

Prokaryotic Biodiversity of Lonar Meteorite Crater
Soda Lake Sediment and Community Dynamics
During Microenvironmental pH Homeostasis by
Metagenomics

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“A man should look for what is, and not for what he thinks should be.”

- *Albert Einstein*

Dedicated to my family and friends.

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ABBREVIATIONS

Abbreviation	Description
°C	Degree Celsius
μF	Microfarad
μl	Microliter
16S rDNA	16S ribosomal DNA
A	Adenine
bp	Base Pairs
BSA	Bovine Serum Albumin
C	cytosine
CTAB	Cetrimonium Bromide
dATP	Deoxyadenosine triphosphate
dATPαS	α-thiotriphosphate
dH ₂ O	Deionized water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside Triphosphate
<i>E. coli</i>	Escherichia coli
<i>EcoRI</i>	<i>E. coli</i> restriction enzyme I
EDTA	Ethylenediaminetetraacetic Acid
EtBr	Ethidium Bromide
F	Forward
g	Gram
G	Guanine
GITC	Guanidinium Thiocyanate
gm	Gram
h	Hour
hrs	Hours
kb	Kilobase Pairs
kV	Kilovolt
LB Medium	Luria – Bertani Medium
LMP	Low Melting Point
MID	Multiplex Identifier

Abbreviation	Description
min	Minute
ml	Milliliter
mM	Millimolar
ng	Nanogram
nm	Nanometer
No.	Number
OTU	Operational Taxonomic Units
PCR	Polymerase Chain Reaction
PPi	Pyrophosphate
R	Reverse
R	Purines (adenine or guanine)
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Revolutions per Minute
RT	Room Temperature
S.O.C.	Super Optimal Broth with Catabolite Repression
SDS	Sodium Dodecyl Sulfate
Sec	Second
StMQ	Sterile MilliQ
T	Thymine (5-methyluracil)
TAE	Tris-Acetate-EDTA-Buffer
Taq	<i>Thermus aquaticus</i>
Tris	Tris (hydroxymethyl) -aminomethane
U	Unit
UV	Ultraviolet radiation
V	Volt
x g	9.8 meter/second ² (acceleration caused by gravity)
Y	Pyrimidines (cytosine, thymine or uracil)

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SUMMARY

This thesis is on the *Archaea* and *Bacteria* from a hypersaline hyperalkaline lake sediment of a meteorite impact crater named Lonar. We have surveyed the active and the total biodiversity of the sediment from Lonar. We have detected biogeochemically related important taxa and functional genes from the sediment. We have constructed and screened metagenomic libraries for industrially relevant enzymes. We have also investigated this communities' ability to engineer its microenvironment in terms of pH.

The study site, Lonar crater lake represents a unique environment. As per its origin, Lonar is a meteorite impact crater and as per physicochemical parameters, it is hypersaline and hyperalkaline. This meteorite crater soda lake is located in the southern peninsula of Indian subcontinent known as Lonar lake. Main objectives were to investigate the total (DNA-based) and the active (RNA-based) biodiversity of *Archaea* and *Bacteria*, the presence of different taxa and genes (metagenome-based) with their role in biogeochemical cycles, and the ability of the microbial community to engineer their microenvironmental pH.

A total of 85,668 high-quality partial 16S rRNA gene sequences of archaeal and 182,137 sequences of bacterial origin were recovered and analyzed. In *Archaea*, the total and the active community diversity, a coverage of 74.21 % and 84.07% was observed. The total and the active community diversity of *Bacteria* showed a coverage of 59.78% and 88.98% respectively. Among the *Archaea* at the order level, most dominant taxa was *Halobacteriales*. *Halobacteriales* is mostly represented by *Natronococcus*, which was also the most dominant genera both in the total and the active community diversity. In the case of *Bacteria* most dominant phyla was *Firmicutes* and the genera were *Alkaliphilus* and *Bacillus*. Both of these genera represents *Firmicutes*. Upon comparison of all the previous studies on Lonar lake and this investigation, more than 67 % of all bacterial and archaeal genera are unique to this study and were not observed in previous investigations.

In our study, we have observed 24 genera, for example, *Methanosaeta* and *Methylobacterium*, which may have been involved in methane cycle. In *Archaea*, they contribute to an average of 39.24 % relative abundance in the active community. In the case of *Bacteria*, they contribute to an average of 0.50 % relative abundance in the active

community. A total of 16 genera, for example, *Ammonifex* and *Nitratireductor*, were found which may have been involved in the nitrogen cycle. Among *Archaea*, they contribute to an average of 1.37 % relative abundance in the active community. In the case of *Bacteria*, they contribute to an average of 2.87 % relative abundance in the active community. A significantly high diversity of bacterial genera, totaling 36, involved in the sulfur cycle were recorded, for example, *Desulfococcus* and *Thioalkalivibrio*. They represent an average relative abundance of 0.93 % relative abundance in the active community.

A total of 32 million paired-end reads were obtained from direct metagenome sequencing. Analysis of the metagenome resulted in 588,668 contigs, with a total number of base of 371 Mb (371,120,372 bases). Several ORFs involved in these biogeochemical cycles were detected. The predicted relative abundance of ORFs in relation to methane metabolism, nitrogen metabolism, and sulfur metabolism pathways were found to be 1.49 %, 0.50 %, and 0.68 %. Experimental data mapped on the reference pathways provides a comprehensive overall view of methane, nitrogen and sulfur metabolism in the sediments of Lonar crater lake.

A total of 235,943 archaeal and 1,657,168 bacterial partial 16S rRNA gene sequences were recovered from the different time point of the nonoptimal pH exposure of the sediments. The microbial community, from the Lonar meteorite crater soda lake sediments, was exposed to suboptimal and superoptimal pH conditions. The change of pH of the culture filtrate was monitored. Community dynamics was also measured at a resolution of 5 days for a total of 25 days using high-throughput 16S rRNA gene analysis. We observed an average coverage of 71.04 % in *Archaea* and 85.56 % in *Bacteria*. We have seen a 10-fold change in the initial hydrogen ion concentration difference to a point between suboptimal and superoptimal pH. Several archaeal and bacterial taxa at phylum (*Bacteria*) or order (*Archaea*) level and genus (both *Archaea* and *Bacteria*) level have been identified to modulate significantly upon exposure to nonoptimal pH. Several of them regained their original or extremely close to their original relative abundance with the progression of time. Also, from HPLC analysis, it is evident that metabolism of ammonia and hydroxyproline have a function in this community dynamics and eventual microenvironmental pH homeostasis. However, we were not able to confirm if this

observation is due to correlation or causality. It was also observed, that the dynamics of several archaeal and bacterial genera can be grouped in to different types of dynamic groups based on their changing relative abundances. We found two types of dynamic groups in *Archaea* and four types of dynamic groups in *Bacteria*. Considering, all these observations, it might be safe to speculate that this microbial community can change their microenvironment to a more favorable (hypothetical optimal) one in terms of pH at the same time resisting permanent change in its community structure.

Introduction

1 INTRODUCTION

1.1 STATE OF THE RESEARCH ON PROKARYOTES INHABITING SODA LAKES

This study on the Lonar meteorite crater soda lake at prima facia deals with soda lakes as subject and metagenomics as the approach to investigate this soda lake for getting answers to some fundamental questions. The questions like, what is the microbial community structure, what are the ecologically important functional genes and if this community can engineer their own environment. A survey was conducted to understand the attention of researchers on soda lakes in general and Lonar lake specifically. It was found that although the soda lakes have been subjected to scrutiny from as early as the beginning of the 1990s, the Lonar Crater Lake has been studied thoroughly only since 2006. In 2015, a total of 28 publications were found from all other soda lakes whereas 13 were found on Lonar. Considering the number of available literature from the previous years both are gaining contemporary relevance in recent past (Figure 1).

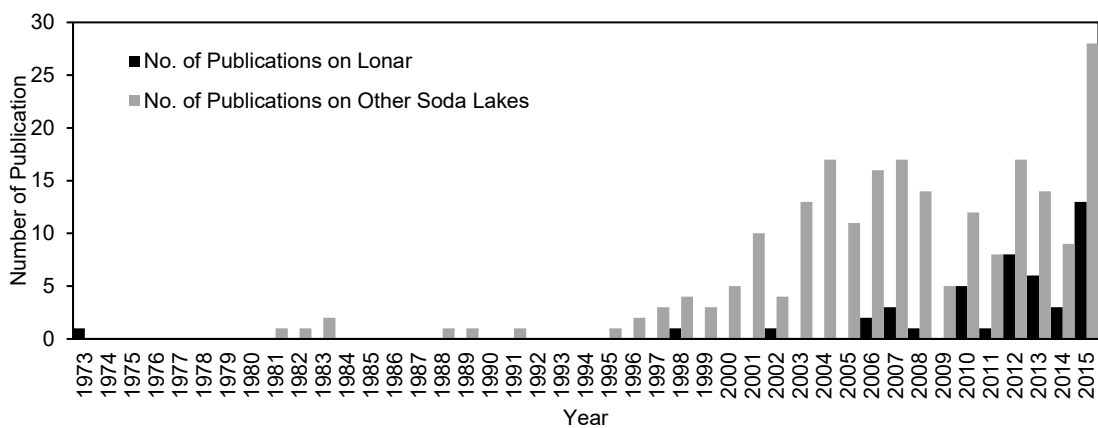


FIGURE 1: A COMPARISON OF INVESTIGATION EFFORT ON LONAR AND OTHER SODA LAKES.

A comprehensive review also revealed the crucial areas of investigations that have been subjected to examination by various researchers; it is tabulated in Table 1 and discussed later in this chapter. Most of the detailed biological analyses have been limnological rather than microbiological. The most vividly studied soda lakes are of the East African Rift Valley where detailed limnological and microbiological investigations have been carried out

since as early as the 1930s. Microbiological studies of Central Asian soda lakes have also been thoroughly documented.

TABLE 1: COMPREHENSIVE REVIEW OF AVAILABLE PUBLICATIONS ON SODA LAKES. The column on the left depicts the crucial areas of investigations and column on the right depicts the relevant studies.

Area of Contribution	Reference
Cultivation and isolation of Bacteria	(Duckworth, Grant et al. 1996, Ochsenreiter, Pfeifer et al. 2002, Surakasi, Wani et al. 2007, Dimitriu, Pinkart et al. 2008, Joshi, Kanekar et al. 2008, Mwirichia, Muigai et al. 2010)
Biodiversity of Soda Lakes	(Duckworth, Grant et al. 1996, Jones, Grant et al. 1998, Ochsenreiter, Pfeifer et al. 2002, Humayoun, Bano et al. 2003, Ma, Zhang et al. 2004, Rees, Grant et al. 2004, Tiago, Chung et al. 2004, Wani, Surakasi et al. 2006, Mesbah, Abou-El-Ela et al. 2007, Surakasi, Wani et al. 2007, Dimitriu, Pinkart et al. 2008, Joshi, Kanekar et al. 2008, Mwirichia, Cousin et al. 2010, Surakasi, Antony et al. 2010, Deshmukh, Pathak et al. 2011, Xiong, Liu et al. 2012)
Carbon cycle/utilization in Soda Lakes	(Oremland, Marsh et al. 1982, Sorokin, Jones et al. 2000, Kaluzhnaya, Khmelenina et al. 2001, Lin, Radajewski et al. 2004, Antony, Kumaresan et al. 2010, Antony, Doronina et al. 2012, Antony, Murrell et al. 2012, Nolla-Ardevol, Strous et al. 2012, Shetty, Marathe et al. 2013)
Nitrogen cycle/utilization in Soda Lakes	(Milford, Achenbach et al. 2000, Sorokin, Gijs Kuenen et al. 2001, Boltianskaia lu, Kevbrin et al. 2007, Shapovalova, Khijniak et al. 2008, Sorokin, van Pelt et al. 2009, Shao, Zhang et al. 2010)

Area of Contribution	Reference
Sulfur cycle/utilization in Soda Lakes	(Sorokin and Kuenen 2005, Zhilina, Zavarzina et al. 2005, Foti, Ma et al. 2006, Asao, Takaichi et al. 2007, Foti, Sorokin et al. 2007, Sorokin, Foti et al. 2007, Banciu, Sorokin et al. 2008, Sorokin, Tourova et al. 2008, Sorokin, van den Bosch et al. 2008, Gorlenko, Bryantseva et al. 2009, Sorokin and Muyzer 2010, Sorokin and Muyzer 2010, Sorokin, Rusanov et al. 2010, Asao, Pinkart et al. 2011, Sorokin, Detkova et al. 2011, Sorokin, Kuenen et al. 2011, Sorokin, Muntyan et al. 2012, Sorokin, Tourova et al. 2012, Sorokin, Tourova et al. 2012, Tourova, Slobodova et al. 2013)
Arsenic cycle in Soda Lakes	(Ciulla, Diaz et al. 1997, Hollibaugh, Budinoff et al. 2006, Kulp, Hoefl et al. 2006, Sorokin, Zhilina et al. 2006, Hoefl, Blum et al. 2007, Kulp, Han et al. 2007, Sorokin, Tourova et al. 2012)

1.1.1 PROKARYOTES ISOLATED FROM SODA LAKES

Prokaryotes have been isolated from various soda lakes as early as the end 1970's. In the year 1979, William D. Grant isolated an alkaliphilic species of *Ectothiorhodospira* from a Kenyan soda lake named Lake Hannington. It resembled *Ectothiorhodospira shaposhnikovii* in some aspects but contrasted in demonstrating more extreme alkaliphily, the pH optimum of pH 9.0 to pH 9.5, and in being obligatory phototrophic (Grant, Mills et al. 1979). Since then numerous prokaryotes have been isolated, and many novel genera and species of *Archaea* and *Bacteria* have also been reported from various soda lakes from around the world.

1.1.1.1 PROKARYOTIC BIODIVERSITY AND BIOGEOCHEMICAL CYCLES IN SODA LAKES

Regardless of the seemingly hostile circumstances enforced by high alkalinity and occasionally high salinity, soda lakes harbour alkaliphilic prokaryotes. These prokaryotes living and flourishing in the soda lakes are representatives of most of the major evolutionary and trophic groups of *Archaea* and *Bacteria* (Duckworth, Grant et al. 1996, Jones, Grant et al. 1998, Grant, Gerday et al. 2006). They are also associated with the active carbon, nitrogen, and sulfur cycling under the aerobic and anaerobic environment (Grant and Sorokin 2011, Sorokin, Berben et al. 2014). Conventionally, investigations on microbial communities were limited to only a few cultured isolates. A species of *Halobacterium* has been isolated from solar evaporation ponds and sodium sesquicarbonate deposits at Lake Magadi, Kenya. It differs from known species of *Halobacterium* in being obligate alkaliphilic with a pH optimum between pH 9.0 and pH 10.0 (Tindall, Mills et al. 1980). A new phototrophic bacterium, new species *Ectothiorhodospira vacuolata*, have been isolated from Jordanian and Kenyan alkaline salt lakes. They use sulfide and thiosulfate as photosynthetic electron donors. During the oxidation of sulfide to sulfate, this new species forms elemental sulfur which accumulates outside the cells. This species is strictly anaerobic, moderately halophilic and alkaliphilic (Imhoff, Tindall et al. 1981). An alpha-amylase-producing haloalkaliphilic archaeon *Natronococcus amylolyticus* sp. nov., has been isolated from Kenyan soda lakes (Kanal, Kobayashi et al. 1995). An extremely haloalkaliphilic, chemoorganotrophic, homoacetogenic bacteria, *Natroniella acetigena* gen. nov., sp. nov., has been isolated from Lake Magadi, Kenya. It is an obligate anaerobic *Bacteria* with optimal growth pH in the range of pH 9.7 to pH 10.0 (Zhilina, Zavarzin et al. 1996). An alkaliphilic, halotolerant microaerophilic bacteria, *Bogoriella caseilytica* gen. nov., sp. nov., with optimal growth pH values between pH 9 and pH 10 has been isolated from Lake Bogoria, Kenya (Groth, Schumann et al. 1997). A new alkaliphilic, sulfate-reducing bacterium, *Desulfonatronovibrio hydrogenovorans* gen. nov., sp. nov., has been isolated from Lake Magadi in Kenya with optimum growth pH between pH 9.5 to pH 9.7 (Zhilina, Zavarzin et al. 1997). An alkaliphilic anaerobic ammonifier, *Tindallia magadii* gen. nov., sp. nov., has been isolated from Lake Magadi, Kenya with optimum growth pH of 8.5 (Kevbrin, Zhilina

et al. 1998). An alkaliphilic acetogenic anaerobe, *Natronoincola histidinovorans* gen. nov., sp. nov., has been isolated from Lake Magadi, Kenya with a pH optimum of pH 9.4 (Zhilina, Detkova et al. 1998). A new strictly anaerobic purple sulfur bacterium, *Thiorhodospira sibirica* gen. nov., sp. nov., has been isolated from Lake Malyi Kasytui, southeast Siberia with pH optima of pH 9.0. This new bacterium under anoxic conditions uses hydrogen sulfide and elemental sulfur as photosynthetic electron donors (Bryantseva, Gorlenko et al. 1999). Two haloalkaliphilic strictly aerobic archaea, *Natronorubrum bangense* gen. nov., sp. nov. and *Natronorubrum tibetense* gen. nov., sp. nov., has been isolated from a soda lake in Tibet in 1999 with optimum growth pH between pH 9.0 and pH 9.5 (Xu, Zhou et al. 1999). An alkaliphilic obligatory phototrophic strictly anaerobic purple sulfur bacterium, *Thioalkalicoccus limnaeus* gen. nov., sp. nov., has been isolated from soda lakes in the steppe of southeast Siberia, Russia with optimal growth pH of pH 9 (Bryantseva, Gorlenko et al. 2000). An alkaliphilic purple nonsulfur bacterium, *Rhodobaca bogoriensis* gen. nov., sp. nov., has been isolated from African Rift Valley soda lakes Lake Bogoria and Crater Lake with the pH optimum for growth at pH 9 (Milford, Achenbach et al. 2000). Two new genera, *Thioalkalimicrobium* and *Thioalkalivibrio*, with two and three new species respectively, *Thioalkalimicrobium aerophilum* gen. nov., sp. nov., *T. sibiricum* sp. nov., and *Thioalkalivibrio versutus* gen. nov., sp. nov., *T. nitratis* sp. nov., *T. denitrificans* sp. nov., has been isolated from soda lakes in south-east Siberia, Russia and Kenya. They are all obligate alkaliphilic and obligate chemolithoautotrophic sulfur-oxidizing *Bacteria* (Sorokin, Lysenko et al. 2001). A novel lithoautotrophic sulfur-oxidizing alkaliphilic moderately halophilic bacterium, *Thioalkalispira microaerophila* gen. nov., sp. nov., has been isolated from Lake Fazda, Wadi Natrun, Egypt with optimum growth pH of pH 10 (Sorokin, Tourova et al. 2002). New alkaliphilic anaerobic fermentative bacteria, *Anoxynatronum sibiricum* gen. nov., sp. nov., has been isolated from the soda lake Nizhnee Belaye, Baikal with pH optima of pH 9.1 (Garnova, Zhilina et al. 2003). A new anaerobic alkaliphilic saccharolytic bacteria, *Alkaliflexus imshenetskii* gen. nov., sp. nov., have been isolated from the alkaline lake, Verkhneye Belaye of Central Asia with an optimum growth pH around pH 8.5 (Zhilina, Appel et al. 2004). Novel aerobic haloalkaliphilic archaea, *Natronolimnobius baerhuensis* gen. nov., sp. nov., and *N. innermongolicus* sp. nov., has been isolated from soda lakes in Inner Mongolia, China growing optimally between pH 9.0 and pH 9.5 (Itoh, Yamaguchi et al. 2005). A novel

alkaliphilic anaerobe, *Anaerovirgula multivorans* gen. nov., sp. nov., has been isolated from Owens Lake, California, USA with optimal growth pH of pH 8.5 (Pikuta, Itoh et al. 2006). An alkalitolerant moderately halophilic bacterium, *Salsuginibacillus kocurii* gen. nov., sp. nov., has been isolated from the Lake Chagannor in the Inner Mongolia Autonomous Region, China with pH optima of pH 8.5 (Carrasco, Marquez et al. 2007). Two novel alkaliphilic moderately halotolerant obligate anaerobes, *Dethiobacter alkaliphilus* gen. nov., sp. nov., and *Desulfurivibrio alkaliphilus* gen. nov., sp. nov., has been isolated from north-eastern Mongolian soda lakes and Wadi al Natrun lakes in Egypt able to grow between pH 8.5 and pH 10.3 (Sorokin, Tourova et al. 2008). One new genus and one new species of anaerobic halophilic alkaliphilic thermophilic bacteria, *Natronovirga wadinatronensis* gen. nov., sp. nov., and *Natranaerobius trueperi* sp. nov., has been isolated from lakes of the Wadi An Natrun, Egypt (Mesbah and Wiegel 2009). An obligate haloalkaliphilic obligate anaerobic dissimilatory sulfur-reducing bacterium, *Desulfurispira natronophila* gen. nov., sp. nov., has been isolated from soda lakes of Kulunda Steppe, Altai, Russia with a pH growth optimum from pH 10 to pH 10.2 (Sorokin and Muyzer 2010). One new haloalkaliphilic heterotrophic sulfate reducing bacterial genera and one species of *Desulfobulbus*, *Desulfonatronobacter acidivorans* gen. nov., sp. nov., and *Desulfobulbus alkaliphilus* sp. nov., have been isolated from hypersaline soda lakes in Kulunda Steppe Altai, Russia (Sorokin, Tourova et al. 2012). An obligate alkaliphilic halotolerant anaerobic bacterium, *Natranaerobaculum magadiense* gen. nov., sp. nov., has been isolated from Lake Magadi, Kenya with pH optima between pH 9.25 and pH 9.5 (Zavarzina, Zhilina et al. 2013). An extremely haloalkaliphilic archaeon, *Halostagnicola bangensis* sp. nov., has been isolated from Lake Bange in the region of Tibet, China (Corral, Corcelli et al. 2015).

With the advent of modern high-throughput sequencing techniques, the study of microbial community composition as a whole become more and more feasible. A significant number of cultured isolates has already been found from soda lakes, as described in the previous section. Culture-independent approaches are also uncovering a much more detailed and diverse microbial community from soda lakes. The complete range of soda lake prokaryotic community structure and the roles played by individual members of the community has not been entirely discovered. It is speculated that yet unrepresented phylogenetic groups will eventually prove to have soda lake members and

perhaps the elusive, exclusively soda lake group will emerge in the future (Sorokin, Berben et al. 2014).

1.2 SODA LAKE ENVIRONMENT

One of the most remarkable features of alkaline soda lakes is that irrespective of their apparent adverse conditions conferred by its extreme alkaline pH and often high salinity, they are one of the most productive aquatic ecosystems on Earth (Melack and Kilham 1974). Soda lakes are naturally occurring alkaline environments. They characterize the most alkaline, natural environments on earth, mostly between pH 8.5 to pH 10, occasionally reaching as high as pH 12 (Grant and Jones 1992). The existence of large amounts of sodium carbonate or complexes of it distinguishes soda lakes. With the progression of evaporative concentration alkalinity develops as the concentration of CO_3^{2-} exceeds the concentration of Mg^{2+} and Ca^{2+} . As a consequence of this process, a shift in the $\text{CO}_2/\text{HCO}_3^-/\text{CO}_3^{2-}/\text{OH}^-$ equilibrium is observed (Grant, Gerday et al. 2006). Soda lakes occur throughout the geological record. At Green River formation in Wyoming and Utah, one of the largest and between 36 and 55 million years old fossil soda lakes are reported. Geological formations at Ventersdorp formation of South Africa advocate up to 2.3 billion-year-old fossil soda lakes (Grant and Jones 1992). Distribution of soda lakes spread throughout the world. Some of them are listed in Table 2.

Several soda lakes have been studied extensively, but they still represent only a small fraction of all the soda lakes distributed on different continents. Among the best studied soda lakes are the soda lakes of East African rift valley (Grant, Mwatha et al. 1990, Duckworth, Grant et al. 1996, Jones, Grant et al. 1998), Mono Lake in California (Ward, Martino et al. 2000, Humayoun, Bano et al. 2003), hyper alkaline spring waters in Maquqrin, Jordan (Pedersen, Nilsson et al. 2004, Tiago, Chung et al. 2004), Inner Mongolian Baer soda lake (Ma, Zhang et al. 2004, Dadheech, Glockner et al. 2013) and Kenyan soda lakes (Rees, Grant et al. 2004, Mwirichia, Cousin et al. 2010).

TABLE 2: GLOBAL DISTRIBUTION OF SODA LAKES

Continent	Country	Name	pH
Africa	Egypt	Wadi El Natrun lakes	8.5-9.5
	Sudan	Malha Crater Lake	9.5-10.3
	Ethiopia	Lake Arenguadi	9.5-9.9
	Ethiopia	Lake Basaka	9.6
	Ethiopia	Lake Shala	9.8
	Ethiopia	Lake Chitu	10.3
	Ethiopia	Lake Abijatta	9.9
	Kenya	Lake Magadi	10
	Kenya	Lake Bogoria	10.5
	Kenya	Lake Turkana	8.5-9.2
	Kenya	Lake Nakuru	10.5
	Kenya	Lake Logipi	9.5-10.5
	Kenya	Lake Sonachi	10.4
	Tanzania	Lake Manyara	9.5-10
	Tanzania	Lake Natron	9-10.5
	Tanzania	Lake Rukwa	8.0-9.0
	Tanzania	Lake Eyasi	9.3
	Botswana	Lake Ngami	9
	Chad	Rombou Lake	10.2
	Asia	Russia	Kulunda Steppe Lakes
Russia		Lake Khatyn	10
Turkey		Lake Van	9.7-9.8
Turkey		Lake Salda	9
Iran		Lake Urmia	7.35-8.45
India		Lonar Lake	9.5-10.5
India		Sambhar Salt Lake	9.5
India		Khyagar Lake	9.5
India		Tso Moriri Salt Lake	9
India		Tso Kar Salt Lake	8.8
Aksai Chin	Lake Surigh Yilganing Kol	NA	
Aksai Chin	Tso Tang Lake	NA	

Continent	Country	Name	pH
	Aksai Chin	Aksayqin Hu Lake	NA
	Aksai Chin	Lake Hongshan Hu	NA
	Aksai Chin	Tianshuihai lake	NA
	Aksai Chin	Guozha lake	NA
	Aksai Chin	North Tianshuihai lake	NA
	India & China	Pangong Salt Lake	9.4
	India & China	Spanggur Tso	NA
	China	Qinghai Lake	9.3
	China	Namucuo Lake	9.4
	China	Lake Zabuye	10
	Mongolia	Taboos-nor	NA
Australia	Australia	Lake Werowrap	9.8
Europe	Hungary	Lake Fehér	NA
	Hungary	Böddi-szék	8.8-9.8
	Austria, Hungary	Lake Neusiedl	9-9.3
	Serbia	Rusanda	9.3
	Hungary	Kelemen-szék	9-9.7
North America	USA	Mono Lake	9.8
	USA	Big Soda Lake	9.7
	USA	Soap Lake	9.7
	USA	Alkali Lake	11
	USA	Summer Lake	NA
	USA	Owens Lake	9.1-9.7
	USA	Borax Lake	NA
	Canada	Manitou Lake	NA
	Canada	Goodenough Lake	10.2
	Mexico	Lake Texcoco	8.8-11.5
	Mexico	Lake Alchichica	8.9
South America	Chile	Antofagasta Lake	NA

1.2.1 ORIGIN OF SODA LAKES

The soda lakes are a class of lakes with waters showing an excess of the total alkalinity, $TA = \text{HCO}_3^- + 2\text{CO}_3^{2-}$, (the total of the charges of the bicarbonate ion and carbonate ion) over the charges of the alkaline earth metal ions magnesium and calcium; $\text{HCO}_3^- + 2\text{CO}_3^{2-} > 2\text{Mg}^{2+} + 2\text{Ca}^{2+}$. When the water of this property evaporates the high concentration of CO_3^{2-} will cause a rise in pH making the water alkaline. The perfect condition for the establishment of a soda lake has a lot in common with those for the creation of a thalassohaline salt lake. However, in soda lakes; carbonate or complexes of it becomes the major anion in the solution. The most significant contributing feature for creation of a soda lake is the lack of alkaline earth metal ions, (cations, Ca^{2+} and Mg^{2+}) in the nearby topography, which basically means a lack of rocks of sedimentary origin. Additional conditions require a formation of a closed drainage basin of shallow depression with a high marginal relief, also with sufficient rainfall to sustain the source of water entering the basin to construct a standing body of water. In arid zones through higher rates of evaporation than the rate of inflow, salts accumulate by evaporative concentration. For example, in the Rift Valley of Kenya-Tanzania, the graben is composed of Pleistocene alkaline trachyte lavas which are high in Na^+ , low in Ca^{2+} and low in Mg^{2+} . Under these conditions in the groundwater of meteoric origin (water derived from precipitation), saturated with CO_2 , the molar concentration of $\text{HCO}_3^- / \text{CO}_3^{2-}$ greatly exceeds that of $\text{Ca}^{2+} / \text{Mg}^{2+}$. Saturation of the alkaline earth metal cations is rapidly achieved as a result of evaporation in this arid tropical zone, and they precipitate out of solution in the form of insoluble carbonates leaving Na^+ , Cl^- , and $\text{HCO}_3^- / \text{CO}_3^{2-}$ as the major ions in solution.

1.2.2 PH AS A MAJOR ENVIRONMENTAL VARIABLE

There has been a detailed investigation of how pH affects bacterial growth. One particular problem with pH-growth models is that they are not 1:1. Two different values of pH can give the same growth rate. Considering the definition of pH, at face value, both a high and low concentration of hydrogen ions influence the growth rate similarly (Lambert 2011).

Initially, the pH-growth models were exponential or square root models later they have been moved to cardinal polynomial models. Most widely used standard model for the effect of pH is known as the CPM (Cardinal pH Model) or based on CPM. Cardinal pH Model was introduced by Rosso et al. in 1995 (Rosso, Lobry et al. 1995). In later year other models were also proposed, in 1997 by Presser et al. (Presser, Ratkowsky et al. 1997), in 2000 by Tienungoon et al. (Tienungoon and Ratkowsky 2000) and another one by Lambert and Pearson (Lambert and Pearson 2000), then in 2003 by Lambert et al. known as extended Lambert–Pearson model (Tienungoon and Ratkowsky 2000, Lambert and Lambert 2003). However, none of them explain the effect of pH at the community level, or they prove to fit accurately at high alkaline pH. Most of these studies were conducted keeping issues of food quality and pathogens in mind.

The pH of naturally existing environments can contrast extensively from approximately pH 0.6 to almost pH 12.6 (Becking, Kaplan et al. 1960). pH value can drive or shape prokaryotic communities. In research of lake sediments of Tibetan Plateau, no correlation between the relative abundance of *Acidobacteria* and *Bacteroidetes* and pH was found (Xiong, Liu et al. 2012). In another study of sediments and other samples from springs in Western Canada (Boström, Pettersson et al. 1989) and the Taupo Volcanic Zone, New Zealand (Giggenbach 1995) by C. E. Sharp et al. found that pH can only explain variability from 13 % to 20 % (Sharp, Brady et al. 2014). These are the reasons; the significance of pH cannot be seen as the lone limiting environmental influence on bacterial communities.

1.3 ELEMENT CYCLING IN THE SODA LAKE ENVIRONMENT

1.3.1.1 CARBON CYCLE

Photosynthetic primary production seems to have an imperative part in the soda lakes in supporting all other microbial community. Oxygenic and anoxygenic haloalkaliphilic phototrophs and chemolithoautotrophs, autotrophic primary producers, living in the soda lakes are capable of fixing inorganic CO₂ into organic polymers. The primary production in most soda lakes is high due to a dense population of haloalkaliphilic *Cyanobacteria*. They

are primitive taxa of photosynthetic prokaryotic microorganisms (Wood, Rueckert et al. 2008). *Cyanobacteria* have existed on this planet for around 2.8 billion years (Olson 2006). Dense blooms of *Cyanobacteria* usually dominate the less alkaline lakes. Hypersaline soda lakes support both *Cyanobacteria* and alkaliphilic anoxygenic phototrophs belonging to the genera *Ectothiorhodospira* and *Halorhodospira* (Grant, Mwatha et al. 1990, Jones, Grant et al. 1998). Several cyanobacteria from the orders *Nostocales* and *Chroococcales* have also been reported from the highly alkaline lake, Santa Olalla, southwest of Spain (Lopez-Archilla, Moreira et al. 2004). The cyanobacterial members mostly, *Oscillatoriales*, *Nostocales* and *Chroococcales*, which are dominant in tropical soda lakes in Kenya and Ethiopia, include the genera *Arthrospira*, *Microcoleus*, *Lyngbya*, *Oscillatoria*, *Trichodesmium*, *Anabaenopsis*, *Cyanospira* and *Synechococcus* (Ballot, Kotut et al. 2009, Krienitz, Dadheech et al. 2012, Dadheech, Glockner et al. 2013, Schagerl, Burian et al. 2015). Cyanobacterial genera *Leptolyngbya* was also reported from Lake Arenguadi of Ethiopia (Lanzen, Simachew et al. 2013). Anoxygenic phototrophic purple bacteria, other than *Cyanobacteria*, also produce organic matter (Kompantseva, Sorokin et al. 2005, Kompantseva 2007, Nuianzina-Boldareva and Gorlenko 2014). The most thoroughly studied alkaliphilic purple *Bacteria* are from Lake Wadi-el-Natron, Egypt (Imhoff, Sahl et al. 2009), the lakes of the Kenyan Rift Valley (Tindall, Mills et al. 1980) and the lakes of the southeastern Transbaikal region (Kompantseva, Sorokin et al. 2005). Also two new strictly anaerobic obligate phototrophic purple sulfur bacteria, *Thiorhodospira sibirica* and *Thioalkalicoccus limnaeus*, were isolated from low saline soda lakes in the steppe of southeast Siberia (Bryantseva, Gorlenko et al. 1999, Bryantseva, Gorlenko et al. 2000). Under anoxic conditions, these *Bacteria* use hydrogen sulfide and elemental sulfur as photosynthetic electron donors. The haloalkaliphilic members of *Chromatiales* like *Thiorhodospira* at moderate salinity and *Ectothiorhodospiraceae* like *Ectothiorhodospira* and *Halorhodospira* at high salinity, also represent anoxygenic phototrophs which contribute to the primary production in soda lakes (Bryantseva, Gorlenko et al. 1999, Gorlenko, Briantseva et al. 2004, Kovaleva, Tourova et al. 2011). Also, aerobic chemolithoautotrophic *Bacteria* contribute to inorganic carbon fixation in soda lakes (Sorokin, Berben et al. 2014). Very few purple nonsulfur *Bacteria* also have been reported from soda lakes with low mineralization. The *Rhodobaca bogoriensis*, is capable of both, phototrophic and chemotrophic growth have been isolated from Lake Bogoria, Kenya,

(Milford, Achenbach et al. 2000). Also, two more alkaliphilic heliobacteria, *Heliorestis daurensis* and *H. baculata* have been isolated from Siberian soda lakes able to grow photoheterotrophically (Bryantseva, Gorlenko et al. 1999, Bryantseva, Gorlenko et al. 2000). Also, *Roseinatronobacter thiooxidans*, another alkaliphilic Bacteriochlorophyll-a containing *Bacteria*, has been isolated from Siberian low-salt soda lakes (Sorokin, Turova et al. 2000).

The primary degradation of organic matter, which is formed by the autotrophic *Bacteria*, is performed by the heterotrophic *Bacteria*. These heterotrophic *Bacteria* comprise of aerobes and fermentative anaerobes. These fermentative anaerobes, in turn, are composed of two subgroups, hydrolytics, and secondary heterotrophs. The hydrolytics accomplish degradation of polymers, and the resulting monomers are utilized by the secondary heterotrophs (Sorokin, Berben et al. 2014). Several known isolates, performing aerobic hydrolytics, were recovered from soda lakes. They mostly include aerobic *Firmicutes*, such as *Bacillus*, *Amphibacillus*, *Clostridium*, *Natronoincola* and several *Actinobacteria*, such as *Cellulomonas*, *Dietzia*, *Rathayibacter*, *Microbacterium* and *Proteobacteria*, such as *Alkalimonas*, *Alcalilimnicola* (Martins, Davids et al. 2001, Yakimov, Giuliano et al. 2001, Sorokin, Tourova et al. 2002, Humayoun, Bano et al. 2003, Grant, Sorokin et al. 2004, Carrasco, Marquez et al. 2007, Wu, Zheng et al. 2010, Grant and Sorokin 2011, Wang, Huang et al. 2014). The occurrence of haloalkaliphilic chitinolytic microbial community in hypersaline soda lakes has been described like, *Marinimicrobium* from hypersaline soda lakes of Kulunda Steppe, Altai, Russia (Sorokin, Tourova et al. 2012). An anaerobic low salt-tolerant cellulolytic *Clostridium* has also been reported from soda lakes (Zhilina, Kevbrin et al. 2005, Zvereva, Fedorova et al. 2006). Pectin utilizing anaerobic haloalkaliphiles *Natronoflexus*, and *Natronovirga* have been isolated from soda lakes of the Kulunda Steppe, Altai, Russia (Sorokin, Panteleeva et al. 2011, Sorokin, Tourova et al. 2012). A fermentative haloalkaliphilic bacteria, *Chitinivibrio alkaliphilus*, specialized in exclusive chitin utilization have been isolated from soda lakes (Sorokin, Gumerov et al. 2014). The most detailed studied groups of aerobic haloalkaliphiles are secondary heterotrophs isolated from soda lakes. They are capable of utilizing monomeric organic compounds, sugars, amino acids, organic acids and alcohols. The genus *Halomonas* from the *Proteobacteria*, *Bacillus* from the *Firmicutes*, and *Actinobacteria* are

the most abundant haloalkaliphilic aerobes (Duckworth, Grant et al. 1996, Grant and Sorokin 2011). Most dominated anaerobic haloalkaliphilic heterotrophic genera found in soda lakes are *Anaerobacillus*, *Alkaliphilus*, *Anoxynatronum*, *Anaerovirgula*, *Anaerobranca*, *Natranaerobius*, *Natranaerobaculum* and *Spirochaeta* (Zavarzin, Zhilina et al. 1999, Zavarzin and Zhilina 2000, Bowers, Mesbah et al. 2009, Grant and Sorokin 2011, Mesbah and Wiegel 2012). The homoacetogens and methanogens are the less exhaustively studied functional groups of secondary anaerobes performing the last stage of organic carbon degradation in soda lake microbial communities (Sorokin, Berben et al. 2014). They mostly include *Tindallia*, *Natronincola*, and *Natroniella* represent heterotrophic fermentative haloalkaliphilic acetogens, utilizing amino acids and alcohols as substrates (Zhilina, Zavarzin et al. 1996, Kevbrin, Zhilina et al. 1998, Zhilina, Detkova et al. 1998, Pikuta, Hoover et al. 2003, Alazard, Badillo et al. 2007). The only culturable haloalkaliphilic obligately anaerobic hydrogenotrophic homoacetogen from soda lake, *Fuchsiella alkaliacetigena* of the order *Halanaerobiales*, have been reported in 2012 from soda lake Tanatar III, Altay, Russia (Zhilina, Zavarzina et al. 2012).

There is sufficient evidence for biogenic methane production in soda lakes. The methane cycle has been studied in soda lakes as a significant part of the microbial carbon cycle. Among the most detailed studied ones are North American and Central Asian soda lakes (Oremland, Marsh et al. 1982, Oremland, Cloern et al. 1988, Khmelenina, Eshinimaev et al. 2000, Lin, Radajewski et al. 2004, Nolla-Ardevol, Strous et al. 2012, Sorokin, Abbas et al. 2015). One carbon compounds are most probably abundant in soda lakes due to anaerobic degradation of cyanobacterial mats. The strains of haloalkaliphilic methanogens have been isolated, as early as the 1980s, from Lake Wadi-el-Natron, Egypt (Boone, Worakit et al. 1986). Most of the prokaryotes, involved in methane cycle, isolated from various soda lakes are related to members of the family *Methanosarcinaceae* within the phylum *Euryarchaeota*. Most of the methanogenic *Archaea* isolated so far are mainly methylotrophic. They use a variety of one-carbon (C1) compounds like methanol and methylamine. These particular methanogens are mostly represented by the genus *Methanocalculus*, *Methanosalsum*, and *Methanolobus* (Antony, Murrell et al. 2012, Sorokin, Abbas et al. 2015, Sorokin, Abbas et al. 2015). Hydrogen-utilizing methanogens, *Methanobacterium* spp, has also been reported from soda lakes (Nolla-Ardevol, Strous et

al. 2012). The bacterial genera like *Methylobacter* and *Methylomicrobium* are methane-oxidizing prokaryotes (Sorokin, Jones et al. 2000, Kaluzhnaya, Khmelenina et al. 2001). They utilize methane in soda lakes under aerobic or microaerophilic conditions and return carbon of methane to the soda lakes organic matter by methanotrophy.

