Addis Ababa University

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Nitrogen Fixing Alkaliphiles from Lake Chitu, a Soda Lake in the Central Rift Valley of Ethiopia

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Abstract

Lake Chitu is a tropical crater lake located in the Ethiopian Rift Valley 287 km south of Addis. It is a small soda lake unusually with high primary productivity. The lake water has high pH, salinity, $HCO^{3-} + CO_3^{2-}$*, alkalinity,* Na^+ *and* Cl^- *ions and frequently limiting levels of inorganic nitrogen compounds due to high rate of denitrification. Microbial* N_2 *fixation is expected to play an important role in the production of bioavailable nitrogen. To test this hypothesis the diversity, abundance and vertical distribution, of Lake Chitu diazotrophic microbial communities were investigated at 6 sampling sites including 0 m, 2.9 m, 5 m, 10 m, 14 m and 17 m depth using cultivation based and molecular methods. .A total of 120 isolates cultivated on nitrogen free medium cultures were analyzed to cultivation-based and molecular analysis. 19 isolates representative of each OTU were analyzed for NifH gene and 16S rRNA gene amplification and sequencing. PCR-DGGE analysis was also carried out from enrichment and environmental samples. 120 isolates clustered in to 9 operational taxonomic group (OTU).The presence of NifH gene was detected by PCR in 11 out of 19 representative isolates. The nifH gene sequences retrieved were closely related with more than 96% sequence similarity to nitrogenase (NifH) genes of uncultured microbial communities, retrieved from various sources. The results of the study indicate that alkaliphilic diazotrophic microbial communities were found along the whole water depth of the lakes including the surface water. However, the diversity of diazotrophic microbial communities was high at the bottom of the lake. The gene diversity depicted the presence of nitrogen-fixing bacteria affiliated phylum Proteobacteria, Firmicutes, and Actinobacteria.*

Keywords: Diazotrophs, Lake Chitu, nitrogenase, nitrogen fixation, Soda Lake

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List of Abbreviations and Acronyms

AAU	Addis Ababa University
ARDRA	Amplified Ribosomal DNA Restriction Analysis
BSA	Bovine serum albumin
CFU	Colony forming unit
G	Gram
IOB	Institute of Biotechnology
L	Liter
LB	Luria broth
NF	Nitrogen fixing
O.D	Optical density
OUT	Operational taxonomic unit
PBS	Phosphate buffered saline
PHA	Polyhydroxyalkanoate
Q.PCR	Quantitative polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RT-PCR	Reverse transcript polymerase chain reaction
SDS	Sodium dodecyl sulfate
SOC	Supplemental Organic Solutions
TMPD	N,N,N',N'-Tertamethyl-p-phenylenediamine
µl	Microliter
Ml	Milliliter

1. Introduction

Nitrogen fixation is an essential biochemical process that sustains life (Falcon *et al.*, 2004; Francis *et al.,* 2007). In the process atmospheric dinitrogen (N_2) is reduced to ammonia (NH_3), a form of nitrogen that can be utilized by living organisms to build nitrogenous compounds (Francis *et al.,* 2007). Nitrogen fixation takes place in industries by Harber–Bosch process and by biological process. Concern over greenhouse gasses emitted by the Harber–Bosch process had resulted in a research focus on nitrogen-fixing bacteria (Saikia and Jain, 2007). The biological fixation includes those microbes that exist as independent free-living organisms, or in associations of different degrees of complexity with other microbes, plants and animals symbiotically (Beneduzi *et al.,* 2008).

Nitrogen fixers are unique and special microorganisms including archaea and bacteria with the enzyme nitrogenase, which have the ability of converting N_2 gas directly from the atmosphere (Lindemann and Glover, 2003). Biological nitrogen fixation (BNF) makes the reduction of atmospheric nitrogen into bioavailable ammonia, using molybdenum- or alternative nitrogenases encoded by *nif*, *anf* or *vnf* genes (Kennedy and Tchan, 1992; Coelho *et al.*, 2009). This enzyme catalyzes the extremely energy expensive process of nitrogen fixation (Postgate, 1982).

The diversity studies of nitrogen fixing microbial species have revealed them to be unicellular cyanobacteria and anaerobic bacteria. It was not confirmed whether a similar phenomenon exists in lake environments or not until Breznik and Harper in 1969 detected small nitrogenase activity in the anoxic and aphotic conditions of dystrophic Lake Mize in Florida (Jäntti, 2007). Similarly, in oligotrophic Lake Michigan amplified *nif* sequences and low natural abundance of ^{15}N indicated that nitrogen fixation might be occurring below the photic zone although the actual rate was not measured (MacGregor *et al.,* 2001).

The methodological difficulties have hindered research in these areas but the development of stable isotope mass spectrometry and molecular biology has broadened the possibilities to investigate nitrogen fixing bacteria (Howarth *et al.,* 1988; Lindemann and Glover 2003; Capone *et al.,* 2006). The genetic potential for diazotrophy has also received increased attention and the methods in molecular biology have enabled these analyses. Currently the presence and the expression of the nitrogenase encoding *nifH* gene are generally analyzed by using the quantative polymerase chain reaction (Q-PCR) and the reverse transcriptase polymerase chain reaction (Shukla *et al.,* 2010).

The rate of nitrogen fixation in lake environment is controlled by various environmental parameters such as oxygen concentration, nutrient concentrations and light availability (Howarth *et al.,*1988). The oxygen concentration is an important regulatory factor because nitrogenase enzyme is easily destroyed by oxygen. This has lead to an evolution of various protective mechanisms such as the heterocyst in the filamentous cyanobacteria and alternation of nitrogenase production with photosynthesis in unicellular diazotrophic bacteria (Postgate, 1982; Wasmund *et al.,* 2005). Non-photosynthetic diazotrophs are less vulnerable to oxygen because they can inhabit the anoxic region of the water column and their energy is provided by other mechanisms than oxygen evolving photosynthesis (Montoya-Pino *et al.,* 2010). Besides oxygen concentration, nutrients are important controlling factors for nitrogen fixation (Howarth *et al.,* 1988; Arrigo, 2005).

One of the striking facts about soda lakes is that, despite apparently inhospitable caustic conditions, these environments are extremely productive. The high buffering capacity, high temperature of the environments, high light intensity and effectively unlimited supply of CO_2 via the HCO_3/CO_2 equilibrium created the unique and extremely productive alkaline environment (Jones *et al.,* 1998; Grant, 2006). The stability of the soda lakes and the environmental factors create an ideal breeding ground for algae. As a result, millions of birds flock to these lakes to feast on the abundant food supply of algae (Kebede *et al.*, 1994) . In case of Ethiopia different soda lakes naming Lake Arenguade, Lake Shalla, Lake Abijata, Lake Chitu and Lake Methara are located in the Rift Valley of Southern Ethiopia. The microbial biomass in soda lakes is highly dominated by prokaryotes but rarely by eukaryotic algae and protozoans (Jones *et al*., 1998; Grant, 2006).

Productive soda lake microbial community contains alkaliphilic representatives of all the major trophic groups of bacteria and Archaea. Between these groups, there is cycling of carbon, sulphur, and nitrogen under aerobic and anaerobic conditions present in the lakes (Rees *et al.,* 2004).

Nitrogen cycle in soda lakes, especially in hyper saline ones, may depend on an externally supplied source of $NO_{x.}$ (Sorokin *et al.,* 2014). However, in closed soda lakes, such as Lake Chitu where there is small input from external source of nitrogen and high rate of denitrification, biological nitrogen fixation is expected to contribute much to nitrogen economy of the lake. Even if nitrogen cycle in alkaline soda lakes is complicated and limited due to the concentration of nitrogen compounds NO_3 and NH_3 are often low or undetectable. Volatilization of NH_3 at high pH, cessation of nitrite production at high salt concentration, conversion of NH_3 mostly to toxic and volatile NH_3 at pH 10 and higher are major causes for nitrogen cycle limitation and product untraceability (Sorokin and Kuenen, 2005a).The capability to fix nitrogen is often considered an advantage in environments where nitrogen limitation is a common phenomenon. The genetic potential for nitrogen fixation has been detected in various organisms inhabiting aquatic environments including those living in the soda lake environment (Zani *et al.,* 2000; Arrigo, 2005).

Lake Chitu is a closed lake where there is no a significant inflow and out flow. There is no much external source of nitrogen compounds to the lake system from Flooding from surrounding farm, fertilizer or from any other means but there is high rate of denitrification. Thus, there should be active alkaliphilic nitrogen fixing bacterial community, which maintains the nitrogen economy of the lake. In this study, we address the diversity and vertical distribution of nitrogen fixing bacteria on different depth of Lake Chitu those win over the complication and provide inorganic nitrogen to alkaline soda lake stabilize nitrogen economy.

2. Objectives of the Study

2.1. General Objective

To assess the diversity of alkaliphilic nitrogen fixing bacterial community in Lake Chitu using culture-dependent and molecular methods.

2.2. Specific objectives

- To enrich nitrogen fixing bacterial community from different depths using nitrogen free media aerobically and anaerobically
- To determine the effect of oxygen on nitrogen fixation process
- To evaluate the interaction between nitrogen fixing and non fixing bacterial communities
- To determine the diversity of pure nitrogen fixing bacterial strains using 16S rRNA gene sequencing
- To detect the presence of NifH gene on specific isolates
- To assess the depth profile of nitrogen fixing bacterial community among different depths of the lake by DGGE.

3. LITERATURE REVIEW

3.1. Soda Lakes

Soda lakes are naturally occurring highly alkaline environments often exhibiting pH values >11.5 (Jones *et al.,* 1998). Soda lakes are characterized by large amounts of Na_2CO_3 and Na_2CO_3 complexes (usually $Na_2CO_3.10H_2O$ or $Na_2CO_3.NaHCO_3.2H_2O$), formed as the result of evaporative concentration under particular conditions of geology, geography and climate (Jones *et al*., 1998). They also contain no or very low concentration of calcium and magnesium ions due to the insolubility of these cations as carbonate minerals at alkaline conditions (Duckworth *et al.,* 1996a). Sodium chloride and other salts may also concentrate leading to the formation of alkaline saline lakes.

Soda lakes are saline ecosystems that are believed to have existed throughout the geological record of earth. They are widely distributed across the globe, but are highly abundant in terrestrial biomes such as deserts and steppes and in geologically interesting regions such as the East African Rift valley (Grant *et al.,* 1990; Jones *et al.*, 1994; Jones *et al*., 1998; Grant and Jones, 2000). Mostly these lakes are closed basins with no obvious outflow where ground water and seasonal streams flowing from the surrounding highlands collect to form (semi) permanent standing bodies of water. Surface evaporation rates exceed the rate of inflow of water allowing the dissolved minerals to concentrate into a caustic alkaline brine with CO_3^{+2} and Cl^- as major anions, creating a pH of 8.5 to >12 (Bryantseva *et al.,* 1999).

The remarkable geochemistry of these lakes supports the growth of an impressive array of microorganisms that are ecologically and economically important. Haloalkaliphilic bacteria and archaea belonging to all major trophic groups have been described from many soda lakes, including lakes with exceptionally high levels of heavy metals (Antony *et al.,* 2012; Antony *et al.,* 2013; Tourova *et al.,* 2013).

Soda lakes are widely distributed throughout the world (Fig. 1).Soda lakes are found in North America, Central America, South America, Europe, Asia (notably in Siberia, Outer Mongolia and Tibet), throughout Africa, and in Australia.

Although highly alkaline lakes are confined to specific geographic regions, more than 80% of all inland waters, by volume, are on the alkaline side of neutrality exhibiting a less intense form of the chemistry seen in the more concentrated and alkaline types (Grant *et al.,* 1990).

Figure 1. Map showing the distribution of soda lakes in the world (Lekang, 2009). Spots show the location of soda lakes.

Ethiopia has a number of soda lakes including lakes Arenguade, Shalla, Abijata, Chitu, and Methara. Most of the Ethiopian soda lakes are located in the floor of Rift Valley of southern Ethiopia. They are closed basins with no significant outflow, where groundwater and seasonal streams are flowing from the surrounding highlands to form permanent standing bodies of water (Gizaw, 1996; Le Turdu *et al.,* 1999). Surface evaporation rates allow the dissolved minerals to concentrate into alkali basins with Na^{+}, $HCO_3^{-,}$ CO_3^{2-}, and Cl^{-} as the major ions, creating saline alkaline lakes with pH values of 9 and above (Wood and Talling, 1988b; Kebede *et al.,* 1994; Gizaw, 1996).

3.2. Microbial Diversity of Soda Lakes

Despite apparently hostile conditions, soda lakes are the most productive aquatic environments in the world, with productivity rate in an order of magnitude greater than the mean rate for all aquatic environments on Earth (Jones *et al.,* 1998). Soda lakes are populated almost exclusively by prokaryotes, which can form dense communities (possibly due to the absence of grazing pressure) even in saturated alkaline brines

(Sorokin and Kuenen, 2005a). Alkaliphilic cyanobacteria drive these systems, providing fixed carbon that is utilized by a vast range of alkaliphilic aerobic and anaerobic chemo-organotrophs, notably *halomonads*, *Bacilli* and *Clostridia*. There is also cycling of nitrogen and sulfur in the lakes, brought about by alkaliphilic groups (Grant *et al.*, 1999).

A characteristic feature of the majority of soda lakes is the presence of permanent or seasonal colored blooms of phototropic microorganisms. Thus, dependent on the water chemistry, the lakes are likely to be green due to cyanobacteria (Fig. 2A), but may also have areas that are orange or red due to other organisms mainly during dry season of the year (Fig. 2B). Unlike other types of lakes, the microbial biomass is dominated by prokaryotes, with only rare instances where eukaryotic algae and protozoan make up a significant fraction of the population usually in the more dilute and less alkaline example of Soda Lake (Sorokin *et al.,* 2014).

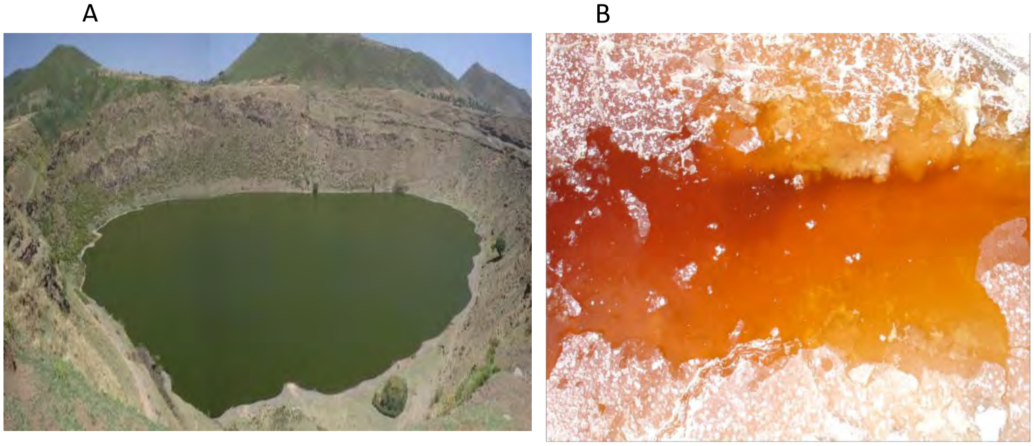

Figure 2.Microbial blooms in soda lakes. A: Green cyanobacteria bloom in Lake Areneguadae, Ethiopia; B: Red pigmentation due to haloalkaliphilic archaea on Trona crusts at Abijata-Shalla Soda Ash Concentration pond, Ethiopia (Simachew, 2015).

