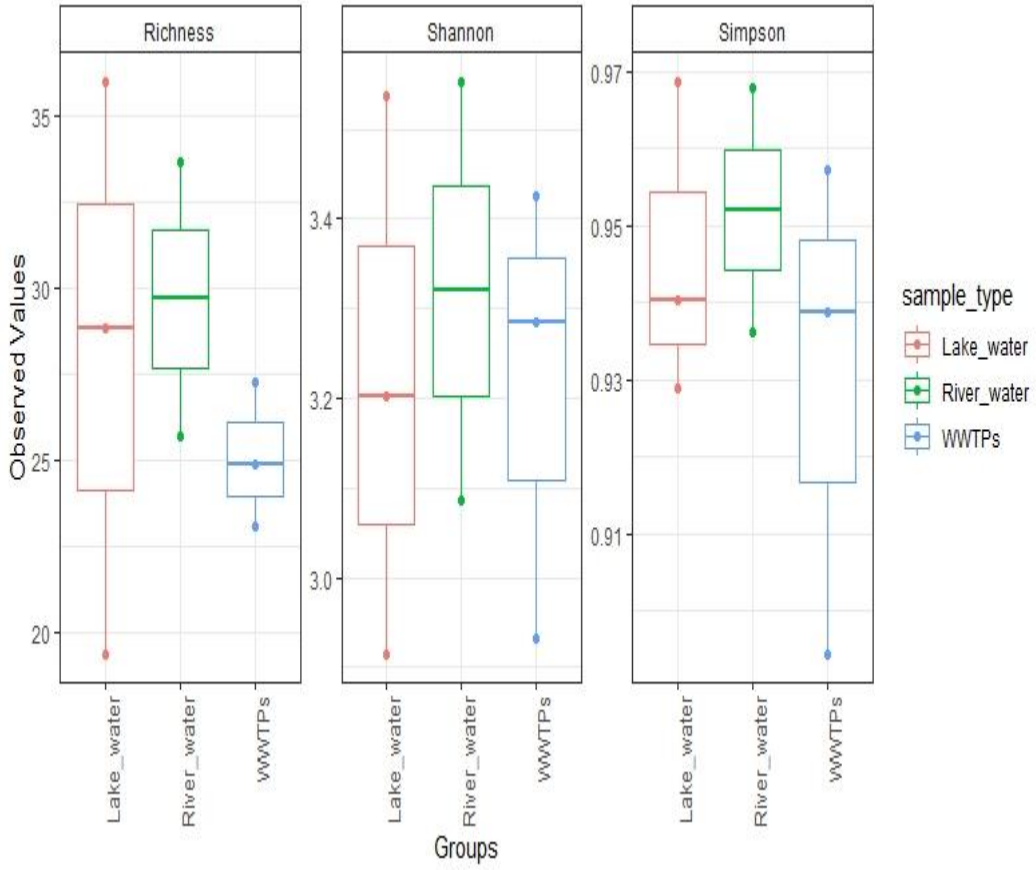

Figure 5: Alpha diversity comparisons between different sample types and sampling sites. Statistical analysis was performed through three species richness estimators: Richness, Simpson, and Shannon diversity index.

Sample J3 had the richest biodiversity with a Chao1 value of 1456.78, while sample J4 (Homabay WWTP) had the least diversity with a value of 1022.38. Sample J7, Kisat treated WWTP, had relatively higher diversity than the two untreated WWTP (Homabay WWTP and Lagoon WWTP) with a Shannon diversity index of 7.11 and richness index of 1032.31 (**Table 2**). Dunga site B showed higher biodiversity than Dunga site A, which had Shannon values of 8.3 and 6.8, respectively. Beta diversity analysis showed that samples with similar water chemistry and microbial diversity

clustered together (**Figure 6**). The lake sample and rivers are clustered together separately, which is a different case in WWTPs. The untreated WWTP (Homabay and Lagoon WWTP) are ecologically far apart from the treated WWTP (Kisat WWTP)

Table 2: Alpha-diversity comparisons between samples using four different Alpha diversity indices: Chao1, Ace, Simpson, and Shannon.

Sample ID	Site	Sequence No	OTUs	Chao1	Ace	Simpson	Shannon
J1	River Sondu	23,730	1,202	1361.56	1355.19	0.99	8.53
J2	Lagoon WWTP	29,531	883	1097.03	1055.39	0.96	6.94
J3	Homabay Beach	23,596	1,241	1456.78	1401.15	0.99	8.30
J4	Homabay WWTP	29,354	732	1022.38	1050.56	0.96	6.28
J5	Dunga site A	29,380	995	1199.48	1191.98	0.97	6.50
J6	Dunga site B	18,205	1,033	1368.41	1392.83	1.00	8.53
J7	Kisat WWTP	29,518	702	1032.31	945.88	0.98	7.11
J8	River Nyando	24,610	1,138	1343.11	1301.36	0.99	8.03

