ACTINOBACTERIAL AND ARCHAEAL DIVERSITY IN LAKE MAGADI, KENYA

BY

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Biotechnological potentials

16S rRNA gene

Clone library

Denaturing gradient gel electrophoresis (DGGE)

Isolation studies

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~	Approximately.
ARDRA	Amplified Ribosomal DNA Restriction Analysis.
APS	Ammonium-persulfate.
ATP	Adenosine triphosphate.
BLAST	Basic local alignment search tool.
bp	Base pair.
BSA	Bovine serum albumin.
CO_2	Carbondioxide.
°C	Degree Celsius.
DGGE	Denaturing gradient gel electrophoresis.
DNA	Deoxyribonucleic acid.
dNTP	2'- deoxynucleoside-5'-triphosphate.
EC	Electrical conductivity.
EDTA	Ethylene diamine- tetraacetic acid.
e.g.	*Exempli gratia* (Latin) for example.
Etc	Etcetera (and so on).

G+C	Guanine + Cytosine.
g	Gram.
hr	Hour.
H_2O	Water.
IPTG	Isopropyl-β-thiogalactoside.
kb	Kilo base.
mmol/kg	Millimoles per kilogram.
MPa	Mega Pascals (unit of atmospheric pressure).
$mScm^{-1}$	milliSiemens per centimeter (unit of electrical conductivity).
NaCl	Sodium chloride.
NaOH	Sodium hydroxide.
O. D	Optical density.
PCR	Polymerase chain reaction.
ppm	Parts per million.
psi	Pound-force per square inch (avoirdupois unit of pressure 1 psi ~ 6,894.757 Pa, where avoirdupois units is a non-metric (weight/ mass based) system which uses pounds of 16 ounces).
rDNA	Ribosomal deoxyribonucleic acid.
Rpm	Revolutions per minute.
rRNA	Ribosomal ribonucleic acid.
SDS	Sodium deodecyl sulphate.
Sec (s)	Seconds.
TAE	Tris-acetic acid EDTA.
TE	Tris-(HCl)- EDTA.
Tris	Tris-(hydroxymethyl-) aminomethane.
U	Unit.
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside.

Abstract

Microorganisms of the class *Actinobacteria* and domain *Archaea* are interesting from a biotechnological perspective owing to their metabolic attributes as producers of secondary metabolites and resilience under harsh environmental conditions respectively.

Lake Magadi is a soda lake well studied in terms of its geology and limnology. Research attention has also been drawn to the microbial populations which thrive in this unique habitat but currently there are no reports on the assessment of its microflora using molecular methods.

This study aimed to assess the actinobacterial and archaeal communities within Lake Magadi, Kenya a hypersaline –highly alkaline habitat using metagenomic methods as a preliminary study to identify potential candidates for exploitative biology

Samples from two sites dubbed Lake Magadi station 2 (LM2) and Lake Magadi salt pan 4 (LMS4) within the Lake Magadi were analyzed using the 16S rRNA gene as a phylogenetic marker.

Cluster analysis of taxon-specific 16S rDNA PCR-DGGE profiles revealed moderately heterogeneous actinobacterial and archaeal populations across the sample sites under investigation which is probably a reflection of the differences in abiotic conditions at the study sites. This observation was also confirmed from the multi-dimensional scaling (MDS) plot.

PCR-based clonal libraries of actinobacterial and archaeal communities of both study sites retrieved a total of thirty-two clones (twenty actinobacterial and twelve archaeal) were sequenced. Analysis of the sequences revealed cultured and uncultured signatures of microorganisms typical of hypersaline and or highly alkaline niches. A few (3) sequences presented novelty (<96%) in identities with any previously identified organism. It was concluded that the species dominance at site LMS4 [situated within the salt flats of Lake

Magadi and site for exploration of trona and its mineralized extensions (nacholite and gayllusite)] is likely to be dictated by anthropogenic stress since most of the microbial signals associated with the study site are typical of saline and or alkaline environmental samples exposed to especially mining but also agricultural and waste management practices.

Isolation studies also revealed previously identified strains peculiar to hypersaline brines and sediments. The strains retrieved were affiliated to the taxonomically diverse genus *Bacillus* and *Halomonas* sp. The true applications and potential opportunities these isolates have for biotechnology have been well documented.

Observations made from the culture dependent and culture independent methods suggests strongly that study site LMS4 is subjected to environmental conditions more severe than at site LM2.

This study is a guide for future studies as it provides primary information on the haloalkaliphilic representatives of the actinobacteria phylum and domain *Archaea* within the soda lake environment. It can serve as a pedestal for investigation into the molecular machinery that supports the haloalkaliphilic lifestyles of inhabiting microorganisms and consequently give leads as to how they can be commercially exploited.

May, 2013.

Declaration/ Indemnity

I, Halimat Olubukola Ashabi Ibrahim, hereby solemnly declare that "*Actinobacterial* and *Archaeal* diversity in Lake Magadi, Kenya" is an original work of mine and has not been submitted for examination or the award of a degree in any other institution. I, also declare that all information sourced are duly and accurately referenced.

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Halimat Olubukola Ashabi Ibrahim

May, 2013

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CHAPTER 1: LITERATURE REVIEW

This chapter provides an overview of extreme environments and extremophiles in general. It also contains information about the relevance of extreme environments and their inhabitants to man in the age of modern science.

In particular, this chapter reveals key information on Lake Magadi, Kenya, the extreme study site around which the aims and objectives of this research revolve. The basis of this diversity study is also highlighted by presenting information on the all-important role of microorganisms, the challenges of assessing the abundance of microbes and the methods that are used, especially those pivotal to this research.

1.1 Extreme environments and their peculiarities

The existence of every living organism is dictated by a combination of physical and chemical environmental factors, biotic and abiotic alike. These varying factors are crucial to the growth and survival of each organism and, for each, fall within prescribed ranges which are defined relative to the "normal" conditions which support human life (Kristjansson and Hreggvidsson, 1995; Satyanarayana *et al.,* 2005) (Table 1.1).

Table 1.1: Limits governing survival in the human physical environment*

Conditions	Lower limit	Upper limit
Temperature	4ºC	40ºC
pH	5	8
Salinity (NaCl)	About 0.1M	<0.5M
Dissolved minerals/ions (Ca^{2+}, Mg^{2+}, Na^{+}, Cl^{-})	100ppm	About 250ppm
Hydrostatic pressure	1atm ≡ 0.101Mpa	About 10Mpa

References: Kristjasson and Hreggividsson, 1995; Satyanarayana *et al.*, 2005; McGenity and Oren, 2012; Pradillon, 2012.

However in various ecosystems, many organisms exist well outside these pre-defined limits (Madigan and Marrs, 1997). Any of the many habitats on planet Earth and beyond with environmental conditions which do not fall within the anthropocentric normalcy are termed extreme and organisms living under such conditions are collectively termed extremophiles (Rainey and Oren, 2006; Canganella and Wiegel, 2011; Bell and Callaghan, 2012).

Severe environmental conditions which were previously thought to be unfavourable to support any form of life include high temperatures (>80°C), intense cold (<4°C), high salt concentrations (>1.0 M NaCl), extreme pH's (<2 and >12), high radiation, anoxic and hypoxic conditions and nutrient deficient environments. Typical examples of extreme environments or extremobiospheres are the deep sea and volcanic vents, hot springs, deserts, the polar regions, saline lakes and soda lakes (Figure 1.1).

Prokaryotes are the dominate life forms in extreme habitats, but higher organisms (plants, vertebrate and invertebrates animals) are also represented (e.g. polar bears and cacti) (Madigan *et al.*, 2000a; Bull, 2004). Not only do organisms of the extremes inhabit their respective niches but they actually thrive under these conditions, the extreme element may even be an obligate necessity for their existence (Rampelotto, 2010). This refers to variation in the degree of adaptation of an organism to severe conditions. While some organisms are capable of tolerating an extreme factor or factors (extremotolerant organisms), others are obligately depedent on an extreme factor or factors and flourish under such conditions (Madigan and Marrs, 1997). These are the true extremophiles.

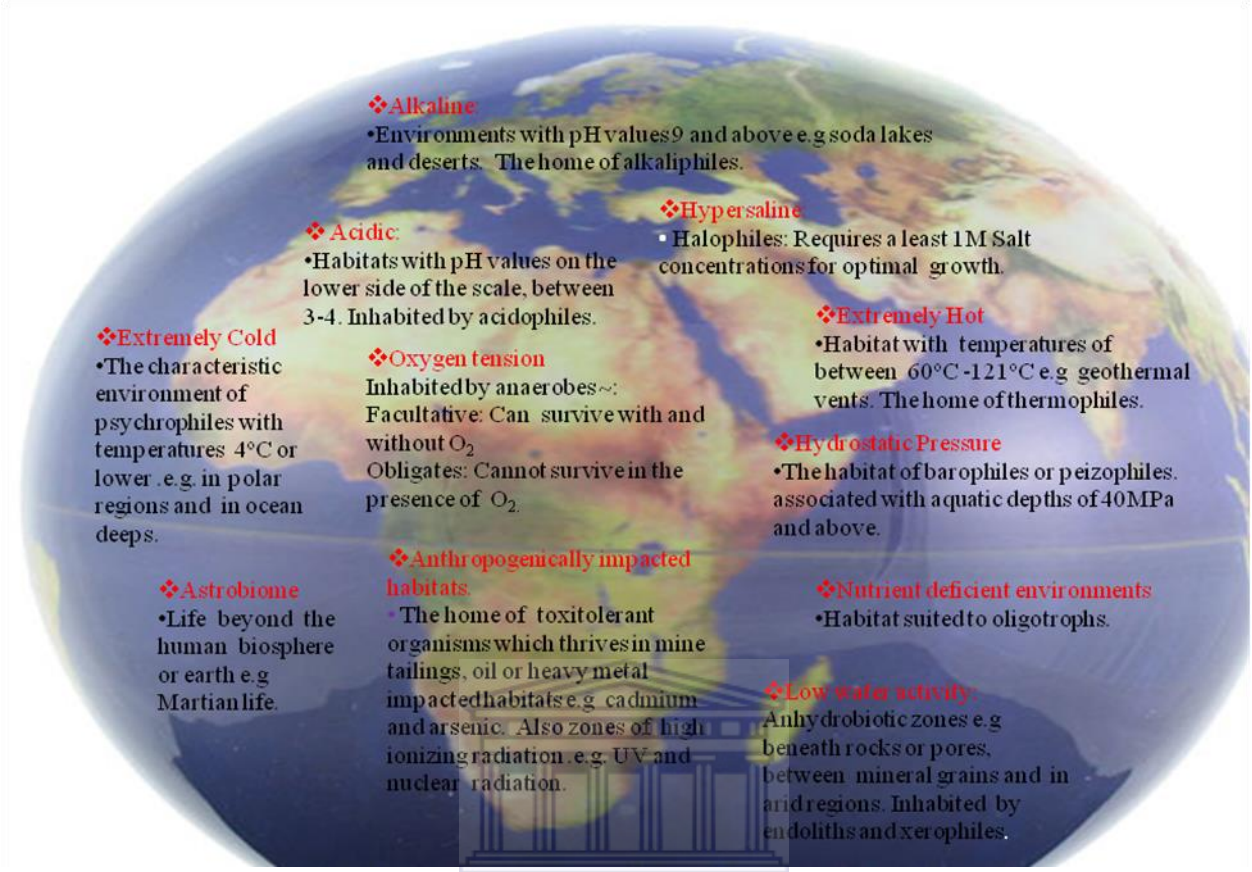

Figure 1.1: The Extremophilic worlds /Extremobiospheres. References: Maloney, 1995; Stetter, 1996; Ovreas, 2000; Satyanarayana *et al.*, 2005; Canganella and Wiegel, 2011.

Some organisms, the polyextremophiles, are adapted to survival simultaneously under more than one environmental extreme. In addition, the extreme nature of an environment is influenced by fluctuations in that environment that may be transient or protracted and that can either be man made or natural (Oren, 2004; Bell and Callaghan, 2012). Placing the concept of extreme environment and its inhabitants into a broader perspective reveals that in reality "one mans meat is another mans poison" (Madigan and Marrs, 1997; Gomes and Steiner, 2004). For example in the context of obligate anaerobes, human beings and other organisms that require oxygen to flourish or survive are extremophiles.

Table 1.2 lists the stress tolerance values and habitats of some record-holding extremophiles.

Table 1.2: Identities of some "record –holding" extremophiles

Environmental factor	Organism	Phylogenetic affiliation	Habitat	Stress tolerance values
Temperature	*Pyrolobus fumarii*	Crenarchaeota	Hot undersea hydrothermal vents	Maximum 113°C, Optimum 106°C Minimum. 90°C
	Polaromonas vacuolata	Bacteria	Sea- ice	Maximum 0°C, Optimum 4°C Minimum 12°C
Salt concentration	*Halobacterium salinarum*	Euryarchaeota	Salt lakes, salted hides, salted fish	Maximum about 35%w/v, Optimum 250g/l Minimum 150g/l
pH	*Picrophilus oshimae* (also a thermophile)	Euryarchaeota	Acidic hot springs	Maximum Ph -0.06, Optimum pH 0.7 Minimum pH 4 Maximum - Temperature 65°C
	Natronobacterium gregoryi (also a halophile)	Euryarchaeota	Soda lakes	Maximum pH 12 Optimum pH 10 Minimum pH 8.5 NaCl to saturation point
Hydrostatic pressure	Strain MT41	Bacteria	Mariana Trench	Maximum >100 MPa, Optimum 70MPa, Minimum 50Mpa
Ultraviolet & ionizing Radiations	*Deinococcus radiodurans*	Bacteria	Isolated from radioactive waste, nasal secretion of elephants, and ground meat.	Resistance to 1.5 kG γ radiation and 1500 Jm^{-2} ultraviolet radiation

Reference: Modified from Rainey and Oren, 2006.

As a result of the discovery of and research into communities thriving under extreme conditions, knowledge of the adaptation, evolution and responses to environmental changes is beginning to

unfold. Extreme environments are considered worthy of exploration as alternate and better option to the fast diminishing resources of normal environments, especially as a range of biotechnological opportunities have been revealed (Stetter, 1996; Satyanarayana *et al.*, 2005). As a result, investigating the diversity of extremophiles has metamorphosed from a mere academic science to an industrially viable technology which has a further advantage of giving possible clues to both the origins of life and as to where and how to seek extraterrestrial life (Bull, 2004; Canganella and Wiegel, 2011; Bell and Callaghan, 2012).

1.2 Microbial diversity, extremophiles and biotechnological potentials

This study focuses on the diversity of microorganisms from an extreme environment, Lake Magadi. The need for microbial diversity studies to be conducted particularly in relation to study of extremophiles and the promise these studies hold for biotechnology is discussed in the following sections.

1.2.1. Microbes: drivers of life

All life is dependent on microbial processes (Øvreås, 2000; Oren, 2010). Although typically microscopical in size, microorganisms are present in large numbers everywhere in the soil, water and even air. And they have evolved the basic biochemistries necessary for life in so their many types of habitat. Microbes are the ancestors of all other life forms as they are an intergral component of every ecosystem where they are at the bottom of the food chain involved in primary production (Bull, 2004; Oren, 2004). Microbes constitute a huge reservoir of resources which drive the functions needed for all other organisms to exist (Whitman *et al.*, 1998; Handelsman, 2007). By breaking down human and other animal wastes, and dead plants and animals, microbes (mineralization) under oxic and anoxic conditions, microorganisms facilitate the release of nutrients and the cycling of energy required by other organisms for survival into the environment. Despite being the causative organisms of some human and animal diseases, microorganisms are responsible for the breakdown of food and production of nutrients in our digestive system and the defense of our body from invasive organisms. So also, in agriculture

and food production microbes play a part where they help convert atmospheric nitrogen into useable form for plants and are used to ferment diary and bakery products respectively.

Man has for a long while realized the metabolic affluence of the microbial world and has been exploiting this for his needs (Pace, 1997; Gomes and Steiner, 2004). The ubiquity, evolutionary history and the ability to harness energy from a range of sources are the basis of the potential biotechnological wealth of the microbial world. This wealth lies in their nucleic acids, cell components or extracts and even in the whole organism (Bull, 2004; Bérdy, 2005).

1.2.2 Assessing microbial diversity

The microbial landscape in its entirety is largely underexplored and poorly understood compared to the better understood diversities of the plant and animal kingdoms (Head *et al.*, 1998; Øvreås, 2000; Bérdy, 2005). A major challenge is that only a minute fraction (less than 1%) of the diversity of microbial life has been identified using traditional culture-based techniques (Pace, 1997; Handelsman, 2004a and b; Goodfellow and Fiedler, 2010). There is absolute need for information on available genetic resources, their relative distribution and functional roles within and outside their microbiota (Øvreås, 2000; Bull, 2004).

Kirk and colleagues (2004) discussed methods for studying microbial diversity and stated that these studies are hindered by:

1. The inability to access the innate heterogeneity of environmental samples and how the component organisms are distributed (spatial and temporal variability) because of the available sampling methods.

2. The inability to assess the huge phenotypic and genetic diversity of viable (metabolically active) but unculturable soil microorganisms.

3 Taxonomic ambiguity in which the definition of prokaryotic species is yet to be defined because the standards available are designed for higher organisms and are essentially not applicable to microbial species.

In recent times new technologies in computational biology, analytical chemistry and in metagenomics have been used in microbial ecology studies. These offer new insights into the role of microorganisms in the ecosystem (Head *et al.*, 1998; Oren, 2004). The identities of a significant number of previously unknown have emerged from their molecular signatures alone (Handelmans, 2004b). The three domains of cellular life, namely Bacteria, Archaea and Eukarya have also been verified by metagenome analysis (Pace, 1997; Head *et al.*, 1998) (Figure 1.2). In addition, the richest assortment of molecular and chemical diversity available in nature is now known to exist in the microbial world (Whitman *et al.*, 1998; Bull, 2004). It is therefore imperative that the "unseen majority" and sustainers of our biosphere be thoroughly investigated using techniques now available (Zinder and Salyers, 2005).

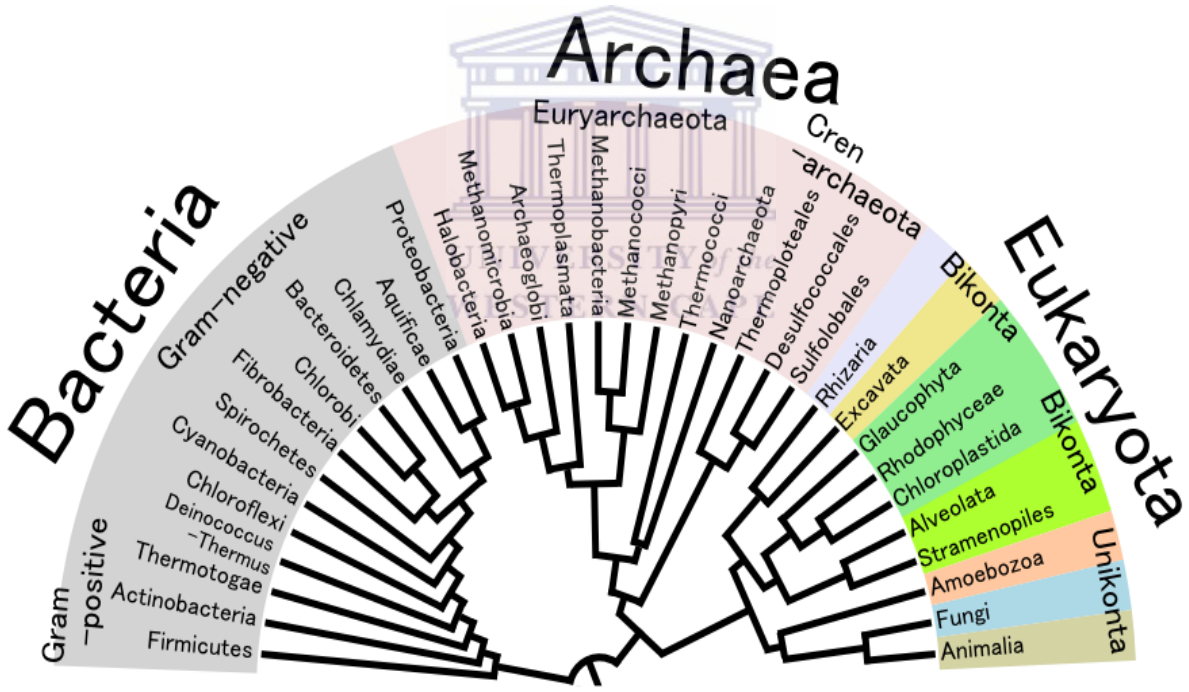

Figure 1.2: The three domains of cellular life indicated by metagenome analysis. Reference: Adapted from Gribaldo and Brochier-Armanet, 2006.

1.2.3 Diversity of extremophiles as a source of biomaterials

Exploration of nature is the best and most versatile means of sourcing new compounds that can be engineered for biotechnological and pharmaceutical purposes. This exploration has extended to the extreme environments (Canganella and Wiegel, 2011; Horikoshi, 1991; 1998). Initial screens for bioactive metabolites or biomolecules focused almost entirely on mesophilic terrestrial habitats (Bentley *et al.*, 2002). A major constraint of enzymes and compounds isolated from neutralophilic environments was that they were generally only active at ambient temperatures and pH. In addition, the stability of mesophiles to proteolytic degradation was often low and they functioned often in narrow organic solvent ranges (Madigan *et al.*, 2000b; Rameplotto, 2010; Bell, 2012). Research into the biology of extremophiles is believed would open new frontiers for the manipulation of their robust biocatalytic properties and unique metabolic capabilities which would in turn pave way for the generation of novel and unknown value added products (Kumar and Singh, 2012).

The discovery of thermostable enzymes which in nature allow thermophiles to survive under high temperatures (Gomes and Steiner, 2004; Canganella and Wiegel, 2011) and which are amenable to many industrial process requirements sparked an interest in the study of the biotechnological potential of other extreme environments. This research interest was further driven by the pressing demand for new antibiotics to combat life threatening infections, the need for biocatalysts to drive industrial processes in a more economical and eco-friendly manner and sthe general need for more sustainable and versatile resources (Whitman *et al.*, 1998). The remarkable structural and chemical adaptations which ensure the survival of extremophiles in hostile environments gives rise to unique extremozymes (enzymes obtained from extremophiles), compatible solutes and metabolites (Gomes and Steiner, 2004). Many of these compounds are been exploited for biotechnological applications (Table 1.3).

Alkaline proteases, lipases and cellulases derived from alkaliphilic species (e.g. *Bacillus* sp strain KSM-K16 (Kobayashi *et al.*, 1995), given their activity and stability under high pH, are used as additives in the making of laundry and dishwashing detergents. These enzymes are active and effective regardless of the presence of chemical ingredients (chelating agents, surfactants,

phosphates) that are present in the detergent formula. This market generates hundreds of millions of dollars annually and constitutes 30% of global total enzyme production (Horikoshi, 1998; 1999; Canganella and Wiegel, 2011).

Another example of an extremophilic material used in biotechnology is bacteriorhodopsin - an integral membrane protein. This retinal based pigment found in the halophilic archaeon *Halobacterium salinarum*, is part of a unique photosynthetic apparatus which functions as a light–driven proton pump. It is fuelled by solar energy (500-650 nm) and assists in the translocation of information and materials across cell membranes. It is a perfect model for energy conversion and its biotechnological use lies in optical information recording, spatial light modulation and holography (Haupts *et al.*, 1999; Margesin and Schinner, 2001).

Table 1.3: Extremophile diversity as a valuable resource for biotechnology

Extremophile	Products and or source organism	Uses (or possible applications)
Acidophiles & Alkaliphiles	Cyclodextrins	Foodstuffs, chemicals, pharmaceuticals (a, d)
	Acidophiles	Fine papers, degumming of ramie fibers, waste treatment, organic acids and solvents (a, d)
	Esterases, keratinases e.g. Alcalase® and Milezyme®.	Dehairing of hides and skin, removal of the gelatinous layer of X-ray film (a, g)
	Sulphur oxidizing acidophiles e.g. *Acidithiobacillus ferroxidans*	Recovery of metals and de-sulphurication of coal (d, e)
Halophiles	Lipids	Liposomes for cosmetics and drug delivery (a,b)
	Compatible solutes, ectoines e.g. from *Ectothiorhodospira halochloris*	Proteins, DNA and cell protectants (a, b, d, e)
	β- carotene and cell extracts e.g. *Spirulina* and *Dunaliella*	Dietary supplements, food additives and feedstock (a, d, e)
Thermophiles	DNA polymerases e.g. from *Thermus aquaticus*	Amplification of DNA via PCR (a, d)
	Xylanases e.g. from *Rhodothermus marinus* Amylases	Paper bleaching and rayon modification, Glucose and fructose for sweetners (c, g)
	Therapeutics from *Bacillus thermodenitrificans* and *Bathymodiolus thermophiles*	Immunomodulatory and antitumor agents respectively (g)
Psychrophiles	Alkaline phosphatase	Molecular biology applications (a, d, f)
	Polyunsaturated fatty acids	Food additives, dietary supplements (c, d, e)
	Ice nucleating proteins or ice minus microbes	Artificial snow, food industry e.g. ice making or frost protectants for sensitive plants (c,d)
	β-galactosidase from *Arthrobacter sp* C2-2	Cheese maturation and milk lactose hydrolysis (c, d, g)
	Therapeutics	Antibacterial and antitumor agents, diagnostics materials (e, g).
Radiophiles/ metallophiles	Genetically modified organisms *Deinococcus radioradurans* and *Deinococcus geothermalis*	Enzymatic transformations, detoxification of toxic metals, halogenated organics (a, c, d)

References: Maloney, 1995[a]; Margesin and Schinner, 2001[b]; Gomes and Steiner, 2004[c]; Satyanarayana *et al.*, 2005[d]; Canganella and Wiegel, 2011[e]; Horikoshi, 2011[f]; Bell, 2012[g].

1.2.4 Challenges of harvesting extremophiles for biotechnology

Ground breaking research into the biology of extremophiles, the resulting advances in our understanding of world and the promises and prospects it holds for biotechnology really cannot be overemphasized. This is so especially because the strong point of the opportunities of extreme environments lies in its applicability in many areas of food, health and ecology. It must however be noted that the implementation of the many true and potential benefits of engineered extremophile products are met by certain challenges, intrinsic and downstream.

Kumar and Singh (2012) discussed the various technological hurdles associated with bringing the theoretical concepts of the potential use of extremophiles in therapeutics to reality. Most of the problems pointed out apply to use of resources from extreme environments for purposes other than for medical and pharmaceutical ends. In summary, there is the primary obstacle of being able to simulate the actual environmental conditions of a candidate organism to initiate the appropriate response for the synthesis of a desired metabolite. In most cases, it is required that dedicated bioreactor systems need to specially developed to create a particular extreme environmental condition. Also, the molecular cascades triggered under a particular extreme condition needs to be well understood as well as is the need to unveil the specific nutritional or other growth requirement to elicit target response. Finally, downstream there is the need for the design of strategies specific for the rapid recovery (extraction, purification and preservation) of a desired metabolite from an extremophile. The functional properties of most secondary metabolites are or may be lost if they are improperly purified or stored as they are likely to be unstable in an inappropriate organic solvent or under environmental conditions outside their native one. According to Chakravorty *et al.* (2012), the introduction of engineered extremophiles into 'normal environments" arouses some concerns of possible negative consequences. For instance, there is concern that they may be impossible to eliminate from the environment and may actually out-compete the real wild type microbial species. There is also the possibility of genetic transfer events (lateral or vertical gene transfer) to wild type and pathogenic organisms which may lead to costly health or environmental consequences. Another possibility is that bioengineered extremophiles may serve as templates for the development of agents of biological warfare.

From the perspective of Bell, (2012), the harvesting of extremophiles and their metabolites must be very well regulated so as not to lead to an irreparable loss to biodiversity as observed with natural resources from many moderate environments. Also, consumers' confidence in the use of products from extreme environments must be instilled as more often than not, the marketability of products obtained from extremophiles or extreme environments might be greatly affected by skepticism from the intended end user. Another matter of concern is that local communities from which biomaterials generally have been harvested have mostly not being well compensated for the resources mined in their domain. The need for strict food and drug administration (FDA) laws and other relevant legislations to be enacted for the commercialization of biosourced products or templates (extreme environment) was also raised.

1.2.5 Culture dependent microbiology and metagenomic analysis

The detailed assessment of microbial population structure, function, evolution and ecology can be determined using either a culture based approach and or a culture independent approach.

The culture dependent method is the traditional approach used in microbiology. It involves the generation of isolates from environmental samples by growth on substrates which mimic the growth conditions of the natural habitat of the target organisms. Phenotypic characterization of isolates is the traditional basis for determining the identity of members of a population (Macrae, 2000; Smalla, 2004). However, some microorganisms cannot be revived using the standard cultivation methods. This method therefore greatly underestimates both the species complexity and microbial load of an ecosystem. A metabolically active component of a niche may be recalcitrant to culturing for a myriad of reasons including an inadequate supply of nutrients, the presence of inhibitory compounds in the growth medium or accumulation of waste products from its own metabolism, or because of an intrinsic slow growth rate (Schleper *et al.,* 2005; Vartoukian *et al.,* 2010).