Recently, microbial diversities of soda lakes in the world have been investigated intensively by cultivation-based and molecular methods (Antony *et al.,* 2012; Banciu and Sorokin, 2013; Lanzen *et al.,* 2013). These studies indicate that soda lakes are habitats of surprisingly diverse microorganisms belonging to the major phylogenetic groups of bacteria including: *Cyanobacteria*, *Actinobacteria*, *Firmicutes*, *Verrucomicrobia*, *Proteobacteria* (including *alpha*, *beta*, *gamma*, and *delta* subdivisions), *Bacteroidetes*, *Spirochaetes*, *Chloroflexi*, *Thermotogae* (Fig. 3) and several candidate divisions of bacteria. Similarly, the result of these studies indicates that haloalkaliphilic members of archaea belonging to *Euryarchaeota* flourished in soda lakes.

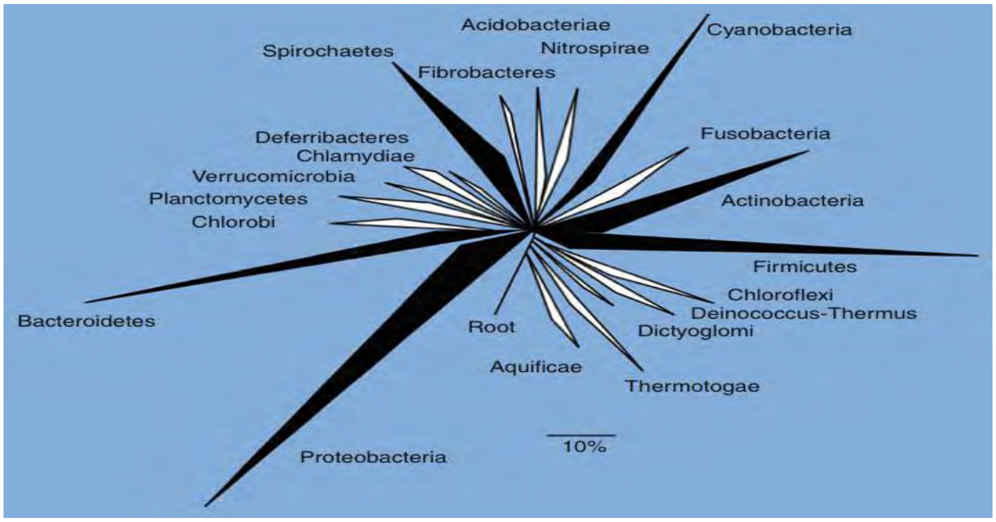

Figure 3.16S rRNA gene sequence tree showing the major bacterial lines of descent. Lines of descent with known soda lake isolates are shown shaded (Grant and Sorokin, 2011).

The majority of previous studies on soda lakes were focused almost exclusively on aerobic chemo-organotrophs (Jones *et al.,* 1998). However, recently there have been increasing numbers of effort to look at other groups, including phototrophs, lithotrophs, spirochetes, and a range of different anaerobes (Mwirichia *et al.,* 2010a). This has allowed the identification of most of the major trophic groups responsible for the cycling of carbon, sulfur and nitrogen in the lakes using the obvious parallels with better characterize aquatic systems (Ohlendieck *et al.,* 2000).

3.3 Adaptation made by microbes

Life on Soda Lake exhibits an enormous adaptive capacity like other living organisms found even in extreme environments. Bactria and archaea use different mechanism to compensate presence of high salinity and pH. To avoid osmotic shock, halophiles made the same ionic strength inside their cells as outside. All their macromolecules, including the proteins, have therefore adapted to remain folded and functional under such ionic strength conditions. As a result, the amino acid composition of proteins adapted to a hyper saline environment is very characteristic: they have an abundance of negatively charged residues combined with a low frequency of lysines (Rothschild and Mancinelli, 2001).

In order to cope with the alkaline conditions, alkaliphilic microbes have many adaptations including the presence of cytoplasmic polyamines with charged amino acids, which allow buffering from extremely alkaline environments. It has been observed that, in addition to peptidoglycan, there are acidic compounds such as galacturonic acid, gluconic acid, glutamic acid, aspartic acid, and phosphoric acid that act as buffers to the alkaline environment, allowing uptake of hydronium ions and exclusion of hydroxide ions (Horikoshi, K. 1999).This is known as passive regulation. Some microbes also actively maintain the pH of their cytoplasm by using sodium ion channels that take in Na+. When there is a buildup in the cytoplasm of Na+, the sodium ions are exported from the cell in exchange for hydrogen ions, which keeps the cell in equilibrium (Jäntti, H. 2007).

3.4. Nutrient cycling in soda lakes

The soda lake microbial community contains alkaliphilic representatives of all the major trophic groups of bacteria and archaea. Between these groups, there is cycling of carbon, sulphur, and nitrogen under aerobic and anaerobic conditions present in the lakes (Rees *et al.,* 2004)**.**Alkaliphilic cyanobacteria are responsible for a generally high level of primary production and nitrogen fixation (Sorokin *et al.,* 2004). The polymers produced by the primary producers are degraded by the aerobic and anaerobic hydrolytic bacteria (Humayoun *et al.,* 2003).

The sulphur cycle is active in the soda lakes by both the anaerobic sulphur reducing and the aerobic sulphur oxidizing bacteria (Sorokin and Kuenen, 2005c; Sorokin *et al.*, 2011). Similarly, the presence of methane cycle is well documented in soda lakes (Sorokin and Kuenen, 2005b). Different nutrient cycle taken place by active interaction of different bacteria as shown in figure 4.

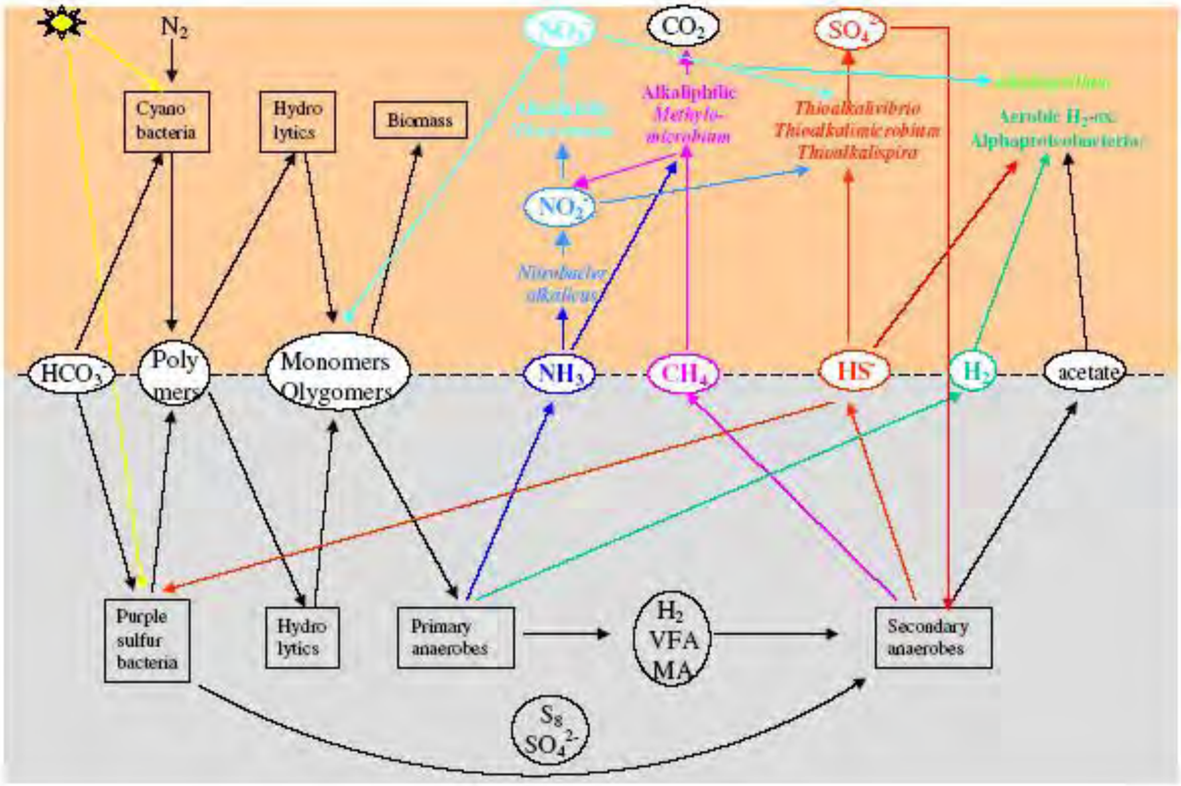

Figure 4.Trophic relationships in the soda lake ecosystem (Sorokin *et al*., 2005b)

3.5. Nitrogen cycle in soda lakes

Nitrogen undergoes many different transformations in the ecosystem, changing from one form to another as organisms use it for growth and in some cases for energy. The major transformations of nitrogen are nitrogen fixation, ammonification, nitrification, denitrification and anammox (Fig. 5). The transformation of nitrogen into its many oxidation states is input to productivity in the biosphere and is highly dependent on the activities of a diverse assemblage of microorganisms, such as Bacteria and Archaea (Vitousek *et al.,* 2002a). The nitrogen cycle is inhibited in hyper saline soda lakes due to cessation of nitrification at high pH and conversion of NH_4 to toxic and volatile NH_3causes potential nitrogen loss from the ecosystem (Zavarzin and Zhilina, 2000; Sorokin and Kuenen, 2005b). Therefore, the nitrogen cycle in soda lakes, especially in hyper saline ones may depend on an externally supplied source of $NO_{x.}$ (Gallon *et al.*, 2002).

3.5.1 Nitrogen Fixation

In the nitrogen cycle atmospheric dinitrogen (N_2) becomes bioavailable when it is reduced into ammonia (NH_3) in a process called nitrogen fixation (Vitousek *et al.,* 2002a;

Capone *et al.*, 2006). Nitrogen gas (N_2) makes up nearly 80% of the Earth's atmosphere, yet nitrogen is often the nutrient that limits primary production in many ecosystems (Galloway *et al.,* 2004). This happens because plants and animals cannot use nitrogen gas in that form. For nitrogen to be available to make proteins, DNA and other biologically important compounds, it must first be converted into a different chemical form.

Nitrogen gas is a very stable compound due to the strength of the triple bond between the nitrogen atoms, and it requires a large amount of energy to break this (Zehr *et al.,* 2003; Capone *et al.,*2006). During biological nitrogen fixation (BNF), molecular nitrogen is reduced in multiple electron-transfer reactions, resulting in the synthesis of ammonia and release of hydrogen (Formula 1) (Kneip *et al.,* 2007). Ammonium is then used for the subsequent synthesis of biomolecule. This reduction of molecular nitrogen to ammonium is catalyzed in all nitrogen-fixing organisms via the nitrogenase enzyme complex in an ATP-dependent, highly energy-consuming reaction.

Formula1: $N + 8H + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$

Nitrogen fixation (NF) is one of the most important indicators of microbial activity. A few studies on NF in soda lakes were identified haloalkaliphilic cyanobacteria as the only active diazotrophic group in water (Herbst, 1998; Zavarzin and Zhilina, 2000). Studies done latter found the potential of NF in a few haloalkaliphilic bacteria isolated from soda lake sediments such as the aerobic *Gamma Proteobacteria , Thialkalispira microaerophila* and *Alkalilimnicolahalodurans* (nifH gene detection) (Tourova *et al.,* 2007) as well as the obligate anaerobes *Clostridium alkalicellulosum* (Zhilina *et al.,* 2005) and *Geoalkalibacter ferrihydritucus* (Zavarzina *et al.,* 2006).

Nitrogen fixation rates vary according to the trophic status of the lake and the highest nitrogen fixation rates have been observed in eutrophic and hyper eutrophic lakes but in haloalkaline soda lake habitats evidence for NF activity is very scarce (Wetzel *et al.,* 2001)(Table 1) .The authors further noted that Mono Lake, the only soda lake shows non NF activity whereas other lakes show better activity based on their productivity. The occurrence of rapid and frequent changes in elevation, volume and solute concentration is familiar to closed-basin inland salt lakes, so the nitrogen fixation activity affected by salinity and other major factors that enclose direct effect on process can lead to the scarcity of NF activity even if alkaline soda lakes are productive (Herbst, 1998).

Table 1.The percent of total nitrogen input to the system contributed by nitrogen fixation in various types of lakes measured by acetylene reduction (AR) (Wetzel, 2001)

Lake	**Trophic State**	**% of annual N loading**	**Measuring Method**	**References**
Lake Erken, Sweden	Eutrophic	82	AR	(Howarth *et al.*, 1988)
Clear Lake, U.S.A	Eutrophic	43	AR	(Horne, 1972)
Lake Võrtsjärv, Estonia	Eutrophic	2.34	AR	(Tõnno, 2005)
Lake Verevi,Estonia	Hyper Eutrophic	Less than 1	AR	(Tõnno, 2005)
Lake Washington, U.S.A	Mesotrophic	0.29	AR	(Howarth *et al.,* 1988)
Pyramid LakeU.S.A	Oligotrophic	81	AR	(Howarth *et al.,* 1988)
Lakes Superior,and Michigan, U.S.A	Oligotrophic	0.02	AR	(Howarth *et al.,* 1988)
Mono Lake, U.S.A	Oligotrophic Haloalkaline	0	AR^{15}N tracer	(Strous, 1999)

3.5.2 Nitrification

Nitrification is the process that supplies oxidized forms of nitrogen for assimilation and anaerobic respiration and contributes to the primary production. Very little was known about its operation in extremely alkaline environments, such as soda lakes. Indirect evidence, such as the presence of active population of denitrifying and nitrate-utilizing bacteria, including heterotrophic aloalkaliphilic *Halomonas sp.* and chemolithoautotrophic sulphur oxidizing bacteria (SOB), indicated that nitrogen oxides might be produced in the alkaline habitats (Sorokin and Kuenen, 2005b).

Most nitrification occurs aerobically and is carried out exclusively by prokaryotes (Sorokin *et al.,* 2014). There are two distinct steps of nitrification that are carried out by distinct types of microorganisms in soda lakes. Ammonium oxidation to nitrite is performed by an extremely alkali-tolerant subpopulation of *Nitrosomonas halophila*, whilst nitrite oxidation can be performed by the moderately alkali-tolerant *Nitrobacter alkalicus* (Sorokin and Kuenen, 2005b). Nitrite production ceased at higher NH_3 concentrations and at maximum salt concentration. Nitrification is come to an end in soda lakes because of maximum concentration for nitrification to occur in soda lakes is 1 M of total Na^+ (Sorokin *et al.,* 2014).