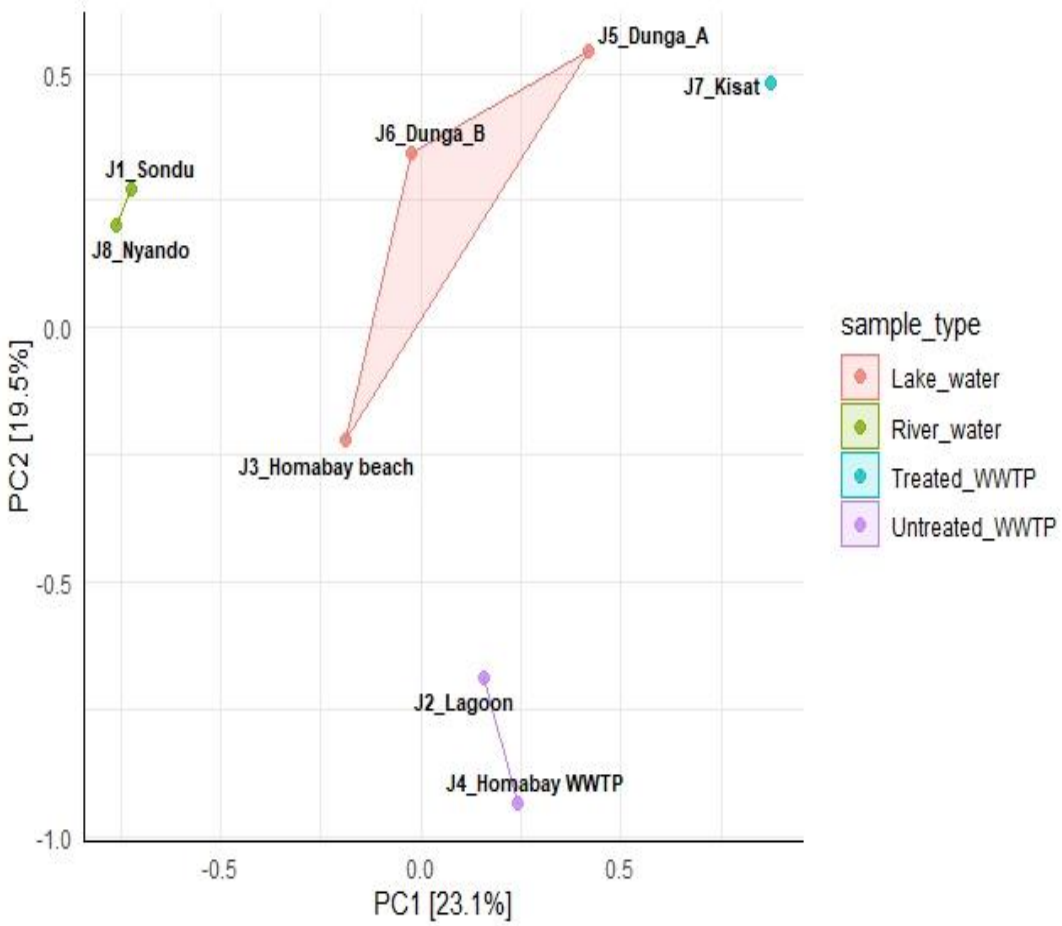

Figure 6: Plots of PC1 and PC2 from a principal component analysis on the x-axes and y-axes showing the ecological distance of sampling sites based on the water chemistry and microbial biodiversity. Similar colour codes reveal samples correspondence in terms of environmental parameters and diversity and vice versa.

4.4 Screening for nitrogenous compounds from the isolates

Twelve bacterial isolates in total were successful in utilizing the three nitrogenous components. However, they had variable mean of absorbance (**Table 3**). Percentage mean of N reduction index on ammonium utilization ranged from 95.32% (JM2) to 79.55% (JM11), nitrate utilization ranged from 93.18% (JM2) to 73.78% (JM4), whereas nitrite usage ranged from 94.69% (JM2) to 77.8% (JM10) (**Figure 7**). Nitrogenous compounds were not utilized in the negative control test. A statistically significant test result was ($P \leq 0.05$) which means that the test hypothesis was false. The test hypothesis was therefore rejected because there was a statistical significance between the sample means.

Table 3: Average mean of absorbance of the bacterial isolates grown in a basal media. Low absorbance indicates that fewer molecules are available to interact with the light, and vice versa.

Isolates Sample ID	Mean±SD of absorbance		
	Ammonium	**Nitrate**	**Nitrite**
JM2	0.0243± 0.0031	0.0240±0.0020	0.0227±0.0021
JM4	0.078±0.00264	0.0923±0.0025	0.0567±0.0015
JM5	0.0753±0.0040	0.0747±0.0035	0.0933±0.0025
JM7	0.1050±0.0020	0.0703±0.0025	0.0850±0.0020
JM10	0.0553±0.0025	0.0827±0.0031	0.0950±0.0020
JM11	0.1063±0.0025	0.0643±0.0032	0.0133±0.0025
JM14	0.0446±0.0015	0.0533±0.0015	0.0757±0.0031
JM15	0.0530±0.0031	0.0530±0.0031	0.0333±0.0023
JM19	0.0370±0.0020	0.0263±0.0015	0.0720±0.0036
JM22	0.0333±0.0015	0.0417±0.0015	0.0337±0.0035
Control	**0.52±0.0039**	**0.352±0.0028**	**0.428±0.0033**

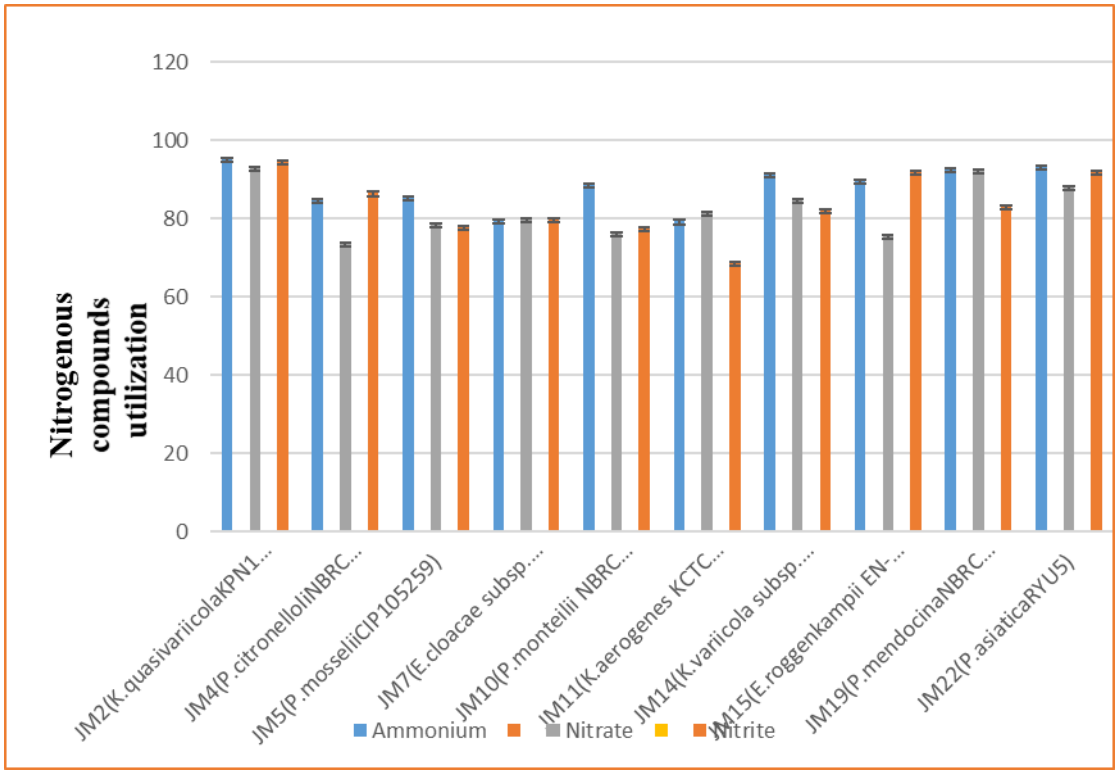

Figure 7: Percentage mean of nitrogenous compounds utilization in basal media after inoculating different bacterial isolates.

