Addis Ababa University Faculty of Science Biotechnology Program

Diversity of culturable alkaliphilic denitrifying bacteria in four soda lakes of Ethiopia

A thesis submitted to the School of Graduate Studies of Addis Ababa University
in partial fulfilment of the Degree of Master of Science in Biotechnology

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Abstract

Denitrifying bacteria (95 in number) were isolated from the four Soda Lakes of Ethiopia namely, Lake Abijata, Lake Arenguade, Lake Chitu and Lake Shalla. Similar species of denitrifying bacteria were identified from the four lakes. The sequence and phylogeny relation of the isolates show that *Halomonas campisalis, Halomonas salina, Halomonas nitritophilus, Bacillus cohnii* as well as *Bacillus pseudofirmus* exhibited high similarities with the isolates studied. In addition, 9 isolates from the four lakes show similarity with the novel bacterial group, *Nitricola lacisaponesis*. Fast denitrifyers were among the isolates that are capable of producing N_2 gas only in 2 hours after inoculation. Molecular, morphological and some biochemical studies conducted on the DN-C18 isolate from L. Chitu, showed high similarity on all accounts with previously obtained isolate BACC180 from L. Chitu. These two isolates showed high denitrification activity and tolerance to high pH and salt concentration. Both isolates were found to be closely related the *Halomonas sp.*

Key words: denitrification; alkaliphilic denitrifiers; soda lakes; Lake Abijata; Lake Arenguade; Lake Chitu; Lake Shalla; *Halomonas; Bacillus; Nitricola lacisaponesis*

1. Introduction

1.1. Soda Lakes

Soda lakes, desert soil and alkaline springs represent the most stable naturally occurring alkaline environments on Earth. Particularly, soda lakes represent a specific type of salt lakes, which contain an alkaline soda carbonate/bicarbonate fraction, among the dominant salts (Sorokin and Kuenen, 2005). The pH of these ecosystems naturally reaches above 10 (Jones and Grant, 1999; Wani *et al.,* 2006; Grant, 2006; Oarga, 2009). Such environments are widely distributed throughout the world and some examples are listed in Table 1.

Table 1: Worldwide distribution of Soda Lakes and Soda Deserts

Continent	**Country**	**Location**
Africa	Libya	Lake Fezzan
	Egypt	Wake Natrun
	Ethiopia	Lake Aranguadi, Lake Kilotes, Lake Abijata, Lake Shala, Lake Chitu, Lake Hertale, Lake Metahara
	Sudan	Dariba Lakes
	Kenya	Lake Bogoria, Lake Nakuru, Lake Elmenteita, Lake Magadi, Lake Simbi, Crater Lake (Lake Sonachi), Lake Oloidien
	Tanzania	Lake Natron, Lake Evasi, Lake Magad, Lake Manyara, Lake Balangida, Bosotu Crater Lake, Lake Kusare, Lake Tulusia, El Kekhooito, Momela Lakes, Lake Lekandiro, Lake Reshitani, Lake Lgarya, Lake Ndutua
	Uganda	Lake Rukwa North Lake Katwe, Lake Mahenga, Lake Kikorongo, Lake Nyamunuka
	Chad	Lake Munyanyange, Lake Murumuli, Lake Nunyampaka, Lake Bodu, Lake Rombou, Lake Dijikare, Lake Monboio, Lake Yoan
Asia	Siberia	Kulunda Steppe, Tanatar Lakes, Karakul, Chita, Barnaul, Slavgerod, Lake Baikal region, Lake Khatyn
	Armenia	Araxes Plain Lakes
	Turkey	Lake Van, Lake Salda
	India	Lake Looner, Lake Sambhar
	China	Outer Mongolia, various "nors", Sui-Yuan, Cha-Han-Nor and Na-Lin-Nor; Heilungkiang, Hailar and Tsitsihar; Kirin, Fu-U-Hsein and Taboos-Nor; Liao-Ning, Tao-Nan Hsein; Jehol, various soda lakes; Tibet, alkaline deserts; Chahar,

		Lang-Chai; Shansi, U-Tsu-Hsein; Shensi, Shen-Hsia-Hsein; Kansu, Ning-Hsia-Hsein, Oinhgai Hu
Australia		Lake Corangamite, Red Rock Lake, Lake Werowrap, Lake Chidnup
Central America	Mexico	Lake Texcoco
Europe	Hungary	Lake Feher
	Fromer Yugoslavia	Pecena Slatina
North America	Canada	Manito
	USA	Alkali Valley, Albert Lake Lenore, Soap Lake, Big Soda Lake, Owen Lake, Borax Lake, Mono Lake, Searles Lake, Deep Springs, Rhodes Marsh, Harney Lake, Summer Lake, Surprise Valley, Pyramid Lake, Walker Lake, Union Pacific Lakes (Green Rivers), Ragtown Soda Lakes
South America	Venezuela	Langunilla valley
	Chile	Antofagasta

The well known Soda lakes are located in Central Asia, in the Western mountain shadowed desert of the USA and in East African Rift valley (Jones and Grant, 1999). The alkalinity of these lakes comes from the dominant soluble salts, Na_2CO_3/ $NaHCO_3$, formed by evaporative concentration under particular conditions of geology, geography and climate (Jones and Grant, 1999; Grant, 2006).

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 and Grant, 1999; 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 (Elizabeth Kebede, 1997; Tenalem Ayenew *et al.*, 2007).

The major Ethiopian Soda Lakes are Lake Arenguade, Lake Kilotes, Lake Shalla, Lake Abijata, Lake Chitu, Lake Hertale and Lake Methara. Most of the Ethiopian Soda Lakes are located in the Rift Valley of Southern Ethiopia (Grove *et al.*, 1975). The existence of these lakes is due to the numerous Late Quaternary central volcanic structures, which often separate the lakes from each other (Tenalem Ayenew *et al.*, 2007). Some Ethiopian Crater Lakes also share similarities with the Rift Valley Lakes concerning their chemical composition. These lakes are alkaline where the pH value ranges from 9 to more than 10 and sodium carbonate and sodium bicarbonate ions are in high concentration (Talling and Talling, 1965).

1.2. Microbial diversity in Soda lakes

The microbial biomass in Soda lakes is highly dominated by prokaryotes but rarely by eukaryotic algae and protozoans (Jones and Grant, 1999; Sorokin and Kuenen, 2005; Grant, 2006). The cell physiology of the microorganisms in this environment is adapted to maintain normal metabolic activity and dwell unaffected by the extreme alkalinity. The intracellular neutrality in alkaliphilic organisms is mainly maintained by adjusting the cell surface and their plasma membrane components (Horikoshi, 1999).

In the moderately saline lakes cyanobacteria, usually *Spirulina plantensis* (*Arthrospira fusiformis*) are the main contributors to primary production. Alkaliphilic anoxygenic phototrophic bacteria also substantially contribute to the primary production where the oxygen level is low in hyper saline lakes (Jones and Grant, 1999; Baumagrite, 2003). Some anoxygenic phototrophs isolated from the East African Soda Lakes include: *Ectothiorhodospira, Halorhodospira, Rhodobacter a, Roseinatronobacter, Alkalisprillum,* and *Heliorestis* sp.

Several hundred strains of non-phototrophic aerobic organotrophs have also been isolated from the environments of Rift valley Soda Lakes on a variety of media (Jones and Grant, 1999). Table 2 shows some of previously isolated prokaryotes in Soda Lakes.

Table 2: List of some prokaryotes growing in Soda Lakes (Source; Grant, 2006)

Groups known to be present	**Strains and species**
Cyanobacteria	*Arthrospira platensis, Cyanospira rippkae, Synechocystis sp., Synechococcus sp., Phormidium sp.*
Corynebacteria	*Bogoriella caseilyticus,Dietzia natronolimnaeaa*
Micrococci/Nesterenkonia	Lake Bogoria isolate 69B4a
Streptomyces/Nocardiopsis	Lake Nakuru isolate 11AG8
Bacillus/Clostridium	*Bacillus agaradhaerens, Bacillus clarkii, Bacillus cohnii, Bacillus pseudalcalophilus, Bacillus horikoshii, Bacillus gibbonsii, Bacillus haloalkaliphilus, Bacillus hortii, Bacillus vedderi, Anaerobranca gottschalkii, Tindallia magadiensis, Natronincola histidinovorans*
Haloanaerobes	*Natroniella acetigena, Halonatronum saccharophilum, Anaerobranca horikoshii*
Heliobacteria	*Heliorestis durensis, Heliorestis baculata, Heliospira daurica*
Sulfur oxidizers	*Thioakalimicrobium sibericum, Thioalkalimicrobium aerophilum, Thioalcalivibrio versutus, Thioalcalivibrio nitratus, Thioalcalivibrio denitrificans, Thioalkalimicrobium cyclum, Thioalkalivibrio jannaschii*
Nitrifiers	*Nitrobacter alkalicus*
Sulfate-reducing bacteria	*Desulfonatronovibrio hydrogenovorans, Desulfonatronum lacustre*
Anoxygenic phototrophs	*Ectothiorhodospira mobilis, Halorhodospira halophila, Thiorhodospira siberica, Roseinatronobacter thiooxidans, Rhodobacter bogoriensis, Alkalispirillum mobile, Ectothiorhodospira vacuolata, Ectothiorhodospira halochloris, Ectothiorhodospira haloalkaliphilus*
Halomonads	*Halomonas magadii, Halomonas campisalis, Lake Bogoria isolates 25B1, WB2, etc, Lake Elmeteita isolates 44E3, WE5, etc., Lake Magadii isolate 27M1, Lake Sonachi isolates 12C1, 75C4, etc*
Methylotrophs	*Methylobacter alcaliphilus, Methylomicrobium sp. AMO1*
Pseudomonads/*Stentrophomonas*	*Lake Elmenteita isolates 45E3, 97NT4*
Spirochaetes	*Spirochaeta alkalica, Spirochaeta asiatica, Spirochaeta africana*
Thermotogales	*Thermopallium natronophilum*
Halobacteria	*Halorubrum vacuolatum, Natrialba magadii, Natronobacterium gregoryi, Natronomonas pharaonis, Natronococcus occultus, Natronococcus amylolyticus, Natronorubrum bangense*
Methanogens	*Methanolobus oregonensis, Methanosalsus zhilinaeae, Methanobacteriun alcaliphilum*

1.3. Nutrient cycling in Soda Lakes

The effort in cultivating alkaline organisms resulted in finding remarkable diverse organisms. It also permitted the identification of most of the major trophic groups responsible for the cycling of carbon, sulphur and nitrogen in the lakes (Jones and Grant, 1999). Different types of alkaliphilic bacteria involved in the biogeochemical cycles of life-essential elements have been previously isolated from soda lakes (Dean and Gorham, 1998; Jones and Grant, 1999).

The methanogens and methylotrophic bacteria, utilizing variety of different one-carbon compounds including methane, methanol and methylated amines, are some of the carbon utilizing organisms isolated (Sorokin and Kuenen, 2005). The sulphur cycle is kept active in the soda lakes by both the anaerobic sulphur reducing and the aerobic sulphur oxidizing bacteria. Three genera of alkaliphilic, chemolithoautotrophic sulphur-oxidizing bacteria have so far been obtained in previous studies (Sorokin and Kuenen, 2005). Furthermore, several strains of alkaliphilic, lithotrophic ammonia and nitrite oxidizers as well as denitrifyers were isolated from different soda lakes of the world, hinting the presence of nitrogen cycle in the systems (Grant, 2006).

