CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Metagenomic Analysis of Ammonia Oxidizing Archaea Affiliated with the Oil Field

by

Shagufta Batool

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Dedicated to Allah Almighty, Hazrat Muhammad (SAW) and my Parents whose utter support and sheer trust helps me to achieve this.

CERTIFICATE OF APPROVAL

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Abstract

World's largest source for energy is crude oil from earth reservoirs and is used as means of economy. Rise in the price of barrel oil leads towards the decline in crude oil reserves, to meet future energy need development of oil resources is crucial. Modern oil recovery technologies that are commonly used are less effective for oil companies due to which there is continuous requirement for development of new technology, that can improve the production and recovery of oil from oil reservoirs. Approximately more than 2 trillion barrels of oil remain in oil reservoirs due to use of conventional oil recovery techniques worldwide. Microbial Enhanced Oil Recovery (MEOR) is found to be significant tertiary oil recovery approach that is cost effective as well as sustainable approach to extract residual oil from oil reservoirs. Microorganisms has been explored and proved successful to dissolve crude oil and minimize viscosity.

In this study physicochemical characteristics of micro and macro nutrients were measured by using AAS method. Six water samples from Nandpur, Panjpir and Bahu oil and gas field and four soil samples from Nourag and Rajian ware taken. Metagenomics analysis was done at sequencing depth of 85000 to 89000. Results shows highest PH (8.5) in RJ1and lowest PH (6.3) in BH1. Alpha and beta-diversity analysis was done that showed highest concentration of nitrates and sulphur found in PN1 and NP2. Archaeal rich diversity of phylum Euryarchaeota and Thaumarchaeota and bacterial phylum Acidobacteria, Actinobacteria, Bacteriodete, Firmicutes, Planctomycetes, Protobacteria and Chloroflexi were found dominating in reported samples.

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Abbreviations

AOA	Ammonia Oxidizing Archaea
AOB	Ammonia Oxidizing Bacteria
BDL	Below Detection Limit
BH	Bahu
CN	Chak Nourang
ER	Enhanced Oil Recovery
MEOR	Microbial Enhanced Oil Recovery
NP	Nandpur
PN	Panjpir
Rj	Rajion
SBR	Sulphate Reducing Bacteria

Chapter 1

Introduction

Oil creation, by which is meant drilling for oil and recovering the item as financially as could be allowed, was good in early days, an unrefined and inefficient procedure. Economic development of the world drives by oil which is a vital source of energy. Oil recovery is composed of primary and secondary phases. In primary recovery method, oil and gas is produced through the usual force energy of the reservoir as well as oil wells are stimulated to inject the fluids in secondary phase to recover the production of petroleum from reservoirs [1]. 5-10% of oil is recovered in primary oil recovery method, while secondary phase of oil recovery which includes other recovery efficiencies recoveries other 10-40% of oil [2]. In this way, raw petroleum (crude oil) which is remained in the reservoirs after ordinary oil recoveries methods formulate 2/3 of the total oil reserves [3-4].

Methods that are currently used in oil industries are Enhanced Oil Recovery (EOR) also called as tertiary recovery. EORs include Chemical flooding, miscible carbon dioxide injection and thermal enhanced oil recovery methods that uses heat as main source for additional oil recovery [55]. Plasma-Pulse technology was introduced in 2013 which improved the 50% of production progress of oil from reservoirs [9]. Factors that are main cause of poor oil recovery from reservoirs include little permeability, high thickness of oil and the high interfacial tension between hydrocarbons and aqueous phase [2][4].

Pakistan has oil and gas resources that are distributed in whole country and include many prospective reservoirs. Approx., total oil reserves in Pakistan are twenty-seven billion barrels and other recoverable reserves are approx. 936 million barrels. The capacity of crude oil refining is 13 million tones. 18.6 million Tones oil is imported in the country [5]. Presently, Pakistan is producing oil with primary and secondary recovery, 69,286 bbl per day approximately. According to EIA, USA 2012 statistics the verified oil reserves of Pakistan are 0.31 billion barrels [6]. Pakistan is blessed with massive resources and it also contains a vast amount of oil and gas wells. In Baluchistan, the Sui place and some areas of Sindh contain a greater amount of these resources. But unluckily, these recourses are not appropriately channelized by the government of Pakistan rather overseas companies are exploring and playing a great part in the production. The demand for oil and gas is increasing day by day and the local production is too low regardless of the fact that Pakistan has its own oil and gas resources and also has the potential to produce it by self [7].