4.5 Nitrogenous compounds utilization in wastewater by the bacterial isolates

The tests indicated that all of the isolates selected after screening could utilize the three nitrogenous chemicals found in the effluent. The isolates revealed varied nitrogen removal activity among the three nitrogenous compounds **(Table 4)**. The percentage mean of each nitrogenous compounds reduction index ranged as follows: The percentage mean of each nitrogenous compound reduction index ranged as follows: Ammonium: 93.03% (JM2) to 66.67% (JM10). Nitrate: 41.51% (JM22) to 11.15% (JM11). Nitrite: 89.09% (JM11) to 8.41% (JM15). (**Figure 8**). A statistically significant test result was ($P \leq 0.05$) which means that the test hypothesis was false. The test hypothesis was therefore rejected because there was a statistical significance between the sample means.

Table 4: Average mean of absorbance of nitrogenous compounds utilization from the wastewater.

Isolates Sample ID	**Mean±SD of absorbance (Absorbance Units)**		
	Ammonium	**Nitrate**	**Nitrite**
JM2-HB	0.0377±0.0021	0.3337±0.0025	0.1857±0.0025
JM4-HB	0.0547±0.0015	0.2743±0.0015	0.1757±0.0025
JM5-HB	0.0687±0.0015	0.2653±0.0035	0.1650±0.0030
JM7-HS1	0.0660±0.0017	0.3843±0.0015	0.1860±0.0030
JM10-HS	0.0750±0.0030	0.2647±0.0015	0.3753±0.0025
JM11-KS	0.0600±0.0200	0.2850±0.0020	0.2033±0.0025
JM14-LS	0.0540±0.0020	0.3253±0.0031	0.2863±0.0031
JM15-LS	0.0753±0.0031	0.3140±0.0020	0.3743±0.0015
JM19-LS	0.0327±0.0021	0.3477±0.0015	0.2733±0.025
JM22-DB	0.0643±0.0025	0.2363±0.0015	0.2270±0.0020
Control Sample ID	**Ammonium**	**Nitrate**	**Nitrite**
Kisat_WWTP(KS)	0.2140± 0.0024	0.4333± 0.0034	0.3470± 0.0040
Homabay_Beach(HB)	0.2247± 0.0027	0.4097± 0.0015	0.3873± 0.036
Homabay_WWTP(HS)	0.2257± 0.0017	0.4400± 0.0028	0.2240± 0.030
Lagoon_WWTP(LS)	0.2867± 0.0012	0.4287± 0.0019	0.4094± 0.0024
Dunga_Beach(DB)	0.2153± 0.0018	0.4040± 0.0015	0.3190± 0.0026

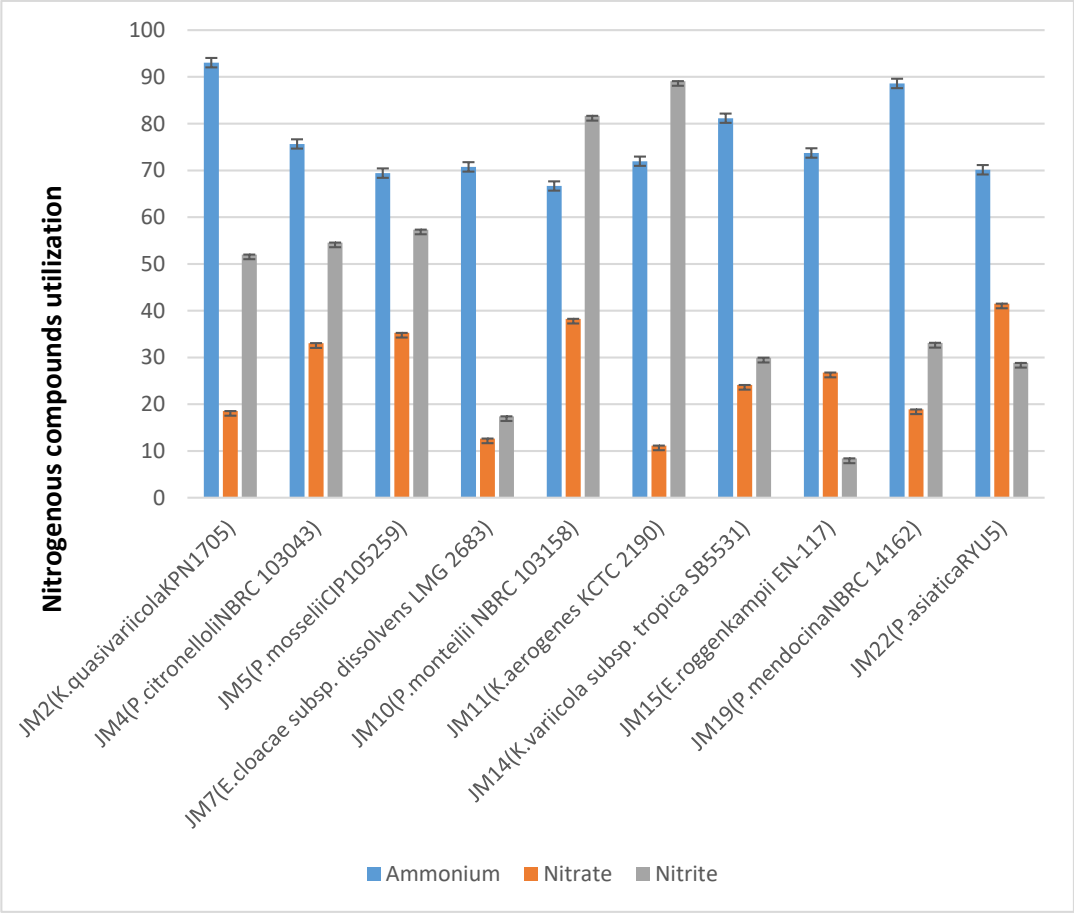

Figure 8: Percentage mean of Nitrogenous compounds utilization in wastewater through bacterial isolates inoculation.