1.3.1.2 NITROGEN CYCLE

The contribution of *Cyanobacteria* in soda lake is not only limited to primary production but also they are well-known nitrogen fixers. Undoubtedly heterocystous *Cyanobacteria* such as *Anabaenopsis*, *Cyanospira* and *Nodularia* as observed in soda lakes of the African Rift Valley are active in atmospheric nitrogen fixation (Florenzano, Sili et al. 1985, Krienitz, Dadheech et al. 2012, Sorokin, Berben et al. 2014). Nonheterocystous *Cyanobacteria*, like *Oscillatoria*, have also been recorded from soda lakes to fix nitrogen as members of diazotrophic microbial communities in alkaline hypersaline Mono Lake, California (Oremland 1990). Heterotrophic anaerobic fermentative haloalkaliphiles, *Bacillus alkalidiazotrophicus* and *Natronobacillus azotifigans*, are known to fix nitrogen actively in soda lakes of the Kulunda Steppe, Altai, Russia and north-eastern Mongolia (Sorokin, Kravchenko et al. 2008, Sorokin, Kravchenko et al. 2008, Sorokin, Zadorina et al. 2008). In some other soda lakes anaerobes show the presence of the *nifH* gene, for example, *Geoalkalibacter ferrihydriticus* and *Clostridium alkalicellulosi* (Zhilina, Kevbrin et al. 2005, Zavarzina, Kolganova et al. 2006). Anoxygenic phototrophs may also contribute to nitrogen fixation in soda lakes as the *nifH* gene has been detected in several cases (Tourova, Spiridonova et al. 2007). Several heterotrophs are known for denitrification in soda lakes. They are represented by extremely halotolerant alkaliphiles of the genus *Halomonas*, facultative anaerobic lithotrophs of the genus *Thioalkalivibrio* and the *Alkalilimnicola*–*Alkalispirillum* group belonging to the class *Gammaproteobacteria* (Sorokin, Zhilina et al. 2006, Shapovalova, Khijniak et al. 2008, Shapovalova, Khijniak et al. 2009, Berben, Sorokin et al. 2015). In soda lakes, for example, Mongolian soda lakes, ammonium produced during nitrogen fixation is oxidized to nitrite by prokaryotes like a subpopulation of *Nitrosomonas halophila* and this nitrite is further oxidized to nitrate by

Nitrobacter alkalicus (Sorokin, Muyzer et al. 1998, Sorokin, Tourova et al. 2001, Sorokin and Kuenen 2005).

1.3.1.3 SULFUR CYCLE

The sulfur cycle has been studied with significant details in soda lakes (Foti, Sorokin et al. 2007, Sorokin, Kuenen et al. 2011, Sorokin, Berben et al. 2014). Sulfidogenesis is a typical and important biogeochemical processes in soda lakes (Sorokin, Rusanov et al. 2010). Oxidized sulfur compounds for example sulfate, sulfite, thiosulfate, and sulfur are converted into sulfide during dissimilatory reduction. Dissimilatory reduction of oxidized sulfur is performed by several obligatory anaerobic and obligatory haloalkaliphilic *Bacteria*. Most investigated ones are represented by the members of the *Deltaproteobacteria* such as genera *Desulfonatrum*, *Desulfonatrovibrio*, and *Desulfonatrospira* represent lithotrophic sulfate-reducing *Bacteria* in soda lakes (Zhilina, Zavarzin et al. 1997, Pikuta, Hoover et al. 2003, Zhilina, Zavarzina et al. 2005, Sorokin, Tourova et al. 2008, Sorokin, Tourova et al. 2011, Sorokin, Tourova et al. 2012, Zakharyuk, Kozyreva et al. 2015). Hydrogen, formate or short chain organic compounds are used as electron donor and sulfate, thiosulfate or sulfite as an electron acceptor by the sulfate-reducing *Bacteria* (SRB) of soda lakes to obtain energy by oxidation. Disproportionation of thiosulfate or sulfite is also used by SRB to obtain energy (Sorokin, Tourova et al. 2008, Sorokin, Kuenen et al. 2011). Incompletely oxidizing heterotrophic SRB utilizes either propionate as in the case of *Desulfobulbus alkaliphilus* or butyrate as in the case of *Desulfobotulus alkaliphilus* as electron donor or carbon source and sulfate or thiosulfate as electron acceptor, in the process they form acetate as the end product (Sorokin, Detkova et al. 2010, Sorokin, Tourova et al. 2012). Completely oxidizing SRB is also reported from soda lakes, for example, *Desulfonatrobacter acidivorans*. It can oxidize several volatile fatty acids completely to CO₂ with sulfate or thiosulfate as an electron acceptor (Sorokin, Tourova et al. 2012). Reduction of elemental sulfur in soda lakes is accomplished by diverse obligatory anaerobic haloalkaliphiles. They can use polysulfide formed abiotically at high pH as an electron acceptor. They include *Desulfurispira natronophila* of the phylum *Chrysiogenetes*, *Desulfuribacillus*

alkaliarsenatis and *Natroniella sulfidigena* of the phylum *Firmicutes* (Sorokin and Muyzer 2010, Sorokin, Detkova et al. 2011, Sorokin, Tourova et al. 2012).

Alkaliphilic anaerobes from soda lakes have been reported to be able to grow chemolithoautotrophically by sulfur or polysulfide disproportionation, for example *Dethiobacter alkaliphilus* and *Desulfurivibrio alkaliphilus* (Sorokin, Tourova et al. 2008, Poser, Lohmayer et al. 2013).

Phototrophic sulfur oxidizing *Bacteria* (SOB) oxidize sulfide, produced by sulfidogens, to elemental sulfur or sulfate (Sorokin and Kuenen 2005). They are dominated by anoxygenic purple sulfur *Bacteria* mostly represented by members of the genera *Ectothiorhodospira*, *Halorhodospira*, *Thiorhodospira*, *Thioalkalicoccus*, and *Ectothiorhodosinus* (Imhoff, Tindall et al. 1981, Bryantseva, Gorlenko et al. 1999, Bryantseva, Gorlenko et al. 2000, Gorlenko, Briantseva et al. 2004, Gorlenko, Bryantseva et al. 2009, Sorokin, Kuenen et al. 2011). Other than the phototrophic SOB in soda lakes chemotrophic SOB are also dominant. They can use reduced sulfur compounds such as sulfide, polysulfide, thiosulfate, polythionates, and elemental sulfur as the electron donor (Sorokin, Kuenen et al. 2001, Sorokin, Tourova et al. 2002, Banciu, Sorokin et al. 2004). Several chemotrophic SOB has been reported to be haloalkaliphilic *Gammaproteobacteria* such as *Thioalkalimicrobium*, *Thioalkalispira*, *Thioalkalivibrio* and *Thioalkalibacter* (Sorokin, Lysenko et al. 2001, Sorokin, Tourova et al. 2002, Banciu, Sorokin et al. 2004, Foti, Ma et al. 2006, Banciu, Sorokin et al. 2008, Sorokin, Banciu et al. 2013).

1.4 UNIQUE NATURE OF LONAR

The Lonar crater lake is a 50,000 year (Approximately) old impact structure it is situated in the Buldhana district of Maharashtra, India (Nayak 1972, Fredriksson, Dube et al. 1973). This lake is a roughly circular depression in the basalt flows of the Deccan Traps. It is 1830 m across and is almost 150 m deep; a shallow alkaline saline lake occupies the majority of the floor. In the region of most of the circumference, the rim is raised about 30 m above the nearby plain (Fudali, Milton et al. 1980). A view of the Lonar Crater Lake is provided in Figure 2.



FIGURE 2: VIEW OF THE LONAR CRATER LAKE FROM THE TOP OF THE IMPACT EJECTA. Photograph by Soumya Biswas.

This comparatively small crater is one of only two acknowledged (so far) terrestrial craters to be created in basaltic target rock surface by a meteorite impact; the other one is the Logancha crater in Russia (Feldman, Sazonova et al. 1983). The target rocks are mostly classified as quartz-normative tholeiites that contain a moderate level of iron enrichment. These tholeiitic basalts comprise higher total iron, lower MgO, lower Al₂O₃, and higher CaO content than do other terrestrial tholeiitic provinces (Roy and Chatterjee 1998). The unusual compositional characteristics of the Lonar crater strongly match the composition of Martian basalts, which also have higher Fe and lower Al abundances than most terrestrial basalts (McSween 1994, McSween 2002). Another fascinating characteristic of the Lonar crater was documented in 1996 by Nayak, who suggested that the groundwater underlying the Lonar crater was heated by remnant impact energy, thus resulting in the establishment of post-impact hydrothermal activity. The validity of this hydrothermal hypothesis is supported by evidence of impact-induced hydrothermal systems in other terrestrial craters (Newsom 1980, Allen, Gooding et al. 1982, Crossey and McCarville 1993).

As per our observation on available literature regarding Lonar, we found some general trend. There is a paucity of available literature on ecologically important taxa and ecologically important functional genes present in Lonar. A lack of using metagenomics in general or high throughput sequencing specifically to survey the microbial diversity is prominent. Therefore, it is reasonable to consider this study a timely endeavor to investigate Lonar Meteorite Crater Soda Lake using metagenomics.

1.5 BIODIVERSITY AND RIBOSOMAL RNA GENES

The most exclusive quality of the planet Earth is the existence of life, and the most amazing characteristic of life is its diversity. Approximately two decades ago, at the first Earth Summit (The United Nations Conference on Environment and Development held in Rio de Janeiro on 3rd June to 14th June 1992) the vast majority of the world's nations acknowledged that human actions were dismantling the Earth's ecosystems, eliminating species, biological traits and genes at a shocking rate (Cardinale, Duffy et al. 2012). To be able to study diversity or the number and variety of organisms found within a specified geographic region it is of utmost importance to identify, classify and catalog these variations and this is the sole objective of biosystematics.

The principal purpose of bacterial systematic is the founding of a classification that spans the whole of the prokaryote kingdom. However, classification by conventional techniques has been proved to be difficult for prokaryotes, due to the relative morphological simplicity. Diverse molecules have been discussed, for a long time, in relation to their suitability for providing the ground for a molecular phylogeny. rRNA genes had been used widely to identify and classify prokaryotes. The ribosome is undoubtedly of a very ancient origin and is essentially everywhere. The primary structures of these rRNA molecules are satisfactorily constrained that on the whole, they have not altered rapidly over time (Fox, Pechman et al. 1977). They include regions of both extremely conserved regions (Woese, Fox et al. 1975) and hypervariable regions (Sogin, Sogin et al. 1971) so that both distant and close relationships can be investigated using rRNA genes. Earlier alignments of bacterial 16S rRNA gene sequences have discovered nine separate hypervariable regions, which was termed as V1 to V9 in relation to previous nomenclature (Van de Peer, Chapelle et al. 1996).

1.6 COMMUNITY DYNAMICS AND MICROENVIRONMENTAL PH HOMEOSTASIS

Microorganisms can endure and rapidly adapt to non-optimal and fluctuating environmental situations. They are small in size, ubiquitous in distribution, versatile in metabolism, flexible in adaptation and demonstrates genetic plasticity. Prokaryotes sense their environment and react to specific environmental challenges individually as well as cooperatively, demonstrating communal activities. In most of the microbial ecosystems, the functionally the active unit is not a solitary species but a consortium of many species sharing a common microenvironment (Guerrero and Berlanga 2006). Rich biodiversity stabilizes microbial ecosystems. Richness stabilizes communities mainly by increasing community biomass and reducing the strength of demographic stochasticity and also with asynchrony in the responses of the community dwellers to environmental fluctuations (de Mazancourt, Isbell et al. 2013). Various environmental variables largely shape community structure and community dynamics. However, one issue concerning this question is apparently under investigated; that is, if and to what extent microbial community can affect its environment or microenvironment in the context of microbial ecology. The microenvironment is a comparatively small, often noticeably specialized and effectively isolated biophysical environment surrounding a living organism. pH is one of the most significant of these environmental variables.

1.7 METAGENOMICS

A revolution took place in the field of microbiology during the last 30 years, which has changed microbiologist's view of microorganisms and how to study them. Accepting the fact that most microorganisms cannot be grown willingly in pure culture (till date) and the credible demonstration that the uncultured microbial world is greater than the cultured world along with evidence that these uncultivable microorganisms can be studied was at the heart of this revolution (Olsen, Pace et al. 1985, Pace, Stahl et al. 1985, Pace 1995). In the following years of this essential addition to the revolutionary approach to studying microbial life forms, microbiologists devoted earnest attempt to describe the

phylogenetic diversity of exotic and usual environments like sea surfaces, deep sea hydrothermal vents, hot springs, soils, animal rumens and guts, human oral cavity and intestine. Several new lineages were classified exclusively based on their molecular signatures. The next development that followed was to reveal the functions of these new phylotypes and conclude whether they epitomized new species, genera, or phyla of prokaryotic life. These questions triggered the development of diverse techniques, including metagenomics itself.

During the last ten years, the science of metagenomics has been transformed by the use of whole genome shotgun sequencing technology that a decade or so earlier revolutionized the field of the single organism genomics (Fleischmann, Adams et al. 1995). More current advances in the next generation sequencing technologies, causing a remarkable drop in the price of DNA sequencing, resulted in efforts on the scale considerably surpassing the scale permissible by the conventional technologies in DNA (genome, genes, cDNA and whole transcriptome) sequencing. Previously unfeasible questions in microbiology, turn out to be possible to address, due to these developments. They have also been demonstrated to be useful to accelerate considerably genome-based detection for medical and biotechnological applications of microbial, previously untapped, resources by delivering a comprehensive and high-resolution blueprint of a variety of biochemical transformations that has been evolved and fine-tuned by nature since the emergence of life itself. In the last few years, the study of uncultured microorganisms had expanded from just the study of diversity to their function and application.

Objective and Importance

2 OBJECTIVE AND IMPORTANCE

The overview of the workflow from sediment sample to objectives is provided in a simple flow chart below (Figure 3).

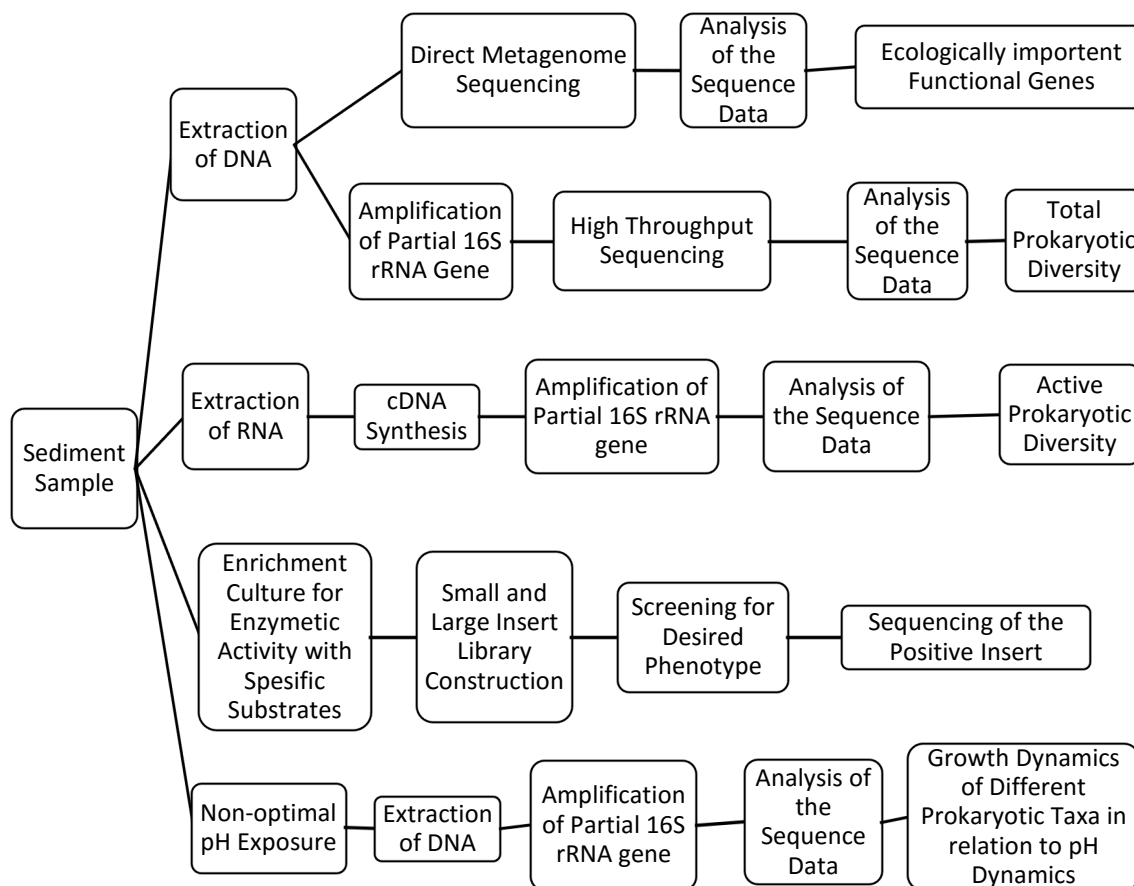


FIGURE 3: OVERVIEW OF THE OBJECTIVES AND WORKFLOW. The figure shows the workflow from samples to objectives.

2.1 OBJECTIVES OF THE INVESTIGATION

Our primary objectives of the current investigation were-

1. To assess the total prokaryotic biodiversity and the active prokaryotic biodiversity using high-throughput sequencing-based techniques,
2. To assess the relative abundance and presence of different ecologically important functional genes using high-throughput sequencing based direct metagenome analysis,

3. To study the community dynamics during microenvironmental pH homeostasis of the prokaryotic community from the hyperalkaline and saline Lonar meteorite crater lake in India.
4. To investigate any possibilities of exploiting the polyextremophilic adaptations of the microbial community for industrial use.

2.2 IMPORTANCE OF THIS INVESTIGATION

Sediment microbial communities play important roles in ecosystem functioning and processes such as biogeochemical cycles and nutrient transformation (Nissenbaum 1975, Whitby, Saunders et al. 2001, Foti, Sorokin et al. 2007, Emmerich, Bhansali et al. 2012, Melton, Schmidt et al. 2012, Zeng, Zhao et al. 2012, Li, Jin et al. 2013, Vissers, Anselmetti et al. 2013, Eyice, Namura et al. 2015). Microorganisms have indispensable roles in influencing and regulating nearly all ecosystems. *Archaea* and *Bacteria* exist in different metabolic states in these ecosystems. They could be actively growing, dormant and recently deceased. These various metabolic states can have a differential influence on their environment. Consequently, it is of substantial importance to truly associate microbial taxa with its metabolic state. So that it is possible to have any inference of the relationships between ecosystem functions and microbial community structure (Blazewicz, Barnard et al. 2013). However, most of the metagenomic exploration used DNA-based approaches. As a result, they concentrate only on the total bacterial community, which contains dormant microorganisms, dead cells and extracellular DNA (Lennon and Jones 2011). There is no known literature about overall diversity on the potentially metabolically active archaeal and bacterial communities in Lonar sediment. Although there are very few reports on the active community study in sediments of other types; from the sea (Sorensen and Teske 2006, Edlund, Hardeman et al. 2008, Mills, Reese et al. 2012, Kormas, Pachiadaki et al. 2015), lakes (Nercessian, Noyes et al. 2005) and estuary (Li, Wang et al. 2012, Chen, Wang et al. 2013). These populations can be evaluated by analysis of 16S rRNA transcripts (Pace, Stahl et al. 1985). The abundance of rRNA serves as an index of activity but not as a direct measure of activity (Blazewicz, Barnard et al.

2013). Thus, variations in the relative abundance essentially do not mirror variations in the activity of the studied microbial group.

Studying microbial diversity of hyperalkaline hypersaline meteorite impact crater lake ecosystems is valuable for several reasons. Research on the microbial community of soda lakes may provide clues to the evolution of life on earth; as some of the earliest microbial life on Earth might have been haloalkaliphiles (Kunte, Trüper et al. 2002). Due to the presence of hypersaline conditions on Mars, terrestrial saline environments can be used as an appropriate analog for investigation (Mancinelli, Fahlen et al. 2004). Lonar, specifically, is preferable over other soda lakes as Mars analog due to its strong resemblance with martian basalts. The origin of Lonar lake, the impact event itself, makes Lonar even more unique to all other soda lakes. The post impact crater and hydrothermal activity could mimic the prebiotic chemistry (Cockell 2006) in relation to the origin of life itself. Impact structures are a scarce ecosystem on earth, but wherever they are present, they can potentially have a significant influence on the local ecology. Impact events are one of the processes that can cause localized obliteration to ecosystems, understanding the behavior of recolonization of these impact structures is of ecological interest. Impact craters are a universal phenomenon on solid planetary surfaces, and so they are of possible biological importance on other planetary habitats (Cockell, Osinski et al. 2003).

Materials and Methods

3 MATERIALS AND METHODS

3.1 SEDIMENT SAMPLES

The sediment samples used in this study were collected on 14th of September 2014 from three different places of Lonar Lake, Buldhana district, Maharashtra, India. A total of 3 sediment samples were collected from various locations in triplicate. These triplicates were mixed in equal weight to obtain the working samples which will be referred as Lonar 1, Lonar 2 and Lonar 3 in the rest of the article.

3.2 HANDLING OF EQUIPMENT AND REAGENTS

All glassware, culture media, buffers and other solutions were autoclaved (20 min, 121°C) to sterilize except for the heat labile substances. Non-autoclavable or heat labile substances (e. g. lysozyme, glucose) were dissolved in sterile buffers or water and filter-sterilized. Tools that were not autoclavable were first rinsed with 70 % (w/v) ethanol and subsequently with sterile dd H₂O.

3.3 MEDIA, MEDIA SUPPLEMENTS AND ANTIBIOTICS

All the reagents and recipes of preparation are provided bellow.

Modified Horikoshi	Amount
Glucose	6.75 g
<u>Component (A)</u>	
Soluble Starch	6.25 g
Peptone	6.25 g

Modified Horikoshi	Amount
Yeast Extract	6.25 g
KH ₂ PO ₄ (Potassium dihydrogen phosphate)	1.25 g
MgSO ₄ · 7 H ₂ O (Magnesium sulfat)	0.25 g
NaCl (Sodium chloride)	12.50 g
<u>Component (B)</u>	
Na ₂ CO ₃ (Sodium carbonate)	12.50 g

TABLE 3: COMPOSITION OF MODIFIED HORIKOSHI MEDIUM.

Enrichment Medium for Protease	Amount
Casin	10.00 g
NaCl	20.00 g
NaCO ₃	10.00 g
K ₂ HPO ₄	0.50 g
CaCl ₂	0.025 g
MgSO ₄	0.005 g
Deionized H ₂ O	1000 ml

TABLE 4: COMPOSITION OF PROTEASE ENRICHMENT MEDIUM

Enrichment Medium for Lipase	Amount
Olive oil	3.5 ml
NaCl	20.00 g
NaCO ₃	10.00 g
K ₂ HPO ₄	0.50 g

Enrichment Medium for Lipase	Amount
CaCl ₂	0.025 g
MgSO ₄	0.005 g
Deionized H ₂ O	1000 ml

TABLE 5: COMPOSITION OF LIPASE ENRICHMENT MEDIUM

Enrichment Medium for Cellulase	Amount
Cellulose	10.00 g
NaCl	20.00 g
NaCO ₃	10.00 g
K ₂ HPO ₄	0.50 g
CaCl ₂	0.025 g
MgSO ₄	0.005 g
Deionized H ₂ O	1000 ml

TABLE 6: COMPOSITION OF CELLULASE ENRICHMENT MEDIUM

Luria-Bertani (LB) medium (Sambrook, 1989)	Amount
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Deionized H ₂ O	1000 ml

TABLE 7: COMPOSITION OF LURIA-BERTANI MEDIUM

Supplement	Stock solution	Working concentration
Ampicillin	50 mg/ml 50 % Ethanol	0 50-100 mg/ml
Kanamycin	25 mg/ml dd H2O	50 mg/ml
IPTG	25 mg/ml dd H2O	50 mg/ml
X-Gal	20 mg/ml Dimethylformamide	40 mg/ml

TABLE 8: LIST OF SUPPLEMENTS AND THEIR CONCENTRATIONS.

3.4 ORGANISMS, OLIGONUCLEOTIDES, ENZYMES, AND BUFFERS

3.4.1 STRAIN DESCRIPTION

E. coli strain	Genotype	Source
DH5α	F- ϕ 80lacZ Δ M15 Δ (lacZYA-argF) U169 endA1 recA1 hsdR17 (rk-, mk+) supE44 thi -1 gyrA96 relA1 phoA	Invitrogen
TOP10	F- mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80lacZ Δ M15 Δ lacX74 recA1 araD139 Δ (ara-leu) 7697 galU galK rpsL (StrR) end A1 nupG	Invitrogen
EPI300-T1	F- mcrA Δ (mrr-hsdRMS-mcrBC) (StrR) ϕ 80dlacZ Δ M15 Δ lacX74 recA1 endA1 araD139 Δ (ara, leu) 7697 galU galK λ -rpsL nupG trfA tonA dhfr	Epicentre

TABLE 9: LIST OF E.COLI STRAINS WITH DESCRIPTION.

3.4.2 DESCRIPTION OF PRIMERS

3.4.2.1 PRIMERS FOR ROCHE PLATFORM SEQUENCING

The V3-V5 region was amplified with the following set of primers containing the Roche 454 pyrosequencing adaptors (blue), key (red) and MID or multiplex identifier (10 bp long) in the case of forward primer only. Modified bacterial primers were S-D-Bact-0343-a-S-15 (Bac343F) (Nossa, Oberdorf et al. 2010) and S-D-Bact-0907-a-A-20 (Bac907R) (Muyzer, Teske et al. 1995). Modified archaeal primers were S-D-Arch-0340-a-S-18 (Arch340F) (Ovreås, Forney et al. 1997) and S-D-Arch-0911-a-A-20 (Arch915R) (Stahl and Amann 1991).

Primers for bacterial 16S rRNA gene

S-D-Bact-0343-a-S-15 (forward)	CCATCTCATCCCTGCGTGTCTCCGACTCAG-MID-TACGGRAGGCAGCAG
--	--

S-D-Bact-0907-a-A-20 (reverse)	CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCCGTCAATTCMTTGGAGT
--	--

Primers for archaeal 16S rRNA gene

S-D-Arch-0340-a-S-18 (forward)	CCATCTCATCCCTGCGTGTCTCCGACTCAG-MID- CCCTAYGGGGYGCASCAG
--	---

S-D-Arch-0911-a-A-20 (reverse)	CCTATCCCCTGTGTGCCTTGGCAGTCTCAGGTGCTCCCCGCCAATTCCT
--	---

TABLE 10: LIST OF 16S rRNA PRIMERS FOR ROCHE PLATFORM SEQUENCING

3.4.2.2 PRIMERS FOR ILLUMINA PLATFORM SEQUENCING

The V3-V4 region was amplified with the following set of primers containing the Illumina overhang adapter (blue). Primer set used for amplification of bacterial V3-V4 was S-D-

Bact-0341-b-S-17 (forward) and S-D-Bact-0785-a-A-21 (reverse) (Klindworth, Pruesse et al. 2013) with modifications. For *Archaea* 514Fa (Reed, Fujita et al. 2002) and In-house were used.

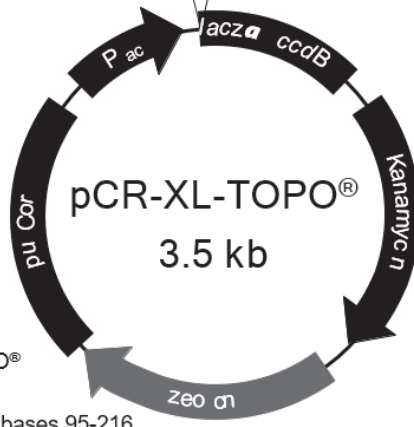
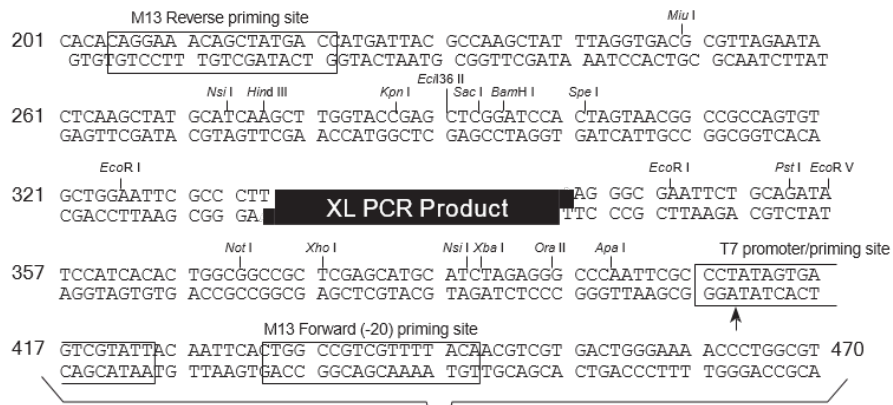
Primers for bacterial 16S rRNA gene	
S-D-Bact-0341-b-S-17 (forward)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
S-D-Bact-0785-a-A-21 (reverse)	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC
Primers for archaeal 16S rRNA gene	
514Fa (forward)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGTGBACGCCGCCGCGGTAA
In-house	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCCGCCAATTYCTTTAAG

TABLE 11: LIST OF 16S rRNA PRIMERS FOR ILLUMINA PLATFORM SEQUENCING.

3.4.3 VECTORS

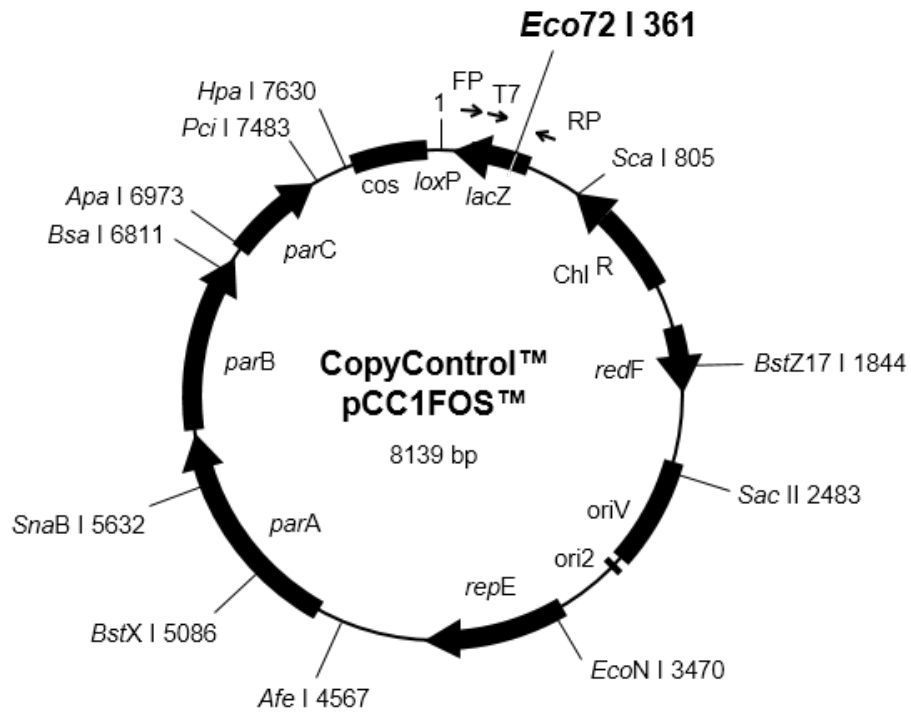
Vector Name	Purpose	Source
pCR-XL-TOPO	Small insert Library (Plasmid Library)	Invitrogen
pCC1FOS	Large insert Library (Fosmid library)	Epicentre

TABLE 12: LIST OF VECTORS USED FOR CONSTRUCTION OF METAGENOMIC LIBRARIES



Comments for pCR-XL-TOPO®
 3519 nucleotides
 Lac promoter/operator region: bases 95-216
 M13 Reverse priming site: bases 205-221
 Lac Za ORF: bases 217-576
 Multiple Cloning Site: bases 248-399
 TOPO® Cloning site: bases 336-337
 T7 promoter priming site: bases 406-425
 M13 Forward (-20) priming site: bases 433-448
 Fusion joint: bases 577-585
 ccdB lethal gene ORF: bases 586-888
 Kanamycin resistance ORF: bases 1237-2031
 Zeocin resistance ORF: bases 2238-2612 pUC
 origin: bases 2680-3393

FIGURE 4: VECTOR MAP OF PCR-XL-TOPO (FROM INVITROGEN)



Note: Not all restriction enzymes that cut only once are indicated above.
See Appendix E for complete restriction information.
Primers are not drawn to scale.



FP = pCC1™/pEpiFOS™ Forward Sequencing Primer 5' GGATGTGCTGCAAGGCGATTAAGTTGG 3'
 RP = pCC1™/pEpiFOS™ Reverse Sequencing Primer 5' CTCGTATGTTGTGTGGAATTGTGAGC 3'
 T7 = T7 Promoter Primer 5' TAATACGACTCACTATAGGG 3'

FIGURE 5: VECTOR MAP OF pCC1FOS (FROM EPICENTER)

3.4.4 DETAILS OF THE ENZYMES

Enzymes	Usage
Antarctic Phosphatase (5U)	Dephosphorylation of DNA ends
<i>EcoRI</i> Restriction enzyme	Plasmid Digestion
Lysozyme	Cell lysis during DNA extraction
Phusion DNA Polymerase (2U)	PCR for cloning
T4 Polymerase (5U)	Generation of blunt-end DNA
<i>Taq</i> Polymerase (5U)	Temperature gradient PCR

TABLE 13: LIST OF ENZYMES USED.

3.4.5 COMPOSITION OF BUFFERS AND STOCK SOLUTIONS

Reagent	Amount
CTAB	10 g
StMQ H ₂ O	100 ml

TABLE 14: 10 % CTAB

Reagent	Amount
Na ₂ EDTA.2H ₂ O	186.1 g
NaOH	20 g
<i>Adjust pH to 8.0</i>	
StMQ H ₂ O	1000 ml

TABLE 15: EDTA (0.5M, pH 8.0)

Reagent	Amount
Tris HCl (100mM; pH 8.0)	100 ml
Sodium Phosphate Buffer (2M)	50 ml
Sodium EDTA (100mM; pH 8.0)	200 ml
NaCl (1.5 M)	300 ml
CTAB 1 %	100 ml

TABLE 16: DNA EXTRACTION BUFFER

Reagent	Amount
NaCl	292 g
StMQ H ₂ O	1000 ml

TABLE 17: NaCl (5M)

Reagent	Amount
Na ₂ HPO ₄	141.95 g
NaH ₂ PO ₄	119.97 g
StMQ H ₂ O	1000 ml

TABLE 18: SODIUM PHOSPHATE SOLUTION (2M)

Reagent	Amount
Tris	121 g
<i>Adjust pH with con. HCl to 8.0</i>	
StMQ H ₂ O	1000 ml

TABLE 19: TRIS-HCL (1M)

Reagent	Amount
Tris-HCl	0.157 g
NaCl	0.584 g
MgCl ₂ .6H ₂ O	0.203 g
StMQ H ₂ O	100 ml

TABLE 20: PHAGE DILUTION BUFFER.

Reagent	Amount
Tris	242 g
Acetate	57 ml
EDTA (0.5 M)	100 ml
StMQ H ₂ O	Add 1000 ml

TABLE 21: 50X TAE

3.5 COMMERCIAL KITS

Kit	Provider
GeneRead Size Selection Kit	Qiagen GbH, Germany
peqGOLD Gel Extraction Kit (Safety-Line)	Peqlab Biotechnologie GbH, Germany
peqGOLD Plasmid Miniprep Kit I	Peqlab Biotechnologie GbH, Germany
PowerClean DNA cleanup kit	MoBio Laboratories, Inc., USA
Quant-iT dsDNA BR assay kit	Invitrogen GbH, Germany
RNA PowerSoil DNA elution accessory kit	Mo Bio Laboratories, Inc., USA
RNA PowerSoil total RNA isolation kit	Mo Bio Laboratories, Inc., USA
RNeasy MinElute Cleanup kit	Qiagen GbH, Germany
SureClean Kit	Bioline GbH, Germany
TOPO XL PCR Cloning Kit	Invitrogen GbH, Germany
TURBO DNA-free kit	Ambion Applied Biosystems, Germany

TABLE 22: LIST OF KITS USED

3.6 COMPUTATIONAL AND STATISTICAL ANALYSIS TOOLS

Software/Services	Source
NanoDrop 1000, version 3.8.0	ThermoFisher Scientific
USEARCH version 8.1	http://drive5.com/usearch/
UPARSE	http://drive5.com/uparse/ Reff. (Edgar 2013)
UCHIME	http://drive5.com/usearch/manual/uchime_algo.html Reff. (Edgar, Haas et al. 2011)
QIIME, Version 1.9 (Quantitative Insights into Microbial Ecology)	http://qiime.org/ Reff. (Caporaso, Kuczynski et al. 2010)
R version 3.2.3	https://www.r-project.org Reff. (R Core Team 2015)
SigmaPlot, Exact Graphs and Data Analysis	Systat Software, Inc., San Jose California USA, www.sigaplot.com
Trimmomatic v.0.30	http://www.usadellab.org/cms/?page=trimmomatic Reff. (Bolger, Lohse et al. 2014)
SPAdes version 3.7.1	http://bioinf.spbau.ru/en/spades Reff. (Bankevich, Nurk et al. 2012)
Prodigal version 2.6.0 (Prokaryotic Dynamic Programming Genefinding Algorithm)	http://prodigal.ornl.gov/ Reff. (Hyatt, Chen et al. 2010)
GhostKOALA version 2.0	http://www.kegg.jp/ghostkoala/ Reff. (Kanehisa, Sato et al. 2016)

TABLE 23: LIST OF TOOLS USED.

3.7 DNA AND RNA EXTRACTION METHODS

Extraction of DNA was carried out by two general methods with minor modifications. The first method is Phenol–chloroform extraction. It is a liquid–liquid extraction method in biochemistry. It is broadly used in molecular biology for isolating DNA, RNA, and protein. Equal volumes of phenol : chloroform mixture and an aqueous sample are mixed, forming a biphasic mixture. This method can take longer than a column-based system, such as the silica-based purification, but has the benefit of high recovery of DNA. It was formerly used by Piotr Chomczynski and Nicoletta Sacchi (referred to as guanidinium thiocyanate-phenol-chloroform extraction) and published in 1987 (Chomczynski and Sacchi 1987). In 1998, this basic Phenol-Chloroform extraction was adapted by Yeats (Yeates, Gillings et al. 1998). In this thesis, we have used the method revised by Yeates et al. with some more modifications. It is described in next two chapters. The second method is column based. The key to these systems is the binding matrix/column that avidly, but reversibly, binds under certain optimal conditions allowing proteins and other contaminants to be removed. Then the nucleic acids can be easily eluted with deionized water.

3.7.1 EXTRACTION OF DNA AND RNA FOR THE TOTAL AND THE ACTIVE PROKARYOTIC BIODIVERSITY ASSESSMENT BY ILLUMINA PLATFORM

Co-extraction of total environmental RNA and DNA were performed from 0.5 g of sediment of each of the Lonar 1, Lonar 2 and Lonar 3. The RNA PowerSoil total RNA isolation kit and the RNA PowerSoil DNA elution accessory kit were used respectively, as per the recommendation of the manufacturer (MoBio Laboratories, USA). The extracted RNA was purified from residual DNA with the TURBO DNA-free kit (Ambion Applied Biosystems, Germany). The success of purification was confirmed by PCR to determine the absence of DNA as described by Wemheuer et al. (Wemheuer, Wemheuer et al. 2012). Purification and concentration of the DNA-free RNA were achieved with the RNeasy MinElute Cleanup kit (Qiagen GbH, Hilden, Germany). Isolated DNA was cleaned with the PowerClean DNA cleanup kit (MoBio Laboratories). Concentrations of DNA and RNA were

determined using a NanoDrop ND-1000 spectrophotometer (Peqlab Biotechnologie GbH, Erlangen, Germany). cDNA synthesis was performed, from approximately 500 ng of purified RNA, with the SuperScript™ III reverse transcriptase as recommended by the manufacturer (Invitrogen, Karlsruhe, Germany) and the reverse primer S-D-Bact-0785-a-A-21 (reverse) (Klindworth, Pruesse et al. 2013) and in-house (reverse) of the next PCR reaction separately. These DNA and cDNA will be used to assess the biodiversity of the total and the active bacterial and archaeal community.

3.7.2 EXTRACTION OF DNA FOR ADDITIONAL BIODIVERSITY ASSESSMENT BY ROCHE PLATFORM

DNA extraction protocol for lake sediments in use was modified after Yeates (Yeates, Gillings et al. 1998). 10g of sediment was suspended in 20ml of wash buffer (50mM Tris-HCl, 50mM EDTA, and 25 % sucrose), it was vortexed briefly to disperse the soil particles and centrifuged at 10,000g for 3 minutes. The supernatant was discarded. This helps eliminate the salts and exopolysaccharides. The sediment was resuspended in 15 ml of DNA extraction buffer [100 mM Tris-HCl (pH 8.0) , 100 mM sodium EDTA (pH 8.0) , 1.5 M NaCl, CTAB 1 % w/v]. 75 µl of lysozyme (from a 100mg/ml stock solution) and 75 µl of RNaseA (from a 100 mg/ml stock solution) were added and incubated at 37 °C for 1 h. It was frozen using liquid nitrogen and then thawed in a water bath at 65 °C for 30 minutes. This freeze-thaw cycle was repeated one more time. 1.6 ml of 20 % (w/v) SDS and 0.7 ml of 6M GITC was added, mixed gently by inversion and incubated for 2 hrs at 65 °C with occasional gentle mixing. After 2 hrs, the mixture was centrifuged at 15,000 rpm for 20 min at 10 °C to remove soil residue, and the supernatant was transferred, which contains the crude DNA, into a clean tube. An equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently on a slow shaker for 10 minutes. The mixture was centrifuged at 15,000 rpm for 20 min at 10 °C and the supernatant was transferred to a clean tube. The 0.7x volume of isopropanol was added and allowed to mix on a slow shaker for 10 minutes. The mixture was centrifuged at 15,000 rpm for 40 min at 10 °C to pellet the DNA, and all traces of the supernatant were removed. The DNA pellet was resuspended in a

minimum amount (2 ml) of St.MQ and aliquoted into Eppendorf tubes using a wide bore pipette tip. The DNA was purified by using an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). One was extracted with Chloroform: isoamyl alcohol (24:1) to remove traces of phenol. The mixture was centrifuged at 15,000 rpm for 20 min at 10 °C and the supernatant was transferred to a clean tube. The 0.7x volume of isopropanol was added and allowed to mix on a slow shaker for 10 minutes. The mixture was centrifuged at 15,000 rpm for 20 min at 10 °C to pellet the DNA and remove all traces of the supernatant. The pellet was washed twice with 80 % (v/v) ethanol, air-dry and dissolves in 2 ml St.MQ. The quality and quantity were checked by 0.8 % agarose gel electrophoresis and Nanodrop (NanoDrop ND-1000, Thermo Scientific). This DNA will be used for additional surveying of the total diversity to confirm the coverage.