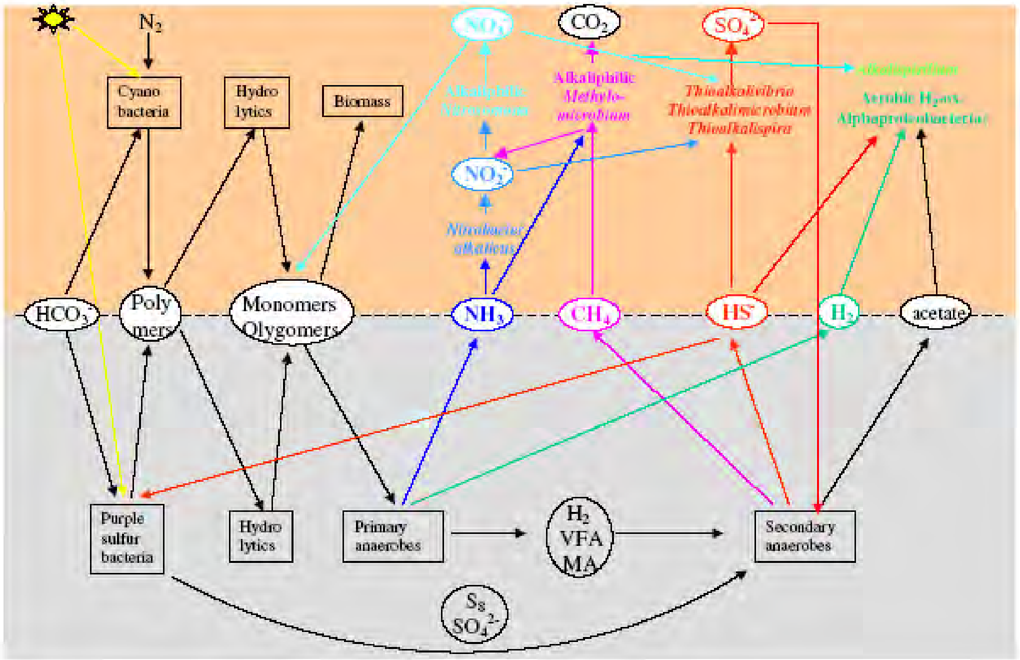


Figure 1: Trophic Relation in Soda Lake ecosystem (Source; Sorokin and Kuenen, 2005)

1.4. The Nitrogen shift in Soda lakes

High accumulation of the degraded products of the *S. plantensis*, bird's feather and other diverse organisms found in the soda lake supply the organic protein needed for the cycle

(Elizabeth Kebede, 1997; Grant, 2006; Tafesse Kefyalew, 2008). The amino acids and proteins are degraded, releasing carbon dioxide and ammonia in the system (Gerardi, 2007). Figure 2 illustrates the different sources of organic proteins and the microorganisms that are involved in the nitrogen cycle at different stages.

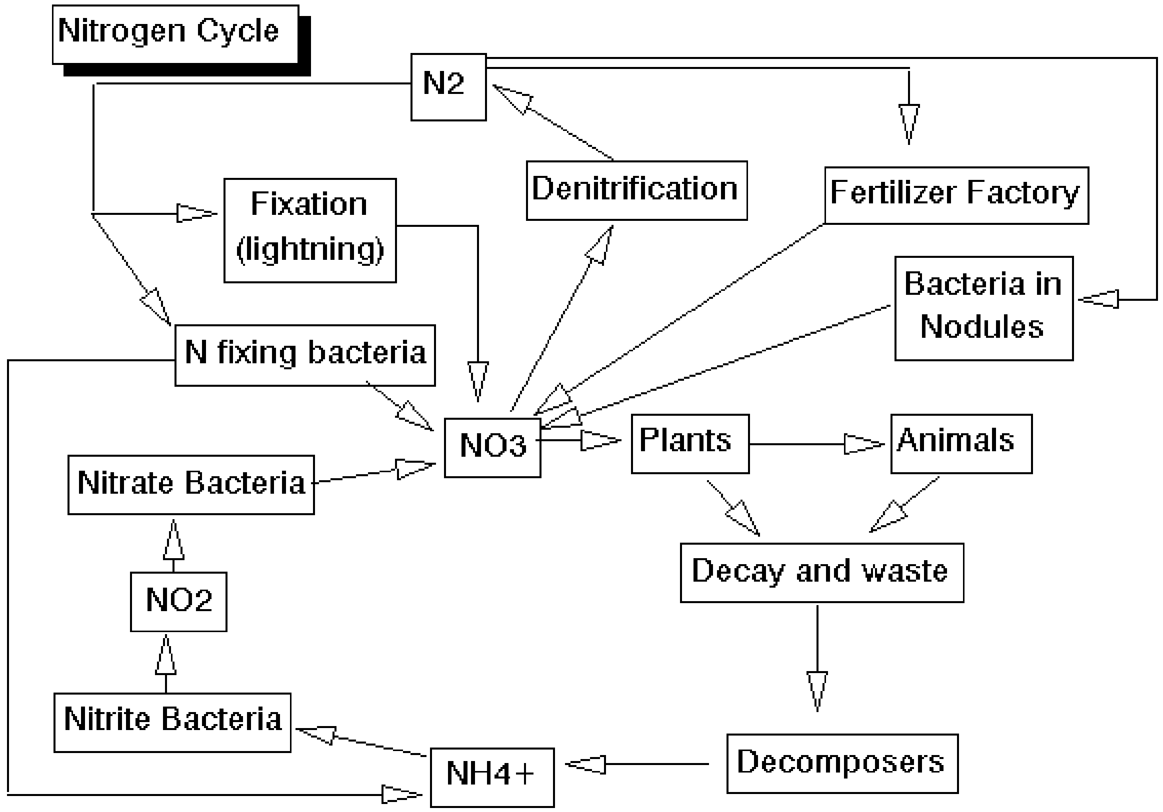


Figure 2: The Nitrogen cycle (Source: http://www.marietta.edu/~biol/102/ecosystem.html)

Assimilation and dissimilation are the two major processes in the nitrogen cycle. In the assimilation process, ammonification, nitrogen fixation and nitrification are present while denitrification and anaerobic ammonium oxidation are grouped under the dissimilation process. The assimilatory processes release NO_2 because of reduction of N_2 and/or N_2O by nitrogenase, and NO_3 by oxidation of ammonium (Bothe *et al.*, 2002). On the contrary, denitrification converts nitrate to nitrogen gas through a series of reactions that utilize the oxygen molecules as electron acceptors. The recently discovered path in the nitrogen cycle by the anaerobic ammonium oxidizing bacteria (ANAMMOX) is another process that produces either nitrogen gas or ammonium as an end product (Kartal, 2008).

1.4.1. Nitrification

Nitrification is one of the essential activities in natural and man-made environments. This process supplies oxidized forms of nitrogen for assimilation and anaerobic respiration and contributes to the primary production (Sorokin and Kuenen, 2005). Before nitrification occurs, decomposers convert the organic proteins found in living cells of any organisms into NH_4^+ through hydrolysis and decomposition. The other way of obtaining fixed nitrogen is through nitrogen fixers. The nitrogen fixers are unique and special microorganisms with the enzyme nitrogenase and the ability of converting N_2 gas directly from the atmosphere (Lindemann and Glover, 2003). The fixation of molecular nitrogen in soda lakes is accomplished by the phototrophic heterocystous cyanobacteria (*Cyanospira rippkae*), and probably by some of the aerobic and anaerobic chemo-organotrophs (Grant, 2006). The fixed nitrogen is released into the environment when the bacteria die and the nitrifiers use the ammonia produce in the process (Lindemann and Glover, 2003).

Ammonia oxidizing bacteria and Nitrite oxidizing bacteria are termed as Nitrifiers. Both types of organisms produce nitrate as their end product. Nitrification and production of nitrate occurs in both aerobic and anaerobic conditions by the nitrifiers. Ammonia oxidizing bacteria and Nitrite oxidizing bacteria perform well in the presence of oxygen but can also be found in anoxic environment (Abeliovich and Vonhak, 1992). The development of nitrifying bacteria in saline alkaline lakes is controlled by the salt content, rather than high pH values, in such a way that the nitrogen cycle in hyper saline soda lakes might get impaired (Sorokin and Kuenen, 2005). Several strains of lithotrophic and alkaliphilic ammonia and nitrite oxidizers are known to be found in Siberian and Kenyan soda lakes (Grant, 2006).

1.4.2. Anaerobic ammonium oxidation

In the Anammox process, ammonium is converted to dinitrogen gas with nitrite as electron acceptor (Kartal, 2008). This process comprises an ammonium oxidation reaction which is similar to nitrification, and a nitrite reduction reaction which is comparable with denitrification (Baolan *et al.,* 2006). The anaerobic ammonium oxidizing bacteria (ANAMMOX) are different from the ammonia oxidizers in such a way that, they do not need oxygen to oxidize but are highly dependent on nitrite. In contrast to denitrifying bacteria, ANAMMOX bacteria are chemolithoautotrophic, where ammonium is oxidized under anoxic conditions using nitrite as electron acceptor and form biomass by CO_2 fixation (Mulder *et al.,* 1995; Ward *et al.,* 2009). These bacteria are very sensitive to oxygen and nitrite. Oxygen and nitrite levels as low as 2µM and 5-10mM respectively stop the activity completely (Schmidt *et al.,* 2001).

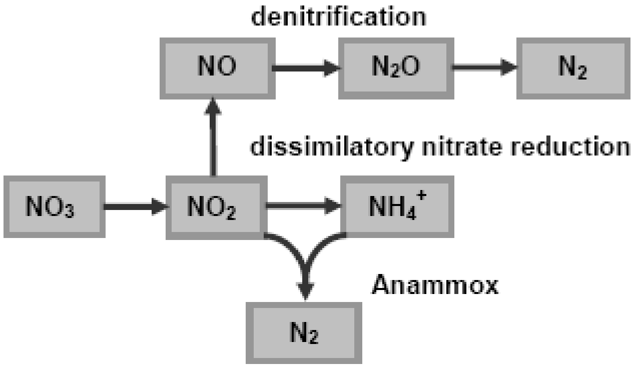


Fig.3: Two possible routes of nitrate in dissimilatory process: reduction by Anammox bacteria and denitrifying bacteria (Source: Kartal, 2008).

1.4.3. Denitrification

Denitrification is a dissimilatory process of denitrifying bacteria in which oxidized nitrogen compounds are used as alternative electron acceptors for energy production (Braker *et al.,* 1998; Flores-Mireles *et al.*, 2007). Normally, denitrification process occurs under anoxic conditions, where both nitrate as well as soluble carbon enriches the environment (Shapleigh, 2006). However, denitrifiers can also utilize oxygen as the terminal electron acceptor when it is available at higher concentration. The capacity to utilize nitrate as a terminal oxidant, when oxygen becomes limiting, allows denitrifying bacteria to continue respiration using an alternative electron acceptor (Shapleigh, 2006). Hence these bacteria derive the energy for cell division from the conversion of nitrate to nitrogen gas.

Denitrification is catalyzed by enzymes that strip the oxygen, one molecule at a time, where NO, N_2O and N_2 gases are released along with as end products (Braker *et al.*, 1998). The crucial reaction in denitrification is where nitrite is reduced to gaseous nitric oxide. This dissimilatory conversion of a fixed, oxidized, non gaseous form of nitrogen to a reduced gaseous forms that is lost to the environment led the process to be termed as denitrification (Shapleigh, 2006; Flores-Mireles *et al.*, 2007).

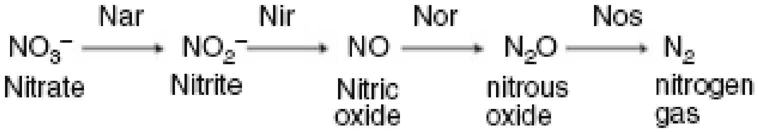


Figure 4: the four steps of denitrification involving the four important reductase enzymes (Nar- nitrate reductase; Nir- nitrite reductase; Nor- nitric reductase; Nos- nitrous reductase)

Many studies have shown the presence of denitrification in Soda lakes. The results obtained from previous tests done on N_2O reductase activity on samples taken from an alkaline and saline lake suggests the presence of denitrification activity in such environments (Miller *et al.*, 1986). Consequently, this finding together with the actual isolation of denitrifyers from Soda lakes (Jones and Grant, 1999; Sorokin and Kuenen, 2005; Grant, 2006; Rajiha Abubeker, 2008) provides enough evidence to assure the presence of active denitrification process in Soda lakes.

1.5. Previously studied Denitrifiers

Denitrifyers are one of the cosmopolitan organisms found on earth. Their habitats tend to range from hyper saline deep sea (Heitzer and Ottow, 1976) to an artificially synthesized “Mars” like environment (Julian *et al.*, 1995). Prokaryotes, mostly bacteria but a few archaea constitute the vast majority of organisms capable of denitrification (Shapleigh, 2006).