Table 1.1: Pakistan's Oil Sector Overview

Total Proved Reserves	**Total Oil Production**	**Total Crude Oil production**	**Total Oil Consumption**
0.31 billion barrels	62.09 thousand barrels/day	59.08 thousand barrels/day	426.72 thousand barrels/day
Imports	**Refinery Capacity**	**Exploratory**	**Wells Up to 2012 Discovered**
634.43 thousand barrels/day	286 thousand barrels/day	785	69 (OIL)

Usage of the EOR process is inevitable in Pakistan. Implementation of the EOR processes in Pakistan requires extensive research, development, and expertise. The decision to initiate the EOR projects depends more on an evaluation of economics. Extensive laboratory and numerical simulation work are however necessary to

check the feasibility of the EOR Project. The first step to evaluating the feasibility is analytical technical screening of the EOR process for a particular reservoir. Current main and subordinate oil recovery methods leave about 2/3 of the original oil in the reservoir so residual oil in huge amount is recovered by these EOR methods.

New technologies are needed for recovering trapped oil and slowing down production declines in the limited wells to upsurge oil reserves [8]. Recently, the MEOR technique has become prevalent in recovering trapped oil, therefore a considerable knowledge of the multi-phase flow characteristics of reservoir rock as well as the different strategies are important for the achievement of any MEOR project, including microbial ones [9]. Several classes of microbes were discovered from oil reservoirs with varying physiological and metabolic capacities and phylogenetic relationships. The presence of indigenous microbial communities in oil reservoirs can tolerate an underground deep biosphere, that is sovereign of primary productivity above the surface [10], coupled with the proven ability of anaerobic micro-organisms to consume multiple oil components [11], It is currently a well-established scientific datum that retains and harbors numerous bacterial and archaeal species in oil reservoirs [12]. This research focuses on the occurrence of one of the various groups of microbes in oil fields that are sulfate-reducing SRB bacteria, their role in MEOR, and the potential to mediate various metabolic processes that occur in oil fields.

Ammonia-Oxidizing bacteria (AOBs) were observed as most prevalent microorganism for the ammonia oxidation process [Zhixuan Yin et al 2018]. It was found that amoA gene that act as ammonia oxidation gene present in enormous number in archaea that are widely dispersed in the marine ecosystem which suggest that archaea has potential of oxidizing ammonia at metabolic level [Zhixuan Yin et al 2018]. Due to ammonia oxidizing potential of archaea they are named as ammonia-oxidizing archaea (AOA). Ammonia oxidizing archaea are found to be habitants of hot springes, soils, lakes, sediments, wetlands and oceans and have amoA gene due to which they play a significant role in global nitrogen cycle. Improvements in petroleum and mechanical technology significantly increased the efficiency and

increase in oil yield from oil reservoirs. The microbiologist has researched for bacterial corrosion, plugging of oil reservoirs, fermentation of drilling fluids so that oil recovery can be increased by use of bacterial action in petroleum reservoirs.