4.6 Phylogenetic analysis of 16S rRNA gene libraries

Phylogenetic study suggests that the isolates were split into two clusters: the first cluster consist of members of the *Enterobacter* and *Klebsiella* and the other cluster has the members of *Pseudomonas* (**Figure 9**). Analysis of the sequences revealed that the recovered isolates had closest matches to Gammaproteobacteria group.

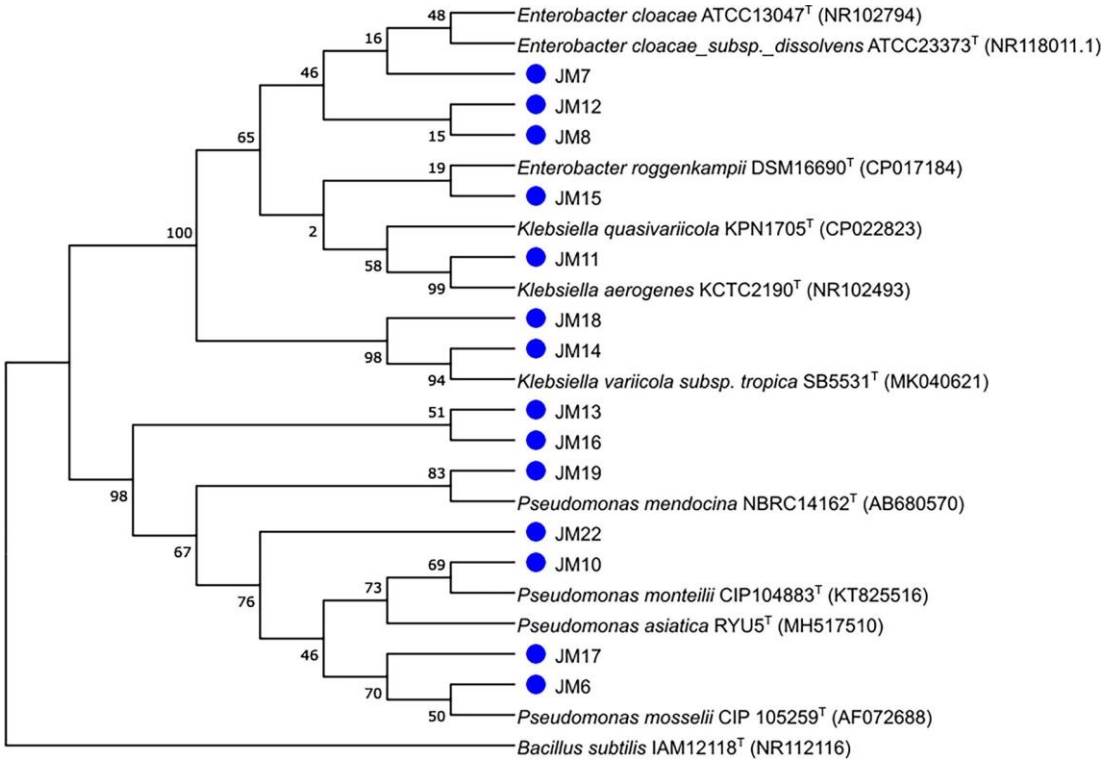

Figure 9: Evolutionary relationships of taxa based on 16S rRNA gene sequences, inferred using the neighbour-joining method. The ideal tree is displayed. The branches are accompanied with the proportion of replicate trees in which the linked taxa clustered during the bootstrap test (500 replicates). The evolutionary distances, which are measured in base substitutions per site, were assessed using the Maximum Composite Likelihood technique. MEGA X program was used to conduct evolutionary studies, with *Bacillus subtilis* IAM12118 serving as the out-group.

Table 5: Isolates obtained through culture dependent methods and their similarities to known type strains.

Sample Name & Isolates Origin	**Sample Accession Number**	**Related Type Strain**	**Type Strain Accession Number**	**Similarity to Type Strain**	**Sequence Size (bp)**
JM2 (Homabay Beach)	ON227428	*Klebsiella quasivariicola*	KPN1705	99.84	670
JM4 (Homabay Beach)	ON227429	*Pseudomonas citronellolis*	NBRC 103043	100	457
JM5 (Homabay Beach)	ON227430	*Pseudomonas mosselii*	CIP 105259	100	468
JM6 (Homabay Beach)	ON227431	*Pseudomonas mosselii*	CIP 105259	100	531
JM7 (Homabay WWTP)	ON227432	*Enterobacter cloacae subsp. dissolvens*	LMG 2683	99.86	715
JM8 (Homabay WWTP)	ON227433	*Enterobacter cloacae subsp. dissolvens*	LMG 2683	100	850
JM10(Homabay WWTP)	ON227417	*Pseudomonas monteilii*	NBRC 103158	100	788
JM11 (Kisat WWTP)	ON227418	*Klebsiella aerogenes*	KCTC 2190	100	834
JM12 (Lagoon WWTP)	ON227419	*Enterobacter cloacae subsp. dissolvens*	LMG 2683	100	751
JM13 (Lagoon WWTP)	ON227420	*ATKM_s*	P818	100	759
JM14 (Lagoon WWTP)	ON227421	*Klebsiella variicola subsp. tropica*	SB5531	99.84	626
JM15 (Lagoon WWTP)	ON227422	*Enterobacter roggenkampii*	EN-117	100	660
JM16 (Lagoon WWTP)	ON227423	*ATKM_s*	P818	99.11	803
JM17 (Homabay Beach)	ON227424	*Pseudomonas mosselii*	CIP 105259	100	568
JM18 (Lagoon WWTP)	ON227425	*Klebsiella variicola subsp. tropica*	SB5531	99.87	801
JM19 (Lagoon WWTP)	ON227426	*Pseudomonas mendocina*	NBRC 14162	100	710
JM22 (Dunga Beach)	ON227427	*Pseudomonas asiatica*	RYU5	100	748

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

Lake Victoria is the world's second-largest freshwater reservoir and it's highly threatened by uncontrolled release of pollutants into its waters. The release of these pollutants to the waters has greatly affected the freshwater ecosystems through eutrophication (Kabenge *et al*., 2016). Studies on microorganisms from these habitats will offer a new insight on the potential use of pure cultures in treating nitrogen-rich wastewater. The study explored a detailed experimental comparison of bacterial communities between eight water samples; from rivers and WWTP that drains to Lake Victoria. Water analysis showed that WWTP were highly polluted compared to the rivers and the lake. Elevated levels of pollution in WWTP caused a significant decline of the bacterial density in those areas. Similar study reported a significant change in microbial density as well as community functionality due to excessive accumulation of pollutants in WWTPs (Wang *et al*., 2012; Chakraborty & Bhadury, 2015).