3.5.3. Anammox

Traditionally, all nitrification was thought to be carried out under aerobic conditions, but recently a new type of ammonia oxidation occurring under anoxic conditions was discovered. Anammox is carried out by prokaryotes belonging to the phylum *Planctomycetes* of bacteria (Francis *et al.,* 2007). Anammox bacteria oxidize ammonia by using nitrite as the electron acceptor to produce gaseous nitrogen (Strous *et al.,* 1997; Kuenen, 2008).

3.5.4. Ammonification

When an organism excretes waste or dies, the nitrogen in its tissues is in the form of organic nitrogen (e.g. amino acids, DNA). Various fungi and prokaryotes then decompose the tissue and release inorganic nitrogen back into the ecosystem as ammonia in the process known as ammonification. The ammonia then becomes available for uptake by plants and other microorganisms for growth (Jetten, 2008).

3.5.5. Denitrification

Denitrification is the process that converts nitrate to nitrogen gas, thus removing bioavailable nitrogen and returning it to the atmosphere. Dinitrogen gas (N_2) is the ultimate end product of denitrification, but other intermediate gaseous forms of nitrogen exist (Figure 5). Some of these gases, such as nitrous oxide (N_2O), are considered greenhouse gasses, reacting with ozone and contributing to air pollution. Similar to nitrogen fixation, denitrification is carried out by a diverse group of prokaryotes, and there are evidences that some eukaryotes are also capable of denitrification (Risgaard-Petersen, 2006). Denitrification is common in Ethiopian soda lakes.Diverse denitrifying bacterial strains found in Lake Abijata, Lake Arenguade, Lake Chitu and Lake Shalla.The *Bacillus* species and *Proteobacteria* species are dominant in Chitu Lake (Lulit,T., 2010).

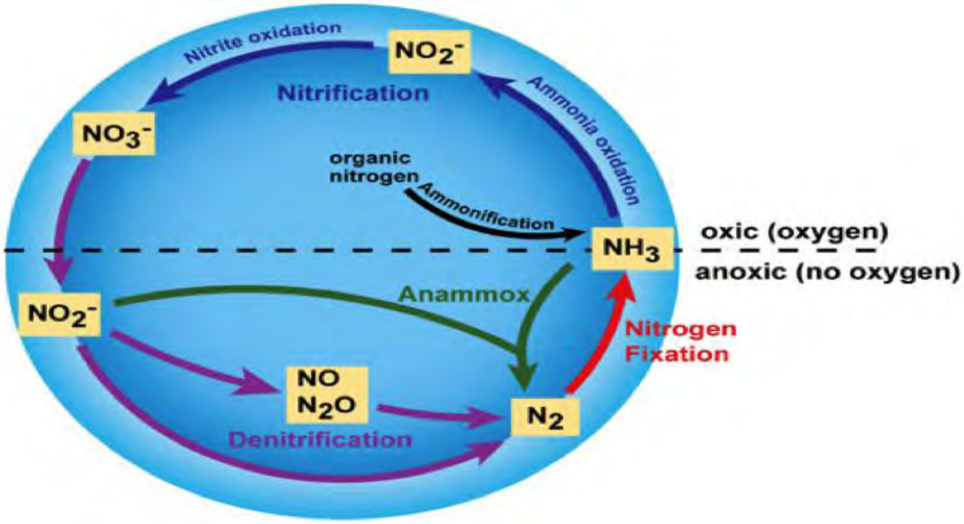

Figure 5. Major transformations in the nitrogen cycle (van Breemen, 2002)

3.6. Physical structure of the nitrogenase enzyme

The nitrogenase enzyme, which exists in nitrogen fixing bacteria, consists of distinct proteins, dinitrogenase and dinitrogenase reductase (Fig. 6). The larger protein, dinitrogenase (shades of blue), consists of four sub-units that can be divided into two types, and the small protein, (shades of green), consists of two sub-units those are similar with each other.

Both proteins contain iron and sulfur. Dinitrogenase has additional molybdenum. The molybdenum and iron in dinitrogenase are contributed by the cofactor known as FeMoCo and the actual reduction of dinitrogen involves participation of this iron-molybdenum center (Postgate, 1982). During molybdenum limitation so called alternative-nitrogenases are formed. Alternative nitrogenases do not contain molybdenum but instead vanadium and iron or only iron. The alternative nitrogenases are presumed to be a back-up mechanism in molybdenum limiting conditions (Postgate, 1982; Howarth *et al.,* 1988).

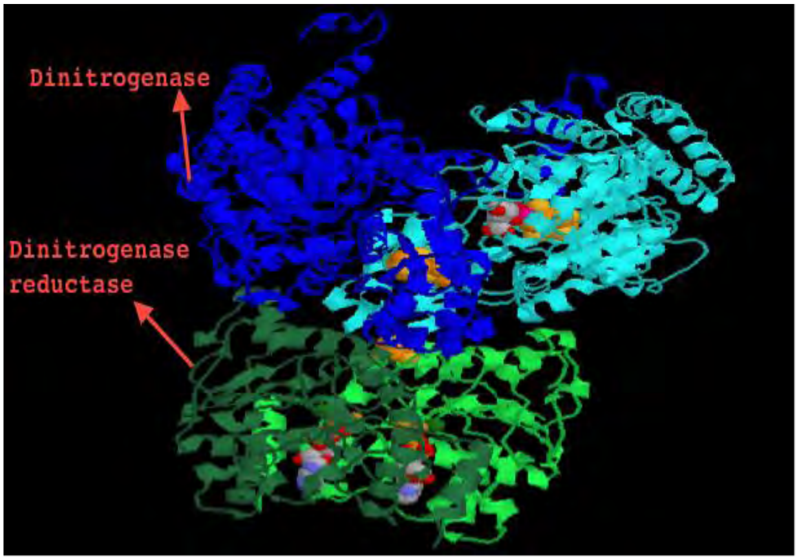

Figure 6. Structure of Nitrogenase from the free-living bacterium *Azotobactervinelandii*stabilizedby ADP-tetrafluoroaluminate(Khalsa-Moyers, 2010).

3.7. Genetic structure of the nitrogenase enzyme

The biological reduction of dinitrogen to ammonia is an extremely complex process that genetically controlled by the *nif* regulon, which encodes for the nitrogenase enzyme. Nitrogenase is large (up to 300kDa) compared to many other enzymes, and relatively slowly reacting, with one enzyme taking 1.25s to form two NH_3^+ molecules (Postgate, 1982).

The formation and maintenance of the nitrogenase enzyme complex require a major investment of protein up to 30% of total cell protein, energy (ATP) and trace metals (Mo and Fe) (Hagen, *et al.,* 1987). The trace metals incorporate into the active core of two different components associated with nitrogenase, a Mo-Fe protein known as dinitrogenase and a Fe protein known as dinitrogenase reductase (Haaker and Klugkist, 1987).

The genetic information of the *nif* regulon has been determined mainly by sequencing the nitrogen fixing organism *Klebsiella pneumoniae*. The regulon contains over 30 genes including 24206 base pairs that encode for nitrogenases structural units, FeMoCo, and regulatory genes (MacGregor *et al.,* 2001; Johnston *et al.,* 2005). What is peculiar about the *nif* regulon is that some of the *nif* genes overlap and it has several binding sites in different operons for the main regulatory *nif A* protein (Johnston *et al.,* 2005).

Table 2. The identified subunits and their function in the *nif* regulon (Johnston *et al.*, 2005)

nif D**:** The α subunit of the dinitrogenase

nif K**:** The β subunit of the dinitrogenase

nif H**:** the dinitrogenase reductase protein dimmer

***nif N, E, B, V* and *Q*:** FeMoCo

nif A**:** positive regulatory protein activates transcription of other *nif* genes.

nif L**:** negative regulatory protein, represses transcription of other *nif* genes

nif F**:** flavodoxin, physiological reductant of the Fe protein

nif J**:** Pyruvate-flavodoxin-oxidoreductase

nif M**:** activation of the Fe protein

nif S, U and Y**:** Processing of FeMo protein

nif W and Z**:** Required for full activity of FeMo protein

3.8. Mechanism of the reduction of N2to ammonia

The reduction process is catalyzed by the enzyme complex nitrogenase (Masepohl *et al.,* 2002). Dinitrogenase reductase accepts electrons from a low-redox donor, such as reduced ferrodoxin or flavodoxin, and binds two MgATP. It transfers electrons, one at a time, to dinitrogenase. Dinitrogenase reductase and dinitrogenase form a complex, the electron is transferred and two MgATP molecules are hydrolyzed to two MgADP+Pi. Dinitrogenase reductase and dinitrogenase dissociate and the process is then repeated. When dinitrogenase has collected enough electrons, it binds a molecule of dinitrogen, reduces it, and releases ammonia. Dinitrogenase then accepts additional electrons from dinitrogenase reductase to repeat the cycle (Fig.7).

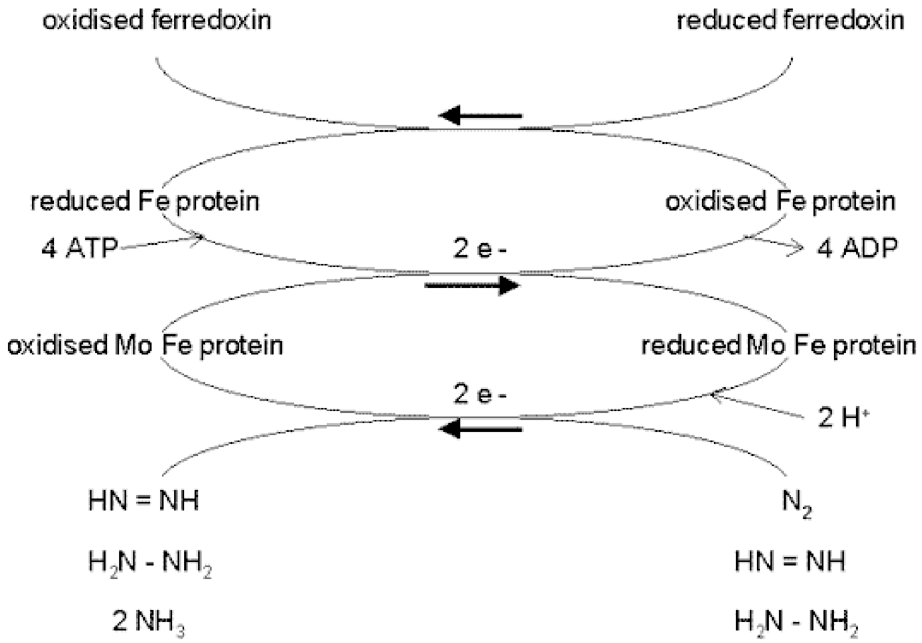

Figure 7. Mechanism of biological nitrogen fixation (Masepohl *et al.*, 2005)

3.9. Factors controlling nitrogen fixation

3.9.1. Oxygen

Nitrogen fixation is a very oxygen sensitive process and diazotrophic species have developed various mechanisms to protect the nitrogenase enzyme (Postgate, 1982). Numerous strategies have been adopted to avoid O_2 including (1) life without O_2 (2) high rates of O_2 respiration, (3) conformational protection, and (4) heterocyst utilization (Madigan, 2003). For example filamentous cyanobacteria physically separate the nitrogen fixing unit from the rest of the cell with heterocysts. Heterocysts are separate cell units with thick cell walls that exclude oxygen and protect the oxygen sensitive nitrogenase enzyme so that photosynthesis can be maintained during nitrogen fixation. This is very beneficial because photosynthesis supplies energy for nitrogen fixation. However, not all phototrophic diazotrophs have heterocysts. Several unicellular phototropic diazotrophs have developed a cycle, where nitrogen fixation is separated temporarily so that the oxygen evolving photosynthesis occurs during the day and nitrogen fixation during respiration in low oxygen concentrations at night (Postgate, 1982). Some nitrogen fixers are also able to maintain undamaged nitrogenase under oxic conditions by conformational protection. In this case, nitrogenase proteins undergo a reversible conformational change to a state that is unaffected by O_2. In this way, the enzyme becomes inoperative but remains undamaged, and can readily resume nitrogen fixation as soon as O_2 disappears (Postgate, 1982).

3.9.2. Light

More robust mechanisms often involve adjusting the nitrogenase activity with the light availability instead of regulating the light harvesting pigments (Lewis and Levine, 1984).Depending on the environment and the species adaptations, light can either inhibit or stimulate nitrogen fixation rate. Heterocyst bearing cyanobacteria exhibited strong dial variation in their nitrogen fixation behavior. Increasing light intensities stimulated nitrogen fixation and cyanobacteria began migrating towards light. The cyanobacteria migrated to the surface during the highest light intensities and descended when light availability decreased. The migration and nitrogen fixation pattern varied between species (Lewis and Levine, 1984).

3.9.3. Temperature

Extreme temperatures affect nitrogen fixation adversely. This is easy to understand because nitrogen fixation is an enzymatic process. However, there are differences between microorganisms in their ability to tolerate high (>35°C) and low (<25°C) temperatures. In addition to effect on enzymatic process, it also increase occurrence of volatilization as water temperatures increase.

Diazotrophs are well adapted to different kinds of temperatures. Although the temperature optimum for most diazotrophs is well above 15°C, exception are found even from the Antarctic, where temperatures are extremely low and the light availability is very restricted. Cyanobacterial strains isolated from Antarctica were able to perform their metabolic functions normally in temperatures as low as 5°C and their temperature optima was 10°C lower than for their counterparts in the tropics (Pandey *et al.,* 2004).

3.9.4. PH

The pH level exists in soda lakes affects nitrogen cycle. As alkalinity increases a pH greater than 7.5 allows for considerable loss of ammonium in the water converted to toxic ammonia at higher rate. Ammonia is volatile so alkaline condition will lead to loss of ammonia from the ecosystem due to volatilization (Sorokin *et al.,* 2001).In particular, the availability of metal cofactors is very important for activity of nitrogenase enzyme like zinc, iron, manganese and boron could be limited at highly alkaline conditions and confines activity of nitrogen fixation. Fortunately, the carbonate ion forms alkaline complexes with the metal ions, which are much more soluble than the respective hydroxides (Johannesson and Lyons, 1994).

3.9.5. N: P Ratio

It is a widely accepted assumption that the most important nutrients that determine primary production in aquatic ecosystems are nitrogen and phosphorus. The net primary production in lake and estuarine environments is typically considered to be phosphorus limited with periodic nitrogen limitation during midsummer (Wetzel, 2001).It has been proposed that nitrogen fixation by cyanobacteria is triggered by low N: P ratio and there are various examples where nitrogen limitation is observed during Cyanobacterial blooms (Howarth *et al.,* 1988). However, low N: P ratio does not always trigger nitrogen fixation.

3.9.6. Salinity

The effect of salinity on nitrogen fixation is an essential consideration to the nitrogen budget and dynamics of soda lake ecosystems. Salinity exerts a powerful control on the fate of nitrogen introduced to system. Salinity controls nitrogen cycling through several mechanisms. First, salinity directly affects ammonium release from sediment particles through ion exchange(Herbst, 1998; Howarth and Marino, 2006). Under conditions of low salinity in more nitrogen released through decomposition but remains associated with sediment particles. As salinity rises, ammonium is released from the sediments and enters the overlying water. Second, salinity has a large effect on rates of microbial processes because of biological stress to microbial assemblages that resulted in smaller and less efficient microbial communities (Rietz and Haynes, 2003; Jackson and Vallaire, 2009). Nitrification, the oxidation of ammonium to nitrate, is especially sensitive to salinity changes. As salinity rises nitrification rates decrease. The numbers of ammonium oxidizing bacteria also decrease although the overall structure of the ammonia oxidizing bacteria (AOB) community does not change (Sorokin and Kuenen, 2005b).