3.7.3 EXTRACTION OF DNA FROM AGAROSE GEL

Gel extraction of DNA with peqGOLD Gel Extraction Kit (Safety-Line, Cat. No.12-2500-01). The sonicated sample was mixed with Loading Dye and loaded on a 0.8 % agarose gel. The electrophoresis was performed for 90 min at 90 V. Afterward, the sides with the marker were cut out and stained in Ethidium Bromide (EtBR). The gel with DNA from 6 to 10 kb was excised out under UV to mark the region of interest. The rest of the unstained gel was added in between the marked stained gel pieces and was cut as well (it was not stained with EtBR or exposed to UV). For DNA Extraction from the gel, the peqGOLD Gel Extraction Kit provided by Peqlab Biotechnology was used along with the provided protocol described in the next paragraph.

The approximate volume of the gel slice was determined (by weighing) to add an equal volume of the provided Binding Buffer. The mixture was incubated for 7 min. at 65°C. During this time, it was inverted from time to time to mix thoroughly until the gel was completely dissolved. A PerfectBindDNA Column was placed in a 2 ml Collection Tube (both provided with the kit) and loaded with the DNA/agarose solution. The column/tube was centrifuged for 1 min. at 10.000 x g. The flow-through was discarded, and the column was loaded again until there was no solution left. Afterward, the Binding Buffer was added

to the column, and another centrifugation step (same conditions) was performed. Then, the column was washed with CG Wash Buffer (diluted with ethanol). Again, the column/tube was centrifuged for 1 min at 10.000 x g. The centrifugation step was repeated to dry the column and remove ethanol. The column was placed in a fresh tube, and prewarmed Elution Buffer was added directly to the filter. The column/tube was centrifuged for 1 min. at 5.000 x g to elute the DNA. The success of the DNA gel extraction was verified by gel electrophoresis.

3.7.4 EXTRACTION OF DNA FOR DIRECT METAGENOME ANALYSIS

The DNA extraction protocol is same as described in the chapter 3.7.2. The only difference was, before storing the extracted DNAs were further cleaned using SureClean Kit (Bioline, Germany).

3.7.5 EXTRACTION OF DNA FOR THE STUDY OF COMMUNITY DYNAMICS DURING MICROENVIRONMENTAL PH HOMEOSTASIS

Cultures from non-optimum pH exposures were subjected to centrifugation as 15 ml aliquots. The cell pellet was resuspended in 15 ml of DNA extraction Buffer and 75 µl of lysozyme were added. The mixture was incubated at 37°C for 1 h. Afterward, 1.6 ml of 20 % SDS and 0.7 ml of 6 M GITC were added. The sample incubated for 2 hrs and 65°C. It was inverted from time to time. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and mixed on a slow shaker for 10 min. Followed by a centrifugation step for 20 min. at 15,000 rpm and 10 °C. The supernatant was transferred to a clean tube. To remove traces of Phenol; Chloroform/Isoamylalcohol (24:1) was added. The mixture was centrifuged at 15.000 rpm for 20 min at 10°C and the supernatant was placed in a clean tube. The 1X volume of Isopropanol was added, and the solution was mixed gently and kept overnight at -20 °C. On the next day, the mixture was centrifuged at 15.000 rpm for 40 min at 10°C to precipitate the DNA as a pellet. All traces of supernatant were

removed. The pellet was washed twice with 80 % ethanol, air-dried and dissolved in 1 ml StMQ water.

3.7.6 EXTRACTION OF DNA FOR CONSTRUCTION OF METAGENOMIC LIBRARIES

Enrichment cultures (as described in 3.9.1) were subjected to centrifugation as 15 ml aliquots. Rest of the protocol is as described above (3.7.2).

3.7.7 EXTRACTION OF PLASMID DNA

3.7.7.1 ISOLATION OF PLASMID USING PEQGOLD PLASMID MINIPREP KIT

For plasmid isolation the peqGOLD Plasmid Miniprep Kit I was used, offered by Peqlab Biotechnology. The cultures were transferred to 2 ml tubes and centrifuged 2 times for 10 min at 5.000 x g. The bacterial pellet was resuspended in Solution I (with RNase A) to lyse the *Bacteria*. Solution II was added and the mixture was mixed by inverting. The lysate was neutralized by adding Solution III and inverting. A white flocculent precipitate was observed. The solution was centrifuged at 10.000 x g for 10 min at room temperature. The supernatant was transferred to a column collection tube. The column and the tube were centrifuged 1 min at 10.00x g at room temperature, and the flow through was discarded. Then, three washing steps followed: HB Buffer was added, and the Solution was centrifuged for 1 min at 10.000 x g. Then, Wash Buffer completed with ethanol was added two times to the column. The Centrifugation was repeated. For drying, the column was centrifuged one more time with same conditions. Afterward, DNA was eluted by placing the column to a fresh tube adding Elution Buffer and centrifuging at 5.000 x g for 1 min. An analytical gel electrophoresis was performed to check the extraction success.

3.7.7.2 ISOLATION OF PLASMID USING QIAGEN PLASMID PLUS MIDI KIT

Pelleted cell mass was resuspended in 2 ml Buffer P1 (with RNase A). The pellet was resuspended completely by vortexing. 2 ml of Buffer P2 was added and gently mixed by inverting. The mixture was incubated at 25°C for 3 min. 2 ml of Buffer S3 was added to the lysate and mixed immediately by inverting several times. The lysate was transferred immediately to the QIAfilter Cartridge and incubate at room temperature for 10 min. During incubation, the vacuum manifold, and the QIAGEN Plasmid Plus Midi spin columns were prepared. The plunger was inserted gently into the QIAfilter Cartridge and filter the cell lysate into a new tube, allowing space for the addition of Buffer BB. Filtration was performed until all of the lysates has passed through the QIAfilter Cartridge. 2 ml Buffer BB was added to the cleared lysate and mix by inverting several times. The lysate was transferred to a QIAGEN Plasmid Plus Midi spin column with a tube extender attached on the vacuum set. The solution was drawn through the QIAGEN Plasmid Plus Midi spin column using -300 mbar pressure. 0.7 ml of Buffer ETR was added to the DNA to wash. The tube extenders were discarded and the QIAGEN Plasmid Plus Midi Spin Column was placed into the 2 ml collection tube, provided with the kit. The column was washed by centrifuging for 1 min at 10,000 x g. The flow-through was discarded. 0.7 ml of Buffer PE was added. The column was washed again by centrifuging for 1 min at 10,000 x g. The flow-through was discarded. The column was again centrifuged for 1 min at 10,000 x g in a microcentrifuge to remove the residual wash buffer completely. The QIAGEN Plasmid Plus Midi spin column was placed into a clean 1.5 ml microcentrifuge tube. The DNA or Plasmid was eluted by adding 200 µl of stMQ Water to the center of the QIAGEN Plasmid Plus Midi spin column. The setup was allowed to stand for 1 min and then centrifuged for 1 min at 10,000 x g. The plasmid was stored at -20°C.

3.8 QUANTIFICATION OF DNA

Nucleic acids absorb ultraviolet light in a precise pattern. In a spectrophotometer, a sample is exposed to ultraviolet light at 260 nm and a photodetector measures the light that travels through the sample. The more light absorbed by the sample, the higher the nucleic acid concentration in the sample. Using the Beer-Lambert Law, it is achievable to correlate the quantity of light absorbed to the concentration of the absorbing molecule.

In this thesis the DNA concentration was measured with two instruments one is with Nano-Drop Spectrophotometer (Cat No. ND-1000) by Thermo Fisher Scientific and the other is Qubit 2.0 Fluorometer (Cat No. Q32866) by Life Technologies. Qubit 2.0 Fluorometer unlike Nano-Drop Spectrophotometer uses fluorescence rather than absorbance.

3.8.1 DETERMINATION OF DNA CONCENTRATION BY NANO-DROP

SPECTROPHOTOMETER

Before every measurement, the sample plate was cleaned with 5 μl of StMQ water and dried with a tissue. Afterward, a blank was measured using 2 μl of the solution in which the DNA was dissolved (StMQ water or any other DNA Elution Buffer). The sample plate was cleaned again, and 2 μl of the sample were placed on the plate. The concentrations were determined using the computer program NanoDrop 1000 (version 3.8.0) provided by ThermoFisher Scientific. The concentration was stated in $\text{ng}/\mu\text{l}$. In addition, other values were available, which contains calculations of absorbance ratios and allows drawing conclusions about the quality or purity of the examined DNA. One of the most important values was the A260/A280 ratio (1.8 can be considered as pure or clean).

3.8.2 DETERMINATION OF DNA CONCENTRATION BY QUBIT FLUOROMETER

The principle of measurement of DNA concentration by Qubit is based on fluorescent dyes that bind specifically to DNA, RNA or protein. The dye releases a detectable fluorescent signal when it is bind to its target (DNA, RNA or Protein). Therefore, the Qubit measurement is more accurate than the NanoDrop measurement. NanoDrop is based on absorbance, where it is possible that other molecules can exist in the DNA solution, which is measured along with the DNA and provide inaccurate measurement compared to Qubit. Before starting the measurement, a Working Solution has to be prepared. 199 μl of provided buffer is mixed with 1 μl of dye for every sample. The solution is mixed by vortexing. 190 μl of it were aliquoted for standards. They were completed with 10 μl of standard solution (2 per measurement). The other working solution was used for the samples. Depending on the amount of sample which is used for measurement, 198 μl -199 μl Working Solution were aliquoted in assay tubes. In the end, the total volume should be 200 μl . So, 1 μl -2 μl of the sample were added. The solution was mixed by vortexing and incubated for 2 min at room temperature. Then the measurement starts by choosing the kind of the sample and blanking the Qubit with Standard Solution 1 and 2 by putting the assay tubes in the designated place. Afterward, the samples are measured using the provided computer program.

3.9 CONSTRUCTION AND SCREENING OF METAGENOMIC LIBRARIES

3.9.1 ENRICHMENT CULTURES

An enrichment culture is a medium with definite and known character that positively discriminates the growth of a specific microorganism above others. The microbiologist (and botanist) Martinus Willem Beijerinck (March 16, 1851 – January 1, 1931) is credited with developing the first enrichment cultures (King-Thom and Hunter 1996). We established three different enrichment cultures to get the population of protease, lipase

and cellulase producing microorganism vastly obtainable. The recipe as described in Chapter 3.3 (Table 4, Table 5, Table 6).

3.9.2 CONSTRUCTION OF SMALL INSERT LIBRARY

For the construction of small insert library TOPO XL PCR Cloning Kit provided by Invitrogen was used in this experiment.

3.9.2.1 PREPARATION OF ELECTROCOMPETENT CELLS

For the preparation of electrocompetent cells, a 5 ml overnight culture was added to 250 ml growth medium. LB supplemented with kanamycin was used for *E. coli* DH5 α . The cells were incubated at 30 °C until an optical density (OD₆₀₀) of 0.5 – 1. The OD was measured with an Ultraspec 3300 pro photometer (Amersham Pharmacia Biotech Europe GbH). The *E. coli* culture was then incubated 20 min on ice before being centrifuged for 10 min (5000 x g, 4 °C). The pellet was washed two times with equal volume of sterile H₂O and subsequently washed one time with 10 ml glycerol (10 %). Resuspension was done with 0.5 ml of glycerol (10 %) and for further use aliquots of 40 μ l were prepared and frozen using liquid nitrogen before storage at -70 °C.

3.9.2.2 SMALL INSERT LIBRARY

Small-insert libraries were constructed using the TOPO XL PCR Cloning Kit (Invitrogen GbH, Karlsruhe, Germany) with plasmid pCR-XL-TOPO as a vector. For each enrichment culture, approximately, 10 μ g extracted DNA was separated by agarose gel electrophoresis. DNA fragments of more than 6 kb in size were selected and purified from the gels using the peqGold Gel Extraction Kit (Peqlab Biotechnologie GbH, Germany). The purified DNA fragments were subjected to sticky end repairing using T4 DNA polymerase (MBI

Fermentas, Germany) as suggested by the manufacturer. Afterward, the DNA was purified using SureClean Kit (Bioline GbH, Germany) and the resulting DNA pellet was suspended in 35 μ l H₂O. Then, a deoxyadenosine was added to the 3' end of the DNA to facilitate the TA cloning method. For this purpose, 1 μ l dATP solution (100 mM) , 6 μ l MgCl₂ solution (25 mM) , 7 μ l of 10X Taq DNA polymerase buffer containing (NH₄)₂SO₄ (Fermentas) , 1 μ l of Taq DNA polymerase (5 U) , and 20 μ l of H₂O were mixed with the DNA solution, incubated at 72 °C for 30 min, and purified using SureClean solution (Bioline GbH). The resulting DNA pellet was suspended in 15 μ l H₂O and dephosphorylated using 5U Antarctic Phosphatase (NEB, Ipswich, MA). The blunt-end-DNA with 3' A-overhang was mixed with 1.5 μ l of Antarctic Phosphatase Buffer (10x) and 1 μ l of Antarctic Phosphatase (5U). The sample was incubated for 15 min. at 37 °C and inactivated by another incubation step at 65 °C for 15 min.

Finally, the recovered DNA fragments were inserted into pCR-XL-TOPO using the TOPO XL PCR cloning kit (Invitrogen) by adding 1 μ l of the pCR-XL-TOPO vector to 4 μ l of prepared enrichment culture DNA (protease and lipase separately). The mixture was incubated for 5 min at room temperature. Then, 1 μ l Stop solution (provided with the kit) was added, and the ligation mixture was kept on the ice.

3.9.2.2.1 Transformation Of *E. Coli* TOP10 And *E. Coli* DH5 α Cells By Electroporation

Electroporation is a quick transformation method (Dower, Miller et al. 1988). It is based on the permeability of the cell membrane. The rapid breakdown of the membrane potential allows the absorption of DNA, mostly plasmids.

The TOPO-Cloning reactions were used to transform *Escherichia coli* TOP10 cells with the electroporation method. 2 μ l of the TOPO-Cloning reaction was added to one vial of OneShot electrocompetent *E. coli* cells and mixed gently. The cells with the DNA were transferred to a chilled 0.1 cm electroporation cuvette. Electroporation occurred at 25 μ F, 200 Ω and 2.5 kV with the pulse controller II and the gene controller II manufactured by BioRad. Immediately after electroporation 450 μ l of S.O.C. medium (room temperature) was added and mixed well. The solution was transferred to a 2 ml tube and incubated for

one hour at 37 °C to allow expression of the antibiotic resistance genes. During incubation, the solution was inverted from time to time. Afterward, the cells were spread on four plates, one of 50 µl, two of 100 µl and one of 150 µl. The plates were incubated overnight at 37 °C.

The metagenomic libraries were used to transform *E. coli* DH5α cells in the same way as described above. Instead of the TOPO-Cloning reaction the library itself is used.

3.9.2.2.2 Detection Of Recombinant Cells By Blue-White Screening

Kanamycin provided the selection for successful transformation in addition of X-Gal and IPTG. The presence of X-Gal (bromo-chloro-indolyl-galactopyranoside) allows a selection of insert-carrying plasmids. Galactoside is linked to indole. Galactose and 5-bromo-4-chloro-3-hydroxyindole emerge from cleavage by β-galactosidase. Oxidation of 5-bromo-4-chloro-3-hydroxyindole results in a blue product. Since the aminoterminal region of the lacZ gene is situated in the multiple cloning site, an insert-carrying clone is not able to produce a functional β-galactosidase, assumed that the host cells carry an inactive lacZ gene or lack the aminoterminal region. IPTG (Isopropyl β-D-1-thiogalactopyranoside) served as an inducer to ensure the activity of possible presence of β-galactosidases.

3.9.2.2.3 Library Preparation

The white and blue *E. coli* TOP10 clones resulting from TOPO-XL-cloning were counted and removed from plates, followed by a QIAGEN Plasmid Plus Midi preparation as described in 3.7.7.2. The plasmids were then collected in one tube, stored at -20 °C, and referred to as a metagenomic library.

All the procedures were performed three times with three enrichment culture DNA resulting into three small insert libraries.

3.9.2.3 SCREENING OF SMALL INSERT LIBRARIES

3.9.2.3.1 *Protease Activity Screening*

LB agar plates containing skimmed milk were used for detection of proteolytic activity. Skimmed milk can be degraded by proteolytic enzymes leading to the formation of a halo around a positive clone. After transforming the protease enrichment library into *E. coli* DH5 α , recombinant cells were plated and incubated at 37 °C for 3 to 14 days.

3.9.2.3.2 *Lipase Activity Screening*

LB agar plates containing glycerol tributyrates were used for detection of lipolytic activity. Recombinant cells expressing lipolytic enzymes can be detected by halo formation caused by the degradation of short-chain triglycerides. After transforming the lipase enrichment library into *E. coli* DH5 α , recombinant cells were plated and incubated at 37 °C for 3 to 14 days.

3.9.3 LARGE INSERT LIBRARY

For the construction of large insert library, Copy Control Fosmid Library Production Kit provided by Epicentre was used in this experiment.

3.9.3.1 PREPARATION OF INFECTION CELLS

Infection cells were prepared before packaging. 1 μ l of *E. coli* EPI300 cells were inoculated into 50 ml of LB (+ 10 mM MgSO₄). The flask was incubated at 37 °C for overnight on a shaker. 5 ml of overnight grown culture was transferred into 50 ml of LB (+ 10 mM

MgSO₄). The flask was incubated at 37 °C with shaking until OD₆₀₀ reaches 0.8 – 1.0. The culture was stored at 4 °C for later use (maximum of 5 days).

3.9.3.2 SHEARING THE INSERT DNA

DNA extracted from enrichment culture (for cellulase) was sufficiently sheared as a result of the purification process, and additional shearing was not necessary.

3.9.3.3 END-REPAIR OF THE SHEARED DNA

For each reaction, 60 µl of sheared DNA (up to 20 µg), 8 µl of 10x End-Repair Buffer, 8 µl of 2.5 mM dNTPs, 8 µl of 10mM ATP and 4 µl of End-Repair Enzyme Mix were mixed gently and incubated for 2 h. After incubation, the insert DNA was purified with Bioline SureClean and eluted with 12 µl of sterile water.

3.9.3.4 LIGATION REACTION

For each reaction 2 µl of 10X Ligation Buffer, 2 µl of 10 mM ATP, 2 µl of Copy Control pCC1FOS Vector (0.5 µg/µl), 12 µl of insert DNA and 2.0 µl of DNA Ligase were mixed and incubated at 16 °C for overnight. Following overnight ligation additional 1 µl of DNA ligase was added to the ligation mix and incubate at room temperature for 2 hrs. The reaction was inactivated by heating at 70 °C for 10 minutes. The reaction mix was cooled on ice for 10 min.

3.9.3.5 PACKAGING OF FOSMID CLONES

The entire ligation mix was used in this step. 25 µl of thawed Max Plax packaging extract was added to the ligation mix (the remaining 25 µl of the Max Plax Packaging Extract was

kept at -70 °C). The mix was incubated at 30 °C for 120 min. After incubation additional 25 µl of the phage packaging extract was added to the sample and incubated again for 120 min at 30 °C. Phage dilution buffer was added to a final volume of 1 ml, and 25 µl of chloroform was added to the sample. The sample was stored at 4 °C until further use.

3.9.3.6 INFECTION

During infection, different volumes (e.g. 10 µl / 30 µl / 50 µl) of the packaged sample were added to 100 µl of infection cells. The cells were incubated at 37 °C for 90 min in a heating block. The library was plated onto LB (+ 12.5 µg/ml Chloramphenicol) plates. The plates were incubated at 37 °C overnight.

3.10 PCR AMPLIFICATION OF PARTIAL 16S RRNA GENE

The polymerase chain reaction (PCR) is the fundamental laboratory technique of molecular biology. It is one of the most influential laboratory techniques that have been discovered ever; PCR combines the distinctive properties of being very sensitive and specific with an immense degree of flexibility. With the PCR, it is achievable to address a specific DNA sequence and to amplify this sequence to an extremely high number of copy. Since its initial development in the early 1980's, several variations on the fundamental idea of PCR have successfully been carried out.

3.10.1 AMPLIFICATION OF PARTIAL 16S RRNA GENE FOR THE TOTAL AND THE ACTIVE COMMUNITY DIVERSITY ASSESSMENT

The V3-V4 region of the 16S rRNA gene was amplified by PCR (polymerase chain reaction). Every sample was amplified in triplicates. Every sample was amplified 3 times in replica along with negative controls (no template) resulting in a total of 40 reactions; 50µl each

(36 samples, 4 controls). 3 sediment samples (Lonar 1, Lonar 2 and Lonar 3) , two type of template (DNA and cDNA) , two set of primers (Archaeal and Bacterial) and 3 replicas for each resulting in 36 reactions in total. Each PCR reaction (50µl) contained 31 µl nuclease-free demineralized sterile H₂O, 10µl 5X reaction buffer (Phusion GC Buffer for *Archaea* and Phusion HF buffer for *Bacteria*, ThermoFisher Scientific) , 1µl of 10 mM of deoxynucleoside triphosphates (all four) , 2.5 µl DMSO, 2 µl of BSA [20 mg ml⁻¹ in 10 mM Tris-HCl (pH 7.4 at 25o C) , 100 mM KCl, 1 mM EDTA and 50 % (v/v) glycerol] (ThermoFisher Scientific) , 1µl of 10µM primer each (forward and reverse) , 0.5µl of 2U/µl Phusion high-fidelity DNA Polymerase (ThermoFisher Scientific) , 1µl of isolated DNA diluted to 50 ng/µl concentration as template. The V3-V4 hypervariable region was selected as the parameter for identification of *Bacteria* (Vasileiadis, Puglisi et al. 2012, Vilo and Dong 2012). Primer set used for amplification of V3-V4 region was S-D-Bact-0341-b-S-17 (forward) and S-D-Bact-0785-a-A-21 (reverse) (Klindworth, Pruesse et al. 2013) with modifications(3.4.2.2). The V3-V4 hypervariable region was also selected as the parameter for identification of *Archaea*. Primer set used for amplification of V3-V4 region was 514Fa (forward) and In-house (reverse) with modifications(3.4.2.2). The scheme of thermal cycling was- initial denaturation at 98 °C for 5 min, 25 cycles of denaturation at 95 °C for 45 s, annealing at 57 °C for 45 s, and extension at 72 °C for 90 s followed by a final extension for 10 min at 72 °C. The PCR products were visualized on 0.8 % agarose gel and cleaned using GeneRead Size Selection Kit (QIAGEN) following the protocol as suggested by the manufacturer. Quantification of the cleaned PCR products was performed using the Quant-iT dsDNA BR assay kit and a Qubit fluorometer (Invitrogen) as suggested by the manufacturer. PCR products were pooled in an equal amount of DNA as per sediment sample, primer and type of template resulting in 6 samples for *Archaea* (3 each for DNA and cDNA) and 6 for *Bacteria* (3 each for DNA and cDNA). The Goettingen Genomics Laboratory determined the sequences of the partial 16S rRNA genes by using a MiSeq System (Illumina) and the instructions of the manufacturer for amplicon sequencing.

3.10.2 AMPLIFICATION OF PARTIAL 16S rRNA GENE FOR ADDITIONAL TOTAL DIVERSITY ASSESSMENT

The V3-V5 region of the 16S rRNA gene was amplified by PCR (polymerase chain reaction). Every sample was amplified 3 times in replica along with negative controls (no template) resulting in a total of 20 reactions; 50µl each (18 samples, 2 controls). 3 sediment samples (Lonar 1, Lonar 2 and Lonar 3), one type of template (DNA), two set of primers (archaeal and bacterial) and 3 replicas for each resulting in 18 reactions in total. Each PCR reaction (50µl) contained 31 µl nuclease-free demineralized sterile H₂O, 10µl 5 fold reaction buffer (Phusion GC Buffer, Thermo Scientific), 1µl of 10 mM of deoxynucleoside triphosphates (all four), 2.5 µl DMSO, 2 µl of BSA, 1µl of 10µM primer each (forward and reverse) , 0.5µl of 2U/µl Phusion high-fidelity DNA Polymerase (Thermo Scientific) , 1µl isolated DNA of 50 ng/µl as template. The V3-V5 region was amplified with the following set of primers containing the Roche 454 pyrosequencing adaptors, key and MID or multiplex identifier in the case of forward primer only. For *Bacteria*, the primer sequences were modified Bac343F(Nossa, Oberdorf et al. 2010) primer and modified Bac907R(Muyzer, Teske et al. 1995); for *Archaea* modified Arch340F(Ovreås, Forney et al. 1997) primer and modified Arch915R(Stahl and Amann 1991). Details of the primers are provided in 3.4.2.1. The scheme of thermal cycling was- initial denaturation at 98 °C for 5 min, 25 cycles of denaturation at 95 °C for 45 s, annealing at 64 °C for 45 s, and extension at 72 °C for 90 s followed by a final extension for 10 min at 72 °C.

All amplified triplicate samples were pooled in equal amounts resulting in 6 samples (3 for *Bacteria* and 3 for *Archaea*, and purified using the peqGold gel extraction kit as recommended by the manufacturer (Peqlab Biotechnology). Quantification of the PCR products was performed using the Quant-iT dsDNA BR assay kit and a Qubit fluorometer (Invitrogen) as suggested by the manufacturer. PCR products were pooled in an equal amount of DNA as per sediment sample and primer resulting in 3 samples for *Archaea* and 3 for *Bacteria*. The Goettingen Genomics Laboratory determined the sequences of the partial 16S rRNA genes by using a Roche GS-FLX 454 pyrosequencer (Roche) and the instructions of the manufacturer for amplicon sequencing.

3.10.3 AMPLIFICATION OF PARTIAL 16S rRNA GENE FOR COMMUNITY DYNAMICS STUDY DURING MICROENVIRONMENTAL PH HOMEOSTASIS

The V3-V4 region of the 16S rRNA gene was amplified by PCR (polymerase chain reaction). Every sample was amplified 3 times in replica along with negative controls (no template) resulting in a total of 208 reactions; 50µl each (186 samples, 22 controls). Each PCR reaction (50µl) contained 31 µl nuclease-free demineralized sterile H₂O, 10µl 5X reaction buffer (Phusion GC Buffer for *Archaea* and Phusion HF buffer for *Bacteria*, ThermoFisher Scientific), 1µl of 10 mM of deoxynucleoside triphosphates (all four), 2.5 µl DMSO, 2 µl of BSA [20 mg ml⁻¹ in 10 mM Tris-HCl (pH 7.4 at 25o C), 100 mM KCl, 1 mM EDTA and 50 % (v/v) glycerol] (ThermoFisher Scientific), 1µl of 10µM primer each (forward and reverse), 0.5µl of 2U/µl Phusion high-fidelity DNA Polymerase (ThermoFisher Scientific), 1µl of isolated DNA diluted to 50 ng/µl concentration as template.

The V3-V4 hypervariable region was selected as the parameter for identification of *Bacteria* (Vasileiadis, Puglisi et al. 2012, Vilo and Dong 2012). Primer set used for amplification of V3-V4 region was S-D-Bact-0341-b-S-17 (forward) and S-D-Bact-0785-a-A-21 (reverse) (Klindworth, Pruesse et al. 2013) with modifications (3.4.2.2). The V3-V4 hypervariable region was also selected as the parameter for identification of *Archaea*. Primer set used for amplification of V3-V4 region was 514Fa (forward) and In-house (reverse) with modifications (3.4.2.2).

The scheme of thermal cycling was- initial denaturation at 98 °C for 5 min, 25 cycles of denaturation at 95 °C for 45 s, annealing at 57 °C for 45 s, and extension at 72 °C for 90 s followed by a final extension for 10 min at 72 °C. The PCR products were visualized on 0.8 % agarose gel and cleaned using GeneRead Size Selection Kit (QIAGEN) following the protocol as suggested by the manufacturer. Quantification of the cleaned PCR products was performed using the Quant-iT dsDNA BR assay kit and a Qubit fluorometer (Invitrogen) as suggested by the manufacturer. The Goettingen Genomics Laboratory determined the sequences of the partial 16S rRNA genes by using a MiSeq System (Illumina) and the instructions of the manufacturer for amplicon sequencing.

3.11 NONOPTIMAL MICROENVIRONMENTAL PH EXPOSURE

To investigate how this microbial community responds to nonoptimal microenvironmental pH. We used Horikoshi media (with modification) which is the most commonly used culture medium for isolating alkaliphiles designed by Koki Horikoshi (Horikoshi 1999). The prokaryotic cultures were set up using modified Horikoshi media, which is essentially a 50 % v/v Horikoshi-I and Horikoshi-II. This modified Horikoshi media contains glucose 5g, soluble starch 5g, peptone 5g, yeast extract 5g, KH_2PO_4 1g, $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ 0.2g, Na_2CO_3 10g and NaCl 40g for every 1000 ml of media. The pH was adjusted to pH 9 (suboptimal) and pH 10 (superoptimal) with sterile NaOH, respectively. A total of 8 cultures of 300 ml modified Horikoshi were set up 4 for each initial pH; 3 samples and 1 control. The modified Horikoshi medium was inoculated with 3 g of the sediment mix. Controls were used to see the extent of the effect of aeration due to shaking and the impact of the sediment itself. Two controls, one for each pH, were inoculated with 3 g of double autoclaved sediment. The cultures were incubated at 30 °C on a shaker at 100 rpm. 50 ml of culture from each flask was taken every 5 days until 25 days. The samples were centrifuged for 15 min at 10,000 rpm and 10 °C (Sorvall RC 6 Centrifuge, Thermo Scientific). The supernatants were used for pH measurement and HPLC analysis. The resulting cell pellets were used for DNA extraction.

3.12 HPLC ANALYSIS OF CULTURE FILTRATE FROM NONOPTIMAL PH EXPOSURE BY VARIAN STAR PLATFORM

Culture filtrates were collected and centrifuged at low rpm resulting supernatants were filter sterilized and lyophilized, stored in the freezer until required. HPLC was performed on a Varian Star instrument where channel was A=Fluores 1V, detector type was ADCB (1 Volt), bus address 16, sample rate 5.00 Hz and run time was 50.003 min. Run mode was analysis, peak measurement was peak area and calculation type was percent.

3.13 SEQUENCING

In this study, we have used high-throughput next generation sequencing (NGS) techniques to explore the total and the active prokaryotic biodiversity of sediment from Lonar crater lake. High-throughput sequencing is also deployed to follow the community dynamics of prokaryotes during microenvironmental pH homeostasis and direct metagenome analysis. With the advance in high-throughput sequencing techniques, it is possible to produce an enormous volume of data cheaply (Metzker 2010). In this study, we have used two different high-throughput sequencing platform, both based on 'sequencing by synthesis' principle of Melamade(Melamade 1985, Hyman 1988). One is Roche GS-FLX 454 pyrosequencer, and the other is Illumina MiSeq.

3.13.1 SEQUENCING BY ILLUMINA MISEQ

In the case of Illumina, solid-phase amplification PCR is used for clonal amplification of templates to yield arbitrarily distributed clusters on a glass slide. Forward and reverse primers are covalently attached to the slide. The primer-template ratio delimits density of the amplified clusters. This process results in hundreds of millions spatially distinct template clusters. Illumina uses a four-color cyclic reversible terminator (CRT) chemistry that includes four different fluorescently labeled nucleotide incorporation, fluorescence imaging, and cleavage repeatedly (Metzker 2005). A DNA polymerase, attached to the primed template, adds only one fluorescently modified nucleotide, which represents the complement of the template base. The DNA synthesis is termination after the addition of a single nucleotide. The remaining unincorporated nucleotides are discarded. After that imaging is performed to identify the incorporated nucleotide. Then a cleavage step removes the terminating or inhibiting group along with the fluorescent dye. After washing, next cycle is performed. In this way, one nucleotide per cycle, the sequence is determined. Four colors are detected by total internal reflection fluorescence imaging using two lasers (Metzker 2010).

3.13.2 SEQUENCING BY ROCHE GS-FLX 454 PYROSEQUENCER

In the case of Roche, emulsion PCR (emPCR) is used to amplify the templates clonally. Universal priming sites of adaptors are ligated to the target ends; the DNA strands are separated and arrested onto beads as one DNA molecule per bead. After emPCR beads are amplified and enriched, they are deposited on individual picotiter plate (PTP) wells (Leamon, Lee et al. 2003, Metzker 2010). The NGS chemistry is performed in these wells. Smaller beads with attached sulfurylase and luciferase are loaded into wells surrounding the larger template beads. Different dNTPs are then flowed through the wells and dispensed in a predetermined chronological order. The sequencing chemistry of Roche is a non-electrophoretic, bioluminescence scheme that measures the release of inorganic pyrophosphate, that is pyrosequencing, by proportionately translating it into a visible light signal using a series of enzymatic reactions (Ronaghi, Karamohamed et al. 1996, Ronaghi, Uhlen et al. 1998). This bioluminescence is imaged with a charge-coupled device camera.

3.14 ANALYSIS OF SEQUENCING DATA

3.14.1 ANALYSIS OF SEQUENCING OF PARTIAL 16S rRNA GENE DATA FROM ILLUMINA PLATFORM

Raw sequence data from samples of the total and the active community diversity was extracted from Illumina MiSeq. Datasets, generated from MiSeq sequencing, of partial 16S rRNA gene, were processed with Usearch version 8.0.1623 (Edgar 2010, Edgar and Flyvbjerg 2015). Paired-end reads were merged and quality filtered. Filtering included the removal of reads shorter than 400bp. Processed sequences of all samples were joined and clustered into operational taxonomic units (OTUs) at 3 % genetic dissimilarity using the UPARSE algorithm (Edgar 2013) implemented in USEARCH. A denovo chimera removal was included in the clustering step. Afterward, putative chimeric sequences were removed using UCHIME (Edgar, Haas et al. 2011) in reference mode. It was done with the

SILVA database (Silva SSURef 123 NR) (Quast, Pruesse et al. 2013, Yilmaz, Parfrey et al. 2014) as reference dataset (Camacho, Coulouris et al. 2009). Subsequently, processed sequences were mapped to OTU sequences to obtain an OTU table. Taxonomy was determined by the representative sequence of each OTU classified by BLAST alignment against the most recent SILVA database (see above). All non-Bacterial or non-Archaeal OTUs were removed. Alpha diversity indices and rarefaction curves were calculated with QIIME version 1.9 (Caporaso, Kuczynski et al. 2010) as described by Wemheuer et al. (Wemheuer, Taube et al. 2013).

3.14.2 ANALYSIS OF SEQUENCING OF PARTIAL 16S RRNA GENE DATA FROM ROCHE PLATFORM

Raw sequence data from samples of additional total diversity survey was extracted from Roche GS-FLX 454 pyrosequencer. Sequences with shorter read than 300bp, with an average quality value below 25, sequences with number of ambiguous base exceeding 6, possessing long homopolymer stretches (>8bp) and primer mismatches (>3) were removed. These sequences were denoised using Acacia denoiser (Bragg, Stone et al. 2012). Remaining primer sequences were truncated from the obtained reads with Cutadapt (Martin 2011). Chimeric sequences were also removed using UCHIME and the current greengenes core set as reference dataset (DeSantis, Hugenholtz et al. 2006, Edgar, Haas et al. 2011). Processed sequences of all samples were joined, sorted by decreasing the length, and clustered employing the UCLUST algorithm (Edgar 2010). Sequences were clustered into operational taxonomic units (OTUs) at 3 % genetic divergence according to Simon et al. (Simon, Wiezer et al. 2009). OTUs at 3 % divergences represent species level (Schloss and Handelsman 2005). The phylogenetic composition was determined using the QIIME (Version 1.9) assign_taxonomy.py script (Caporaso, Kuczynski et al. 2010). A BLAST alignment against the Silva SSURef 123 NR database (Pruesse, Quast et al. 2007) was thereby performed. Sequences were classified as per the Silva taxonomy of their best hit. Rarefaction curves, Shannon indices (Shannon 2001) and Chao1 indices (Chao and Bunge 2002) were calculated.

3.14.3 ANALYSIS OF DIRECT METAGENOME SEQUENCING DATA FROM ILLUMINA PLATFORM

Reads were obtained from the direct sequencing of the Lonar metagenome and filtered with Trimmomatic v.0.30 (Bolger, Lohse et al. 2014). Assembling of this metagenomic data was performed with SPAdes, v 3.7.0, (Bankevich, Nurk et al. 2012, Nurk, Bankevich et al. 2013) using its metaSPAdes function and default settings. Resulting contigs were analyzed for predicted ORFs (Open Reading Frame) with Prodigal (Prokaryotic Dynamic Programming Gene Finding Algorithm) , v 2.6.0 (Hyatt, Chen et al. 2010) using recommended mode for metagenome, Anonymous mode. All the protein translation results of these ORFs from Prodigal are annotated and analyzed using GhostKOALA, v 2.0 (Kanehisa, Sato et al. 2016), a KEGG (Kyoto Encyclopedia of Genes and Genomes) tool for functional characterization of the genome and metagenome sequences. Reconstruction of Genes or proteins (KEGG GENES) , KO groups (KEGG ORTHOLOGY) , KEGG modules, functional hierarchy (KEGG BRITE) and pathway maps (KEGG PATHWAY) were also obtained from the GhostKOALA service by KEGG.

Results and Discussion

4 RESULTS AND DISCUSSION

4.1 DESCRIPTION OF SEDIMENT SAMPLES FROM LONAR CRATER LAKE

The coordinates of Lonar 1, Lonar 2 and Lonar 3 (as described in 3.1) were 19°58'20.65"N 76°30'22.74"E, 19°58'36.70"N 76°30'45.19"E and 19°58'47.82"N 76°30'17.50"E. Their respective pH and temperature were found to be pH 9.7, pH 10.2, pH 10.3 and 27.2 °C, 28.5 °C, 28.3 °C. Lonar 1, Lonar 2 and Lonar 3 were mixed thoroughly w/w and analyzed to determine an overview of physicochemical parameters of Lonar lake sediment (Table 24). All samples were stored at -80 °C until further processing.

TABLE 24: PHYSICOCHEMICAL PARAMETERS OF LONAR SEDIMENT

Parameter	Unit	Measuring Value	Detection Limit
Ash 815 °C (FG)	% of Wet Weight	57.9	
Ash 815 °C (FG)	% of Dry Weight	97.3	
Carbon	% of Wet Weight	0.8	
Carbon	% of Dry Weight	1.3	
Hydrogen	% of Wet Weight	0.1	
Hydrogen	% of Dry Weight	0.1	
Nitrogen	% of Wet Weight	0.2	
Nitrogen	% of Dry Weight	0.3	
Oxygen	% of Wet Weight	0.6	
Oxygen	% of Dry Weight	1	
Sulphur	% of Wet Weight	0	
Sulphur	% of Dry Weight	0	
Aluminium	mg/L	0.18	0.02
Antimony	mg/L	< 0.010	0.01
Arsenic	mg/L	< 0.010	0.01

Parameter	Unit	Measuring Value	Detection Limit
Barium	mg/L	< 0.0050	0.005
Beryllium	mg/L	< 0.001	0.001
Boron	mg/L	0.89	0.02
Cadmium	mg/L	< 0.001	0.001
Calcium	mg/L	6	0.1
Chromium	mg/L	< 0.005	0.005
Copper	mg/L	0.089	0.005
Iron	mg/L	< 0.005	0.005
Lead	mg/L	< 0.01	0.01
Lithium	mg/L	< 0.02	0.02
Magnesium	mg/L	0.56	0.1
Manganese	mg/L	< 0.005	0.005
molybdenum	mg/L	0.029	0.005
Nickel	mg/L	< 0.005	0.005
Phosphorus	mg/L	1.57	0.05
Potassium	mg/L	4.3	0.1
Selenium	mg/L	< 0.01	0.01
Sodium	mg/L	374	0.1
Strontium	mg/L	0.0289	0.005
Thallium	mg/L	< 0.005	0.005
Tin	mg/L	< 0.0	0.005
Titanium	mg/L	< 0.02	0.02
Vanadium	mg/L	0.98	0.005
Zinc	mg/L	0.039	0.01

The physicochemical parameters of the sediments samples that we collected differ from some of the other studies in certain aspects, particularly Sulphur, Iron and Nickel. In our analysis, we failed to detect these elements whereas in the studies from 2006 (Wani, Surakasi et al. 2006) and 2015 (Paul, Kumbhare et al. 2015) these elements have been detected. Sulfates as high as 53.84 mg/L, Iron as high as 7.9 g/L and Nickel as high as 8.25 mg/L has been reported from these studies from 2006 and 2015.

4.2 THE TOTAL AND THE ACTIVE PROKARYOTIC DIVERSITY OF LONAR CRATER LAKE

4.2.1 GENERAL ANALYSES OF THE TOTAL AND THE ACTIVE COMMUNITY SEQUENCING DATA

The significant benefit of illumine sequencing technique is the assembly of paired-end reads. This assembly prominently decreases the number of erroneous sequences included in downstream analyses (Bartram, Lynch et al. 2011). Significantly low total error rates, between 0.0030 and 0.0049, has been reported for Illumina MiSeq (Ross, Russ et al. 2013). As described in 3.10.1 and 3.13.1, V3-V4 region of the 16S rRNA gene was amplified and sequenced. Reads shorter than 400bp was removed. Processed sequences of all samples were joined and clustered into operational taxonomic units (OTUs) at 3 % genetic dissimilarity using the UPARSE algorithm (Edgar 2013) implemented in USEARCH. The analysis method is provided in 3.14.1. A total of 12 samples (3 sediments X 2 types of target domains X 2 types of template DNA) as described in 3.10.1 and 3.10.2. In 6 samples of the total diversity assessment, a total of 31,650 and 59,079 of archaeal and bacterial sequences were found. It ranges from 11,664 to 5,567 with an average of 10,550 in *Archaea* and in *Bacteria* it varies from 26,430 to 13,979 with an average of 19,693. In the case of 6 samples of the active community diversity, a total of 20,724 and 71,205 of archaeal and bacterial sequences were found. It ranges from 9,691 to 4,907 with an average of 6,908 for *Archaea* and for *Bacteria* it is 28,270 to 16,079 with an average of 23,735.