Metagenomics analysis of the *16S rRNA* gene pyrosequencing reported more details on bacterial density. Applying diversity indexes; Richness, Simpson and Shannon's analysis, both lake and river samples showed high significance compared to WWTP samples. At sample level, diversity indexes recorded more richness in sample J3 (Homabay B) and less in sample J4 (Homabay WWTP). Between the two samples, there was a divergence in terms of biodiverversity, which may have been caused by different influent wastewater composition. Physicochemical analysis gave more details on water pollution, where biochemical oxygen demand was higher in WWTP compared to rivers and lake waters. Based on the analysis of physicochemical parameters **(Table 1)**, WWTP had higher levels of biochemical oxygen demand than rivers and lake, this factor may lower the amount of dissolved oxygen hence resulting to the anoxic environment which may significantly reduce and regulate the bacterial density in the WWTPs (Xu *et al*., 2019). A higher level of chloride ions revealed high salinity levels, which is an important factor in shaping the bacterial

community in WWTP (Wang *et al*., 2014). Additionally, *Planktothrix agardhii* was very limited to one sample, being extremely high in sample J2 (Lagoon WWTP) accounting for 18.9% of the resulting OTUs, and being extremely low in other samples accounting for less than 0.1%. *Cyanobacteria* that produce toxins, like *Planktothrix agardhii,* can cause ecosystem degradation because they thrive in environments that are frequently low in dissolved oxygen and abundant in nutrients (Monteagudo *et al*., 2016). *Cyanobacteria* are a diverse and widespread community that can serve as a bioindicator to monitor eutrophication (Soltani *et al*., 2012).

The three phyla that predominated in all of the samples were *Proteobacteria, Bacteroidetes,* and *Firmicutes,* and these findings were consistent with previous research on wastewater treatment (Paiva *et al*., (2015). *Proteobacteria,* which accounted for almost 59% of all bacterial sequences, was the most abundant phylum. On the phylum *Proteobacteria, Betaproteobacteria* were rich in rivers and lake waters compared to WWTPs accounting for 54.5%. Other study reported the same on class *Betaproteobacteria,* showing high significance in freshwater due to nutrients and a favorable pH (Newton *et al*., 2011). *Gammaproteobacteria* were also available and they were dominant in WWTPs compared to other samples **(Figure 3).** Bacterial communities enriched in WWTPs were mainly limited to *Gammaproteobacteria* and studies have reported their competence to perform denitrification process (Chen *et al*., 2018). *Bacteriodetes* attributed 19% of all bacterial sequences and was the second most abundant phylum. Within the phylum *Bacteriodetes,* the two classes*; Bacteroidia* and *Sphingobacteria,* were the most abundant among other classes within the phylum, and they were dominant in WWTP compared to rivers and lake **(Figure 3)**. The most dominant class was *Bacteroidia* accounting for < 0.7% to 8.8% of bacteria in all the samples. The results were consistent with the study done by Niestępski *et al*. (2020), where *Bacteroidia* accounted for < 0.1% to 8% of all the bacterial communities. In other study, *Bacteroidia* and *Sphingobacteria* were isolated and they were reported to poses the ability to decompose complex organic compounds from anaerobic treatment of the wastewater. Phylum *Nitrospirae* was also detected in freshwater samples, which performs a crucial role in the nitrogen cycling as nitrite oxidizers (Mehran *et al*., 2020).

Lakes and rivers had a higher richness of major genera than treated and untreated WWTPs, owing to higher levels of organic pollutants from WWTPs (Wang *et al*.,

2012). Statistical analysis showed that genus *Dechromonas spp* dominated all samples accounting for 5% of bacterial in all samples. *Dechromonas spp* are facultative anaerobes, nitrite reducers and they were originally isolated as aromatic compounds degraders (Coates *et al*., 2001). *Dechloromonas spp* have been detected from different studies done in aerobic and anaerobic WWTPs (Terashima *et al*., 2016). *Dechloromonas spp* is very important in the removal of nitrogenous compounds through denitrification as well as the breakdown of organic compounds in both oxic and anoxic habitats. *Hydrogenophaga spp* was the second richest genus with 4% of bacteria in all the samples. The genus had a higher richness in sample J4 (untreated WWTP) and low in sample J7 (treated WWTP), and it accounted for 12% and 0.8% respectively. One possible reason for its dominance is because of higher levels of organic compounds present in untreated WWTP than in treated WWTP. It is well-documented that *Hydrogenophaga spp* can utilize organic carbon through denitrification metabolism in both anaerobic and aerobic settings (Gonzalez-Martinez *et al*., 2016). *Planktothrix agardhii*, *Thauera mechernichensis*, and *Hydrogenophaga sp* were more ubiquitous in untreated WWTP than in treated WWTP due to elevated levels of pollutants and favorable environmental conditions in untreated WWTPs (Wear *et al*., 2021).

PCA ordination graph revealed the ecological distance between the sampling sites according to the water chemistry and microbial diversity. Clustering of sampling sites was due to their similarities with respect to diversity and physicochemical parameters. In comparison, the treated and untreated WWTP had a different tendency. The ecological distance between the two sample types was far apart due to the variation in microbial diversity and water chemistry.

Following 16S rRNA gene sequencing, culture-dependent analysis revealed that all bacterial isolates matched to the genera *Enterobacter*, *Pseudomonas*, and *Klebsiella*, and BLAST results confirmed this. All isolates were allocated to phylum *Proteobacteria*, class *Gammaproteobacteria*. As compared to other genera, *Pseudomonas* bacteria dominated. This study is consistent with the study done from the Michelson water reclamation plant (MWRP) showing high dominance in the members of *Pseudomonas* species (Fang *et al*., 2020). *Pseudomonas* is one of the most prevalent genera of Gram-negative rod bacteria in soils as well as waterways, and it is well-adapted to diverse habitats. *Pseudomonas* has the potential to tolerate

variety of organic pollutants and other stressors within the environment. It has a high biodegradation capacity hence making it play crucial role in wastewater treatment (Yong *et al*., 2015). *Klebsiella spp* was present in the samples analyzed. *Klebsiella sp,* which is a Gram-negative bacteria genus, was detected in a WWTP in Damascus, with an ability to biodegrade chlorpyrifos (Ghanem *et al*., 2007). Few of the isolates were detected from genus *Enterobacter*, which is a Gram-negative, rod-shaped bacteria of the family *Enterobacteriaceae*. *Enterobacteria sp* was isolated from the wastewater from olive oil extraction, revealing its ability to break simple aromatic compounds.