The metagenomic method is a culture independent method and one of the novel fields of investigation in microbial ecology (Su *et al.,* 2012). It involves subjecting the entire microbial

population within an environmental sample to DNA based analysis. The direct isolation of bulk DNA representative of all microbes in an environmental sample bypasses the culturing step. The total community DNA is subjected to further molecular analysis which involves the use of next generation sequencing to identify individual components (Riesenfield *et al.,* 2004).

Since metagenomic DNA is comprised of the genomes of various organisms, its analysis could be daunting. Analysis of the DNA is achieved by cloning fragments of interest in the DNA into a suitable vector and transforming the clones into a host cell, often *Escherichia coli* (*E.coli*). Clones are screened using phylogenetic markers such as the 16S rRNA and 18S rRNA genes, the internal transcribed spacer (ITS) regions, the beta subunit of ATPase and the 23S rRNA gene or sampled fragments of whole genomes or transcriptomes (Smalla, 2004; Handelsman, 2004a, 2007). Screening or genetic analyses of genomes is done in either of two ways. In sequenced based analysis, the entire genetic sequence is studied with reference to the sequences of the nucleotide bases in the DNA of the samples. Using functional expression screening, genes are screened based on the properties they confer on the host organism such as the production of secondary metabolites (Pace *et al.,* 1986).

Metagenomic sequencing technologies entails the direct random shotgun sequencing of cloned DNA on platforms which have shifted from the classical Sanger sequencing technology to next-generation sequencing (NGS) such as Roche 454 or Ilumina/ Solexa systems which have led to acceleration of sequenced-based metagenomics through rapid and substantial cost reduction (Mardis, 2012; Thomas *et al.*, 2012). Metagenomics has lended easier access to functional gene composition and the evolutionary profiles of community function and structure while showing the interaction of microbial communities with the host. In addition, metagenomic sequencing has provided genomic linkages between the phylogeny and function of non-culturable organisms, genetic information on novel enzymes or biocatalysts.

The metagenome approach has by far surpassed the limitation posed by classic microbiology. Interestingly, metagenome analysis may also provide drive and direction for better culturing strategies (Amman *et al.,* 1995; Schleper *et al.,* 2005). Extinction dilution or the use of dilute nutrient media, prolonged incubation, density gradient centrifugation or elutriation and the

labeling of nucleic acids with fluorescent probes include some of the culturing strategies hinted by culture independent approaches and that have facilitated the assay of enrichment and growth quantitatively (Handelsman, 2004; Vartoukian *et al.,* 2010).

No single means of assessment is exhaustive for a diversity study. A combination of microbiological, molecular and geochemical techniques (i.e. a polyphasic approach) offers better chances of understanding the complexity of the microbial world (Head *et al.,* 1998; Kirk *et al.,* 2004).

1.2.6 The 16S rRNA gene as a phylogenetic marker in microbial ecology studies

In the mid-1980's, the era of metagenomics emerged with techniques which have enhanced research and our knowledge about the role of biological communities in the biosphere (Xu, 2006). In microbial ecology, experimental methods which explore genomes have circumvented the anomalies of traditional culturing methods (Head *et al.,* 1998; Bull, 2004). The use of the ribosomal RNA genes, a section of prokaryotic DNA present in all cellular life forms, as a molecular marker has created awareness of the "large pool" of uncultivatable, rarely isolated, poorly described or phenotypically aberrant strains from environmental samples which may actually underpin key functions in ecological processes (Clarridge, 2004; McAuliffe *et al.,* 2005).

1.2.6.1 Strengths

The 16S rRNA genes and other ribosomal RNA genes (5S, 18S and 23S genes) have been functionally and evolutionarily conserved owing to their essential role in protein translation in cells (Yarza *et al.,* 2010). Moreover, certain regions within these genes are more conserved than others across all the domains of life due to the manner of ribosome folding. This folding results in intrinsic bond formation in some places whilst the other parts remain free from bonds. The bonded regions constitute the conserved regions of the RNA genes while the unbonded regions

are the hypervariable regions (Yarza *et al.,* 2010). The conserved regions are sites for primer directed PCR and are the hybridization targets in cloning procedures. Hybridization studies have established that the folds of the ribosomal RNA genes are phylogenetically conserved and are not mere scaffoldings for the molecule. Instead they are essential for messenger RNA selection, ribosomal subunit association, tRNA binding and antibiotic sensitivity or resistance (Woese *et al.,* 1980; Muzyer *et al.,* 1993; Yarza *et al.,* 2010).

A good phylogenetic marker possesses both a reasonable number of informative positions and a reasonable number of conserved regions. The number of informative positions is dictated by the number of variable sites and the actual number of nucleotides possible per site as per functional and evolutionary constraints (Macrae, 2000). A reasonable number of conserved regions is a necessity for gene homology identification (Ludwig and Klenk, 2001). The 23S rRNA gene is a better phylogenetic chronometer than the 16S rRNA gene because it is about double of the size 16S rRNA gene and consequently contains double the informational content of the 16S rRNA gene. For technical and economic considerations however, the 16S rRNA genes are the most widely used phylogenetic markers in prokaryotic studies (Huguenoltz *et al.,* 1998; Macrae, 2000).

Nucleotide sequence analysis of the 16S rRNA genes is used to make genealogical inferences because it establishes relatedness between organisms and can estimate the rate of species divergence (Woese *et al.,* 1980b; Wang and Qian, 2009; Morris *et al.,* 2002; Yarza *et al.,* 2010). Moreover, the 16S rRNA gene is comparable not only among all bacteria but also with the 16S rRNA gene of archaea and the 18S rRNA gene of eukaryotes (Riesenfeld *et al.,* 2004). In addition, the number of partial or complete 16S rRNA gene sequences available in public databases is continuously on the increase and this facilitates its use as a comparative tool. The Ribosomal Database Project (RDP), Genbank (NCBI) and Greengenes are some of the repositories containing vast volumes of 16S rRNA gene data (Macrae, 2000, Baumgarte, 2003; Xu, 2006).

16S rRNA gene sequences are obtained by direct extraction of the nucleic acid from a biological sample. The genomic DNA is PCR amplified using consensus primers which target the regions

within the ribosomal complex which are evolving at a consistent rate and which accurately reflect divergences amongst species in the sample. The amplicons of the conserved regions are sequenced, and informative taxonomic assignment is drawn from the sequences (Wang and Qian, 2009). The use of taxon specific oligonucleotides (degenerate primers) which flank the region of interest in a desired taxon is crucial to the successful amplification of the target regions (Reysenbach *et al.*, 1992).

For environmental samples, analysis of component microorganisms begins with the separation of amplicons, either by cloning and sequencing or using molecular fingerprinting techniques such as denaturing or temperature gradient gel electrophoresis (DGGE/TGGE), terminal restriction fragment length polymorphism (T-RFLP), amplified ribosomal DNA restriction analysis (ARDRA), single strand conformation polymorphism analysis (SSCP) and ribosomal intergenic spacer analysis (RISA) or its automated variant (ARISA) (Watanabe *et al.*, 2001). While molecular fingerprinting methods permit the study of spatial and temporal variations within microbial communities, inferences about phylogenetic kinship can only be established by sequencing the ribotypes identified (Kirk *et al.*, 2004; Xu, 2006).

1.2.6.2 Limitations

The 16S rRNA gene is a valuable index for elucidating the taxon composition of a microbial community but the fidelity of its use is plagued by shortcomings peculiar to molecular-based methods (Farelly *et al.*, 1995, Stackebrandt *et al.*, 1999; Gonzalez *et al.*, 2012).

The shortcomings of the use of 16S rRNA gene in microbial diversity studies include errors due to methodological and innate biases and errors due to the interpretation of the 16S rRNA gene generated data.

1 Methodological biases include:

i) Sampling and sample handling: The relative ratio of taxa in an environmental sample is altered by the removal of a sample from its natural niche, and even more by the transportation of the sample to and the storage of the sample in the laboratory. For this

reason, only a semi-quantitative reflection of the naturally occurring diversity is obtained (Stackebrandt *et al.,* 1999; Zinder and Salyers, 2005).

ii) Nucleic acid extraction is the cardinal step in molecular analysis (Macrae, 2000). The major challenge in achieving this is the recovery good quality nucleic acid and ascertaining if the DNA is indicative of the microflora of its source sample. The current methods of cell lysis may be too mild to penetrate certain cells leading to incomplete lysis. Alternatively they may be too rigorous and detrimental to the integrity of the DNA, resulting in no amplification of the target DNA and possibly the formation of chimeric sequences (von Wintzingerode *et al.,* 1997; Stackebrandt *et al.,* 1999; Xu, 2006; Su *et al.,* 2012).

iii) In environmental samples the co-extraction of soil components such as humic acids interferes with downstream applications. Subsequent purification procedures such as polyvinylpolypyrollidone (PVPP) clean up and column chromatography may remove the humic matter, but these steps are elaborate and drastically reduce DNA yield (Kirk *et al.,* 2004). An indirect extraction protocol has been suggested to circumvent this bias. It involves the selective removal of microorganisms from the soil matrix prior to nucleic acid extraction. However, the efficacy of this protocol is dependent on the efficient recovery of microorganisms from the sample (Riesenfield *et al.,* 2004).

iv) The various issues associated with PCR amplification such as random fluctuations in priming efficiencies, polymerase specificity, different copy numbers of target genes and hybridization efficiency are the causes of selective amplification of certain regions of DNA. For example, GC rich priming sites are preferably amplified over AT rich sites (Reysenbach *et al.,* 1992; Ishii and Fukui, 2001). The differences in clonability and sequence diversity due to contaminating reagents have also been reported (Stackebrandt, *et al.,* 1999). These disparities limit the reliability of data obtained from PCR or 16S rRNA based methods (Sipos *et al.,* 2007; Gonzalez *et al.,* 2012).

2 Innate biases:

i) Innate biases refer to biases introduced by differences in genome size and the number of ribosomal RNA (*rrn*) operons present in genomic DNA. This can be explained by

considering the PCR amplification of *rrn* operons from a microbial assemblage which consists of the DNA of strains with large genomes but small number of *rrn* operons, and DNA originating from strains with small sized genomes but large numbers of *rrn* operons. Strains with large *rrn* operons would be favorably amplified resulting in a significant taxon composition misrepresentation (Farelly *et al.,* 1995).

ii) The microheterogeneity of the *rrn* operons from a microbial assemblage may also lead to taxon composition misrepresentation. Although heterogeneity is minute between related organisms, PCR amplification would favour any strain bearing the slightest difference if its cell numbers are high in the sample analyzed or if it is artificially amplified by any of the aforementioned biases of PCR amplication. Conclusions about sequence diversity based on results so obtained are misleading (von Wintzegerode *et al.,* 2007).

3. The interpretation of 16S rRNA gene data may introduce a further source of error.

The size of the 16S rRNA gene (about 1500bp) represents a small fraction of an organism's genome and can therefore not be an exhaustive index for microbial ecology deductions (Stackebrandt *et al.,* 1999). In addition, deduction of evolutionary relationships based on sequence manipulation and reconstruction of phylogenetic trees is dependent on differences in base composition, the selection of an outgroup and the stretches of sequences to be analyzed. The number of organisms present on the phylogenetic tree is at the discretion of the researcher as there is as yet no definitive protocol for sequence quality refinement (Clarridge, 2004; Gonzalez *et al.,* 2012). Even though the number of full and partial 16S rRNA genes sequences available in databases is increasing daily they reflect only a miniscule portion of the global diversity (Macrae, 2000; Xu, 2006). Thus the quality of results from comparative sequence analysis is dependent on the available data set which is currently incomplete. Many rRNA genes sequences from environmental samples show low sequence similarities to sequences of known organisms. It is therefore necessary to ascertain whether the sequences reflect the presence of novel uncultured soil organisms or whether they are affiliated to known taxa because available comparative sequences are of low quality (Head *et al.,* 1998).

DNA-DNA hybridization studies have shown that the resolving power of the 16S rRNA sequence alone is inadequate for bacterial species delineation. These studies demonstrate that organisms sharing identical SSU rRNA moieties may be genomically more divergent than those having similar SSU moieties differing in a few variable positions (Stackebrandt and Goebel, 1994).

Irrespective of these shortfalls, the use of the 16S rRNA gene as a molecular marker in ecological studies is one of the techniques which have proved to aid the identification of miroorganisms at a genetic level and leads to an understanding of the complex interactions between environmental components. This potential can only be fully harnessed if information from physico-chemical analysis of natural samples is obtained and matched with the information of the diversity present in the environment. Following diversity studies, information on population size and microbial activity (gene expression from mRNA and protein studies) should be obtained so as to provide a broader and more accurate view of the functioning of the ecosystem (Farelly, 1995; Stackebrandt *et al*., 1999; Smalla, 2004).

1.2.7 Molecular methods used in this research

1.2.7.1 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE is a genetic fingerprinting technique used in environmental microbiology to assess microbial populations, structure and dynamics (Morris *et al.,* 2002). It is a practical approach for the analysis of various organisms within larger communities since the technique is capable of detecting non-restriction fragment length polymorphisms thus permitting changes within these communities to be monitored (Helms, 1990). The technique is amenable for the examination of temporal succession and spatial variations of microbial community diversity (Yu *et al.,* 2008). The 16S rRNA gene is the suitable genetic marker for DGGE because its sequence (1500bp of which only 200-700bp are used for DGGE) are the highly conserved sequences and specific identifier regions of the target organism (McAuliffe *et al.,* 2001).

The use of DGGE as an analytical tool is based on the electrophoretic separation (at constant heat of about 60°C) of PCR generated DNA fragments in an environment of increasing polyacrylamide gel denaturants. The fragments are similar in length but differ by as little as a single base pair substitution. As with other electrophoretic techniques, the electrical charge, molecular weight and shape of the molecules are critical to resolution of fragments. In DGGE, the positive electrode attracts the negative charges of DNA molecules facilitating migration through the polyacrylamide gel pores. The migration of double stranded DNA within the acrylamide environment is fast and the speed is altered only at the point in the increasing urea-formamide gradient at which G-C base pairs within the DNA molecule denatures (Muzyer, *et al.,* 1993).

The denaturation of the component DNA fragments hinges on differences in the stability of the G-C rich sequence at the 3' end of the primers (conferred by H=H=H bonds as against H= H bonds of A-T regions) and as influenced by temperature. Any variation in the DNA sequence results in different denaturation rates with concomitant migration of different sequences at different positions within the gel. Hence, DGGE is suitable for identifying minute differences in closely related organisms (Watanabe *et al.*, 2001).

1.2.7.2 Recombinant DNA technology- The cloning strategy

Recombinant DNA technology essentially is the engineering of a host cell to produce copies of a genetic material of interest in its daughter cells as it grows and undergoes cell division. It features the isolation of DNA or RNA or segments of interest within these genetic molecules and the sustenance of this in a carrier molecule, the vector; –which is another DNA molecule that is capable of self-replicating. The vector and gene of interest are joined by covalent linkage facilitated by complementary base pairing at their overhanging or cohesive single-stranded tails in the presence of a DNA ligase. This joining is the cloning step in which the recombinant DNA is generated. The recombinant DNA molecules are incorporated into host cell often *E. coli,* for propagation of clones. Clones are screened for correct insert conformation, and the inserts are purified using enzymes (restriction endonucleases) , which cuts the vector at the precise sites

flanking the cloning regions, that is, the point of insertion of fragment of interest (Boffey, 1987; Madigan *et al.*, 2000b).

In this study clones are generated by inserting PCR (and not restriction digest) generated 16S rDNA fragments into a plasmid (Table 2.6) and then incorporated into the *E. coli* strain as described in Section 2.3.7.

1.2.7.3 Amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA is a genotype-based identification method. Also referred to as PCR-RFLP analysis of the rRNA gene, it encompasses molecular techniques such as DNA extraction, PCR, cloning and restriction digestion of amplified fragments, thus enabling the rapid screening of isolates or phylotypes for sequence polymorphisms (Vaneechoutte & Heyndrickx, 2001).

It involves the incubation of amplified rDNA with different restriction enzymes separately and the generation of various restriction patterns which are discriminatory between representative isolates or phylotypes which are then characterized by sequencing (Kirk *et al.*, 2004; Su *et al.*, 2012).

1.3 Lake Magadi, Kenya- an arid zone soda lake

1.3.1 Geographical location and other features

Lake Magadi, Kenya is a soda lake and is considered to be an extreme environment due to the combination of condition of extreme pH (often above pH 9), extreme salinity (4M NaCl concentration) and certain sites of extreme temperature (up to 80°C) present in the lake (Jones *et al.*, 1994; Davies, 1997). Located on the floor and at the lowest point of the East African Rift Valley which runs from Ethiopia through Kenya to Tanzania, Lake Magadi lies in the southernmost part of Kenya about 120 km southwest of Nairobi (Figure 1.3). It covers an area of about 90 km^2 bounded by latitudes 1°40′S and 2°10′S and longitudes 36°00′E and 36°30′E (Ibs-von Seht *et al.*, 2001; Denson *et al.*, 2010).

The bedrock of Lake Magadi is largely volcanic and its metamorphosed extensions. It is primarily fed by meteoric inputs and its groundwater has no obvious outflow (Davies, 1997; Warren, 2006, Denson *et al.*, 2010).

On the shores of Lake Magadi fresh water springs arise, with saline hot springs being found in the southern regions (Denson *et al.*, 2010). The southern region is characterised by extensive salt flats which account for Lake Magadi being the site of the worlds' second largest source of sodium carbonate. The sodium carbonate is harvested for use in the production of salt and glass for local use and export (Bell, 2012). In a global context soda lakes are sites for the industrial exploitation of borax, borates, halites, lithium, trona, uranium and zeolites (William, 1981; Warren, 2006a). In aquaculture, soda lakes are the sources of the alga *Spirulina sp* which is used in the pharmaceutical industry (Kebede, 1997).

Figure 1.3: Geographical location of Lake Magadi within the East African Rift Valley. Adapted from http://www.webkenya.com/eng/safari/gif_map.

Soda lakes and their arid counterparts, the soda deserts, are uncommon and are geographically widely dispersed (Table 1.4). They are the best representatives of stable naturally occurring high pH environments on Earth. This is in sharp contrast to the transiently alkaline environments which arise from either biological activities (photosynthesis, ammonification or sulfate reduction) or from the industrial activities of hide processing and cement manufacturing (Grant, 2006). Soda lakes are also often remotely situated, confined to subtropical and continental interiors and rain shadow zones as such they have been underexplored for microbiological research (Kristjánsson and Hreggvidsson, 1995; Jones and Grant, 1999). Studies have focused mostly on three soda lakes: Mono Lake (U.S.A), Lake Wadi An-Natrun (Egypt) and Lake Magadi (Kenya) (Litchfield and Gillevet, 2002; Baumgarte, 2003). According to Zhilina *et al.* (2012), like other extreme environments, soda lakes are attracting research attention because of the perception that the microbial life forms they harbor may provide valuable biotechnological innovations (Horikoshi, 1999). Additionally, they may provide new perspectives on the true extent of microbial diversity and clues to the evolution of life on Earth (Canganella and Wiegel, 2011).

Despite the extreme chemistry of high pH and high salt concentrations in soda lakes which imposes severe abiotic stress on its inhabiting organisms, the lakes are home to relict microbial communities which are deemed to be possible centres of the origin of microbial diversity (Zarvazin, 1993). The extreme conditions are also responsible for the formation of a specialized niche of microorganisms more precisely described as natronophilic and of which only a few true representatives have been discovered (Antony-Babu and Goodfellow, 2008; Bowers *et al.*, 2009, Zhilina *et al.*, 2012).

Table 1.4: Distribution of alkaline and/or saline lakes across the globe

Region of the World	Country	Identity
Africa	Central: Chad	Lake Yoan, Lake Nunyampaka, Lake Murumuli, Lake Munyanyange, Lake Dijikare.
	East: Kenya	Lake Magadi, Lake Sonachi,
	Ethiopia	Lake Chitu, Lake Shala, Lake Abjiata, Lake Aranguadi, Lake Kilotes, Lake Hertale
	Uganda	Lake Mahenga, Lake Kikorongo, Lake Nyamunuka.
	Tanzania	Lake Tulusia, Lake Lgarya, Lake Ndutu, El Kekhooito, Lake Balangida, Lake Eyasi, Bosotu crater lake, Lake Magad.
	North: Egypt	Lake Wadi an-Natrun
	Libya	Lake Fezzan
	Sudan	Dabira Lakes
North America	USA	Mono Lake, Searles Lakes, Ragtown Soda Lakes, Big Soda Lake, Union Lake.
	Canada	Manitou Lake
South America	Venezuela	Langunilla Valley Lakes.
	Chile	Lake Antofagasta.
	Mexico	Lake Texcoco
Asia	India	Lonar Soda Lake, Lake Sambhar.
	China	Inner Mongolia Lakes, Lake Bange.
	*Siberia	Lake Khatyn, Tanatar Lakes, Lake Karakul, Lake Chita.
Australia	Australia	Red Rock Lake, Lake Corangamite, Lake Chidnup, Lake Eyre and Lake Werowrap.
Europe	Hungary	Lake Feher.
	Serbia-Montenegro	Pecena Slatina Lake.

References: Modified from Jones and Grant, 1999; Grant, 2006; Wani *et al*., 2006; Yumoto *et al.*, 2011. *Siberia is not a country, but the vast region comprising almost the whole of Northern Asia and part of the defunct Soviet Union.

1.3.2 The genesis of the haloalkaline conditions

Lake Magadi is situated in an arid zone of largely volcanic and metamorphic bedrock which contributes calcium-sodium-bicarbonate (Ca-Na-HCO_3) to the groundwater. Its landscape is a shallow depression surrounded by high marginal relief limits inflow from overlands. This trough with no obvious outflow sits at the lowest point in the East African Rift valley and is exposed to intense solar penetration which results in maximum evaporite productivity (Jones and Grant, 1999; Bell, 2012). The combination of these features climate, topography and geology are the mechanisms which leads to the evolution of the hypersaline highly alkaline conditions of Lake Magadi.

Athalassahaline lakes are saline water bodies that are derived from evaporative deposits and not from sea water. The chemistry of the development of briny conditions in hypersaline athalassahaline lakes is dictated by the prevailing geology (Jones *et al.,* 1998; Warren, 2006a). The absence or presence of calcium and or magnesium ions (from basaltic minerals and antiquated microbial activities) determines on which side of neutrality the pH of the waters lies (Eugster, 1986; Grant, 2006). Most present day aqueous environments are saturated with calcium ions' which precipitate as calcite often in association with magnesite and dolomite (Jones *et al.,* 1994). If the concentration of calcium and magnesium exceeds that of carbonate, then carbonate is removed from solution and an essentially neutral condition is generated. This hypersaline neutral condition is found in Great Salt Lake in the United States of America (Eugster and Hardie, 1978; Grant and Tindall, 1986).

Hypersaline acidic environments such as that of the Dead Sea develop due to the presence of an excess of magnesium ions from dissolution of its evaporite (from earlier geological events) and also from the precipitation of minerals such as sepiolite which generates hydrogen ions during the evaporative process (Figure 1.4).

Soda lakes (Figure 1.4) are also of athalassahaline origin and according to Zhilina *et al.* (2012) they are the end reservoir of subaerial carbondioxide (CO_2) weathering in semi-arid climates which may be regarded as the best model of the Early Proterozoic terrestial environment. Lake Magadi, Kenya is a typical example of a soda lake. Combinations of climatic, geochemical and topographical factors are responsible for the highly alkaline and hypersaline conditions within Lake Magadi (Eugster, 1970; Warren, 2006b).

Climatic conditions control how much water enters the closed basin system as rainfall, as surface runoff or by inflow from surrounding escarpments, and the rates at which the groundwater leaves the system (Grant, 2004). The surface water becomes carbonate charged from weathering of the sodium carbonate in the surrounding strata and from biological activities at the surface and near-surface zones. Ion exchange occurs between the carbon dioxide in solution leading to the production of a bicarbonate/carbonate solution. This leaches the minerals in the surrounding rocks and determines the ionic composition of the groundwater (Eugster, 1970). The absence of significant amounts of calcium and magnesium ions in the groundwaters (Ibs-von Seht *et al.*, 2001) and the presence of exceedingly high molar concentrations of carbonate and bicarbonate ions, accounts for the alkalinity of the soda lake.

Evaporative concentration of carbonate ions leads to a shift in the carbon dioxide/ bicarbonate/ carbonate equilibrium in favour of carbonate formation (Equation 1) (William, 1981).

$$\underset{\text{carbondioxide}}{CO_2} + H_2O \rightleftharpoons \underset{\text{carbonic acid}}{H_2CO_3} \rightleftharpoons \underset{\text{bicarbonate}}{H^+ + HCO^-_3} \rightleftharpoons \underset{\text{carbonate}}{2H^+ + CO_3^{2-}}$$

Equation 1: The shift in the equilibrium of bicarbonate/ carbonate formation favouring the formation of carbonate.

As evaporative concentration of carbonate progresses, sequential precipitation of the insoluble carbonates of calcium and then magnesium occur providing buffering capacity in the waters. The elimination of these alkaline earth cations from the waters permits the accumulation of the more soluble carbonates of sodium and potassium (Jiang *et al.*, 2006; Litchfield *et al.*, 2009). This results in the evolution of an alkaline sodium carbonate brine.

Unlike Lake Magadi which is surrounded by high marginal relief, the other two well studied lakes -Mono Lake and the lakes of the Wadi Natron depression in Egypt are bounded by low relief (<5m) (Botkin *et al.*, 1988; Taher, 1999). Also, the pH and salinities of these two lakes are lower compared to that at Lake Magadi. Mono Lake has a pH of 10 while the saline-alkaline lakes in Egypt have an 8.5-9.5 pH range and both salinities are not close to saturation like at Lake Magadi. The waters of Mono Lake are calcium carbonate rich unlike Magadi while those of the Wadi An-Natron are rich in chlorides and sulfates in addition to carbonate. However, the three lakes share common grounds being endorrheic (closed- no obvious outflow) basins located

in arid regions with low and seasonal rainfall hence are characterized by evaporative concentration of brine.

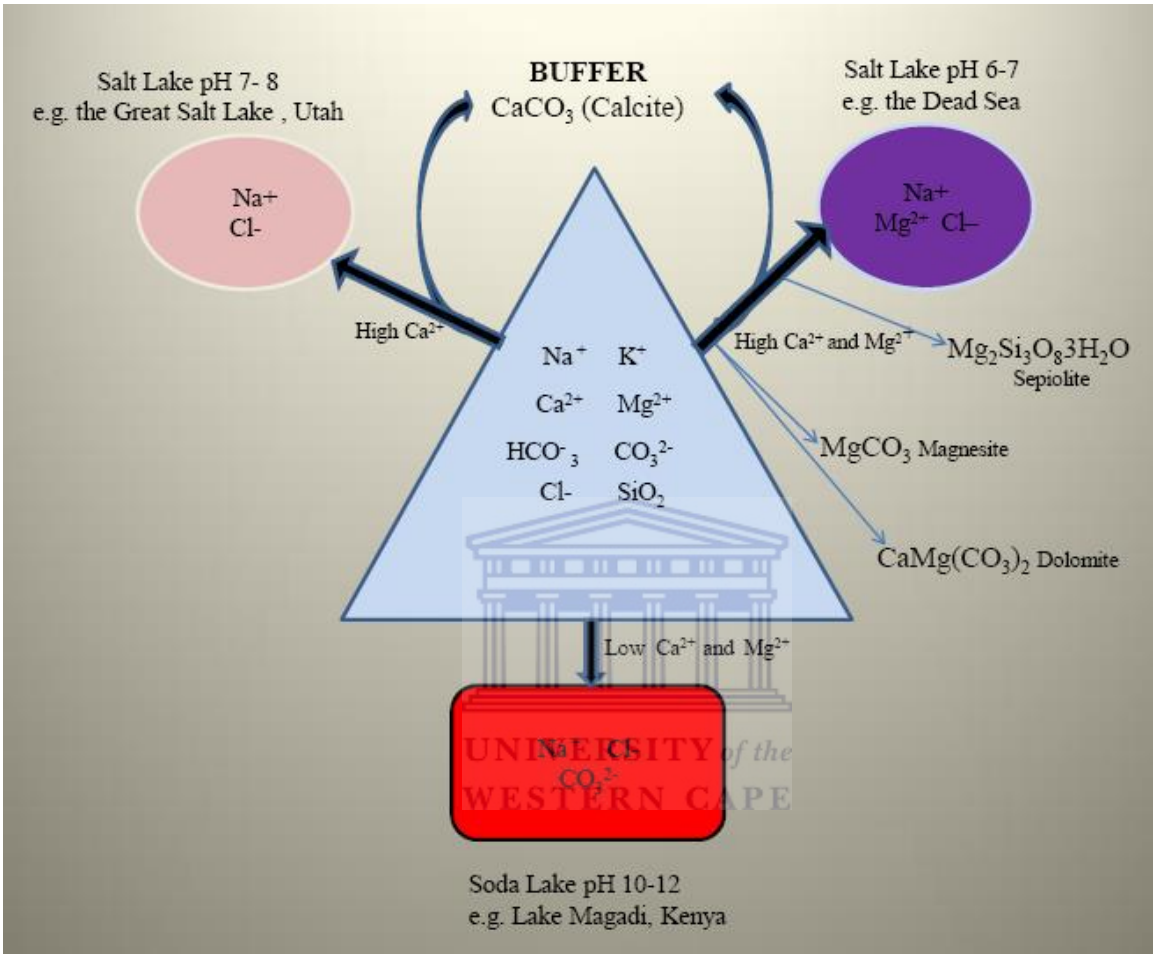

Figure 1.4 Schematic representation of the genesis of saline and alkaline lakes. References: Modified from Grant, (2004; 2006).