4.2.2 PROKARYOTIC DIVERSITY AND RICHNESS

All the OTUs were identified at genetic distances of 3 % by using 4,907 randomly selected and denoised sequences per sample for *Archaea* and 13,979 sequences per sample for *Bacteria* were used. These OTUs were used to determine rarefaction curves, richness, and diversity. At 3 % genetic distance all rarefaction curves attain a significant level of saturation, indicating that the surveying effort covered a substantial range of taxonomic diversity present in the samples at this genetic distance (Figure 6).

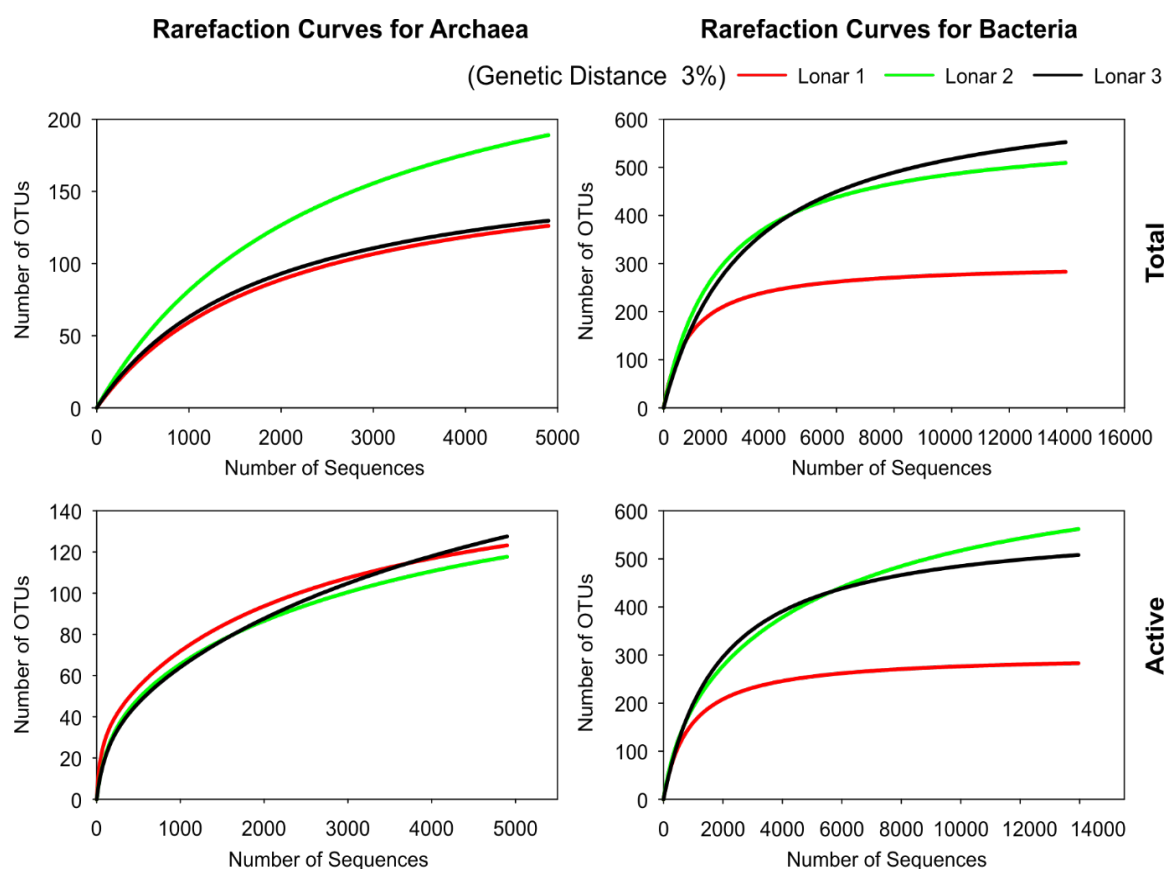


FIGURE 6: RAREFACTION CURVES INDICATING THE OBSERVED NUMBER OF OPERATIONAL TAXONOMIC UNITS (OTUS) IN THE TOTAL AND THE ACTIVE COMMUNITY STUDY. Three different samples are indicated in red, green and black respectively for Lonar 1, Lonar 2 and Lonar 3. The curves indicate gradual saturation in the number of OTUs with an increase in the number of sequences examined. On the left rarefaction curves for *Archaea* and on the right for *Bacteria*, all the curves are at 3 % genetic distances.

Evaluation of the rarefaction analyses with the number of OTUs determined by Max. clusters (n_{max}) revealed that among *Archaea*, the total and the active community diversity covered 74.21 % (± 1.83) and 84.07 % (± 3.52) of the estimated complete diversity. In the case of *Bacteria* the total and the active community diversity covered 59.78 % (± 2.24) and 88.98 % (± 3.96). Highest coverage in *Bacteria* among all samples was observed in the active community of Lonar 1, 93.95 %. Lowest coverage was found in the total diversity of Lonar 2 for Bacteria, 56.61 %. Thus, we did not survey the full extent of taxonomic diversity at these genetic distances, but the surveying efforts assessed a substantial fraction of the prokaryotic diversity within individual sediment samples. In samples of the total and the active community diversity, variation in the number of OTUs is observed (Figure 7).

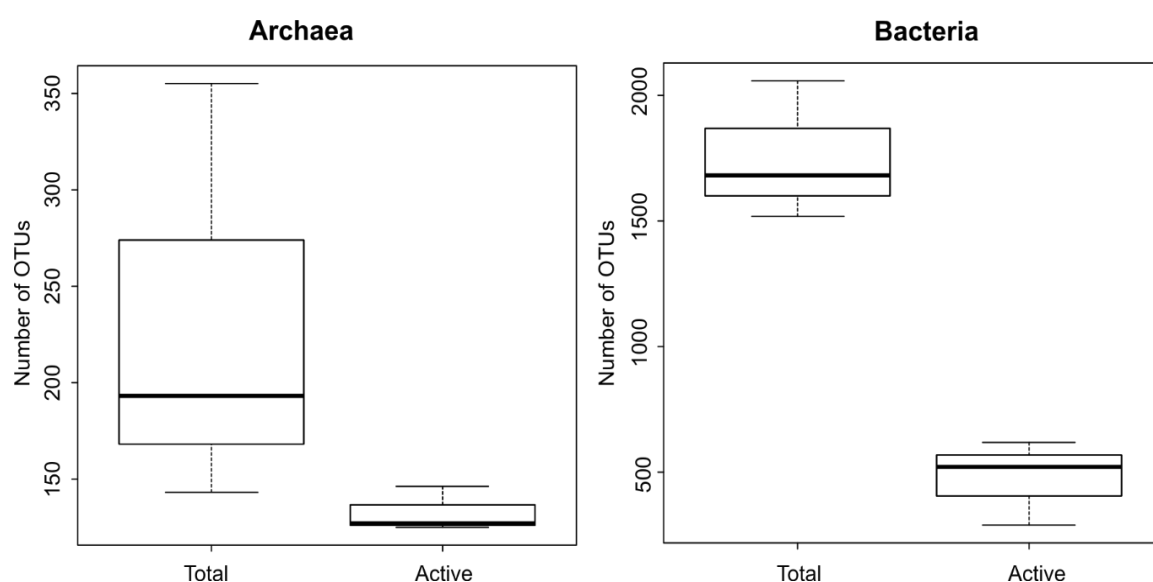


FIGURE 7: BOXPLOT DIAGRAM OF THE NUMBER OF TAXONOMIC UNITS (OTUs) AT THE SPECIES LEVEL IN DIFFERENT SAMPLING EFFORTS. On the left is the boxplot diagram for *Archaea* and on the right is for *Bacteria*.

In the survey of the total diversity, the highest number of unique OTUs in *Archaea* and *Bacteria* were found from Lonar 2 and Lonar 1 of 355 OTUs and 2,057 OTUs respectively. Among the active community, the highest number of OTUs in *Archaea* and *Bacteria* were observed in Lonar 1 and Lonar 3 of 146 OTUs and 618 OTUs respectively. Most of the OTUs derived from all the samples shows that majority of them are not cultured (Figure 8). One noteworthy observation in this regard is that the total archaeal diversity has more cultured representatives than the active archaeal diversity. However, in the case of *Bacteria*, the active community diversity is better represented in terms of closely related

cultured members than in the total diversity. In archaeal samples, the average percentage of cultured OTUs in the total and the active community diversity was found to be 32.11 % (± 13.21) and 17.6 % (± 4.56). In the case of *Bacteria*, we observed, the average percentage of cultured OTUs in the total and the active community diversity of 26.25 % (± 8.14) and 48.75 % (± 8.04). Among all the observations highest percentage of cultured prokaryotes, 59.95 %, was found in the active bacterial community of Lonar 3 sediment. Lowest percentage of cultured prokaryotes were observed in the active archaeal community of Lonar 3 sediment. These observations indicate that bacterial community of soda lakes are better investigated than archaeal community. Also, the majority of the prokaryotes do not have a cultured representative.

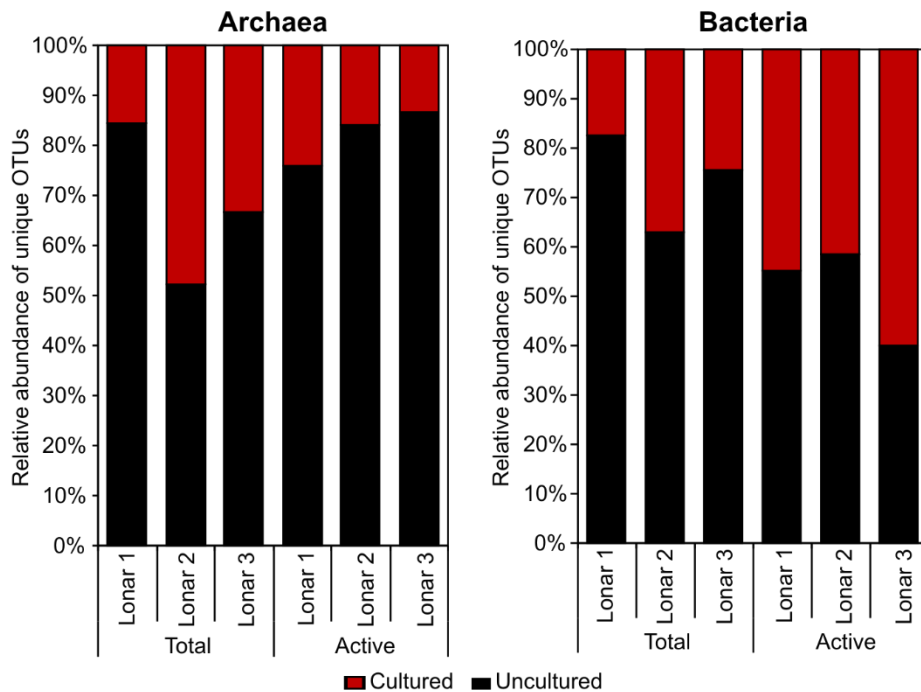


FIGURE 8: RELATIVE DISTRIBUTION OF UNCULTURED AND CULTURED UNIQUE OTUs. On the left distribution of uncultured and cultured unique OTUs among *Archaea* is shown. On the right distribution of uncultured and cultured unique OTUs among *Bacteria* is shown.

Details of all 12 individual observations with their Observed clusters, Max. clusters (n_{max}), Coverage (%), Shannon index (H') and Chao1 are given in Table 25. The comparison of coverage of all the 12 observations highest 5 covered samples shows that all 5 are from the active community. It indicates that, in our survey, the active community is more widely covered than the total diversity. Also, this observation signifies that the

total diversity is more diverse than the active community, and all the prokaryotes of the sediment may not be active at the time of collection. Out of these 5 observations, only 2 are from *Archaea* and 3 from *Bacteria*. In our assessment *Bacteria* is better covered than *Archaea*. It may be a result of recovering more bacterial sequences than Archaeal. The coverage of these samples ranged from 93.96 % to 84.27 %. The comparison of Shannon index from all 12 observations revealed that out of highest indexed 5 samples 3 of them were from the total diversity and 2 of them were from the active community. It indicates that the total diversity is more diverse than the active community. Also, we noticed that all of these 5 observations were from *Bacteria*, which signifies that bacterial diversity is more than archaeal in this habitat. Highest Shannon index was observed to be 7.762 in the total diversity of Lonar 3 *Bacteria*. In the case of Chao 1 among the 5 with highest values are all from *Bacteria*. 3 of which are from the total diversity and other 2 are from the active community diversity. In these 5 samples, Chao 1 values are between 3,755.51 to 545.27. From all the Observed clusters, Max. clusters (n_{max}), Shannon index (H') and Chao1, sediments of Lonar 3 and Lonar 2 tends to be more diverse than Lonar 1 sediments.

TABLE 25: GENERAL ANALYSIS OF THE SEQUENCE DATA.

Sample		Observed clusters	Max. clusters (n _{max})	Coverage (%)	Shannon index (H')	Chao1
Total	Lonar 1 (Archaea)	131.6	177.49	74.14	3.01	225.52
	Lonar 2 (Archaea)	191.9	266.52	71.99	3.75	453.88
	Lonar 3 (Archaea)	135	176.47	76.49	3.67	226.82
	Mean	152.83	206.83	74.21	3.48	302.08
	SD	27.65	42.21	1.83	0.33	107.34
	CV	0.18	0.20	0.02	0.09	0.35
	Lonar 1 (Bacteria)	1405	2292.51	61.28	7.50	3491.55
Lonar 2 (Bacteria)	1405.3	2482.00	56.61	6.75	3557.27	

Sample			Observed clusters	Max. clusters (n _{max})	Coverage (%)	Shannon index (H')	Chao1
Total	Lonar	3	1518	2469.82	61.46	7.76	3755.51
	(Bacteria)						
	Mean		1442.76	2414.77	59.78	7.33	3601.44
	SD		53.19	86.59	2.24	0.42	112.19
Sample			Observed clusters	Max. clusters (n _{max})	Coverage (%)	Shannon index (H')	Chao1
	CV		0.03	0.03	0.03	0.05	0.03
Active	Lonar	1	123.3	140.31	87.87	4.41	145.78
	(Archaea)						
	Lonar	2	118.2	139.13	84.95	3.79	157.32
	(Archaea)						
	Lonar	3	127	159.98	79.38	3.71	157.51
	(Archaea)						
	Mean		122.83	146.47	84.07	3.97	153.54
	SD		3.60	9.56	3.52	0.31	5.48
	CV		0.02	0.06	0.04	0.07	0.03
	Lonar	1	283.7	301.93	93.95	5.69	291.22
(Bacteria)							
Lonar	2	512.4	577.52	88.72	6.23	545.27	
(Bacteria)							
Lonar	3	559	663.35	84.26	5.74	628.31	
(Bacteria)							
Mean		451.7	514.27	88.98	5.89	488.27	
SD		120.30	154.17	3.96	0.24	143.36	
CV		0.26	0.29	0.04	0.04	0.29	

4.2.3 DISTRIBUTION OF TAXA AND PHYLOTYPES

4.2.3.1 THE TOTAL AND THE ACTIVE COMMUNITY DIVERSITY OF ARCHAEA

Among *Archaea*, we were able to classify 99.91 % and 98.94 % of all the sequences above domain level in the total and the active community diversity respectively. All the archaeal OTUs were distributed to 10 Order or order-level taxa and 47 Genera. In the total and the active community 45 and 27 genera were observed. Relative abundances of archaeal orders are presented in Figure 9 and Figure 10. The genera are presented in Figure 11 and Figure 12. Details of the relative abundances of the orders and genera are provided in Table 33 and Table 34. Most dominant orders were *Halobacteriales*, *Methanobacteriales*, *Methanomicrobiales*, *Thermoplasmatales*, *Methanosarcinales*.

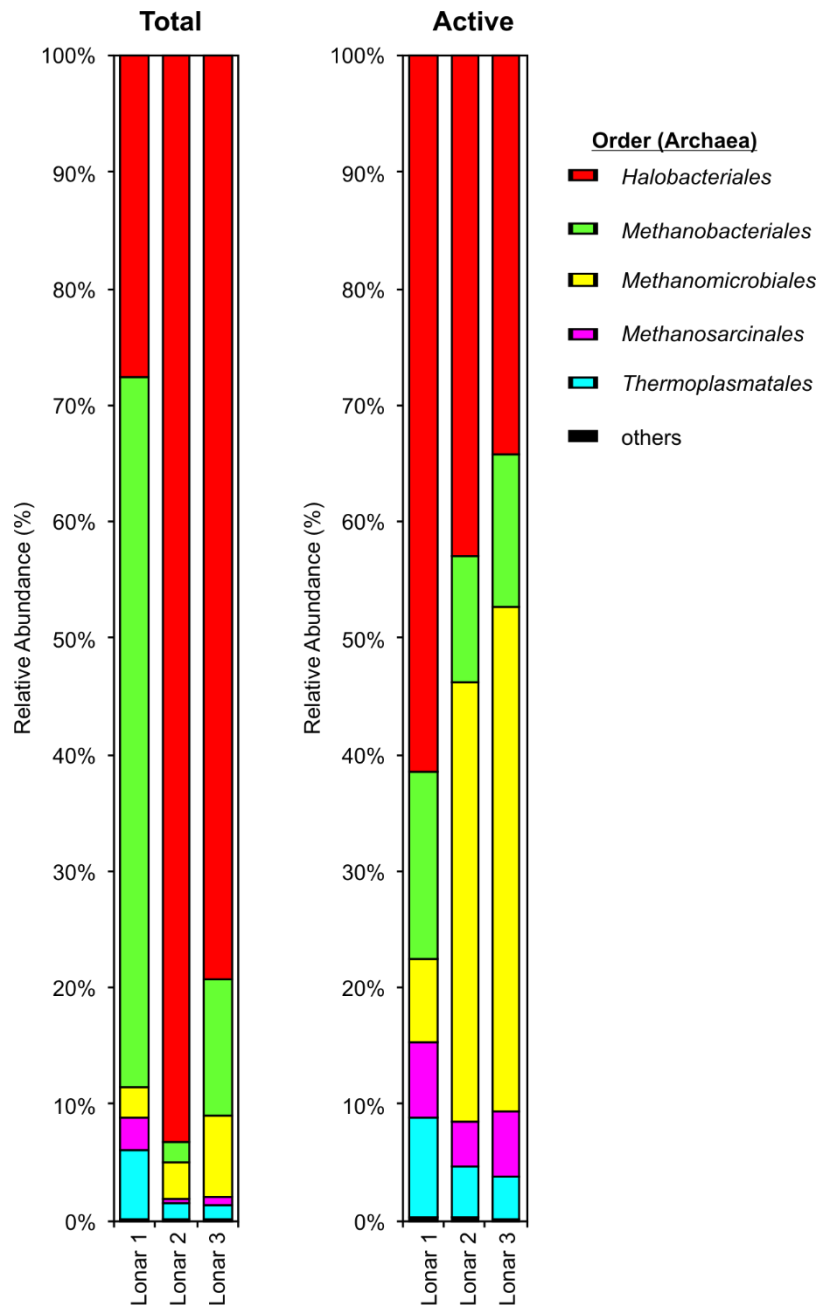


FIGURE 9: RELATIVE ABUNDANCES OF ARCHAEOAL ORDERS IN LONAR SEDIMENTS. Sample numbers indicating the different sediment samples are given below the respective bars. Orders are accounting for <1 % of all classified sequences are summarized in the artificial group 'others.'

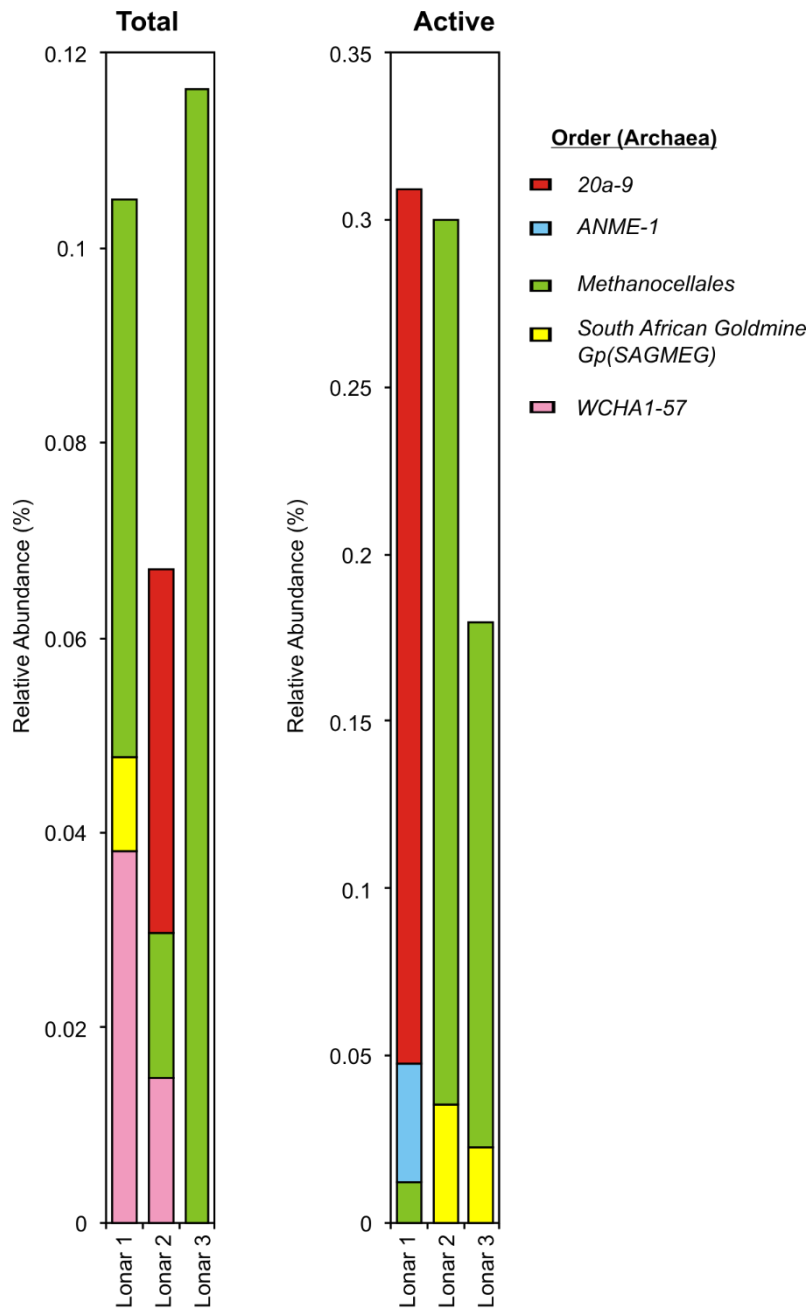


FIGURE 10: RELATIVE ABUNDANCES OF THE RARE ARCHAEOAL ORDER IN LONAR SEDIMENTS. Sample numbers indicating the different sediment samples are given below the respective bars. Only the phylogenetic groups are accounting for <1 % of all classified sequences are shown.

In the total and the active community, *Halobacteriales* alone contribute to the average relative abundance of 66.73 % and 46.18 %. Highest relative abundance, 93.31 %, of *Halobacteriales* was observed in the total diversity of Lonar 3 sediments, and lowest of 27.59 % was found in the sediments of Lonar 1. In the total diversity, the *Halobacteriales* were mostly represented by *Natronococcus* (72.28 %), *Haloterrigena* (10.71 %), *Natrialba*

(2.66 %) , *Halovivax* (1.34 %) , and *Natronorubrum* (0.59 %). In case of the active community diversity, *Natronococcus* (50.78 %) , *Haloterrigena* (5.87 %) , *Halovivax* (1.44 %) , *Natrialba* (0.82 %) , and *Natronorubrum* (0.22 %) represents the order *Halobacteriales*. Two novel haloalkaliphilic archaeon species of *Natronococcus*, *Natronococcus amylolyticus* sp. nov., and *Natronococcus roseus* sp. nov., has been isolated from Kenyan soda lake, Lake Magadi and sediments of the soda lake Chagannor, Inner Mongolia, China, they are able to grow at pH as high as pH 11 (Kanal, Kobayashi et al. 1995, Corral, Gutierrez et al. 2013). Members of the genus *Haloterrigena* are mostly halophilic and often alkaliphilic as well. Novel species of this genus has been isolated from soda lakes and are known to grow at NaCl concentrations as high as 4.5 M, pH as high as pH 11.(Ventosa, Gutierrez et al. 1999, Selim and Hagagy 2016). The novel genus *Halovivax* are mostly extremely halophilic and moderately alkaliphilic, they have been isolated from salt lakes of Iran and Inner Mongolia, China, they usually are able to grow at pH lower than pH 10.(Castillo, Gutierrez et al. 2006, Castillo, Gutierrez et al. 2007, Amoozegar, Makhdoumi-Kakhki et al. 2014, Amoozegar, Makhdoumi-Kakhki et al. 2015). However, *Halovivax* has also been reported from soda lakes, for example, Lake Elmenteita in Kenya(Mwirichia, Cousin et al. 2010). Novel species of *Natrialba*, *Natrialba hulunbeirensis* sp. nov., and *Natrialba chahannaensis* sp. nov., has been reported from soda lakes of Inner Mongolia, China(Xu, Wang et al. 2001). Two novel haloalkaliphilic species of novel genus *Natronorubrum*, *Natronorubrum bangense* gen. nov., sp. nov., and *Natronorubrum tibetense* gen. nov., sp. Nov., have been isolated from the soda lakes of Tibet, with the ability to grow at pH as high as pH 11(Xu, Zhou et al. 1999).

In the total and the active community diversity the relative abundance of the order *Methanobacteriales* was found to be 24.75 % and 13.34 % respectively. Sediments of Lonar 1 shows the highest relative abundance of, 60.99 %, *Methanobacteriales* in the total diversity and lowest relative abundance, 1.66 %, was found in the sediments of Lonar 2 in the total diversity. The order *Methanobacteriales* was represented by the genera *Methanobacterium* (0.68 %) , *Methanobrevibacter* (0.03 %) , *Methanosphaera* (0.01 %) in the total community and *Methanobrevibacter* (16.90 %) , *Methanobacterium* (2.01 %) , in the active community diversity. We were unable to detect any *Methanosphaera* in the active community. Several methylotrophic novel species of *Methanobacterium* has been

observed from various lake sediments. For example *Methanobacterium movens* sp. nov., *Methanobacterium flexile* sp. nov., *Methanobacterium lacus* sp. nov., (Zhu, Liu et al. 2011, Borrel, Joblin et al. 2012, Schirmack, Mangelsdorf et al. 2014). Although they are not of significance in terms of alkaliphily, they are well-known methanogens. We found a paucity of available literature about the presence of *Methanobrevibacter* and *Methanosphaera* in soda lakes. Which is not surprising as there is a general lack of investigations on *Archaea* of soda lakes as compared to *Bacteria*. However, they are both well known for their methanogenic activity (van de Wijngaard, Creemers et al. 1991).

The order *Methanomicrobiales* was observed to be of 4.30 % and 29.39 % relative abundance in the total and the active community. Sediments of Lonar 3 showed the maximum abundance of *Methanomicrobiales*; that is 43.21 % in the active community and lowest value of 2.58 % was observed in sediments of Lonar 1 in the total diversity assessment. The order *Methanomicrobiales*, in the total diversity, was represented by the genera *Methanocalculus* (2.74 %), *Methanoculleus* (0.05 %) and *Methanogenium* (0.004 %). In case of the active diversity, *Methanomicrobiales* was represented by *Methanocalculus* (5.87 %), *Methanocorpusculum* (0.17 %), *Methanospirillum* (0.12 %) and *Methanoculleus* (0.08 %). Haloalkaliphilic methanogenic novel species, *Methanocalculus alkaliphilus* sp. nov., and *Methanocalculus natronophilus* sp. nov., of the genus *Methanocalculus* has been isolated from hypersaline soda lakes including Lonar with optimum growth pH between pH 9 and pH 9.5 (Surakasi, Wani et al. 2007, Zhilina, Zavarzina et al. 2013, Sorokin, Abbas et al. 2015). Several novel methanogen species of genus *Methanocorpusculum* has been isolated from various waterbodies but not from any soda lake (Zellner, Stackebrandt et al. 1989). Novel species of the genus *Methanoculleus* has been isolated from marine sediment as well as from Lonar before and they are methanogenic (Mikucki, Liu et al. 2003, Dabir, Honkalas et al. 2014, Weng, Chen et al. 2015). New methanogenic species, for example, *Methanogenium frigidum* sp. nov., *Methanogenium marinum* sp. nov., *Methanogenium boonei* sp. Nov., of the genus *Methanogenium* has been isolated mostly from marine and lake sediments, however, none has been isolated from soda lakes (Franzmann, Liu et al. 1997, Chong, Liu et al. 2002, Kendall, Wardlaw et al. 2007). Novel methanogenic species, *Methanospirillum lacunae* sp. nov., and *Methanospirillum psychrodurum* sp. nov., of the genus *Methanospirillum* has

been isolated from soils but not from soda lakes mostly able to grow between pH 6.5 and pH 8 (Iino, Mori et al. 2010, Zhou, Liu et al. 2014).

Relative abundance of the order *Thermoplasmatales* was observed in the total and the active community to be 2.82 % and 5.48 % respectively. The highest relative abundance of 8.53 % was found in the active community of Lonar 1 sediments, and lowest relative abundance of 1.18 % was found in the sediments of Lonar 3 in the total diversity assessment. The order *Thermoplasmatales* is represented by the genus *Methanomassiliicoccus* in both the total and the active community diversity. Higher relative abundance was observed in the active community (0.72 %) than in the total diversity (0.004 %). Species of the genus *Methanomassiliicoccus* has been reported from sediments of estuary but not from any soda lake (Zhou, Chen et al. 2014).

In the total and the active community diversity the relative abundance of the order *Methanosarcinales* was found to be 1.27 % and 5.32 % respectively. Maximum relative abundance was noticed in the active community of Lonar 1 sediments to be 6.49 %. The sediments of Lonar 2 in the total diversity showed the lowest relative abundance of 0.32 %. They are mostly represented by *Methanosaeta* (4.93 %), *Methanosarcina* (0.32 %), *Methanolobus* (0.01 %), *Methanococcoides* (0.004 %) and *Methermicoccus* (0.004 %) in the total diversity. In the active community, they are mostly represented by *Methanosaeta* (14.89 %), *Methanosarcina* (1.88 %) and *Methanolobus* (0.07 %). *Methanosaeta* has been reported from the soda lakes of Kulunda Steppe, Altai, Russia able to function as methanogenic *Archaea* between pH 8 and pH 10.5 (Sorokin, Abbas et al. 2015). Several methanogenic species of *Methanosarcina* has been isolated and reported from various lake sediments including Lonar (Cairo, Clarens et al. 1992, Simankova, Parshina et al. 2001, Antony, Kumaresan et al. 2013, Ganzert, Schirmack et al. 2014). Methanogenic species of *Methanolobus* has been found in soda lakes of Kulunda Steppe, Altai, Russia and also from Lonar (Antony, Murrell et al. 2012, Sorokin, Abbas et al. 2015). Methanogenic species of *Methanococcoides* has been isolated from marine, lake and estuarine sediments but not from soda lakes (Singh, Kendall et al. 2005, Lyimo, Pol et al. 2009, Ticak, Hariraju et al. 2015). The novel methanogenic genus *Methermicoccus* with only one species, *Methermicoccus shengliensis* gen. nov., sp. nov.,

has only been isolated from Shengli oilfield, China. No species of *Methermicoccus* have been isolated or reported from soda lakes.

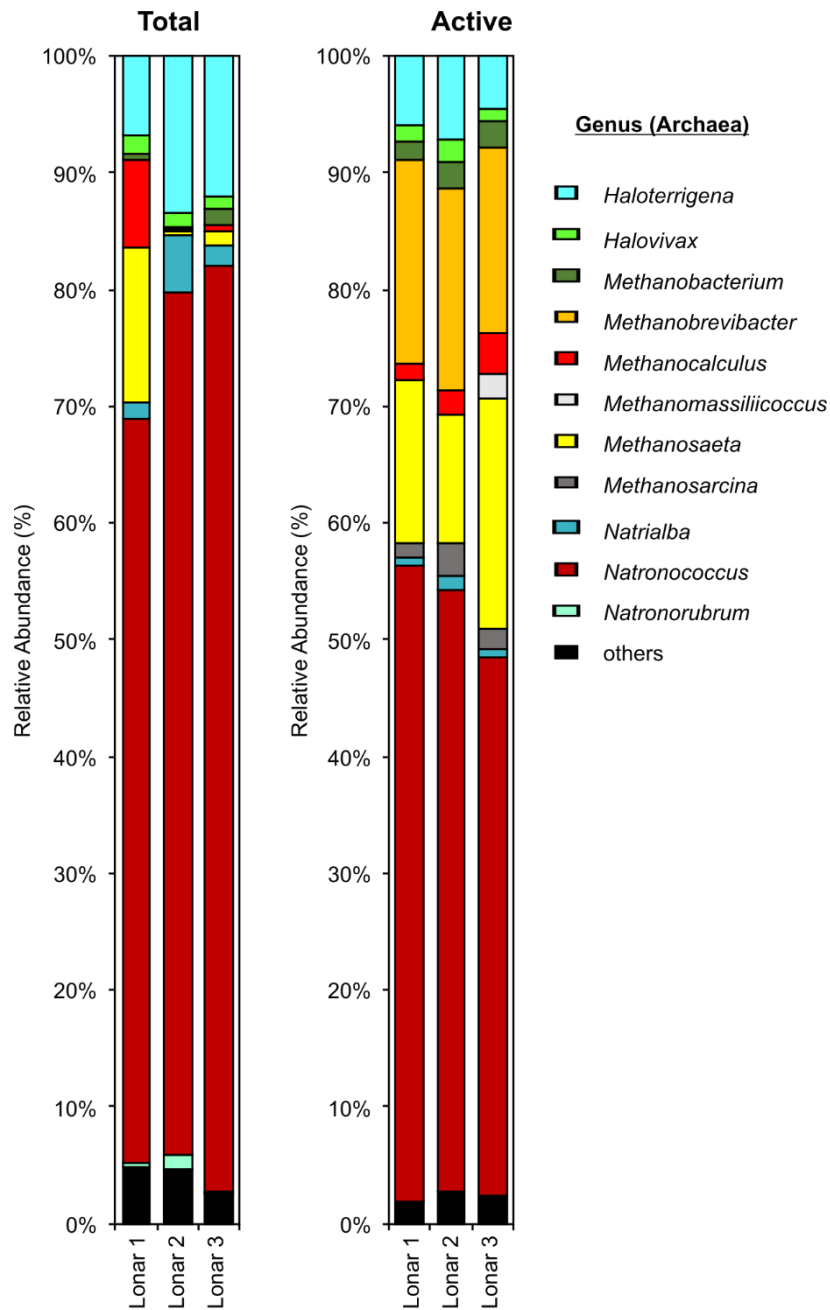


FIGURE 11: RELATIVE ABUNDANCES OF THE MOST ABUNDANT ARCHAEOAL GENERA IN LONAR SEDIMENTS. Sample numbers indicating the different sediment samples are given below the respective bars. Only the phylogenetic groups accounting for >1 % of all classified sequences are shown.

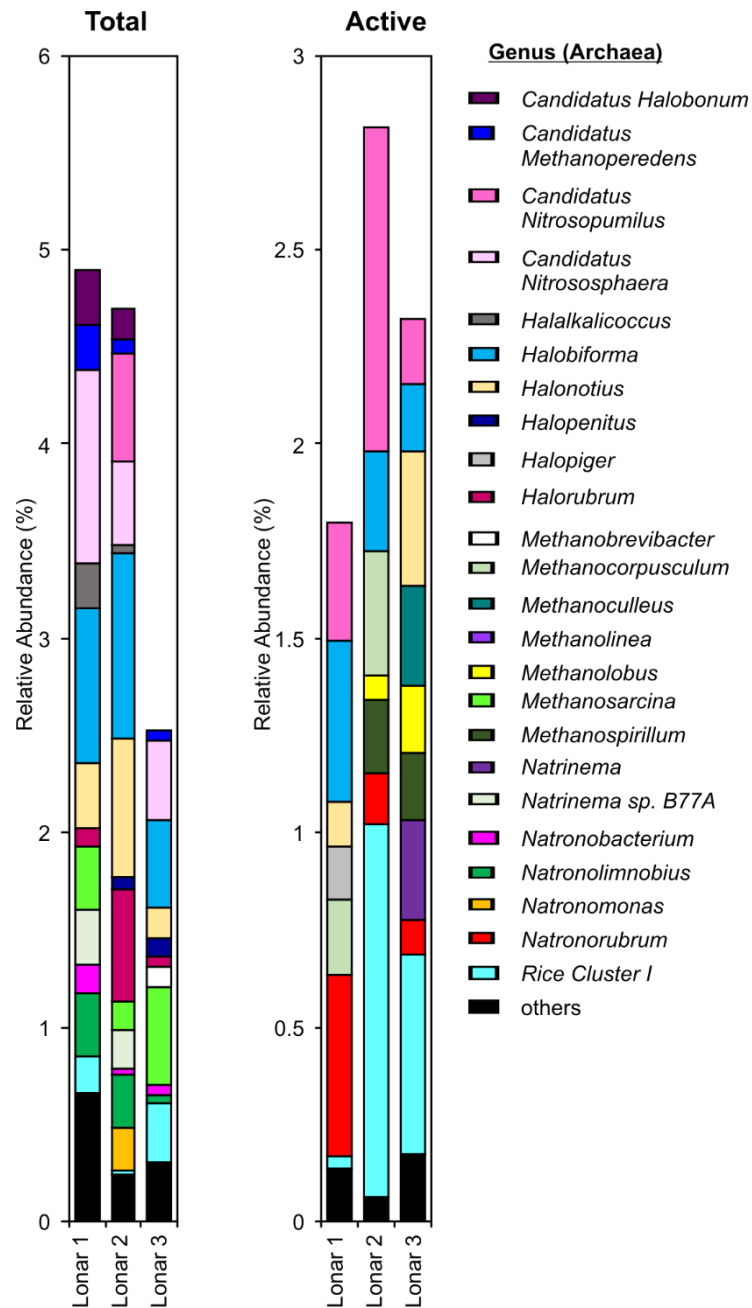


FIGURE 12: RELATIVE ABUNDANCES OF THE RARE ARCHAEOAL GENERA IN LONAR SEDIMENTS. Sample numbers indicating the different sediment samples are given below the respective bars. Only the phylogenetic groups, accounting for <1 % of all classified sequences are shown.

4.2.3.2 THE TOTAL AND THE ACTIVE COMMUNITY DIVERSITY OF BACTERIA

All the bacterial sequences have been classified above domain level. All the bacterial OTUs were distributed to 47 phylum or phylum-level taxa and 386 genera or genera level taxa in the total diversity. In the case of the active community, all the OTUs were distributed to 40 phylum or phylum-level taxa and 236 genera or genera level taxa. Relative abundances of these phyla and genera are presented in the Figure 13, Figure 14 and Figure 15, Figure 16. Details of the relative abundances of the phyla and genera are provided in Table 35 and Table 36. Most dominant phyla in the total diversity are *Firmicutes*, *Actinobacteria*, *Chloroflexi*, *Proteobacteria* and *Gemmatimonadetes*. In the active community, most dominant phyla are *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Chloroflexi* and *Deinococcus-Thermus*.