3.10. Methods for Measuring Nitrogen

3.10.1. Measurement of Nitrogen Fixation with the Growth in nitrogen-free Medium

The traditional method for detection of biological nitrogen fixation had been the ocular assay based on observation of growth of microorganisms on what is alleged to be nitrogen-free medium. Though not a quantitative procedure, this method deserves some consideration because of its usefulness and historically it has led to the investigation of many microorganisms known or alleged to fix nitrogen (Mague and Burris, 1972).

3.10.2. Measurement of Nitrogen Fixation by Stable Isotope (15N) Method

The most definitive measurements of biological nitrogen fixation make use of the stable, heavy isotope, ^{15}N, and require access to a mass spectrometer. In this method, incorporation of $^{15}N_2$ (labeled dinitrogen) into plant or microbial cells is measured, being a straightforward method. Samples are exposed to an atmosphere of about 10% $^{15}N_2$, usually in a balance of argon or helium to eliminate competition from $^{14}N_2$. Following incubation, the samples are digested and the ^{15}N content of the materials is determined using a mass spectrometer. Detection of ^{15}N in tissues or cells provides definitive proof of N_2 fixation and allows very accurate quantification of the amount of dinitrogen fixation (Montoya *et al.*, 1996).

3.10.3. Measurement of Nitrogen Fixation by the Acetylene Reduction Assay

The acetylene reduction assay is a convenient, indirect method to assay the activity of nitrogenase enzyme system. It was developed based on the fact that the nitrogenase system can reduce several alternative substrates, such as acetylene (C_2H_2), which is reduced to ethylene (C_2H_4) (Jensen and Cox, 1983).The assay is performed by incubating the nitrogen fixing organism (a bacterial culture, a soil sample, or root nodules) in a gas-tight vessel and 5–10% of acetylene is added to the vessel atmosphere. At this acetylene concentration, the nitrogenase system stops reducing dinitrogen and turns completely into an acetylene-reducing system, because the affinity of the enzyme for acetylene is very high. Since the organisms do not require the product of the reaction, it is released to the atmosphere of the vessel. By sampling at regular intervals and analyzing the gas samples by gas chromatography, it is possible to estimate the activity of the nitrogenase(Havens *et al.*, 2003).

The acetylene reduction rate has to be converted into the dinitrogen reduction rate. The theoretical conversion ratio is 3 moles of acetylene reduced per 1 mole of dinitrogen reduced, thus the six moles of reductant required to reduce one mole of dinitrogen would instead reduce three moles of acetylene. The hydrogen evolution occurring during nitrogen fixation involves additional electron transfers hence it is generally assumed that the conversion factor is 4:1 (Galloway *et al.*, 2004). However, it should be noted that this conversion factor is known to vary, especially in natural environments (Kayanne *et al.*, 2005).

3.10.4. Measurements by applying modern molecular biological methods

The development of molecular biology has enabled investigations of the genetic potential and the genetic expression of nitrogen fixation. This is generally done by investigating the *nifH* gene fragments with quantitative polymerase chain reaction (Q-PCR) and quantitative reverse transcriptase polymerase chain reaction (Q-RT-PCR). These methods measure the potential and the activity of the *nifH* gene but not the actual volumetric rate of nitrogen fixation (Church *et al.,* 2005). Besides *nifH* gene activity measurements, lots of emphasis has been put on to quantify the diversity of the diazotrophic species. This is generally done by constructing phylogenetic trees from amplified 16S rRNA and comparing *nifH* gene sequences (Mazard *et al*., 2004).

4. Materials and methods

4.1. Description of the study area

Lake Chitu is part of the Abijata-Shalla Lakes National Park located in the Oromiya region, Ethiopia. It is located at about 287 km south of Addis Ababa and lies at about 1.5 Km south of lake Shalla at 7°24'0"N and 38°25'0.02"E (Fig. 8). Lake Chitu is a cup like with a diameter of 1.6 x 1.2 Km.The lake is a small, relatively shallow, volcanic crater that is caused by an explosion when ground water comes into contact with hot lava or magma (Legesse *et al.,* 2002). The resulting crater is filled with water to form relatively shallow crater Lake. It covers approximately 800 m^2, has a maximum depth of 21 m and highly saline (around 6% salinity). The estimated terrain elevation above sea level is 1573 m. The lake is within the closed basin and lacks surface outflow and inflows, and receives water from direct precipitation and a few hot springs located at its shores. The lake region has semi-arid to sub-humid type of climate with mean annual precipitation and air temperature of 600 mm and 25°C, respectively. It experiences high evaporative concentration, which is the major factor for its saline–alkaline nature (Wood and Talling, 1988a).In the lake there is high rate of denitrification in absence of much external source of nitrogen, due to this its selected to study active nitrogen fixing bacteria which balance nitrogen loss of the lake and make it productive lake.

Figure 8: Satellite image of Lake Chitu showing its surroundings and location (Source: Google Earth)

4.2. Sample collection

Water and mud samples were collected from Lake Chitu at 07^{0}24’15.7”N, 38^{0}25’17.2”E at elevation of 1556 m.a.s.l. Water samples were collected at six depths including the surface (0 m). Mud sample was also collected at the depth of 17 m. 2.9 m depth chosen because its transition zone from aerobic to anaerobic .0m and 17m to perceive the surface and bottom nitrogen fixing bacteria .The rest depth favor randomly to see middle nitrogen fixing community (Table 3). The samples were collected using bottle sampler and Ekmangrab for water and mud samples, respectively. The aphotic zone of the lake was measured using a Secchi disc. Then the samples were transferred to sterilized sample bottles and Falcon tubes in replicates. Some physicochemical parameters including pH, salinity, conductivity and temperature were measured on site. The samples were transported to the laboratory of Institute of Biotechnology, Addis Ababa University in icebox containing cooling elements on the same date of sampling. Then the samples were preserved at 4 ºC for further analysis.

Table 3: Sampling site and sample types

Sample type	Water depth(meter)	Sample designation
Water	0	NF0m
Water	2.9	NF2.9m
Water	5	NF5m
Water	10	NF10m
Water	14	NF14m
Mud	17	NFM

4.3. Microbial analysis

4.3.1. Enrichment cultures

To isolate nitrogen fixing bacterial isolates, water samples were enriched on nitrogen free broth and solid media prepared according to Jensen’s media with slight modification (Jensen, 1942). The enrichment media with a composition 55 g/l NaCl,0.2 g/l, $MgSo_4.7H_2O$,0.1 g/l k_2HPO_4, 0.26 g/l $CaCl.2H_20$ and 0.1 g/l $FeSo_4$.Trace metals compositions 0.03 g/l H_3Bo_3, 0.003g/l $NaMo_4.2H_2O$ and $MnCl_2.4H_2O$ plus 0.01g/l

$ZnSo_4.7H_2O$ and $CoCl.6H_2O$ with 0.002g/l $NiCl.6H_2O$, 0.001g/l $CaCl.2H_2O$. The media components were mixed and boiled in a hot plate until the crystals of the components dissolved. Then pH was adjusted to 7.6 by 1M NaOH and allowed to cool. Next to this all the media components were autoclaved at 121°c for 15 min. Na_2CO_3 (1%) and glucose (0.5%) autoclaved separately were added to the medium after cooled approximately 50 to 60 ºC. Trace metals solution (1 ml/100 ml) was added to the medium before pouring. Then it was poured to sterilized 100ml culture bottles and 250ml Erlenmeyer flasks for anaerobic and aerobic cultivation, correspondingly. In case of anaerobic cultivation the media was filled up to top of the culture bottle to minimize the incidence of oxygen.

Well-mixed water sample was inoculated (1%) to the enrichment broth media under aseptic condition. For anaerobic cultivation the culture bottles was sealed by Parafilm and incubated after removing the air by a burning candle in an anaerobic jar. Then the culture bottles were incubated at 30 ºC for seven days in a culture box (Electrical heat Thermostatic Culture Box DH5000B) while Erlenmeyer flasks were incubated aerobically on the rotary shaker (120 rpm) at 30 ºC for a week. To capture the dominant nitrogen fixing bacterial community, 1 ml of 7 day old enrichment culture was transferred to a fresh broth medium (passage one) then to passage two.

4.3.2. Isolation of nitrogen fixing bacterial strains

To isolate pure strains solid medium with the same composition as that of enrichments except contained 2% agar was used. Enrichment culture samples taken at the maximal growth of the last passage (passage two) were serially diluted 10^1 to 10^5 using filter-sterilized 1X PBS buffer, pH 10. Then 100 µl serially diluted enrichment culture was spreaded onto agar plates. The plates were incubated at 30 ºC until clear distinct colonies formed. In case of anaerobic sample the plate was sealed by Parafilm and incubated after removing the air by a burning candle in an anaerobic jar. When the colonies grew very well a single distinct colony was isolated randomly and streaked onto agar plates. At the same time total viable count or colony forming units (CFUs) was determined from plates contained colony numbers in the range of 30 to 300 . On average 30 isolates were picked from each enrichment agar plate and a total of 180 isolates were picked. All these isolates were kept on nitrogen rich agar slants at 4 ºC for further characterization.

Formula 2: (Number of colonies) X (dilution factor) X (vol. of inoculums) = CFUs/ml

4.3.3. Effect of oxygen on nitrogen fixing strains

The effect of oxygen on the nitrogen fixing strains was determined by cultivating the abundant pure nitrogen fixing strains in the presence and absence of oxygen on nitrogen free medium. To do so a loopful culture of the three isolates selected from abundant OTU groups, namely NF0m15, NF5m19 and NFM6 were taken and inoculated into a liquid nitrogen free media under aerobic and anaerobic conditions. Then their growth was monitored as optical density (600 nm, UV-7804C spectrophotometer) every 24 hour for fifteen days. In the case of anaerobic cultivation a single culture bottle was used for every 24 measurement to avoid the effect of oxygen entering the bottle while it was opened during measurement.

4.3.4. Co-culture of nitrogen fixing bacterial isolates with non fixing isolates

Co-culture of nitrogen fixing bacteria with known non-fixing bacteria was carried out.To test the nitrogen fixation potential of fixer and release of ammonia to surrounding. The non-fixing experimental bacterial isolates were obtained from the culture collection of bacterial isolates maintained in the Microbial Biotechnology Laboratory of the Institute of Biotechnology, Addis Ababa University (IOB – AAU). The non-fixing experimental bacterial isolates were isolated previously from water samples of Lake Abijata, Ethiopian Rift Valley Soda Lake, and studied for their biotechnological potentials (Addis Simachew, 2015).

First the non-fixing experimental bacterial isolates were screened for co-culturing based on their ability to use ammonia as nitrogen source, their distinct colony color compared to the nitrogen fixing bacterial isolates and their ability to grow in anaerobic condition. Then a single bacterial isolate from nitrogen fixers and non-fixers each were co-cultured using three set ups. In the first set up the nitrogen fixing bacteria (NF10m6) alone was cultured in nitrogen free medium broth. In the second set up, the nitrogen fixing bacteria (NF10m6) and non nitrogen-fixing bacteria isolate, *Alkalibacterium sp*. 3.5R*1 (JX434738), were co-cultured in a nitrogen free broth medium. And in the third set up non-nitrogen fixing bacteria isolate, *Alkalibacterium sp*. 3.5R*1 (JX434738), was cultured alone. All cultures were incubated at 30 ºC for ten days under anaerobic condition.

Following the incubation, a loopful of culture from each set up was serial diluted and spreaded on a nitrogen rich solid medium and incubated as described above. The growth of isolates of the nitrogen fixing and non-fixing was determined based on their colony color after 24 h of incubation.

4.3.5. Biochemical tests

Gram test, Oxidase test and Catalase test were done on the representative 20 isolates of each OTU group. Gram test was carried out based a method describe previously (Pacarynuk *et al.,* 2005). Briefly, a loopful of freshly grown colonies were picked from agar plates were added on a clean glass slide contained a drop of 3% potassium hydroxide (KOH) solution. The colonies were stirred for about a minute occasionally lifting the loop to check for the thickening and elasticity of the slurry. The Oxidase test was performed using N,N,N',N'-tertamethyl-p-phenylenediamine (TMPD) to check whether the isolates could produce certain cytochrome C oxidases or not by adding 3-5 drops of TMPD on the filter paper and observed the results within 2 min. Catalase test was done using 3% H_2O_2.

4.3.6 Colony morphology analysis

To identify the colony shape, the plate was placed on a dissecting microscope. The shape of the colonies of the isolates was determined according to the standard characteristics described in the ninth edition of Bergey's Manual of Systemic Bacteriology (Garrity *et al.,* 2004).

4.4. Molecular-based analysis

4.4.1. Biomass collection

Environmental microbial biomass was harvested from 100 ml water sample by filtration on a 0.2-µm polycarbonate filters using Bio RAD vacuum pump. The filter papers contained biomass were put in a sterile Eppendrof tubes and preserved at -20 ºC for DNA extraction. Enrichment microbial community biomass was harvested from enrichment cell culture collected into sterile 14 ml Falcon tubes. The culture was centrifuged at 3700 rpm for 20 min at 4 ºC (OrtoaresaCosult 21 R). The pellet was resuspended in 5ml

1%PBS buffer (filter-sterilized) and centrifuged again as described above. Cell pellets were preserved at -20 ºC for DNA extraction.

4.4.2. Microbial community genomic DNA extraction

Community DNA was extracted from cell pellets and polycarbonate filters (0.2 μm polycarbonate filters) from six depth using a modified CTAB method (Bengtsson *et al.*, 2010). The filters were transferred into new Eppendorf tubes aseptically. The cell pellets and cells on the filters were suspended in a solution of lysoszyme (1mg/ml) in TE buffer (10 mMTris-HCl, 1 mM EDTA, pH 8) and incubated at 37 ºC for 30 min shaking horizontally at 200 rpm. Proteinase K was added to a final concentration of 40 µg $ml^{-1}$and samples were incubated for additional 30 min under the same conditions described for lysoszyme.

Sodium dodecyl sulphate (SDS) was added to a final concentration of 0.5 mg $ml^{-1}$and samples were incubated at 55 °C for 1h, gently inverting the tubes at 15 min interval. Then a 5M NaCl solution was added to give a final concentration of 1M and CTAB (from a stock solution of 10% of cetyltrimethylammonium bromide in 0.7% NaCl) to a final concentration of 1.6%. After 10 min incubation at 65 ºC, 500 µl of Phenol: chloroform: isoamylalcohol (25:24:1) was added and the samples were shaken on vortexer for 10 min at room temperature. The mix was then centrifuged at 10000 rpm for 10 min (Wagtech, K2 series). Thereafter, 600 µl of the aqueous phase was collected into a new tube and precipitated in 0.6 volumes of isopropanol. Then the samples were centrifuged at 13000 rpm for 10 min and the supernatant was discarded.