Previously studied denitrifying bacteria including the faculitative denitrifiers such as *Paracoccus denitrificans*, *Alcaligenes faecalis, Comamonas sp., Diaphorobacter sp.* (Robertson *et al.* 1988), *Acidovorax delafieldii, Corynebacterium variabile* (Xiao *et al.*, 2009), the *Halomonas sp.* (Jones and Grant, 1999; Yoshie *et al*, 2004), *Pseudomonas sp.* (Baolan *et al*, 2006) and so forth, are mostly from the taxons γ -proteobacter and the Firmicitus. Some species from three families namely the Pseudoalteromonadaceae, the Shewanellaceae and Ferrimonadaceae of the γ-proteobacter show the ability to reduce nitrate to nitrite, where only a few species from Shewanellaceae were reported to be denitrifyers (Lin and Shieh, 2006). Furthermore, many denitrifyers from the genus bacillus had also been isolated from major Soda lakes of the world (Berber and YenidÜnya, 2004). However, according to Anders *et al.*, (1995), when it comes to the property of denitrification, there is a change in the generic definition, since it could be present or be absent as a characteristic property of denitrification between two species of organisms within the same genera

1.6. Significance of Denitrification

1.6.1. Ecological importance of denitrifiers

Excess nitrate has negative impact on the environment. Residual, biologically active forms of nitrogen may be nitrified to NO_3^- and move from the terrestrial nitrogen pool into inland and coastal waters (aquatic/oceanic nitrogen pool) (Kemp et al., 2005). The high solubility and easy travelling nature of NO_3^--N through soil helps it to reach water bodies and promote algal growth, which can block sunlight to bottom- dwelling plants and suck oxygen from the water when the algae die (Xu *et al.,* 2009). The denitrifiers in aquatic and marine sediments return NO_3^- to the atmospheric pool as N_2. In addition to returning N to the atmospheric pool, the ecological significance of denitrification is that it permanently removes NO_3^- from a system that would otherwise be available for primary production (Kemp et al. 2005). This loss is of special interest in eutrophic, nitrogen-limited systems where substantial anthropogenic nitrogen inputs support high primary productivity, with a host of ecological and economic implications (Kemp *et al.* 2005).

1.6.2. Nitrate waste management

From the agricultural point of view, the removal of nitrate is not desirable (Celen and Kilic, 2003). Yet nitrate becomes a problematic pollutant of ground and surface water, creating global dilemma in the supply of drinking water. According to the WHO, the standard limit of NO_3^- in water supplies is 10mg NO_3^- -N/l. In reality however, the amount of NO_3^- in the ground water that is used as municipal water supplies exceeds this standard put forward by the WHO (Ergas and Reuss, 2001). Sources of NO_3^- contamination include the use of synthetic fertilizers, industrial and food processing operations and animal and human waste disposal (Ergas and Reuss, 2001; Xu *et al.*, 2009).

The ingestion of NO_3^- causes reduction of oxygen carrying capacity of the blood by forming methemoglobin when it is reduced to NO_2^-, and reacts with haemoglobin. Mehtaemoglobinaemia or blue baby syndrome is a toxic response to NO_3^- exposure in small children (Ergas and Reuss, 2001). The other serious consequences of NO_3^- wastes are; adverse impact on chlorine disinfection efficiency, unpleasant odours and accumulation of unsightly biomass, which are evidently seen in and around cities (UNESCWA, 2003).

There are physical, chemical, as well as biological ways to remove nitrogen waste (UNESCWA, 2003). From the three waste treatment methods, the biological removal system is frequently used because of its cost effectiveness and environmental friendliness (Ishizuka *et al.*, 1995: Akunna *et al.*, 1992). In the biological treatment system, nitrogen is normally removed out by different bacteria groups in two steps: the conversion of NH_4^+ to NO_3^- during nitrification followed by the conversion of NO_3^- to N_2 gas in denitrification.

Various nitrification/denitrification process technologies have been applied to waste water treatment in order to achieve nitrogen removal. Conventional technologies which are widely used for the biological nitrogen removal from waste waters include activated sludge and trickling filter systems (Nhlapo *et al.*, 2002) as well as sequencing batch biofilm reactor (SBBR) (Xiao *et al.*, 2009).

In the anaerobic digestion stages of anaerobic reactors, different groups of bacteria are found working in a chain like fashion, with the product of one group serving as the substrate of another group (Gerardi, 2002). The acceptable enzymatic activity of most of the different bacteria involved in the anaerobic digestion is achieved at pH range of 6.8 to 7.2 (Gerardi, 2002; Wen *et al*., 2009). Hence, sufficient alkalinity is essential for proper pH control since digester performance is influenced by pH. A study on the sludge from waste water treatment plant showed the optimum pH for N_2 gas production to be 9.5, which was equal to the pH in the reactor from which the sludge was collected (Buys *et al,* 2000). And at pH 11, the denitrification activity was very slow taking more than 40 hours though all the nitrate added was fully converted to N_2 (Buys *et al,* 2000). Hence, this and other studies done indicated that denitrifiers that can perform well in alkaline situations are preferred in nitrate waste treatment because of the increased pH of anaerobic digester (Gerardi, 2002).

2. Objectives

Previous studies confirmed the existence of denitrifying bacteria from East African Rift Valley Soda lakes (Grant, 2006; Rajiha Abubeker, 2008), which are characterized by high alkalinity and salinity (Elizabeth Kebede, 1996; Kemp et al. 2005; Grant, 2006; Xu *et al.*, 2009). Considering the fact that this lake is a closed basin, number of questions could be raised on the nitrogen economy of the lake, which enables to sustain such a high primary productivity. Hence, the detection of denitrifiers with such characteristics provides promising prospect in enhancing performances of waste treatment plants. However, the study on the diversity of the denitrifyers in Ethiopian Soda lakes is not completed; with the only information put in so far is the existence of denitrifyers in Lake Chitu (Rajiha Abubeker, 2008).

Therefore, the main objective of the research is to confirm the existence of denitrifying bacteria in some of the Soda Lakes of Ethiopia, namely; Lake Abijata, Lake Arenguade, Lake Chitu and Lake Shalla. Specifically, the study will focus on:

- The distribution of denitrifyers among the four Soda lakes of Ethiopia
- The abundance of denitrifyers in the mud and water samples of the four Soda lakes
- The similarity/diversity of the denitrifyers among the Soda lakes of Ethiopia
- The phylogenetic relation of the denitrifying Isolates obtained from the Soda lakes

3. Materials and methods

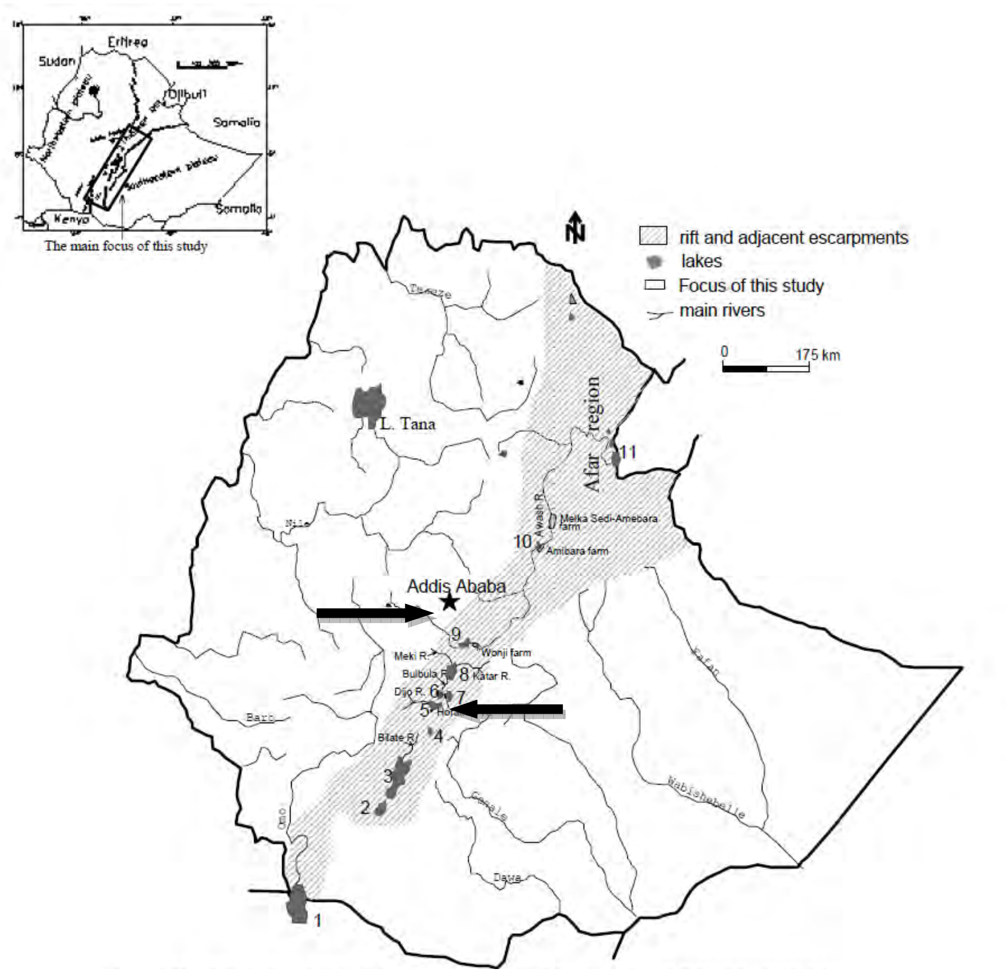
3.1. Description of study areas

The four lakes included in this study are: Lake Abijata, Lake Arenguade, Lake Chitu and Lake Shalla. Lakes Abijata, Chitu and Shalla are found adjacent to each other in the Rift Valley system, and are located about 180 km south from Addis Ababa. Lake Arenguade, however is found far away from the other lakes and is located about 50 km southeast from Addis Ababa. Previously recorded physical and chemical data on the lakes is listed in Table 3.

Table 3: Some physical and chemical properties of the study areas

	Lake Arenguade	Lake Shalla	Lake Abijata	Lake Chitu
Altitude (m)	1900	1570	1580	1540
Area (Km2)	0.54	409	205	0.8
GPS	080 41’ 33”N 0380 58’ 44”E	070 28’ 40”N 0380 38’ 00”E	070 32’ 06”N 0380 37’ 50”E	07^0 24’ 26”N 038^0 25’ 33”E
Maximum depth (m)	32	266	14.2	6.0
Mean pH	10.3	9.9	9.5 (increasing)	10.2
Alkalinity (meq l-1)	51-57	218	349	581
Primary producers	*S.paltensis*	Diatoms	*Anabaenopsis abijatae*	*S.paltensis*
Mean conductivity(µScm^{-}1)	6039	23450	28685	48000
Dissolved oxygen	<2m	<20m	Highly oxygenated	-

(Source: Baxter *et al.,* 1965; Grove *et al*., 1975; Green, 1986; Elizabeth Kebede and Willen, 1996; Elizabeth Kebede, 1997; Baxter, 2002; Tafesse Kefyalew, 2008)



Lakes: 1. Chew Bahir, 2. Chamo, 3. Abaya, 4. Awasa, 5. Shalla, 6. Abijata, 7. Langano, 8. Ziway, 9. Koka, 10. Beseka, 11. Abhe

Figure 5: Map of Ethiopia indicating the two different locations of the sample sites (Source: Tenalem Ayenew, 2007)

Figure 6. Satellite image of Lake Arenguade showing its surroundings and location (Source: Google Earth)

Figure 7. Satellite image of the Rift valley lakes in the study showing their surroundings and locations (Source: Google Earth)

3.2. Sample collection

Samples of water and mud were collected from Lake Chitu, Lake Abijata, Lake Shalla and Lake Arenguade. The samples were kept in a sterilized container and transported to the laboratory and kept at room temperature for analysis

Table 4: Sample collection: sampling site and the sample types

Name of sample place	Sample type	Depth (meter)	#Designated codes for samples
Lake Chitu	Mud	0-0.5m	DN-$C_{0.5}$m
	Mud	0.5-1m	DN-C_1m
	Water	0m(surface)	DN-Cw
Lake Abijata	Mud	0-0.5m	DN-A
Lake Shalla	Mud	0-0.5m(surface)	DN-Ss
	Water	0m(surface)	DN-Sw
Lake Arenguade	Mud	0-0.5m	DN-As
	water	0m(surface)	DN-Aw

3.3. Isolation of denitrifying bacteria

For isolating denitrifying bacteria, an enrichment media was prepared (Dr. Amare Gessesse, personal communication). The pH of the media was adjusted to 10.5 by adding 25% Na_2CO_3 solution. The compositions of the trace metal composition and the enrichment media is listed in Tables 5a and 5b respectively.