1.4 Haloalkaliphily

1.4.1 Biodiversity of haloalkaliphiles

In aquatic ecosystems, salinity is a major determinant of microbial community structure (Horikoshi, 2011). In the soda lake environment, despite the elevated saline conditions which are coupled with high pH and low ammonium ion concentrations (McGenity and Oren, 2012), systematic studies have demonstrated that this niche is home to a specialised groups of

organisms. Although strain or species diversity is often lower in extreme environments (Kristjánsson and Hreggvidsson, 1995) such as Lake Magadi (Jones *et al.*, 1994), the degree of specialization of microbes in this carbonate rich environment is thought to have arisen from long and complex evolutionary processes (Zarvazin *et al.*, 1999; Bell, 2012). Oren (1999), postulates the interplay between salt tolerance and efficient energy metabolism in natronophiles as the partial reason for the decline in community composition and the study of Zhilina and co-workers, (2012) established that total mineralization rather than high alkalinity is the main limiting factor of the complete trophic system present in soda lakes. The microorganisms inhabiting this specialized environment may offer considerable potential for biotechnological innovations and applications (Stackebrandt *et al.*, 1999).

The hypersaline brines of Lake Magadi harbor a unique population of prokaryotes (compared to the more dilute saline lakes) as the dominant microbial biomass. Many of these microbes have been reported from systematic studies as being obligately alkaliphilic or alkalitolerant (Grant and Sorokin, 2011) which represent separate alkaliphilic lineages within pre-established taxa (Zavarzin *et al.*, 1999). These microbes are extremely prolific due to high ambient temperatures, high light intensities, availability of phosphates and an unlimited supply of carbon dioxide from carbonates in solution (Melack and Kilham, 1974; Grant and Tindall, 1986). Production yields within this haloalkaline environment stand at >10 g carbon m^{-2} day^{-1}, against the average primary production in streams and non-alkaline lakes which stands at 0.6g cm^{-2} day^{-1}. This turnover makes soda lakes globally the most productive amongst aquatic ecosystems (Jones *et al.*, 1998).

Seasonally, Lake Magadi exhibits the pinkish-red coloration of the haloalkaliphilic archaea due to the synthesis of C_{50} carotenoids by these microorganisms (Figure 1.5). In addition the colorful blooms (green, pink or red) of cyanobacteria and alkaliphilic anoxygenic phototrophs are also visible periodically due to dilution of the brines (Rodriguez-Valera *et al.*, 1981; Mwatha and Grant, 1993). Gram-negative bacteria are reported to be the principal populations in this soda lake environment. Jones and co-workers (1994) reported that variabilities in conductivity, alkalinity, phosphate and nitrogen levels dictate species dominance during a particular season.

Figure 1.5: The bright coloration of Lake Magadi, Kenya, due to synthesis of C_{50} carotenoids by halobacteria. References: Adapted from Rodrgíuez-Valera *et al.,* 1981; Mwatha and Grant, 1993.

Cultivation of strains from this environment is a major research objective and many haloalkaliphilic strains have been retrieved using nutrient rich media supplemented with appropriate concentrations of sodium carbonate and sodium chloride (Jones and Grant, 1999; Mwirichia *et al.,* 2010).

Biochemically reactive isolates producing an assortment of extracellular hydrolytic enzymes such as lipases, proteinases and cellulases have been isolated from haloalkaline lakes. Many of the cultivable population are assignable to existing taxonomic groups, although most fall into new generic groupings. 16S rRNA gene based studies of the soda lakes have revealed novel taxa yet to be cultivated. However there is currently no separate and exclusive taxon for isolates from the soda lake environment (Grant, 2004)

1.4.2 Nutrient and element cycling under haloalkaline conditions

Active nutrient and energy cycling coordinated by a well structured haloalkaliphilic prokaryotic assemblage in soda lakes is well documented (Zavarzin *et al.,* 1999; Zavarzin, 2007). These microbial communities are reported to be especially active at low to moderate salt concentrations (Sorokin and Muyzer, 2010) and are an integral element of the universal biogeochemical cycle (Sorokin, *et al.,* 2011). The primary production in most of the East African soda lakes is driven

principally by the phototrophic cyanobacterium -*Arthrospira platensis* and to some extent by the anoxygenic phototrophic purple bacteria (Zarvazin *et al.*, 1999). This activity produces organic matter which supports the dense organotrophic bacterial population (>10^6 cfuml^{-1}).

In Lake Magadi, the presence of representatives of all the main trophic groups involved in the active cycling of nutrients (carbon, sulfur and nitrogen) under both aerobic and anaerobic conditions has been reported (Grant and Sorokin, 2011). The basis of primary production within Lake Magadi is however unclear, since the trona beds are often dominated by organotrophic archaea (Grant, 2006). Grant and Tindall (1986) suggest that primary productivity is probably driven by the genus *Halorhodospira*, a typical soda lake component. A representative of this genus the sulfur oxidizing *Halorhodospira halophila* has been isolated from Lake Magadi. The obligately halophilic and alkaliphilic archaea present at densities of 10^6-10^7 ml^{-1} are presumed to be vital for secondary productivity. Table 1.5 lists representatives of trophic groups identified under aerobic and anaerobic conditions within Lake Magadi.

1.4.2.1 The Carbon cycle

Due to evaporative concentration, relatively high organic and inorganic matter levels have been reported in the brines of Lake Magadi (Jones *et al.*, 1994; Grant, 2004). The availability of these nutrients is thought to be sufficient to serve the substantial organotrophic community dominated by haloalkaliphilic archaea of the genera *Natronobacterium* and *Natronococcus.* These genera represent distinct archaeal halophilic lineages and have physiological requirements of high salt (>10 M), high pH (8.5-11) and low magnesium ion concentrations (<10 mM) (Tindall *et al.*, 1984). Moderately halophilic Gram-negative bacteria of the *Halomonadaceae* family are reported to be especially abundant in Kenya soda lakes (Tindall, 1988; Duckworth *et al.*, 2000). They utilize a wide range of sugars and amino-acids and are capable of hydrolyzing proteins and polymeric carbohydrates. These soda lakes halomonads are also reported to be far less nutritionally demanding than the halomonads of moderate environments (Jones *et al.*, 1998).

Table 1.5: Representatives of trophic groups identified within Lake Magadi

Trophic level representatives	Examples
Aerobes	*Arthrospira platensis* (Grant, 2006) *Oscillatoria limnetica* and
Phototrophs	*Synechocytis salina* (Dubinin *et al.*,1995)
Eukaryotic microalgae	*Chlorella minutissima* (Gerasimenko *et al.*, 1999)
Sulfur-oxidisers	*Halomonas magadiensis* (Duckworth *et al.*, 2000)
	Thioalkalivibrio nitratis (Sorokin *et al.*, 2011)
Haloalkaliphilic archaea	*Natrialba magadii; Natronobacterium pharaonis* and
	Natronobacterium gregoryi (Tindall *et al.*, 1984)
Anaerobic alkaliphiles	*Tindallia magadiensis* (Kevbrin *et al.*, 1998)
Anoxygenic phototrophs	*Halorhodospira halophila* (Grant & Tindall, 1986)
Sulphate-reducing bacterium	*Desulfonatronovibrio hydrogenovorans* (Zhilina *et al.*, 1997)
Saccharolytic anaerobes	*Halonatronum saccharophilum* (Zhilina *et al.*, 2001a)
	Amphibacillus fermentum and *Amphibacillus tropicus*
	(Zhilina *et al.*, 2001b)
Saccharolytic spirochetes	*Spirochaeta alkalica* and *Spirochaeta africana* (Zhilina *et al.*, 1996a)
Acetogenic organotroph	*Natroniella acetigena* (Zhilina *et al.*, 1996b)
Methanotrophic methanogen	*Methanohalophilus zhilinae* strain Z7936
	(Zhilina & Zavarzin, 1994; Kevbrin *et al.*, 1997)

References: Mwatha and Grant, 1993; Jones and Grant, 1999; Zavarzin *et al.*, 1999.

Gram-positive bacteria present in soda lakes are haloalkaliphilic strains related to the sodium requiring species of the taxonomically broad *Bacillus* group. These isolates are quite

phenotypically distinct, and grow well in 25-30% w/v sodium chloride with 15% w/v minimum requirement (Duckworth *et al.,* 1996).

The hydrolysis of various complex organic polymers is thought to yield substrates for aerobic organotrophs as well as for the chemo-organotrophic alkaliphilic anaerobes (Grant, 2004). In the latter group, the saccharolytic *Spirochaeta* spp. have been identified by viable counts of anoxic Lake Magadi sediments. The anaerobic fermenters identified within this saline–alkaline environment utilize various pentoses, hexoses, and disaccharides to produce acetate, lactate, ethanol and hydrogen (Grant, 2006). Obligately haloalkaliphilic fermenters found in Lake Magadi belong to a separate well-defined group of a new genus of the *Clostridiaceae* family (Jones *et al.,* 1998). Physiologically they have a high tolerance to sodium chloride (25% w/v) with 12-16% w/v as a minimum requirement at pH 9.5. These microorganisms ferment a range of sugars to produce iso-valeric, iso-butyric and acetic acids (Mesbah and Wiegel, 2008). The products generated by the fermentative bacteria are substrates for acetogenic bacteria, anoxygenic phototrophs and sulfate-reducing bacteria.

1.4.2.2 The Nitrogen cycle

At the apex of the nitrogen cycle, fermentative anaerobes produce ammonia which is utilized by methanotrophs and nitrifiers (Rees *et al.,* 2003). Although alkaline pH conditions do not favour the formation of ammonia, which is volatile and leads to loss of nitrogen from the system, the methane oxidizers convert ammonia to nitrite to achieve a balance in the nitrogen flux (Jones and Grant, 1999). Isolated methane-oxidizing haloalkaliphiles in culture belong to the type I methanotroph group of the *Gammaproteobacteria* mostly in the genus *Methylomicrobium* (Khmelenina *et al.,* 2008; Sorokin *et al.,* 2001).

Shapalova *et al.* (2008) reported the presence of acetate utilizing denitrifying populations at *in situ* haloalkaline conditions and from anaerobic enrichment cultures grown at pH 10 and a 4 M sodium ion concentration. The dominance of *Gammaproteobacteria* belonging to the genus *Halomonas* was observed. The culture had acetate as an electron donor, with nitrate, nitrite and nitrogen oxide serving as double electron acceptors. This finding confirms earlier observations of

Jones *et al.* (1998) and Mesbah *et al.* (2007) that the Gram-negative bacteria of genus *Halomonas* are the dominant microorganisms in the soda lake environment. The authors postulated that soda lake halomonads are likely to be integral players in the nitrogen cycle since the strains obtained in their work uniquely possess the ability to reduce nitrate to nitrite. The report also indicated that one group of the isolates is capable of further reducing nitrite and is able to grow anaerobically with or without nitrate.

Under hypersaline soda lake conditions nitrifying bacteria are represented by a new species belonging to the *Alphaproteobacteria* division, *Nitrobacter alkalicus* and by the alkaliphilic species *Nitrosomonas halophila* which belongs to the *Betaproteobacteria* division. Only the metabolically diverse genus *Thioalkalivibrio* (chemolithotrophic sulfur oxidizers) contains a species - *T. denitrificans,* capable of denitrification at alkaline hypersaline conditions (Sorokin and Kuenen, 2005).

1.4.2.3 The Sulfur cycle

The sulfur cycle is one of the most active microbial cycles in soda lakes even at elevated saline and highly alkaline conditions (Zavarzin, 2007). The extent of the activity of this cycle is evident from the often highly acid labile sulphide contents of the sediments of the soda lakes and the efficient high-energy of dissimilatory inorganic sulfur transformations under oxidative as well as sulfidogenic states. The cycle is coordinated by sulfur-oxidizing bacteria present in high viable numbers (10^6 viable cells cm^{-3}) (Sorokin *et al.,* 2011) and by sulfate-reducing bacteria whose presence is indicated by substantial reduction rates of between 12 to 423 µmol sulfate dm^{-3} day^{-1}. These sulfur-oxidizing bacteria and sulfate-reducing bacteria are unique to the soda lake habitat (Foti *et al.,* 2007).

At high alkaline and saline conditions the culturable sulfur-oxidizing bacteria (more than 100 strains of which have so far been identified) from soda lakes are found in the surface sediments (Sorokin *et al.,* 2011). Jones and Grant (1999) report that while some strains appear to be related to the genus *Thiomicrospira,* others are new lineages of the *Gammaproteobacteria*. The culturable forms so far identified includes members of the the genus *Halorhodospira* (Imhoff *et*

al., 1979) which are the source of glycine, betaine, ectoine and trehalose- the osmolytes used for the balance of intracellular osmotic pressure (Jones and Grant, 1999; ExPasy bioinformatics resources portal, 2007). Other culturable forms belong to the four novel genera of *Thioalkalimicrobium*, *Thioalkalispira*, *Thioalkalivibrio* and *Thioalkalibacter* (Sorokin *et al.*, 2011). All four are obligate chemolithoautotrophs utilizing various reduced inorganic sulfur compounds as electron donors (Foti *et al.*, 2007). *Thioalkalispira* and *Thioalkalibacter*, unlike the other two genera, are occasionally found and each is represented by just a single species. The genus *Thioalkalivibrio* is the most physiologically diverse (Sorokin and Muyzer, 2010). Its major subgroup possesses the unique attribute of being the only known aerobic chemolithoautotroph capable of growing in saturated alkaline brines of 4M total sodium ion concentration. In addition, some species within the genus *Thioalkalivibrio* can utilize thiocyanate as a sole source of energy and some can grow under anaerobic conditions using nitrogen oxides as electron acceptors (Sorokin and Kuenen, 2005; Sorokin *et al.*, 2011).

Sulfidogenesis is active in the anoxic layers of the sediments of soda lakes, and is hindered only by salt-saturated conditions (Foti *et al.*, 2007; Sorokin and Muyzer, 2010). Unlike sulfur oxidation under extremely alkaline-saline conditions, sulfidogenesis is not clearly understood. Although, high rates of sulfate reduction have been reported from *in situ* measurements and laboratory experiments, only a few species of haloalkaliphilic autotrophic sulfate-reducing bacteria have thus far been isolated. These isolates belong to the *Deltaproteobacteria* lineages, order *Desulfovibrionales* and include the genera *Desulfonatronovibrio*, *Desulfonatronum* and *Desulfonatronospira* and were all obtained at low salt concentrations. A few representatives of the order *Desulfobacterales* which are heterotrophs growing on volatile fatty acids and alcohols at high pH and moderate salinities have also been found (Grant, 2006; Foti *et al.*, 2007; Sorokin *et al.*, 2011). A fully characterized example of an alkaliphilic sulfate reducing bacteria, *Desulfonatronovibrio hydrogenovorans* which utilizes hydrogen as an electron donor has been isolated from the trona beds of Lake Magadi, functioning optimally at pH 9.5 and 3% w/v sodium chloride (Zhilina *et al.*, 1997). Non-sulfur reducing sulfidogens from soda lakes are represented by the sulfur/thiosulfate reducing *Desulfurivibrio alkaliphilus* and *Dethiobacter alkaliphilus* (Sorokin and Kuenen, 2005). Figure 1.6 summarises the major nutrient cycles present in the soda lake environment.

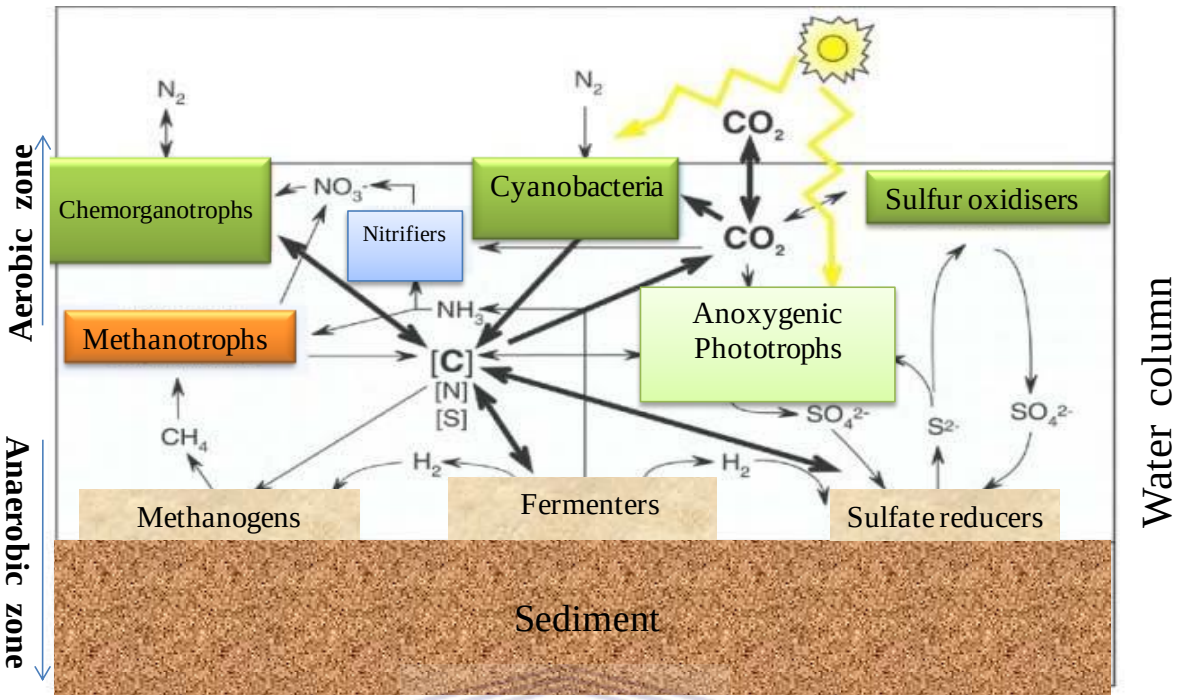

Figure 1.6 Schematic representation of nutrient cycling in the soda lake environment. Reference: Modified from Grant, 2006.

1.4.3 Adaptation to high salt and high pH conditions

The highly specific molecular mechanisms of adaptation amongst the classes of extremophiles can be cited as their respective evolutionary trademarks (Niemetz *et al.*, 1997). The elucidation and comparison of physiological and structural differences between neutralophiles and extremophiles would offers insights into the basis of adaptation to harsh survival conditions displayed by microbes of the extreme environments (Horikoshi, 1999; Krulwich, 2001; Konings *et al.*, 2002; Chakravorty, *et al.*, 2012). Although the biochemical adaptive mechanisms of survival under hypersaline alkaline conditions are well established, strategies for adaptation at a molecular level remain to be unravelled (Singh, 2010; Karan and Khare, 2011). Due to the high ionic strength at haloalkaline conditions, inhabiting organsims need to overcome osmotic effects leading to water loss and dehydration in order to survive.

Here the maintenance of balance between internal and environmental conditions displayed by microbes of soda lakes are discussed relative to those of broader alkaliphiles and halophiles and

then specifically the haloalkaliphiles (where possible) within both the bacterial and the archaeal domains.

1.4.3.1 Cell wall composition

The cell walls of alkaliphilic *Bacillus* are made up of peptidoglycan (murein) similar to that of the neutralophile- *Bacillus subtilis* (Reistad, 1972; Horikoshi, 2011). The peptidoglycan in alkaliphilic strains is characterized by excess hexosamines and amino acids lacking in the neutralophilic strains. A higher rate of cross-linkage in the peptidoglycans of these organisms at higher pH was discovered and is presumed to provide a shielding effect on the cell (Satyanarayana *et al.,* 2005).

Smithies and colleagues (1955) reported that the cell wall of the halophilic *Micrococcus halodenitrificans, Vibrio costicolus and Pseudomonas salinaria* contains relatively high amounts of nitrogen which is an indication that the cell material is predominantly protein. The protein structure is prevented from denaturation, aggregation and precipitation by the high ratio of acidic to basic amino acids which they often contain, thus giving the surface of the proteins a negative charge. The negative charge is believed to allow the proteins to be solvated in the hypertonic environment (Das Sarma and Arora, 2001). In addition, the presence of certain proteins such as bacteriorhodopsin in the membranes of halophiles supports phototrophic growth by serving as a light dependent proton pump (Haupts *et al.,* 1999; Antrakinian, 2001). The presence of novel gas vesicles in halophilic bacteria also permits flotation to depths where oxygen is more available or where the salt concentration is near neutral as a survival strategy (Das Sarma and Arora, 2001).

The presence of certain acidic sugars such as gluconic acid, phosphoric acid, galacturonic acid, glutamic acid and aspartic acid was also detected. The ability to adsorb Na^+/H^+ ions and repulse OH^- which in turn may assist cells to grow under alkaline conditions is thought to be conferred by the negative charges on the acidic non-peptidoglycans of the cell surface. A combination of these features is perceived to maintain internal conditions on the neutral side (Horikoshi, 1999; Yumoto *et al.,* 2011).

Across the archaea domain, the bacterial peptidoglycan is lacking in the cell wall (Kandler and König, 1993; 1998). Rather, diverse polymers of chemically unrelated structures are found

amongst the various archaeal lineages. While the majority Gram-negative archaea have proteinaceous or glycoproteinaceous cell envelopes (S-layers) or some reinforcement of their cytoplasmic membranes similar to the glycocalyx of eukaryotes, the lesser majority Gram positive archaea possess rigid cell walled sacculi made up of various polymers.

In the extremely halophilic *Halococcus morrhuae* a highly sulphated heteropolysaccharide was found to form the rigid cell wall (Schleifer *et al.,* 1982). The heteropolysaccharide consists of a mixture of amino and neutral sugars, uronic acids and gulosaminuronic acid with significant amounts of glycine. It is presumed that glycyl bridges exist between the glucosamine and uronic acid residues of the glycan strands. In the highly alkaliphilic *Natronococcus occultus*, the rigid cell wall polymer was found to be made of a novel glycoconjugate consisting of two types of oligosaccharide linked to a backbone of poly-γ-L-glutamine via an amide group (Niemetz *et al.*, 1997). The cell wall composition and structure of mesophilic archaea such as the pseudomurein of the *Methanobacteriales* and *Methanopyrus* and the Methanochrondroitin in *Methanosarcinales* differ considerably from those of halophilic and alkaliphilic archaea representatives earlier mentioned. The heteropolysaccharides found in the extremophilic archaea group are evolutionarily unique landmarks and are the structural barrier which protects their cytoplasm from their external environmental conditions (Kandler and König, 1993).

1.4.3.2 Osmoadaptation

A characteristic feature of halophily is the maintenance of at least an isoosmotic balance of the cytoplasm with the surrounding medium (Desmarais *et al.,* 1997; Das Sarma and Arora, 2001; Empadinhas and Da Costa, 2008; Bowers *et al.,* 2009). This mechanism is necessary because the biological membranes of halophiles are permeable to water and it is energetically not feasible to conduct active energy-dependent inward transport of water to compensate for water loss in environment of increased (or increasing) osmolality. Turgor pressure is essential for cell division to proceed but this is threatened under hypersaline conditions by drastic water loss hence, cells combats this by obtaining osmotically active molecules either from their surrounding medium or by *de novo* synthesis (Roesser and Muller, 2001). Osmoregulation in neutraphilic halophiles and alkaliphilic halophiles has been demonstrated to be similar and independent of the mechanisms

of pH homoestasis (Boltianskaia *et al.*, 2005; DetKova and Boltianskaia, 2007). To show the osmoregulatory adaptations in non-alkaliphilic halophile and an alkaliphilic halophiles, the study of Boltianskaia *et al.* (2005) revealed the regularities of osmoregulation using *Halomonas campisalis* strain Z-7398-2 and *Halomonas* sp AIR-2 as representative of alkaliphilic moderate halophiles and the alkaliphilic obligate haloanaerobe *Natroniella acetigena*, as a representative of its described group and two fundamentally different strategies for osmoregulation were established

In the halomonads, intracellular inorganic ion concentrations were insufficient to counterbalance environmental osmotic pressure balance was rather attained by the accumulation of organic osmoregulators such as ectoine and betaine. Oren (2006, 2008) reports that the 'organic-solutes-in' adaptation spans a broad salt concentration range. It involves the exclusion of salt from the cytoplasm by synthesis and or accumulation of organic 'compatible' solutes which do not hinder enzymatic activity but require a few adaptations of the cell's proteome. Glycine, betaine and ectoine are the main organic solutes found in nature, but, β-amino acids and their derivatives (β-glutamine, β-glutamate and Nε-acetyl-β-lysine) have been detected in *Methanohalophilus* species. Belitsky *et al.* (2001) reports the acquisition of proline using glutamate as precursor as a compatible solute in *Bacillus subtilis* to ensure cellular osmoadaptation. Sulfotrehalose is reported as a compatible solute in a few alkaliphiles (*Natronobacterium sp*) but only members of the extremely halophilic archaea *Halobacteriaceae* family accumulated these in substantial concentrations of up to 1M in addition to KCl (Desmarais *et al.*, 1997; Horikoshi, 1999).

The presence of the 'organic-solute in' mechanism of adaptation in obligately haloalkaliphilic *Thioalkalivibrio* species has also been reported. *Thioalkalivibrio* species characteristically withstand high salt concentrations up to 4.3M total NaCl, and in order to tolerate this hypersaline condition, glycine-betaine is synthesized as the main compatible solute. Banciu *et al.* (2005) demonstrated a positive correlation between salinity and the intracellular glycine-betaine concentration in *Thioalkalivibrio* strains. Glycine-betaine constituted 9% of cell dry weight at 4M of sodium in the culture medium. Betaine is synthesized mainly from choline by a two-step oxidation pathway or by a series of methylation reactions (Banciu *et al.*, 2008). In the reports of Muyzer *et al.* (2011) from the genome of *Thioalkalivibrio sulfidophilus* strain HL-EbGr7, genes

coding for glycine sarcosine N-methyltransferase and sarcosine dimethylglycine methyltransferase which catalyze betaine synthesis from glycine in a three-step methylation process were found. The sequences of these enzymes have high similarities to sequences found in the close relatives of HL-EbGR7 namely *Halorhodospira halophila* and *Nitrococcus mobilis.* Sucrose is also produced as a minor compatible solute (up to 2.5% of cell dry weight at 2M of sodium) by *Thioalkalivibrio* species and associated enzymes in its synthesis were found in *T. sulfidophilus*. In contrast to other members of the *Ectothiorhodospiraceae (Alkalilimnicola ehrlichii* and *Halorhodospira halodurans*) no genes for ectoine synthesis were found in the genome of *T. sulfidophilus* strain HL-EbGr7 (Banciu *et al.,* 2008).

The model alkaliphic anaerobic halophile, *N. acetigena* in the study conducted by Boltianskaia and co-workers (2005) revealed the use of potassium ion (K^+), sodium ion (Na^+) and chlorine ion (Cl^-) for osmoregulation. High intracellular concentrations of salts was observed to provide cell turgor and counterbalance external osmotic pressure arising from increasing molar concentration of Na^+ in the growth medium.

The other strategy the 'salt-in-strategy' is exclusive to members of the archaeal domain as the intracellular enzymes of halophilic or halotolerant eubacteria do not have special tolerance for salt (Margesin and Schinner, 2001; Oren, 2006; Detkova and Boltianskaia, 2007). This strategy involves the accumulation of molar concentrations of potassium chloride and for which an extensive adaptation of the intracellular enzymatic machinery is required in order to ensure that proteins maintain a proper conformation and activity at saturating salt concentrations. The proteome of obligate halophiles is highly acidic and most proteins denature when suspended in low salt condition, hence the microorganism cannot survive in low salt media.