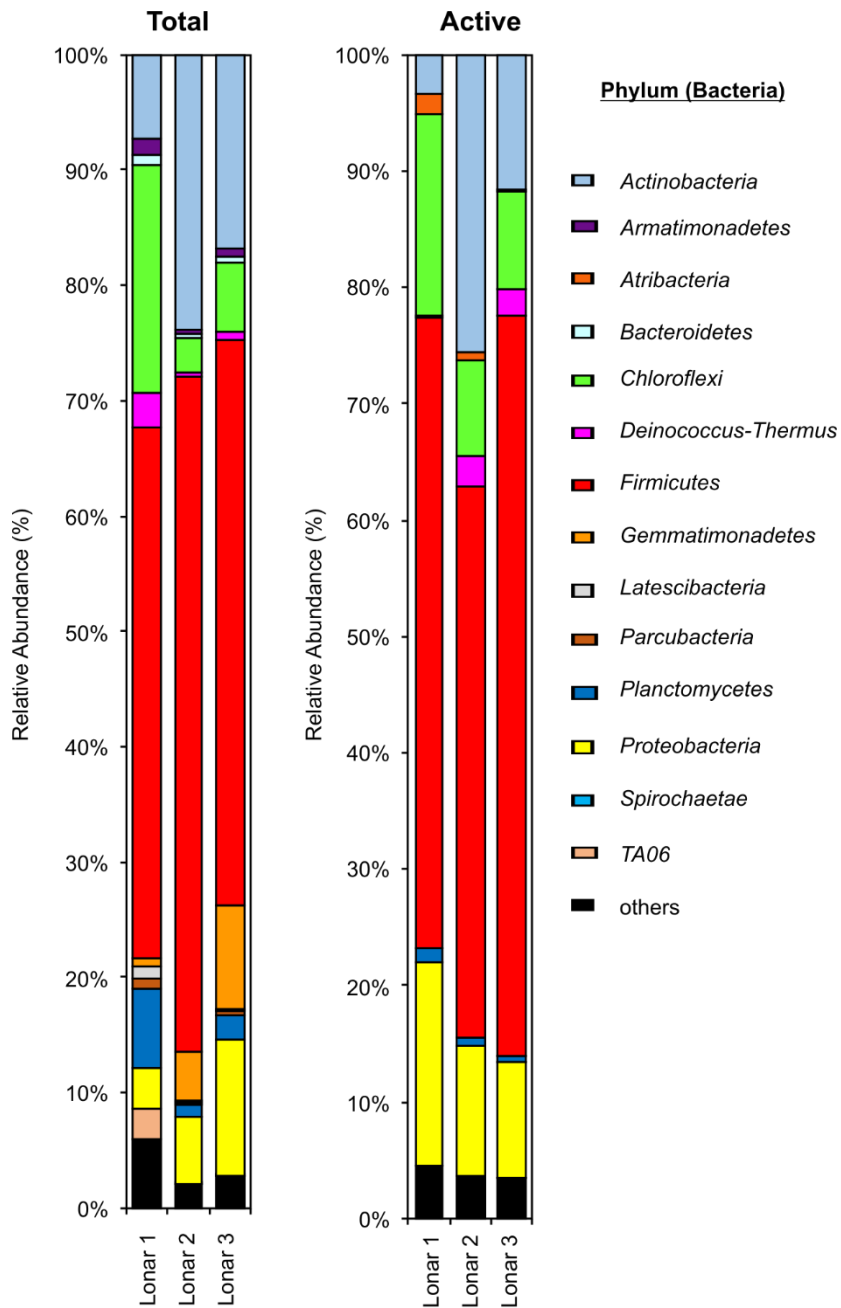


FIGURE 13: RELATIVE ABUNDANCES OF DOMINANT BACTERIAL PHYLUM GROUPS IN LONAR SEDIMENTS. Sample numbers indicating the different sediment samples are given below the respective bars. Phylogenetic groups accounting for <1 % of all classified sequences are summarized in the artificial group 'others.'

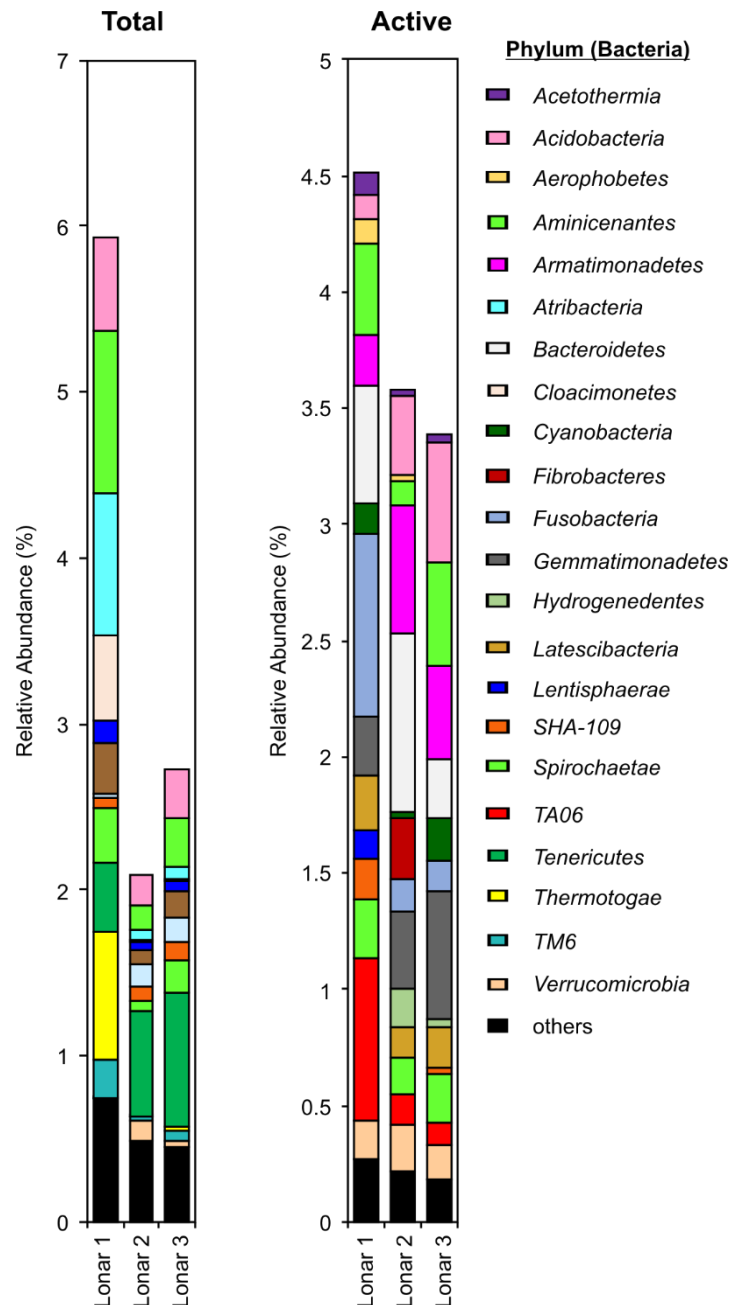


FIGURE 14: RELATIVE ABUNDANCES OF THE RARE BACTERIAL PHYLUM IN LONAR SEDIMENTS. Sample numbers indicating the different sediment samples are given below the respective bars. Only the phylogenetic groups, accounting for <1 % of all classified sequences are shown.

In the active and the total community diversity phylum *Firmicutes* contributes to 51.16 % and 55.03 % relative abundance respectively. The highest relative abundance of 63.55 % was observed in the sediments of Lonar 3 in the active community. Lowest abundance of 45.96 % was found in the sediments of Lonar 1 in the total diversity. *Firmicutes*, in the total diversity, are mostly represented by *Bacillus* (34.83 %) , *Alkaliphilus* (10.22 %) ,

Dethiobacter (8.55 %) , *Anaerobacillus* (7.28 %) and *Natronincola* (3.58 %). In the case of the active community *Firmicutes* are mostly represented by *Alkaliphilus* (24.02 %), *Anaerobacillus* (20.08 %), *Bacillus* (12.78 %), *Natronincola* (9.22 %) and *Gelria* (0.38 %). Several species including novel species (*Bacillus bogoriensis* sp. nov., *Bacillus chagannorensis* sp. nov., *Bacillus daliensis* sp. nov., *Bacillus lonarensis* sp. nov., *Bacillus caseinilyticus* sp. nov.) of the genus *Bacillus* has been isolated from various soda lakes around the world including Lonar(Vargas, Delgado et al. 2005, Carrasco, Marquez et al. 2007, Zhai, Liao et al. 2012, Reddy, Thirumala et al. 2015, Vishnuvardhan Reddy, Thirumala et al. 2015). They are mostly alkaliphilic and often halotolerant, some are able to grow at pH 11. Novel species of the genus *Alkaliphilus* has been isolated from saline lake (*Alkaliphilus halophilus* sp. nov.) and soda lake (*Alkaliphilus peptidofementans* sp. nov.) (Zhilina, Zavarzina et al. 2009, Wu, Shi et al. 2010). They are halophilic and alkaliphilic with pH optima ranging from pH 8 and pH 9.1. Novel reductive sulfur cycling genus *Dethiobacter* (*Dethiobacter alkaliphilus* gen. nov. sp. nov.) has been isolated from north-eastern Mongolian soda lakes, with the ability to grow at pH 10.3(Sorokin, Tourova et al. 2008). Novel strictly anaerobic diazotrophic genus *Anaerobacillus* (*Anaerobacillus alkalilacustre* gen. nov., sp. nov.) has been isolated from soda lake Khadyn, Russia. They are mesophilic halotolerant obligate alkaliphilic and able to grow between pH 8.5 to pH 10.7(Zavarzina, Tourova et al. 2009). Two novel obligate alkaliphilic species, *Natronincola ferrireducens* sp. nov., and *Natronincola peptidovorans* sp. nov., of the genus *Natronincola* with pH optima of pH 8.4 to pH 8.8, have been isolated from soda lakes(Zhilina, Zavarzina et al. 2009). *Gelria* are obligately anaerobic, obligately syntrophic, in pure culture shows saccharolytic growth and they transfer formed hydrogen to methanogenic partner. However, the novel genus *Gelria* (*Gelria glutamica* gen. nov., sp. nov.) has not been isolated or reported from soda lake and they are able to grow in pH up to pH 8 (Plugge, Balk et al. 2002).

Phylum *Actinobacteria* contributes to 15.97 % and 13.49 % in the total and the active community diversity respectively. They show maximum and minimum relative abundance of 25.56 % and 3.37 % in the active community diversity of the sediments of Lonar 2 and Lonar 1 respectively. Phylum *Actinobacteria* is mostly represented by *Longispora* (5.51 %), *Luedemannella* (0.51 %), *Nitriliruptor* (0.43 %), *Micromonospora* (0.23 %) and *Euzebya*

(0.16 %) in the total diversity. In the active community *Actinobacteria* is mostly represented by *Longispora* (5.05 %), *Euzebya* (2.10 %), *Nitriliruptor* (1.53 %), *Micromonospora* (0.59 %) and *Luedemannella* (0.58 %). Two novel species of novel aerobic genus *Longispora* (*Longispora albida* gen. nov., sp. nov., and *Longispora fulva* sp. nov.) has been isolated from soils but not from soda lakes (Matsumoto, Takahashi et al. 2003, Shiratori-Takano, Yamada et al. 2011). *Longispora albida* gen. nov., sp. nov., can utilize glucose, inositol, xylose and sucrose as only carbon sources. *Longispora fulva* sp. nov., can utilize glucose but can not utilize inositol, xylose and sucrose. Two aerobic novel species of novel genus *Luedemannella*, represented by *Luedemannella helvata* sp. nov. and *Luedemannella flava* sp. Nov., have been isolated from soil but not from any soda lakes. They can grow in pH up to 9 (Ara and Kudo 2007). Novel haloalkaliphilic genus, *Nitriliruptor alkaliphilus* sp. nov., has been isolated from soda lakes of Kulunda Steppe, Altai, Russia, with the ability to grow at pH as high as pH 10.6 (Sorokin, van Pelt et al. 2009). Several species of *Micromonospora* including some novel species have been isolated from marine and mangrove sediments, but not from any soda lakes (Huang, Lv et al. 2008, Ohlendorf, Schulz et al. 2012, Xie, Qu et al. 2012, Supong, Suriyachadkun et al. 2013, Li and Hong 2015, Phongsopitanun, Kudo et al. 2015). Novel genus *Euzebya* has been isolated from the ventral epidermis of the *Holothuria edulis* (sea cucumber) but not from soda lakes. *Euzebya tangerina* gen. nov, sp. nov., are known to grow at pH of up to pH 9 (Kurahashi, Fukunaga et al. 2010).

In the total and the active community diversity, the relative abundance of phylum *Chloroflexi* was found to be 9.57 % and 11.27 % respectively. The highest relative abundance of *Chloroflexi* was found to be 19.68 % in the sediments of Lonar 1 in the total diversity assessment and lowest, 3.07 %, was observed in Lonar 2 sediments of the total diversity. Phylum *Chloroflexi* is mostly represented by genera *Nitrolancea* (1.12 %), *Sphaerobacter* (0.06 %), *Pelolinea* (0.04 %), *Dehalococcoides* (0.01 %) and *Ornatilinea* (0.006 %) in the total diversity and by *Nitrolancea* (1.10 %), *Sphaerobacter* (0.14 %), *Dehalococcoides* (0.02 %), *Pelolinea* (0.003 %) in the active community. A chemolithoautotrophic nitrite-oxidizing novel genus, *Nitrolancea hollandica* gen. nov., sp. nov., was isolated from a nitrifying bioreactor with pH optima between pH 6.8 to pH 7.5 (Sorokin, Vejmelkova et al. 2014). However, they have not been reported from any soda

lake. Chemoorganotrophic *Sphaerobacter thermophilus* gen. nov., sp. nov., has been isolated only from sewage sludge (Demharther, Hensel et al. 1989). Obligate anaerobic novel genus *Pelolinea submarina* gen. nov., sp. nov., has been isolated from marine subsurface sediments near Japan in 2014, none has been reported from any soda lakes. They can grow in pH up to pH 8.5 (Imachi, Sakai et al. 2014). *Dehalococcoides mccartyi* gen. nov., sp. nov., are obligate anaerobic hydrogenotrophic, organohalide-respiring *Bacteria* isolated from digester sludge of a wastewater treatment plant (Löffler, Yan et al. 2013). However, they are not isolated or reported from soda lakes. A new anaerobic organotrophic cellulolytic genus *Ornatilinea* (*Ornatilinea apprima* gen. nov., sp. nov.) able to grow at pH up to pH 9 has been reported from Russia, but not from any soda lake in 2013 (Podosokorskaya, Bonch-Osmolovskaya et al. 2013).

Phylum *Proteobacteria* contributes to 7.00 % and 12.93 % in the total and the active community diversity respectively. The highest relative abundance of *Proteobacteria*, 17.24 %, was observed in sediments of Lonar 1 of the active community diversity and lowest, 3.51 %, was found in the total diversity of the Lonar 1 sediments. In the total diversity, phylum *Proteobacteria* is mostly represented by *Hyphomicrobium* (2.22 %), *Haliangium* (1.01 %), *Desulfovibrio* (0.81 %), *Geoalkalibacter* (0.79 %) and *Vulgatibacter* (0.40 %). Phylum *Proteobacteria*, in case the active community is mostly represented by *Pelomonas* (2.35 %), *Caulobacter* (2.10 %), *Acinetobacter* (2.09 %), *Sphingomonas* (0.97 %) and *Brevundimonas* (0.81 %). Methylophilic species of *Hyphomicrobium* has been isolated from alkaline soil of coal mine, China and gold mine debris, Portugal, some of them are able to grow at pH as high as pH 12 (Marco, Pacheco et al. 2004, Han, Chen et al. 2009). Two obligate aerobic and moderately halophilic novel species, *Haliangium ochraceum* sp. nov., and *Haliangium tepidum* sp. nov., of the novel genus *Haliangium* gen. nov., has been isolated from coastal seaweed or sea grass of Japan (Fudou, Jojima et al. 2002). Several novel species of sulfate-reducing bacterial genus *Desulfovibrio* has been isolated from various freshwater, marine, coastal and estuarine sediments of all over the world (Bale, Goodman et al. 1997, Reichenbecher and Schink 1997, Sass, Berchtold et al. 1998, Sun, Cole et al. 2000, Takii, Hanada et al. 2008, Suzuki, Ueki et al. 2009). Members of the novel genus *Geoalkalibacter* (*Geoalkalibacter ferrihydriticus* gen. nov. sp. nov.) has been isolated from soda lakes of Russia. They are obligate alkaliphilic, capable of

dinitrogen fixation and able to grow at pH as high as pH 10 (Zavarzina, Kolganova et al. 2006). In 2014, an obligate aerobic organotrophic new genus *Vulgatibacter* (*Vulgatibacter incomptus* gen. nov., sp. nov.) has been isolated from a forest soil of Yakushima Island, Japan with the ability to grow at pH as high as pH 9 (Yamamoto, Muramatsu et al. 2014). However, none has been reported from any soda lake. Two novel species of *Pelomonas*, *Pelomonas aquatica* sp. nov., and *Pelomonas puraquae* sp. nov., with nitrogen fixing ability has been isolated in 2007, but not from soda lakes (Gomila, Bowien et al. 2007). *Caulobacter* has been reported from a soda lake of Kulunda Steppe, Altai, Russia (Tourova, Grechnikova et al. 2014). Members of the genus *Acinetobacter* has been reported and isolated from soda lakes of the Kulunda Steppe, Altai, Russia and high-altitude shallow lakes and salterns (Andean lakes) of dry Central Andes region of South America (Foti, Sorokin et al. 2008, Di Capua, Bortolotti et al. 2011, Kurth, Belfiore et al. 2015). They are known for their tolerance to high UV radiation, high salinity, and high heavy metal content. Several members of the genus *Sphingomonas* has been isolated from freshwater lakes as well as alkaline lake some are associated with environments of high UV irradiation, hypersalinity, drastic temperature changes (Farias, Revale et al. 2011, Chen, Jogler et al. 2013, Salka, Srivastava et al. 2014, Wei, Wang et al. 2015). Some of them can grow at pH as high as pH 10. Several species of the genus *Brevundimonas* including few new species, for example, *Brevundimonas terrae* sp. nov., *Brevundimonas kwangchunensis* sp. nov., *Brevundimonas abyssalis* sp. nov., *Brevundimonas denitrificans* sp. nov., has been isolated from deep subseafloor sediments and alkaline soils, some are able to grow at pH 10 (Yoon, Kang et al. 2006, Yoon, Kang et al. 2006, Tsubouchi, Shimane et al. 2013, Tsubouchi, Koyama et al. 2014).

In the total diversity, the relative abundance of phylum *Gemmatimonadetes* was found to be 4.69 % and in the case of the active community, it was 0.37 %. The maximum relative abundance of the phylum *Gemmatimonadetes*, of 9.02 % was observed in Lonar 3 of the total diversity and minimum was observed (0.25 %) in Lonar 1 sediments of the active community assessment. It was represented in the total diversity mostly by *Gemmatimonas* (only cultured representative) and the genus *Gemmatimonas* was not detected in the active community diversity. The new aerobe genus *Gemmatimonas aurantiaca* gen. nov., sp. nov., have been described in 2003 from a laboratory scale

wastewater treatment process with the ability to grow between pH 6.5 and pH 9.5 (Zhang, Sekiguchi et al. 2003).

In the active community, the relative abundance of the phylum *Deinococcus-Thermus* was found to be 1.72 % and in the total community, it was 1.3 %. Sediments of Lonar 1 in the total diversity shows highest relative abundance of the phylum *Deinococcus-Thermus*, 3.00 % and lowest of 0.21 %, was observed in Lonar 1 sediments in the active community diversity. The phylum *Deinococcus-Thermus* was mostly represented by *Deinococcus* (0.01 %) and *Truepera* (2.07 %) in the active community and by *Truepera* (2.79 %) in the total community. We were unable to detect any *Deinococcus* in total diversity. *Deinococcus* is a well known chemoorganotrophic *Bacteria* with one of the most radiation resistant cell (vegetative). Few species of the genus *Deinococcus* (*Deinococcus enclensis* sp. nov., *Deinococcus radiopugnans*) have been isolated from soil and marine sediments. However, none has been reported from any soda lake (Masters, Murray et al. 1991, Thorat, Mawlankar et al. 2015). New chemoorganotrophic aerobic alkaliphilic moderately thermophilic and moderately halophilic genus *Truepera radiovictrix* gen. nov., sp. nov., has been isolated from hot springs with the ability to grow in pH up to pH 11.2 (Albuquerque, Simoes et al. 2005). They are also radiation resistant.

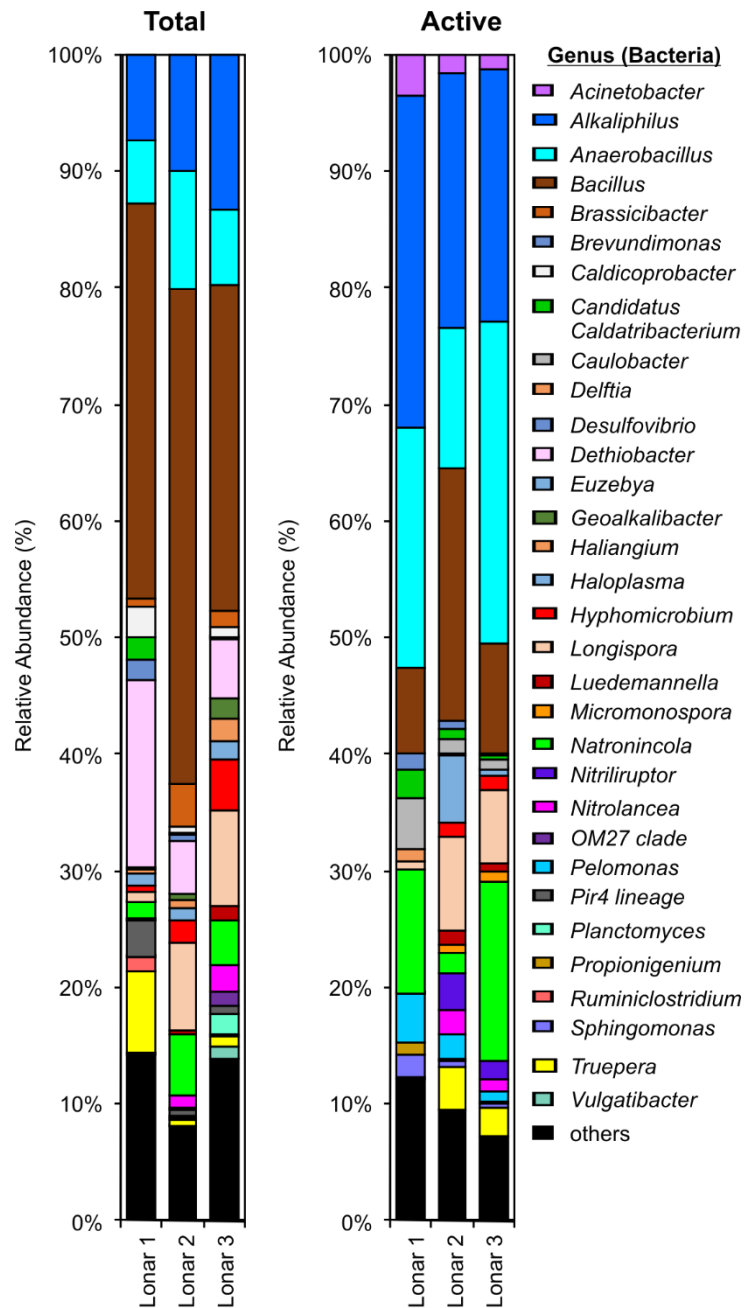


FIGURE 15: RELATIVE ABUNDANCES OF THE MOST ABUNDANT BACTERIAL GENERA IN LONAR SEDIMENTS. Sample numbers indicating the different sediment samples are given below the respective bars. Only the phylogenetic groups accounting for >1 % of all classified sequences are shown.

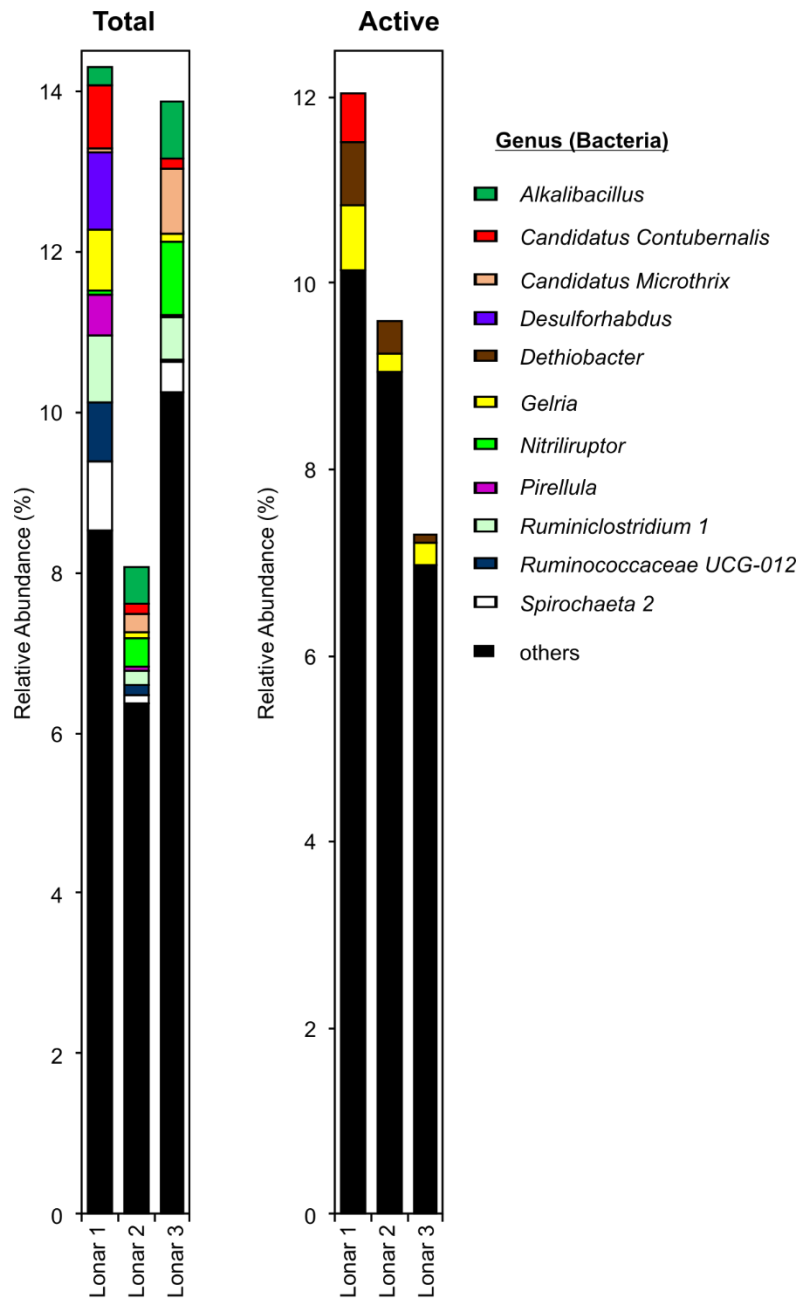


FIGURE 16: RELATIVE ABUNDANCES OF THE RARE BACTERIAL GENERA IN LONAR SEDIMENTS. Sample numbers indicating the different sediment samples are given below the respective bars. Only the phylogenetic groups accounting for <1 % of all classified sequences are shown.

4.2.3.3 RELATIVE COVERAGE OF THE TOTAL AND THE ACTIVE COMMUNITY

Relative coverage of the total and the active community was analyzed using R version 3.2.3 (R Core Team 2015) and gplots (v 2.17.0) (Gregory R. Warnes 2015), plotrix (v 3.6.1) (Lemon 2006) packages. Among *Archaea* at order level 80 % are shared between the total and the active community. The total and the active community both shows 10 % unique order or order-level taxa. At the genus level, the ubiquitously shared genera represent 53.2 % in *Archaea*. The total diversity shows 42.6 % unique genera and in the active community 4.3 % unique genera were observed. Among *Bacteria*, we observed unique phylum or phylum-level taxa of 16.7 % and 2.1 % in the total and the active community diversity respectively. The total and the active community share 81.2 % phylum. At the genus level, they show 28 % common genera between the total and the active diversity. In the total and the active community diversity, we observed unique genera of 51.4 % and 20.6 % respectively. All the mutual overlap of coverages are shown in Figure 17. It is safe to conclude that individual surveying effort (total or active) does have an effect on detection of taxa.

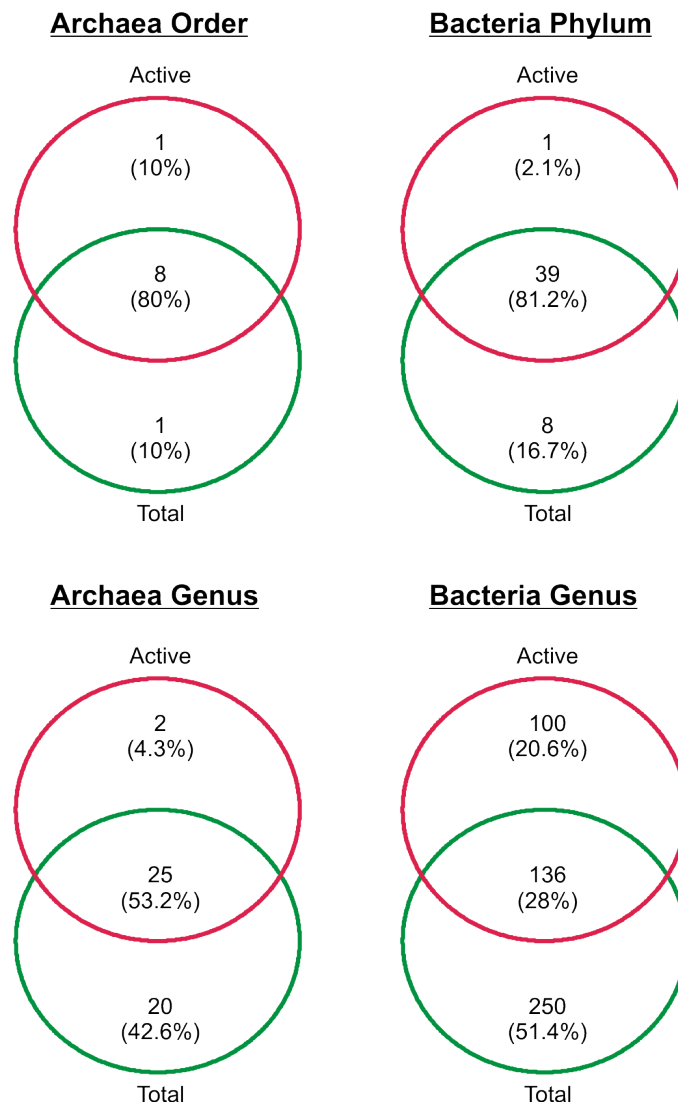


FIGURE 17: VENN DIAGRAM OF RELATIVE COVERAGE BETWEEN DIFFERENT SURVEYING EFFORTS. Upper left- Order level, *Archaea*, lower left- Genus level, *Archaea*, upper right- Phylum level, *Bacteria*, lower right- Genus level, *Bacteria*.

4.3 GENERAL ANALYSES OF THE ADDITIONAL SEQUENCING OF THE TOTAL DIVERSITY DATA FROM LONAR

High-throughput pyrosequencing of 16S rRNA gene sequences provides more sequence data compared to traditional Sanger sequencing of 16S rRNA gene clone libraries for exploring phylogenetic diversity and community composition (Sogin, Morrison et al. 2006). The error rate in terms of error per base in pyrosequencing of 16S rRNA genes is no greater than that of Sanger sequencing (Huse, Huber et al. 2007). The inherent rate of error of pyrosequencing may cause overestimation of the number of rare phylotypes. To minimize the overestimation of rare phylotypes, quality control filtering of the pyrosequencing-derived dataset was used. For clustering and diversity estimates genetic divergences of 3 % were used (Kunin, Engelbrektsen et al. 2010). The analysis method is provided in 3.14.2. The pyrosequencing-based analysis of the V3-V5 region of the 16S rRNA genes resulted in the recovery of 61,362 high-quality sequences for *Bacteria* and 37,968 for *Archaea*; across all 6 samples, 3 samples each for *Bacteria* and *Archaea*. All the sequences were filtered for sequences with a read length between 300 to 1000 bp, the maximum number of allowed ambiguous bases limiting to 6 and a maximum number of allowed mismatches in primer limiting to 3. The average read length was 469.78 bp for *Bacteria* and 467.53 for *Archaea*. The number of sequences per sample for *Bacteria* ranged from 16,298 to 25,450 with an average of 20,454 and for *Archaea*, it ranges from 7,754 to 16,084 with an average of 12,656. Alpha diversity was analyzed at the same level of surveying effort. For *Bacteria*, it was 13,540 sequences per sample and for *Archaea* 6,640 sequences per sample. Additionally, denoising of every sequence subset was performed to avoid overestimation of operational taxonomic units (OTUs) and diversity (Bragg, Stone et al. 2012). We were able to assign 51,853 sequences out of 61,362 to the domain *Bacteria* (84.5 %) and to classify all of these sequences below the domain level (84.5 %). In the case of *Archaea*, 33,294 sequences out of 37,968 were assigned to the domain *Archaea* (87.68 %), and we were able to classify 33,266 of these sequences below the domain level (87.61 %).

All the OTUs were identified at genetic distances of 3 % by using 6,640 randomly selected and denoised sequences per sample for *Archaea* and 13,540 sequences for *Bacteria*.

These OTUs were used to determine rarefaction curves, richness, and diversity. At 3 % genetic distance the rarefaction curves attain a significant level of saturation Figure 18.

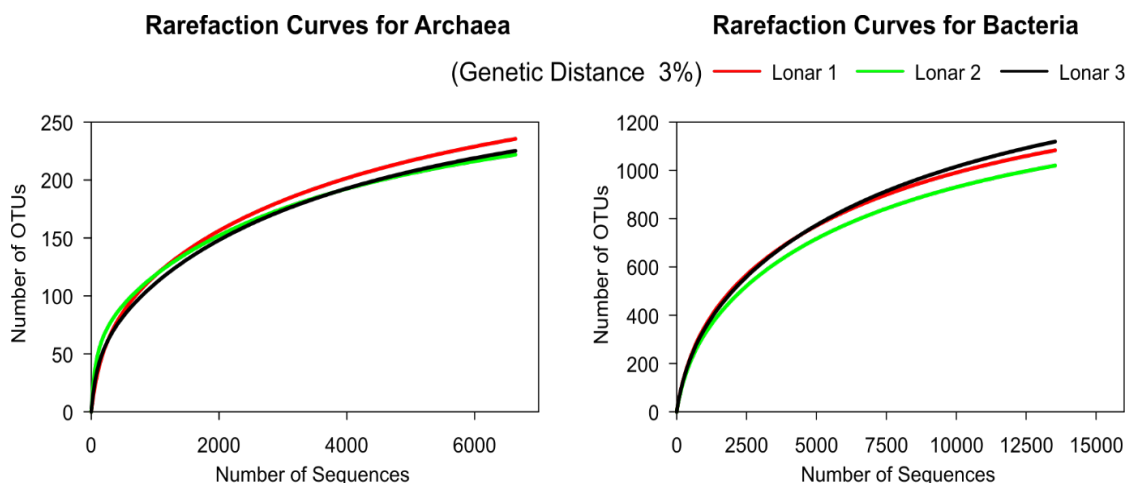


FIGURE 18: RAREFACTION CURVES INDICATING THE OBSERVED NUMBER OF OPERATIONAL TAXONOMIC UNITS (OTUS) IN ADDITIONAL ASSESSMENT OF THE TOTAL DIVERSITY. Three different samples are indicated in red, green and black respectively for Lonar 1, Lonar 2 and Lonar 3. The curves indicate gradual saturation in the number of OTUs with an increase in the number of sequences examined. On the left rarefaction curves for *Archaea* and on the right for *Bacteria* at 3 % genetic distances are shown.

As per rarefaction analyses with the number of OTUs determined by Max. clusters (n_{max}) revealed that *Archaea* covered 86.48 % (± 1.86) of the estimated total diversity. In the case of *Bacteria*, the coverage was observed to be 81.24 % (± 0.89). Thus, we did not survey the full extent of taxonomic diversity at these genetic distances, but the surveying efforts assessed a substantial fraction of the prokaryotic diversity. Details of the Observed clusters, Max. clusters (n_{max}), Coverage (%), Shannon index (H') and Chao1 of individual samples are given in Table 26. The coverage was more than the previous total diversity assessment, where we have surveyed both the total and the active community diversity using Illumina MiSeq. Due to the increased coverage of this additional total diversity assessment furnished several taxa at order or phylum and genus level, which was not detected by our previous total diversity assessment. The proportion of uncultured and cultured OTUs were observed to be 83.24 % and 16.75 % in *Archaea*. In the case of *Bacteria*, it was found to be 87.26 % and 12.73 % for uncultured and cultured OTUs respectively.

TABLE 26: GENERAL ANALYSIS OF THE ADDITIONAL TOTAL DIVERSITY SEQUENCING DATA

Sample	Observed clusters	Max. clusters (n _{max})	Coverage (%)	Shannon index (H')	Chao1
Lonar 1 (Archaea)	235.6	274.33	85.88	5.34	286.06
Lonar 2 (Archaea)	222	249.39	89.01	5.61	260.07
Lonar 3 (Archaea)	225.7	266.91	84.55	4.91	272.35
Mean	227.76	263.54	86.48	5.29	272.82
SD	5.74	10.45	1.86	0.28	10.61
CV	0.02	0.03	0.02	0.05	0.03
Lonar 1 (Bacteria)	1081.9	1314.24	82.32	8.06	1288.14
Lonar 2 (Bacteria)	1019	1253.70	81.27	7.64	1223.63
Lonar 3 (Bacteria)	1114.9	1391.32	80.13	7.91	1357.78
Mean	1071.93	1319.75	81.24	7.87	1289.85
SD	39.78	56.31	0.89	0.17	54.77
CV	0.03	0.04	0.01	0.02	0.04

Due to the increased coverage of this additional total diversity assessment furnished several taxa at order (for *Archaea*) or phylum (for *Bacteria*) and genus level, which was not detected by our previous total diversity assessment. Details are provided in Table 37 of supplements.

This additional assessment of the total diversity furnished 2 archaeal order and 5 archaeal genera that has not been detected by our previous surveying effort. One of order is *Marine Benthic Group E* (0.22 %), and the other is *Archaeoglobales* (0.19 %). Also, 1 bacterial phylum-level taxa, *LCP-89* (0.01 %) has not been detected by our previous assessment. In the case of *Bacteria*, 44 genera or genera level taxa has been observed which was not detected by our previous total diversity assessment.

4.4 COMPARISON OF PRESENT AND PREVIOUS INVESTIGATIONS ON PROKARYOTIC DIVERSITY OF LONAR CRATER LAKE

The present study contributed 52 archaeal genera and 530 bacterial; out of which 36 (69.23 %) archaeal genera and 407 (76.79 %) bacterial genera has never been reported before from Lonar lake before. Abundances of cultivatable aerobic *Bacteria*, as described by Joshi in 2008, in Lonar lake water was found to be 102–104 CFU/ml and for sediment samples were found to be 102–106 CFU/g. Previous research on diversity and abundance of heterotrophic *Bacteria* isolated from the Lonar lake water and sediment showed most dominant phylum to be the *Firmicutes*, followed by *Proteobacteria* and *Actinobacteria*(Wani, Surakasi et al. 2006, Joshi, Kanekar et al. 2008, Antony, Kumaresan et al. 2013); which is also the case for this present study. Study of Lonar lake also resulted in recovery of 4 novel heterotrophs (*Nitritalea halakaliphila*, *Indibacter alkaliphilus*, *Cecembia lonarensis* and *Georgenia satyanarayana*) and one new methylotroph (*Methylophaga lonarensis*) (Anil Kumar, Srinivas et al. 2010, Anil Kumar, Srinivas et al. 2012, Antony, Doronina et al. 2012). DNA stable-isotope probing experiments by Antony in 2010 with Lonar lake sediments showed *Methylomicrobium*, *Methylophaga* and *Bacillus spp* to be the predominant the active methylotrophs utilizing methane, methanol, and methylamine, respectively(Antony, Kumaresan et al. 2010). The previous study of archaeal 16S rRNA genes recovered from Lonar lake sediment showed five crenarchaeotal phylotypes and eight euryarchaeotal phylotypes (Wani, Surakasi et al. 2006). However, we found in this survey that most of the OTUs belong to the phylum *Euryarchaeota* and *Thaumarchaeota* some OTUs affiliated to *Korarchaeota* was also noticed. Methanogenic species associated to *Methanosarcina*, *Methanocalculus* and *Methanoculleus* have been isolated from the Lonar lake sediments(Thakker and Ranade 2002, Surakasi, Wani et al. 2007).

Over the last several years different work, has been undertaken by the various researcher on prokaryotes of Lonar lake resulting in accumulation of hundreds of DNA sequence. We collected all available 16S rRNA gene sequences from NCBI (National Center for Biotechnology Information) Nucleotide database. All sequences with less than 300 bp in size were removed resulting in 1245 number of sequences. These sequences were

processed with the similar protocol (for details see Chapter 3.14) as the one used to process sequences derived from the present study to make the comparison as valid as possible, mainly to avoid issues regarding phylogeny and taxonomy. A total of 17 archaeal and 197 bacterial genera was found in this analysis in contrast with 530 and 52, respective, genera found in our study. Among these only 139 (23.88 %) genera was found in both of the groups (analyzed NCBI nucleotide database sequences and the present high-throughput sequencing data) and 443 (76.11 %) genera were found to be unique to our present study. It is worth mentioning here that these 443 genera have never been reported from Lonar before. Relative coverage at genus level of all the previous surveying efforts and the surveying efforts of the present studies are analyzed using R version 3.2.3 (R Core Team 2015) and gplots (v 2.17.0) (Gregory R. Warnes 2015), plotrix (v 3.6.1) (Lemon 2006) packages. Among *Archaea* both the surveying efforts share 30.2 % genera, and present investigation shows 67.9 % unique genera. In the case of *Bacteria*, at the genus level, the ubiquitously shared genera represent only 20.4 %, and the present investigation reveals 67.4 % unique genera. All the mutual overlaps of coverages are shown in Figure 19. Some of these genera represent a significant relative abundance of the prokaryotic community.