The DNA pellets were then washed with 70% ice-cold ethanol and centrifuged at 13000 rpm for 10 min. Finally the supernatant was discarded and the DNA pellets dried and resuspended in 50 µl of TE buffer, pH 8.0. DNA pellets were stored at -20 ºC for further analysis. The quality and the quantity of the extracted DNA was checked by running 5 µl of DNA onto 0.7% agarose gel electrophoresis for 45 min at 120 v. It was also measured by a Nano drop.

4.4.3 Genomic DNA extraction from pure isolates

Genomic DNA from pure culture of each isolate (120 representative isolates) was extracted by a freeze-thaw method of DNA extraction with a modification of a rapid boiling method designed for preparation of bacterial plasmids (Moore *et al.,* 1999). Few colonies were picked from agar plate using a sterile loop and resuspended in 50 µl of TE buffer, pH 8.0 in 1.5 ml Ependorff tubes. Then the cell suspension was boiled for 5 min in boiling water bath and immediately freezed at -20 ºC for 15 min. The boiling and freezing steps were repeated once again. The cell lysate was stored at -20 ºC and used for PCR analysis.

4.4.4. PCR amplification for pure isolates

For PCR amplification of the 16S rRNA gene of pure isolates a reaction mixture containing 19.625 µl nuclease free H_2O , 2.5 µl PCR buffer ,0.25µl dNTPs mix, 0.125 µl Taq polymerase and 0.25 µl BSA and was prepared. Then, 0.125 µl B338f (5′-ACTCCTACGGGAGGCAGCAG-3) and H1542r (5′- TGCGGCTGGATCACCTCCTT-3′) primers (Amann *et al.,* 1995) and 2 µl template DNA was added to the above 23µl mixture. Finally, the reaction mixture was centrifuged briefly by a micro centrifuge and amplified using the amplification program: Pre-heat lid adjusted at 104°C and initial denaturation at 94 °C for 5 min. Then for each cycle of amplification, the denaturation temperature was 94 °C for 30 s, annealing temperature 55°C for 30 s and extension temperature 72 °C for 1min. This cycle was repeated for 30 times. Final extension temperature used was 72°C for 7 min and the temperature was dropped to 10°C for final hold. This process was done using TECHNE model TC-412 PCR Thermo cycler.

The presence of PCR products was checked by agarose gel electrophoresis. A 5 µl of PCR product was mixed with a drop of 6 x loading dye and was loaded on a 0.9 % agarose gel along side with 1kb ladder. The electrophoresis was run for 5 min at 20v initially, and then for 30 min at 80v.The images of gel was observed using BIO-RAD Gel DocXR$^+$ System and the patterns were analyzed using Quantity One program software. PCR products were stored at 4 °C for further analysis.

4.4.5 . Nested PCR of 16S rRNA gene of microbial community for DGGE analysis

A nested PCR approach using two primer sets were used. The first amplification was carried out using primer set B338f (5′-ACTCCTACGGGAGGCAGCAG-3’) and H1542r (5′- TGCGGCTGGATCACCTCCTT-3′). The second amplification was carried out using a primer set B1055f (5′-ATGGCTGTCGTCAGCT-3′) and H1392r-GC (5′-ACGGGGCGGTGTGTAC3′). A 40 bp GC-clump was attached on the reverse primer of the second primer set. The PCR reaction mixture, amplification program, agarose gel electrophoresis and documentation were carried as described above.

4.4.6. Amplified ribosomal DNA restriction analysis (ARDRA)

The PCR products from pure strains were subjected to restriction digestion for further analysis. First round restriction analysis was carried out for all the 120 amplified PCR products and the second round restriction digestion was carried out for 43 representative strains from each OTU groups of each sampling sites.

Restriction digestion was carried out using restriction enzyme *TaqI* (5'…T^CGA…3'). A 7µl restriction reaction contained 0.4µl *TaqI* (Himedia, India), 1.5µl 10x Hibuffer TaqI (Himedia, India) and 5.1 µl nuclease free water (Qiagen). Then 10 µl PCR products were added to each reaction mixture. The reaction was mixed very well was spinned with a micro centrifuge briefly. The restriction digestion was carried out at 65^{o}C for 1 h on a Thermocycler. Following the restriction digestion the enzyme was inactivated by adding 3 µl 6x gel loading buffer (Himedia, India). Then the entire restriction digest was loaded onto 3% agarose gel contained 7.5 µl ethidium bromide. Electrophoresis was run for 2 h at 80v. The image of the DNA fragments on the gel after electrophoresis seen using BIO-RAD Gel Doc XR^{+}System and the patterns analyzed used Quantity One program software.

4.4.7. Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE analysis was performed with a 700-0175 kuroGel vertical 2020 DGGE system. Equal amounts of PCR products (20 µl) were loaded onto 8% (w/v) polyacrylamide gels in 0.5 x TAE (20 mM, 10 mM acetate, 0.5 mM Na_2-EDTA) with denaturant-gradient of 40–70% (100% denaturant is 7 M urea and 40% (w/v) deionized formamide). The gel electrophoresis was run at 60 °C first for 20 min at 20 v and subsequently at a constant voltage of 70 v and 60^{o}C for 12 h. After electrophoresis completed, the DGGE gel was stained with ethidium bromide (1:10) for 30 min and observed using BIO-RAD Gel Doc XR^{+}System. The intense DGGE bands were excised with sterilized pipette tips and put in a clean Eppendrof tubes contained 50 µl of TE buffer (10 mMTris and 1 mM EDTA, pH 8.0) and left at 4°C overnight to let DNA to passively diffuse into the buffer. Then 5µl of elute was used as a template for PCR with the primers and conditions described above.

Following amplification the PCR products were analyzed by DGGE to confirm their electrophoretic mobility relative to the fragment from which they were excised.

4.4.8. Genomic DNA Extraction and amplification for sequencing

Twenty representative isolates were selected from each overall OTU group and refreshed on nitrogen free media for extraction of DNA. DNA was extracted by CTAB extraction method as described in (4.4.3).Concentration and quality of the DNA extracted was measured using a Nano drop (Appendix 3). Then the extracted genomic DNA was sent to University of Kiel, Germany for sequencing of 16S rRNA gene, detection of genes associated with nitrogen fixation.

4.4.9. PCR for sequencing

PCR amplification of genomic DNA extracted from twenty isolates was done using a PCR mix having 1.25μl of each primers H1542 and A8f(10pM),8.875 μl Milli Q water,4 μl 5*PCR buffer,0.5 μl dNTPs,2 μl $MgCl_2$(125mM) ,0.125 μl Taq polymerase and 2 μl template DNA. Amplification program similar with previously used one.

4.4.10. Sequencing and phylogenetic analysis

Sequences were edited manually using CLUSTAL W (Thompson *et al.,* 1994). The program Bellerophon on Detection of chimeric sequences in multiple alignments (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl) was applied to the studied sequences in order to detect possible chimeric artifacts (Huber *et al.,* 2004). Reference 16S rRNA sequences were retrieved from NCBI GenBank database available at http://www.ncbi.nlm.nih.gov using BLAST (Basic Local Alignment Search Tool) analysis to provide the identity of the sequence (Altschul *et al.*, 1990). Phylogenetic relationship of sequences to closest matches in public database based on 16S RNA gene sequences was constructed by using Maximum likelihood method using distances calculated with Jukes-Cantor correction (Jukes and Cantor, 1969). The stability and reliability of the relationships of lineages on the inferred trees was tested by bootstrap analysis for 1000 replicates (Felsenstein, 1985). Sequences with sufficient length were included in the alignment; the sequence alignments were then corrected manually and sequences with ambiguous alignment positions were removed from the analysis.

4.4.11. PCR detection of NifH gene

PCR detection of NifH gene was carried on the nineteen of the representative isolates selected randomly from each OUT groups. PCR detection of NifH gene was carried by a nested PCR approach using NifH primer sets nifH4 (*A. vinelandii* positions 546 to 562; 5'-TTY TAY GGN AAR GGN GG-3') and nifH3 (*A. vinelandii* positions 1018 to 1002; 5'-ATR TTR TTN GCN GCR TA-3'). One ml of the template DNA was added to 49 ml of the PCR mixture (4 mM MgCl2, 10× reaction buffer, 10 mMdNTPs, 100 pmol each of nifH3 and nifH4 primers, and 2.5 U of *Taq* polymerase). The PCR was carried out with 30 cycles of denaturation at 95°C (1 min), annealing at 55°C (1 min), and extension at 72°C (1 min). The second round of the nested PCR was performed with 1 ml of the first-round product in a mixture of 4 mM $MgCl_2$, 10× reaction buffer, 10mMdNTPs, 100 pmol each of nifH1 (corresponding to *Azotobactervinelandii* nucleotide positions 639 to 655; 5'-TGYGAY CCN AAR GCN GA-3') and nifH2 (*A. vinelandii*positions1000 to 984; 5'-AND GCC ATC ATY TCN CC-3'), and 2.5 U of *Taq* polymerase, with 30 cycles of the same temperature and time conditions as in the first step of amplification. Presence of nifH gene was detected by running the amplicons on 2.5% agarose gel electrophoresis at 120v for 30 min. The gel was stained by ethidium bromide for 30 min and then transilluminated on gel documentation system. Bands, which correspond to nifH gene around 360 bp, were excised with a cleaned scalp. The excised gel suspended in 20 µl and incubated at 4 °C overnight. Fragment of NifH gene was gel purified by using gel Nucleospine purification kit according manufactures instruction (Nucleospine extraction kit 2004). Lastly the elute was preserved at 4 ºC for cloning and further analysis.

4.4.12. Cloning of NifH gene

Depending of the band intensity 3-4 µl of the gel purified fragment of PCR amplified NifH gene (approximately 360 bp) were was ligated into the plasmid vector (pCR® 2.1-TOPO® vector, 3.9 kb) using TOPO® TA Cloning Kit (Invitrogen, 2006) according to the manufacture's instruction. The ligated product was inserted into TOP10 OneShot® chemically competent *E. coli* cells. using TOPO® TA Cloning Kit (Invitrogen, 2006) according to the manufacture's instruction with a composition 4 µl PCR product,1 µl of salt solution, nuclease free water and topovector.

Heat-shock method of transformation (Sambrook *et al.,* 1989) was used; 2 µl of the TOPO® cloning reaction (construct) was added to 50 µl of One Shot® Chemically Competent *E. coli* cells (TOPO10) and mixed gently. Then the cloning reaction mixture was incubated on ice for 5 min. After incubation, transformation was carried by heat-shocked the cells in a water bath equilibrated at 42 °C for 30 seconds and then the cells were transferred immediately to ice. On heat-shocked cell, 250 µl of room temperature S.O.C. medium was added, incubated at 37°C for 1 h shacking horizontally at 200 rpm in Gyrotory shaker (New Brunswick Scientific Corporation). After one hour of incubation, 10 µl, 20 µl, 30 µl, 40 µl and 50 µl of transformed cells were spreaded on a pre warmed selective Luria-Bertani (LB) plate containing 50 µg/ml ampicillin. 40 µl X-Gal (4 mg/ml) was spreaded onto the plates before 30 min of inoculation and finally the plates were incubated upside-down overnight at 37°C. X-Gal was used to screen colonies containing a recombinant plasmid. When the blue colour develops, white colonies were picked and inoculated in a liquid LB medium prepared as described in TOPO TA Cloning Kit (Invitrogen, 2006).

4.4.13. Amplification of cloned NifH gene products

Cloned PCR products with the right insert approximately 1.5 kb, were screened by PCR amplification using M13 primers directly from overnight cultured transformed *E. coli* cells (Tables 7) and running the amplified PCR products on agarose gel (3% w/v) electrophoresis. Accordingly, 25 µl reaction mixture containing the following components was used for amplification of the overnight-cultured clones (Table 4).

Table 4 . PCR reaction master mix used for amplification of clones

Reagents	**Stock solution**	**Working solution**	**Amount added (in µl) per 25 µl**
Nuclease free water[1]	-	-	20.5
BSA[2]	2%	0.25%	0.25
10x buffer[3]	10x	1x	2.5
dNTP[3]	10 µM	0.1 µM	0.25
M13f	100 µM	0.5 µM	0.10
M13r	100 µM	0.5 µM	0.10
DNA polymerase	2 U/µl	0.02 U/µl	0.25

5. Results

5.1. Physicochemical parameters of samples

Physicochemical parameter measurement showed that pH of the lake was around 10 (Table5).Temperature ranges 24.2ºC up to 24.9ºC, salinity almost 5.5% throughout the depth and conductivity ranges 62.5-66.9 MScm^2(Table 5). The Secci depth measurement showed that the photic zone was up to 39 cm. The depth where oxic -anoxic transition took place was at 2.9 m. The maximum depth found was 17 m at the site of sample collection. Generally, the physicochemical characteristics were almost similar along the depth.

Table 5. Physicochemical parameters during sample collection and total viable count from each depth.

Depth (meter)	Sample Type	Temperature (ºC)	pH	Salinity (%)	Conductivity MScm^2	Sample designation	Total viable count (CFUs/ml)
0	Water	24.8	10.25	5.5	64.3	NF0m	2.17×10^3
2.9	Water	25.2	10.24	5.5	66.9	NF2.9m	1.82×10^3
5	Water	24.2	10.18	5.5	62.5	NF5m	6.50×10^2
10	Water	23.7	10.16	5.6	64.2	NF10m	1.72×10^4
14	Water	24.9	10.16	5.6	63.1	NF14m	1.31×10^5
17	Mud	24.9	10.18	-	-	NFM	1.48×10^5

5.2. Enrichment and total viable count

The culture medium incubated at anaerobic condition changed to turbid at the seventh day of incubation. On the other the culture medium incubated at aerobic condition did not show change in turbidity. Total viable count obtained from second passage indicated that higher colony forming units (CFUs/ml) (1.48×10^5) were found at bottom of lake in the mud sample followed by 14 m (1.31×10^5). The least number of CFUs was found at 5m (6.50×10^2) (Table 5).

5.3. Isolation of pure alkaliphilic nitrogen fixing bacterial strains

Out of 180 distinct colonies tested, 125 isolates were able to grow on nitrogen free medium (Fig. 9). Of these 25 isolates were obtained from mud sample (NFM) at depth of 17 m. Among water sample isolates, large numbers of nitrogen fixing isolates were obtained from NF 14m (23 isolates) and NF10m (20isolates). Thus more isolates were capable of growth on nitrogen free medium from samples collected at lower depth.
(Fig 9).