Table 5a: Enrichment media composition

No.	**Components**	**Amount (per litter)**
1	K_2HPO_4	0.1g
2	$MgSO_4.7H_2O$	0.1g
3	$CaCl_2$	0.15g
4	$FeCl_3.6H_2O$	0.02g
5	$MnSO_4.4H_2O$	0.01g
6	$C_2H_3NaO_2$	2g
7	$NaNO_3$	2g
8	Nutrient Broth	13g
9	Nutrient Agar	28g
10	Trace metal solution	10ml
11	25% Na_2CO_3	10g

Table 5b: Composition of Trace metal

No.	**Component**	**Amount (g/l)**
1	EDTA	0.05
2	$ZnSO_4.7H_2O$	0.02
3	$MnCl_2.4H_2O$	0.055
4	$(NH_4)Mo_7O_2. 4.H_2O$	0.01
5	$CaSO_4. 2H_2O$	0.1142
6	$CoCl_2.6H_2O$	0.02

Mud and water sample collected from the different lakes was mixed and serially diluted from 10^{-1} to 10^{-5} using sterile saline solution (0.85% NaCl). A 100 µl of serially diluted sample was spread on agar plates, incubated at 30°C, and checked for the emergence of colonies after 24 and 48 h. Each distinct colony was picked and purified through repeated streaking and transferred to agar slants for further characterization.

3.4. Test for denitrification

The test for denitrification was adopted from Gerhardt *et al.* (1981). Plates of enrichment media were prepared to grow fresh pure colonies of isolates. Those that grew under 24hrs were tested first and those that grew between 24hrs to 48hrs were tested next. Two types of media were prepared for the test and their composition is listed in Table 6a and Table 6b.

Media A, in the amount of, 4ml was poured first into a sterilized screw up test tube. After inoculation of one loop full of the pure colonies in each test tube, 1ml of media B was poured slowly not to produce bubble. The test tubes were left for about 30min for the solution to stand. Another 1ml of Media B was added into the test tubes as a top layer to make a two phase solution. Each test tube was labeled by writing the dilution the designated code of sample site and the replication and then was incubated at 30^0 until gas bubble was noticed.

Table 6a: Composition of Media A for N_2 gas test

Component	Amount (g/l)
Nutrient broth	8
$NaNO_3$	1.5
25% Na_2CO_3	40ml

Table 6b: Composition of Media B for N_2 gas test

Component	Amount (g/l)
Nutrient broth	8
$NaNO_3$	1.5
1% Agar	10
25% Na_2CO_3	40ml

3.5. DNA extraction

For DNA extraction, a freeze and thaw method (Moore *et al.,* 1999) was modified and used. In this method, a loop full of colonies was placed in the eppendrof tubes and 50µl 1xTE buffer was added. Then the eppendrof tubes were put in a 90˚C water bath for 5min and then in a -20˚C for 20min. The step was repeated three times before equal amount of Phenol: Chloroform: Isoamyl in a 25:24:1 ratio was added and centrifuged at 14,000 rpm for 5min. The presence of DNA was checked by electrophoresis using 0.70% Agarose gel. The presence of DNA on gel after electrophoresis was observed using BIORAD Gel Doc XR+System and the patterns were analyzed using QuantityOne program software.

3.6. Gel electrophoresis

The Agarose was dissolved in 1xTAE buffer by boiling it on hot plate. Once it was boiled it was left to cool down until it can be hold by hand (about 40˚C) before Ethidium bromide was added into it in the amount of 5µl/100ml of Agarose. The Agarose gel was then poured on electrophoresis plate with a comb for making loading wells. When the gel was set the plate was transferred to the electrophoresis for the running. The thickness of the gel was determined by adjusting the concentration of the Agarose according to the purpose of the electrophoresis.

3.7. Amplification of DNA using Polymerase chain reaction (PCR)

The method for the synthesis of DNA using PCR was adapted and done according to Mullis and Floona, (1987). The extracted DNAs from were grouped in batches. The first three batches each composed of ten isolates were amplified using HOT START PCR master mix. The taq polymerase and all the other components except for the forward and reverse primers, the RNase free water and the DNA templates were provided as a mixture from the QIAGEN (Germany). This process was done using TECHNE model TC-412 PCR.

The sequences of the forward and reverse primers are:

Forward primer (E9$_f$): 5´- GAG TTT GAT CCT GGC TCA G -3´

Reverse primer (UI5IO$_r$): 5´- GGT TAC CTT GTT ACG ACT T-3´

Table 7a: PCR components used in the reaction

	PCR component	**Amount/one reaction volume**
Hot Start PCR	Taq hot start	12.5 µ l
	Primer (E9$_f$)	1.6 µ l
	Primer (UI5IO$_r$)	1.6 µ l
	RNase free H_2O	7.3 µ l
	DNA template	2.0 µ l
	Total reaction volume	25.0 µ l

PCR-program for Hot Start: Pre heat lid was adjusted at 104°C and then Pre denaturation at 94 °C for 5 min. For each cycle of amplification, the denaturation temperature was 94 °C for 30 sec, annealing temperature was55 °C for 30 sec and extension temperature was 72 °C for 1min. The total cycles adjusting for amplification were 30 cycles. The final extension was hold for 7 min at 72°C and the temperature dropped to 10°C for final hold. The PCR products were then stored at 4 °C.

The remaining 46 DNA extracts were amplified using different PCR components with different amount and PCR-program. For this reaction mix the taq polymerase, the DNtps mix and the PCR buffer were provided separately as the two forward and reverse primers.

Table 7b: Amounts of PCR components used for non hot start PCR reaction

	PCR component	**Amount/one reaction volume**
Non Hot Start PCR	PCR H_2O	17.975 μ l
	PCR buffer	2.5μ l
	DNtp mix	1μ l
	Primer ($A8_f$)	0.2μ l
	Primer ($H1542_r$)	0.2μ l
	Taq polymerase	0.125μ l
	DNA template	3μ l
	Total reaction volume	25.0 μ l

The sequences of the forward and reverse primers were:

Forward primer ($A8_f$): 5´- GAG AGT TTG ATC CTG GCT CAG -3´

Reverse primer ($H1542_r$): 5´- AAG GAG GTG ATC CAG CCG CA-3´

The temperature and the time for denaturation, annealing and extension were adjusted according to the optimization process undergone. This process was done using TECHNE model TC-412 PCR.

PCR-program for non Hot Start: Pre heat lid was adjusted at 105°C and then Pre denaturation at 94 °C for 5 min. For each cycle of amplification, the denaturation temperature was 94 °C for 30 sec, annealing temperature was 56 °C for 30 sec and extension temperature was 72 °C for 1min. The total cycles adjusting for amplification were 35 cycles. The final extension was hold for 7 min at 72°C and the temperature dropped to 10°C for final hold. The PCR products were then stored at 4 °C.

The presence of PCR product was checked by running electrophoresis. A 3 μ l of PCR product mixed with loading dye was put on wells made on 1% agarose gel along side with

1kb ladder. The electrophoresis was running for 1hr at an electric current of 80volt. The images of the PCR product were observed using BIORAD Gel Doc XR+System and the patterns were analyzed using QuantityOne program software.

3.8. Restriction Digest

The obtained PCR products were subjected to restriction digestion using different types of restriction enzymes for further analysis. The two types of enzymes used separately in this procedure were Alu I and Rsa I. The amount of the enzymes and their buffers used are listed in Table 8. The procedure for this analysis was adapted from Current Protocols in Molecular Biology ringbou edition (2003).

Table 8: Amount of Restriction Digest components

Types of enzyme	**Amount of enzyme (10u/ µ l)**	**Amount of 10x buffer (Tango yellow)**	**Amount of H_2O (distilled and sterilised)**	**Amount of PCR product**	**Total reaction volume**
Alu I	0.4 µ l(4U)	1.5 µ l	5.1 µ l	8 µ l	15 µ l
Rsa l	0.4 µ l(4U)	1.5 µ l	5.1 µ l	8 µ l	15 µ l

Once the PCR product and the two enzymes were mixed in a separate eppendrof tubes and sealed carefully using a parafilm, the restriction mixture was incubated at 37°C incubator overnight. The overnight mixture was blended with loading dye and was put on 3% agarose gel along side with a 1kb ladder and run electrophoresis for 1hr and 20min at an electric current of 80volt. The purpose of the 1kb ladder was to look at the size of the fragments. The image of the DNA fragments on the gel after electrophoresis was seen using BIORAD Gel Doc XR+System and the patterns were analyzed using QuantityOne program software.

3.9. Construction of Amplified Ribosomal DNA Restriction Analysis (ARDRA) table

The approach for screening numbers of isolates using restriction enzymes for amplified rDNA restriction analysis (Upholt, 1977) was applied. The patterns obtained from the restriction digest were used as ‘fingerprint’ to compare isolates with each other and with the corresponding fragments of 1kb ladder used. The isolates were then grouped according to the information obtained in the Restriction analysis. ARDRA table was constructed to show which isolates were grouped together.

3.10. Sequencing of isolates and construction of phylogenic tree

Representatives from the constructed ARDRA groups were sequenced and the phylogenic tree using the 16s rRNA was made. The sequencing was done using the Exozap reaction (cleaning PCR-products), for removing left over primers and other reagents from the PCR-reaction followed by BigDye reaction, to label the nucleotides in the DNA fragments that will be sequenced with fluorescence. The principles used for this process was adopted from Sanger *et al.,* (1977). The products from the BigDye reaction were delivered to the sequencing lab at University of Bergen.

The sequences obtained were edited using sequence editor, BioEdit. Sequences that were not “Bad” were compared using BLAST (Basic Local Alignment Search Tool) at the National Center for Biotechnology Information (NCBI) database. The alignment scores and the percent sequence identity were determined for the closest identity of the sequences obtained to attain similarities with other 16S rDNA nucleotide in the database. Further sequencing could not be conducted for isolates which had percent similar identity less than 97%; hence, isolates with less than 97% similarity were excluded from further molecular investigations. The first 3 hits with maximum percentage similarities were taken as reference. The phylogenic tree was constructed first by aligning the sequences using ClustalW program. The aligned sequences were then run to construct phylogenic tree using MEGA4 software by downloading from the internet.

3.11. Biochemical tests for selected Operational Taxonomic Units (OTU)

Gram test, Oxidase Test and Catalase Test were done on the representatives of the ARDRA groups to check their biochemical nature. Gram Test was done for the representative OTUs of each constructed ARDRA groups by adapting Pacarynuk (2005) method. The Oxidase test was done using N,N,N',N'-tertamethyl-p-phenylenediamine (TMPD) to check whether the isolates could produce certain cytoochrome c oxidases or not by adding 3-5 drops of TMPD on the filter paper and observing the results with in 2 minutes. Catalase Test for the representative isolates from each OTU groups was done using 1% H_2O_2. The air bubble is an indicative of the production of O_2 when H_2O_2 is catalyzed by the enzyme Catalase.

3.12. Morphological analysis

For further characterization of the isolates, microscopic observations of the colonies as well as slides were undertaken. The slide of the isolates were prepared and examined under 40 x and 100 x magnification. Then to have clear view, oil staining was used under light microscope. For studying the colony shape, the plate was placed on a dissecting microscope to analyze the form of a single colony. The shape of the colonies of the isolates were determined according to the standard characteristics described in the ninth edition of Bergey's Manual of Systemic Bacteriology (Holt *et al.,* 1994)

3.13. Determination of Growth Curve

For additional description of the isolates and selecting one with relative high denitrification ability, qualitative test using the test for denitrification (Gerhardt *et al.* 1981) and determination of growth curve were done respectively. For determination of growth rate, one isolate was chosen after looking at the performance on the qualitative test. Hence an overnight culture of isolate with relative high denitrification ability was inoculated into a liquid enrichment media early in the morning and the optical density was measured every hour until enough data was obtained. The OD was measured using UV-7804C spectrophotometer at 600nm.