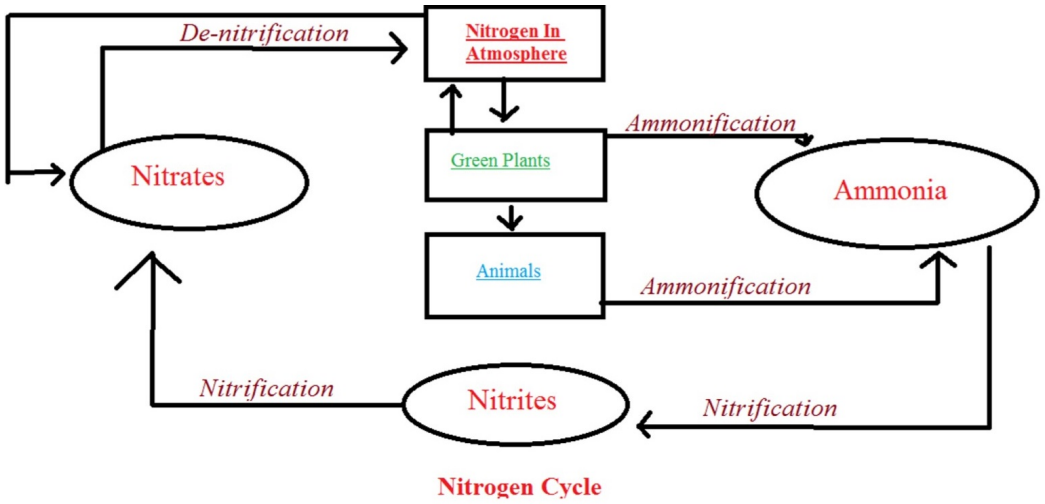

FIGURE 1.1: Nitrogen cycle

1.0.1 Nitrifying Bacteria

Chemolithotrophs also include nitrifying bacteria of genera Nitrosomonas, Nitrosococcus, Nitrobacter and Nitrococcus. Species of these genera get their energy by oxidizing inorganic nitrogen compounds [5]. Ammonia-oxidizing bacteria (AOB) and Nitrite oxidizing bacteria (NOB) are microbes that play a significant role for conversion of soil ammonia into nitrates in nitrogen cycle. Complex internal membrane system that are sites for key enzymes of nitrification are found in many species of nitrifying bacteria. These enzymes include ammonia monooxygenase, hydroxylamine oxidoreductase monooxygenase, and nitrite oxidoreductase [6].

Ammonia oxidizing archaea (AOA) and Ammonia-oxidizing bacteria (AOB) are the bacteria that are key contributors to oxidation of ammonia in marine sediments and also contribute relatively to the one of the process that is important in nitrogen cycle issues. The two groups of bacteria that are involved in the cycle of nitrification involves: bacteria that convert ammonia to nitrites (Nitrosomonas, Nitrosospira, Nitrosococcus, and Nitrosolobus) and bacteria that convert nitrites

into nitrates (Nitrobacter, Nitrospina, and Nitrococcus). In agriculture, introducing dilute ammonia solutions via irrigation leads to an increase in soil nitrates by nitrifying bacteria.

1.1 Cell Structure and Metabolism Physiology of Ammonia Oxidizing Archaea

Ammonia oxidizing archaea's cell volumes are smaller than (10-100 times) than ammonia-oxidizing bacteria's. Nitrosopumilus maritimus SCM1 is a ammonia oxidizing archaea whose ammonia oxidation level is 10-folds lower than ammonia-oxidizing bacteria's [30]. Contribution to ammonia oxidation from ammonia oxidizing archaea and ammonia-oxidizing bacteria could be considered by relative abundance of cells as well as cell activity. ammonia oxidizing archaea are less ion permeable than ammonia-oxidizing bacteria that enables them to tolerate intense environment [40]. Cryoelectron tomography data showed the exponential growth of Nitrosopumilus maritimus SCM1 due to increase in ribosomes number in bacterial cells. This increase in ribosomes enables them to react rapidly in changing environmental conditions. Majority of archaea are highly adapted to energy-stressed conditions [40]. Data on stability of ammonia oxidizing archaea's mRNA ammonia monoxygenase (AMO), and ribosomal protein is not available but it could be important for understanding ammonia oxidizing archaea's ecological adaptations to ammonia-oxidizing bacteria. AMO require NH3 as substrate however substrate for archaeal AMO is still not known [43]. AMO catalyzes NH3 to hydroxylamine, in this way NH3 is oxidized to NO2- via periplasmic hydroxylamine oxidoreductase (HAO) [44,45]. It is not clear weather AMO catalyzes the same reaction as ammonia-oxidizing bacteria. It is reported that AMO could produce nitoxyl hydride (HNO) that is further oxidized to NO2- via nitroxyl oxidoreductase (NxOR) [45]. O_2 activation for monooxygenase reaction could be possible by nitric oxide, nitrite reductase reaction that is responsible for generation of nitrogen gas [2].

Copper based biochemistry may explain the biological performance of aquatic ammonia oxidizing archaea because Archaeal nirK gene consist of copper-dependent NIR, enzymes and proteins for electron transport [46, 45, 42]. Petroleum based products are main energy sources for manufacturing product that are used in everyday life. When crude oil and petroleum products are being explored, leaks and spills occurs while production processing transportation and storage. The daily consumption of oil is 600000 metric ton per year worldwide with uncertainty of 200000 metric ton per year. Release of hydrocarbon in environment via any process (accidently or human activity), is one of major cause for water, soil and air pollution. Pollution due to hydrocarbons cause damage to local system, animals and plant tissues that leads to lethal mutation and cause death. Soil remediation techniques include mechanical, burial, evaporation, dispersion and washing. These methods are very costly and are not much effective. Bioremediation is the use of microbes to remove or convert pollutants through the metabolic abilities. Bioremediation is now emerging technology that is currently used for removing or detoxifying pollutants from petroleum products [12] due to its economical and non-invasive characteristic [15].