The findings reveals that the three genera were distributed into 10 isolates and they all exhibited positive activity in both screening media and from the wastewater. These isolates showed varied nitrogen removal activity among the three nitrogenous compounds. The test hypothesis is false because $P \leq 0.05$ and hence there is a statistical significant in all observations. The most significant finding involved isolate JM2 (*Klebsiella quasivariicola*), which consumed a significant amount of nitrate as well as ammonium from both wastewater and screening media. *Klebsiella quasivariicola* displayed the highest utilization of nitrate and ammonium from the basal media, with low absorbance values of 95.32% and 93.18%, respectively, as well as 93.03% of total ammonium in the effluent. According to a comparative study on *Klebsiella sp*., co-cultured strains elevated total nitrogen removal potential, with ammonia removal efficiencies of 99.64% (Zhang *et al*., 2019).

The research has demonstrated that novel strains of bacteria exist in Lake Victoria as well as WWTPs, which may efficiently exploit nitrogenous substances through coupled nitrification and denitrification mechanisms. For instance, the findings of the present study demonstrated that *Pseudomonas mendocina* strain GL6 had significant aerobic denitrification capacity, with a nitrate removal rate of 6.61 mg (N) L 1 h 1. The denitrification genes *napA*, *nirK*, *norB*, and *nosZ* have been discovered in strain *Pseudomonas mendocina* GL6 through sequence amplification (Zhang *et al*., 2019). In an experiment on *Enterobacter cloacae*, simultaneous heterotrophic nitrification and aerobic denitrification processes were found to be effective at removing phosphorus and denitrifying nitrogen successfully (Wan *et al*., 2017). Another study suggests *Pseudomonas asiatica,* which may be used for affordable biological

remediation of p-nitrophenol (PNP) polluted reservoirs and holds the most intense reported breakdown PNP capacity from the Ganges water of India (Alam & Saha, 2022). According to findings, *Klebsiella aerogenes* and *Klebsiella quasivariicola* can bioremediate effluent through coupled heterotrophic nitrification and aerobic denitrification process. (Padhi *et al*., 2013; Feng *et al*., 2018). *Pseudomonas citronellolis* has been revealed to be capable of achieving 100% nitrate and nitrite removal efficiency by utilizing sodium succinate as a carbon source in aerobic and anaerobic environments (Wang *et al*., 2020). *Pseudomonas mosselii* K17 has also been well documented for its capacity to display exceptional effectiveness in heterotrophic nitrification and aerobic denitrification process under a broad spectrum of ammonia concentrations (Hu *et al*., 2022)

5.2 CONCLUSION

Proteobacteria was the most abundant phylum, and within that phylum, *Betaproteobacteria* was rich in freshwater, whereas *Gammaproteobacteria* dominated in WWTPs samples. From the findings, freshwaters (lake and rivers) had higher bacterial diversity compared to WWTPs, which was likely caused by the high concentration of organic and inorganic contaminants present in WWTPs. All positive isolates were assigned to the phylum *Proteobacteria*, class *Gammaproteobacteria*. Members of *Pseudomonas, Enterobacter* and *Klebsiella* were able to perform coupled heterotrophic nitrification and aerobic denitrification process. All 10 bacterial isolates were able to effectively remove nitrogenous substances from wastewater, demonstrating their capacity to utilize oxygen and nitrogen as electron acceptors during the screening and the effluent phases. Bacterial isolate JM2 (*Klebsiella quasivariicola*) removed a significant amount of nitrate and ammonium from both wastewater and screening media samples.

5.3 RECOMEDATIONS

- Potential isolates should be preserved and utilized to remove extra nitrogen from wastewater effluents.
- It is necessary to conduct empirical investigation on all potential microorganisms isolated from eutrophic ecosystems, including whole genome sequencing and amplification of nitrogen cycle genes.

- Further investigation is necessary to identify and characterize more promising strains that are competent to carry out the SND process. This will be important for future treatment of contaminated sites since it will broaden our knowledge on the structure, function, and mechanisms of aerobic denitrifiers available in polluted aquatic habitats.
- In situ bioremediation techniques such as biostimulation technique can be applied to boost aerobic degradation of microbes involved in SND process.
- Several eutrophic coastal and marine waterbodies should be sampled at different seasons of the year in order to comprehend the microbial density and activity in various water reservoirs in a given period of time.

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APPENDICES

Appendix 1: Basal media preparation

The screening medium (pH 7.2) included the following reagents per liter: sodium succinate, 2.84 g: $NaNO_3$, 10 mM; KH_2PO_4, 1.36 g; $(NH_4)2SO_4$, 0.27 g; Yeast Extract, 1g; $MgSO_4.7H_2O$, 0.19 g; TE (trace element) solution, 1 ml. Solid BTB (pH 7.0-7.3) included the following reagents per liter: agar, 20g; KNO_3, 1g; KH_2PO_4, 1g; $FeCl_2.6H_2O$, 0.5g;, $CaCl_2.7H_2O$, 0.2g; $MgSO_4.7H_2O$, 1 g; sodium succinate, 8.5g and BTB reagent (1% in alcohol) 1ml.

Appendix 2: DNA extraction solution

Stock solution:

M Tris-HCl

0.5 M EDTA

5 M NaCl

Solution A:

1 M Tris-HCl pH 8.5

4 ml- 0.5 M EDTA pH 8.0

10 g- Sucrose

Adjust the volume to 50 ml using sterile distilled water and filter.

Solution B (lysis buffer):

20 ml- 400 mM Tris-HCl pH 8.5

6 ml- 60 mM EDTA pH 8.0

1.5 ml- 5 M NaCl

0.5 g- SDS

Heat the solution to dissolve SDS and adjust the volume to 50 ml using sterile distilled water.