1.4.3.3 Membrane lipid composition

The biophysical property of the membrane lipids of bacteria and archaea are also crucial to their sustenance in a wide variety of physical environment (Zhang and Rock, 2008). The lipid composition in halophiles (alkaliphilic and non-alkaliphilic alike) do not only have evolutionary

consequences as a tool for delineating taxons at the generic level, comparative studies have further implicated the membrane phospholipids and glycolipids of halophiles as an important adaptation to salinity (Kushwaha *et al.,* 1982; Asker *et al.,* 2002; LoBasso *et al.,* 2008; Dawson *et al.,* 2012). The lipids of halophiles are entirely derived from isoprenoid (saturated or unsaturated or polyunsaturated) glycerol diether (DGD's) 'archeaol' and or its dimer, dibiphytanyldiglyceroltetraether. In some halobacteria phytanyl (C_{20}) and sesterpanyl (C_{25}) archaeol are also found. The glycolipids found in halophiles are either sulfated and /or unsulfated glycosyl archaeols with diverse carbohydrate structure (Kates, 1993; Upasani *et al.,* 1994, Dawson *et al.,* 2012). Also they possess any combination of carotenoids such as p-carotene, 3-hydroxyechinenone, y-carotene, squalene, cis-astaxanthin, lycopene, bacterioruberin canthaxanthin trisanhydro-bacterioruberin, monanhydro-bacterioruberin, bacterioruberin isomer) as their non-polar lipids. Extreme halophiles possess the archaeol analogue of phosphatidylglycerolmethylphosphate (PGP-Me) as their major phospholipid. The study conducted recently by Dawson and co-workers (2012) using *Halorhabdus utahensis, Natronomonas pharaonis, Haloferax sulfurifontis* and *Halobaculum gomorrense* representing four haloarchaeal genera and different salinity optima, grown at salinity values between 10% and 30% NaCl (w/v) established a strong linear correlation between optimal growth salinity and fraction of unsaturated DGD's. The researchers observed that the proportion of unsaturated DGD's increased with increasing NaCl concentration in the medium and that the strains with higher NaCl concentration optima (*N. pharaonis* and *H. utahensis*) had higher proportion of unsaturated DGD's. Also the occurrence of $C_{(25–20)}$ DGD's was observed in the two strains with higher salinity optima. The study concluded that halophilic archaea regulate membrane lipid unsaturation in response to environmental salinity change, regardless of their salinity optima from a further observation that in three of the four strains investigated, the fraction of unsaturated DGD's increased above a salinity threshold or in response to increasing salinity in the medium. This observation had earlier been reported by Kushwaha and co-workers (1982) in their study of the lipids of a halophilic bacterium

1.4.3.4 Bulk protein composition

Various studies have reported that the amino-acid composition of the proteins of halophilic bacteria and haloarchaea differ considerably from the amino-acids of the proteins in mesophilic bacteria and archaea. While capable of eliciting similar physiological response as the enzymes of mesophiles, the proteins of halophiles have been shown to be comprised of a lesser proportion of hydrophobic amino acids but higher proportion of acidic amino acids in comparison to those in neutralophiles (Mevarech *et al.*, 2000; Detkova and Boltianskaia, 2007). The structural and functional intergrity of mesophilic proteins are disrupted at high ionic strength. However, in halophiles under saline environmental conditions, salt ions exerts charge screening on the acidic amino acid residues at the protein surface and reduces electrostatic repulsion among the groups. The interaction of hydrated salt at the acidic group also enhances hydrophobic interactions which in turn favors the formation of a compactly folded structure. Proteins which fold at high salt concentrations are made up of polypeptide chains and a tight solvation shell. The folding involves specific interactions (leading to greater surface charge) between the water molecules, the salt ions and the polypeptide. The activity at the protein surface results in greater surface charge, enhanced molecular motion and consequently, solubility and stabilization of protein subunits (Hutcheon *et al.*, 2005; Karan *et al.*, 2012).

The study by Karan and Khare, (2011) demonstrated the salt dependent resistance of the protease of haloalkaliphilic *Geomicrobium* sp. against organic solvents and thermal inactivation. It was observed that gradual withdrawal of salt from the medium led to loss of active conformation. The structure of the protein investigated was also observed to remain intact (more soluble and flexible) when exposed to denaturants at high salt concentrations. Salt-dependent stability in a haloarchaeal enzyme (an alpha-amylase of *Haloarcula hispanica*) to urea denaturation had also been reported by Hutcheon and co-workers (2005).

1.4.3.5 pH homeostasis

Alkaliphiles have to maintain a cytoplasmic pH that is much lower than the external pH (Ivey *et al.*, 1998; Bell, 2012). This is necessary since most of their cytoplasmic enzymes function

optimally at near neutral pH (Van de Vossenberg *et al.*, 1995; Krulwich *et al.*, 2011). Horikoshi (1991; 1999) reports that as revealed by measurement of the optimal pH of intracellular enzymes, as well as measurement of non active weak bases inside and outside cells in alkaliphilic organisms in comparison to those of neutralophiles the maintenance of cytoplasmic pH in the neutral spectrum is achieved by one of two mechanisms. The acidic polymers of the cell walls function as a negatively charged matrix which may reduce the pH value at the cell surface. Alternately, the plasma membranes use the Na^+/H^+-antiporter system and the ATPase-driven hydrogen ion (H^+) expulsion to maintain at least neutrality (or below neutral –cytoplasmic acidification)of the intracellular milieu (Yumoto *et al.*, 2011). Sodium ion (Na^+) plays a vital role in the growth of alkaliphiles as shown by studies into membrane transport across membranes. According to the reports of Van de Vossenberg and colleagues (1999), their study of the salt and pH dependence of ion permeability across lipid membranes in extreme halophilic and alkalihalophilic archaea (*Halobacterium salinarum* and *Natronobacterium vacuolatum* respectively) showed that the membranes of the model organisms were impermeant to hydrogen ions and sodium ions (H^+ and Na^+) at a wide range of salt concentrations and elevated pH values thus permitting proliferation at such environmental conditions. An electrogenic Na^+- H^+ (Na^+/ H^+ ratio < 1) antiport reaction was reported to facilitate the extrusion of sodium ions as well as the uptake of protons. Although this sodium ion-coupled trasnport is useful for halo(alkalithermo)philes since they are subjected to a large sodium ion gradient, it is actually the stability of their lipid membranes that allows them to thrive at high salt concentrations.

1.4.3.6 Bioenergetics

When internal pH homeostasis is achieved within a cell, a secondary biological problem of creating a pH gradient across a cytoplasmic membrane oriented in the reverse of electron transport must be resolved (Ivey, *et al.*, 1998). This inverted chemiosmotic proton gradient must be attained in order to drive the cellular processes of ion-coupled solute uptake, prokaryotic motility and oxidative phosphorylation.

Krulwich and co-workers (1998a & b; 2001), using the facultatively alkaliphilic *Bacillus pseudofirmus* strain OF4 grown in continuous culture containing malate at pH 7.5 or 10.5 as a

model, shed light on the mechanisms of alkaliphily. The research focused on the elucidation of characteristics and energetics of membrane-associated proteins that must catalyze inward proton movements. One of the two proteins found was the Na^+/H^+ antiporter system on the plasma membranes which plays a pivotal role in the maintenance of pH homeostasis in *B. pseudofirmus* strain OF4. The system enables cells to adapt to a sudden upward shift in pH and to maintain a cytoplasmic pH that is 2- 2.3 units below the external pH in the most alkaline range of pH for growth. The efficiency of attaining this pH balance in turn sets the threshold for optimum growth. On one hand the alkaliphile sodium cycle consists of Na^+/H^+ antiporters which achieve a net proton accumulation that is coupled to sodium ion efflux. Three genes with putative roles in this system were identified. The *Mrp (Sha)* - encoded antiporter which may function as a complex is the major antiporter for pH homoestasis. The *NhaC* is the other of the two supporting antiporters identified. The additional antiporters may be essential to maintain pH homeostasis at low sodium ion concentration or at near neutral pH. On the other hand, the sodium cycle facilitates sodium ion re-entry via $Na^{(+)}$/solute symporters which are also the ion channels responsible for sodium ion dependent flagellar motor. The genes identified by Krulwich and colleagues have homologues in *Bacillus halodurans* C-125, an extreme alkaliphile (Horikoshi, 1999; Takami *et al.,* 1999).

The second protein identified is the ATP synthase, a proton-translocating enzyme which catalyzes the production of ATP under conditions in which the external proton concentration and the bulk chemiosmotic driving force are low (Ivey *et al*., 1998; Krulwich *et al.,* 1998b; Antranikian, 2001). Alkaliphilic *Bacillus* species, according to the report of Gomes and Steiner (2004), conduct oxidative phosphorylation via an exclusively proton coupled ATP synthase unlike the sodium-dependent ATP synthases found in non-alkaliphilic species. ATP generation is in this alkaliphile is reported to be more rapid and reaches a higher phosphorylation potential at highly alkaline pH than neutral pH.

Insights into energy metabolism of halophiles have been inferred from the genome sequence of *Thioalkalivibrio sulfidophilus* strain HL-EbGr7 (Muyzer *et al.,* 2011). No sodium pump driven synthase was detected but a proton-driven F_1 F_0-type ATP synthase was found. In addition the 7 genes (*mnhA-G*) for the multisubunit Na^+/H^+-antiporter *Mrp* were detected. This assembly is

thought to play a role in the regulation of internal pH. Genes encoding proton-driven flagellar motors *(motA/B)*, and sodium-driven flagellar motors *(pomA/B)* were also found. Phylogenetically, *motA/B* and *pomA/B* sequences grouped with sequences of other members of the family *Ectothiorhodospiraceae* such as *Halorhodospira halophila* and *Alkalilimnicola ehrlichii*. Cardiolipin synthase and squalene synthase genes previously identified in the cell membranes of *Thioalkalivibrio sulfidophilus* strain ALJ15 were also discovered in *T. sulfidophilus* strain HL-EbGr7 (Muyzer *et al.,* 2011). These compounds contribute indirectly to an efficient energy metabolism as the negatively charged cardiolipids trap protons at the cell membrane preventing them from diffusing into the environment. Additionally, squalene lowers the proton permeability of the lipid bilayer.

The studies of Gonzalez *et al.* (2009) using the extremely halophilic archaea- *Halobacterium salinarum*, revealed that usable energy is generated by an interplay of the bioenergetic mechanisms of amino acids fermentation (an alternate energy source in most cases) and respiration or photosynthesis simultaneously. It was reported that the fermentation of arginine contributes energy in levels comparable to the primary bioenergetic modes which should generate surplus energy on their own. In addition, although considerable amount of nutrients was found to be consumed, this nutrient was neither incorporated in to *H. salinarum's* biomass nor was it used as respiratory substrates and also with extremely low total carbon incorporation under anaerobic conditions. This observation is strongly believed to be an adaptation of the organism to its otherwise lethal environmental condition in which nutrient availability is often erratic for protracted periods.

1.5 Microbes of interest to this study.

Two groups of prokaryotic microorganisms were targeted in this study - actinobacteria and the *Archaea.* These groups were chosen because of the pharmacologic importance and biocatalytic potential of the former (Garrity and Holt, 2001b; Gao and Gupta, 2005; and Nikaīdo, 2007; Kikani, *et al.,* 2010) and the reputation of the latter group of being the dominant life forms in most extreme ecosystems (Oren, 2004; Oren, 2010; Brochier-Armanet *et al.,* 2011)

Prokaryotes representing different taxa and requiring diverse nutrient and growth conditions have been identified from isolation and sequence based studies in haloalkaliphilic environments (Jones *et al.*, 1998; Horikoshi, 1999; Sorokin *et al.*, 2008). Isolates obtained are reported to have varied morphologies and reactions to Gram stain. Horikoshi (2011) reports that hypersaline soda lakes are particularly populated by alkaliphilic representatives of halophilic archaea.

1.5.1 Class *Actinobacteria*

Within the bacterial kingdom, the class *Actinobacteria* represent an ubiquitous but distinctive phylum of diverse microorganisms with respect to their morphology, physiology, relationship to oxygen (Stackebrandt *et al.*, 1997; Stach *et al.*, 2003) and genomic composition (Garrity and Holt, 2001b; Gao and Gupta, 2005).

Actinobacteria represent one of the largest taxonomic units among the 18 major lineages recognized currently within the domain *Bacteria*. The actinobacteria are Gram positive prokaryotes with the base composition of their DNA containing above 50 mol% G + C (Gao and Gupta, 2005; Goodfellow and Fiedler, 2010). Regardless of the seemingly low sequence diversity within this class as inferred from genomic studies compared to most other phyla (Garrity and Holt, 2001b), these microorganisms form a distinct phyletic line in the 16S rRNA tree (Glazer and Nikaidō, 2007; Ventura *et al.*, 2007). The phylogenetic tree of class *Actinobacteria* at the suprageneric level based on 16S rRNA gene sequence shows thirty-five families belonging to six orders, Figure 1.7.

Actinobacteria are aerobic microorgansims with specialized respiratory metabolisms (Garrity and Holt, 2001b). They are the predominant life forms in soil where they play major roles in decomposition and humus formation (Mehrotra and Sumbali, 2009).

The phenotypes peculiar to members within the phylum are of two broad forms: the unicellular, non-sporulating and the filamentous sporulating sporoactinomycete (Mehrotra and Sumbali, 2009). The unicellular non sporulating species may be spherical (e.g. *Micrococcus*) or rod shaped (e.g. members of the *Arthrobacter* and *Cellulomonas* genera). The filamentous forms are rods with either rudimentarily fragmented hyphae (e.g. *Nocardia*) or permanent and highly

differentiated branched mycelium (e.g. *Streptomycetes, Micromonospora* and *Corynebacterium*) (Glazer and Nikaidō, 2007). The superficial radial or starlike mycelia resemble those of the fungi with which they were previously classified (Garrity and Holt, 2001b). The elaborate mycelium confers a higher surface to volume area which is advantageous in the highly competitive soil biosphere. Spore forms of the actinomycetes range from motile zoospores to specialized propagules which are resistant to desiccation and mild heat but lack the complexity and resistance of bacterial endospores (Goodfellow and Williams, 1983).

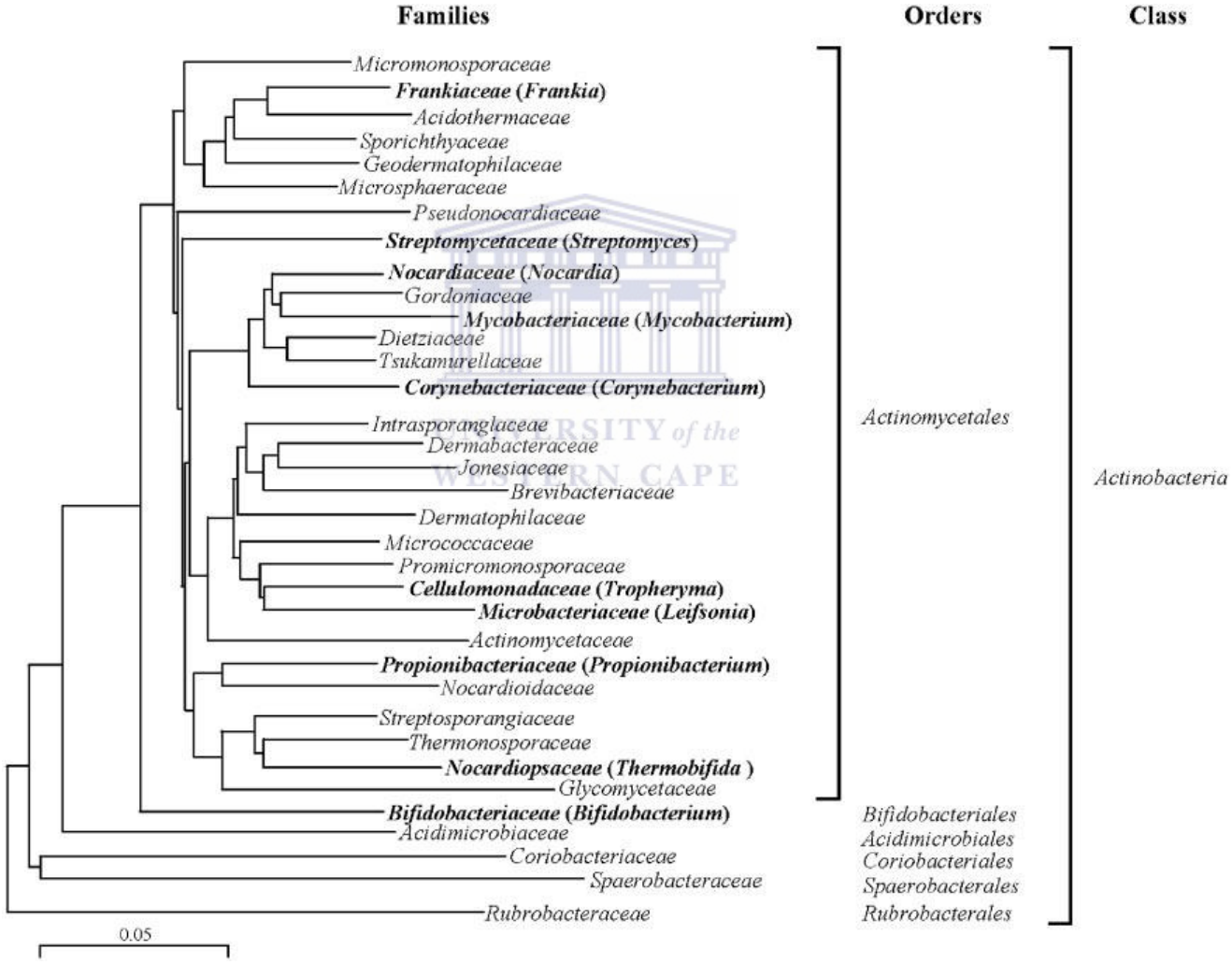

Figure 1.7: Phylogenetic tree of class *Actinobacteria* at the suprageneric level as determined by 1,500 nucleotides of 16S rRNA gene. Modified from Ventura *et al.* 2007; Zhi *et al.*, 2009.

The metabolic diversity of actinobacteria is evident from the variety of enzymes and or metabolites produced many of which are of considerable biotechnological and economic importance (Dworkin, 2006a). Members of the order *Actinomycetales*, notably the genus *Streptomyces* are antibiotic producers. This order accounts for 80% of the 20% total output of bioactive metabolites obtained from natural resources (Bentley *et al*., 2002; Babalola *et al.*, 2009). Some members of the actinobacteria are also pathogens of plants, man and other animals e.g. Mycobacteria (*Mycobacterium tuberculosis* and *M. leprae*) and *Corynebacterium diphtheria (Diphtheriae).*

Representative of the actinobacterium phylum identified in hypersaline alkaline habitats include the thermophilic actinomycetes, *Streptomyces rimosus* R6-554W (Abrami *et al.,* 1999) and *Thermoactinomyces sp* HS682 (Tsuchiya *et al.,* 1991). The biotechnological potential of some of these haloalkaliphiles have also been elucidated (Kikani *et al.,* 2010; Horikoshi, 2011).

1.5.2 Domain *Archaea*

This is an ancient group of prokaryotes. In 1977, Carl Woese and George Fox identified the archaea as a distinct kingdom in the classification of living organisms (Oren, 2010). Archaea are the dominant species in extreme environments but have also been found in mesophilic habitats (Oren, 2010) and even in the gut of mammals (Garrity and Holt, 2001a).

It is suggested that archaea have a great influence on the global energy cycle since they represent a considerable fraction of the terrestrial and marine ecosystems (Oren, 2004; Schleper, 2005).

Although archaea are microscopic unicellular organisms found everywhere in nature they are genetically and metabolically different from bacteria and all other forms of life, sharing some molecular traits with both the prokaryotic and the eukaryotic worlds (Gribaldo and Brochier-Armanet, 2006).

Members of this uniquely diverse group of microorganisms have a single circular DNA molecule which is associated with unique binding proteins (often not histones) similar to those found in higher organisms and possess ribosomes which are sensitive to diphtheria toxin, an eukaryotic characteristic (Dworkin, 2006b). They also lack membrane bound organelles (no nucleus, chloroplasts or mitochondria) within their cells like prokaryotes (Dworkin, 2006a). The

membranes lipids of archaea are not ester-linked to glycerol as found in organisms of other kingdoms but are ether-linked. Some archaea are capable of nitrogen fixation and nitrification like some bacteria, but only one group has been reported to be capable of denitrification – members of the order *Halobacteriales* of the euryarchaeote phylum: e.g. *Haloarcula marismortui* (Prakash, 2012). Translation and elongation factors in archaeal species are eukaryotic-like and their transcription proceeds via TATA-binding proteins (Woese *et al.*, 1990; Brochier- Armanet *et al.*, 2011). Unlike bacteria, archaea are insensitive to antibiotics such as chloramphenicol, kanamycin and streptomycin (Speer and Waggoner, 2001; Gribaldo and Brochier-Armanet 2006). Table 1.6 summarises other characteristic of the archaea.

The taxonomic groups recognised within the domain archaea based on phyletic lines in the 16S rRNA tree are the *Euryarchaeota* and *Crenarchaeota* (Woese *et al.*, 1990). A third smaller group, the *Korarcheota* (Barns *et al*., 1996) was identified on the basis of PCR and 16S rRNA genes analysis (Dawson *et al.*, 2006). Members of the group have only recently been cultivated (Schleper, 2005). The *Nanoarchaeaota* and *Thaumarchaeota* group have also been established as the fourth and fifth archaeal divisions (Brochier *et al.*, 2011).

Table 1.6: Characteristics of Archaea

Cell structure	Relatively small, mostly between 0.1-15µ diameter. Some form filaments or colonies of up to 200µm long. Various shapes: lobed, spiral, spherical, rod-shaped or rectangular. They lack murein in their cell wall**.**
Growth	Most have a doubling time of 20minutes after cell division.
Reproduction	Budding, fragmentation, binary and multiple fission.
Movement	Motile forms have multiple flagella for movement.
Response to stimulus	Phototaxis, chemotaxis, thermotaxis and sensitivity to other organisms.
Energy mechanisms	No photosynthesis with electron transport chain. Some e.g. halobacteria use a light mediated mechanism for ATP generation.
Homeostasis	Heat shock proteins, chaperonins for protein refolding and reactivity, di-bi-phytanyl di-ether lipid cell membrane which confer heat resistance..
Nutrition	Phototrophic, chemoautotrophic and their heterotrophic variants

References: Schepler *et al*., 2005; Gribaldo and Brochier-Armanet, 2006; Jurgens *et al*., 2000; Oren, 2010.

The phylum *Euryarchaeota* includes members of the class *Halobacteriales, Thermoplasmales, Methanomicrobia, Halobacteriales, Thermococcales* and *Archaeoglobales,* while the phylum *Crenarchaeota* includes members of the class *Thermoproteales, Igneococcales, Acidilobales* and *Sulfolohale* (Ciccarelli *et al.*, 2006; Prakash, 2012).

Korarchaeote, Nanoarchaeote and Thaumarchaeote groups each contain single species (Satyanarayana *et al.,* 2012). Figure 1.8 shows the evolutionary relationship between in the domain Archaea as deduced from 16S rRNA gene sequence. The Thaumarchaeote group is not depicted in the figure.

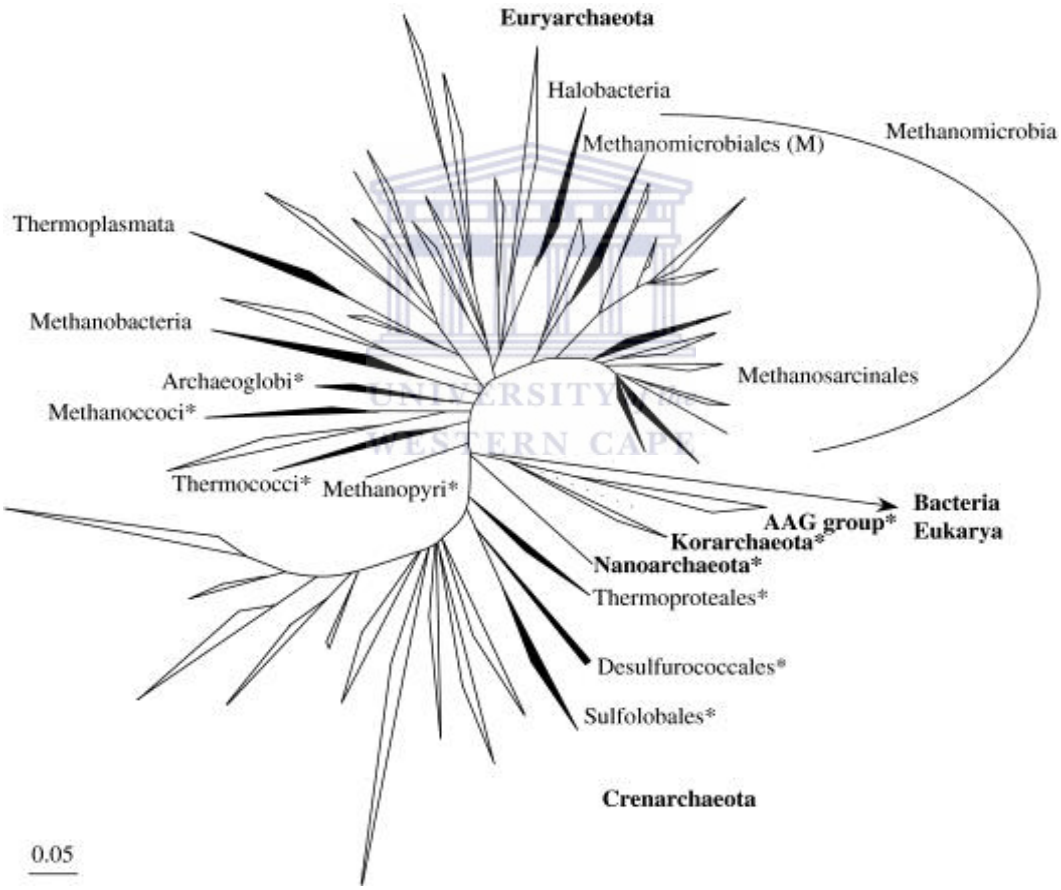

Figure 1.8: Domain Archaeota based on small 16S rRNA subunit. Reference: Schleper *et al.,* 2005.

1.6 Justification

Many microbes have been isolated and cultured from various ecosystems on earth but the identities of many remain to be revealed. Moreso, various regions of our diverse planet have yet to be explored for natural products (Bérdy, 2005).

In order to retrieve metabolites from the microbial world suitable for biotechnological applications, it is imperative to understand the diversity of microorganisms, both in terms of richness and distribution as a function of their ecology. This is also true for the communities inhabiting extreme environments. Advanced molecular techniques can be employed to increase the likelihood of recovery of recalcitrant components of environmental samples bypassing the limitations imposed by microbial culturing approaches (Stach *et al.,* 2003). The unique adaptations of extremophilic microorganisms which enable them to survive in the harsh habitats have made extreme environments the prime target in the hunt for biocatalysts, biotherapeutics and biomimetics (Canganella and Wiegel, 2011).

The extreme habitat, Lake Magadi, characterized by halophilic, alkaliphilic and thermophilic gradients has been extensively studied in terms of limnology, geology and ecology (Jones *et al.,* 1998; Ibs-von Seht *et al.,* 2001; Warren, 2006a) yet a sound metagenome based study is lacking. Moreover, cloning, sequencing and gene expression studies of haloalkaliphilic archaea and actinomycetes are quite limited (Singh *et al.,* 2012). This project therefore employs culture dependent and culture independent molecular methods, using the 16S rRNA gene as a phylogenetic marker to explore the actinobacterial and archaeal diversity of Lake Magadi, with the following objectives:

- To determine the community structure of the actinobacterial and archaeal species within Lake Magadi
- To identify novel microorganisms (culturable and uncultured) within the class actinobacteria and domain archaea as possible sources of extremozymes
- To increase the knowledge base on haloalkaliphilic or haloalkalithermophilic microorganisms in Lake Magadi as a framework for future study.

CHAPTER 2 MATERIALS AND METHODS

This chapter details the experimental approaches employed towards achieving the aims of the study of Lake Magadi, Kenya. It presents information on the methods, instruments, media and other types of reagents used in this study.

2.1 Environmental sampling

From two points within Lake Magadi, Kenya, East Africa, identified as Lake Magadi station 2 (LM2) and Lake Magadi Salt pan 4 (LMS4) sediments and moist soil samples respectively were collected for analysis. The geographical position of the sample sites were determined using standard methods (global positioning systems –GPS). The location of sites LM2 and LMS4 are shown in Figure 2.1.

Samples were collected using sterile scoops and stored in sterile 300 ml Nalgene® bottles which were capped, labelled and preserved on dry ice and later at 4°C for transport to the University of the Western Cape, South Africa.

2.2 Determination of physico-chemical parameters

2.2.1 Field measurements

The pH, electrical conductivity (EC), temperature and total dissolved solids (TDS) of stations LM2 and LMS4 were determined *in situ*.

A Jenway 3520 probe was used to determine the pH, while the EC, TDS and temperature were determined using an EcoScan Temp JKT thermocouple thermometer (Thermo Scientific Inc., Singapore).