4. Results

4.1. Isolation of alkaliphilic denitrifyers

A total of 229 distinct colonies were picked from all the sample sites. Out of these distinct colonies, 95 isolates (42%) gave positive results after N_2 gas test. After the N_2 gas test, different numbers of denitrifying colonies from both the mud and water samples were obtained from the four lakes. Relative high percentage of denitrifying isolates was attained from Lake Abijata. Lakes Chitu and Shalla ranked second and third, in the total abundance of denitrifiers from both sample types (Table 9).

Table 9: Total amount of denitrifying bacteria obtained indicating the relative abundance of each studied lake.

Sample site	**# of distinct colonies from enrichment media**	**# of isolates positive for N_2gas test**	**% of denitrifying isolates in each lakes**
Lake Chitu	65	35	54%
Lake Abijta	19	14	70%
Lake Shalla	60	27	45%
Lake Arenguade	85	19	22%
Total	**229**	**95**	**42%**

Relatively similar percent of denitrifying isolates in the mud samples of Lakes Chitu and Shalla were obtained (Table 10). The water samples from Lakes Chitu and Shalla also exhibited similar ranks as with their mud samples (Table 11). However, the total number of denitrifying isolates obtained from Lake Arenguade was relatively the least even though the number of distinct colonies picked from the enrichment media for the gas test was the highest. The scarcity of denitrifiers from the water samples of this lake contribute significantly for the total minimum percent attained despite the fact that the mud samples contained high percentage of denitrifying isolates.

Table 10: Amount of denitrifying bacteria obtained from the mud samples.

Place of mud sample taken	**# of colonies from enrichment media**	**# of isolates positive for N_2 gas test**	**% of denitrifying isolates in each sample types**
Lake Abijata	19	14	**70%**
Lake Chitu	41	22	**54 %**
Lake Shalla	30	16	**54%**
Lake Arenguade	35	18	**51%**
Total	**125**	**70**	**56%**

Table 11: The amount of denitrifying bacteria obtained from the water samples of the lakes

Place of water sample taken	**# of colonies from enrichment media**	**# of isolates positive for N_2 gas test**	**% of denitrifying isolates in water sample**
Lake Chitu	24	13	**54%**
Lake Shalla	30	11	**37%**
Lake Arenguade	50	1	**2%**
Total	**104**	**25**	**24%**

4.2. Amplification of extracted DNA

Enough DNA was obtained from all the tested denitrifying isolates. The amplification of the 16SrRNA gene was highly successful for the batches that were analyzed using the Hot Start Taq. In Hot Start activation, primer extension is blocked until the reaction mixture reaches an elevated, Hot Start temperature, where the stringency of the primer/target hybridization is optimal for specificity and primer complexes are dissociated (Lebedev *et al.,* 2008).

However, the amplification of this DNA region was not as simple for the Non Hot Start Taq PCR- Programme as it was for the Hot Start Taq. Off target amplification occurred during this procedure which leads to the formation of “Mis- Priming” and/ or “Primer Dimer” extension products. All the same, the DNA or 75 isolates were successfully amplified, where one isolate (BACC118), was from previous study and used as a positive control.

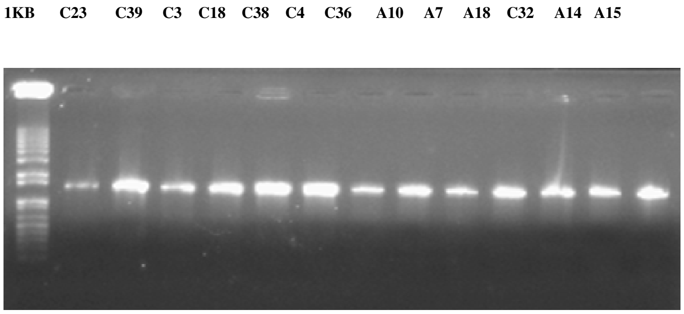
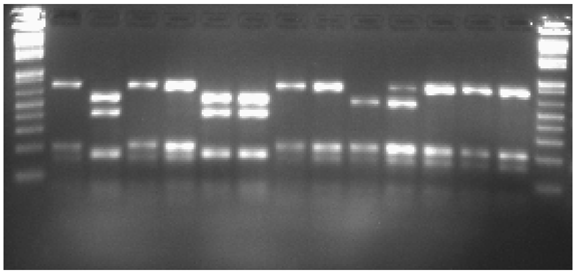


Figure 8. Sample image for amplified 16rRNA gene using PCR

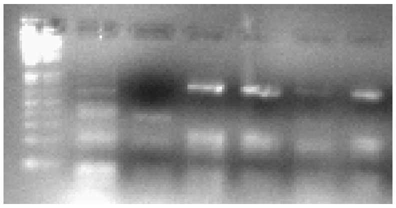
4.3. Restriction Digest

The results obtained after restriction digestion using the two enzymes, AluI and RsaI are shown in Figure 9. The patterns observed indicated that the two restriction enzymes cleave the DNA at different site generating different length of DNA fragment. As a result, groups of different patterns of DNA fragments were obtained indicating different types of isolates. These different patterns suggest the dissimilarity of organisms from which the 16SrRNA gene was obtained. On the other hand similar fragment patterns indicate close relationship between the organisms from which 16rRNA gene is obtained.

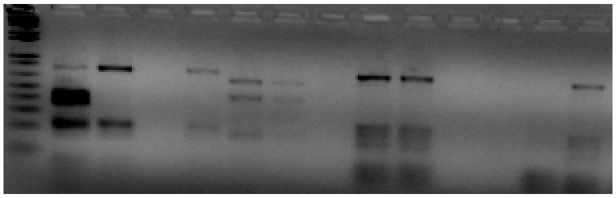
Alu I



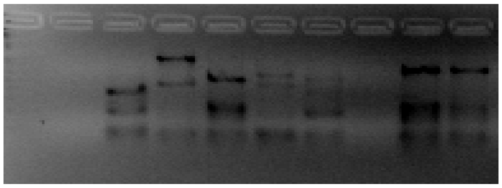
Alu I



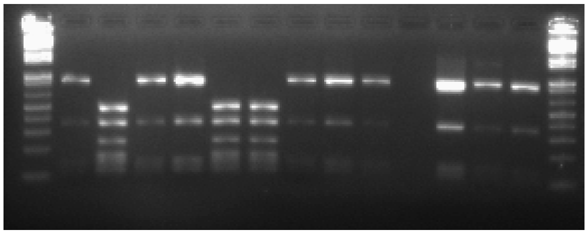
AluI



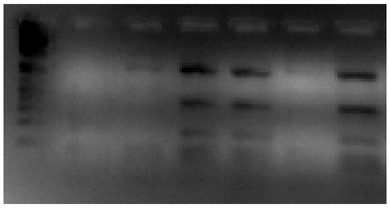
AluI



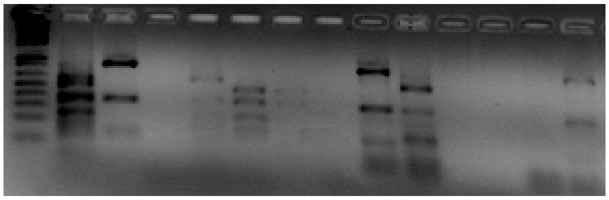
Rsa I



Rsa I



RsaI



RsaI

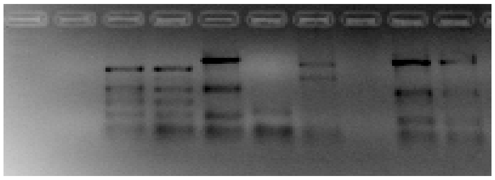


Figure 9: Images of restriction fragment patterns obtained using AluI and RsaI enzymes

4.4. Amplified Ribosomal DNA Restriction Analysis (ARDRA) grouping

According to the 16SrDNA ARDRA map, the total possible operational taxonomic unit (OTU) number values obtained were 31. These OTUs were classified according to Alu I and RsaI cleavage patterns. Each ARDRA group encloses isolates with identical fragment pattern, hence indicate the similarity or close association of the isolates. However, Group 1 and Group 1.1 showed same restriction pattern but differed in morphological appearance. BACC118 is a denitrifying bacterial isolate that was obtained from previous study on Lake Chitu.

Table 12: ARDRA table: the table shows the isolates forming groups according to the cleavage patterns of the two restriction enzymes used. In addition, the sample place is included to crosscheck the lake distribution of the isolates.

OUT Group no	**SAMPLE PLACE**	**SAMPLE NAME(DN-)**
1	**Chitu and Abijata mud , Chitu water**	**$C13_{1}$, A17, C8, C19, C14, C9, (BACC118), C3, C18, C36, CW16, CW18, CW12, C32**
1.1	**Chitu and Abijata mud , Chitu water**	**A14, A15, C23, A10, CW15**
2	**Chitu and Abijata mud**	**$C13_{2}$, A13**
3	**Abijata mud**	**A2**
4	**Abijata mud**	**A8**
5	**Chitu mud**	**C41**
6	**Chitu mud**	**$C17_{1}$, ($C17_{3}$)**
7	**Chitu and Arenguade mud**	**C39, C38, C4, AS47, $AS58_{C}$, AS72**
8	**Abijata mud**	**A7**
9	**Abijata mud**	**A18**
10	**Shalla and Arenguade mud & Chitu water**	**SS55 SS57 AS68 AS67 AS51 CW19**
11	**Shalla and Arenguade mud**	**SS44 AS11**
12	**Shalla mud**	**SS36**
13	**Shalla water**	**SW21 SW28**
14	**Shalla water**	**SW23**
15	**Shalla water**	**SW26**
16	**Arenguade mud**	**AS12**
17	**Shalla water**	**SW36**
18	**Abijata mud**	**A12**
19	**Arenguade mud**	**AS58**

20	**Chitu water**	**CW14**
21	**Chitu water**	**CW22**
22	**Arenguade water and Arenguade mud**	**AW25 AS42**
23	**Shalla mud**	**SS38**
24	**Chitu water**	**(CW5)**
25	**Shalla water**	**SW 29**
26	**Shalla mud**	**SS32**
27	**Chitu and Abijata mud**	**A19 C40 C22 C11 A9**
28	**Chitu Mud**	**A3**
29	**Shalla and Arenguade mud**	**SS41 SS31 SS56 SS35 SS48 AS69 SS46 SS40**
30	**Chitu water**	**(CW17)**
31	**Chitu water**	**CW11**

4.5. Analysis of 16SrRNA sequence and construction of phylogenic relationship

Sequencing of selected representative isolates from each group was done to further classify the diversity of the bacteria. Of the 31 ARDRA groups, 30 representatives were able to be sequenced. Six sequence results were considered “Bad” because there were many chains of unidentified nucleotides (high noise). Hence these sequences were not included in the construction of the phylogenic tree. In addition, 4 sequences were moderately short (~800bp) with the exception of one that was about 600bp (Annex 1). Therefore, the pair wise as well as the multiple alignments and the tree construction processes was done with and without this very short sequenced OTU. The nucleotide sequence similarities between the 16SrRNA genes of the representative isolates and those in the database from the NCBI ranged from 93-100%.

Consensus phylogenetic tree based on partial 16S rDNA sequences of Denitrifying bacteria isolates. Maximum parsimony method was applied to find the simplest and most reasonable relation. *Archaeoglobus infectus* was used as an out group. The significance of each branch is indicated by a bootstrap value calculated for 1000 replicates. Values of 50% or greater are shown.