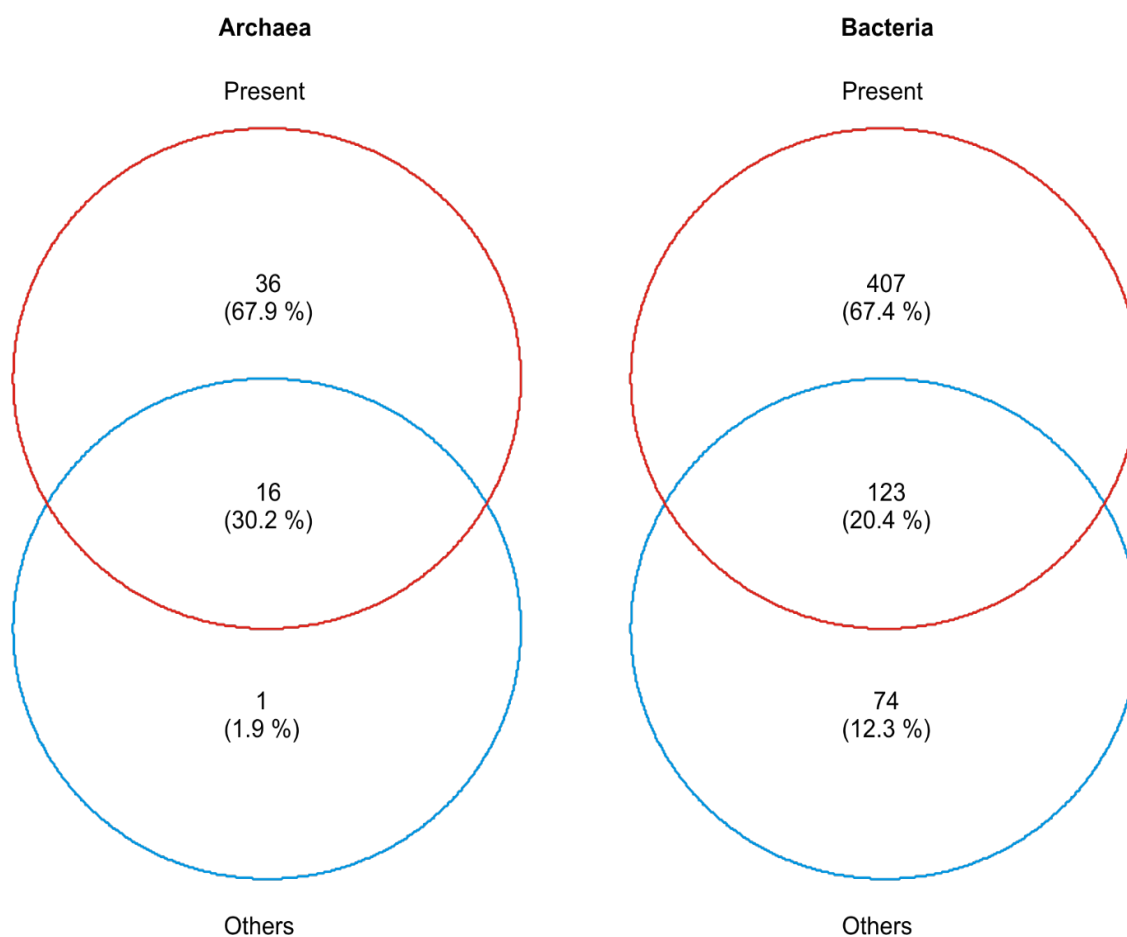


FIGURE 19: COMPARISON OF CURRENT AND PREVIOUS INVESTIGATIONS IN DETECTION GENERA. The figure shows mutual coverage of detected Genera in all previous investigations (Others) and current investigation (Present). The red circle represents this study and blue represents all other studies.

As per this study these unique archaeal genera together represent the average relative abundance of 3.62 % (± 0.51) and 21.54 % (± 0.68) in the total and the active community respectively. Among these unique archaeal genera, the highest 3 abundant genera in the active community include *Methanobrevibacter*, *Methanobacterium* and *Methanomassiliicoccus* representing relative abundance of as high as 17.54 %, 2.32 % and 2.15 % respectively. In the total diversity assessment, 3 most abundant of these unique genera are *Halobiforma*, *Methanobacterium* and *Natronorubrum* showing relative abundance as high as 0.96 %, 1.41 % and 1.24 % respectively. A rarity of available literature about the presence of *Methanobrevibacter* in soda lakes was noticed. Which is in accordance with the fact that there is a general lack of investigations on *Archaea* of soda lakes. They obtain energy by reducing CO₂ to CH₄, using H₂ and sometimes formate

as the electron donor, but not acetate, methanol, methylamines (Leadbetter, Crosby et al. 1998, Tokura, Tajima et al. 1999, Savant, Shouche et al. 2002, Ng, Kittelmann et al. 2015, Zhou, Zeitz et al. 2015). *Methanobacterium* are also hydrogen-utilizing methanogens, and they have been reported from soda lakes of Central Asia (Nolla-Ardevol, Strous et al. 2012). *Methanomassiliicoccus* are obligatory anaerobic methanogenic and moderately alkaliphilic (Dridi, Fardeau et al. 2012). The presence of the genus *Methanomassiliicoccus* has been reported from sediments of the estuary (Zhou, Chen et al. 2014). However, none has been reported from soda lakes. Members of aerobic extremely halophilic novel genus *Halobiforma* (*Halobiforma haloterrestris* gen. nov., sp. nov., and *Halobiforma lacisalsi* sp. nov.) has been isolated from hypersaline soil, Aswan, Egypt and Salt Lake in Xinjiang, China (Hezayen, Tindall et al. 2002, Xu, Wu et al. 2005). *Natronorubrum* is strictly aerobic and haloalkaliphilic with optimum growth pH between 9.0 and 9.5. Two species, *Natronorubrum bangense* gen. nov., sp. nov., and *Natronorubrum tibetense* gen. nov., sp. nov., has been isolated from Tibetan soda lake. (Xu, Zhou et al. 1999). Some novel species have also been isolated from salt lakes of Xin-Jiang, China and soils of lake Texcoco, Mexico with most of them able to grow till pH 10 (Cui, Tohty et al. 2006, Cui, Tohty et al. 2007, Ruiz-Romero, Valenzuela-Encinas et al. 2013).

In the case of *Bacteria* these unique genera cumulatively contribute to 22.21 % (± 4.69) and 17.75 % (± 3.22) average relative abundance in the total and the active bacterial diversity respectively. Among these unique bacterial genera, highest 3 abundant genera in the total diversity were found to be *Longispora*, *Hyphomicrobium* and *Brassicibacter* representing as high as 8.23 %, 4.22 %, and 3.61 % respectively. In the active community diversity of *Bacteria*, 3 most abundant of these unique genera were *Longispora*, *Pelomonas* and *Caulobacter* showing relative abundance of 5.05 %, 2.35 % and 2.10 % respectively. Few members of aerobic genus *Longispora* has been isolated from soils. They are not reported from soda lakes, but they are known to be able to grow at pH 9 (Matsumoto, Takahashi et al. 2003, Shiratori-Takano, Yamada et al. 2011). Members of *Hyphomicrobium* are able to grow at pH as high as pH 12. They are methylotrophic and has been isolated from alkaline soil of coal mine, China and gold mine debris, Portugal (Marco, Pacheco et al. 2004, Han, Chen et al. 2009). Novel species of obligate anaerobic chemoorganotrophic *Brassicibacter* (*Brassicibacter thermophilus* sp. nov.) has been

isolated from coastal marine sediment. Some members are also known to grow in pH of up to pH 9 (Fang, Zhang et al. 2012, Wang, Ji et al. 2015). Few nitrogen-fixing members of *Pelomonas*, *Pelomonas aquatica* sp. nov., and *Pelomonas puraquae* sp. nov., has been reported (Gomila, Bowien et al. 2007). However, they are not from soda lakes. Some aerobic chemoorganotrophic species of *Caulobacter* of are known to grow in pH up to pH 9. They have been reported from a hypersaline soda lake of Kulunda Steppe, Altai, Russia (Tourova, Grechnikova et al. 2014).

4.4.1 GENERA DETECTED IN LONAR CRATER SODA LAKE WITH SIGNIFICANCE IN BIOGEOCHEMICAL CYCLES

In this survey of the prokaryotic diversity of the lake sediment from Lonar showed rich biodiversity despite its extreme physicochemical parameters. The presence of different ecologically appealing functional groups was found. Most of these genera had not been reported from Lonar before. A comprehensive list of these genera is provided in Table 27.

TABLE 27: GENERA INVOLVED IN VARIOUS BIOGEOCHEMICAL CYCLE

Methane cycle	Nitrogen cycle	Sulfur cycle
Archaeal genera	Archaeal genera	Bacterial genera
<i>Candidatus Methanoperedens</i>	<i>Candidatus Nitrosopumilus</i>	<i>Candidatus Desulforudis</i>
<i>Methanobacterium</i>	<i>Candidatus Nitrososphaera</i>	<i>Desulfatiglans</i>
<i>Methanobrevibacter</i>	Bacterial genera	<i>Desulfatitalea</i>
<i>Methanocalculus</i>	<i>Ammonifex</i>	<i>Desulfitibacter</i>
<i>Methanocella</i>	<i>Ammoniphilus</i>	<i>Desulfitispora</i>
<i>Methanococcoides</i>	<i>Azoarcus</i>	<i>Desulfobacca</i>
<i>Methanocorpusculum</i>	<i>Azospirillum</i>	<i>Desulfobulbus</i>
<i>Methanoculleus</i>	<i>Bradyrhizobium</i>	<i>Desulfococcus</i>
<i>Methanogenium</i>	<i>Candidatus Anammoximicrobium</i>	<i>Desulfomonile</i>

Methane cycle	Nitrogen cycle	Sulfur cycle
<i>Methanolinea</i>	<i>Mesorhizobium</i>	<i>Desulfonatronobacter</i>
<i>Methanolobus</i>	<i>Nitratireductor</i>	<i>Desulfonatronospira</i>
<i>Methanomassiliicoccus</i>	<i>Nitriliruptor</i>	<i>Desulfonatronum</i>
<i>Methanosaeta</i>	<i>Nitrincola</i>	<i>Desulforhabdus</i>
<i>Methanosarcina</i>	<i>Nitrolancea</i>	<i>Desulforhopalus</i>
<i>Methanosphaera</i>	<i>Nitrosococcus</i>	<i>Desulfosarcina</i>
<i>Methanospirillum</i>	<i>Nitrospira</i>	<i>Desulfosporosinus</i>
Bacterial genera	<i>Rhizobium</i>	<i>Desulfotomaculum</i>
<i>Methylobacterium</i>		<i>Desulfovibrio</i>
<i>Methylobacterium</i>		<i>Desulfovirga</i>
<i>Methylocaldum</i>		<i>Desulfuribacillus</i>
<i>Methyloceanibacter</i>		<i>Desulfurispora</i>
<i>Methylocella</i>		<i>Desulfurivibrio</i>
<i>Methylohalomonas</i>		<i>Desulfuromonas</i>
<i>Methylonatrum</i>		<i>Desulfuromusa</i>
<i>Methylonatrum</i>		<i>Dethiobacter</i>
		<i>Sulfitobacter</i>
		<i>Thioalkalispira</i>
		<i>Thioalkalivibrio</i>
		<i>Thiogranum</i>
		<i>Thiohalobacter</i>
		<i>Thiohalocapsa</i>
		<i>Thiohalomonas</i>
		<i>Thiohalophilus</i>
		<i>Thiohalospira</i>
		<i>Thiomicrospira</i>
		<i>Thioprofundum</i>

In our study, we have observed 24 genera which may have been involved in methane cycle both from *Archaea* and *Bacteria*. In *Archaea*, they contribute to an average of 39.24 % relative abundance in the active community and an average of 8.97 % in the total community. In the case of *Bacteria*, they contribute to an average of 0.50 % relative

abundance in the active community and an average of 0.24 % in the total community. Most dominant genera involved in methane cycle was found to be *Methanosaeta* (*Archaea*) exhibiting relative abundance as high as 19.79 % in the active community of Lonar 3 sediments. Several methanogenic species of *Methanosaeta* has been isolated from rice paddies, natural wetlands, and lake sediments from around the world including marine and estuarine sediments as well as hypersaline soda lakes of Kulunda Steppe, Altai, Siberia (Mizukami, Takeda et al. 2006, Carbonero, Oakley et al. 2010, Mori, Iino et al. 2012, Sorokin, Abbas et al. 2015).

A total of 16 genera were found which may have been involved in nitrogen cycle both from *Archaea* and *Bacteria*. Among *Archaea*, they contribute to an average of 0.46 % relative abundance in the active community and an average of 0.79 % in the total community. In the case of *Bacteria*, they contribute to an average of 2.87 % relative abundance in the active community and an average of 1.95 % in the total community. Most dominant genera involved in nitrogen cycle was found to be *Nitriliruptor* (*Bacteria*) with the relative abundance of 2.97 % in Lonar 2 sediments in the active community. Members of obligate aerobic nitrile degrading genus *Nitriliruptor* are aerobic alkaliphilic and moderately halotolerant. For energy and carbon source they use short-chain organic acids, amides, and aliphatic nitriles. *Nitriliruptor alkaliphilus* gen. nov., sp. nov., has been isolated from soda lakes which can grow in pH of up to pH 10.6 (Sorokin, van Pelt et al. 2009).

A significantly high diversity of bacterial genera, totaling 36, involved in the sulfur cycle were recorded. They represent an average relative abundance of 0.93 % relative abundance in the active community and an average of 10.84 % in the total community. The most dominant genus was found to be *Dethiobacter* representing relative abundance as high as 16.11 % in the total community from Lonar 1 sediments. In the case of the active community, it was found to be also *Dethiobacter*, from sediments of Lonar 1, with the relative abundance of 0.66 %. In reductive sulfur cycling genus *Dethiobacter*, H₂ is used as the electron donor and thiosulfate, elemental sulfur and polysulfide are used as the electron acceptors. Obligate anaerobic chemolithoautotrophic *Dethiobacter alkaliphilus* gen. nov., sp. nov., has been isolated from soda lakes, northeast Mongolia, able to grow in pH between pH 8.5 and pH 10.3 (Sorokin, Tourova et al. 2008).

4.5 DIVERSITY OF FUNCTIONAL GENES WITH EMPHASIS ON ECOLOGICAL ROLE

In the previous chapter several genera potentially involved in the nitrogen cycle, sulfur cycle and methane cycle were detected. This analysis is an attempt to detect functional genes involved in those geobiochemical cycles and also relative abundance of different genes of the different functional category.

4.5.1 GENERAL ANALYSIS OF THE METAGENOME FROM THE SEDIMENTS OF LONAR LAKE

A total of 32 million paired-end reads were obtained from the sequencing effort. These sequences were analyzed as described in the Chapter 3.14.3. Sequences were assembled with metaSPAdes v 3.7.0 resulting in to 588,668 contigs, total number of base 371 Mb (371,120,372 base). These contigs were analyzed with Prodigal v 2.6.0 for ORF prediction and protein translation resulting in to 790,245 proteins. All the sequences shorter than 150 amino acids were discarded leaving 236,182 deduced protein sequences. These ORFs were annotated and analyzed with GhostKOALA, v 2.0. A total of 104,143 translations (41 %) were successfully annotated using KEGG database.

The metagenome shows the assignment of the taxonomic composition of the metagenome in accordance with our 16S rRNA gene based assessment of the sediments from Lonar crater lake (Figure 20). Most dominant phyla were *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Chloroflexi* and *Deinococcus-Thermus* in our 16S rRNA gene based assessment as described in 4.2.3. We observed that the metagenome also shows *Firmicutes* (30.65%), *Proteobacteria* (25.87%), *Actinobacteria* (8.78%), *Chloroflexi* (7.24%) and *Deinococcus-Thermus* (1.89%).

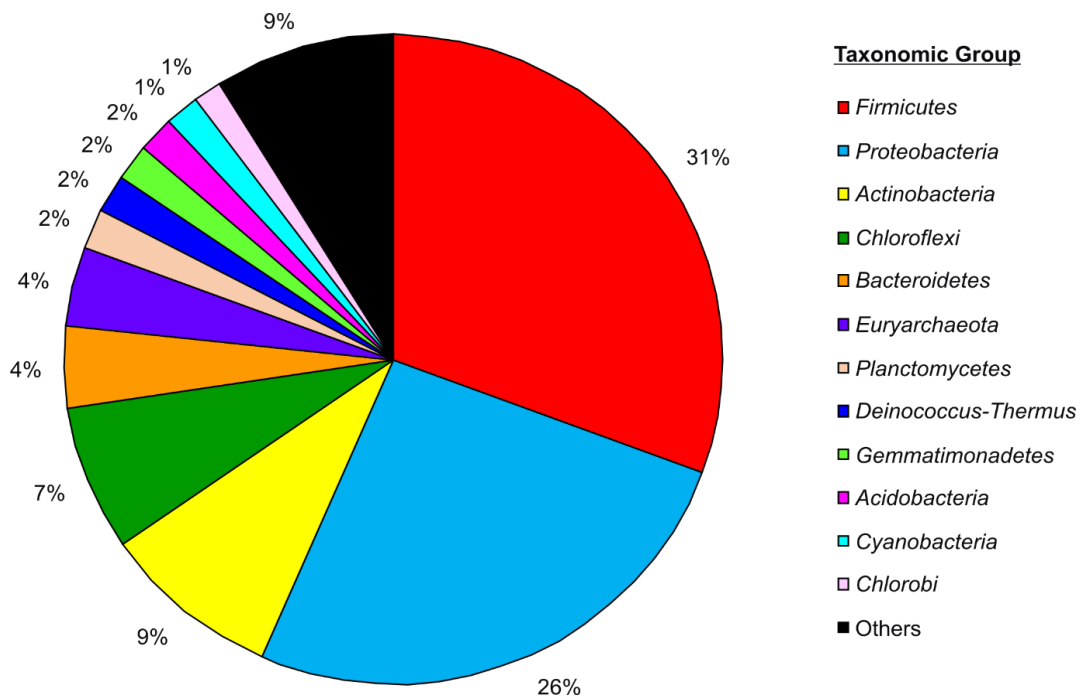


FIGURE 20: TAXONOMIC COMPOSITION OF THE METAGENOME. The figure shows relative abundance of the taxonomic groups in the Lonar metagenome.

An overview of reconstruction of Genes or proteins (KEGG GENES), KO groups (KEGG ORTHOLOGY), KEGG modules, functional hierarchy (KEGG BRITE) and pathway maps (KEGG PATHWAY) are provided in Supplement-F: Diversity of Functional genes observed by direct metagenome analysis.

4.5.1.1 RELATIVE ABUNDANCE OF FUNCTIONAL CATEGORY

The detected ORFs were distributed in 17 (excluding Unclassified) distinct functional categories. Out of these categories most, abundant categories were Genetic Information Processing, Environmental Information Processing and Amino acid metabolism with the relative abundance of 16.72 %, 13.53 %, and 10.33 % respectively. One of the lowest abundant categories was found to be Metabolism of terpenoids and polyketides, Xenobiotics biodegradation & metabolism and Biosynthesis of other secondary metabolites with an abundance of 1.55 %, 1.49 %, and 1.17 % respectively. Also, it was

observed that abundance of category Unclassified was significant, 9.58 %. Details of the relative abundance of each category are provided in Figure 21.

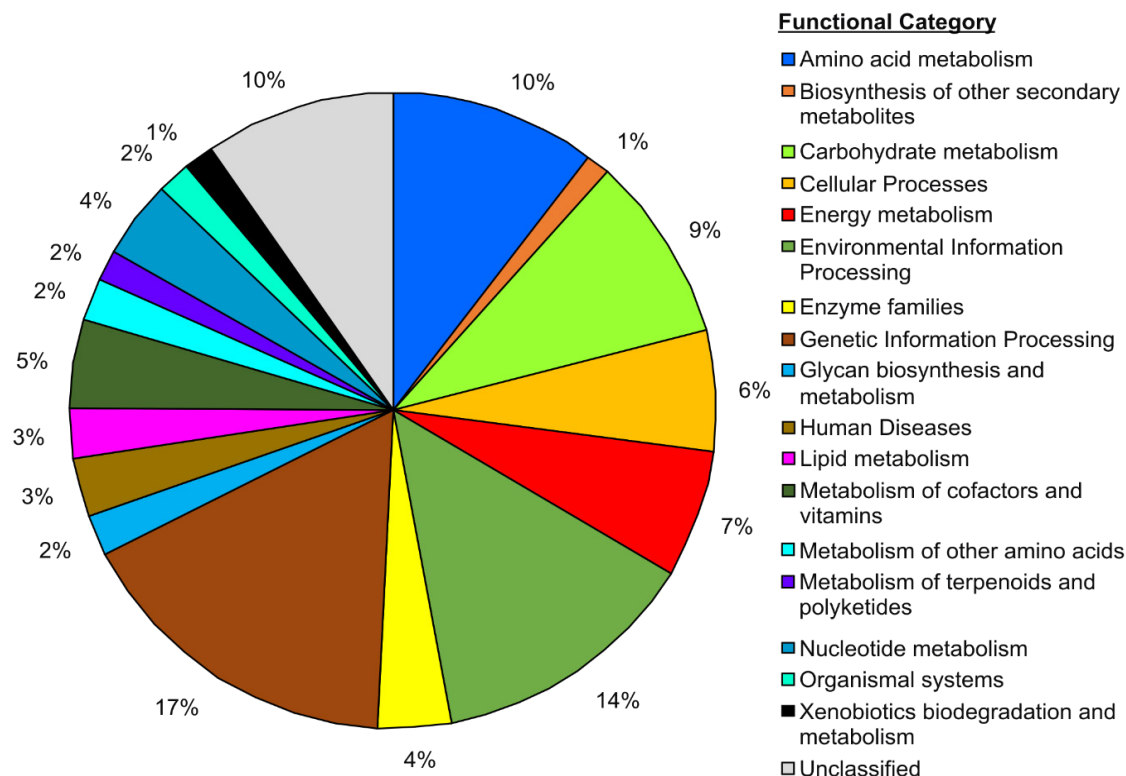


FIGURE 21: RELATIVE ABUNDANCE OF FUNCTIONAL CATEGORIES IN THE LONAR SEDIMENT. The pie diagram shows the relative abundance of detected and annotated ORFs in the Lonar sediment with respect to its functional category.

4.5.2 BIOGEOCHEMICAL CYCLE AND METABOLISM OF METHANE, NITROGEN, AND SULFUR

Methane, nitrogen, and sulfur metabolism contribute to 54.73 % of the general functional category Energy metabolism as per the abundance of the KEGG ortholog. This methane, nitrogen, and sulfur metabolism together contribute to 2.69 % of all the detected KEGG ortholog.

4.5.2.1 METHANE METABOLISM

In the global carbon cycle methane metabolism is predominantly accomplished by methanotrophs and methanogens. Methane metabolism alone represents 30.52 % of Energy metabolism and 1.49 % of all metabolism in Lonar lake sediment metagenome. 9 pathway modules have been detected in this study. Out of these 9, 3 were complete, and 6 were partial. Details are provided in Table 28. Methane is consumed as the solitary carbon source by methanotrophs and methane is produced as a metabolic by-product by methanogens. Also, there are methylotrophs, able to acquire energy by oxidizing one-carbon compounds, such as methane and methanol.

TABLE 28: MODULE COVERAGE OF METHANE METABOLISM.

Module No. / Description	Path	No. of KEGG Ortholog	Coverage
M00357 Methanogenesis (acetate => methane)	map01200 map00680	678	2 blocks missing
M00356 Methanogenesis (methanol => methane)	map01200 map00680	347	2 blocks missing
M00358 Coenzyme M biosynthesis	map00680	28	1 block missing
M00608 2-Oxocarboxylic acid chain extension	map01210 map00680	7	1 block missing
M00346 Formaldehyde assimilation (serine pathway)	map01200 map00680	351	2 blocks missing
M00345 Formaldehyde assimilation (ribulose monophosphate pathway)	map01200 map00680	227	complete
M00344 Formaldehyde assimilation (xylulose monophosphate pathway)	map01200 map00680	83	1 block missing
M00378 F420 biosynthesis	map00680	53	complete
M00422 Acetyl-CoA pathway	map00680	83	complete

Experimental data mapped on the reference pathway provides a comprehensive overall view of methane metabolism. Figure 22 and Figure 23 shows the pathway and coverage. There are different categories of methanogenic pathways observed in prokaryotes; CO₂ to methane, methanol to methane, and acetate to methane. Coenzyme M is used by

methanogens as the terminal methyl carrier in methanogenesis. Coenzyme M biosynthesis requires 4 enzymes. The presence of 3 out of these 4 enzymes for Coenzyme M biosynthesis were detected in Lonar sediment metagenome shown in the Figure 23 as enzyme commission (EC) number 4.4.1.19 (phosphosulfolactate synthase), 3.1.3.71 (2-phosphosulfolactate phosphatase) and 4.1.1.79 (sulfopyruvate decarboxylase). CoenzymeB-CoenzymeM heterodisulfide reductase (Hdr) is required for the final reaction steps of the methanogenic pathway (Madadi-Kahkesh, Duin et al. 2001, Mander, Duin et al. 2002, Hedderich, Hamann et al. 2005). We were able to detect this Hdr in Lonar sediment metagenome, shown in the Figure 22 as EC number 1.8.98.1. One of the key enzymes of methanogenesis is methyl-coenzyme M reductase, which catalyzes the final step in methanogenesis (Rospert, Breitung et al. 1991, Hallam, Girguis et al. 2003, Dhillon, Lever et al. 2005, Ferry 2011, Zeleke, Lu et al. 2013). We have detected this methyl-coenzyme M reductase in Lonar sediments, shown as E.C. 2.8.4.1 in the Figure 22.

Formaldehyde is formed by oxidation of methane by methanotrophs and methylotrophs. This formaldehyde proceeds further by oxidation to CO₂ for an energy source or assimilation for biosynthesis of organic matter. Different pathways convert formaldehyde to two-carbon or three-carbon compounds. These pathways are serine pathway and ribulose monophosphate pathway. The enzymes known as methane monooxygenases, oxidize methane to methanol (Nielsen, Gerdes et al. 1996, Hakemian and Rosenzweig 2007, Sirajuddin and Rosenzweig 2015). In our sample, we were unable to detect the presence of methane monooxygenases as shown in Figure 22 as EC number 1.14.13.25 and 1.14.18.3. However, methanol dehydrogenase (EC 1.1.2.7) was observed in Lonar sediment. Methanol dehydrogenase acts on methanol and other primary alcohols (including ethanol, duodecanol, chloroethanol, cinnamyl alcohol) resulting in the formation of formaldehyde (Patel and Felix 1976, Dumont and Murrell 2005).

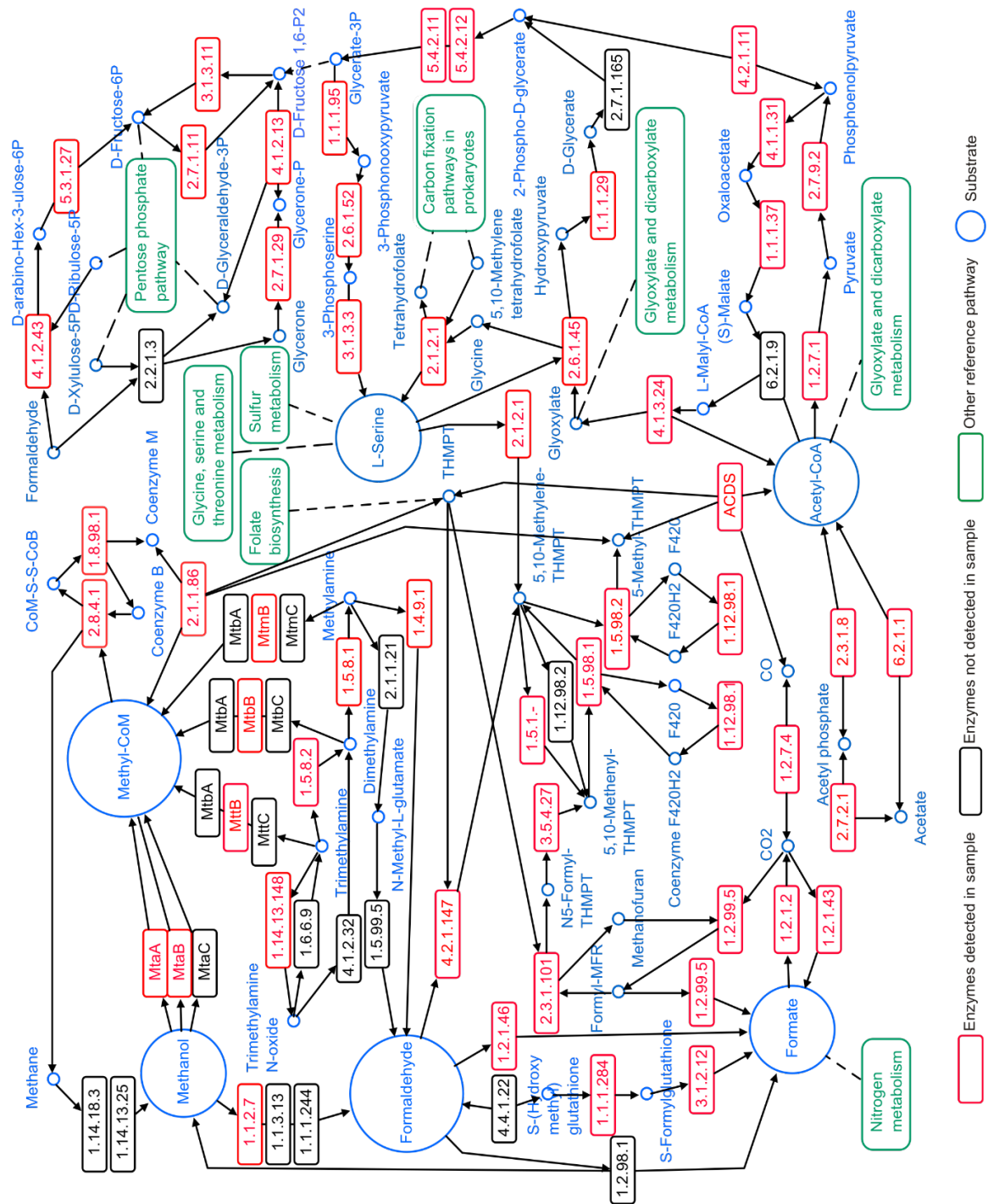


FIGURE 22: METHANE METABOLISM (A). The figure shows experimental data (red) mapped on the reference pathway.

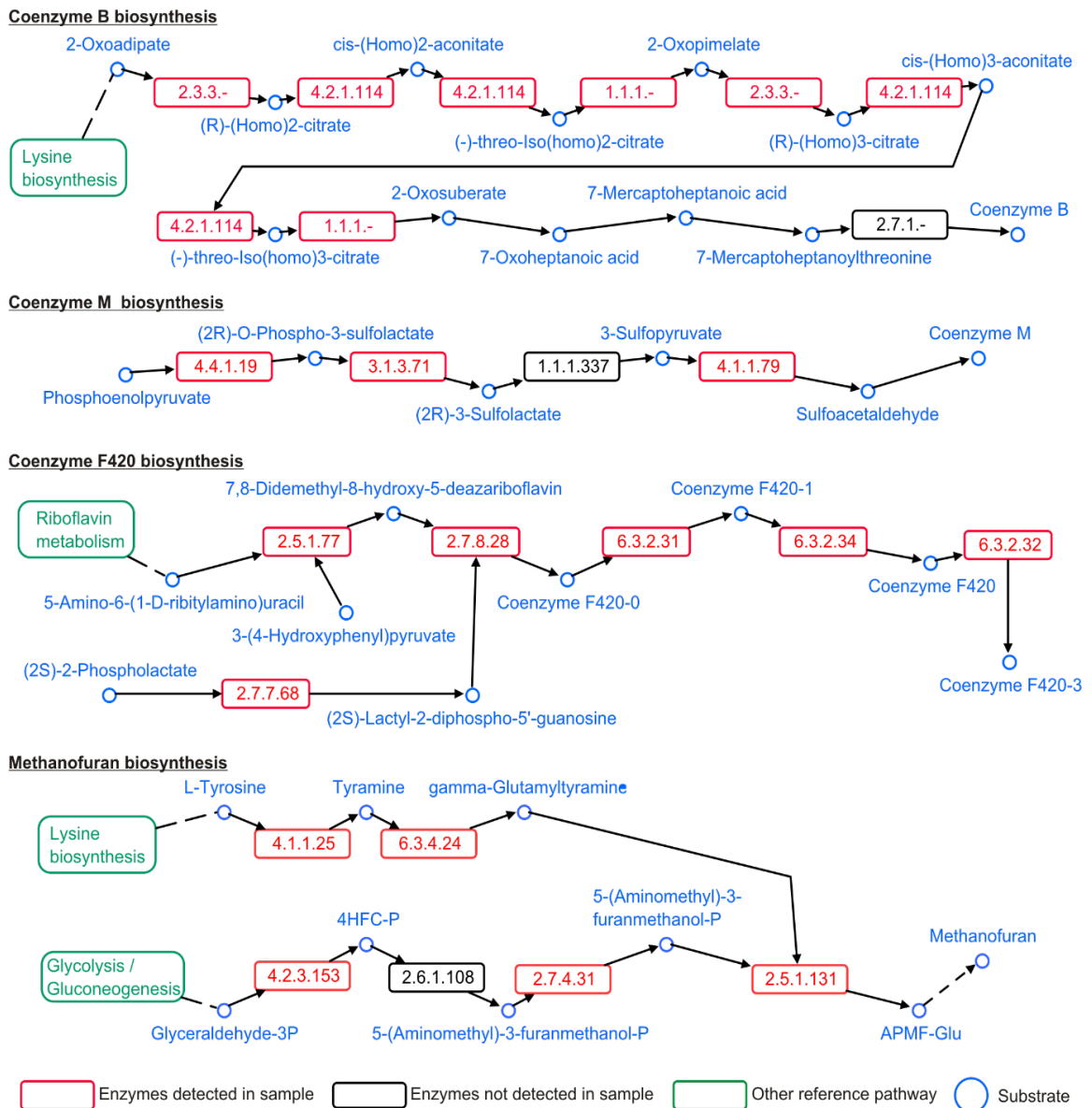


FIGURE 23: METHANE METABOLISM (B). The figure shows experimental data (red) mapped on the reference pathway.

4.5.2.2 NITROGEN METABOLISM

The multifaceted process of biological nitrogen cycle involves diverse reactions performed by prokaryotes. Nitrogen metabolism alone represents 10.26 % of Energy metabolism and 0.50 % of all metabolism. 5 pathway modules have been detected in this study. Out of these 5, 3 were complete, and 2 were partial. Details are provided in Table 29. During the

process nitrogen changes its oxidation state varies between +5 in nitrate and -3 in ammonia. There are 4 reductions (Nitrogen fixation, Assimilatory nitrate reduction, Dissimilatory nitrate reduction, Denitrification) and 2 oxidations (Nitrification, Anaerobic ammonium oxidation) pathway involved in the nitrogen cycle.

TABLE 29: MODULE COVERAGE OF NITROGEN METABOLISM.

Module No. / Description	Path	No. of KEGG Ortholog	Coverage
M00175 Nitrogen fixation	map00910	2	1 block missing
M00531 Assimilatory nitrate reduction	map00910	42	complete
M00530 Dissimilatory nitrate reduction	map00910	184	complete
M00529 Denitrification	map00910	177	complete
M00804 Complete nitrification	map00910	63	2 blocks missing

Experimental data mapped on the reference pathway provides a comprehensive overall view of nitrogen metabolism. Figure 24 and Figure 25 shows the pathway and coverage. Atmospheric molecular nitrogen is reduced to ammonia by nitrogen fixation. The reaction is catalyzed by the nitrogenase enzyme system (Kim and Rees 1994, Fani, Gallo et al. 2000, Henson, Watson et al. 2004). In our sample, we detected nitrogenase (genes coding for nitrogenase) as shown in Figure 24 as EC number 1.18.6.1 and in Figure 25 as NifDHK. Assimilatory nitrate reduction and dissimilatory nitrate reduction convert nitrate to ammonia. In the process of assimilatory nitrate reduction, nitrate reductase (EC 1.7.99.4) and ferredoxin nitrite reductase (EC 1.7.7.1) are important enzymes (Pino, Olmo-Mira et al. 2006, Martinez-Espinosa, Lledo et al. 2007, Imamura, Terashita et al. 2010). Both of them have been detected in our assessment as NasAB and NirA as shown in the Figure 25. Also in the Figure 24, nitrate reductase is shown as EC number 1.7.99.4. The process of dissimilatory nitrate reduction requires nitrate reductase (EC 1.7.5.1 and EC 1.7.99.4,) which converts nitrate to nitrite and nitrite reductase (EC 1.7.1.15 and EC 1.7.2.2) which converts nitrite to ammonia (Bursakov, Carneiro et al. 1997, Smith, Nedwell et al. 2007, Tamegai, Ikeda et al. 2007, Dong, Smith et al. 2009, Kuroki, Igarashi et al. 2014, Mauffrey, Martineau et al. 2015). In the Lonar sediment, we observed all of these enzymes, shown in the Figure 25 as NarGHU, NapAB, NirBD and NrfAH. Denitrification reduces nitrate or nitrite by using them as a terminal electron acceptor and returning N₂, NO and N₂O to the atmosphere (Yan, Yang et al. 2005, Barth, Isabella et al. 2009, Torres, Rubia et al. 2011,

Torres, Rubia et al. 2014). We observed the presence of all the enzymes (genes coding for the enzymes) in sediments of Lonar meteorite crater soda lake shown in the Figure 25 as NarGHU, NapAB, NirK, NirS, NorBC and NosZ. During nitrification ammonia is oxidized with oxygen into nitrite and nitrite into nitrate. In this process ammonia monooxygenase (EC 1.14.99.39), hydroxylamine dehydrogenase (EC 1.7.2.6) and nitrite reductase (EC 1.7.99.4) catalyzes the reactions converting ammonia to hydroxylamine, hydroxylamine to Nitrite and finally nitrite to nitrate. However, we were able to detect only the nitrate reductase, shown in the Figure 25 as NxrAB. Oxidation of ammonium (NH_4^+) to N_2 is performed by anaerobic ammonium oxidation (Anammox), where nitrite is used as an electron acceptor. The key enzyme of this process is hydrazine oxidoreductase (EC 1.7.99.8) that converts hydrazine to nitrogen (Jetten, Strous et al. 1998, Schalk, de Vries et al. 2000, Jetten, Slijkers et al. 2003, Smith, Bohlke et al. 2015). In our sample, we were unable to detect the presence of hydrazine oxidoreductase. However, we detected nitrite reductase (EC 1.7.2.1) shown in the Figure 25 as NirK and NirS that converts nitrite to nitric oxide. This nitric oxide combined with ammonia forms hydrazine.

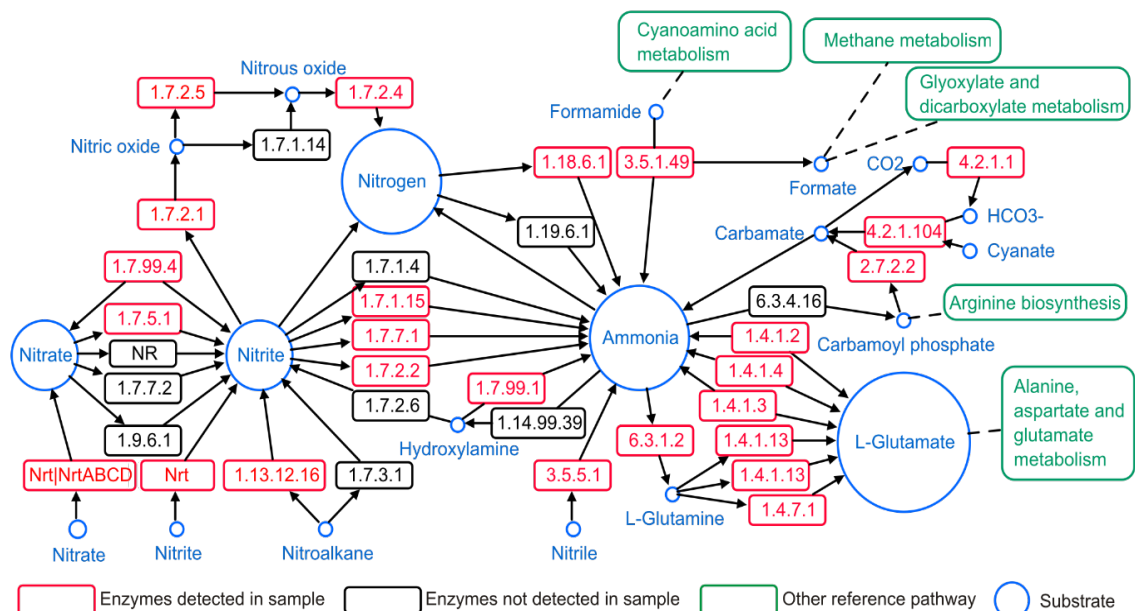


FIGURE 24: NITROGEN METABOLISM (A). The figure shows experimental data (red) mapped on the reference pathway.