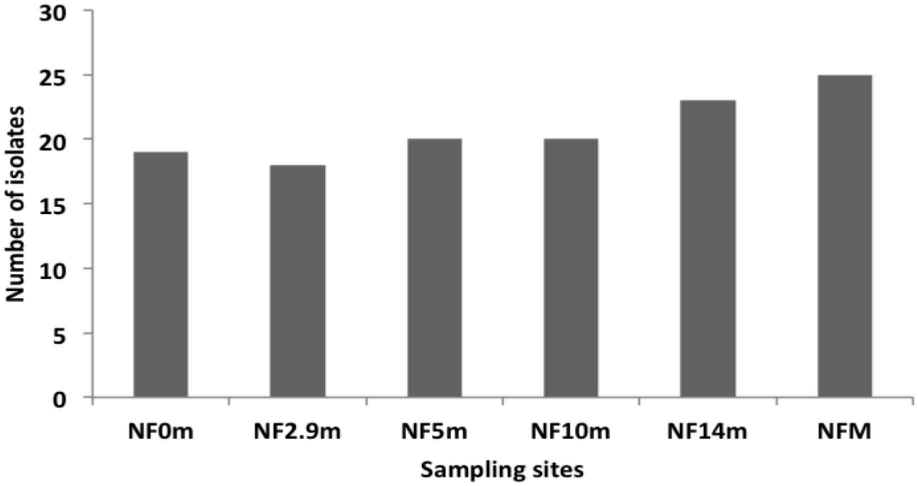

Figure 9: Number of nitrogen fixing bacterial isolates obtained from each depth

5.4. Effect of oxygen on the growth of nitrogen fixing strains

The result obtained after the OD measurements of NF0m15, NFM6 and NF5m7 under aerobic and anaerobic condition indicated that those nitrogen fixing bacteria isolates grew on both aerobic and anaerobic condition(Fig.10) The growth in anaerobic condition was better compared to the aerobic one (Fig. 10). The growth indicates that the selected three isolates and isolates represented by those three share similar OTU growth better in anaerobic condition because the study isolates selected from major OUT groups.

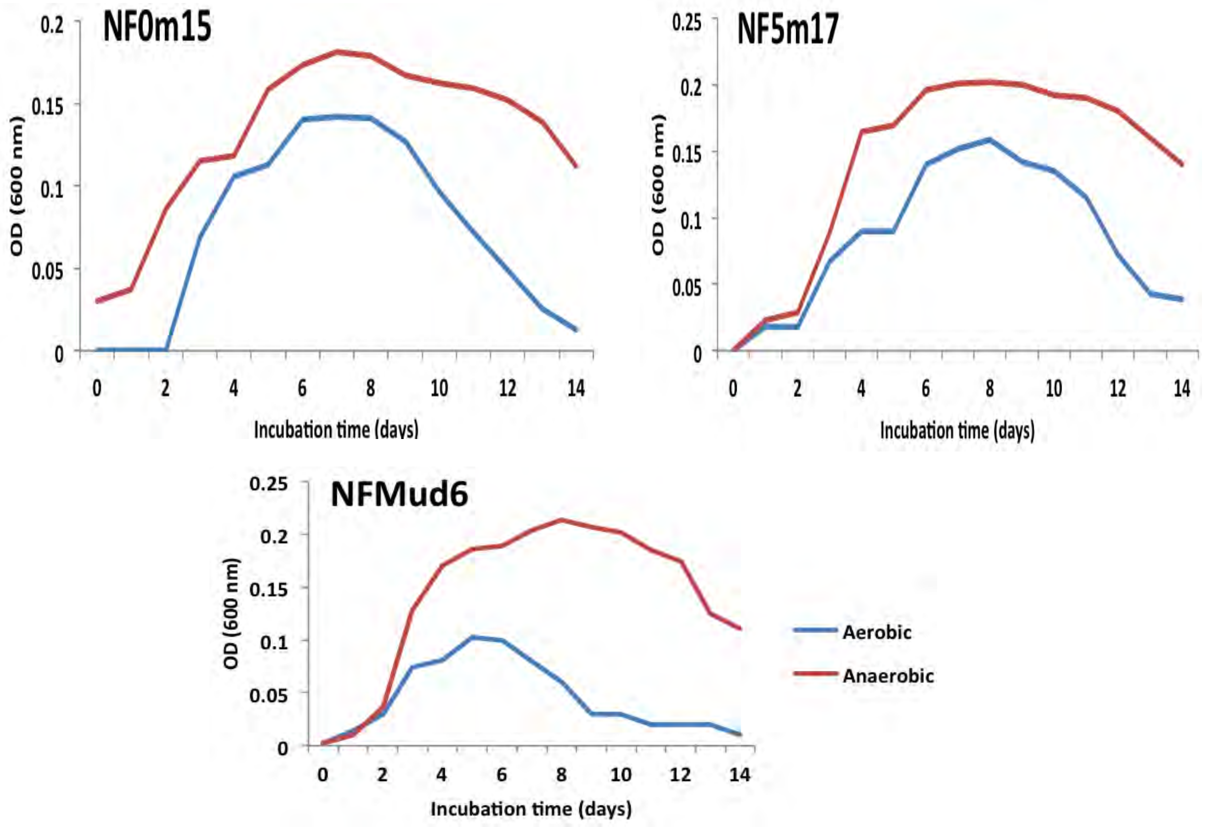

Figure 10. Effect of oxygen on the growth of nitrogen fixing bacterial strains.

5.5. Co-culture of nitrogen fixing isolates with the non-fixer isolates

The viable count for co-culturing of the nitrogen fixing isolates with non-fixing isolates is given in table 6. The viable count of the non nitrogen fixer isolate (3.5*R 1) was high when it was co-cultured with the nitrogen fixing isolate compared to when cultured alone. While viable cell count for nitrogen fixing isolate (NF10m6) is higher when it grew in single culture than on co-culture condition.

Table6.Colony count for co culture

Culture type	**CFU/ml**		
	Nitrogen fixing strain (NF10m6)	**3.5R*1 (Non-nitrogen fixing strain)**	**Control**
Monoculture	5.2×10^3	0	0
Co-culture	4×10^2	3×10^2	0

5.6. Biochemical and morphological tests for selected Operational Taxonomic Units

The biochemical test and morphological analysis performed is presented in tables 7 and 8. Most of the representative isolates have the enzyme Catalase. Except for few isolates most of them did not have Oxidase activity. The Gram test revealed that the majority of the isolates were Gram negative. The colony morphology of the majority of the isolates was similar.

Table 7. Biochemical characteristics of representative isolates

No	Isolate code	OxidaseTest	CatalaseTest	Gram Test (+ or-)
1	NF0m15	+	+	+
2	NF0m18	+	+	+
3	NF2.9m12	-	+	-
4	NF2.9m15	-	+	-
5	NF2.9m18	+	+	-
6	NF5m11	-	+	-
7	NF5m17	-	+	-
8	NF5m19	-	+	-
9	NF10m6	+	+	-
10	NF10m9	+	+	-
11	NF10m12	-	-	+
12	NF14m10	-	-	+
13	NF14m10.4	-	-	+
14	NF14m11	-	+	-
15	NFMud1.4	-	+	+
16	NFMud6.4	-	+	-
17	NFMud7.4	-	+	-
18	NFMud10.4	+	+	+
19	NFMud18	-	+	-
20	NFMud19	-	-	-

Table 8: Colony morphology of representative isolates

No	Isolate code	Form	Elevation	Margin
1	0m15	Circular	Flat	Entire
2	0m18	Circular	Flat	Entire
3	2.9m12	Spindle	Flat	Entire
4	2.9m15	Spindle	Flat	Entire
5	2.9m18	Spindle	Flat	Entire
6	5m11	Circular	Flat	Entire
7	5m17	Spindle	Flat	Entire
8	5m19	Spindle	Flat	Entire
9	10m6	Circular	Flat	Entire
10	10m9	Spindle	Flat	Entire
11	10m12	Spindle	Flat	Entire
12	14m10	Circular	Flat	Entire
13	14m10.4	Spindle	Flat	Entire
14	14m11	Spindle	Flat	Entire
15	Mud1.4	Filamentous	Flat	Lobate
16	Mud6.4	Spindle	Flat	Entire
17	Mud7.4	Circular	Flat	Entire
18	Mud10.4	Circular	Flat	Entire
19	Mud18	Spindle	Flat	Entire
20	Mud19	Circular	Flat	Entire

5.7. Diversity of haloalkaliphilic nitrogen fixing microbial community

5.7.1. ARDRA analysis

Figure 11 shows the ARDRA pattern of the 43 representative isolates digested with restriction enzyme Taq I. The ARDRA pattern observed indicated that the restriction enzyme was efficient in digesting the PCR products. All the 43 representative isolates digested were clustered into 9 operational Taxonomic Units (OTUs).

Isolates from the bottom Mud sample (17 m) were distributed across the five of the OTUs where as isolates from surface water samples (0 m) were grouped into a single OTU (Table 9).

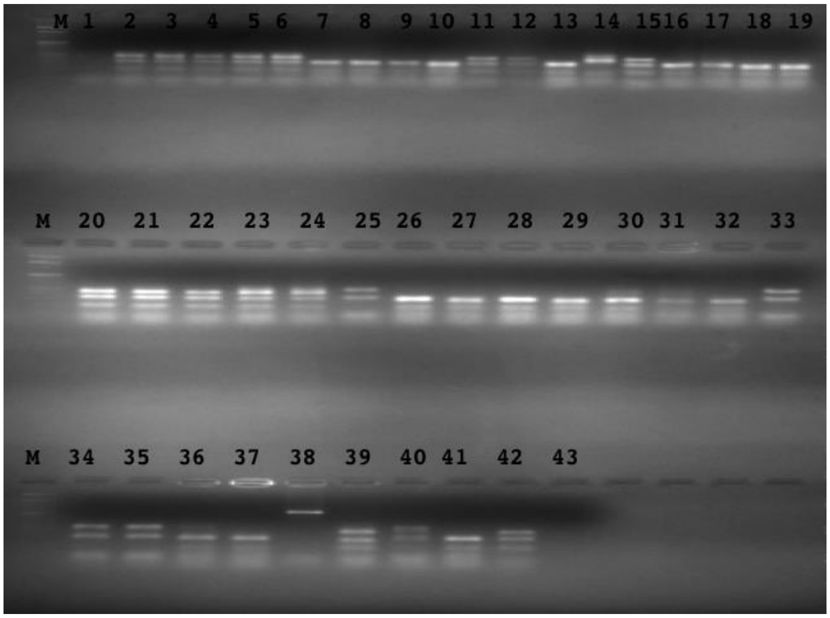

Figure 11. ARDRA profile of restriction enzyme Taq1

Table .9 Depth based OTU distribution

Depth (m)	Number of isolates growing on N-free medium	Number of OTUs
0	**6**	**1**
2.9	**7**	**4**
5	**6**	**4**
10	**6**	**4**
14	**7**	**2**
17	**11**	**5**

5.7.2. DGGE profiling

Analysis of DGGE gel (Fig. 12) showed a total of 48 detectable bands in 9 different positions. The number of bands per sample varied between 3 and 6. Single band repletion level was 1-18.The difference in the repetition level shows that there are bacteria which are very common and rare on the lake environment. Based on the result, bacteria at band position 3 and 7 are very common ones that exist throughout the lake at all depth at different oxygen and nutrient level. Bactria isolates represented at position 1, 2,4,5,6 and 9 existed only on direct environmental sample not in enriched samples.

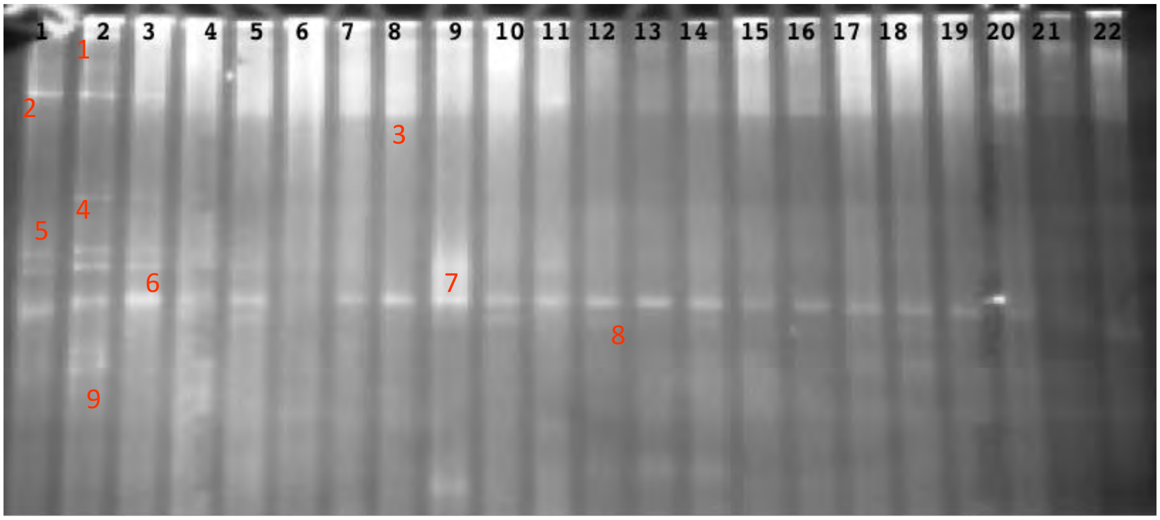

Figure 12.DGGEprofiles sampling from direct environment and different enrichment.
Key: 1: 0 m environmental sample (ES) 2: 5 m ES 3: 10 m ES 4: 14 m ES 5: 17 m ES 6: 0m Initial enrichment (IE) 7: 2.9m IE 8: 5m IE 9 :10m IE 10: 14m IE 11: 17m IE12: 0m passage one(P1) 13: 2.9mP1 14: 5mP1 15:14mP1 16: 17mP1 17 :0m passage two (P2) 18:2.9mP2 19:5mP2 20:10mP2 21:14mP2 22:17mP2

5.7.3 Analysis of 16SrRNA gene sequence and construction of phylogenic relationship

Figure 13 shows phylogenetic analysis based on 16S RNA gene sequences of representative isolates from each ARDRA group. Nineteen representative isolates were sequenced from 9 ARDRA groups resulted from 98 isolates. The sequences of all representative isolates except one were high quality, which was removed from further analysis. These strains were grouped into three clusters, affiliated with the three major bacterial phyla namely *Proteobacteria*, *Actinobacteria* and *Firmicutes.*

The majority of the isolates, 69% were members of *Proteobacteria* followed by members of phylum *Firmicutes* (23%). However, the remaining 6 strains were members of phylum *Actinobacteria*. All the strains affiliated with phylum *Proteobacteria* were closely related to alkaliphilic strains of the genus *Halomonas* isolated from various soda lakes of the world. On the other hand those isolates affiliated with phylum *Firmicutes* were closely related to alkaliphilic members of genera *Bacillus* and *Alkalibacterium*. Six strains affiliated to genus *Nesterenkonia* of *Actinobacteria.*