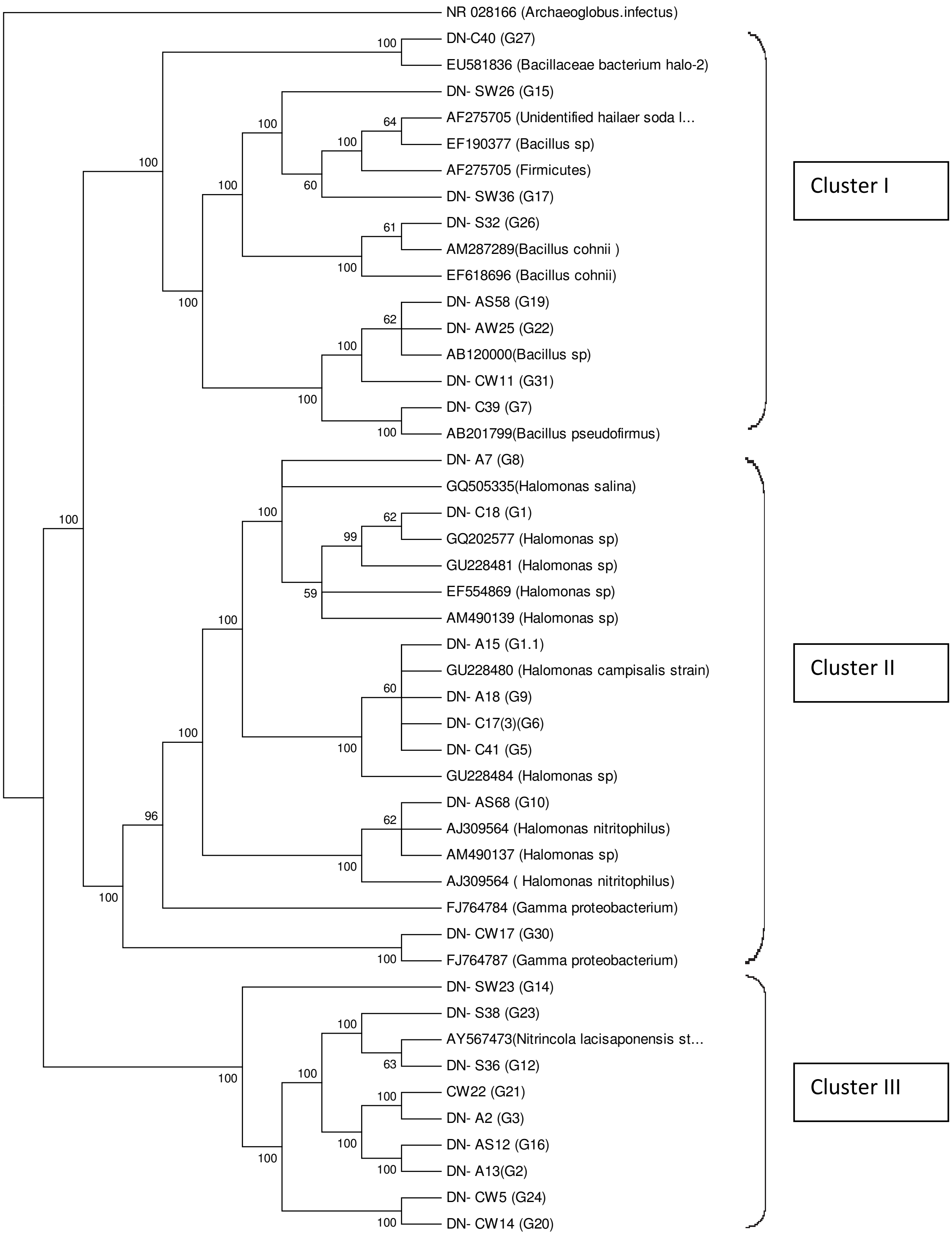


Figure 10: Phylogeny tree: Evolutionary relationships of 47 taxa

{The evolutionary history was gathered using the Maximum Parsimony (MP) method. The consensus tree inferred from 104 most parsimonious trees is shown. Branches corresponding to partitions reproduced in less than 50% trees are collapsed. The percentage of parsimonious trees in which the associated taxa clustered together is shown next to the branches. The MP tree was obtained using the Close-Neighbor-Interchange algorithm in which the initial trees were obtained with the random addition of sequences (10 replicates). All alignment gaps were treated as missing data. There were a total of 1169 positions in the final dataset, out of which 817 were parsimony informative. Phylogenetic analyses were conducted in MEGA4 software. }

4.6. Results of biochemical and morphological analysis

The biochemical tests and the morphological analysis performed helped to enforce the results obtained from the molecular characterization. The results obtained from these tests are listed in Tables 13 and 14 respectively. Almost all the representatives contained the enzymes Oxidase and Catalase, except for few isolates that did not have Oxidase. The Gram test revealed that most of the isolates are Gram negative.

Table 13: Results of biochemical tests

Group number	**Code of isolates**	**Test result of**		
		Oxidase	**Catalase**	**Gram staining**
G1	DN-C18	+	+	Gram -
G1.1	DN-A15	-	+	Gram -
G2	DN-A13	+	+	Gram -
G3	DN-A2	+	+	Gram -
G4	DN-A8	+	+	Gram -
G5	DN-C41	+	+	Gram -
G6	DN-C171	+	+	Gram -
G7	DN-C39	+	+	Gram +
G8	DN-A7	+	+	Gram -
G9	DN-A18	+	+	Gram -
G10	DN-AS68	+	+	Gram -
G11	DN-SS44	+	+	Gram +
G12	DN-SS36	-	+	Gram -
G13	DN-SW28	+	+	Gram -
G14	DN-SW23	+	+	Gram +
G15	DN-SW26	+	+	Gram +
G16	DN-AS12	+	+	Gram -
G17	DN-SW36	+	+	Gram +
G18	DN-A12	-	+	Gram +
G19	DN-AS58	-	+	Gram +
G20	DN-CW14	+	+	Gram -
G21	DN-CW22	+	+	Gram -
G22	DN-AW25	+	+	Gram +
G23	DN-SS38	+	+	Gram -
G24	DN-CW5	-	+	Gram -
G25	DN-SW29	+	+	Gram +
G26	DN-SS32	+	+	Gram +
G27	DN-C40	-	+	Gram +
G28	DN-A3	+	+	Gram -
G29	DN-AS69	+	+	Gram -
G30	DN-CW17	+	+	Gram -
G31	DN-CW11	-	+	Gram +

Table 14: Result of Microscopic observation

Group no.	**Name of isolates**	**Microscope observations**	
		Slide (cell structure)	**Colony**
G1	DN-C18	Rod	Circular, translucent mucoid
G1.1	DN-A15	Rod	Circular, dark middle with translucent edge
G2	DN-A13	Rod	Circular, translucent mucoid
G3	DN-A2	Rod	Circular, dark middle with translucent edge
G4	DN-A8	Rod	Circular, translucent mucoid
G5	DN-C41	Rod	Circular, dark middle with translucent edge
G6	DN-C171	Rod	Circular, translucent mucoid
G7	DN-C39	Rod	Circular, translucent mucoid
G8	DN-A7	Rod	Circular, dark middle with translucent edge
G9	DN-A18	Rod	Circular, dark middle with translucent edge
G10	DN-AS68	Rod	Circular, translucent mucoid
G11	DN-SS44	Rod	Circular, translucent mucoid
G12	DN-SS36	Rod	Circular, translucent mucoid
G13	DN-SW28	Rod	Circular, translucent mucoid
G14	DN-SW23	Rod	Circular, translucent mucoid
G15	DN-SW26	Rod	Circular, translucent mucoid
G16	DN-AS12	Rod	Circular, translucent mucoid
G17	DN-SW36	Rod	Non circular “star like” translucent mucoid
G18	DN-A12	Rod	Non circular “star like” translucent mucoid
G19	DN-AS58	Rod	Circular, translucent mucoid
G20	DN-CW14	Rod	Circular, translucent mucoid
G21	DN-CW22	Rod	Circular, translucent mucoid
G22	DN-AW25	Rod	Circular, translucent mucoid
G23	DN-SS38	Rod	Circular, translucent mucoid
G24	DN-CW5	Rod	Non circular “star like” translucent mucoid

G25	DN-SW29	Rod	Circular, translucent mucoid
G26	DN-SS32	Rod	Circular, translucent mucoid
G27	DN-C40	Rod	Non circular “star like” translucent mucoid
G28	DN-A3	Rod	Circular, translucent mucoid
G29	DN-AS69	Rod	Circular, translucent mucoid
G30	DN-CW17	Rod	Circular, translucent mucoid
G31	DN-CW11	Rod	Circular, translucent mucoid

4.7. Determining Growth Curve

This test helped in the ranking of the isolates according to the time each representative isolate took to produce visible air bubble in the test tube. The qualitative test was done by adapting the procedure from (Gerhardt *et al.* 1981). Table 15 shows the result of this test. From these results, it was possible to select the fast gas producer for further analysis on its growth. In addition, the table also gives indication about the denitrification activity of each representative isolates (highly intense gas bubble indicated high denitrification activity of the isolate while little gas bubble indicated low denitrification activity) where the gas bubbles signified NO_3^- changing in to gaseous form.

For determining late Log phase, an isolate with the fastest gas production from the representative OTU groups was chosen. The isolate with the designated code DN-C18 was found to produce gas faster than the others thus was chosen for further analysis. The result for determination of the growth curve of the chosen isolate showed the time needed for the bacteria to reach stationary phase to be 8hrs after inoculation (Annex 2).

Table15: Result of qualitative test of representative isolates using N_2 Gas test intensity.

NAME OF ISOLATES	**GROUP NO.**	**TIME**										
		0hr	**1hr**	**2hr**	**3hr**	**4hr**	**5hr**	**6hr**	**7hr**	**>8hr&<24hr**	**>24hr&<48hr**	**>48hr&<96hr**
DN-C18	G1	-	-	+	+	+*	+*	+*	+*	+*	+*	+*
DN-A15	G1.1	-	-	-	-	-	-	-	-	-	-	+
DN-A13	G2	-	-	-	-	+	+*	+*	+*	+*	+*	+*
DN-A2	G3	-	-	-	-	-	-	-	-	+	+	+
DN-A8	G4	-	-	-	-	-	-	-	-	-	-	+
DN-C41	G5	-	-	-	-	-	+	+	+	+	+	+
DN-C171	G6	-	-	-	-	-	-	-	-	-	-	+
DN-C39	G7	-	-	-	-	-	-	-	-	-	+	+
DN-A7	G8	-	-	+-	+-	+-	+-	+-	+-	+-	+-	+-
DN-A18	G9	-	-	-	-	-	-	-	-	+*	+*	+*
DN-AS68	G10	-	-	-	-	-	-	-	-	+	+	+
DN-SS44	G11	-	-	-	+	+	+	+	+*	+*	+*	+*
DN-SS36	G12	-	-	-	-	-	-	-	-	+	+	+
DN-SW28	G13	-	-	-	-	-	-	-	-	+	+	+
DN-SW23	G14	-	-	-	-	-	-	--	-	-	+	+
DN-SW26	G15	-	-	-	-	-	-	-	-	-	+	+
DN-AS12	G16	-	-	+	+	+	+	+	+	+	+	+
DN-SW36	G17	-	-	-	-	-	-	-	-	-	+	+
DN-A12	G18	-	-	-	-	-	-	-	-	-	+	+
DN-AS58	G19	-	-	-	-	-	-	-	-	-	+	+
DN-CW14	G20	-	-	-	+	+	+*	+*	+*	+*	+*	+*
DN-CW22	G21	-	-	-	+	+	+*	+*	+*	+*	+*	+*
DN-AW25	G22	-	-	-	-	-	-	-	-	-	-	+
DN-SS38	G23	-	-	-	+	+	+*	+*	+*	+*	+*	+*
DN-CW5	G24	-	-	-	-	-	-	-	-	-	-	+
DN-SW29	G25	-	-	-	-	-	-	-	-	-	+	+
DN-SS32	G26	-	-	-	-	-	-	-	-	-	+	+
DN-C40	G27	-	-	-	-	-	-	-	-	-	+	+
DN-A3	G28	-	-	-	-	-	-	-	-	+-	+	+
DN-AS69	G29	-	-	-	+-	+-	+-	+-	+	+	+	+
DN-CW17	G30	-	-	-	-	-	-	-	-	+	+	+
DN-CW11	G31	-	-	-	-	-	-	-	-	+	+	+

- +*indicates highly intense gas bubble
- + indicates medium gas bubble
- +- indicate low amount of gas bubble
- +/+*indicates relatively fast and intense gas production

5. Discussion

Out of the total 229 alkaliphilic bacterial islolates tested from the four studied sites, 42% were positive for denitrification. The percentage of isolates obtained from each sample sites of the lakes indicated the comparative abundance of denitrifying bacteria in the four soda lakes. The availability of organic nutrients is one factor that determines the abundance of denitrifying bacteria in any habitat (Parkin and Robinson, 1989; McCutchan and Lewis, 2008). Hence, in two of the four lakes (L. Chitu and L. Arenguade), which are highly productive and *Spirulina* is the main primary producer, the organic nitrogen is assumed to be released primarily from dead *Spirulina* cells after degradation (Elezabeth Kebede, 1997; Zinabu Gebremariam and Taylor, 1997; Seyum Mengistou *et al.*, 2003). In the case of Lakes Abijata and Shalla, however, the *Spirulina sp* is absent. Yet, the two lakes' organic contents can be supplied by: the discards (feathers and wastes) of the flocks of birds that reside on them (Tafesse Kefyalew, 2008), the remains of the relatively abundant planktonic bacteria and other microorganisms inhabiting them (Elizabeth and Willen, 1996; Elizabeth Kebede, 1997; Zinabu Gebremariam and Taylor, 1997), or the rivers Bulbula and Dijo that feed the two lakes respectively (Tenalem Ayenew, 2007).