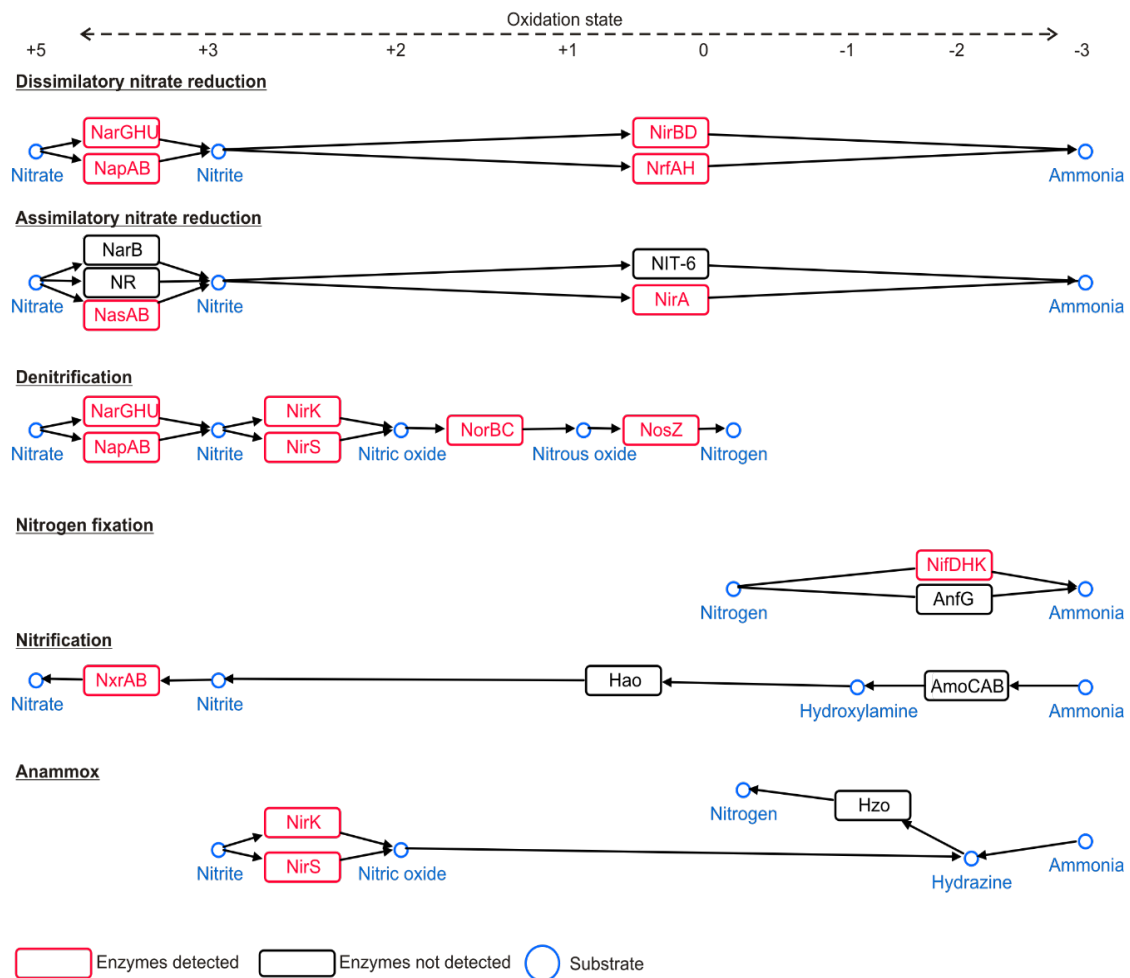


FIGURE 25: NITROGEN METABOLISM (B). The figure shows experimental data (red) mapped on the reference pathway.

4.5.2.3 SULFUR METABOLISM

In the global sulfur cycle, a significant role is played by organic sulfur compound metabolism. Sulfur metabolism alone represents 13.95 % of Energy metabolism and 0.68 % of all metabolism in Lonar sediments. 3 pathway modules have been detected in this study. Out of these 3, 2 were complete, and 1 was partial. Details are provided in Table 30. Different oxidation states of sulfur are present in the sulfur cycle, between +6 in sulfate and -2 in sulfide.

TABLE 30: MODULE COVERAGE OF SULFUR METABOLISM.

Module No. / Description	Path	No. of KEGG Ortholog	Coverage
M00176 Assimilatory sulfate reduction	map00920	197	complete
M00596 Dissimilatory sulfate reduction	map00920	79	complete
M00595 Thiosulfate oxidation by SOX complex	map00920	20	1 block missing

Experimental data mapped on the reference pathway provides a comprehensive overall view of nitrogen metabolism. Figure 26 and Figure 27 shows the pathway and coverage. Sulfate is reduced by assimilatory pathway and dissimilatory pathway (Peck 1961). During biosynthesis of sulfur-containing amino acids, sulfur compounds are reduced by assimilatory pathway (Cuhel, Taylor et al. 1981, Cuhel, Taylor et al. 1982, Daniels, Belay et al. 1986, Liu, Beer et al. 2012). In the case of the dissimilatory pathway, sulfate or sulfur is used as the terminal electron acceptor and inorganic sulfide is produced. The initial step of the assimilatory and dissimilatory pathway is the activation of sulfate that forms adenylyl-sulfate(APS) using ATP (Neumann, Wynen et al. 2000). Sulfate adenylyltransferase (EC 2.7.7.4) is the enzyme that activates sulfate and forms adenylyl-sulfate(APS) was detected in our sample and shown in Figure 27 as Sat and CysND. This APS, by assimilatory pathway, is transformed to 3'-phospho-adenylyl-sulfate (PAPS) and further reduced to sulfite. This sulfite is again reduced to sulfide. Some of the other enzymes of assimilatory sulfate reduction were also observed. For example, adenylyl-sulfate kinase (EC 2.7.1.25), phosphoadenylyl-sulfate reductase (EC 1.8.4.8) and assimilatory sulfite reductase (EC 1.8.1.2) were detected and shown in Figure 27 as CysC, CysH, CysJI and Sir. In the case of the dissimilatory pathway, APS is directly reduced to sulfite. This sulfite is again reduced to sulfide (Basen, Kruger et al. 2011, Bradley, Leavitt et al. 2011, Barton, Fardeau et al. 2014). We were able to detect all the enzymes involved in the process such as sulfate adenylyltransferase (EC 2.7.7.4), adenylyl-sulfate reductase (EC 1.8.99.2) and dissimilatory sulfite reductase (EC 1.8.99.5) in the Figure 27 as Sat, AprAB and DsrAB. The sulfur oxidation capacity of the SOX system (sulfur oxidation) is a well-recognized sulfur oxidation pathway (Wodara, Kostka et al. 1994, Quentmeier, Hellwig et al. 2003, Ghosh and Dam 2009, Grimm, Franz et al. 2011, Yin, Zhang et al. 2014). All the enzymes of SOX system were detected in our sample as shown in the Figure 27. During

anoxygenic photosynthesis by green and purple sulfur bacteria, reduced sulfur compounds such as sulfide, elemental sulfur, and thiosulfate are used as the electron donor.

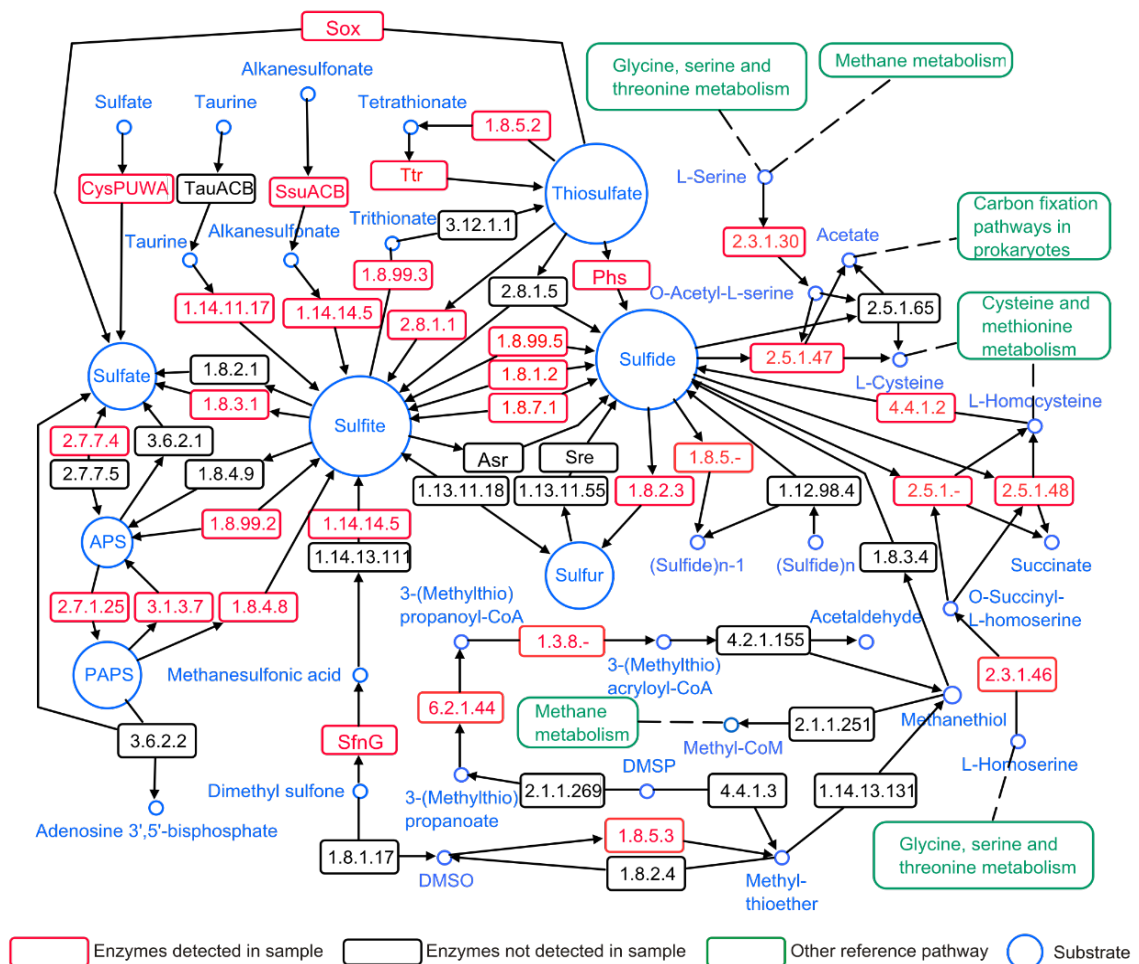


FIGURE 26: SULFUR METABOLISM (A). The figure shows experimental data (red) mapped on the reference pathway.

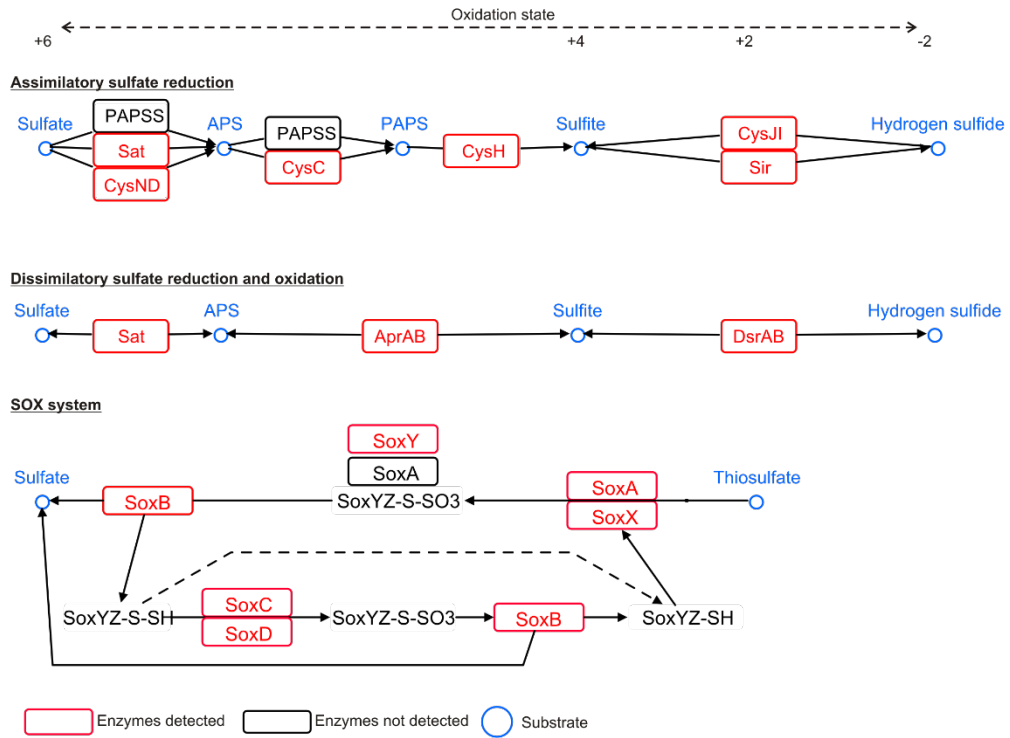


FIGURE 27: SULFUR METABOLISM (B). The figure shows experimental data (red) mapped on the reference pathway.

4.6 COMMUNITY DYNAMICS DURING MICROENVIRONMENTAL PH HOMEOSTASIS

4.6.1 SHIFT OF EXTRACELLULAR PH

The measured pH of the surface water of Lonar Meteorite Crater Soda Lake ranged between pH 9.19 to pH 10.5, indicating pronounced seasonality (Badve, Kumaran et al. 1993, Siddiqi 2007). pH of the sediment itself was recorded at pH 9.65, and in our sample we found it to be pH 9.9, but there is no record of seasonality or maximum and minimum pH variation (Kanekar, Sarnaik et al. 1998). The objective of this experiment was to investigate how this microbial community responds to nonoptimal microenvironmental pH. The prokaryotic cultures were set up using modified Horikoshi media as described in 3.11. The pH was adjusted to pH 9 (suboptimal) and pH 10 (superoptimal) with sterile NaOH, respectively.

All the measured pH values from both the superoptimal and suboptimal systems in triplicates at all the time points from t0 to t5 (day 0 to day 25 with a resolution of 5 days) were recorded (Figure 28). The modified Horikoshi medium contains glucose; as a result, we observed a rapid acidification in the beginning (Solé, Lorén et al. 2010). This phenomenon was observed till t1 in both the suboptimal and superoptimal systems. In superoptimal system pH dropped from pH 10.094 (± 0.049) to pH 8.193 (± 0.075) and in suboptimal system pH dropped from pH 9.054 (± 0.024) to pH 7.7 (± 0.087). From t2 to t5 in both systems pH gradually increased to pH 9.253 (± 0.066) and pH 9.286 (± 0.053) in the superoptimal system and suboptimal system respectively. In this study, we found that the initial pH difference between both the systems of 1.04 unit of pH decreased to 0.032 unit of pH. Which in terms of H⁺ ion concentration translates to more than 10 fold. However, the interesting fact is that in both systems, irrespective of initial pH, the final pH is approximately pH 9.3 (9.253 and 9.286), which is close to the reported pH of the sediment of pH 9.65.

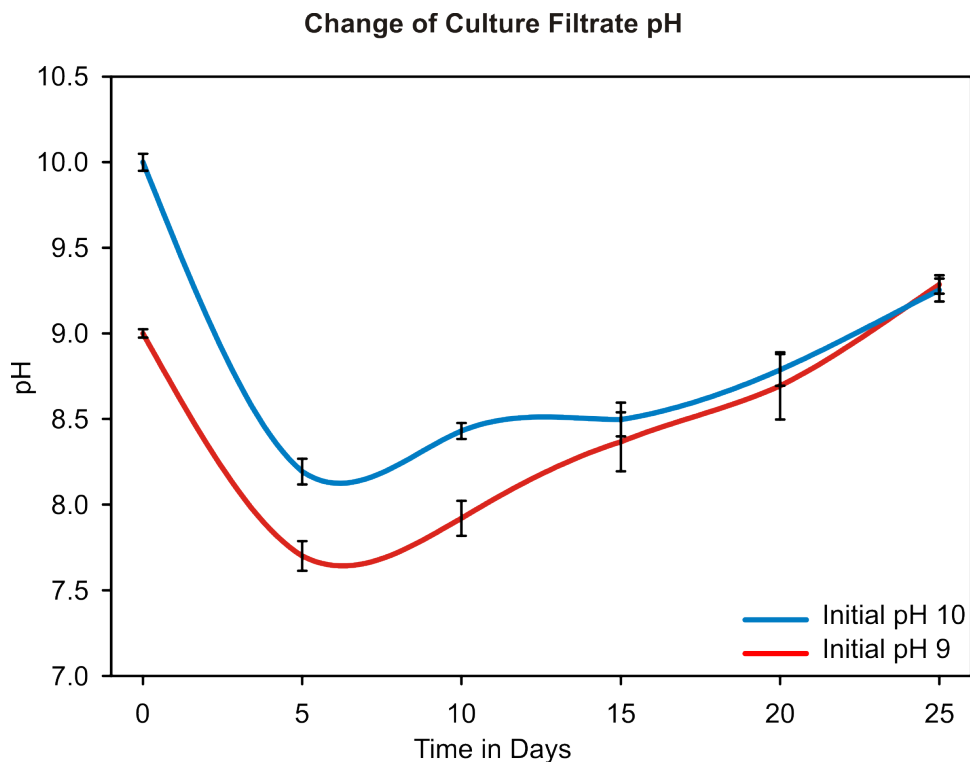


FIGURE 28: CHANGE OF EXTRACELLULAR pH. The Figure Shows Changes in Microenvironmental pH with Time. The Blue line depicts the Changes of pH in the Superoptimal pH System and Red line depicts the Changes in the Suboptimal pH System.

4.6.2 DIFFERENTIAL UTILIZATION OF DIFFERENT AMINO ACIDS IN SUBOPTIMAL AND SUPEROPTIMAL pH

Amino acid content of the culture filtrate at t_0 was considered as baseline and culture filtrate at t_4 was compared in both the pH system to detect any changes. We observed that NH_3 and hydroxyproline behave oppositely depending on the pH system they were exposed to. The amount of NH_3 increases in the medium when exposed to suboptimal pH but decreases when exposed to superoptimal pH. Similarly, hydroxyproline also increases in suboptimal pH but increases in superoptimal pH. Details are shown in Figure 29. From these observations, it is evident that NH_3 and hydroxyproline metabolism does have some effect on the pH dynamics.

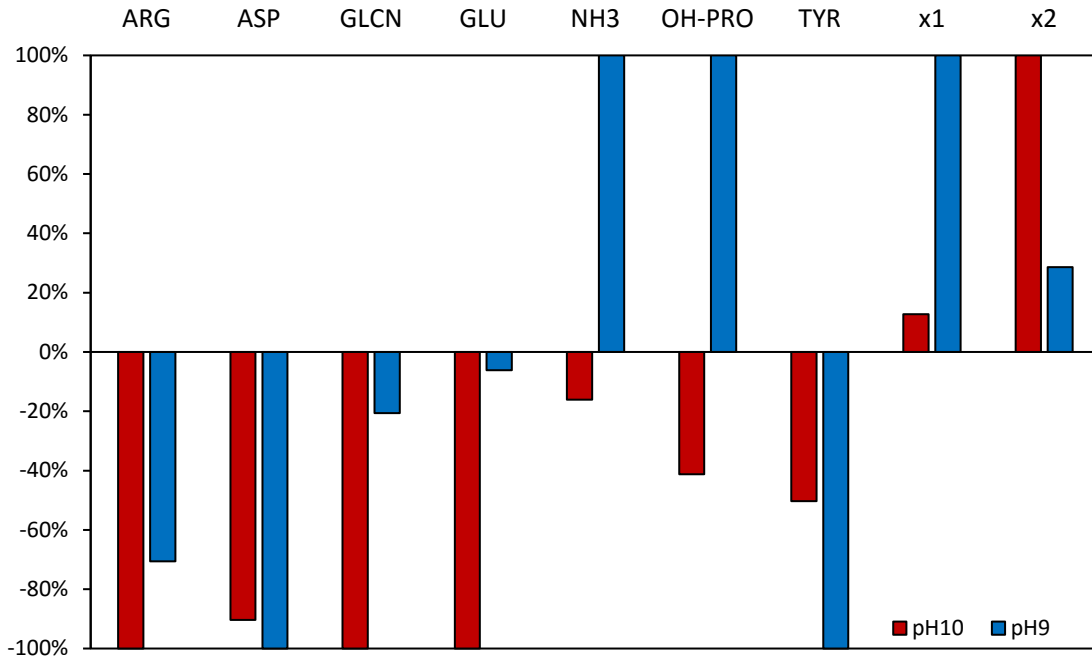


FIGURE 29: CHANGES IN THE MICROENVIRONMENT IN RESPONSE TO SUBOPTIMAL OR SUPEROPTIMAL pH. Amino acids are given above the respective bars. pH 9 is considered as suboptimal, and pH 10 is considered as superoptimal.

4.6.3 PROKARYOTIC DIVERSITY, RICHNESS AND DISTRIBUTION OF TAXA

Two pH systems (suboptimal and superoptimal), 5 time points (t1 to t5) in triplicates and t0 (original sediment) resulted in a total of 33 samples. For details see 3.11. From these 33 samples a total of 235,943 archaeal and 1,657,168 bacterial sequences were found. We observed an average coverage of 71.04 % (± 2.5) in *Archaea* with maximum and minimum coverage as 73.96 % and 65.7 %. For *Bacteria*, the average coverage was 85.56 % (± 8.3), maximum and minimum coverages were 95.13 % and 63.17 % (Table 31).

TABLE 31: GENERAL ANALYSIS OF SEQUENCE DATA.

Archaea (1,038 Sequences / Sample)						
	chao1	Michaelis-Menten Fit	Observed OTUs	Shannon Index	Simpson Index	Coverage
Average	228.61 3	155.892	110.444	4.836	0.936	71.04 %
Maximum	287.66 7	185.696	122	4.913	0.94	73.96 %
Minimum	188.07 9	138.719	102.6	4.763	0.934	65.70 %
Standard Deviation	34.039	14.178	6.201	0.055	0.001	2.506
Bacteria (19,920 Sequences / Sample)						
	chao1	Michaelis-Menten Fit	Observed OTUs	Shannon Index	Simpson Index	Coverage
Average	381.25 3	262.765	206.975	3.616	0.833	85.56 %
Maximum	1584.9 1	1058.121	668.4	4.205	0.914	95.13 %
Minimum	146.34 1	112.489	101	2.957	0.721	63.17 %
Standard Deviation	374.11 5	245.141	146.073	0.359	0.054	8.3

In the case of *Archaea*, 1,071 OTUs were unique. The most dominant genus was *Methanocalculus* representing 11.77 % of all sequences. In the case of *Bacteria*, in a total of 33 samples, 3,259 OTUs were unique. The most dominant genus was *Exiguobacterium* representing 20.4 % of all sequences. There is also an overall initial drop of total number of OTUs in both pH systems and increase at the end (Figure 30).

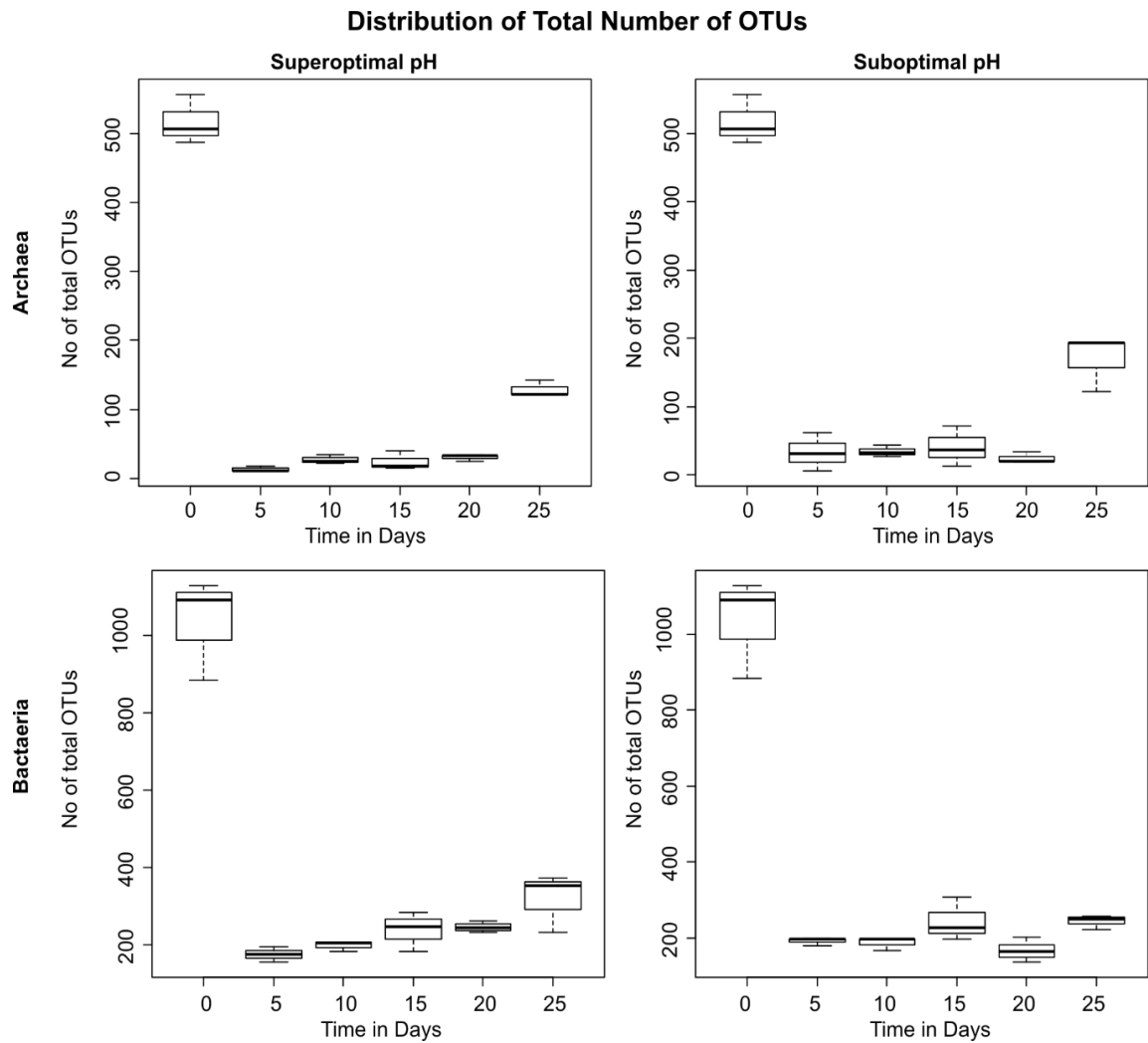


FIGURE 30: CHANGES IN THE NUMBER OF OTUs WITH TIME. The Figure shows an overall initial drop of the total number of OTUs and increase at the end in both Superoptimal and Suboptimal pH Systems.

4.6.4 COMMUNITY DYNAMICS OF PROKARYOTES

The overarching objective of this experiment was to investigate the community dynamics or changing biodiversity from a temporal perspective as the pH of the culture changes. Upon reviewing the relative abundance of all the samples at higher level taxa, we can see, there is a fundamental difference in how archaeal and bacterial community responds to exposure to superoptimal or suboptimal pH (Figure 31).

Overview of Prokaryotic Diversity

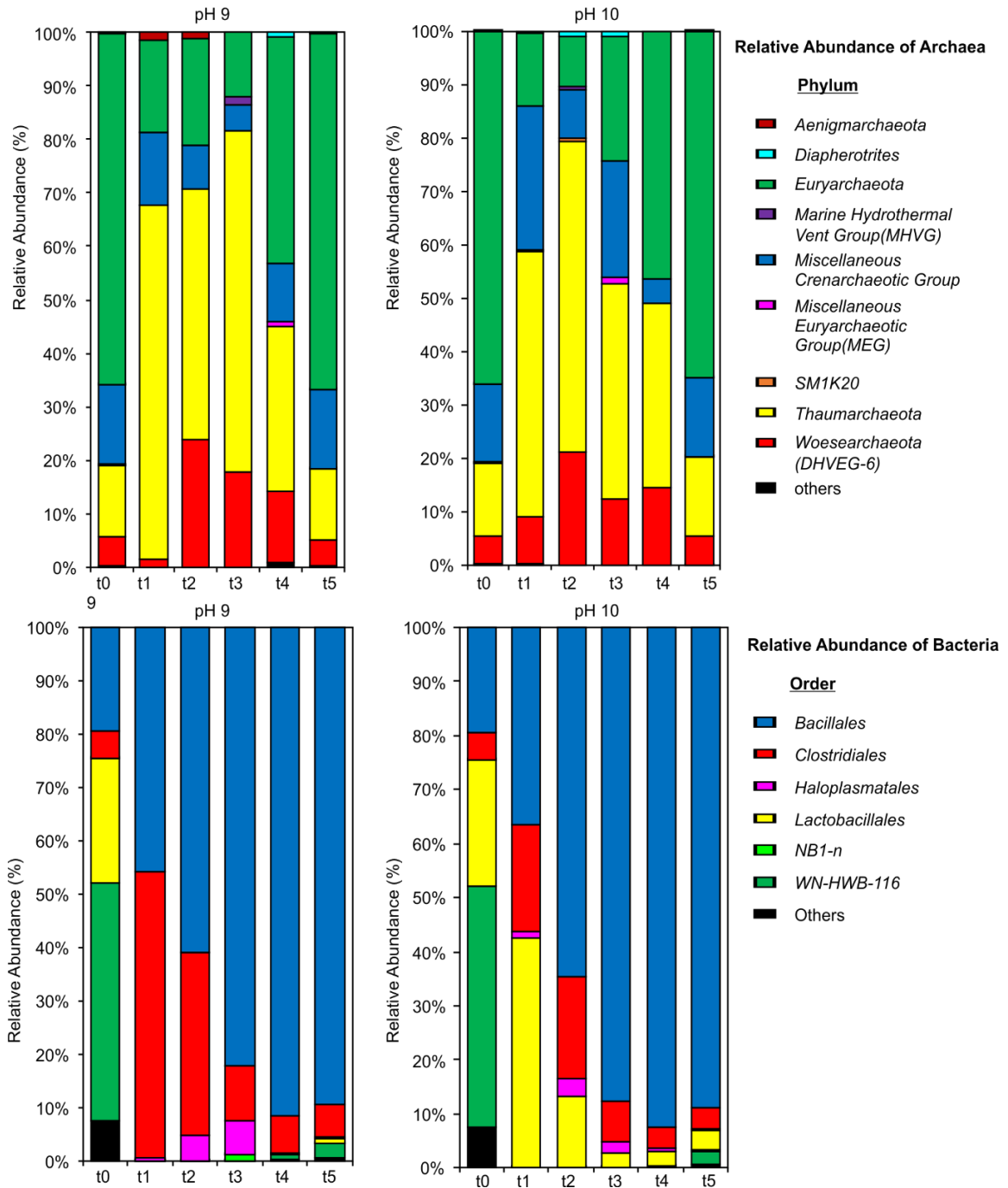


FIGURE 31: CHANGES IN THE PROKARYOTIC DIVERSITY WITH TIME. The figure shows changes in relative abundance of archaeal Phylum and bacterial Order in Suboptimal and Superoptimal pH in relation to time.

Archaeal community changes significantly from t0 to t1 but at t5 in both pH systems they change gradually and exhibit relative abundance similar to t0 or original profile. The bacterial community also changes significantly from t0 to t1 but like the archaeal

community at t5 in both pH systems, they do not show any gradual overall changes in relative abundance to very close relative abundance values of t0 or original profile. It is noticed that after 25 days of incubation *Clostridiales* and *Haloplasmales* were able to regain its relative abundance closer to its original values in the suboptimal pH system than in superoptimal pH system.

Looking at the changes in relative abundances of different genera in details, we were able to identify several archaeal and bacterial genera which shows variation. For the purpose of emphasizing on the relative change in population sizes, relative abundance at t0 in the case of both pH systems was considered as 1 or 100 % and variations were plotted as log₂ values along the y-axis (Figure 32 to Figure 37). Among the *Archaea* the genera showing significant variations were *Candidatus Nitrosopumilus*, *Natronorubrum*, *Methanolobus*, *Halorubrum*, *Haloterrigena*, *Halovenus* and *Halonotius*. Among the bacterial genera showing significant variations were *Alkalibacterium*, *Lysinibacillus*, *Natronincola*, *Anaerobacillus*, *Paenibacillus*, *Anaerovirgula*, *Desulfitispora*, *Salimesophilobacter* and *Clostridium sensu stricto* 7.

First of all, we must acknowledge that neither the culture medium nor the culture condition exactly represents the microenvironment of the sediment. Also, the duration of incubation might not be sufficient to let the community work on their microenvironment. However, nonetheless, it can be seen by looking at the dynamics of the above mentioned archaeal and bacterial genera that they can be grouped in to different types of dynamic groups.

4.6.4.1 DYNAMICS OF ARCHAEAL GENERA:

After scrutinizing the growth dynamics of archaeal genera, we were able to identify 7 genera showing significant variation in relative abundance. We were able to group them into 2 groups based on their changing relative abundance pattern.

4.6.4.1.1 Dynamic Group Type-AI:

Five of them namely *Natronorubrum*, *Haloterrigena*, *Halovenus*, *Halorubrum* and *Candidatus Nitrosopumilus* show a significant increase in relative abundance in between t2 and t4 compared to t0. Then again at t5, they tend to get closer to the original relative abundance of t0 (Figure 32).

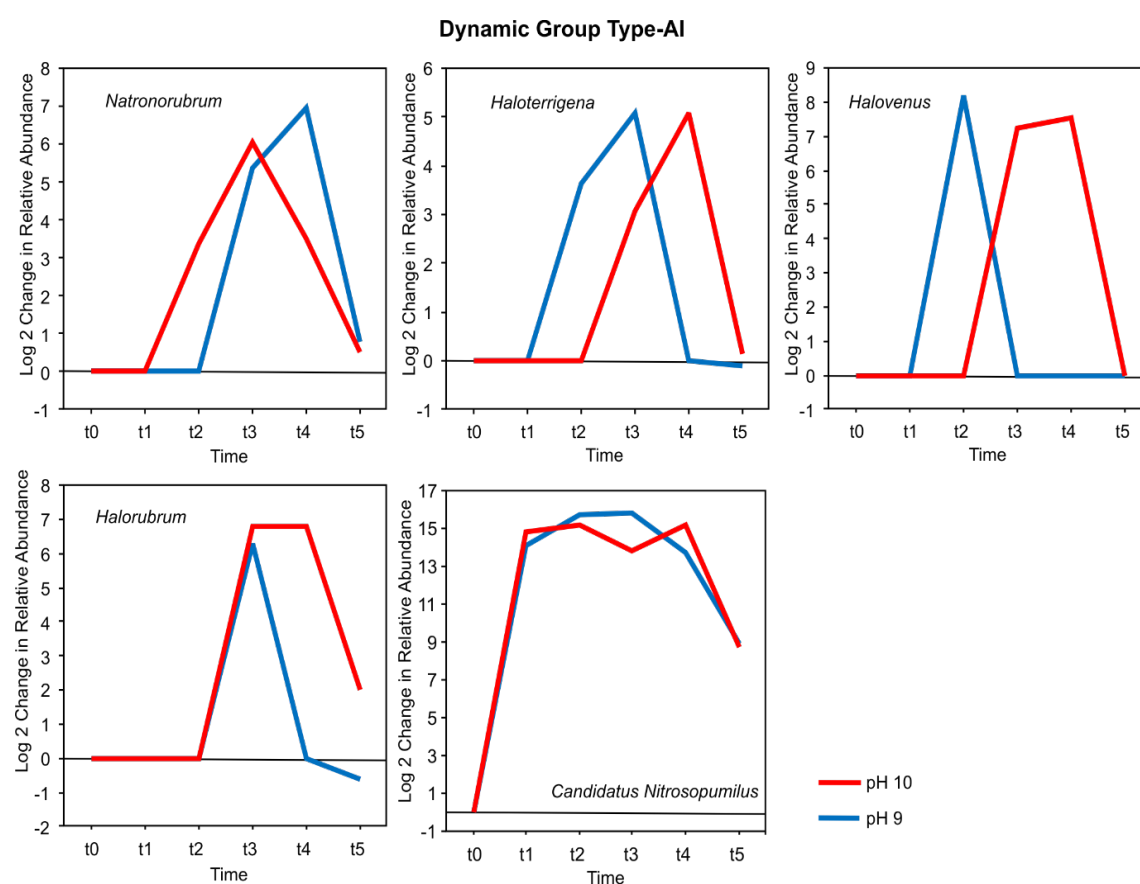


FIGURE 32: DYNAMIC GROUP TYPE-AI. The figures show changes in relative abundance in Suboptimal and Superoptimal pH in relation to time.

Natronorubrum reaches its highest relative abundance of 33.333 % in the suboptimal pH system and 17.777 % in the superoptimal pH system at t4 and t3 respectively. Then decrease to 0.462 % and 0.374 % at t5 which is close to 0.264 % at t0. *Natronorubrum* have been found and isolated from various habitats like, a soda lake in Tibet , Aibi salt lake in Xin-Jiang (Xu, Zhou et al. 1999), China (Cui, Tohty et al. 2006), Aiding salt lake in Xin-Jiang, China (Cui, Tohty et al. 2007), from sediment of the hypersaline Lake Chagannor in

Inner Mongolia (Gutierrez, Castillo et al. 2010) and former Lake Texcoco in Mexico (Ruiz-Romero, Valenzuela-Encinas et al. 2013). Most, if not all, of them are known haloalkaliphiles showing growth at pH as high as 11 with optimal pH for most of them being between 8.5 to 9.5 and for some of them optimal NaCl concentration is 3.4 M.

Haloterrigena has been reported from different so called extreme habitat, solar salterns of Cabo Rojo, Puerto Rico (Montalvo-Rodriguez, Lopez-Garriga et al. 2000); Aibi salt lake, Xin-Jiang, China (Xu, Liu et al. 2005); Fuente de Piedra salt lake, Spain (Romano, Poli et al. 2007); salt lake Xilinhot in Inner Mongolia, China (Gutierrez, Castillo et al. 2008); saline-alkaline soil from Daqing, Heilongjiang Province, China (Wang, Yang et al. 2010). Most of them are extremely halophilic and extremely alkaliphilic, able to grows at a salinity of 5 M NaCl (Gutierrez, Castillo et al. 2008) and pH 10.5 (Wang, Yang et al. 2010).

Halovenus has been observed in the Aran-Bidgol salt lake, Iran (Makhdoumi-Kakhki, Amoozegar et al. 2012); Isla Bacuta saltern, Huelva, Spain (Infante-Dominguez, Corral et al. 2015). Most of them are extremely halophilic and alkaliphilic, able to grows at a salinity of 5.1 M NaCl (approximately, actual 30 % w/v) (Infante-Dominguez, Corral et al. 2015) and pH 9.

Halorubrum has been observed in extremely diverse but polyextremophilic habitats, in Lake Tebenquiche, situated in the northern part of the Atacama Saltern, Chile (Lizama, Monteoliva-Sanchez et al. 2002); from Lake Zabuye, on the Tibetan Plateau; China (Fan, Xue et al. 2004); soda lake in Xinjiang, China (Feng, Zhou et al. 2005); Lake Ejinor, a saline lake in Inner Mongolia, China (Castillo, Gutierrez et al. 2006); Aibi salt lake and Aiding salt lake in Xin-Jiang, China (Cui, Tohty et al. 2006); Ayakekum salt lake on the Qinghai-Tibet Plateau (Xu, Wu et al. 2007); Laguna Antofalla in the Argentinian Puna (Burguener, Maldonado et al. 2014); hypersaline lake Aran-Bidgol in Iran (Corral, de la Haba et al. 2016); water and silt sample from Burlinskoye Lake, Altai Krai, Russia (Rozanov, Bryanskaya et al. 2015); subterranean salt mine in Yunnan, China (Chen, Liu et al. 2015). Most of the members of *Halorubrum* genus are extremely halophilic and often alkaliphilic growing at salinity as high as 5 M NaCl (Gutierrez, Castillo et al. 2011) and pH 10 (Hu, Pan et al. 2008).

Candidatus Nitrosopumilus have been isolated and reported before from varies kind of environment; marine sediment from Svalbard, the Arctic Circle (Park, Kim et al. 2012),

estuary of San Francisco Bay (Mosier, Allen et al. 2012), pelagic redoxcline in the central Baltic Sea (Labrenz, Sintes et al. 2010) similar sequences has also been reported from underwater cave systems, beneath Australia’s vast, dry Nullarbor Plain (Tetu, Breakwell et al. 2013) and the Yellowstone geothermal complex, USA (Kan, Clingenpeel et al. 2011). *Candidatus Nitrosopumilus* is a known ammonia-oxidizing archaeon.

4.6.4.1.2 Dynamic Group Type-All:

In two other genera, *Methanolobus* and *Halonotius* similar behavior was observed in the beginning as they show an increase in relative abundance in between t1 and t4. Later at t5, although they tend to decrease to the original relative abundance, the similarity is not as close as the first discussed group of archaeal genera. Most logically significant feature to group them apart is that *Methanolobus* and *Halonotius* do not behave similarly in suboptimal and superoptimal pH system. Unlike Type-AI, they show these dynamics only in one system (Figure 33).

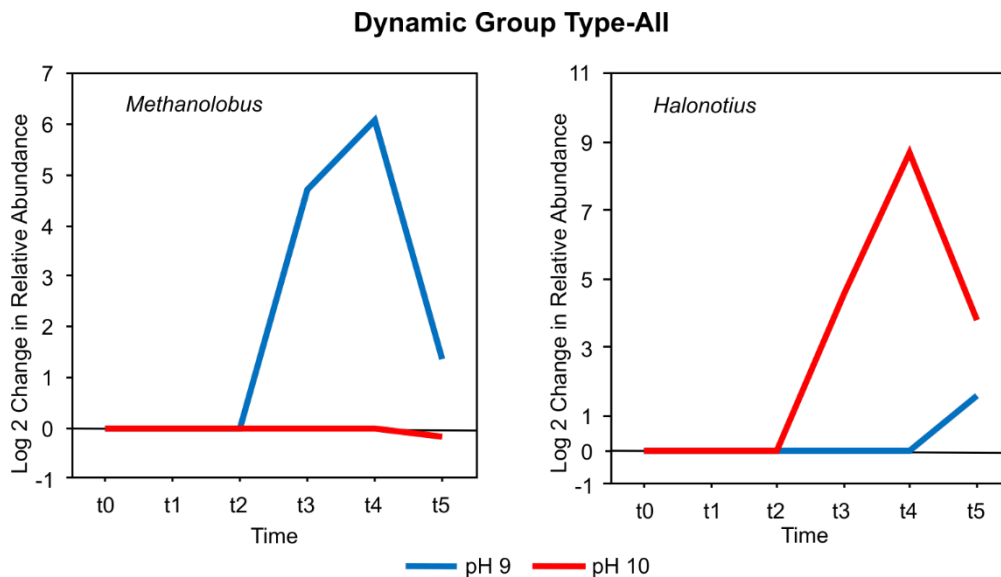


FIGURE 33: DYNAMIC GROUP TYPE-AII. The figures show changes in relative abundance in Suboptimal and Superoptimal pH in relation to time.