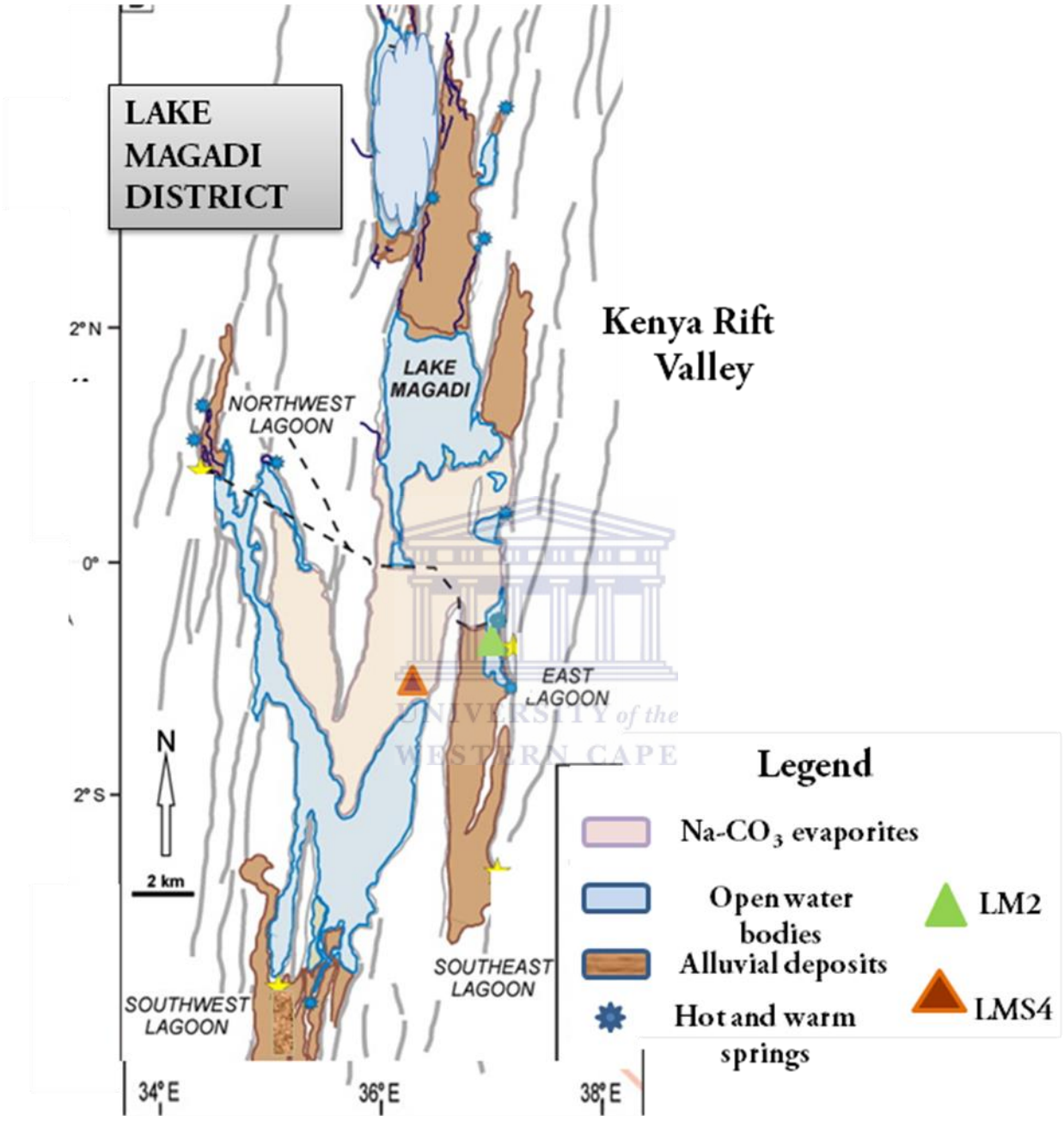

Figure 2.1 Map of the Lake Magadi district showing the geographical positions of sites LM2 and LMS4. Reference: Modified from Scott *et al.*, 2012.

The salinity index total dissolved solids (TDS) and electrical conductivity (EC) are similar parameters which can often be correlated. TDS readings represent the contribution of both

inorganic (minerals, dissolved ions, salts, metals and other chemicals) and organic matter. TDS is measured gravimetrically and values represent the contribution of four major cations (calcium (Ca^{2+}), magnesium, (Mg^{2+}), sodium (Na^{+}) and potassium (K^{+})) and three anions (bicarbonates (HCO^{3-}/CO_3^{2-}), chlorides (Cl^{-}) and sulfates (SO_4^{2-})). TDS is expressed in units of mgl^{-1} or parts per million.

EC is a measure of the ability of water to conduct an electric current and its value is directly proportional to the concentration of the ions in solution (higher concentrations equates to a higher EC), the temperature of the solution (higher temperature equates to higher EC) and the specific nature of the ions (higher specific ability and higher valence results to higher EC). EC estimates the concentration of salts relative to the rate of ionic dissociation.

2.2.2 Laboratory determinations

Station LM2 and LMS4 samples were sent to BEMLAB, an analytical laboratory located in Somerset West, South Africa for physico-chemical analysis. The following analytical methods were used to analyse the chemical composition of the samples.

i) The cation exchange capacity (CEC) method, which is an index of acidity of the sample, was used to determine ionic composition. The CEC method is a standard reference method which uses 1N ammonium acetate at neutral pH (Sumner and Miller, 1996). The values obtained for the exchangeable cations (Ca^{+2}, K^{+1} and Na^{+1}) per kilogram sample are interpreted such that the higher the value of exchangeable base cations, the higher the extent of neutralization of acidic cations (H^{+} and Al^{+3}). The test is therefore an indication of the pH of the sample.

ii) The Walkley Black method estimates the total organic matter from the dissolved carbon dioxide and carbonates present in a sample (Walkley, 1947).

2.3 Molecular Analysis

The focus of this study is to investigate the diverse microbial population in the sediment and soil samples from Lake Magadi. Methods that would best reveal information about community composition were attempted.

Community DNA was extracted from sediment and soil obtained from stations LM2 and LMS4 respectively using the method for total environmental DNA extraction described by Wang *et al*. (1996). This method is suitable for genomic DNA recovery from both Gram positive and Gram negative microorganisms.

2.3.1 Metagenomic DNA extraction

In duplicate, 100 mg of lakes sediments were suspended in an equal volume of lysozyme buffer. Samples were duplicated to increase the quantity of nucleic acid recovered. RNase A in a final concentration of 50 μgml^{-1} was added to the lysis solution to eliminate the co-extraction of RNA. The solution was incubated at 37ºC overnight to facilitate efficient lysis. Proteinase K was added to a final concentration of 1 $mgml^{-1}$ and the solution was incubated at 37ºC for 1 hour. Sodium deodecyl sulphate was added in a final concentration of 1% followed by incubation of the samples at 65ºC for 30 minutes. An equal volume of phenol was added and mixed by inversion. The aqueous phase was removed and transferred to a new tube. The phenol extraction was repeated until protein and cell debris was visibly absent. An equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added to the tube to remove any trace of phenol and the aqueous phase was transferred to a new tube. One volume of isopropanol was added and the solution was gently inverted. DNA pellets were collected by centrifugation at 10 000 rpm for 10 minutes and the supernant was discarded. An equal volume of 70% ethanol was used to wash the pellets (twice) prior to air drying for 10 minutes in a laminar flow cabinet. The DNA was suspended in 100 µl 1X TE buffer and stored at 4ºC.

2.3.2 Determination of DNA concentration

The concentration and purity of the genomic DNA, 16S rRNA gene amplicons and plasmid DNA preparations was determined spectrophotometrically using a Nanodrop ND-1000-v3.3 spectrophotometer (Nanodrop Technologies Inc., Delaware). This instrument evaluates nucleic acid quality using the absorbance ratios A_{260}/A_{280} and A_{230}/A_{260} for DNA and RNA respectively. The A_{260}/A_{280} (nm) ratios are ideal at 1.8-2.0 while the A_{230}/A_{260} (nm) values should be between 0.3-0.9 (Marmur, 1961).

2.3.3 Amplification of target sequences

2.3.3.1 Polymerase chain reaction (PCR)

Template DNA was incubated with a thermostable DNA polymerase, dNTPs and a set of primers to initiate the synthesis of complementary strands of DNA. Amplifications were carried out in a MultiGene thermocycler (Labnet International Inc.) or an Eppendorf Gradient Mastercycler (Eppendorf International). The standard protocols used for PCR in this study are shown in Table 2.1.

Genomic DNA templates were serially diluted (10^{-1}, 10^{-2}, 10^{-3}) prior to amplification of 16S rRNA genes as initial amplification attempt using undiluted DNA was unsuccessful. The 10^{-1} dilution template was most suitable for amplification. Initial attempts to use polyvinyl polypyrollidone (PVPP) to clean up the genomic DNA resulted in extensive DNA degradation (Figure 4.2 A). Positive and negative controls were included in all amplification reactions. The negative controls contained all PCR reagents but no DNA template. Genomic DNA from the following sources was used as templates in positive controls reactions for the different primer combinations used:

E coli strain DSM 30083^T genomic DNA for all universal bacterial specific amplification reactions, genomic DNA of *Sulfolubus acidocaldarius* strain DSM 639 for all archaeal PCR reactions and that of *Dietzia sp* BS12 (unpublished) for all actinobacterial specific amplifications.

Table 2.1 Standard protocol for PCR in this study

Compound	**20 µl reaction volumes (µl)**	**Final concentration.**
Sterile dH_2O	9	
DreamTaq buffer or 10 X PCR Buffer for LabTaq	2	1X
dNTPs (2 mM)	2	0.2 mM
Forward primer (100 µM)	2	5 µM
Reverse primer (100 µM)	2	5 µM
*DNA Polymerase (DreamTaq™ or Labtaq) (5U/µl)	1	
Template DNA (25 ng)	1	1.25ng
BSA (1mg/ml)	0.2	1%
Glycerol (50%)	0.8	2%

The polymerases used in this study were DreamTaq™ and Labtaq. DreamTaq™ (Fermentas International) was used for all routine PCR amplifications and for the generation of amplicons with 3'-dA overhangs. LabTaq [a recombinant enzyme prepared by members of the Institute for Microbial Biotechnology and Metagenomics (IMBM), University of the Western Cape, South Africa], was used for all colony PCR amplifications including amplification of 16S rRNA genes from isolates.

2.3.3.2 Amplification of phylogenetic marker genes

The 16S rRNA genes of actinobacteria and archaea from samples LM2 and LMS4 were PCR amplified using degenerate primers. Actinobacterial and archaeal amplicons were obtained by two rounds of (nested PCR) targeted amplification of community DNA. Table 2.2 summarises data on the primers and cycling conditions used in this study. All primers were supplied by Inqaba Biotechnical Industries, Pretoria, South Africa.

Table 2.2 Primer combinations, specificity of primer sets and PCR cycling parameters used in this study

Primer Set	Sequence (5' to 3')	Target (l6S rRNA gene)	Cycling conditions	Reference
E9F U1510R	GAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	Universal for bacteria	94°C/4min 30X(94°C/30 s-52°C/30 s-72°C/105 s) 72°C/10 min	Hansen *et al.*, 1998
S-C-Act 235-a-S-20 S-C-Act-878-a-A-19	CGCGGCCTATCAGCTTGTTG CCGTACTCCCCAGGCGGGG	Universal for Actinobacteria	95°C/4 min 10x (95°C/45 s-72°C/45 s (-0.5°C) 72°C/60 s) 15x (95°/45 s-68°C/45 s-72°C/60 s) 72°C/5 min	Stach *et al.*, 2003
A341F A534R	*CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	Most bacteria DGGE primer	94°C/4 min 20x (94°C/45 s-65°C/45 s-72°C/60 s) 20x (94°/30s-55°C/30 s-72°C/60 s) 72°C/10 min	Muyzer *et al.*, 1993
A340F A533R	*CTACGGGGYGCASCAG TTACCGCGGKGCTG	Universal for Archaea	94°C/4 min 30x (94°C/30 s-53.5°C/30 s-72°C/60 s) 72°C/10 min	Øvreas *et al.*, 1997
A3Fa Ab927R	TCCGGTTGATCCYGCCGG CCCGCCAATTCCTTTAAGTTTC	Archaea except nanoarchaea	94°C/4min 20x(94°C/45s-55°C/45s -72°C/75 s) 10x (94°C/30 s-55/30 s-72°C/75 s 72°C/10 min	Jurgen *et al.*, 1997; 2002
M13Fw M13Rev	CCGTACTCCCCAGGCGGGG AGCGGATAACAATTTCACACAGG	Vector Primers (pTZ57R)	94°C/4 min 35x (94°C/30 s-64°C/30 s-72°C/[var]) 72°C/10 min	Yanisch-Perron *et al.*, 1985

The universal 16S rRNA gene region of actinobacteria and archaea was targeted in the first round of amplification using primer sets S-C-Act 235-a-S-20/S-C-Act-878-a-A-19 and A3Fa /Ab927R respectively. This was followed by amplification using the primer set A341F /A534R and A340F /A533R for actinobacteria and archaea respectively. For resolution by DGGE, a GC-rich sequence (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCCC) (the (GC-clamp) was attached to the 3' end (* in Table 2.2) of the forward primer for the second round of amplification. The GC-rich clamp serves to enhance the melting behaviour of the samples and their migration within the polyacrylamide gel.

2.3.4 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using standard methods (Sambrook *et al.,* 1989). Community DNA and all PCR products were resolved on 0.7% and 1.5% agarose gels respectively while ligation and all restriction digests were analyzed on 2.5% agarose gels. Reaction samples were mixed with 6X agarose loading dye (Appendix I) prior to electrophoresis.

Horizontal TAE gels were run at at 90 -100 V for 30 minutes in 1X TAE buffer (40 mM Tris-HCl, 10 mM glacial acetic acid, 1 mM EDTA, pH 8.0). The gels were stained with 50 μgml^{-1} ethidium bromide in 0.5X TAE buffer. The DNA profiles were visualized and recorded using an Alphaimager 2000 (Alpha Inotech, San Leandro, CA) digital imaging system. DNA fragments sizes were estimated relative to that of the band sizes of the DNA molecular weight markers: λ *Pst*1 or GeneRuler^{TM} 100 bp or GeneRuler low range marker (Fermentas International).

2.3.5 Denaturing gradient gel electrophoresis (DGGE)

In order to characterise the actinobacterial and archaeal communities of stations LM2 and LMS4 16S rRNA gene sequences of actinobacteria and archaea obtained from the gDNA of the sample (Section 2.2.3) were analyzed using DGGE. The procedure was performed using the DCode™ Universal Mutation Detection System (Bio-Rad, Hercules, USA) as described by Muyzer *et al.* (1993) with some modifications.

The PCR products amplified with primers containing a GC clamp were separated on 9% (w/v) polyacrylamide gels containing a chemical gradient. Denaturing gels were poured using a gradient former (Bio-Rad, Hercules, USA) containing "high" (maximum denaturant) and "low" (minimum denaturant) gels solutions. "High" and "low'' denaturant solutions were prepared by mixing 0% and 100% denaturant stock solutions to give the required denaturant concentrations. The 0% solution contained 40% acrylamide: N, N' bis-acrylamide (37.5: 1) and 1X TAE while the 100% solution included the addition of 7 M urea and 40% (v/v) deionised formamide as the denaturants. APS (0.5% w/v) and TEMED (0.02% w/v) were added to acrylamide: bis-acrylamide solutions to catalyze gel polymerization prior to gradient formation.

DGGE plates were thoroughly cleaned with methanol, then twice with ethanol to remove all traces of grease from previous use. Urea formamide gel denaturing gradients were formed using the Bio-Rad gradient former. Using a 70- 40% urea-formamide gradient, amplicons of the second round amplification were resolved on 16.5 mm x 16.5 mm x 1 mm thick 9% (w/v) polyacrylamide gels at 100 V and 60^{o}C for 16 hrs in 1X TAE buffer. After electrophoresis the gels were stained in 1X TAE containing 0.5 $\mu g ml^{-1}$ ethidium bromide for 15 minutes and destained in 1X TAE buffer for 30 minutes. The gel was visualized and its image captured using an Alphaimager 3400 Imaging System UV transilluminator (Alphatech Corporation™, San Leandro, CA).

A non-commercial molecular marker constituted by members of the Institute for Microbial Biotechnology and Metagenomics (IMBM) using DNA (of identical length), sourced from organisms of mesophilic and extremophilic environment was used as a standard in the reaction. The different GC –pairing content of the constituent rDNA in the molecular marker are unknown.

The digitised gel image was stored as tagged image file format (TIFF) and analysed using the GelCompar® II software (Applied Maths NV, Belgium). The presence or absence of bands in the samples was recorded as a matrix which was used to generate similarity indices between similar taxa from two study sites. The similarity matrix was interpreted into dendograms and multi-dimensional scaling plots using the Dice co-efficient and UPGMA (Unweighted Pair-Group Method with Arithmetic Mean) methods in order to establish the differences in actinobacterial and archaeal communities within the study sites.

2.3.6 Clone library construction

2.3.6.1 Cloning of phylogenetic marker genes

Amplicons (10 µl) specific for actinobacterial and archaeal sequences were separated on 1.5% agarose gels to verify the purified amplicons were of the correct size (about 640 -900 bp for actinobacterial and 200 bp for archaeal sequences). The purified amplicons were quanitified using a Nanodrop ND-1000 v3.3 spectrophotometer (Section 2.5).

Amplicons were directly cloned into the pGEM –T-Easy vector (Promega, Madison, Wisconsin). The pGEM –T-Easy vector is suitably serves the objective of this research which is to generate clones with no requirement for expression. Ligation reactions (10 µl) were prepared as per the manufacturers' instructions and incubated at 16ºC overnight to maximize ligation efficiency. Positive control reactions containing insert DNA (supplied with the vector system) and negative controls (no insert DNA) were included in the ligation procedures.

The actinobacterial and archaeal specific amplicons (Section 2.3.3.3) were purified using the Illustra™ GFX™ PCR DNA and gel band purification kit (GE- Healthcare, UK) according to the manufacturers' instructions.

2.3.6.2 Drop dialysis

In order to increase the efficiency of transformation the standard ligation reaction mixes were drop dialyzed using 0.025 µm cellulose filter papers floating on sterile distilled water at 4ºC for one hour (Phillips and Signs, 2005) prior to electroporation.

2.3.6.3 Media

2.3.6.3.1 Luria-Bertani (LB) broth and agar (Luria et al., 1960; Sambrook et al., 1989)

LB [tryptone 10 g, yeast extract 5 g, NaCl 10 g, agar (for LB agar) 15 g and sterile distilled H_2O, 950 ml] was used for the cultivation and sustenance of the host organism [*Escherichia coli (E.*

coli GeneHogs®)]. The pH of the medium was adjusted to 7.0 with 1 M NaOH and made up to 1 litre with deionised water. The medium was autoclaved for 20 minutes at 15 psi and allowed to cool to 55°C before antibiotics or induction agents were added.

2.3.6.3.2 Super optimal broth (SOB) (Hananas, 1983)

This is a variant of LB medium with additional salts (2.5 ml 1 M KCl 10 ml 1 M $MgCl_2$ 10 ml 1 M $MgSO_4$ per litre). The pH was adjusted to 7.0 with 1 M NaOH and the volume made up to 1L with distilled water. The medium was used to grow overnight culture of *E.coli* used for making competent cells.

2.3.6.3.3 Super Optimal Broth with catabolite repressor (SOC) (Hananas, 1983)

Filter sterilized 1 M glucose (20 ml) and 1 M $MgCl_2$ (5 ml) were added to SOB (1 L). Equal volumes of SOC were added to transformation mixes to allow cells to recover after electroporation.

2.3.6.3 4 Selective markers/ Induction agents

Ampicillin in a final concentration of 100 $mgml^{-1}$ was used as a selectable marker for recombinants. Isopropyl- β-D -thiogalactopyranoside (IPTG) and X-gal (Fermentas lnternational) in final concentrations of 20 μgml^{-1} and 30 μgml^{-1} respectively were used as induction agents.

2.3.6.4 Generation of electrocompetent GeneHogs® cells

GeneHogs® (Invitrogen, USA) is a DH10B™-derived *E coli* strain especially designed to take up large vector fragments.

A single *E.coli* GeneHogs® colony was innoculated into 10 ml LB broth containing ampicillin (100 $mgml^{-1}$) and grown overnight at 37˚C in a shaking incubator. The fresh overnight culture was diluted 1: 200 in SOB and grown at 37˚C at 220 rpm until the $OD600_{nm}$ of 0.5 was reached. The culture was placed on ice. The cultures were harvested by centrifugation at 1000 xg (~2400 rpm) in a Beckman JA-10 rotor for 15 minutes at 4˚C. The supernatant was decanted and the cells were suspended in sterile ice cold water in a volume equal to the volume of the original

culture. The suspension was centrifuged at 1000 xg for 10 minutes at 4˚C. Half of the supernatant was decanted and the cell pellets were resuspended in an equal volume of 10% glycerol followed by centrifugation for 15 minutes at 1000 xg. The supernatant was aspirated and the pellet was resuspended in a solution containing 15% glycerol and 2% sorbitol in distilled water. The volume used was 2 ml per litre of initial culture volume. Aliquots (50 µl) of the suspension were snap frozen in liquid nitrogen and preserved at -80˚C.

2.3.6.5 Transformation of electrocompetent GeneHogs® cells

Transformation into GeneHogs® cells was achieved by electroporation using a Micropulser™ electroporation system (Bio-Rad, Hercules, Ca) as follows:

Electrocompetent cells (50 µl; Section 2.3.6.4) were thawed on ice. Aliquots of cells (30 µl) were pipetted into microcentrifuge tubes and placed on ice. The dialysed ligation mix (1 µl; Section 2.3.6.2) was added to the cells in the microcentrifuge tubes and the contents were gently mixed. The transformation mix was transferred to pre-chilled electrocuvettes with 0.1 cm gaps (Bio-Rad) and placed in electroporation chamber for delivery of electric pulse (25 µF capacitor, 600 Ω resistance at a field strength of 1.8 $kVcm^{-1}$). After electroporation, the cuvette was removed from the chamber and 1 ml aliquot of prewarmed SOC broth at 37ºC was added to the cuvette. The transformation reactions were transferred to a sterile 2.5 ml culture tube for cells to recover at 37ºC with shaking at 220 rpm for one hour. Suitable positive and negative controls (control vector and no vector) were included for each round of transformation.

2.3.6.6 Screening for positive transformants

LB agar plates containing ampicillin (100 $mgml^{-1}$) and supplemented with IPTG (20 μgml^{-1}) and X-gal (30 μgml^{-1}) was prepared. Aliquots (200 µl) of serial dilutions (10^{-1}, 10^{-2} and 10^{-3}) of the transformation reactions were plated in duplicate on the LB agar plates containing ampicillin, IPTG and X-gal. The plates were incubated overnight at 37ºC. White colonies were selected, and using sterile tooth picks, they were streaked on LB plates containing ampicillin (100 $mgml^{-1}$) and incubated at 37ºC overnight. The resulting white colonies were selected and innoculated into 50 µl 1X TE buffer for colony PCR.

2.3.6.7 Storage of clones

All clones were cultured overnight in LB broth containing ampicillin (100 $mgml^{-1}$) and archived in 40 µl LB broth containing 50% glycerol at -80ºC. The culuring and storage of the clones were done in 96-well microtitre plates.

2.3.6.8 Size screening for recombinant plasmids by M13 colony PCR

Two microlitres of clones in 1X TE buffer served as the template for colony PCR amplification using M13 forward and reverse primers and the described cycling parameters (Table 2.2). Amplification was catalyzed by LabTaq polymerase in 20 µl reaction volumes as detailed in Table 2.1. The PCR products were resolved by agarose gel electrophoresis (Section 2.3.5). All accurately sized 16S rDNA were used directly as template for ARDRA without prior purification.

2.3.6.9 Amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA is a genotype-based identification method. Actinobacterial and archaeal 16S rRNA gene amplicons that were successfully cloned and shown by M13 PCR to contain the correct insert size were subjected to ARDRA.

The restriction enzymes *Alu*I, *Hae*III and *RSa*I (Fermentas International) were selected for the digest because they recognise short nucleotide sequences (tetranucleotide cutters). The enzymes were selected after *in silico* digestion trials using the DNAMAN software application (Heyndrickx *et al.*, 1996). Numerical analysis was performed by computer simulation using GelCompar® II software (Applied Maths, NV, Belgium).

Enzyme profiles were obtained after 1 – 1.5 hours incubation at 37^0C and separation on 2.5 % agarose gels. The reaction conditions of restriction digest is as outlined in Table 2.3.

Table 2.3 Standard restriction digest protocol used in this study

Reagent	Volume (µl) per 20 µl reaction
Distilled H_2O	9
10X respective buffer	1X
Restriction endonuclease (10U/µl)	1 (10U)
PCR product	9

2.3.6.10. Plasmid purification (plasmid mini-prep)

Clones containing inserts that generated distinctly different ARDRA banding patterns were re-amplified and purified using the Zippy™ plasmid mini-prep kit (Qiagen, Hilden) according to the manufacturer's instructions.

2.3.6.11 Nucleotide sequence determination of phylogenetic marker genes

Cloned insert DNA were sequenced by the central analytical facility of the University of Stellenbosch, South Africa using the Hitachi 3730x1 DNA analyzer (Applied Biosystems). The Big Dye Terminator v3.1 system based on the Sanger method was used. M13 forward and reverse oligonucleotide primers (Table 2.2) were used to determine the nucleotides sequences of the clones.

2.3.6.12 Nucleotide sequence data analysis

DNA sequences were edited using BioEdit v7.09 software package (Hall, 1999). Homology searches were conducted via the database of the National Centre for Biotechnological Information, (NCBI) by BLAST search (http://www.ncbi.nlm.nih.gov /BLAST) (Altschul *et al.*, 1997). Sequences from this study and those of their respective closest relatives (at least one cultured and one uncultured relative for each) and then an outgroup for each case were aligned using the ClustalW (Thompson *et al.,* 1994) algorithm implicated in the molecular evolutionary genetics analysis (MEGA) software package version 5.0 (Tamura *et al.,* 2011). The aligned

sequences were screened for chimeras using Mallard program implicated in the Bioinformatic tool kit (Ashelford *et al.*, 2006).

2.3.6.13 Phylogenetic tree construction

Aligned partial sequences were used to construct a phylogenetic tree. Phylogenetic relationships were determined using the neighbor joining and maximum likelihood analyses conducted in MEGA 5.0. Evolutionary distances were computed using the maximum composite likelihood method.

2.4 Isolation of microorganisms from sites samples

2.4.1 Media

Culturing of microorganisms from sites LM2 and LMS4 was done using three selective media. The media were Medium Alkaliphilic (MA) which contains carbon sources and salts suitable (classical) for the isolation of alkaliphilic haloarchaea (Arahal *et al.*, 1996, Ochsenreiter *et al.*, 2002; Yildiz *et al.*, 2012), *Streptomyces* General Defined Medium (SGDM) to favour the growth of actinobacteria and Yeast Extract-Malt Extract (YEME) medium as the control. The compositions of media are given in Tables 2.4 A –C.

Table 2.4 A: Medium Alkaliphilic (Sato *et al.*, 1983)

Constituent	**Per litre medium**
Glucose	10 g
Peptone	5 g
Yeast	5 g
K_2HPO_4	1 g
Na_2CO_3	10 g
$MgSO_4\,7H_2O$	0.2 g

Distilled H_2O was added to make the volume up to 1L and the pH was adjusted to 8.5, 9.5 or 10.5 with $NaHCO_3$/ $NaCO_3$ buffer. The medium was autoclaved at 20 psi for 15 minutes.

Table 2.4 B *Streptomyces* General Defined Medium (SGDM)

Constituent	Per litre medium
$NaHPO_4.2H_2O$	0.17 g
KH_2PO_4	0.14 g
$MgSO_4.\ 7H_2O$	0.05 g
$FeSO_4.\ 7H_2O$	0.01 g
Agar	15 g
100mM glucose,	100 ml
50mM $(NH_4)_2SO_4$	50 ml
50mM Na-L-glutamic acid	50 ml
Cycloheximide	0.5 ml.

} Filter sterilized into sterile 100ml cylinder and added post-autoclave.

Constituents were dissolved in ~800ml distilled water, the solution was adjusted to pH 9 with $NaHCO_3$ and was autoclaved.at 15 psi (103·5 kPa) for 15minutes.

Table 2.4 (C): Yeast Extract-Malt Extract (YEME) International *Streptomyces* Project Medium 2.

Constituent	Per litre medium
Yeast extract	4.0 g
Malt extract	10.0 g
Glucose	4.0 g
H_2O to	1 litre

The pH was adjusted to pH 7, 9 or 10.5 with $NaHCO_3$. 20 g agar was added to make a solid medium. The solutions were autoclaved at 15 psi for 15-20 minutes. 0.5 ml. cycloheximide (50 $\mu g ml^{-1}$) was added after the medium had cooled to 55°C to eliminate fungal growth.

2.4.2 Growth conditions

In an attempt to provide cultivation conditions similar to those experienced at sites LM2 and LMS4, various combinations of pH and NaCl were used in this study. In addition plates were incubated at temperatures of 37ºC, 45ºC and 55ºC. Table 2.5 gives the various growth conditions that each meduim was poised at.

Table 2.5 Media and growth conditions used for culturing in this study

Medium	pH	NaCl	Temperature
MA	8.5, 9.5, 10.5	1.5%, 5%, 15%	37ºC, 45ºC, 55ºC
SGDM	9	1.5%, 5%	37ºC, 45ºC
YEME	7, 9, 10.5	1.5%, 5% 10%	37ºC, 45ºC, 55ºC

Each medium (~20ml) variant (pH and NaCl) were poured into sterile petri dishes in a laminar flow hood and made to solidify. One gram of sample from sites LM2 and LMS4 was serially diluted (10^{-3}, 10^{-4} and $10^{-5,}$ 10^{-6}) and streaked (one for each medium variant) unto the plates and made to incubate at the respective conditions as given in Table 2.5.

2.4.3 Storage of pure cultures

All pure cultures obtained were stored in 20% glycerol stocks at -20ºC and -80ºC.

2.4.4 Characterization of isolates

All isolates with distinctive colony morphologies were selected for further screening.

2.4.5 Genomic DNA extraction / colony PCR

A sterile tooth pick was used to recover a sample of selected isolate. The recovered sample was used as template for colony PCR by suspending it into the PCR mix for amplification of 16S rRNA genes. The universal bacterial primer set E9F and U1510R and described cycling parameters (Table 2.2) were used for the amplification catalyzed by LabTaq polymerase as stated in Table 2.1.

2.4.6 ARDRA

Colony PCR products from isolates were directly analyzed using ARDRA as previously described (Section 2.3.6.9).

2.4.7 Sequence data generation and analysis

Isolates selected on the basis of their unique ARDRA patterns were identified by sequence determination as described in Section 2.3.6.10 but using the universal bacteria primer set E9F and U1510R. Sequence analysis was done using the BLASTn program of the NCBI.