Relatively a high percents (56%) of denitrifying bacteria isolates were found from all the mud samples of the lakes. This distribution was expected since the anaerobic sediments of Soda lakes are commonly inhabited by an active and diverse population of haloalkaliphilic anaerobic bacteria (Sorokin and Kuenen, 2005). The water samples of Lakes Chitu and Shalla contained moderately high percentages of planktonic denitrifying isolates but hardly any were found from Lake Arenguade. The high disparity observed in the number of planktonic denitrifying isolates between the three lakes could be due to the differences in salt contents of the three lakes' water (Table 3). The salt content of soda lakes were previously reported to be influential in the distribution of organisms in such environments (Jones and Grant, 1999; Baumagrite, 2003).

During the construction of ARDRA table, isolates from the different lakes come together and form groups. Finding similar species of denitrifying bacteria was anticipated in the three Rift valley lakes since their origin was believed to be from the same place (Baxter *et al.,* 1965; Grove *et al*., 1975; Baxter, 2002; Cash and Klemperer, 2007). In addition, since these lakes are physically close with one another, it is possible that the bacteria can easily be transported from one lake to another through human as well as animal contacts (Tafesse Kefyalew, 2008). However, as Lake Arenguade is hundredth kilometers far from the other Lakes and differs from their origination, the most likely explanation for showing similarity in the bacterial species could be due to the migratory birds (lesser flamingos and pelicans) that feed on the *Spirulina* and thus provide the mode for transferring the bacteria during their migration (Elizabeth and Willen, 1996; Elizabeth Kebede, 1997; Zinabu Gebremariam and Taylor, 1997; Seyoum Mengistou *et al.,* 2003).

In addition, the ARDRA table also showed that isolates from mud samples of the different lakes aggregating together. For example, isolates from Lakes Chitu and Abijata, aggregated in Group 1 (G-1), Group 2 (G-2) and Group 27 (G-27), while isolates from mud samples in Lakes Arenguade and Chitu were collectively put in Group 7 (G-7). Besides the distribution, the aggregations of isolates also hint on the expressed enzyme activity present in the microorganisms and the specific denitrification activities in the areas. The detail that shows the spatial denitrification activity governing the dispersion of denitrifying bacteria in soil/mud is well documented in the study conducted by Parkin and Robinson, (1989).

On the other hand, isolates from mud sample of Lake Shalla were mostly grouped by themselves and rarely with isolates of Lakes Arenguade and Chitu in Groups 10 (G-10) and 11(G11). The explanation for Lake Shalla’s isolates to be grouped alone can be related to the lake’s total depth of the lake which contributes for the mixing of nutrients in the lake, the types of primary source and organic content as well as general chemistry of the lake that are listed in Table 3.

However, ARDRA mapping can only tell so much as the similarity and differences in band patterns and has limitation in determining differences between highly similar bacterial communities in samples. This is because the PCR-based ARDRA approach has low resolution display of bacterial DNA fragments on gel (Liu *et al*., 2008). For this reason, it is difficult to state all OTUs in each ARDRA groups to be identical yet they are highly similar. For example, OTUs of G1 and G1.1 were identitical during ARDRA mapping, while in reality, these two groups show difference in their morphological appearance and in some biochemical characteristics.

The result of the partial 16SrRNA gene sequence revealed the isolates taken from the different ARDRA groups were in fact highly similar. The result of neucleotide blasting of the sequences on the NCBI database indicated that about 68% of the blasted and aligned sequences belonged in the sub group γ –proteobacter. This subdivision was previously identified from culture-based studies on Soda lakes (Wani *et al.,* 2006). The group *Firmicutes* covered the other 32%. These two findings show resemblance with the results obtained in other Soda Lakes (Jones and Grant, 1999; Sorokin and Kuenen, 2005; Grant, 2006).

Halomonas campisalis, Halomonas salina and *Halomonas nitritophilus* from Halomanaceae and *Bacillus cohnii* as well as *Bacillus pseudofirmus* from Bacillaceae are the species from the database that showed high similarity with some of the studied isolates. Most of the species obtained in this study were previously isolated from; the Lonar Soda Lake in India (Wani *et al.*, 2006), most East African Soda lakes (Jones and Grant, 1999), the China Sea (Li, 2009) as well as other marine mud (Gontang *et al.*, 2007). A novel species previously identified as *Nitricola lacisaponesis* (Dimitriu *et al.*, 2005) with only 90-94 % similarity to *γ -proteobacter*, was also found to be similar with the isolates obtained in this study. This motile, strict alakaliphilic bacteria was separated to a novel lineage, excluded from previously described genera to which it was phylogenetically related because of its different phenotypic, molecular and biochemical characteristics (Dimitriu *et al.,* 2005).

The constructed phylogenic tree (figure 10) showed that the sequenced isolates created three clusters. Cluster 1 shows substantial similarity that range between 98-99% to members of the genus Bacillus. The *Bacillus sps* are popular in Soda lakes and some even have dormant stages that survive drying in marginal areas around the lakes (Grant, 2006). Denitrifying bacteria from this taxa group were represented in this study from all the four lakes and are well distributed. The *Bacillus pseudofirmus,* showed very close relation with the isolated denitrifying bacteria from Lakes Chitu and Arenguade, while *Bacillus cohnii* showed distant relation with isolates from Lakes Chitu and Abijata. Both *B. pseudofirmus* and *B. cohnii* were previously identified from the Lakes Abijata, Shala, and Arenguade (Martin *et al.*, 2001). Other unidentified strains of the Bacillus sps that have close evolutionary relations with the denitrifying bacteria obtained from the four studied lakes were also obtained.

In cluster 2 most of the isolates from Lakes Abijata, Shala, Chitu and Arenguade samples formed relationship with Halomonas of the γ *-proteobacter*. The genus Halomonas exhibited high similarity with the isolates in this cluster that range between 97-99%. With in this cluster, three different species of the Halomonas; *H. salina, H. campisalis* and *H. nitritophilus* correlated with 8 groups of isolates. The high salt enduring γ *-proteobacter*, *H. salina,* (Jiang *et al.*, 2007) was found to have an early and distant evolutionary relationship with Lake Abijata and Lake Chitu isolates respectively. Most of the isolates from Lakes Chitu and Abijata mud samples, however, had 99% to 100% similarity and convergent evolutionary history with the *H. campisalis.* This modernately haloalkalophilc-denitrifying bacterium (Mormile *et al.,* 1999) was previously isolated from the East African Soda Lakes and found to be well distributed (Grant, 2006). The *H. nitritophilus* that was previously isolated from a haloalkaline lake (Gilvanova *et. al*, 2001) was happened to be highly similar with the isolates from Lakes Arenguade and Shalla mud samples as well as Chitu waters. This result showed that the Halomonas are well distributed among the Ethiopian Soda lakes.

Cluster 3 was also made up of 9 groups, representing all four Lakes and the two sample types, showed association with a recently identified novel species *N. lacisaponesis*. In previous study, this alkalophilic strain was revealed to posses the ability to reduce NO_2^- in the presence of acetate (Dimitriu *et al.*, 2005). During the construction of the phylogenic tree, denitrifying isolates with equal to or less than 98% similarity with the databank reference sequences appeared to be grouped with the *N. lacisaponesis* in this cluster. This cluster represented the evolutionary relationships of isolates that made groups by themselves during the ARDRA mapping and mostly taken from water samples. Hence, this evidence show that denitrifying strains isolated with closer evolutionary relationship with this novel taxa are well spread in the Ethiopian Soda lakes.

Generally, all the Clusters showed high similarities and low divergence in the phylogenic relationship between the isolates and their respective reference sequences from the database. These outcomes lead to the assumption that they could be of the same species with different traits. However, according to Fox *et al.,* 1992, 16SrRNA gene identity may not be sufficient to guarantee species identity and as a result the isolates could indeed be different. For instance, isolates A18 and $C17_3$ were set together as *Halomonas sp.*, whereas the ARDRA mapping put them as different OTUs. Tracing the different nucleotide base pairs in the aligned sequences can show that these isolates are not 100% identical. Figures 10 and 11 showed difference in neculeotide type at same site of gene sequence.

Site no.	**1 21----**	**937 946- -**	**997 1005- -**
DN-A 15 (G1.1) -	GGGCGAA-GCCTGATC-AGCC----	CTAATCCCATAAA- -	NGNAATCGCT- -
GU228480 -	GGGCGAAAGCCTGATCCAGCC---	CTAATCCCATAAA- -	CGGAATCGCT--
DN- A 18 (G9) -	GGGCGAA-GCCTGATC-AGCC-----	CTAATCCCATAAA- -	CGG- -CCGCT- -
DN-C17$_3$ (G6) -	GGGCGAA-GCCTGATCCAGCC----	CTAATCC-ATAAA- - -	CGNAATCGCT- -

Figure 11: Fragment of aligned sequences of γ-proteobacters that show difference in base pairs

Sites	**962 974--**	**992 999--**	**1016 1032**
DN-A 15 (G1.1)-	CCNNATCGGANTC—	AAGTNGNA--	AATCANAATGTCACGGT
GU228480 -	CCGGATCGGAGTC—	AAGTCGGA---	AATCAGAATGTCACGGT
DN-A 18 (G9)-	CCNGATCGNANTC- -	AAGTCGG-----	AATCANAATGTCACNGT
DN-C17$_3$ (G6)-	CCGGATCGNAGTC--	AAGTCGNA---	ANTCANAATGTCACGGT

Figure 12: Potential different sites of aligned sequences of γ-proteobacters with unknown base pairs.

Earlier Rajiha Abubeker, (2008) isolated two highly active denitrifying bacteria from Lake Chitu. Similarly, some of the isolates tested in this study showed rapid gas formation. This shows that the nitrate released by ammonia oxidizers in the studied lakes is used as a terminal electron acceptor by a large group of autotrophic bacteria as well as by organotrophic denitrifying bacteria. The isolate designated as BACC 118 was isolated from Lake Chitu in previous study (Rajiha Abubeker, 2008) and was used in this study as a reference to some activity tests.

The isolate BACC 118, was previously found to be Gram-negative and fast growing. Optimum denitrification activity for this bacterium was reached at concentrations of 0-2M of NaCl, 0.5-3.5% of Na_2CO_3 and a pH from 8.5 to 12 in an anaerobic environment (Rajiha Abubeker, 2008). The partial ARDRA map of BACC 118 put it in G-1 and the sequence result showed 99% similarity with *Halomonas sp.* (Data not shown). The representative isolate from G1 designated as DN-C18 started denitrification after 2 hrs of inoculation (Table 15) where the pH was 10.5. The maximum growth recorded for this isolate took place only after 8 hrs of inoculation. This isolate showed high similarity with that of BACC118 (Rajiha Abubeker, 2008) in its morphological, bichemical as well as molecular features. Hence, the denitrifying bacteria from Lakes Abijata and Chitu aggregated in G-1 represents potentially active denitrifyers of *Halomonas* species than the rest of the other groups.