Methanolobus show a highest relative abundance of 19.576 % in the suboptimal pH system at t4; regaining 0.727 % relative abundance at t5, which is similar to 0.286 %

relative abundance at t₀. In the superoptimal pH system, there is no significant change in the relative abundance of *Methanolobus*. *Methanolobus* has been isolated from estuarine sediments (Ticak, Hariraju et al. 2015), Tibetan plateau wetland (Chen, Feng et al. 2015), natural gas field in Japan (Mochimaru, Tamaki et al. 2009), from a coal seam located 926 m below the surface, Monroe, Louisiana, USA (Doerfert, Reichlen et al. 2009), from the Zoige wetland of the Tibetan plateau (Zhang, Jiang et al. 2008), from gas and oil wells in the Gulf of Mexico (Ni and Boone 1991). Most of them are moderately halophilic and extremely alkaliphilic methanogen, grows over a wide pH range, from 6.8 to 9.0.

Halonotius has been isolated from a crystallizer pond of Cheetham Salt Works, Geelong, Victoria, Australia (Burns, Janssen et al. 2010); hypersaline Deep Lake, Antarctic (Burns, Camakariz Hm Fau - Janssen et al. 2004). These are extremely halophilic known to grow at near saturation concentration.

4.6.4.2 DYNAMICS OF BACTERIAL GENERA:

Regarding the complexity of the dynamics, bacterial genera show more variational pattern than in archaeal genera. However, they do not share as high total relative abundance as in archaeal genera. After scrutinizing their dynamics, we identified nine genera that show most variation in relative abundance. Generally speaking, they can be grouped into four groups based on their changing relative abundance pattern.

4.6.4.2.1 Dynamic Group Type-BI:

In the first group of genera namely *Lysinibacillus* and *Desulfitispora*, it is observed that like Type-AI their relative abundance increases in both suboptimal and superoptimal pH system at first. Also, at t₅, it tends to decrease but does not successfully reaches the original relative abundance of t₀ (Figure 34).

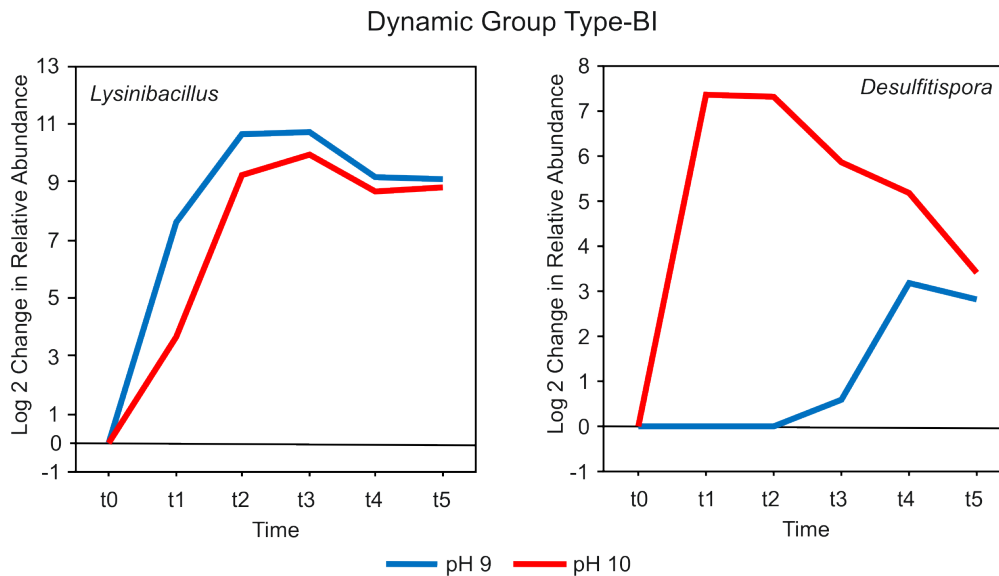


FIGURE 34: DYNAMIC GROUP TYPE-BI. The figures show changes in relative abundance in Suboptimal and Superoptimal pH in relation to time.

Lysinibacillus show the highest increase in relative abundance of 8.255 % and 4.829 % in suboptimal and superoptimal pH system respectively at t3. At t5, it decreases to 2.598 % and 2.153 % in suboptimal and superoptimal pH system respectively but not very close to near zero value at t0. *Lysinibacillus* has been isolated from both extreme as well as not so extreme habitats. It is reported from saline-alkaline soil samples from Lingxian County, Shandong Province, China (Kong, Wang et al. 2014); agricultural soil (Ahmed, Yokota et al. 2007, Liang, Lu et al. 2009); marine sediments (Zhao, Dong et al. 2016); arsenic contaminated lands (Rahman, Nahar et al. 2015); tropical soil, Malaysia (Chan, Chen et al. 2015). Most of them are not extremophiles. However, some of the members of this genus are known haloalkaliphiles are growing at the alkaline condition as high as pH 10 (Zhao, Feng et al. 2015).

Desulfitispora has been found in soda lakes in south-eastern Kulunda Steppe, Altai, Russia (Sorokin and Muyzer 2010). Only one species has been described so far, *Desulfitispora alkaliphila*. They are obligately alkaliphilic with a pH range for growth between 8.5 and 10.3 and an optimum at pH 9.5. It is also reported to have some effect on the initial pH of the culture, but it has not been studied in details.

4.6.4.2.2 Dynamic Group Type-BII:

In another group a general trend in the dynamics of *Alkalibacterium*, *Natronincola*, *Anaerobacillus* and *Paenibacillus* was observed. They exhibit an increase in one pH system and a decrease in the other pH system of relative abundance between t1 and t4 with a decrease or increase in relative abundance at t5 towards its original relative abundance or t0. However, never reaching as close as it was observed in the case of *Archaea* (Figure 35). For example, *Alkalibacterium*, the increase of its highest relative abundance is seen in the superoptimal system, at t1 reaching as high as 42.182 %. Then at t5, they decrease to 3.698 %, but although the progression is towards the values observed at t0, it is not as close as we have seen in the case of archaeal genera. In the case of *Paenibacillus*, the observation is in the suboptimal pH system instead of the superoptimal pH system. It shows an increase in relative abundance at t1 to 0.202 % then a decrease to 0.075 % which is relatively closer to the t0 relative abundance value of 0.002 %.

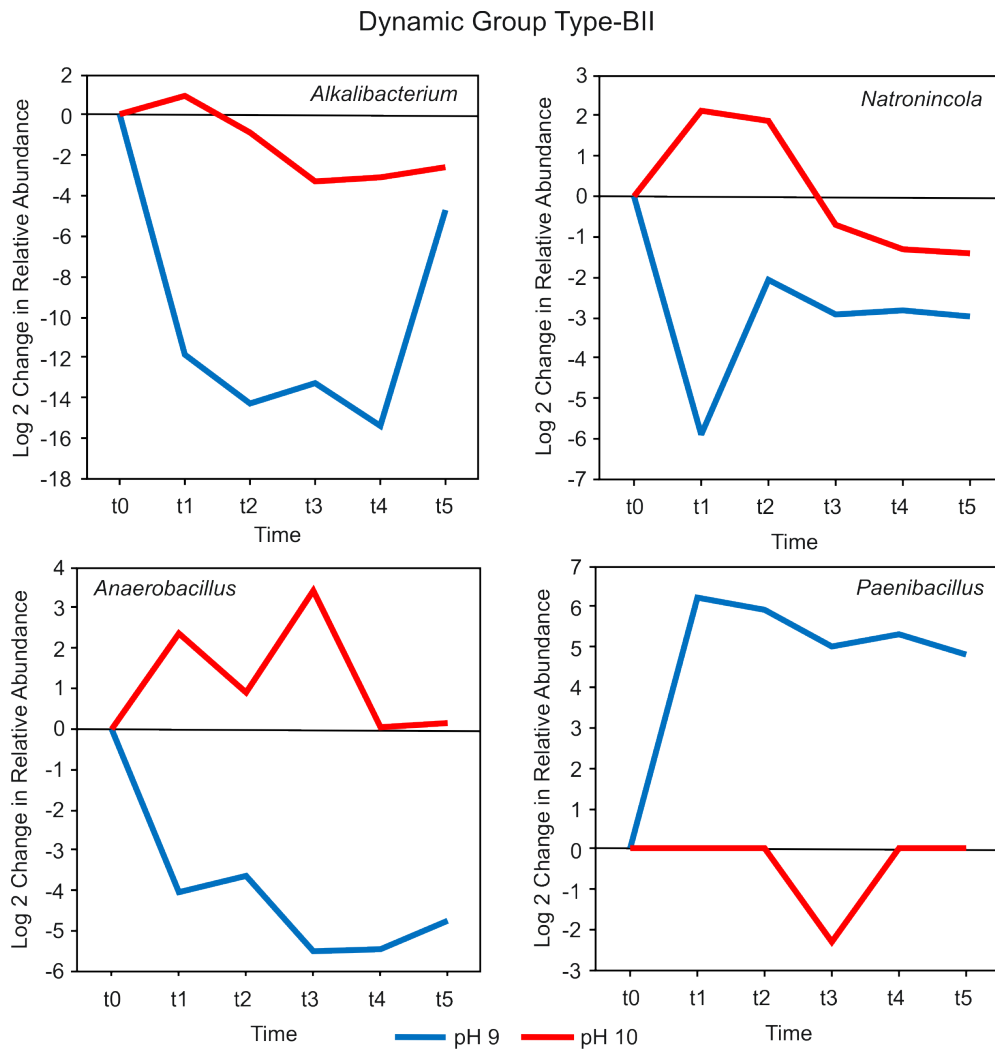


FIGURE 35: DYNAMIC GROUP TYPE-BII. The figures show changes in relative abundance in Suboptimal and Superoptimal pH in relation to time.

Alkalibacterium has been observed in different habitat, decaying marine algae, decaying seagrass, raw fish, salted fish and salted and fermented shrimp paste, temperate area of Japan and Thailand (Ishikawa, Tanasupawat et al. 2009); from wash-waters during the preparation of edible olives (Ntougias and Russell 2001); They are reported to grow at pH as high as 12. *Paenibacillus* has been isolated from various environments, from the alkaline soil, Korea (Yoon, Kang et al. 2005); desert sand sample, Gansu Province, China (Jeon, Lim et al. 2009); soil sample, Assam, India (Rai, Roy et al. 2010). Some of them are extremely haloalkaliphilic able to grow at pH as high as pH 12 (Lee, Lee et al. 2002) and at a salinity of up to 5M NaCl (Raddadi, Cherif et al. 2013).

Natronincola has been reported from bottom sediment of a coastal lagoon of the soda lake Verkhnee Beloe, Buryatia and Lake Khadyn, Tyva (Zhilina, Zavarzina et al. 2009); from soda deposits in Lake Magadi, Kenya (Zhilina, Detkova et al. 1998). Most of them if not all are obligate alkaliphiles growing at pH as high as 10.5. *Anaerobacillus* has been observed in various habitats, from soda lake Khadyn, Tuva upper Yenisey region, Russia (Zavarzina, Tourova et al. 2009); the arsenic contaminated environment in Bendigo, Victoria, Australia (Wang, Liu et al. 2015). Most of them are halotolerant or moderately halophilic, obligate or moderately alkaliphilic.

Anaerobacillus has been observed in various habitats, from soda lake Khadyn, Tuva upper Yenisey region, Russia (Zavarzina, Tourova et al. 2009); the arsenic contaminated environment in Bendigo, Victoria, Australia (Wang, Liu et al. 2015). Most of them are halotolerant or moderately halophilic, obligate or moderately alkaliphilic.

Paenibacillus has been isolated from various environments, from the alkaline soil, Korea (Yoon, Kang et al. 2005); desert sand sample, Gansu Province, China (Jeon, Lim et al. 2009); soil sample, Assam, India (Rai, Roy et al. 2010). Some of them are extremely haloalkaliphilic able to grow at pH as high as pH 12 (Lee, Lee et al. 2002) and at a salinity of up to 5M NaCl (Raddadi, Cherif et al. 2013).

4.6.4.2.3 *Dynamic Group Type-BIII:*

The third group consists of *Clostridium sensu stricto 7*, *Salimesophilobacter*. They increase in their relative abundance in both the pH system with highest obtained relative abundance at a very early stage of the incubation and not at later stages and at t5 they are farther away from regaining their original relative abundance (Figure 36). For example *Clostridium sensu stricto 7* reaches a relative abundance of 23.494 % at t1 in suboptimal pH and 0.009 % at t2 in the superoptimal pH system from near zero relative abundance at t0. At t5, they change to 0.688 % in suboptimal and near zero in the superoptimal pH system.

Dynamic Group Type-BIII

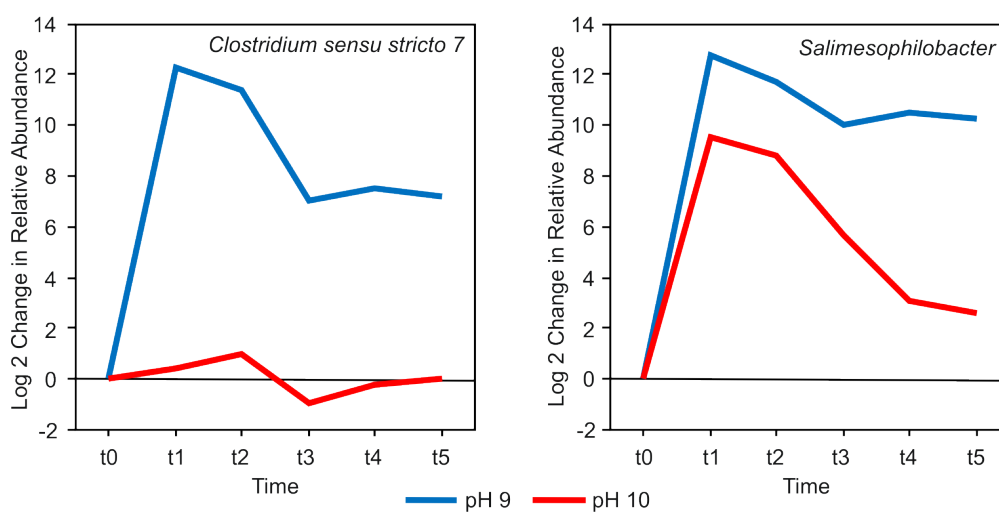


FIGURE 36: DYNAMIC GROUP TYPE-BIII. The figures show changes in relative abundance in Suboptimal and Superoptimal pH in relation to time.

Clostridium sensu stricto 7 The species of *Clostridium* comprise a very heterogeneous assemblage of *Bacteria* that do not form a phylogenetically coherent group (Gupta and Gao 2009). They are same as the Group-C of Rainey et al. and mostly thermophiles (Rainey, Ward et al. 1993). In our study, we found *Clostridium botulinum* BKT015925, *Clostridium sp. 17cr1*, and some uncultured bacterium. Their known role in ecology is not as well studied or understood as its pathology.

Salimesophilobacter has been isolated from the wastewater of a paper mill in Zhejiang, China (Zhang, Fang et al. 2013). So far only one species has been described, *Salimesophilobacter vulgaris*, they can grow at up to pH 9.5.

4.6.4.2.4 Dynamic Group Type-BIV:

Finally, there is *Anaerovirgula*, where there is an opposite trend at first (t1) in suboptimal and superoptimal pH. After that, it behaves similarly to Type-BI. *Anaerovirgula* reaches a relative abundance of 0.023 % at t4 in suboptimal pH and 0.022 % at t2 in the

superoptimal pH system from near zero, 0.003 % relative abundance at t0 (Figure 37). At t5, it changes to 0.011 % in suboptimal and near zero in the superoptimal pH system.

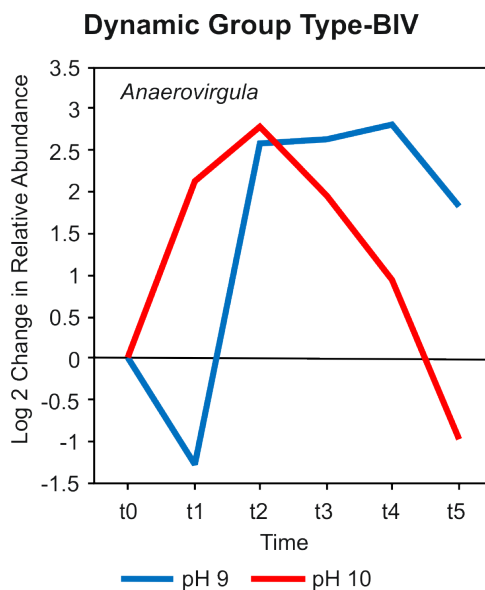


FIGURE 37: DYNAMIC GROUP TYPE-BIV. The figures show changes in relative abundance in Suboptimal and Superoptimal pH in relation to time.

Anaerovirgula has been reported from mud sediments of a soda lake, California, USA (Pikuta, Itoh et al. 2006). Only one species is described so far, *Anaerovirgula multivorans*, able to grow at pH 10.

There are many reports and detailed investigations on how the external microenvironmental pH can change the community dynamics of microbial community as mentioned in the introduction section of this article. Even more, information is available on how single species of numerous genera are affected by its microenvironmental pH (Raevuori and Genigeorgis 1975, Russell and Dombrowski 1980, Chow and Russell 1990, McKay and Peters 1995, Adamberg, Kask et al. 2003, LeBlanc, Garro et al. 2004, Oladipo, Adeleke et al. 2010, Mtimet, Guegan et al. 2016). Also, there is a tremendous amount of literature investigating the response of individual species on their mechanism of sensing and intracellular homeostasis (Krulwich, Guffanti et al. 1982, Nakamura, Kawasaki et al. 1992, Follmann, Becker et al. 2009, Quinn, Resch et al. 2012, Soemphol, Tatsuno et al. 2015). However, there is no significant accumulation of scientific literature on how the microbial community can change their external pH. However very few, although not regarding community but as a single species, literature is available on how

their growth effect the pH of their culture media or immediate external microenvironment. It is not bilateral, or acidification and alkalization simultaneously do not happen for one individual species. It is reported that some bacterial species can acidify their microenvironment by producing organic acids(Solé, Lorén et al. 2010). Alkalization of microenvironment is also well known(Anderson 1984, Athmann, Zeng et al. 2000, Shenderov 2013, Cepl, Blahuskova et al. 2014, Yang, Meng et al. 2015). However, irrespective of their initial external pH, a stabilization of pH, which is also as high as pH 9.3, is not reported before.

4.6.4.3 MICROENVIRONMENTAL PH HOMEOSTASIS AS A FUNCTION OF GROWTH

We observed that there was a substantial variation in initial pH, pH of the culture filtrate, the number of OTUs and incubation duration. All these variables were analyzed with R version 3.2.3. In order to find out which variable predicts best the microenvironmental pH we performed a linear model fit and plotted as a partial residual plot (Figure 38) with the R package car version 2.1.0. A partial residual plot is a graphical method that shows the relationship between a given independent variable and the response variable assumed that other independent variables are also in the model. From the Figure 38, we can see that incubation time (Time) have the greatest impact on microenvironmental pH change (Ini.pH) followed by the number of OTUs and lastly exposure to suboptimal or superoptimal pH with no significant impact. It can be explained as the duration of incubation, and associated community dynamics is the most probable cause of microenvironmental pH homeostasis.

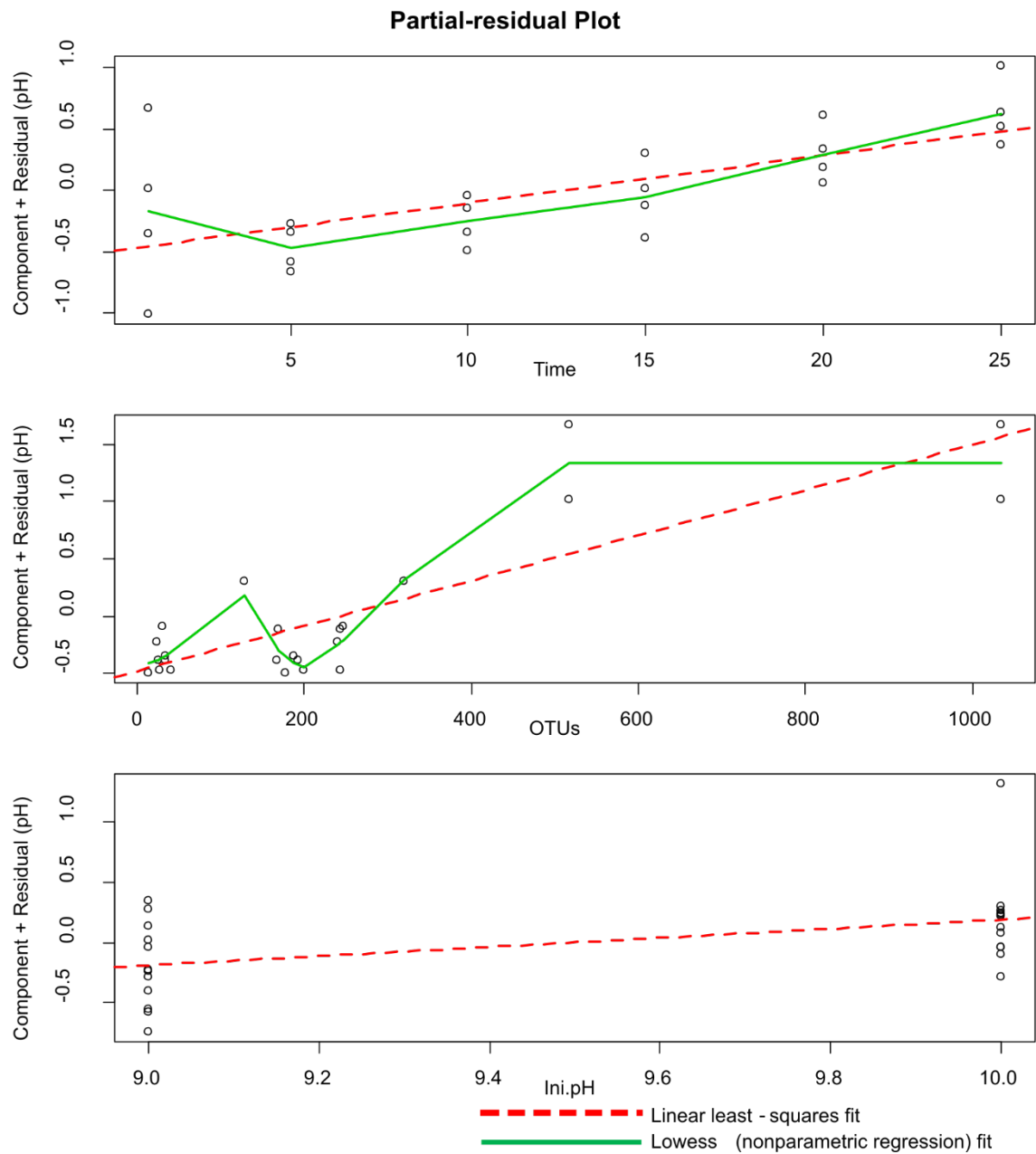


FIGURE 38: PARTIAL-RESIDUAL PLOT SHOWING MICROENVIRONMENTAL PH WITH TIME, OTUs AND NONOPTIMAL INITIAL PH.

Conclusion
&
Future Direction

5 CONCLUSION AND FUTURE DIRECTION

In this thesis, we have found that irrespective of the cataclysmic history of the origin and apparent hostile environmental conditions the prokaryotic community of this meteorite crater soda lake, Lonar, is significantly diverse. We observed Max. clusters (n_{max}), Shannon index (H') and Chao1 as high as 2482.00, 8.06 and 3755.51 respectively. The hypersaline and hyperalkaline ecosystem flourishes with archaeal and bacterial life. We successfully surveyed the prokaryotic biodiversity at taxa level as low as species (genetic distance of 3%) and discussed at taxa level as low as genus. We observed coverage (%) as high as 93.96 % and 89.01 % in *Bacteria* and *Archaea* respectively. This investigation contributed 52 archaeal genera and 530 bacterial genera; out of which 36 (69.23 %) archaeal genera and 407 (76.79 %) bacterial genera has never been reported from Lonar lake before. The difference between the total (DNA-based) diversity and the active (RNA-based) diversity is also noticed. In this investigation, we have observed 24, 16 and 36 genera which may have been involved in methane cycle, nitrogen cycle and sulfur cycle respectively. This methane, nitrogen, and sulfur metabolism together contribute to 2.69 % of all the detected KEGG ortholog in the metagenome. The microbial community of Lonar Crater Lake sediment, when exposed to the nonoptimal pH condition, can change their microenvironment to a more favorable (hypothetical optimal) one in terms of pH, simultaneously resist permanent change in its community structure. 6 different and distinct changing relative abundance patterns of groups of different genera (Dynamic Groups) was also observed. More detailed investigation is needed to confirm their limit and mechanism to modify their microenvironment.

Most of the soda lakes, as described in Table 2, create a kind of clustering at the points where two or more continental plates meet. This distribution pattern has never been reported or observed before. Almost all of them are on or proximity to Rift, Step, Tectonic contact, and Thrust-fault.

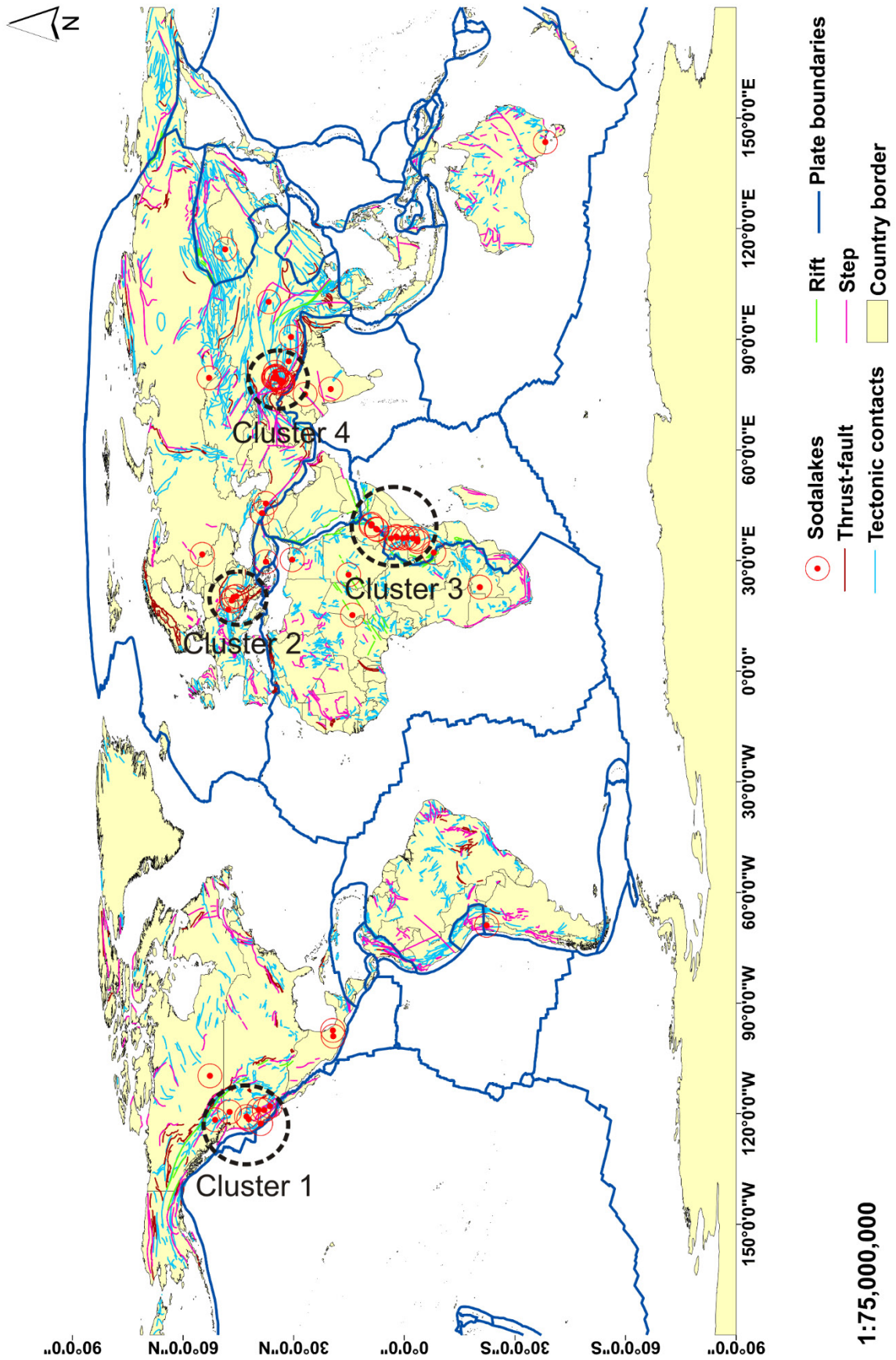


FIGURE 39: FIGURE SHOWS THE GLOBAL DISTRIBUTION OF SODA LAKES AND THEIR LOCATION.

The map was prepared by overlaying the country boundaries and plate boundaries on a GIS platform. The data used is available from <https://github.com/fraxen/tectonicplates>. It was originally published in the paper titled, An updated digital model of plate boundaries in 2003 (Bird 2003). The map is prepared using UTM projection, and the scale of the map is 1: 75,000,000.

From the map, it is comprehensible that there are 4 major clusters. One cluster was observed at the meeting point of Juan de Fuca plate, Pacific plate and North American plate. The second cluster is located near the contact of African plate and Eurasian plate. The third cluster is at the junction of Arabian plate and African plate. The fourth cluster is near the meeting point of Indian plate and Eurasian plate. It seems there is a possibility of some correlation of plate tectonic activity and soda lakes. One reasonable question to ask can be if the biodiversity of the soda lakes is also related to its origin in relation to plate tectonic activity.

We at this moment propose a hypothesis that the origin or geological history of the soda lake plays a significant role in shaping the prokaryotic biodiversity of the soda lakes. Thus, these soda lakes can be categorized as follows-

1. **Geothermally Active Plate Tectonic Dependent Soda Lake (GAPDSL)** - Lakes at the contact points of continental plates with current geothermal activity.
2. **Geothermally Inactive Plate Tectonic Dependent Soda Lake (GIPDSL)** - Lakes at the contact points of continental plates without current geothermal activity/with past geothermal activity.
3. **Plate Tectonic Independent Soda Lake (PISL)** - Lakes away from the contact points of continental plates.

For a hypothesis to be valid, it has to be falsifiable. We did a trial analysis from available information to test the hypothesis. We made a comparison, of 10 soda lakes distributed through 4 continents from 40 Short Read Archive (Table 32) and present investigation on Lonar using QIIME in a similar way as described in Chapter 3.14.

TABLE 32: PROPOSED TYPES OF SODA LAKES WITH EXAMPLES.

Type of Soda Lake	Geothermally Inactive Plate Tectonic Dependent Soda Lake (GIPDSL)		Geothermally the active Plate Tectonic Dependent Soda Lake (GAPDSL)	Plate Tectonic Independent Soda Lake (PISL)
Name and Location	Abhijata, Arenguadi, Beseka, Chitu, Shalla- Africa	Silber, Unterer, Zicklacke - Europe	Heart Lake Geyser Basin - North America	Lonar- Asia
Data used (Short Read Archaive, NCBI)	SRR618362	SRR1519340	SRR900430	Present investigation
	SRR618380	SRR1519333	SRR900451	
	SRR618367	SRR1519332	SRR900428	
	SRR618375	SRR1519335	SRR900433	
	SRR618366	SRR1519337	SRR900431	
	SRR618354	SRR1519338	SRR900426	
	SRR618352	SRR1519334	SRR900437	
	SRR618356	SRR1519336	SRR900457	
	SRR618374	SRR1519339	SRR900439	
	SRR618360			
	SRR618358			
	SRR618370			
	SRR618372			
	SRR618376			
	SRR618373			
	SRR618378			
	SRR618377			
	SRR618353			
	SRR618364			
	SRR618382			
	SRR618368			
	SRR618379			

QIIME analysis provided a relative abundance of different OTUs with assigned taxonomy. We selected only the genera from the three groups of Soda lakes (GAPDSL, GIPDSL, and PISL) and analyzed in R, as described in Chapter 4.2.3, to find out their mutual coverage in terms of presence or absence of the different genera. The analysis shows that irrespective of the fact that all of these lakes are Soda lakes they share a very small percentage of genera, 0.3 %, which is mutually universal. On the other hand, these groups mutually show a higher number of genera to be unique to each group, and the percentage is much greater if considered individually. GAPDSL, GIPDSL, and PISL show 2.1 %, 47.6 %, and 21.6 % genera respectively exclusive when considered mutually. Individually the percentage of exclusive genera for GAPDSL, GIPDSL and PISL shows 60.5 %, 62.4 %, and 43.7 % Figure 40.

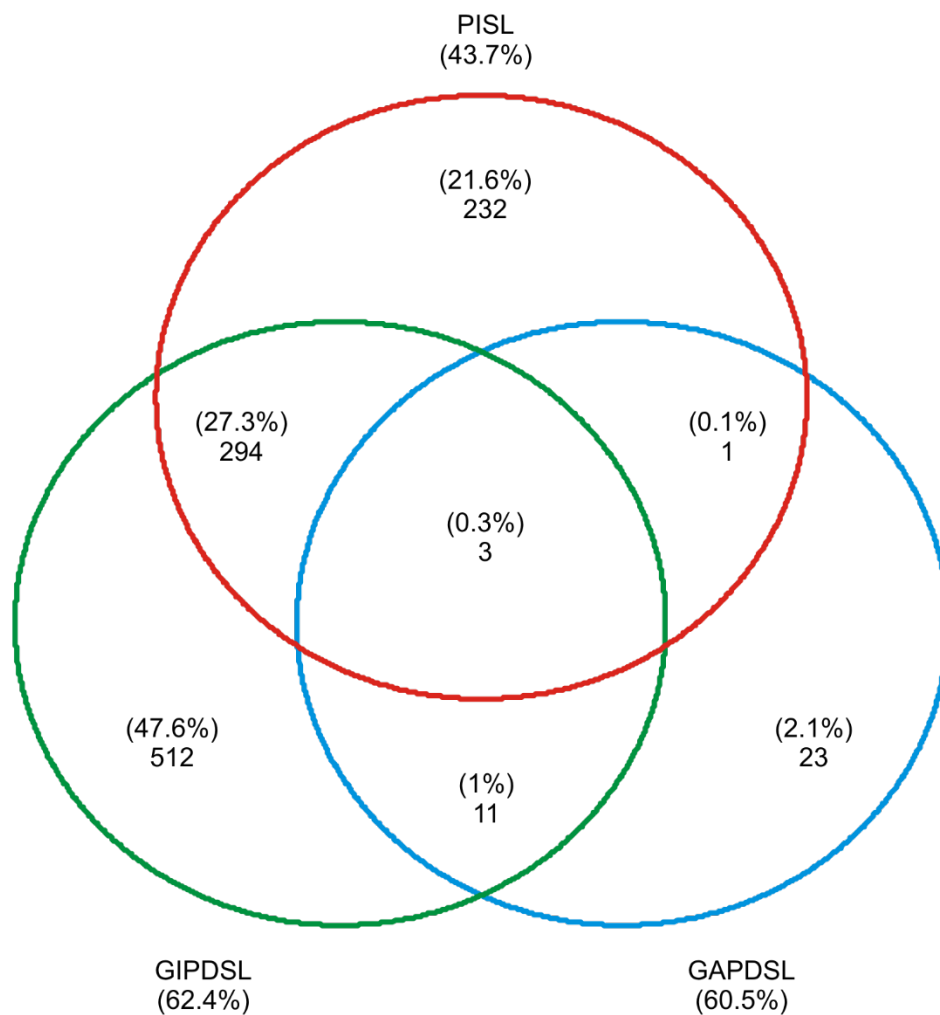


FIGURE 40: MUTUAL AND EXCLUSIVE COVERAGE OF GENERA BY GROUPS OF SODA LAKES.

From the above observations, it seems possible that some taxa are exclusive to each soda lake group. If so then the plate tectonics do predict its prokaryotic diversity (to some extent), and the hypothesis on the types of soda lakes is true. Otherwise, there is no effect of the fact that in general most of the soda lakes happen to be near geologically the active zones, and the hypothesis is false. However, to reach any conclusion about it, there has to be more investigation in a uniform approach; as the nature of samples, 16S rRNA gene primers, sequencing platform or chemistry and coverage (surveying effort) were not identical.

To progress our understanding of the alkaliphiles, of Lonar Meteorite Crater Soda Lake specifically and soda lakes in general, we can investigate two broad question.

1. To investigate the mechanisms by which the microbial community of Lonar Crater Lake sediment can modulate their microenvironment to favorable (hypothetical optimal) one in terms of pH, concurrently repelling permanent alteration in its community structure.
2. To investigate if prokaryotic biodiversity of the soda lakes and the proximity of the most of the soda lakes to the geologically the active have any correlation and if the grouping of soda lakes based on its proximity to plate tectonically the active area is reasonable.

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Supplements

SUPPLEMENTS

SUPPLEMENT-A: AUTHORIZATION FOR SAMPLING



सत्यमेव जयते

National Biodiversity Authority

(An Autonomous and Statutory Body of the Ministry of Environment and Forests, Government of India)

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Secretary

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NBA/ Tech Appl/9/ 520/12 /14-15/2146

08.09.2014

To

Shri. Soumya Biswas,
C/o. Prof. Prabir Kr. Biswas,
Simantapalli (North),
Santiniketan, Birbhum,
West Bengal,
Pin- 731235.

Sir,

Sub: Approval for Access of Bioresource for Research/Bio- survey and Bioutilization (Form-I) application under Section 3 read with Section 19(1) of the Biological Diversity Act, 2002 and Rule 14 of the Biological Diversity Rules, 2004-reg.

Ref- Your application in Form - I dated 01.06.2012.


With reference to your application cited in reference on the subject cited above to facilitate "Collect the soil sediment (Microbial life form) from Lonar lake and carrying to Germany for research purpose" has been approved by the National Biodiversity Authority subject to the condition that the applicant should not transfer any material to other persons without obtaining prior approval of National Biodiversity Authority and no application for patents in any country should be submitted without obtaining prior permission from the National Biodiversity Authority.

I am enclosing herewith one mutually signed stamp paper Agreement executed between National Biodiversity Authority and the applicant for the applicant's reference compliance. It is also to inform you that breach of the terms

of agreement and provisions of the Biological Diversity Act, 2002 and Biological Diversity Rule, 2004 made there under will invite imposition of penalties as per Section 55, 56 & 57 of the Biological Diversity Act, 2002.

Please acknowledge receipt of this communication.

Yours faithfully,


(T. Rabikumar)
Secretary, NBA

Encl: One Mutually Signed agreement (Original)

Copy to: Member Secretary, Shri. Jose. T. Mathew, IFS, West Bengal Biodiversity Board, Poura Bhawan (4th Floor), FD-415A, Sector - III, Bidhan Nagar, Kolkata - 700106.

SUPPLEMENT-B: THE TOTAL AND THE ACTIVE ARCHAEOAL DIVERSITY TABLE

TABLE 33: RELATIVE ABUNDANCE OF THE TOTAL AND THE ACTIVE ARCHAEOAL ORDER. Lonar 1, Lonar 2 and Lonar 3 represent 3 different samples, and the values are average of 3 separate experiments.

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TABLE 34: RELATIVE ABUNDANCE OF THE TOTAL AND THE ACTIVE ARCHAEOAL GENERA. Lonar 1, Lonar 2 and Lonar 3 represent 3 different samples, and the values are average of 3 separate experiments.

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SUPPLEMENT-C: THE TOTAL AND THE ACTIVE BACTERIAL DIVERSITY TABLE

TABLE 35: RELATIVE ABUNDANCE OF THE TOTAL AND THE ACTIVE BACTERIAL PHYLUM. Lonar 1, Lonar 2 and Lonar 3 represent 3 different samples, and the values are average of 3 separate experiments.

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TABLE 36: RELATIVE ABUNDANCE OF THE TOTAL AND THE ACTIVE BACTERIAL GENERA. Lonar 1, Lonar 2 and Lonar 3 represent 3 different samples, and the values are average of 3 separate experiments.

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SUPPLEMENT-D: ADDITIONAL TOTAL DIVERSITY OF *ARCHAEA* AND BACTERIA

TABLE 37: RELATIVE ABUNDANCE OF *ARCHAEA* AND *BACTERIA* FROM ADDITIONAL ASSESSMENT. Lonar 1, Lonar 2 and Lonar 3 represent 3 different samples, and the values are average of 3 separate experiments.

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SUPPLEMENT-E: CHANGING RELATIVE ABUNDANCE OF *ARCHAEA* AND *BACTERIA* IN SUBOPTIMAL AND SUPEROPTIMAL PH SYSTEMS

TABLE 38: RELATIVE ABUNDANCE OF PROKARYOTES. t0 to t5 represents time points from day zero to day 25. All the values are average of 3 separate experiment and only up to 5 decimal points are shown.

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SUPPLEMENT-F: DIVERSITY OF FUNCTIONAL GENES OBSERVED BY DIRECT METAGENOME ANALYSIS

TABLE 39: RELATIVE ABUNDANCE OF ORF IN FUNCTIONAL CATEGORIES

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TABLE 40: RESULTS OF RECONSTRUCTED BRITE ANALYSIS.

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TABLE 41: RECONSTRUCTED PATHWAY MODULE

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TABLE 42: RESULTS OF RECONSTRUCTED PATHWAY ANALYSIS.

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SUPPLEMENT-G: DETAILS OF THE SEQUENCE SUBMITTED TO NCBI

All the sequences generated during this thesis will be uploaded to NCBI Sequence Read Archive under accession number SRA437941.

SUPPLEMENT-H: RESULTS FROM CONSTRUCTION AND SCREENING OF METAGENOMIC LIBRARIES

**TABLE 43: CHARACTERIZATION OF CONSTRUCTED METAGENOMIC LIBRARIES (SMALL INSERT LIBRARY)
AND SCREENING FOR FUNCTIONAL GENES**

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