1.1.1 Archaea

Five major ammonia oxidizing archaea clusters were proposed:

1. *Nitrososphaera,*
2. *Nitrosocosmicus,*
3. *Nitrosocaldus,*
4. *Nitrosotelea Nitrosopumilus*
5. *Nitrosotenuis and Nitrosopelagicus*

Ammonia oxidizing archaea species of neutral pH are from genus Nitrosophaera. In acidic soil Nitrosophaera and Nitrosotelea are common ammonia oxidizing archaea. Hot springs include nitrosocaldus and nitrososphate. Of the ammonia oxidizing archaeas in the Nitrosopumilus family, the Nitrosopumilus family and the Nitrosopelagicus family are found mainly in aquatic ecosystems and Nitrosoarchaeum and Nitrosotenuis family in fresh water.

1.2 Biotransformation

1.2.1 Oil Recovery

An additional trapped oil from oil wells is extracted by the last phase of oil recovery which is tertiary oil recovery technique and it includes microbial enhanced oil recovery. As MEOR process has some advantages such as it forms an emulsion among two liquid states which decreases an interfacial stiffness and blocking the high porous zones.

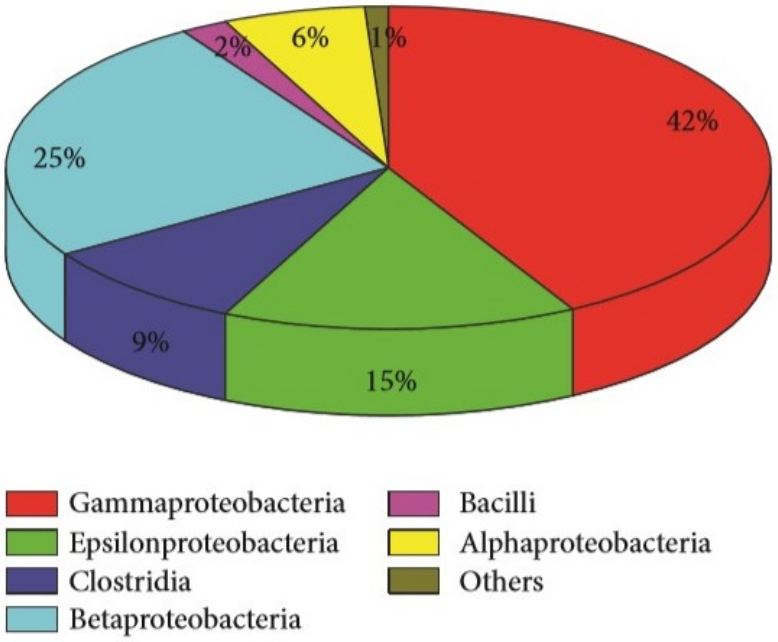

FIGURE 1.2: The Diversity of Bacteria in Oil Reservoir

As bacterias are smaller in size and they have negative charge on their cell surface so in In situ microbial enhanced oil recovery initially bacterias which are injected with water into the wells move into the high porous regions and then they grow and block those high porous zones. Consequently an additional effective retrieval of oil can be attained by this development as it increases the sweep efficiency [44,45].

1.2.2 Strategies

Chemical enhanced oil recovery is an expensive technology therefore MEOR can substitute CEOR. Microbes are able to produce beneficial products by fermentation of inexpensive substrates or raw materials.

TABLE 1.2: Applications of products and MEOR agents produced by microbes [20].

MEOR agents	Microbes	Product	Possible MEOR application
Biomass, i.e. flocks or biofilms	*Bacillus sp.*	Cells and EPS (mainly exopoly saccharides)	Selective plugging of oil depleted zones and wettability
	Leuconostoc		
	Xanthomonas		
Gases	*Clostridium*	Methane and hydrogen	Increased pressure, oil swelling, reduction of interfacial section
	Enterobacter		
	Methanobacterium		
Surfactants	*Acinetobacter*	Emulsan and alasan	Emulsification and de-emulsification through reduction of interfacial tension
	Bacillus sp.	Surfactin, rhamnolipid, lichenysin	
	Pseudomonas	Rhamnolipid, glycolipids	
	Rhodococcus sp.	Viscosin and trehaloselipids	
	Arthrobacter		