The test done on qualitative determination of N_2 gas production for the representative OTUs showed, more than quarter of the 31 isolates from each ARDRA group was fast gas producers. Considering the range of salinity of the habitats they were isolated from (Table 4), the potential of these denitrifyers in waste treatment is tremendous. Especially in an anaerobic reactor of waste treatment where the microbial ecology of moderately halophilic denitrifying bacteria has hardly been reported (Yoshie *et al*, 2004), the *Halomonas* were found to be dominant denitrifying bacteria in an acetate-fed saline waste water treatment system (Yoshie *et al*, 2004). Thus, the diverse denitrifing bacterial strains found in the studied Ethiopian Soda Lakes together with their different potential activities can provide new information to improve and utilize them in different technologies.

6. Conclusion

Denitrifying bacteria exist in all the four Soda lakes studied, namely Lake Abijata, Lake Arenguade, Lake Chitu and Lake Shalla. Similar isolates were obtained from the four lakes. The sequencing of the isolates show that *H. campisalis, H. salina, H. nitritophilus, B.cohnii* as well as *B.pseudofirmus* exhibited high similarities with the isolates studied. In addition, 9 isolates from the four lakes show relation with the novel bacterial group, *Nitricola lacisaponesis*. The lesser flamingos and the pelicans are the possible means of connecting the four lakes physically. Molecular, morphological studies and some biochemical tests conducted on the DN-C18 isolate, a representative of the first ARDRA group, showed high similarity on all accounts with previously obtained isolate from L. Chitu, which exhibited high denitrification activity and tolerance to high pH and salt concentration. Both isolates were found to be closely related the *Halomonas sp.*

7. Recommendation

The isolates obtained in this study showed great potential in their denitrification ability. Further analysis on the type of Nitrate reductase gene present in the isolates will help in the identification of the types of enzyme produced in such environment and their application in biotechnological fields, especially in wastewater treatment. Moreover, a study on the nitrate reductase gene in relation to rate of denitrification and the preference of isolates to different substrates and growth conditions for optimal growth and performance would make the analysis on alkaliphilic denitrifiers found in Ethiopian Soda lakes to be complete.

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Annex 1: NCBI blast result of the denitrifying isolates

Name of isolate	Similar spps	Taxon	Habitat	Alignment score	Query coverage (%)	% similarity	Accession number	Nucleo. sequence (#letters)
DN-C18	Halomonas sp	Gammaprot.	Bioreactor biofilm	1790	100	99	GU228481	982
	Halomonas sp	Gammaprot.	Soda Lake	1790	100	99	GQ202577	
	Halomonas sp	Gammaprot.	Alkaline env.	1790	100	99	EF554886	
DN-A15	Halomonas sp.	Gammaprot.	Bioreactor biofilm	1821	100	98	GU228484	1009
	Halomonas campisalis strain	Gammaprot.	Bioreactor biofilm	1821	100	98	GU228480	
	Halomonas campisalis strain	Gammaprot.	Bioreactor biofilm	1821	100	98	GU228479	
DN-A13	Gamma proteobacterium	Gammaprot.	Alkaline env.	1692	97	98	EF554869	994
	Gamma proteobacterium	Gammaprot.	Salt lake	1692	97	98	AM490139	
	Gamma proteobacterium	Gammaprot.	Salt lake	1692	97	98	AM490135	
DN-A2	Gamma proteobacterium	Gammaprot.	Volcano mud	1685	94	99	EU432573	989
	Gamma proteobacterium	Gammaprot.	Volcano mud	1677	94	98	GQ505335	
	Gamma proteobacterium	Gammaprot.	Alkaline env.	1677	94	98	EF554869	
DN-C41	Halomonas sp	Gammaprot.	Bioreactor biofilm	1808	100	98	GU228484	999
	Halomonas campisalis	Gammaprot.	Bioreactor biofilm	1808	100	98	GU228480	
	Halomonas campisalis	Gammaprot.	Bioreactor biofilm	1808	100	98	GU228479	
DN-C17_3	Halomonas sp	Gammaprot.	Bioreactor biofilm	1825	100	99	GU228484	1003
	Halomonas campisalis	Gammaprot.	Bioreactor biofilm	1825	100	99	GU228480	
	Halomonas campisalis	Gammaprot.	Bioreactor biofilm	1825	100	99	GU228479	
DN-C39	Bacillus pseudofirmus	Firmicutes		1847	98	99	AB201799	1031
	Bacillus pseudofirmus	Firmicutes		1847	98	99	AB201795	
DN-A7	Halomonas sp	Gammaprot.	Bioreactor biofilm	1842	100	99	GU228481	1013
	Halomonas salina	Gammaprot.	Volcano mud	1842	100	99	GQ505335	
	Halomonas sp	Gammaprot.	Soda lake	1842	100	99	GQ202577	
DN-A18	Halomonas sp.	Gammaprot.	Bioreactor biofilm	1851	100	98	GU228484	1034
	Halomonas campisalis strain	Gammaprot.	Bioreactor biofilm	1851	100	98	GU228480	
	Halomonas campisalis strain	Gammaprot.	Bioreactor biofilm	1851	100	98	GU228479	
DN-As68	Halomonas sp	Gammaprot.	Haloalkalne lake	1855	100	99	AM490137	1017

	Halomonas nitritophilus	**Gammaprot.**	**-**	**1855**	**100**	**99**	**AJ309564**	
	Halomomas sp	**Gammaprot.**	**-**	**1849**	**100**	**99**	**EU541470**	
DN-Ss36	**Uncultured bacterium clone**	**Gammaprot.**	**Salt lake**	**1624**	**97**	**96**	**HM127225**	**988**
	Alteromonadales bacterium	**Gammaprot.**	**Alkaline env.**	**1543**	**98**	**94**	**EF554892**	
	Nitrincola lacisaponensis strain	**Gammaprot.**	**Haloalkaline lake**	**1517**	**98**	**94**	**AY567473**	
DN-Sw23	**Unidentified Hailaer soda lake bacterium**	**Firmicutes**	**Haloalkaline lake**	**1773**	**98**	**99**	**AF275705**	**992**
	Bacillus cohnii	**Firmicutes**	**-**	**1760**	**100**	**98**	**AM287289**	
	Bacillus sp	**Firmicutes**	**Marine mud**	**1760**	**100**	**98**	**DQ448750**	
DN-Sw26	**Unidentified hailaer soda lake bacterium**	**Firmicutes**	**Haloalkaline lake**	**1626**	**99**	**99**	**AF275705**	**900**
	Uncultured low G+C Gram+ bacterium clone	**Firmicutes**	**Alkaline lake**	**1604**	**99**	**98**	**AY642557**	
	Bacillus sp.	**Firmicutes**	**Salt lake**	**1598**	**98**	**98**	**EF190377**	
DN-As12	**Halomonas sp**	**Gammaprot.**	**Alkaline env.**	**1799**	**99**	**99**	**EF554869**	**990**
	Halomonas sp	**Gammaprot.**	**Salt lake**	**1799**	**99**	**99**	**AM490139**	
	Halomonas sp	**Gammaprot.**	**Soda lake**	**1799**	**99**	**99**	**AY347310**	
DN-Sw36	**Unidentified hailaer soda lake bacterium**	**Firmicutes**	**Haloalkaline lake**	**1559**	**99**	**98**	**AF275705**	**875**
	Bacillus sp.	**Firmicutes**	**Salt lake**	**1550**	**99**	**98**	**EF190377**	
	Uncultured low G+C Gram+ bacterium clone	**Firmicutes**	**Alkaline lake**	**1539**	**99**	**98**	**AY642557**	
DN-As58	**Bacillus sp**	**Firmicutes**	**Alkaline envt.**	**1657**	**100**	**99**	**AB120000**	**899**
	Bacillus sp	**Firmicutes**	**Haloalkaline lake**	**1652**	**100**	**99**	**FJ764778**	
	Uncultured bacterium clone	**Firmicutes**	**Hot springs**	**1652**	**100**	**99**	**AY559416**	
DN-Cw14	**Gamma proteobacterium**	**Gammaprot.**	**Haloalkaline lake**	**1744**	**100**	**98**	**FJ764784**	**984**
	Gamma proteobacterium	**Gammaprot.**	**Haloalkaline lake**	**1744**	**100**	**98**	**FJ764783**	
	Gamma proteobacterium	**Gammaprot.**	**Haloalkaline lake**	**1744**	**100**	**98**	**FJ764782**	
DN-Cw22	**Gamma proteobacterium**	**Gammaprot.**	**Bioreactor biofilm**	**1687**	**94**	**99**	**GU228484**	**989**
	Gamma proteobacterium	**Gammaprot.**	**Bioreactor biofilm**	**1687**	**94**	**99**	**GU228480**	
	Gamma proteobacterium	**Gammaprot.**	**Bioreactor biofilm**	**1687**	**94**	**99**	**GU228479**	
DN-Aw25	**Bacillus sp**	**Firmicutes**	**Alkaline envt.**	**1513**	**97**	**98**	**AB120000**	**862**
	Bacillus sp	**Firmicutes**	**Haloalkaline**	**1507**	**97**	**98**	**FJ764778**	
	Uncultured bacterial clone	**Firmicutes**	**Hot springs**	**1507**	**97**	**98**	**AY559416**	
DN-Cw5	**Gamma proteobacterium**	**Gammaprot.**	**Haloalkaline lake**	**1432**	**98**	**93**	**FJ764784**	**947**
	Gamma proteobacterium	**Gammaprot.**	**Haloalkaline lake**	**1432**	**98**	**93**	**FJ764783**	
	Gamma	**Gammaprot.**	**Haloalkaline**	**1432**	**98**	**93**	**FJ764782**	

	proteobacterium		**lake**					
DN-Ss32	**Bacillaceae bacterium**	**Firmicutes**	**Alkaline lake**	**1622**	**98**	**99**	**EU596931**	**900**
	Bacillus sp	**Firmicutes**	**-**	**1622**	**98**	**99**	**EU004566**	
	Bacillus cohnii	**Firmicutes**	**Salt lake**	**1622**	**98**	**99**	**EF618696**	
DN-C40	**Bacillaceae bacterium halo-2**	**Firmicutes**	**Soda lake**	**1555**	**97**	**98**	**EU581836**	**888**
	Bacterial sp	**Firmicutes**	**Soda lake**	**1550**	**97**	**98**	**X92164**	
	Bacterium isolate	**Firmicutes**	**Soda lake**	**1522**	**97**	**98**	**X92163**	
DN-Cw17	**Gamma proteobacterium**	**Gammaprot.**	**Haloalkaline lake**	**1677**	**99**	**97**	**FJ764787**	**981**
	Gamma proteobacterium	**Gammaprot.**	**Haloalkaline lake**	**1594**	**99**	**95**	**AY730243**	
	Indomarina sp.	**Gammaprot.**	**Haloalkaline lake**	**1574**	**99**	**95**	**FJ764791**	
DN-Cw11	**Bacillus sp**	**Firmicutes**	**Alkaline envt.**	**1206**	**99**	**99**	**AB120000**	**671**
	Uncultured bacterial clone	**Firmicutes**	**Hot springs**	**1206**	**99**	**99**	**AY559416**	
	Bacillus sp	**Firmicutes**	**Haloalkaline lake**	**1203**	**99**	**98**	**FJ764778**	
DN-Ss38	**Alteromonadales bacterium**	**Gammaprot.**	**Alkaline env.**	**1347**	**85**	**95**	**FJ838764**	**976**
	Nitrincola lacisaponensis strain	**Gammaprot.**	**Alkaline env.**	**1347**	**85**	**95**	**AY567473**	
	Nitrincola sp.	**Gammaprot.**	**Haloalkaline lake**	**1343**	**85**	**95**	**FJ764760**	

Annex 2 Graphical representation of the growth curve of isolate DN-C18