Appendix 3: Working concentrated stock of the Electrophoresis buffer

50× TAE buffer

Chemical	Volume
Trisma base	242 g
EDTA	18.61 g
Glacial acetic acid	57.1 ml

Stir to dissolve Trisma base and EDTA into 700 ml of distilled water followed byaddition of Glacial acetic acid and adjust the volume to 1 liter.

1× TAE buffer: Dissolve 20 ml of 50× TAE buffer with 980 ml of distilled water.

Appendix 4: Light absorbance of bacterial isolates in screening media

Nutrients	Replication	JM2	JM4	JM5	JM7	JM10	JM11	JM14	JM15	JM19	JM22
Ammonium	**AB1**	0.039	0.056	0.067	0.065	0.075	0.06	0.054	0.076	0.031	0.067
	AB2	0.035	0.053	0.069	0.068	0.078	0.08	0.052	0.072	0.035	0.069
	AB3	0.036	0.055	0.07	0.065	0.072	0.04	0.056	0.078	0.032	0.7
Nitrate	**AB1**	0.334	0.273	0.262	0.386	0.266	0.283	0.322	0.314	0.346	0.327
	AB2	0.331	0.276	0.269	0.384	0.263	0.285	0.326	0.316	0.348	0.325
	AB3	0.336	0.274	0.265	0.383	0.265	0.287	0.328	0.312	0.349	0.323
Nitrite	**AB1**	0.186	0.173	0.165	0.186	0.378	0.201	0.283	0.374	0.27	0.241
	AB2	0.183	0.176	0.162	0.189	0.373	0.206	0.289	0.376	0.25	0.243
	AB3	0.188	0.178	0.168	0.183	0.375	0.203	0.287	0.373	0.3	0.246

Appendix 5: Light absorbance of bacterial isolates in wastewater

Nutrients	Replication	JM2	JM4	JM5	JM7	JM10	JM11	JM14	JM15	JM19	JM22
Ammonium	**AB1**	0.021	0.079	0.071	0.107	0.055	0.106	0.043	0.053	0.037	0.032
	AB2	0.025	0.075	0.076	0.105	0.053	0.104	0.046	0.056	0.035	0.035
	AB3	0.027	0.08	0.079	0.103	0.058	0.109	0.045	0.05	0.039	0.033
Nitrate	**AB1**	0.022	0.092	0.078	0.07	0.082	0.063	0.053	0.087	0.026	0.043
	AB2	0.024	0.095	0.075	0.073	0.086	0.068	0.055	0.085	0.028	0.042
	AB3	0.026	0.09	0.071	0.068	0.08	0.062	0.052	0.084	0.025	0.04
Nitrite	**AB1**	0.022	0.057	0.091	0.085	0.095	0.011	0.073	0.032	0.071	0.034
	AB2	0.025	0.055	0.093	0.083	0.097	0.013	0.075	0.036	0.069	0.03
	AB3	0.021	0.058	0.096	0.087	0.093	0.016	0.079	0.032	0.076	0.037

Appendix 6: **Nucleotide sequences of the isolates**

JM2_907-R_E01_13-*Klebsiella quasivariicola* KPN1705

TTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTC
GCACCTGARCGTCAGTMTTTGTCCAGGGGGCCGCCTTCSCCACCGGTRTT
CCTCCCTATCTCTACGCATTTCACCGCTACACCKGAAATTCTACCCCCCTC
TACAAGACTCTAGCCTGCCAGKTTYGAATGCAGTTCCCAGGTTGAGCCCG
GGGATTTCACAT

JM4_907-R_F01_16- *Pseudomonas citronellolis* NBRC 103043

ACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCC
ACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGYCGCCTTCGCCA
CTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCA
CCACCCTCTACCRTACTCTAGYYAGGCAGTTATGGATGCMGTTCCCAGGT
TGAGCCCGGGGATTTCACATCCATCTTACCAAACCACCTACGCGCGCTTT
ACGCCCAGTAATTCCGATTAACGCTTGCACCCTYYGTATTACCGCGGCTG
CTGGCACRAAGTTAGCCGGTGCTTATTCTGKTGGTAACGTCAAAACAGCA
AGGYATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCG
AAACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGT
CCAATA

JM5_907-R_G01_19-*Pseudomonas mosselii* CIP 105259

TGCGTTAGCTGCGCCACTAAAATCTCAAGGATTCCAACGGCTAGTTGAYA
TCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACG
CTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCGCCACTG
GWGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACC
ACCCTCTACCATACTCTAGCTCGCCAGTTTTGGATGCAGTTCCCAGGTTG
AGCCCGGGGCTTTCACATCCAACTTAACGAACCACCTACGCGCGCTTTAC
GCCCAGTAATTCCGATTAACGCTTGCACCCTCTGTATTACCGCGGCTGCT
GGCRCAGAGTTARCCGGTGCTTATTCTGTCGGTAACGTCAAAACAGCAAG
GTATTARCTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGAAG
ACCTTCTTCACACACGC

JM7_907-R_A02_02-*Enterobacter cloacae* subsp. dissolven LMG 2683

TACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAATCG
ACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCC
ACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCA
CCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTA
CCCCCCTCTACAAGACTCTAGCCTGCCAGTTTCGAATGCAGTTCCCAGGT
TGAGCCCGGGGATTTCACATCCGACTTGACAGACCGCCTGCGTGCGCTTT
ACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTG
CTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATYGMTG
WGGTTATTAACCWCAACRCCTTCCTCCCCGCTGAAAGTRCTTTACAACCC
GAAGGCCTTCTTCAYACACGCGGSATGGCTGCATCAGGCTTGCGCCCATT
GTGCAATATTCCCCACTGCTGCCTCCCGTAGGARTCTGGACCGTGTCTCA
GTTCCAGTGTGGSTGGTCATCCTCTCAGACCAGCTAGGGATCGYCGCCTA
GGTGAGCCGTTACCCCACCTACTAGCTAATCCCATCTGGGCACATCTGAT