CHAPTER 3: RESULTS AND DISCUSSION CULTURE INDEPENDENT STUDIES

Introduction

The 'great plate count anomaly' restricts the diversity of microorganisms that can be detected using conventional culturing techniques, and results in a misrepresentation of true microbial richness and evenness (Staley and Konopka, 1985). Identification of microbial cells without cultivation is possible and it offers improved chances of detection and provides positive identification of microorganisms at different levels of taxonomy (Amann *et al.*, 1995; Hugenholtz *et al.*, 1998). Extensive microbial diversity previously undetected by culture dependent techniques have been revealed by methods which incorporate the analysis of nucleic acid representative of microbial genomes extracted from environmental sample and screening of its components based on molecular differences of target regions. As a further advantage, molecular analytical techniques offers easier and comprehensive identification of microorganisms which flourish under dual or multiple extreme environmental conditions in contrast to the laborious and time consuming culture based methods. Fingerprint analysis and DNA sequencing are such avenues for the reliable assessment of existing diversity even within extreme environments (Reisenfield, 2004; Smalla, 2004; Oren, 2006). The determination of geographical factors and physico-chemical parameters of samples give more credence to the investigations because it makes it a more comprehensive research.

Microorganisms inhabiting the Lake Magadi soda lake environment are subjected to abiotic stress. In order to unveil the presence and evolutionary structure of representatives of the phylum actinobacteria and domain archaea which prevail under the combination of hypertonic and highly alkaline conditions at Lake Magadi, samples from study site LM2 and LMS4 were subjected to non-culture based analysis (geophysico-chemical and 16S rRNA gene-based determinations).

The geographical data captured at sample sites and the environmental conditions prevailing at the sites were determined using standard methods.

A snapshot of the variation in actinobacterial and archaeal communities between the two study sites was investigated by 16S rDNA PCR- denaturing gradient gel electrophoretic analysis.

Comprehensive information on phylogenetic affiliations of targeted communities was obtained by analysis of partial 16S rDNA clone constructs.

This chapter presents the geographical and physico-chemical data of the study sites, the results of 16S rDNA primer directed actinobacterial and archaeal community profiling by DGGE and clonal libraries.

3.1 Geophysico- chemical data of study sites LM2 and LMS4

3.1.1 *In situ* data

The values recorded for environmental conditions prevailing at study sites LM2 and LMS4 are presented in Table 3.1.

Table 3.1: Sample site data

Parameter	**Station 2** (LM2)	**Magadi Salt Pan 4** (LMS4)
GPS coordinates	S01º 59'35.6 E36º 15' 36.0	S01º 53'36.4' E36º 17'89.4'
Altitude (m)	1526	1526
Total dissolved solids (TDS)	<2000 mgl^{-1}	ND*
pH	9.63	10.28
Temperature	39.4ºC	44.5ºC
Electrical conductivity	20 $mScm^{-1}$	45 $mScm^{-1}$

ND* - Not determined

The temperature and pH at site LMS4 are higher than at LM2. This finding is in agreement to previous observations that the salt pans are exposed to intense heat and often elevated pH (Jones *et al.,* 1994).

The pH values measured at both sites indicate that the sites are highly alkaline. The pH at site LM2 (9.63) although is within the pH-alkalinity–mineralization gradients within Magadi reported by Zarvazin *et al.,* (1999), the value is however lower than values (10 -11.5) reported in previous studies of the Lake Magadi (Grant and Tindall, 1986; Davies, 1997, Denson *et al.*, 2010). The value obtained for site LM2 could be due to seasonal variation which results in periodic dilution of the brine by rainwater and by the inflow from marginal springs (Renaut *et al.*, 2011). Caution needs to be exercised in data interpretation when using pH metering systems in high salinity solutions. Sorokin (2005) reports that under hypersaline alkaline conditions pH determinations with pH electrodes are prone to fluctuations due to the presence of carbonates in the solution which readily reacts with atmospheric or metabolic carbon dioxide (CO_2). pH determination under hypersaline conditions such as in Lake Magadi, is best determined based on the ionic composition of the sample (Davies, 1997; Grant, 2006), or by using a pH strip *in situ* (Kanekar *et al.,* 2008).

As TDS can only be measured in solution, this data could not be reported for site LMS4 because the site was a salt crust. The higher EC values obtained at station LMS4 over station LM2 maybe as a result of the higher environmental condition (temperature) at LMS4 since EC is strongly dependent on temperature (Hayashi, 2004).

3.1.2 Laboratory determination of physico-chemical parameters

Data obtained from chemical analysis of LM2 and LMS4 are given in Table 3.2.

Table 3.2 Soil analysis report

Sample	Stone volume (%)	Exchangeable cations (+) ($mmolkg^{-1}$)				C%	N%
		Na	K	Ca	Mg		
LM2	1	2481.6	36.0	18.7	2.7	4.4	0.5
LMS4	24	603.0	35.8	10.3	1.6	1.1	0.2

The stone volume percentage is indicative of the proportion of solid particles contained in the samples. The higher stone volume percentage in the sample from LMS4 (24%) compared to the sample from LM2 (1%) reflects the visible difference in the consistency of the samples where LM2 was a sediment- muddy water mixture and the LMS4 sample was moist grainy soil matter.

The ionic composition of the sample shows the exchangeable sodium ion (Na^+) content in the stations LM2 and LMS4 sample as 2481.6 and 603 mmol kg^{-1} respectively. These values account for 97.74% and 93.54%, respectively, of the total ionic composition and are in both cases very high in comparison to the concentrations of other ions. Magnesium and calcium ion concentrations were low in both samples The finding that the samples from stations LM2 and LMS4 are saturated with Na^+ but contain low levels of Ca^{2+} and Mg^{2+} are consistent with previous reports on the hydrochemistry of Lake Magadi (Jones *et al.,* 1998; Warren, 2006a & b; Bell, 2012). The concentration of potassium ion (K^+) (also chlorine and silicate) are reported to remain fairly constant across the gradients within Lake Magadi (Renaut *et al.,* 2011) and as observed in this study the disparity between the values for K^+ (36. 0 and 34.8) obtained is quite low and are indeed the lowest value amongst the ionic parameters analyzed. The ionic composition of LM2 samples is higher than in the LMS4 samples in all cases.

The percentage of carbon and nitrogen in samples (C% and N%) reflects the organic matter content of the samples. The higher values of 4.39% (C) and 0.45% (N) for station LM2 and 1.05% (C) and 0.15% (N) for LMS4 are likely to be due to the contribution of twigs and foliage

which characterize the sample from site LM2, whereas the lower values recorded from site LMS4 is probably a reflection of the contribution of the stones and pebbles which dominated the samples from that site.

3.2 16S rRNA gene based culture independent studies

3.2.1 Molecular weight of genomic DNA obtained

Total community DNA obtained from LM2 and LMS4 samples were verified by agarose gel electrophoresis. Figures 3.1 A and B confirm that high molecular weight DNA (>11kb) was obtained for both samples.

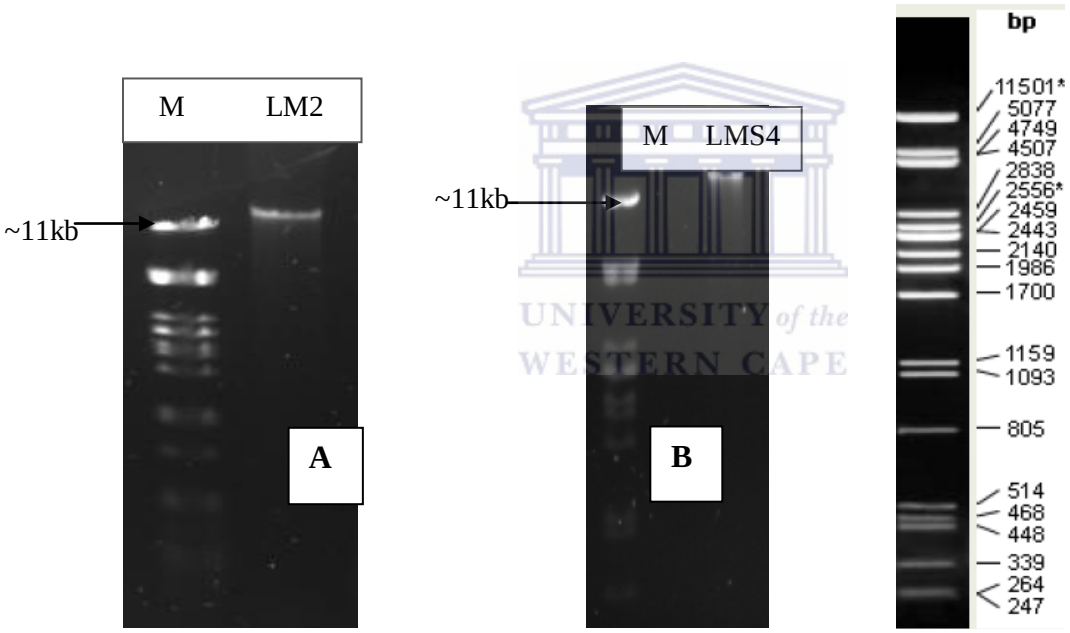

Figure 3.1 A 0.7% ethidium bromide (EtBr) stained agarose gels of genomic DNA profiles of (A) LM2 and (B) LMS4 samples, lane M (molecular ladder), λ *Pst*1 with its largest fragment of approximately 11 kb indicated.

3.2.2 Concentration of total community DNA in samples

The concentration and purity of DNA extracted from samples from sites LM2 and LMS4 as determined spectrophotometrically are presented in Table 3.3.

Table 3.3 Quality and purity values obtained for genomic DNA extracted

Sample	Concentration (ng/µl)	260/280	260/230
LM2	116.7	1.68	1.14
LMS4	79.4	1.59	1.23

The 260/280 ratios (1.68 and 1.59) are acceptable values for relatively pure DNA. The 260/230 ratios (1.14 and 1.23) are therefore low. Ideally, the 260/230 value for pure nucleic acid is often higher than respective 260/280 values (www.nanodrop.com). As this is clearly not the case, the values obatined may be indicative of the presence of contaminants (probably humic acid or salts in the environmental sample or carryover phenol from the genomic DNA extraction procedure) which absorb at 230 nm.

3.2.3 Amplification of target sequences

The profiles of universal 16S rRNA gene and those specific for the respective taxa under investigation are given in Figures 3.2 A-D.

3.2.3.1 Bacterial 16S rRNA gene profiles

The first round PCR amplification targeted at universal bacteria in the study sites samples generated the anticipated 1.5 kb sized fragments for both samples (Figure 3.2 A).

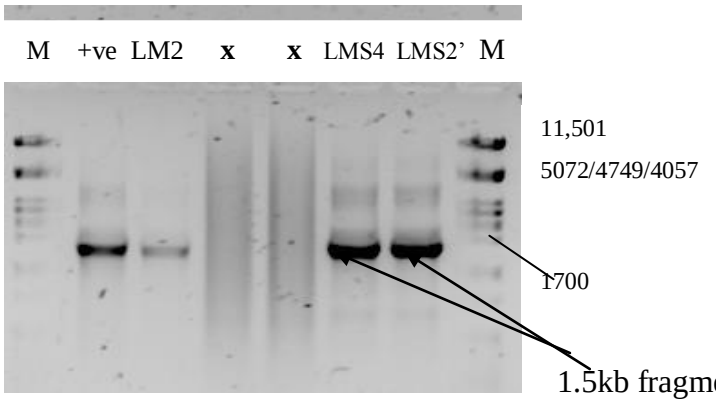

Figure 3.2 (A) 1.5% agarose gel of diluted (10^{-1}) universal bacteria 16S rRNA genes obtained from sites LM2 and LMS4 samples. The expected 1.5 kb sized 16S rRNA gene bands (from 10^{-1}

diluted DNA templates), +ve: positive control, **x**: undiluted DNA template- (Section 2.3.3.1), Lane M: molecular ladder lambda (λ) *Pst*1 are indicated.

Using the S-C-Act 235-a-S-20 and S-C-Act-878-a A-19 primer set, actinobacterial specific products were obtained (Figure 3.2B) from the universal bacterial 16S rRNA gene amplicons shown above (Figure 3.2A).

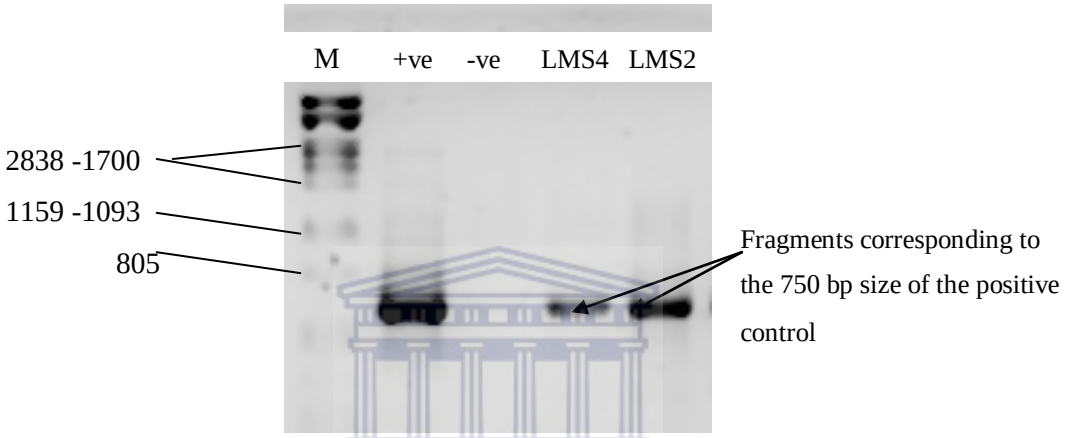

Figure 3.2 (B) 1.5% agarose gel of S-C-Act 235-a-S-20 and S-C-Act-878-a A-19 amplified actinobacterial 16S rRNA genes profile from LM2 and LMS4 samples corresponding to ~750bp fragments of the positive control (+ve):*Dietzia sp* BS12. M: molecular ladder, λ *Pst*1.

3.2.3.2. Archaeal specific 16S rRNA gene profiles

PCR amplifications were targeted firstly at universal archaeal regions (Figure 3.2 C) and then all except nanoarchaeal 16S rRNA gene regions (Figure 3.2 D) as given below.

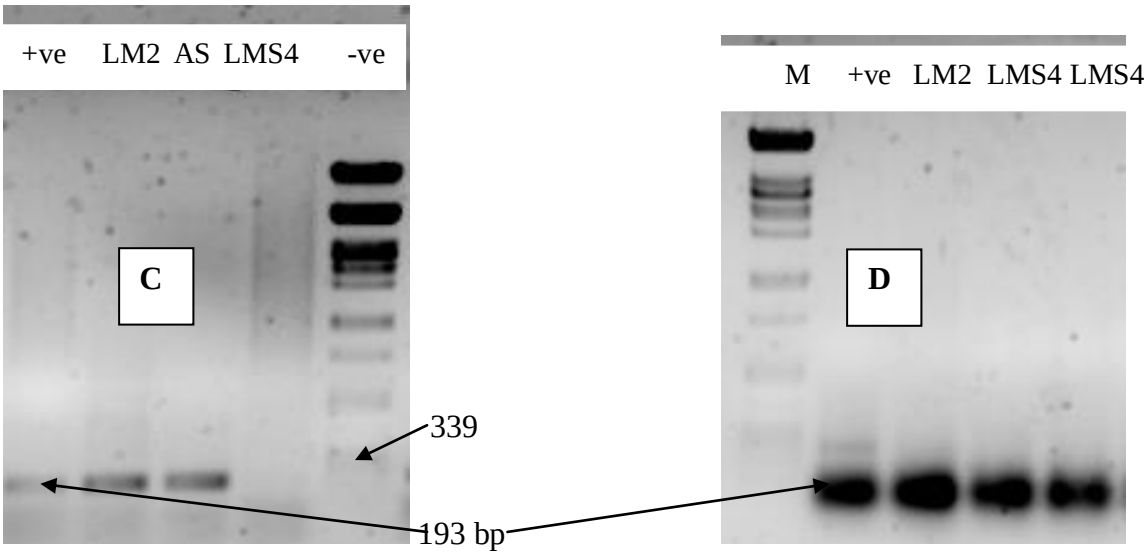

Figure 3.2 (C) and **(D)** 1.5% EtBr stained agarose gel profiles of first round (A340F/A533R) and second round (A3Fa/Ab927R) respectively amplified archaeal phylogenetic marker regions from LM2 and LMS4 samples. LMS4 samples are in duplicate in 3.2C. Lane M: λ *Pst*1, +ve positive control.

The anticipated products sizes (~200bp) obtained are shown relative to the size of the positive control. The sizes of the fragments obtained in both amplifications are very similar but the PCR-bands of the second amplification (obtained using primer set A340F-GC and A533R) are bolder indicating probably that the archaeal population represents a small component within the site samples.

3.3 Phylogenetic characterisation of microbial communities by denaturing gradient gel electrophoresis

3.3.1. PCR amplification of DGGE fragments

Amplification of taxon specific 16S rRNA gene fragments for DGGE analysis was achieved using primers containing GC-clamp and according to the protocol described in Section 2.3. Figures 3.3 (A) and (B) gives the profiles of amplicons obtained for each taxon and study site under examination.

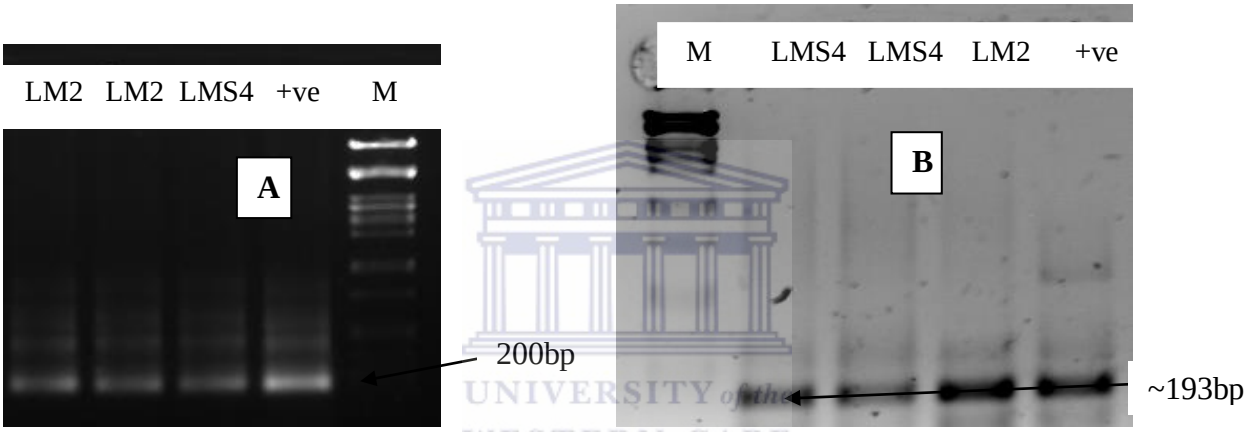

Figure 3.3 2.5% EtBr stained agarose gels of (A) Actinobacteria (A341F/A534R) and (B) Archaea (A3Fa/ Ab927R) primer set bearing a G-C pair rich sequence (GC-clamp) for DGGE profiling. Study site LMS4 sample is in duplicate. The fragment sizes obtained (200 bp and 193 bp respectively) are as indicated.

The profile of the taxon specific amplicons as resolved by denaturing gradient gel electrophoresis is presented in Figure 3.4.

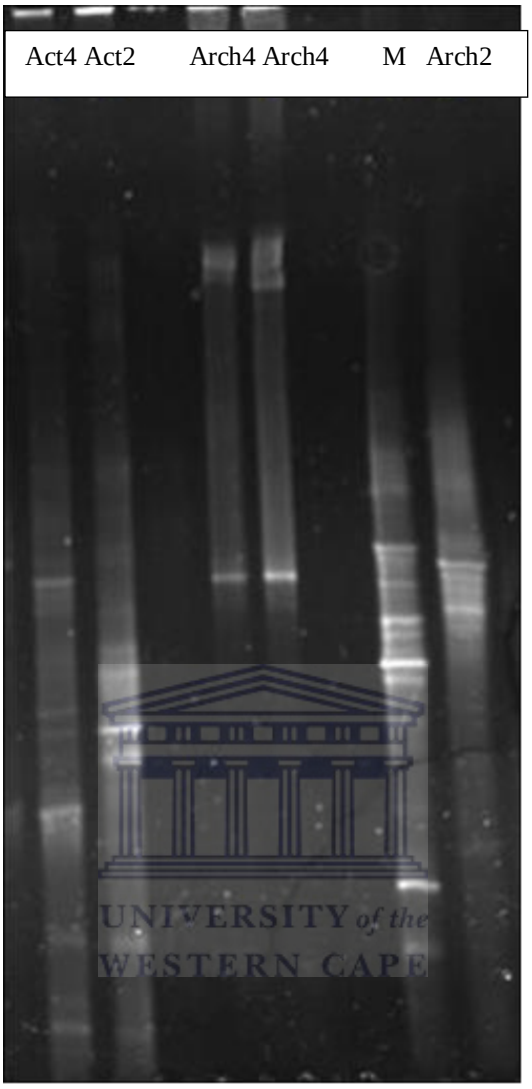

Figure 3.4 DGGE of actinobacterial and archaeal community fingerprints obtained from sites LM2 and LMS4 samples. Act4: actinobacteria LMS4; Act2: actinobacteria LM2; Arch4: archaea LMS4; M: molecular marker; Arch2: archaea LM2.

Gelcompar analysis of the PCR-DGGE profile identified between 1 and 15 distinct bands across the gel. The different number of bands in the lanes of the gel may be an indicator of a variation in sample composition.

A hierarchical cluster analysis of each taxon in the PCR-DGGE profile showing band polymorphism (unique and common bands) is depicted in the dendograms (Figures 3.5A and B).

Each distinct band for a particular taxon per site is declared as an operational taxonomic unit (OTU).

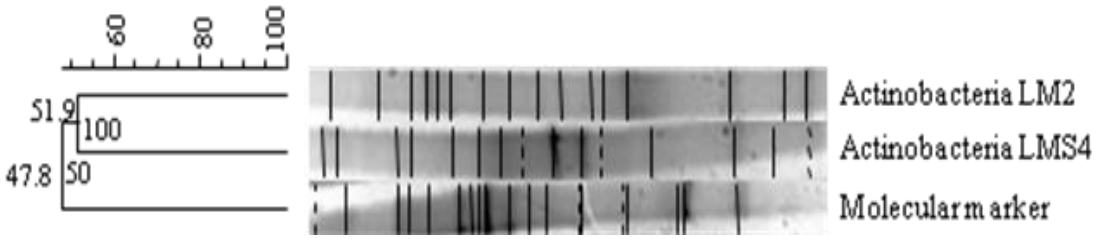

Figure 3.5A A Dice coefficient and UPGMA (Unweighted Pairwise Group Method with Arithmetic Mean) based hierarchial clustering of DGGE fingerprints of actinobacterial communities in LM2 and LMS4 samples. Bands of the experimental samples and the molecular marker are shown. The molecular marker is not part of the comparison but have only been included in this dendogram (and subsequently its MDS plot) for technical requirement by analytical software -GelCompar II.

Cluster significance as calculated by the co-phenetic correlation at each branching level and statistically at all linkage levels are also depicted in the figure. The co-phenetic correlation values are the number shown at the nodes (inside the branches) of the dendogram. They express the consistency of each cluster in the dendogram. It calculates the relatedness between the dendogram-derived similarities and the matrix generated similarities. The similarity values (outside the branches) are indicators of the stability or errors of each subcluster. It expresses the average similarity and exact standard deviation of the derived values against the original value (co-phenetic correlation) as a measure of the internal consistency of the branch. The lesser the similarity score of a group, the more the consistency of the cluster. This implies that the group would not be prone to modifications for example tolerance settings refinements or addition or deletion of entries (www.applied-maths.com).

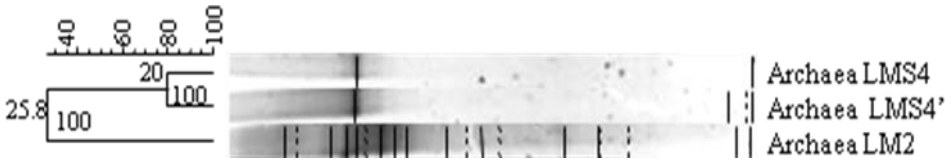

Figure 3.5B A Dice coefficient and UPGMA derived hierarchical clustering of the DGGE resolved archaea communities in LM2 and LMS4 samples.

In Figure 3.5A (of the actinobacterial community fingerprints), it is observed that some OTU's are common to both test samples but those of LM2 are more dominant. A relatedness score of 100% is given for consistency between the dendogram derived and the matrix generated similarities, a statistical value of 51.9% is generated. This is an indication of moderate consistency for that subcluster.

For the archaeal population between the study sites (Figure 3.5B), the duplicate sample of LMS4 clustered together and this was as anticipated The standard deviation (statistical error values) generated for the subclusters (20 and 25.8%) signifies considerable consistence of the clustering and moderate heterogeneity between the taxon at study site samples. The inference from the archaea community DGGE analysis confirms the reliability and reproducibility of DGGE method as a quantitative tool for distinguishing variation between samples.

In order to map out the relationship of the samples, the dendogram clusters for each taxa was superimposed on a multidimensional scaling plot (MDS). The similarity patterns of the OTU's were displayed in 3-D space (XYZ) as depicted in Figure 3.6A and B for actinobacterial and archaeal communities respectively.

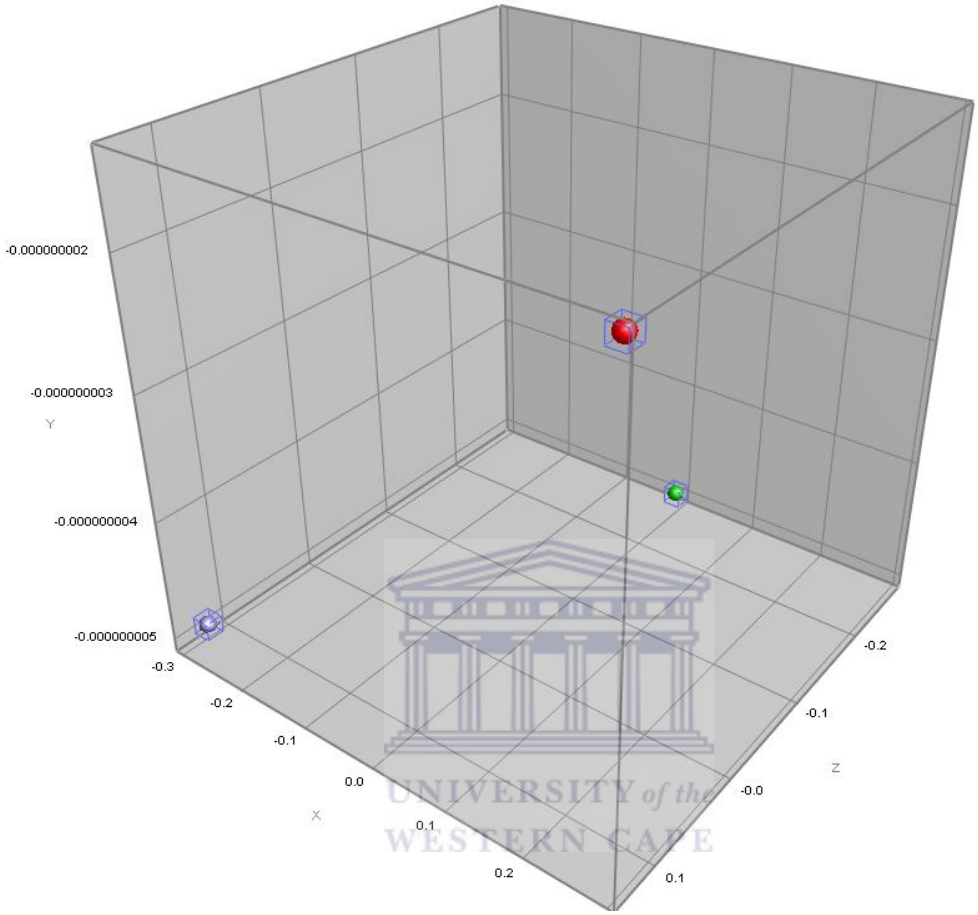

Figure 3.6A Co-ordinate spacing of non-hierarchial MDS analysis of actinobacterial DGGE similarities as inferred from dendogram (Figure 3.5A). Color codes: Red actinobacteria LM2, green actinobacteria LMS4 and blue is the molecular marker.

The distance between any two points in the MDS plot indicates the degree of similarity between the two, that is, the greater the distance the less related they are and vice-versa. In the MDS plot above, although, both test samples are plotted on the same axis (X-Z) they have different degrees of spatial representation which is an indication of sample heterogeneity.