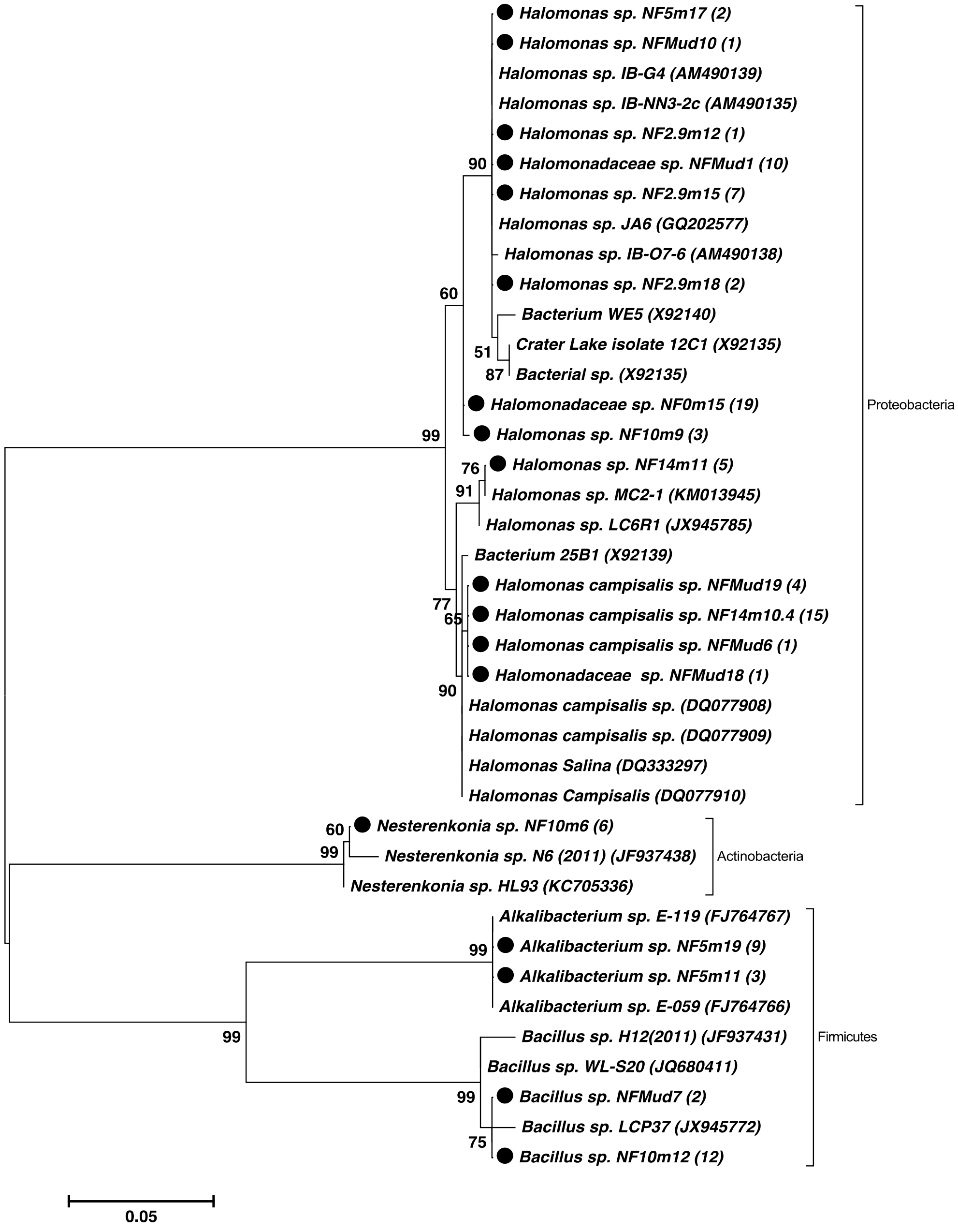

Figure 13: 16S rRNA gene sequence-based phylogenetic tree generated by using the Maximum like hood method showing the relationships between the strains studied and close matches. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees; only values greater than 50% are shown. Bar 0.05 #substitutions per nucleotide position.

5.7.4. Detection of nitrogen fixing gene

Nineteen isolates were analyzed for the presence of genes involved in nitrogen fixation (NifH), using PCR amplification (Fig. 14). The presence of NifH gene was detected in eleven out of 19 isolates. NifH gene was detected from isolates obtained from all depths including the surface water.

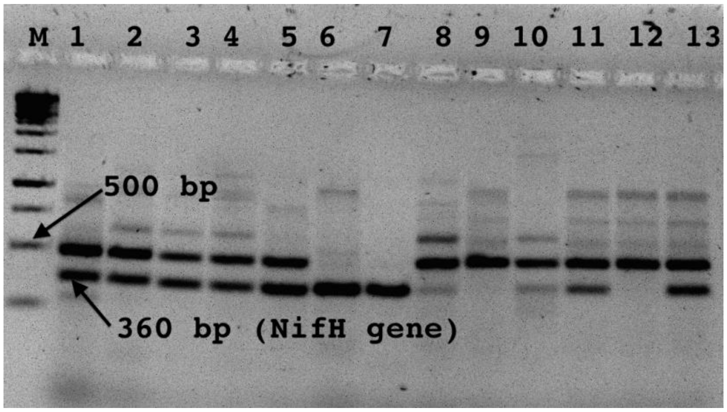

Figure 14. Result for presence nifH gene **Key**: M: Marker, 1:NF0m15, 2:NF2.9m15, 3: NF2.9m18, 4:NF2.9m12, 5: NF10m9, 6: NF10m6, 7: NF14m10, 8: NF14m10.4, 9:NFM1.4, 10 : NFM6.4, 11:NF7.4, 12:NFM18, 13: NFM19

5.7.5. Sequences for nifH

Eleven clone libraries were constructed from the 11 bands corresponding to NifH gene (360 bp) Fig 14. All clone libraries except one (Mud1.4) produced white colonies indicating cloning was successful. A total of 20 NifH gene clones (2 from each clone library) were picked and sequenced. However, only the sequences of six clones were successful and provide best sequence (Fig 15). Accordingly, the phylogenetic relations of the six nifH gene fragments sequenced were closely related to NifH gene fragments of uncultured microbial communities of environmental samples (Fig 15).

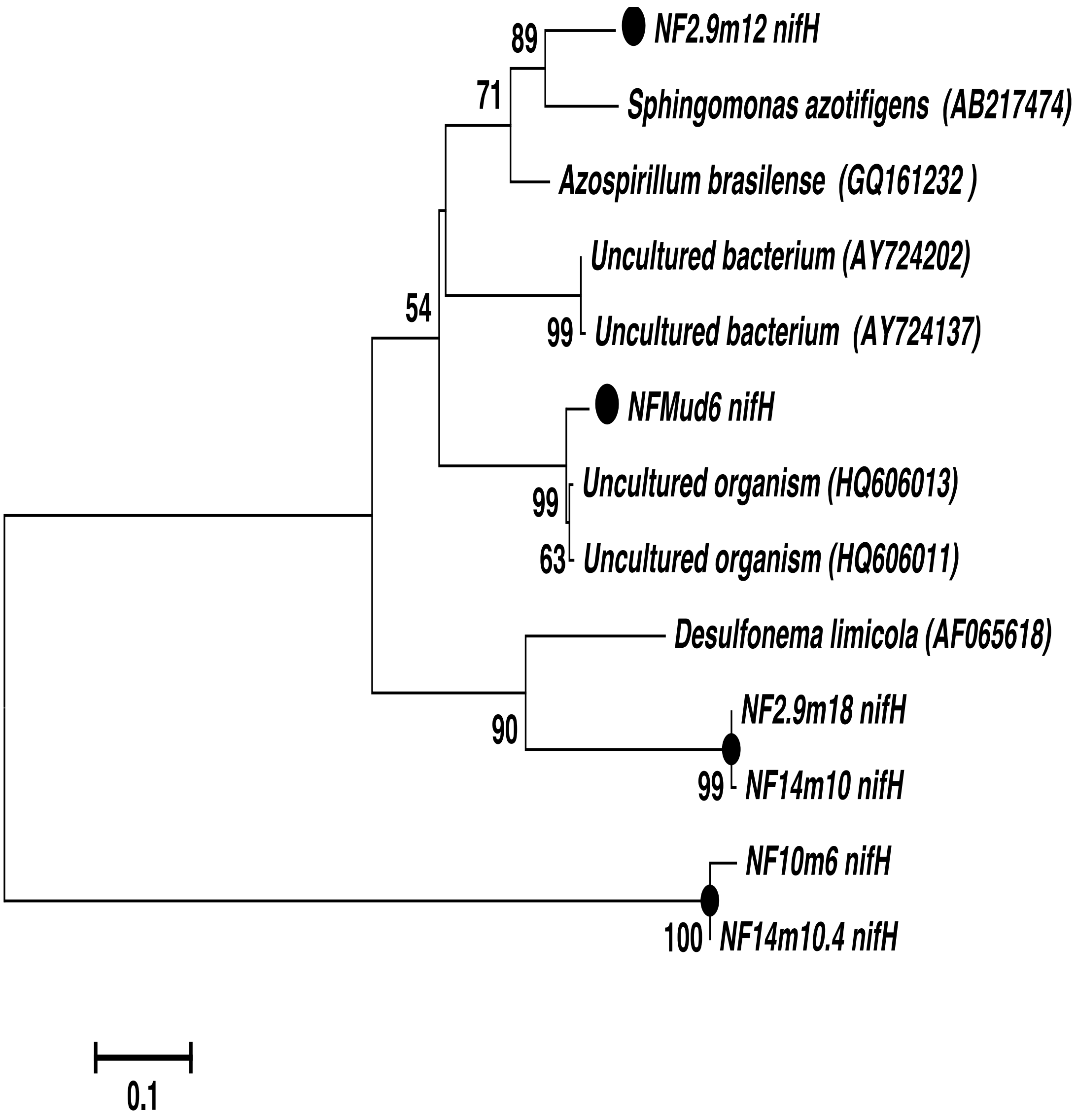

Figure 15: Phylogenetic tree showing representative nifH gene sequences among the diazotrophs. Bootstrap values greater than 50% (out of 100 replicates) are indicated at branch nodes.Bar 0.1 substitutions per nucleotide position.

Discussion

At the time of sampling Lake Chitu had similar temperature, salinity, pH and conductivity throughout the entire depth indicating mixing. The aphotic zone is very close to surface at around 39 cm and below 2.9 m the lake was anoxic. But cell density was higher with increasing depth-suggesting abundance of heterotrophic microbial cells in the anaerobic part of the lake. In the absence of oxygen as the final electron acceptor many prokaryotes probably use nitrate as a final electron acceptor, a process known as denitrification. In an earlier study carried out at Lake Chitu, high abundance of denitrifiers was reported (Lulit Tilahun., 2010). Considering the fact that the lake is a closed ecosystem and the greater depth of the lake is anoxic, abundance of denitrifiers implies that there is a constant loss of nitrogen. Nitrogen fixation by efficient N_2-fixers was therefore expected to support such a high primary productivity. In this study, a total of 9 OTUs capable of growing on nitrogen free media were detected indicating the abundance of nitrogen fixing prokaryotes in the lake.

The 120 isolates that were able to grow on nitrogen free medium were grouped into 9 OTUs based on restriction fragment length pattern. The distribution of the different OTUs along the lake water column was different. Some were common throughout the lake depth while others showed restricted occurrence to a certain depth. Three of the OTUs (7, 8 and 9) were only found from the mud sample, showing that they are restricted to the anaerobic part of the lake. In general most of the nitrogen fixing isolates found in the anoxic or anaerobic part and only one OTU was detected from surface water. This is because absence of O_2 and presence of high nutrients at bottom of the lake probably create a favorable condition for nitrogen fixation and in the bottom. on top of this less rate of predation on the bottom also favor nitrogen fixing bacteria at the bottom (Needoba *et al.,* 2007; Riemann *et al.,* 2010).

Most of the OTUs tested have the *nifH* gene indicating that the OTUs have the genetic make up to fix atmospheric nitrogen. The nitrogenase enzyme, a product of the *nif* gene is highly sensitive to oxygen (Martinez-Romero, 2006). Thus nitrogen fixing prokaryotes either grow anaerobicaly (Klipp, 2004) or develop mechanisms to protect their nitrogenase from exposure to oxygen through different mechanisms (Fay, 1992). The majority of nitrogen fixing alkaliphilic OTUs from Lake Chitu were found at depths below 2.9 m, a region devoid of oxygen. However, some nitrogen fixing isolates were also isolated from the oxic layer and grew well under anaerobic condition like 0m15. The mechanism these microorganisms protect their nitrogenase from oxygen is unknown. It could perhaps involve shielding with a cytochrome oxidase enzyme system that has very high affinity for oxygen and thus scavenge any trace of oxygen around the nitrogenase (Fay, 1992). However, the exact mechanism remains to be studied.

Having the nitrogenase gene may not necessarily mean that these organisms produce a functional nitrogenase enzyme and thus fix nitrogen. To be sure, it is necessary to test for nitrogenase activity and the standard procedure to measure nitrogenase activity is acetylene reduction (David, 1980).Because of lack of facility, in this study co-culturing with another prokaryotic isolate known to be incapable of fixing nitrogen and thus unable to grow on nitrogen free media was used. The logic was that in all nitrogen fixing prokaryotes the nitrogenase gene is highly regulated being turned on only when there is lack of nitrogen for growth in the surrounding environment (Iwata *et al.,* 2012). But when the gene is turned on and prokaryotes fix atmospheric nitrogen it is not only fixing enough for itself but also release excess nitrogen in the form of ammonia for other organisms in its vicinity. This in fact is the basis for the association between the rhizobium bacteria and its leguminous plant hosts where the plant use the excess nitrogen released by the bacteria (Zahran, 1999).

The non-fixing isolate used in this study was selected such that it allows easy detection when grown because of its distinct color that was very different from the nitrogen fixer when the isolate that was not capable of fixing nitrogen grew on nitrogen free medium; no colony was detected but the nitrogen fixer showed abundant growth. However, when the two isolates grew together significant growth of both organisms was detected showing that the non nitrogen-fixing isolate was able to get sufficient nitrogen in the form of ammonia from the nitrogen fixing isolate that grew in close proximity. This is, therefore, an indirect confirmation that these alkaliphilic microorganisms do fix nitrogen.

The number of visible dominant DGGE bands was decreased in enrichment microbial community compared with environmental samples. This shows that a certain group of microbial communities have been enriched and favored. The enrichment cultures were enriched in a nitrogen free medium suggesting that the common and dominant bands obtained in the enrichment cultures might be nitrogen fixing microbial communities. Interestingly, a single dominant band was found along all enrichment microbial communities. This also supported that nitrogen fixing microbial communities were enriched. However, with the drawbacks of PCR-DGGE, it is impossible to rule out that these bands could be artifacts.

The majority of the isolates in the present study were closely related to bacterial strains isolated from various soda lakes widely distributed in the world. Almost all showed 99-100% 16S rRNA gene sequence similarity with alkaliphilic bacterial strains isolated from various soda lakes (Jones *et al.,* 1998; Rees *et al.,* 2004; Sorokin *et al.,* 2011; Simachew, 2015). This suggests that these bacterial stains might play a key role in the ecology of soda lakes such as nitrogen cycle. However, the closest matches to the present isolates were studied from the perspective of diversity and it is difficult to compare and conclude if they have any role in nitrogen fixation.