GGCAAGAGGCCCGAAGGTCCCCCTCTTTGGTCTTGCGACGTTATGCGGTA
TTAGCTACCGTTTCCA

JM10_907-R_C04_07-*Pseudomonas monteilii* NBRC 103158

TTAGCTGCGCCACTAAAATCTCAAGGATTCCAACGGCTAGTYGACATCGT
TTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTC
GCACCTCAGTGTCAGTATCAGTCCAGGKGGTCGCCTTCGCCACTGGTGTT
CCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCCTC
TACCGTACTCTAGCTCGCCAGTTTTGGATGCAGTTCCCAGGTTGAGCCCG
GGGCTTTCACATCCAACTTAACGAACCACCTACGCGCGCTTTACGCCCAG
TAATTCCGATTAACGCTTGCACCCTCTGTATTACCGCGGCTGCTGGCACA
GAGTTAGCCGGTGCTTATTCTGTCGGTAACGTCAAAACAGCAAGGTATTA
RCTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTC
TTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATT
CCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGT
GACTGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTGAGCCAT
TACCYCACCAACTAGCTAATCCGACCTAGGCTCATCTGATARCGCAAGGC
CCGAAGGTCCCCTGCTTTCTCCCGTAGGACGTATGCGGTATTAGCGYTCC
TTTCGAAAACGTTGTCCCCCACTACCAGGCAGATTCCTAGGCATTACTCM
CCCGYCCGCCGCTGAATCAAGGAGMAAGCTCCCGT

JM11_907-R_D04_10-*Klebsiella aerogenes* KCTC 2190

AACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCG
ACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCC
ACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCA
CCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTA
CCCCCCTCTACAAGACTCTAGCCTGCCAGTTTCGAATGCAGTTCCCAGGT
TGAGCCCGGGGATTTCACATCCGACTTGACAGACCGCCTGCGTGCGCTTT
ACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTG
CTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGAGTAACGTCAATCRCYAA
GGTTATTAACCTTAAYGCCTTCCTCCTCGCTGAAAGTACTTTACAACCCG
AAGGCCTTCTTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTG
TGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAG
TTCCAGTGTGGCTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAG
GTGAGCCATTACCCCACCTACTAGCTAATCCCATCTGGGCACATCTGATG
GCATGAGGCCCGAAGGTCCCCCACTTTGGTCTTGCGACRTTATGCGGTAT
TAGCTACCGTTTTCCAGTAGTTATCCCCCTCCATCAGGCAGTTTCCCAGAC
ATTACTCACCCGTCCGCCGCTCGTCACCCGAGAGCAAGSTCTCTGTGYTA
CCGCTCGACTTGCATGTGTTAGGCCTGCCGCCA

JM14_907-R_F04_16-*Klebsiella variicola* subsp. tropica SB5531

CTCAAGGGCACAACCTCCAAATCSAYWTCGTTTACAGCGTGGACTACCA
GGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCT
TTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA
TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACAAGACTCTAGCCTGC
CAGTTTCGAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCGACT
TGACAGACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTT
GCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCT
TCTGCGGGTAACGTCAATCGAYRAGGKTATTAACCTYAYCGCCTTCCTCC

CCGCTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATG
GCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCC
GTAGGAGTCTGGACCGTGTCTCAGTTCCAGKGTGGCTGGTCATCCTCTCA
GACCAGCTAGGGATCGTCGCCTAGGTGAGCCGTTACCCCACCTACCAGCT
AATCCCATCTGGGCACATCTGATGG

JM15_907-R_A03_03-*Enterobacter roggenkampii* EN-117

TAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTC
GACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCC
CACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCC
ACCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCT
ACCCCCCTCTACAAGACTCTAGCCTGCCAGTTTCGAATGCAGTTCCCAGG
TTGAGCCCGGGGATTTCACATCCGACTTGACAGACCGCCTGCGTGCGCTT
TACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCT
GCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATYGCTG
AGGTTATTAACCTCAACRCCTTCCTCCCCGCTGAAAGTACTTTACAACCC
GAAGGCCTTCTTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATT
GTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGAACCGTGTCTC
AGTTCCAGTGTGGCTGGTCCATCCTCTCAGACCAGCTAGGGATCGTYGCC
TAGGTGAGCCGTTACCCCACCTACTAGCCTAATCCCATCTGGGCACATCT
GATGGCAAGA

JM19_907-R_E03_15-*Pseudomonas mendocina* NBRC 14162

ATGCGTTAGCTGCGCCACTAARATCTCAAGGAWTCCAACGGCTAGTYGA
YWTCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCA
CGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCGCCAC
TGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCAC
CACCCTCTACCGTACTCTAGCTCGCCAGTTTTGGATGCAGTTCCCAGGTTG
AGCCCGGGGCTTTCACATCCAACTTAACGAACCACCTACGCGCGCTTTAC
GCCCAGTAATTCCGATTAACGCTTGCACCCTTCGTATTACCGCGGCTGCT
GGCACGAAGTTAGCCGGTGCTTATTCTGTYGGTAACGTCAAAAYACTMA
MGTATTARGTKAATRCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGA
AGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGT
CCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGT
TCCAGTGTGACTGATCATCCTCTCARACCAGTTACGGATCGTCGCCTTGG
TGAGCCATTACCTCACCAACTAGCTAATCCGACCTAGGCTCATCTGATRG
CGCAAGGYCCGAAGGTCCCCTGCTTTCTCCCGTAGGACGTAYGCGGTATT
AGCGTTCCTT

JM22_907-R_G03_21-*Pseudomonas asiatica* RYU5

AATGCGTTAGCTGCGCCACTAAAATCTCAAGGATTCCAACGGCTAGTYGA
CWTCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCA
CGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGKGGTCGCCTTCGCCAC
TGGKGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCA
CCACCCTCTACCGTACTCTAGCTCGCCAGTTTTGGATGCAGTTCCCAGGTT
GAGCCCGGGGCTTTCACATCCAACTTAACGAACCACCTACGCGCGCTTTA
CGCCCAGTAATTCCGATTAACGCTTGCACCCTCTGTATTACCGCGGCTGC
TGGCACAGAGTTAGCCGGTGCTTATTCTGTCGGTAACGTCAAAACAGCAA
GGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGAA

GACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTC
CAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGKGTCTCAGTT
CCAGTGTGACTGATCATCCTCTCARACCAGTTACGGATCGTCGCCTTGGT
GAGCCATTACCTCACCAACTAGCTAATCCGACCTAGGCTCATCTGATAGC
GCAAGGCCCGAAGGTCCCCTGCTTTCTCCCGTAGGACGTATGCGGKATTA
GCGTTCCTTTCGAAACGTTGTCCCCCACTACCAGGGCAGATTCCTA