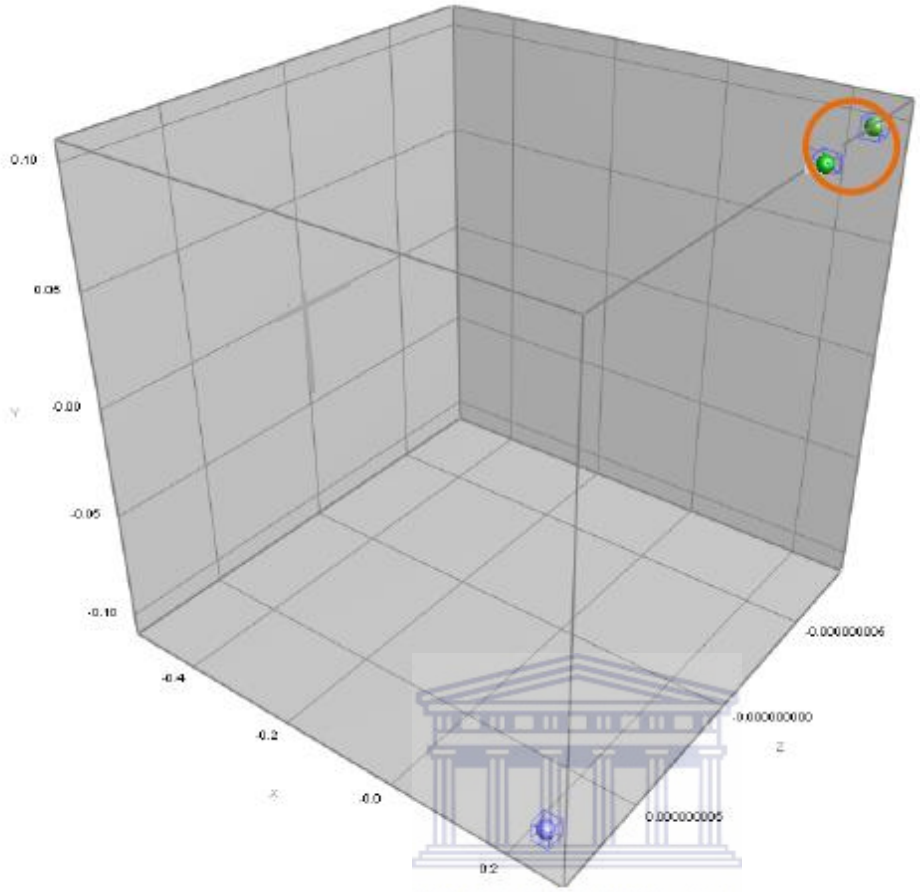

Figure 3.6B Co-ordinate spacing of archaeal DGGE dendogram similarity matrix. Green- Archaea LMS4 duplicates samples and blue: Archaea LM2 sample.

From the MDS plot for the archaeal communities in LM2 and LMS4 samples, the duplicates samples of LMS4 form a cluster as anticipated. This is a confirmation of the reliability of the PCR-DGGE method as tool for monitoring variations in community compositions. Both MDS plots (Figures 3.6A and B) indicate moderate heterogeneities between the populations examined at the two study sites.

3.4 Phylogenetic characterisation of microbial diversity by clonal library screening and amplified ribosomal DNA restriction analysis

Sequenced based metagenomic analysis on LM2 and LMS4 samples was achieved by clone library construction and screening based on unique restriction digestion profiles (ARDRA).

3.4.1 Number of clones obtained per library

Positive clones were identified by blue/white selection. Table 3.4 presents the number of clones picked per taxa per site investigated.

Table 3.4 Clone summary

Sample site	Actinobacteria	Archaea
LM2	396	210
LMS4	300	190

3.4.2 Screening for plasmid bearing correct sized insert

Positive transformants were checked for correct size by M13 colony PCR. Only positive clones containing about 750 bp for actinobacteria and approximately 200 bp for archaea biased amplicons were selected for ARDRA screening. The accurately sized inserts were re-amplified. Figures 3.7 A -and B shows representative clones screened for correct insert conformation.

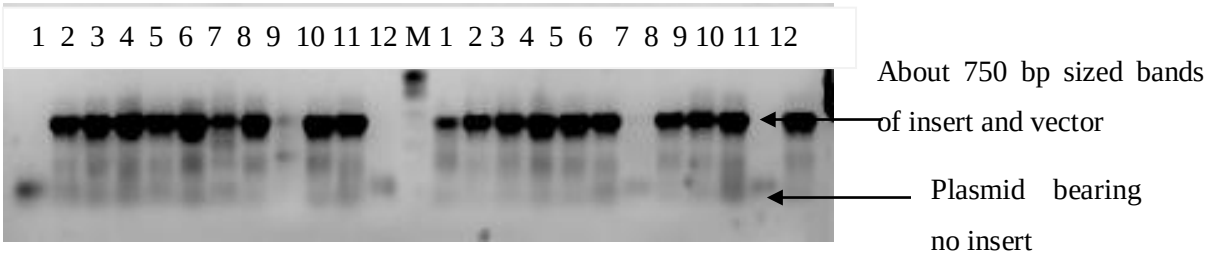

Figure 3.7A: Representative amplicons of actinobacteria-biased M13 colony PCR from site LMS4 samples. M – molecular marker: 100bp marker.

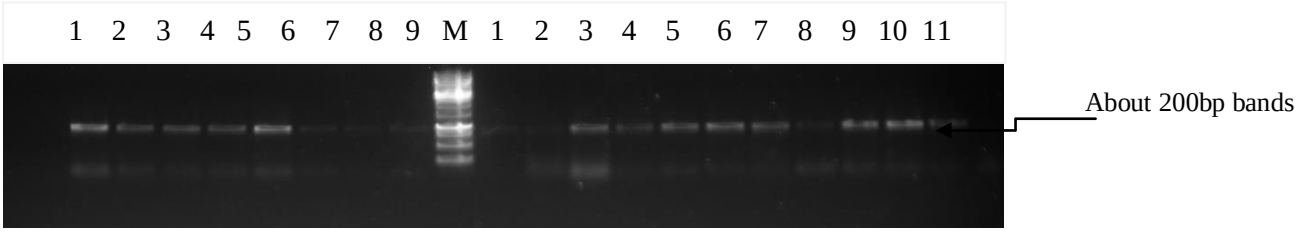

Figure 3.7B: Representative amplicons of archaea biased M13 colony PCR of sample from site LM2 (plates 1A and B). M –molecular marker: GeneRuler low range marker.

3.4.4 ARDRA ribotypes from 16S rDNA libraries

Diversity of the taxa under examination was established by ARDRA profiling. Ribotypes were identified by digest of positive clones with *Alu*I and *Hae*III or *Rsa*I. Unique ribotypes were determined by the size and number of fragments per profile generated. Representative ARDRA patterns for the study sites and taxa in this study are presented in Figures 3.8A – C. Table 3.5 provides a summary of unique ribotypes with respect to accumulated ARDRA patterns.

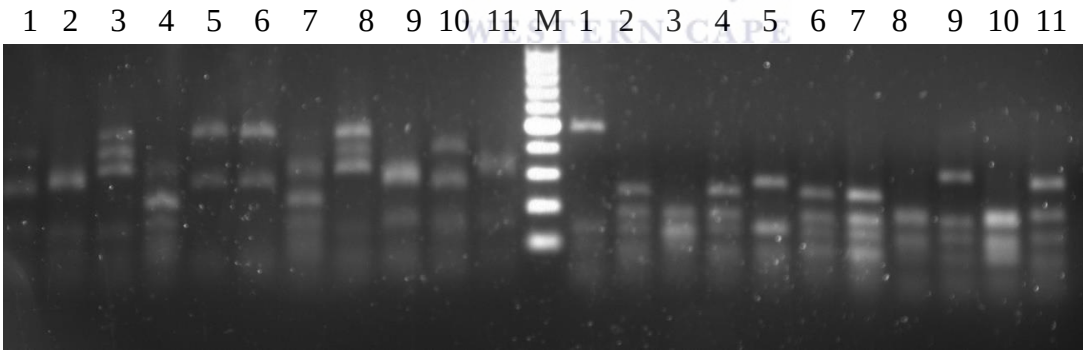

Figure 3.8A: Representatives ARDRA patterns generated from *AluI*/*HaeIII* digestion of cloned LM2 actinobacterial 16S rDNA clones. Lane M, 100 bp molecular marker, lane 3D1-11.

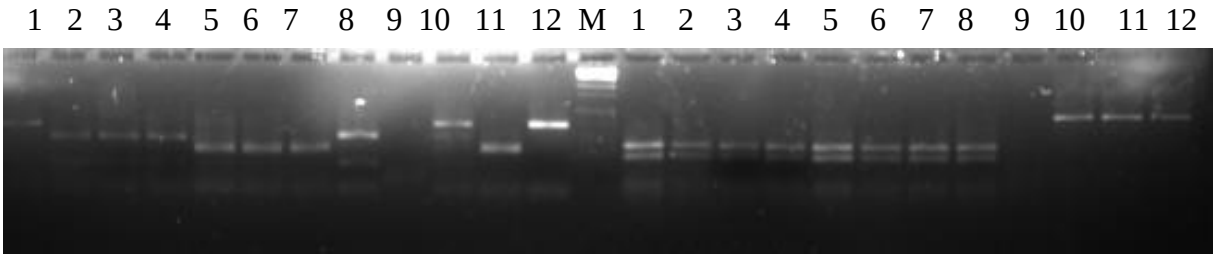

Figure 3.8B: Representative ARDRA profiles generated from *RSa* 1 digest of clones of plate 1B 1-12 LMS4 archaeal 16S rRNA gene fragments. Lane M: GeneRuler low range marker. Double digest with *Alu*I/ *Hae*III were found to discriminate poorly. Improved resolution was obtained using *RSa*I.

Marked heterogeneity was observed in the ribotypes in the actinobacterial library constructs for both sites investigated and for the archaeal library for LM2. The archaea biased libraries were better profiled using the *Alu*I and *Rsa*I. A low ribotype resolution was nonetheless observed in the archaea LMS4 libraries using any combination of the aforementioned endonucleases.

Table 3.5: Ribotypes summary

Site	Actinobacteria	Archaea
LM2	13	9
LMS4	7	5

3.4.2 Nucleotide and phylogenetic analysis of 16S rDNA clone sequences

Codes (clone ID's) were assigned to all sequenced clone insert. The codes for the actinobacterial population are assigned according to sample site, target taxon and then position in microtitre plates such that LM2 Act 1A3 indicates that the clone is from sample site LM2, actinobacterium targetted, in Plate one Lane A and in well number 3. The codes for the archaeal ribotypes are given according to numeric position of sum total of successfully cloned insert obtained for both sites.

All sequences generated were compared to 16S rRNA gene sequences of the closest database relatives by BLAST searches of the NCBI's nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The information thus obtained are presented in Tables 3.6A and B for

actinobacteria LM2 and actinobacteria LMS4 populations respectively, while archaeal sequence similarity information for both study sites are combined in Table 3.6C. All non-target sequences retrieved are highlighted (in red) in the tables.

For the determination of phylogenetic relationship of aligned sequences, bootstrap consensus trees based on 1000 re-samplings were generated as depicted in Figures 3.9 A- C. The optimal tree with the sum of the branch lengths deduced and the percentage of replicate trees in which the associated taxa cluster are displayed next to the branches (Felsentein, 1985). The trees were drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum composite likelihood method (Tamura *et al.,* 2011) and are in units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated in the generation of the trees.

Table 3.6 (A) Phylogenetic affiliations of actinobacterial partial 16S rDNA clones (ca. 750bp) from LM2 as given by BLAST search.

Clone ID Act	Identity of the nearest validly published/ reported genus(partial 16S rRNA gene sequence)	Accession Number	Simila-rity score (%)	Description as inferred from literature
1A3	Uncultured actinomycete clone ZY-98 (Zhang and Zhang, 2012)	JX242838.1	97%	Actinobacteria of salty beach in Tarim University, China
2E3	Uncultured bacterium clone GL81- Act13 (Jiang *et al.*, 2010)	HQ265288.1	98%	Actinobacteria of a Tibetan hotspring.
4E7	Uncultured actinobacterium B101-30 clone (Kim and Cowley, 2005)	DQ 001690.1	98%	Microorganisms associated with moss pillars in East Antartica.
1E4	Uncultured bacterium clone 3-1AE9D5 (Khan *et al.*, 2011)	HQ197617.1	99%	Hypolith of the quartz rocks from Miers Valley, McMurdo dry Valley, Antartica.
1G7	Uncultured actinomycetales clone MLS-180 (Keshri *et al.*, 2012)	JX240864.1	99%	Actinobacteria of the coastal soil of gulf of Khambat, India.
1G9	*Cellulomonas* sp A5-19 (Sun *et al.*, 2012)	JN6271163.1	100%	Culturable actinomycetes associated with soft coral *Scleronephthya* sp
2D2	*Cellulomonas hominis* strain KNUC9071 (Chun *et al.*, 2011)	JF506005.1	100%	Isolate from surface of concrete in South Korea.
2E7	*Cellulomonas* sp Mn10-48 (Cerritos *et al.*, 2010)	HM587952.1	100%	Culturable thermo-resistant aquatic bacteria from an environmental gradient in Cuatro Cienegas, Coahuila, Mexico.
2F4	Uncultured bacterium clone QC34_Act_039 (Li and Jiang, 2012)	JX667863.1	95%	Microorganism of the sediments of four Tibetan hotsprings.
3E2	Uncultured bacterium clone AN0C1BB03	JQ 426222.1	95%	Bacterium of alkaline saline soil spiked with anthracene (a pesticide)
3E9	Low G+C Gram-positive bacterium clone WN-HSB-223 (Mesbah *et al.*, 2007)	DQ432326.1	99%	Firmicute of alkaline hypersaline Lake Wadi an-Natrun Egypt.
2B7	*Demequina aestuarii* strain JC2054 (Yi *et al.*, 2007)	DQ010160.2	93%`	Aerobic slightly halophilic actinomycete of tidal flat sediment in South Korea
4E6	*Cellulomonas flavigena*	AF 140036	95%	Cellulose degrading organisms of forest soils
4E10	*Mycobacterium sp.* JS624 (Coleman and Spain, 2003)	AY162029.1	99%	Non tuberculus mycobacteria of an aquatic system in Mexico.
3G2	*Arthrobacter* sp. IMM11 (Caliz *et al.*, 2011)	FR727708.1	99%	Actinobacteria from an unpolluted acid field in Spain.

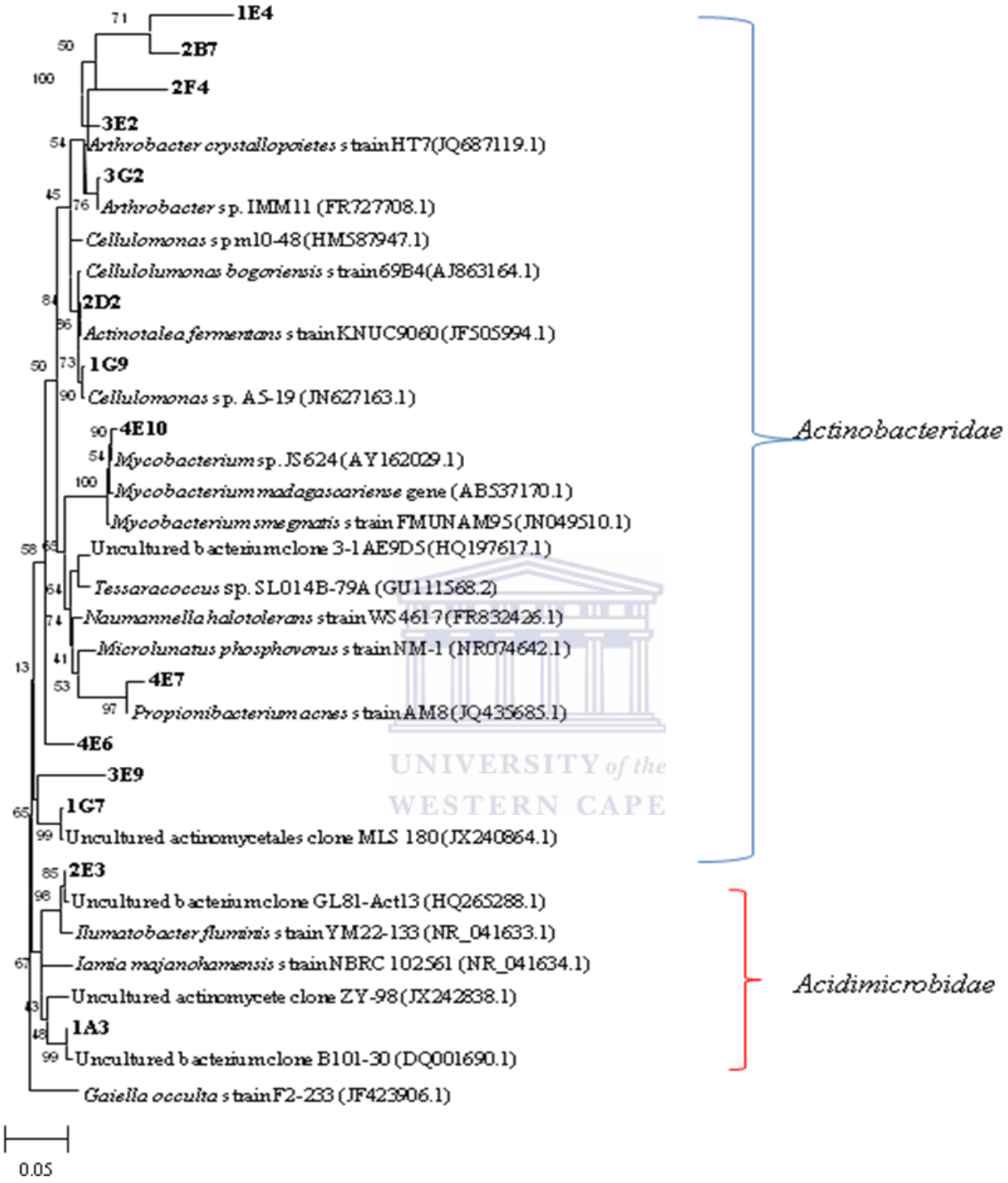

Figure 3.9A Evolutionary relationships of partial 16S rDNA of actinobacterial sequences obtained from LM2 samples with their closest phylogenetic neighbours. Phylogenetic relationship was deduced using the neighbor joining method and the maximum likelihood composite analyses. Values at the nodes indicate bootstrap values derived by 1000 permutations. Sequences obtained in this study are highlighted in bold. A member of the order *Rubrobacterales -Gaiella occulta* was used the outgroup.

Blast analyses of PCR-amplified and sequenced partial 16S rRNA gene identified all the sequences as characteristic signatures of organisms thriving in halophilic and or alkaline environments.

A total of twelve actinobacterial and one bacterium (a firmicute) sequence were retrieved from the cloned 16S rDNA obtained from study site LM2 samples. The non actinobacterial sequences retrieved could have been due to mismatching errors due amplification. Mismatching error refers to replication errors in which artificial base substitutions are present in an amplification product (Reiss *et al.,* 1990). This non-specific nucleotide incorporation leads to inaccurate sequence determination relative to the template strand. This error can be overcome by using a proof reading polymerase (New England Biolabs, 2013). Proof reading polymerases are enzymes with 3'-5'exonuclease activity and are capable of detecting incorrect nucleotide insertion in the template DNA. If a mismatch is found the DNA is transferred to the N'-terminal 3'-5' exonuclease domain of the polymerase from the polymerization domain where the incorrectly incorporated nucleotide is excised and then DNA is returned to the polymerization domain for replication to continue.

Forty-two percent (46.2%) of the actinobacterial sequences obtained from site LM2 have >98% similarities to orgainsms yet to be brought into culture. Sequences affiliated to the subclass *Acidimicrobidae* (*Iamiaceae*, *Ilumatobacter,* and *Acidimicrobium* families) and those of the subclass *Actinobacteridae* order *Actinomycetales* represented by suborder *Corynebacteria* (*Mycobacterium*) and *Propionibacteriaceae*, *Micrococcineae (Micrococcaceae, Cellulomonadaceae* and *Demequina* families) were detected. The latter subclass forms the largest group observed.

Clone Act 1G9 was identified as *Cellulomonas* sp A5-19 (100%) and is described in literature as a culturable actinomycete found to be capable of producing type II polyketides informed by functional gene predictions (Sun *et al.,* 2012). Generally, polyketides are a structural diverse family of bioactive natural products of pharmacological and medicinal importance. The type II polyketides are polycyclic aromatic molecules produced by iterative actions of several enzymes in microorganisms of the actinomycetes (*Streptomyces)* group. Notable amongst these bioactive compounds produced are antitumor and antifungal agents. The metabolites synthesized by these

Streptomyces have been chemically derivitized or bioengineered for drug discovery (Robin, 1991; Moore and Piel, 2000; Das and Khosla, 2009). The identification of signatures with the above described particulars is an indicator to the fact that commercially relevant biomolecules can be sourced from extreme environments and that metagenomics is a viable tool for their primary identification.

Table 3.6 (B) Phylogenetic relationships of actinobacterial 16S rDNA clones (ca. 750bp) from LMS4

Clone ID LMS4 Act	Identity of the nearest validly published/ reported genus (partial 16S rRNA gene sequence)	Accession Number	Similarity score (%)	Description as inferred from literature
1E1	*Cellulomonas* sp. Mn10-48 (Yamaguchi *et al.*, 2011)	AB 637286.1	99%	Culturable thermo-resistant aquatic bacteria from an environmental gradient in Cuatro Cienegas, Coahuila, Mexico
2C4	*Natranaerobius thermophilus* (Bowers *et al.*, 2009)	NR 074181.1	97%	An anaerobic, halophilic alkalithermophilic firmicute isolated from the Kenyan-Tanzanian Rift
2E6	*Bacillus neizouhensis* (Peeters *et al.*, 2011)	FR 694531.1	98%	Cultivable heterotrophs of the brines of Forlidas pond, Antartica
2G7	Uncultured actinobacterium clone HJ10SS74 (Wu *et al.*, 2008)	EU 532513.1	92%	Actinobacteria found in the hypersaline brines in the temperate zone of Yunnan, China
3A8	Uncultured bacterium clone PL35 (Peng and Li, 2011)	FR 853204.1	100%	Bacterium of human skin microbiome
3B10	Uncultured bacterium clone GL81-Act 13 (Tucker *et al.*, 2007)	EU 284188.1	99%	Uncultured bacterium from Tibetan hot spring
3D11	Uncultured bacterium clone FFCH5550 (El-Shahed *et al.*, 2008)	EU 133124.1	97%	Novel and unique rare member of the soil biosphere

N.B All non-target signatures retrieved are depicted in red.

Figure 3.9B Evolutionary relationships of partial 16S rDNA actinobacterial sequences obtained from LMS4 samples with their closest phylogenetic neighbours. Phylogenetic relationship was deduced using the neighbor joining method and the maximum likelihood composite analyses. Values at the nodes indicate bootstrap values derived by 1000 permutations. Act 3D11 is not depicted in the phylogenetic tree above as per the results of multiply sequence alignment. This implies most likely that its sequence is highly identical with one of its representative sequences (uncultured bacterium clone FFCH5550, *Actinobacterium* TC4 or *Aciditerrimonas ferrireducens*). An actinomycete of the *Frankia* genus serves as the outgroup.

Only one of the actinobacterial sequences retrieved from site LMS4 had high sequence similarity (99%) with a microorganism in culture. All signatures found have high homology to thermophilic haloalkaliphiles with the exception of Act 3D11 which had sequence similarity with a moderately thermophilic acidophile (*Actinobacterium* TC4). Nonetheless this finding is consistent with the temperature recorded at site LMS4 (Table 3.1). The actinobacterial sequences identified at LMS4 belong to the subclass *Actinobacteridae* (3) and *Acidimicrobidae* (2) (as observed in LM2) and just one sequence was most closely related to a representative of the subclass *Nitriliruptoridae* (*Euzebya tangerina* with 89% similarity). All the actinobacterial clone

sequences and even the firmicutes identified in this study (LM2 and LMS4) have been reported in previous studies in association with marine or hypersaline sediments including those of the Lakes of the East African Rift Valley. However, it cannot be confidently inferred that these are the only actinobacterial representatives thriving in this ecosystem. This is especially so since a wider variety of genera within this phyllum have been reported in similar environments using both molecular and non molecular strategies (Chen *et al.*, 2004; Delgado *et al.*, 2006; Govender *et al.*, 2012).

It is also observed that the variety of genera retrieved in previous studies on environments with similar conditions as Lake Magadi and using the same actinobacteria specific primer set (S-C-Act 235-a-S-20 and S-C-Act-878-a A-19) are different. While the study conducted by Babalola *et al.* (2009) did not retrieve any member of the subclass *Acidimicrobiales*, the initial study in which this primer set was introduced (Stach *et al*., 2003) had an even broader actinobacterial yield, which has representatives of all four subclasses and members of the order *Acidimicrobiales* were present in 64% of the total actinobacterial phylotypes identified. In comparison to either of the previous studies above, it is observed that this study recovered a lower number of representatives of the subclass *Actinobacteridae*. This variation of course could be due to the total absence of representatives of the order *Acidimicrobiales* in the samples studied by Babalola *et al.* (2009) and the lack of representatives of the other genera detected by Stach and co-workers (2003) in site (LM2 and LMS4) samples in this study.

Low actinobacterial diversity has been generally reported in haloalkaline environments. However, the firmicutes (*Bacillus* and *Clostridium* species) have been identified as dominant microbial populations in many alkaline athalassahaline habitats (Baumgarte, 2003; Mesbah *et al.*, 2007; Sharaf and Al-Fadel, 2012). The reported abundance of these non-targeted species in these environments may also account for their preferential amplification in this study.

One of the firmicute sequences found, Act 2C4, was found to have 97% homology with complete 16S rRNA gene of the poly-extremophile *Natranaerobius thermophilus* JW/NM –KB 411 (Mesbah *et al.*, 2007) isolated from Lake Fazda in Wadi An Natrun, Egypt. Phylogenetically, this organism belongs to a novel lineage within the class *Clostridia*, the novel family *Natranaerobiaceae* and novel order, *Natranaerobiales* (Mesbah *et al.*, 2007) and have

been described as alkalithermophiles (haloalkalithermophilic organisms) with obligate requirements for these conditions.

Table 3.6 (C) Phylogenetic affiliations of archaeal 16S rDNA clones (ca. 193 bp) from LM2 and LMS4 samples

Clone ID	Identity of the nearest validly published/ reported genus(partial 16S rRNA gene sequence)	Accession Number.	Similarity score (%)	Description as inferred from literature
LMS4 Arch 13	Uncultured archaeon hypersaline sediment clone Kasin-A02 E04 (Emmerich *et al.*, 2012)	HE604529.1	96%	Iron (II)-oxidising and Iron (III) reducing archaea from the hypersaline sediments of Lake Kasin, Southern Russia
LM2 Arch 16	Uncultured organism clone SBYT_4837 (Kirk *et al.*, 2013)	JN 482328.1	93%	Bacterium of the hypersaline microbial mat of estuaries of Guerrero Negro, Mexico
17	*Halovivax ruber* (Lucas *et al.*, 2013)	JN 216858.1	96%	Euryarcheote
18	Uncultured euryarchaeon clone WN –FSA-54 (Mesbah *et al.*, 2007)	DQ 432492.1	100%	Novel prokaryote of the alkaline hypersaline lakes of Wadi An Natrun, Egypt
19	*Halopiger xanaduensis* (Anderson *et al.*, 2012)	AB 477974.1	100%	An extremely halophilic archeon isolated from saline Lake Shangmatala, Inner Mongolia, China
20	Uncultured archaeon ARC SB_108 (Anthony *et al.*, 2012)	JQ 738702.1	97%	Prokaryote of Lonar crater basalts, India.
21	Uncultured crenarcheon clone JC- gj5 (Liao, 2011)	JQ013068.1	96%	Uncultured crenarcheon isolated in China.
22	Uncultured archaeon clone GSP_arch158 (Caton *et al.*, 2009)	FJ 696329.1	99%	Archaea isolated from the great salt plains of Oklahoma.
23	*Natronolimnobius baerhuensis gene 1HC – 005* 16S rRNA gene (Itoh *et al.*, 2005).	NR_028161.1	99%	Novel haloalkaliphilic archaeon isolated from soda lakes in Inner Mongolia, China.

Sequence depicted in red indicates a non-target organism.