Members of *Halomonas* were the most abundant microbial communities in the present study. *Halomonas* has been reported to be the most abundant cultivable Gram-negative bacteria in soda lakes and other hyper saline environments (Mwirichia *et al.,* 2011; Simachew, 2015).The authors further noted that, members of *Halomonas* reported that they are adapted to grow in poor medium culture conditions. (Mwirichia *et al.,* 2011; Simachew, 2015). This suggests that they probably have a mechanism to support their nutritional requirements through nitrogen fixation because members in *Halomonas* had been reported as diazotrophs (Yogev *et al.,* 2011). Similarly, members of *Bacillus* and *Alkalibacterium* have been reported as the most abundant cultivable bacterial groups in soda lakes including Ethiopian soda lakes (Simachew, 2015). Members of *Alkalibacterium* have been reported to be facultative anaerobes and halotolerant (Ntougias and Russell, 2001; Yumoto *et al.,* 2004; Nakajima *et al.,* 2005; Yumoto *et al.,* 2008; Ishikawa *et al.,* 2012).

The presence of few isolates affiliated to Actinobacteria genus *Nesterenkonia* is surprising. To the best of our knowledge, so far no members of *Actinobacteria* have been reported to be nitrogen fixer in soda lakes habitat. On the other hand, the closest matches to the present isolates is from hypersaline environments such as Hot Lake, WA, an environment with high concentrations of magnesium Sulfate (Kilmer, 2014) and haloalkaline soil in China (Shi *et al.,* 2012b).

PCR amplification of NifH gene fragments was positive in 11 out of 19 representative isolates studied confirming the presence of active nitrogen fixing bacterial communities in soda lakes. The presence of active nitrogen cycle in soda lakes is well documented (Oremland, 1990; Herbst, 1998). *Cyanobacteria* have been considered as the only group of haloalkaliphiles responsible for nitrogen fixation in soda lakes .However, this potential has also been discovered in a few obligate anaerobes isolated from soda lakes, such as *Clostridium alkalicellum* (Zhilina *et al.,* 2005) and *Geoalkalibacter ferrihydritucus* (Zavarzina *et al.,* 2006). Recently nitrogen fixation activity by a haloalkaliphilic bacterium *Amphibacillus tropicus* and with the obligately anaerobic haloalkaliphile *Bacillus arseniciselenatis* and *Natronobacillus azotifigens* have been reported from soda lakes of Central Asia and Egypt (Sorokin *et al.,* 2008).

The phylogenetic tree based on nifH gene fragment sequences shows that their closest matches were diazotrophic bacterial strains belonging to the phylum *Proteobacteria* and uncultured microorganisms isolated from Mediterranean Sea, Boa Sea and South China Sea. For instance NifH gene fragment sequenced from clone sequence Mud6 nifH showed greater than 96% similarity to nitrogenase (nifH) gene sequence of uncultured organism clone JUN07_Tb01_20m_24h_6 retrieved from the Levantine Basin, East Mediterranean Sea (Yogev *et al.,* 2011).

Three nifH gene fragments sequenced from three clones namely NF10m6nifH, NF2.9m8 nifH, and NF14m10 nifH were 100% similar to putative nitrogenase reductase (nifH) gene of uncultured microorganism clone B10-31,retrieved from the sediment nifH-harboring bacteria in the Bohai Sea, China (Zhou and Dang, 2011). On the other hand these nifH gene fragments showed 75.3% similarity to dinitrogenase reductase (nifH) gene of cultivable bacterial strain, *Desulfonema limicola*, isolated from anaerobic microbial enrichments initiated with marine planktonic invertebrates, including copepods and euphausids (Braun *et al.,* 1999).These three nifH gene sequences were retrieved from isolates obtained at three depths, 2.9 m, 10 m and 14 m at lake Chitu. This suggests that the same type nitrogen fixing bacterial communities survive along the whole depth. Moreover, it could be due to the presence of mixing in the lake so that no stratification in the lake water column.

NifH gene fragment sequenced of 2.9m12 nifH gene fragment showed more than 86% NifH gene similarity to *Sphingomonas azotifigens*, a nitrogen fixing bacterium isolated from the roots of Oryza sativa (Xie and Yokota, 2006) and *Azospirillum brasilense* strain AWB5 (Shukula *et al.,* 2010). Similarly, nifH gene fragment sequence of 14m10.4 NifH showed greater than 92% sequence similarity to B21 dinitrogenase reductase (nifH) gene of uncultured bacterium clone DUN1_nifH_+ and uncultured bacterium clone DUNnif131 (-1B) (OTU#030) (Rosch and Bothe, 2005) both from mixed forest microbial community, Germany. The nifH gene fragment sequences in the present study were cluster with known nitrogen fixing bacterial strains. This clearly demonstrated that the bacterial communities in the present study were nitrogen fixers.

Conclusion

From this study we can conclude that active nitrogen fixation takes place at Lake Chitu. This probably helps to compensate nitrogen loss through denitrification and hence allow the lake to be highly productive. The majority of the nitrogen fixing strains were isolated from a anoxic part of the water column which allow these microorganisms to protect their nitrogenase enzyme from oxygen poisoning.

The alkaliphilic nitrogen fixing prokaryotes isolated in this study belong to three different phyla, Proteobacteria, Firmicutes and Actinobacteria. It can thus be concluded that nitrogen fixers of Lake Chitu are highly diverse.

Recommendation

Any change in water chemistry either through pollution, dilution, or concentration could lead to a change in the ecosystem and the loss of this vital group of organisms. And this could lead to extinction of all life forms in the lake or complete change of the microbial community structure. Therefore, recommended that the lake should be protected from any man made alterations in order to maintain the current equilibrium.

To fell knowledge gap on nitrogen cycle in soda lakes, study on bacteria strains involved on nitrification, ammonification and other nitrogen cycle is optional.

Bactria strains from Soda Lake have potential to degrade wastes from different industries; screening of potential nitrogen fixing bacteria for waste degradation is highly suggested.

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Appendix 1: NCBI blast result of the nitrogen fixing isolates

Name of Isolate	**Nucleo. Sequence #letters**	**Closest matches**	**Accession**	**% similarity**	**Taxon**	**Habitat**	**Reference**
Halomonas sp. NF0m15	658	Bacterium 12C1	X92135 B	98.4%	*Protobacteria*	Lake Bogoria	Duckworth *et al.,* 1996
		Halomonas sp. JA6	GQ202577	98.3%	*Protobacteria*	Lonar soda lake India	Siddharth *et al.,2009*
Nesterenkonia sp. NF10m6	778	*Nesterenkonia* sp. HL93	KC705336	99.4%	*Actinobacteria*	saline margin soil	Kilmer. *et al.,* 2014
		Nesterenkonia sp.N6(2011)	JF937438	98.7%	*Actinobacteria*	Saline-alkali soil. China	Shi, W., Takano, T. and Liu, S. 2011
Halomonas sp. NFMud18	724	*Halomonas sp.* LC6R1	JX945785	98%	*Protobacteria*	Lake Chitu Ethiopia	Mersha. 2012
		bacterium 25B1	X92139	99.3%	*Protobacteria*	Lake Bogoria	Duckworth *et al.,* 1996
Halomonas sp. NF2.9m15	835	*Halomonas sp.* JA6	GQ202577	99.6%	*Protobacteria*	Lonar soda lake India	Siddharth. *et al.* 2009
		bacterium 12C1	X92135	99%	*Protobacteria*	Lake Bogoria	Duckworth. *et al.* 1996
Bacillus sp. NF10m12	687	*Bacillus sp.* LCP37	JX945772	98.9%	*Firmicutes*	Lake Chitu Ethiopia	Mersha. 2012
		Bacillus sp. WL-S20	JQ680411	99%	*Firmicutes*	alkaline soil samples	Meng.*et al.* 2012
Halomonas sp. NFMud10	612	*Halomonas sp.* IB-G4	AM490139	100%	*Protobacteria*	Lake Kuchiger Russia	Gilvanova. *et al.* 2007
		Halomonas sp. IB-NN3-2c	AM490135	100%	*Protobacteria*	Lake Nukhe-nur Russia	Gilvanova. *et al.,* 2007

Halomonas sp. NF2.9m18	841	*Halomonas sp.* IB-G4	AM490139	100%	*Protobacteria*	Lake Kuchiger Russia	Gilvanova. *et al.,*2007
		Halomonas sp. IB-NN3-2c	AM490135	100%	*Protobacteria*	Lake Nukhe-nur Russia	Gilvanova. *et al.,* 2007
Halomonas sp. NF14m11	794	*Halomonas sp.* MC2-1	KM013945	99.5%	*Protobacteria*	Lake Magadi Kenya	Lobo. *et al.,* 2014
		Halomonas sp. LC6R1	JX945785	99%	*Protobacteria*	Lake Chitu Ethiopia	Mersha. *et al.,* 2012
Halomonas Campisalis sp. NFMud19	578	*Halomonas salina*	DQ333297	99.7%	*Protobacteria*	Lonar Lake India	Joshi. *et al.,* 2008
		Halomonas campisalis	DQ077910	99.7%	*Protobacteria*	Lonar Lake India	Joshi. *et al.,* 2007
Halomonas sp. NF2.9m12		*Halomonas sp.* IB-G4	AM490139	99.9%	*Protobacteria*	Lake Kuchiger Russia	Gilvanova. *et al.,* 2007
		Halomonas sp. IB-NN3-2c	AM490135	99.9%	*Protobacteria*	Pacific Ocean	Gilvanova. *et al.,* 2007
Alkalibacterium sp. NF5m19	755	*Alkalibacterium*. E-059	FJ764766B	100%	*Firmicutes*	Lake Elementeia Kenya	Mwirichia. *et al.,*2009
		Alkalibacterium. E-119	FJ764767	100%	*Firmicutes*	Lake Elementeita Kenya	Mwirichia, R. *et al.,* 2009
Halomonas campisalis sp. NF14m10.4	752	*Halomonas salina*	DQ333297	99.6%	*Protobacteria*	Lonar Lake India	Joshi. *et al.,* 2008
		Halomonas campisalis	DQ077910	99.6%	*Protobacteria*	Lonar Lake India	Joshi. *et al.,*2007

Halomonas sp. NF5m17	660	*Halomonas sp.* IB-O7-6	AM490138	99.5%	*Protobacteria*	mud from soda lake	Gilvanova. *et al.,* 2007
		Halomonas sp. IB-G4	AM490139	99.5%	*Protobacteria*	Lake Kuchige Russia	Gilvanova. *et al*., 2007
Halomonadaceae sp. NFMud1	722	bacterium WE5	X92140	99%	*Protobacteria*	Lake Elmenteita Kenya	Duckworth *et al.,* 1996
		bacterium 12C1	X92135	98.5%	*Protobacteria*	Lake Elmenteita Kenya	Duckworth. *et al.*,1996
Alkalibacterium sp. NF5m11	679	*Alkali bacterium sp.* E-119	FJ764767	99.4%	*Firmicutes*	Lake Elmenteita Kenya	Mwirichia. *et al.,* 2009
		Alkali bacterium sp. E-059	FJ764766 B	99.4%	*Firmicutes*	Lake Elmenteita Kenya	Mwirichia. *et al.,* 2009
Halomonas campisalis sp. NFMud6	741	*Halomonas campisalis*	DQ077909	99.8%	*Protobacteria*	Lonar lake India	Joshi. *et al.,*2007
		Halomonas campisalis	DQ077908	99.8%	*Protobacteria*	Lonar lake India	Joshi. *et al.,* 2007
Halomonas sp. NF10m9	559	bacterium 12C1	X92135	98%	*Protobacteria*	Lake Bogoria	Duckworth. *et al*., 1996
		Halomonas sp. JA6	GQ202577	98%	*Protobacteria*	Lonar soda lake India	Siddharth. *et al.,* 2009
Bacillus sp. NFMud7	689	*Bacillus sp.* LCP37	JX945772 B	99.8%	*Firmicutes*	Lake Chitu Ethiopia	Mersha. *et al .,*2012
		Bacillus sp. H12(2011)	JF937431	97.4%	*Firmicutes*	saline-alkali soil	Shi. *et al .,*2011

Appendix 2: NCBI blast result of cloned nitrogen fixing isolates

Name of isolate	**Nucleo. Sequence #letters**	**Similar SPPS**	**Accession**	**% Similarity**	**Taxon**	**Habitat**	**Reference**
NFMud6 nifH	363	Uncultured organism	HQ606013	96.6%	unclassified sequences	Mediterranean Sea	Yogev *et al.,* 2011
		Uncultured organism	HQ606011	96.8%	Unclassified sequences	Mediterranean Sea	Yogev. *et al.,* 2011
NF10m6 nifH	352	Desulfonema limicola str. Jadebusen	AF065618	75.3%	Desulfonema.	Desulfonema limicola str. Jadebusen	Braun. *et al.,* 1999
		uncultured microorganism	KM524479	100%	unclassified sequences	Bohai Sea	Zhou. and Dang. 2012
NF2.9m18 nifH	354	Desulfonema limicola str. Jadebusen	AF065618	75.3%	*Protobacteria*	Desulfonema limicola str. Jadebusen	Braun. *et al.,* 1999
		uncultured microorganism	KM524479	100%	unclassified sequences	Bohai Sea	Zhou. and Dang. 2012
NF14m10 nifH	354	Desulfonema limicola str. Jadebusen	AF065618	75.3%	*Protobacteria*.	Desulfonema limicola str. Jadebusen	Braun. *et al.,*1999
		uncultured microorganism	KM524479	100%	unclassified sequences	Bohai Sea	Zhou and Dang. 2012
2.9m12 nifH	357	Sphingomons azotifigens	AB217474	87.5%	*Protobacteria*	Sphingomonas azotifigen Japan	Xie. and Yokota. 2006
		Azospirillum brasilense	GQ161232	86.7%	*Protobacteria*	uncontaminated soil	Shukla. *et al.*, 2010
14m10.4 nifH	358	uncultured bacterium	KM524474	100%	uncultured bacterium	Bohai sea	Zhou. and Dang. 2012
		Clostridium pasteurianum	X07476	81%	*Firmicutes*	Soil	Johnson. 1998

Appendix 3 Nano drop measurement for isolate selected for sequencing

Sample ID	ng/ul	A260	A280	260/280	260/230
NF0m15	100.01	2.000	1.010	1.98	0.93
NF2.9m15	55.08	1.102	0.538	2.05	0.66
NF2.9m18	61.33	1.227	0.557	2.13	0.67
NF2.9m12	58.87	1.177	0.554	2.13	0.65
NF5m19	46.12	0.922	0.491	1.88	0.70
NF5m17	91.94	1.839	0.868	2.12	0.95
NF5m11	27.28	0.546	0.298	1.83	0.39
NF10m9	160	3.200	1.489	2.15	1.22
NF10m6	63.5	1,270	0.557	2.28	0.72
NF10m12	98.06	1.961	0.902	2.17	0.95
NF14m11	116.93	2.339	1.106	2,11	1.02
NF14m10	0.44	0.009	-0.016	-0.55	0.02
NF14m10.4	334.78	6.696	3.115	2.15	1.70
NFmud1.4	112.68	2.257	1.067	2.11	1.21
NFmud6.4	212.85	4.257	2.002	2.13	1.39
NFmud7.4	131.05	2.621	1.191	2.20	1.18
NFmud18	145.47	2.900	1.339	2.17	1.21
NFmud10.4	151.52	3.030	1.387	2.19	1.23
NFmud19	167.11	3.342	1.539	2.17	1.27