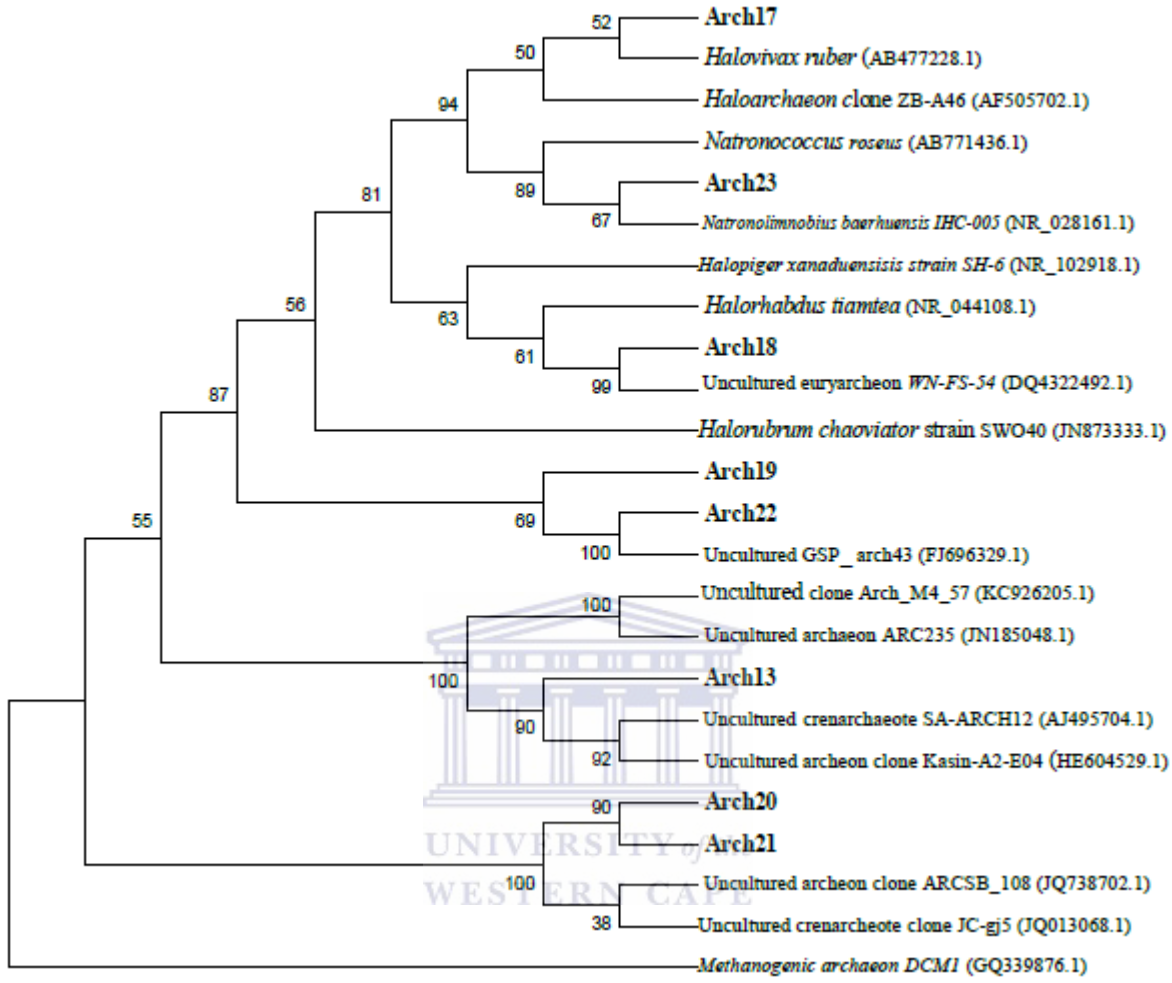

Figure 3.9C Evolutionary relationships of partial 16S rDNA archaeal sequences obtained from LM2 and LMS4 samples with their closest phylogenetic neighbours. Phylogenetic relationship was estimated using the neighbor joining method and the maximum likelihood composite analyses. Values shown at the nodes indicate bootstrap values derived by 1000 permutations. An euryarcheote of the genus *Methanomicrobia*- Methanogenic archeon DCMI- is used as an outgroup.

BLASTn analysis of PCR-amplified and sequenced partial 16S rRNA gene placed the archaeal sequences into crenarchaeal (2) and euryarchaeal (6) phyla which all belong to the subclass *Halobacteria*, genus *Halobacteriales*, a group known to be the dominant life forms in hypersaline environments (Oren, 2004; Singh *et al.*, 2010). Only a single phylotype was identified in LMS4 even after five (5) ribotypes had been randomly chosen from the ARDRA

profile screens. A database search of the sequence identified it as homologous to an uncultured Iron (I oxidising and II reducing) crenarchaeote of hypersaline sediments of Lake Kasin, Southern Russia. A bacterium sequence was also retrieved from LM2 it had 93% sequence identity to a bacterium of a hypersaline microbial mat of estuaries in Mexico. The retrieval of this signature may be attributed to primer mismatch.

The primers used in this study targetted certain groups within the archaeal lineage as such it cannot be affirmed whether the members of the euryarchaeal and crenarchaeal lineages identified are the exclusive archaeal representatives in the the highly alkaline and hypersaline Lake Magadi. The non availabilty of universal archaeal primers because of the lack of complete archaeal 16S rRNA gene sequences poses a great hindrance to phylogenetic studies. The primers used in this study and many such studies are biased towards the classes with cultured representatives leaving out the rare species.

Neither of the two crenarchaeal signatures (Arch 13 aand Arch 21) obtained in this study grouped with organisms in culture. The closest cultured relatives (both had <83% sequence identities) are anaerobic sulphur-dependent or reducing marine halophiles. Kubo *et al.* (2012) and Li *et al*. (2012*)* reported an abundance of a diverse and miscellaneous crenarchaeotal group in marine sediments with molecular signatures different from cultured crenarchaeotes. This finding is also supported by the culture-independent surveys of DeLong (1992) and Karner *et al.* (2005). These authors reported further that marine crenarchaeotes present in estimated populations of 10^{28} cells cm^{-1} may have major roles in global biogeochemical cycles. Also, as indicated in the isotope based studies conducted by Könneke and colleagues (2005), marine crenarchaeotes are involved in nitrogen and inorganic carbon fixation. The detection of such signatures from Lake Magadi samples might be reflecting representatives of the functional microbial population which supports the niche.

The non-archaeal sequences retrieved could well be attributed to mismatching errors of PCR-based techniques. On another hand, it could be as a result of the ubiquity of alkaliphilic or alkalitolerant bacteria, for example, the genus *Bacillus* (Grant *et al.*, 1990; Horikoshi, 1999) and the indications that some especially the haloalkaliphiles are unique to hypersaline lakes such as Lake Magadi (Jones *et al.*, 1994; Litchfield *et al.*, 2009). Hence, the existence of such phylotype in the seemingly hostile Lake Magadi environment cannot be completely overruled.

CHAPTER 4 RESULTS AND DISCUSSION CULTURE DEPENDENT 16S rDNA BASED STUDIES

Introduction

Culture based strategies are the basis for the precise identification of microorganisms. Isolates needed to be obtained for phenotypic, and then biochemical characterization to be conducted for taxonomic inferences to be drawn. However, with recent advances of the application of molecular techniques, particularly the sequence analysis of the 16S rRNA gene in microbial studies, accurate phylogenetic classification of isolates can be achieved without need for tests based on physiology and morphology (Macrae, 2000; Fry, 2004; Priest, 2004).

In this chapter, the results of culturing efforts directed at the actinobacterial and archaeal populations thriving within Lake Magadi, Kenya using the 16S rRNA gene as a phylogenetic marker are presented.

4.1 Microbial isolation studies

4.1.1 Colony characterisation

A total of twenty six isolates were obtained from two of the isolation media used in this study on plates containing variants (pH, NaCl content and incubation temperature) of two of the three media types used for isolation studies. No growth was observed on any of the media inoculated with samples from station LMS4 despite an extended incubation period (20-45 days). Similarly no growth was observed on *Streptomyces* General Defined Media (SGDM) or on any medium with a 15% NaCl concentration or on plates incubated at 55°C. Seven representative isolates were selected for sequencing based on their distinct colony morphologies. Figures 4.1A and B show the colony morphologies of isolates 7 and 11.

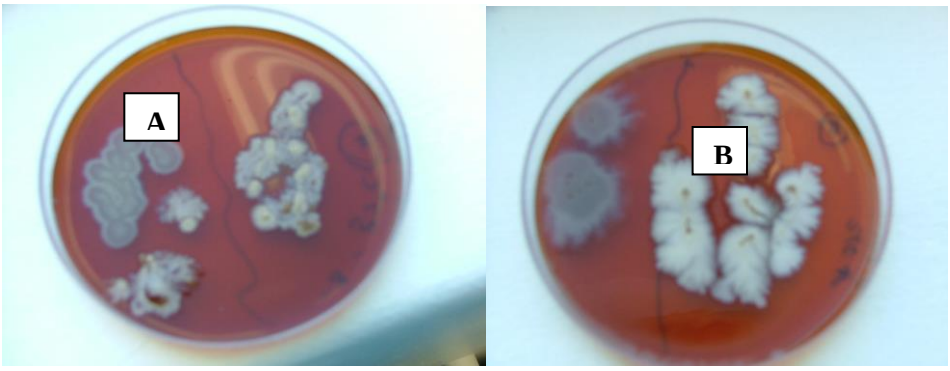

Figure 4.1(A) & (B): Colony morphologies of two of the isolates chosen for further screening.

(A) Isolate 7 and (B) isolate 11.

Table 4.1 outlines the strategies used for the isolation of the seven isolates from site LM2 selected for further investgation.

Table 4.1: Isolates obtained, medium and incubation conditions

Isolates	Medium	Medium conditions		Incubation Temperature
		pH	NaCl	
1	YEME	7	5%	37°C
3	MA	9.5	1.5%	37°C
4	MA	10.5	1.5%	45°C
7	YEME	9	10%	37°C
9	MA	9.5	5%	45°C
11	MA	8.5	5%	45°C
15	YEME	10.5	1.5%	37°C

YEME- Yeast Extract - Malt Extract; MA –Media Alkaliphilic

4.1.2 ARDRA

As expected for the universal bacterial 16S rRNA gene primers fragments of approximately 1500 bp were obtained for the selected isolates (Figure 4.2).

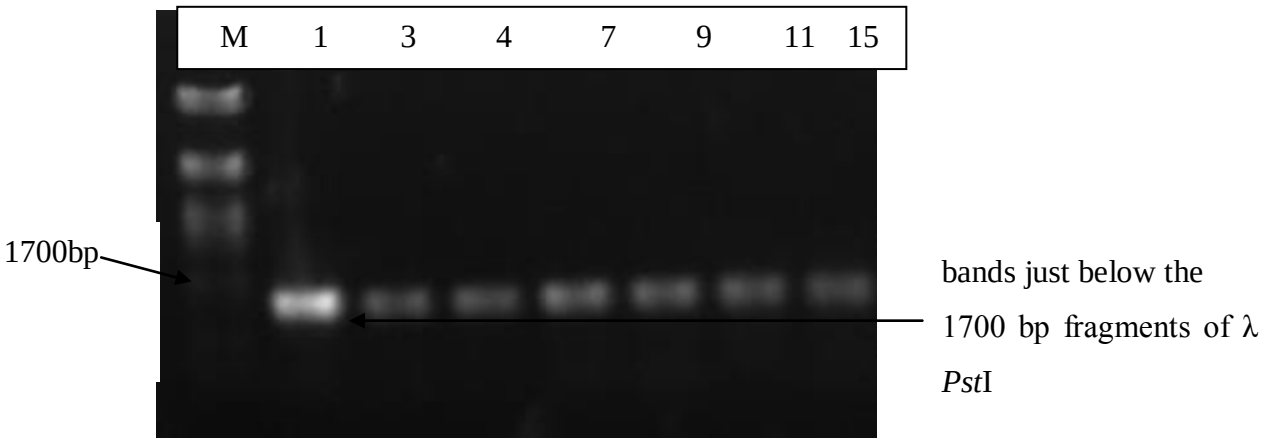

Figure 4.2 1.5% agarose gel depicting 16S rRNA gene amplicons of isolates 1, 3, 4, 7, 9, 11 and 15 from site LM2. The bands are just below the 1700bp (~1500bp) of the molecular marker. (M) *Pst*I digested lambda phage DNA. The colony PCR products were used directly for ARDRA profiling (Figure 4.3).

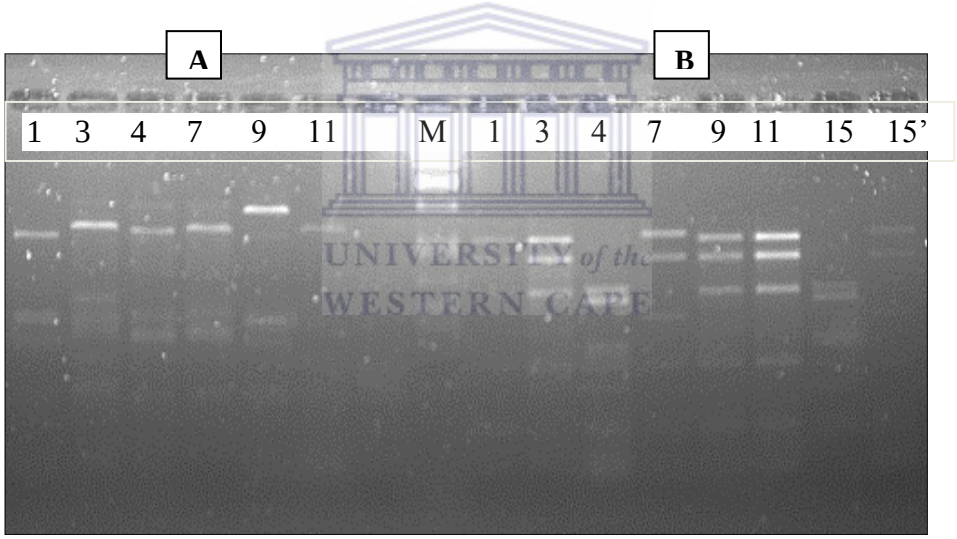

Figure 4.3 ARDRA profiles of 16S rRNA gene colony PCR products generated by *Alu*1 and *Hae*III digestion. The digested products were resolved on 2.5% agarose gels and visualised by EtBr staining. A: *Alu*I digestion, B: *Hae*III digestion, M, lambda phage digest by *Pst*I. 15' is *AluI* digest of isolate 15.

Unique ribotypes (isolates 1, 3, 4, 7 and 9) were identified by the size and number of fragments in each profile. The colony PCR products were purified using the Illustra™ GFX™ PCR DNA and gel band purification kit (GE- Healthcare, UK) according to manufacturer's instructions. The purified amplicons were sequenced using universal bacterial primer set E9F and U1510R.

4.1.3 Analysis of the 16S rRNA gene sequence and phylogenetic determination.

Based on homology searches of sequence data of the isolates against NCBI non-redundant data bases (http:blast.ncbi.nlm.nih.gov/) via BLASTn, the 16S rRNA gene sequences from isolates 3 and 4 were found to have the highest sequence similarities to *Halomonas* species while isolate 7 had the highest sequence similarity to *Bacillus cereus* strain DCB (Table 4.3).

Table 4.2 BLAST analysis of the 16S rRNA gene sequences ampilified from isolates 3, 4 and 7.

Isolate	Identity (partial 16S rRNA gene)	Query length	Similarity score (%)	Accession number	Description as inferred from literature
3	*Halomonas* sp. A_07 (Pradhan *et al.*, 2003)	1152	97%	AY347310.1	A novel alkaliphile of Tanzanian soda lake
4	*Halomonas campisalis* strain HB10.1 (McSweeney *et al.*, 2009)	803	100%	GU 228484.1	Oxalate-degrading microbe in bioreactors treating Bayer liquor organic waste.
7	*Bacillus cereus* strain DCB (Tang and Lian, 2011)	1665	98%	JN 650544.1	Firmicutes from the surface of carbonate rich rocks in China

The quality of sequence data of isolates 1, 9 were too poor for an assignment to be confidently made.

Results from microbiological studies in this research concur with earlier reports on culture based studies of soda lakes in which microorganisms of the gamma *Proteobacteria* subdivision, represented by the genus *Halomonas* and members of the genus *Bacillus* (Grant, *et al.*, 2001; Grant, 2004; Mwirichia *et al*., 2010) were isolated. Jones *et al.* (1994; 1998) and Grant (2006) reported that *Bacillus* species were the second most dominant group isolated after *Halomonas*

species in Lake Magadi. In studies of other lakes in the East African Rift valley, Mwirichia and colleagues (2010) reported that the *Bacillus* species were the most commonly found aerobic eubacteria.

A number of halomonads have been identified in alkaline and saline ecosystems using both culture and culture independent techniques. All reports have indicated that haloalkaliphilic halomonads are denitrifying, moderate halophilic alkaliphiles with NaCl requirements ranging from 0.2 to 4.5 M, temperature optimum ranges of 37ºC to 45ºC and optimal pH of between 9 and 10 (Mormile *et al.,* 1999; Poli *et al.,* 2007; Wang, *et al.,* 2007; Li, *et al.,* 2008).

The production of secondary metabolites (glycine- betaine, exopolysaccharides etc) by these Gram negative anaerobes with potential for biotechnological applications is also well documented. Isolates bearing similar identities to those identified in this study (*Halomonas* sp A_07, *H. campisalis* and *Bacillus cereus*) were recovered amongst others in the study conducted by Joshi and colleagues (2007). The researchers reported the production of polyhydroxyalkanoic acid (PHA) a biodegradable polymer by *Halomonas campisalis* strain MCM b-365. PHA production had only been reported earlier in *H. boliviensis* (Quillaguaman *et al.,* 2004). PHA production is one of the survival strategies exhibited by extremophiles in response to stress (unbalanced nutritional condition during growth) (Kanekar *et al.,* 2008). *H. campisalis* strain MCM b-365 also produces exopolysaccharides which is thought to play a structural role by facilitating attachment to surfaces, nutrient acquisition and protection from environmental stress or may function as a host defence mechanism (Iqbal *et al.,* 2002). In the biotechnology industry, exopolysaccharides are used in the removal of heavy metals from industrial effluents and from potable water (Norberg and Pearson, 1984).

Another *H. campisalis* strain isolated by Mormile and co-workers (1999) is reported to have the potential of being used as a cost-effective alternative in the disposal of the briny-alkaline waste produced from ion-exchange resin regeneration.

The *Halomonas* species (*H. korlensis* and *H. shengliensis*) isolated by Wang *et al.* (2007) and Chi *et al.* (2007) were able to thrive in the presence of crude oil while utilising petroleum products as carbon sources. The triple extreme conditions imposed on *H. korlensis* and *H. shengliensis* and under which they flourish regardless are interesting points for fundamental

scientific research and biotechnology. The strains may be suitable candidates for bioremediation of oil field brines.

The genus *Bacillus* consists of aerobic and facultative anaerobic species, which range from Gram positive to Gram variable endospore forming rods (Claus and Berkeley, 1986) which are able to grow heterotrophically and autotrophically across a broad pH range (Nazina *et al.,* 2001).

Thus far, the *Bacillus* species isolated from haloalkaline environments are reported to have varying morphologies and substrates utilization requirements, but all are capable of substantial production of extracellular alkaline proteases which have established biotechnological values (Weisser and Trüper, 1985; Patel, 2006; Sharaf and Al-Fadel, 2012).

The culturing efforts in this study failed to yield any actinobacterial or archaeal isolates. Jones *et al.* (1994; 1998) reports that microbial isolations from soda lake are particularly difficult and that actinobacteria are only infrequently isolated from soda lakes. The few actinobacteria isolated thus far from haloalkaliphilic environments are reported to have moderate NaCl requirements (Tang *et al.*, 2003; Jones *et al.,* 2005; Sorokin *et al.,* 2009; Mwirichia *et al.,* 2010; Govender *et al.,* 2012). A possible reason for the low recovery of actinobacteria from highly alkaline-hypersaline environments may be that the frequently used medium described by Horikoshi (1990) rich in organic carbon selects for fast growing heterotrophic microbes therefore masking the growth of slow growing organisms (Mwirichia *et al.,* 2010). Another possible reason is that the stringent growth requirements of haloalkaliphiles are difficult to mimic and maintain under laboratory conditions (Sorokin, 2005) or that the microorganisms were not present in the sample analyzed in this study.

Most archaea are also recalcitrant to culturing (Könneke *et al.,* 2005). These microorganisms grow slowly and the residual products of bacterial components in the source sample often create footprints that prevents the retrieval of pure archaeal isolates. Previous studies have shown that archaea have limited physiological diversity which suggests that their metabolic requirements constrains them to environmental niches which are difficult to generate and stably maintain in the laboratory.

However, the isolation efforts in this study would have likely been more successful had enrichment strategies being adopted for both target organisms or had more genus specific media been used for the archaeal culturing attempts. Media for the isolation of the target organsims in this study should have been modified with salts of the major cations (potassium, calcium and magnesium) identified in the physioc-chemical analysis of the samples. To improve the chances of cultivating the actinobacterial population, aerobic enrichment cultures should have been used given the characteristics of most actinobacteria. Previous studies on environments with conditions similar to those at LM2 and LMS4 has reported successful isolation through the modification of the classical growth media for haloarchaea and haloalkaliphiles. The studies of Ochsenreiter *et al.* (2002) and Yildiz *et al.* (2012) to assess the diversity of cultivable halophlic (haloalkaliphilic) archaea reports the supplementation of the media with vitamins solution, casamino acids, pyruvate:2-ketoglutarate:2-ketoisovalerate, ferrous-citrate, sodium-citrate and elements (selenite) identified in the chemical analysis of samples. The studies also showed the use of aerobic and anaerobic enrichment cultures in liquid and solid media. Some isolates (the microaerophiles) were only propagated when poured in agar soft layers and some others had to be propagated in liquid media prior to plating on solid media. Antibiotic e.g Penicillin G should be added to isolation medium to prevent possible masking of archaea growth by bacterial footprints as earlier stated.

It is therefore concluded that future efforts to cultivate microrganisms thriving in extreme habitats such as Lake Magadi, require intensive culturing strategies poised to the conditions of their native habitat of the organisms.

The findings from this isolation study emphasizes the fact that microorganisms are versatile candidates for sourcing secondary metabolites because of their obviously vast biochemical diversity, amenability to mass culture and genetic manipulation. Furthermore, the results from this isolation study also prove that diversity studies are essential for the primary identification of biotechnologically relevant components of our biosphere.

CHAPTER 5 PROJECT SUMMARY AND CONCLUSION

5.1 Project summary

Environmental microbiologists have come to realize and appreciate the true extent of diversity owing to the recent awareness created by metagenomic studies of components within the interactive assemblage of microorganisms most of which are not obtainable by standard culturing techniques (Woese, 1990; Head *et al.*, 1998; Bull, 2004; Handelsman, 2007).

Hypersaline–alkaline ecosystems are one of the microhabitats considered to be home to a physiologically highly diverse group of microorganisms which are adapted to survive under at least dual extreme environmental conditions (Nissenbaum, 1980; Singh *et al.,* 2009). Soda lakes are typical models of haloalkaline habitats. They are unique ecological niches where elevated pH and salinities are coupled with other features such as cationic (Na^+) concentrations, depleted anionic composition in the presence of intense solar penetration. Salt pans are sites for the evolution of halophilic bacteria which dominate while suppressing the less halophilic or halotolerant forms (Ahmad *et al.,* 2008). Lake Magadi, Kenya is an environment where these multiple extremophilic features overlap. Enzymes, compatible solutes, exopolysaccharides amongst all other metabolites derived from microorganisms native to environments such as Lake Magadi may be bioaugumented to drive processes with such development requirements as their natural abode and are likely to offer improved or more versatile bioactive compounds than the existing pool obtained from mesophiles.

Microorganisms belonging to the actinobacterium domain are of pharmacologically relevant being the major sources of secondary metabolites. The domain archaea is the least understood in terms of diversity, physiology, genetics and ecology within the larger prokaryotic world (Ahmad *et al.,* 2008). However, as archaea are the dominant life forms in some extreme environments they are worthy of exploration since their special adaptative mechanisms for survival in this environment may be exploitable for commercial and industrial applications (Egorova and Antranikian, 2005). There is very little information about the processes taking place in salt pans and particularly the phylogenetic diversity of its archaea community is rarely surveyed despite

the thorough investigation of the archaea population in other hypersaline systems (Grant and Larsen, 1989).

The first step necessary in this direction is the identification of component (s) within these environments that can provide such service. The primary identification of this entity can be facilitated by the multifaceted techniques of metagenomics which may provide leads to their cultivability which is a necessity to understanding the role and structure of microflora.

The assessment of prokaryotic diversity using the 16S rRNA gene as a phylogenetic marker offers a means of understanding complex microbial communities via the identification of populations yet to be cultured (Macrae, 2000).

5.2 Conclusion

This study employed the power of 16S rRNA gene based analysis to study the actinobacterial and archaeal diversities within two sites LM2 and LMS4 in the strongly alkaline and hypersaline Lake Magadi, Kenya.

Statistical analyses of PCR-DGGE profile cluster revealed moderate population heterogeneity of both actinobacterial and archaeal populations within the study site samples. This was also confirmed by the multi-dimensional scale plots. The reproducibility of PCR-DGGE and its usefulness as a tool for monitoring community composition was confirmed by the clustering of the duplicates of archaea community of study site LMS4 on the dendogram and MDS plot

Phylogenetic affiliation of actinobacterial and archaeal partial 16S rRNA gene sequences with those of their nearest cultured or uncultured neighbour yielded signatures previously identified in hypersaline and or alkaline environments. The actinobacterial sequences retrieved showed high homology to type strains affiliated to the subclass *Acidimicrobiales, Actinobacteridae and Nitriliputoridae*.

Some of the sequences retrieved had high homology with microorganisms previously identified in cold environments [LM2 (Act 1D3 and 1E4) and Arch 21] and acidophilic environments

(LM2 3G2 and LMS4 Act 3D11). While this finding is quite surprising and interesting, this may be an indication of the presence of a resilient and versatile community of microorganisms capable of adapting to various environmental stresses.

However, the difference in microbial composition is observed between the two study sites, because a broader variety of genera was retrieved from LM2 compared to LMS4.

The archaeal phyloptypes identified in this research spanned only the euryarchaote and crenarcheote domains. Crenarchaeotes are the dominant organisms in hypersaline thermophilic niches with a few novel exceptions (Preston *et al.,* 1996; Karner *et al.,* 2001; Auguet *et al.,* 2010; Kubo *et al.,* 2012). It is thus not surprising that the archaeal sequence identified at the thermophilic site LMS4 is a crenarcheote. However the closest phylogenetic relative of the other crenarcheote identified in this study (at LM2) is a psychrophile capable of growth at temperatures of 15ºC and 30ºC.

Failure to identify other classes of actinobacteria subclasses of actinobacteria and domains of archaea means either these groups are not present or due to errors arising from DNA extractions or PCR amplifications. These reasons may also account for the amplification of the non actinobacterial and non archaeal sequences detected.

It was observed that all sequences (target and non-target) identified in LMS4 samples share the peculiarity of having been previously obtained (isolated or metagenomically detected) from anthropogenically impacted (agricultural practices, waste management and minning activities) environments (Itoh, T *et al.,* 2005; Tucker *et al,* 2007; Aslam and Chung, 2009; Lv *et al.,* 2010; Wang *et al.,* 2010; Davis-Belmar *et al.,* 2011; Jangid *et al.,* 2011; Itoh, H *et al.,* 2011; Itoh, T *et al.,* 2011; Peeters *et al.,* 2011; Peng and Li, 2011; Castro-Silva *et al.,* 2012). This finding is likely tied to the fact that the salt pan of Lake Magadi is a site for the exploration of trona and its mineralized equivalents (Davies, 1997; Warren, 2006b). It also most likely suggests that the evolution and dominance of species in a particular habitat is greatly influenced by the land-use pattern or human activity.

The culture dependent 16S rRNA gene strategies identified one haloalkaliphilic strain belonging to the genus *Bacillus* and two of genus *Halomonas*. The biotechnological opportunity which can be tapped from some of these isolates have been reported (Joshi *et al.*, 2005; Kanekar *et al.*, 2008; Hedi *et al.*, 2009; Peeters *et al.*, 2011; Sharafa and Al-Fadel, 2012).

The need for the development of improved culturing strategies for organisms of specialised (extreme) environments is emphasized by the inability to cultivate any actinobacterial or archaeal isolates in this study.

Furthermore, the substantial occurrence (>98%) of sequences characteristic of halophilic, alkaliphilic and or thermophilic organisms and the correlation with some physico chemical data further indicates the role of the environment in dictating community composition.

The result of all strategies (culture dependent and independent) adopted in this study strongly suggests that the environmental conditions at LMS4 are more extreme than LM2.

While the molecular sequence and phylogenetic data generated in this study can not be used to infer the ecological roles of the microorganisms which they signify, the data obtained is useful as a knowledge base about the actinobacterial and archaeal composition within the sites examined and a starting point for future work.

The outcome of this study also supports previous observations which propose that soda lakes are home for unique microbial communities which may be regarded as centres of evolution of microbial diversity (Zavarzin *et al.*, 1999; Mesbah *et al.*, 2007).

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List of Appendices

Appendix I

Buffers used in this study (Sambrook and Russell, 2001)

Buffer	Components/ml
6X Agarose loading buffer	30% (v/v) Glycerol 0.25% (w/v) Bromophenol 15% (w/v) glycerol
10X Orange G loading buffer	60% Glycerol 0.25% (w/v) Orange G
1X TE	10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)
Lysozyme Buffer	25 mM Tris- HCl pH 8.0; 50 mM glucose; 10 mM EDTA; 25 mg lysozyme
50X TAE	2 M Tris base 10 mM glacial acetic acid 0.5 M EDTA

Appendix II:- Restriction enzyme information

The enzymes used in this work were four base specific restriction endonucleases namely *Alu1, BSuR1 (HaeIII), and Rsa1*. Information specific to each enzyme is as giving below.

Enzyme	Recognition sequence	Ligation efficiency	Thermal inactivation
AluI	5'...A G^C T...3' 3'...T C^G A...5'	95%	65°C in 20mins
BsuRI (HaeIII)	5'...G G^C C...3' 3'...C C^G G...5'	95%	80°C in 20mins
RsaI	5'...G T^A C...3' 3'...C A^T G...5'	95%, cleavage blocked or impaired by overlapping *CpG* methylation	80°C in 20mins

All three enzymes have optimal incubation at 37°C.