Table 1.2 continued from previous page

Biopolymers	*Xanthomonas sp.*	Xanthan gum	Injectivity profile and viscosity modification, selective plugging
	Aureobasidium sp.	Pullulan	
	Bacillus sp.	Levan	
	Alcaligenes sp.	Curdlan	
	Leuconostoc sp.	Dextran	
	Sclerotium sp.	Scleroglucan	
	Brevibacterium		
Solvents	*Clostridium, Zymomonas and Klebsiella*	Acetone, butanol, propan-2-diol	Rock dissolution for increasing permeability, oil viscosity reduction
Acids	*Clostridium*	Propionic and butyric acids	Permeability increase, emulsification
	Enterobacter		
	Mixed acidogens		

Chemicals used in chemical enhanced oil recovery processes are very expensive so in microbial enhanced oil recovery selected microbial strains produces the substances which are similar to those chemicals used in CEOR procedures to rise the oil retrieval from washed-out and peripheral reservoirs. In addition to it microbial products are decomposable and are little toxicant [6]. Microbial innovations are getting to be endorsed all around as valuable and eco accommodating ways to deal with improve oil generation [46,47].

1.2.3 Biopolymers and Biomass

The development of biopolymers and creation of biofilms has been tested and applied to oil recovery technology and showed significant increase in yield of oil in selective plugging. These biopolymers and biofilms are greatly influenced by water chemistry, pH, surface load, microbial physiology, nutrients and fluid flow [22,23]

1.2.4 Biosurfactants

Bio-surfactants are diverse collection of surface-active molecules having both hydrophilic and hydrophobic domains and produced by microorganisms. These waters loving and water hating domains of molecules allow them to screen at the interface between fluid phases with altered amount of polarity such as oil water or air water interfaces. As biosurfactants have all these features that is why they can decrease surface and interfacial tension and form stable emulsions where compounds of hydrogen and carbons can be solubilized in water or water in hydrocarbons [8,9]. Bio-surfactants produced by microbes have the ability to create the low interfacial tension between the compounds which are chief components of petroleum and natural gas and the aqueous phases required to assemble entrapped oil [9]. Biosurfactants play vital role in application of MEOR methods and can be good substitutes of man-made surfactants because of their specific activity low lethality Introduction 3 and high biodegradability and effectiveness at extreme conditions of temperature pressure pH and salinity [10-13].

1.2.5 Uptake of Hydrocarbons by Biosurfactants

Pseudomonads strains has been reported to produce biosurfactant which has the ability to destroy the advanced portions of thick oil then support for improvement of its drift features for a fuel reservoir. P. aeruginosa produce glycolipid type biosurfactant that Degrade lighter chains of carbon (C12-C32) and heavier chain of carbons (C36-C40). These microorganisms have enzymes for degradation of petroleum hydrocarbons. Alkanes such as normal branched cyclic paraffins both paraffinic and aromatics are degraded by some microorganisms [79-81]. Table 1.2 consist the list of microbes and biosurfactants produced by them. Hydrocarbon degrading microbial population is 10% of the entire bacterial population. Metabolic pathways of hydrocarbon bio decomposition have been clarified [83]. Microbes oxidize the oil by an enzyme oxigenases to degrade the hydrocarbons as it is a major step of biodegradation

TABLE 1.3: Biosurfactants produced by microorganisms

Biosurfactants	Microorganisms
Sophorolipids	*Candida bombicola* (Daverey and Pakshirajan, [32]
Rhamnolipids	*Pseudomonas aeruginosa* (Kumar et al. [33]
Lipomannan	*Candida tropicalis* (Muthuswamy et al. [34]
Rhamnolipids	*Pseudomonas fluorescens* (Mahmound et al. [35]
Surfactin	*Bacillus subtilis* (Youssef et al. [36]
Glycolipid	*Aeromonas sp.* (Ilori et al. [37]
Glycolipid	*Bacillus sp.* (Tabatabaee et al. [38]

These are surface-active, microbial-synthesized substances that qualify for more effective biodegradation of hydrocarbons. There are two aspects involved in bioremediation process via biosurfactants.

1. Raise the surface aria of hydrophobic substrates, restrict the microbial growth on hydrocarbon through water oil interface. Emulsifiers produced by microbes may breakup oil droplets that will significantly increase the surface area.

2. Improving the availability of hydrophobic substrate biologically by removing them from surface biosurfactants can increase the availability of bound substrates. Many biosurfactants have low concentrations of essential micelles (CMCs) which improve the obvious solubility of hydrocarbons by sequestration of hydrophobic molecules into micellular centers. [17].

1.2.6 Gas and Solvent

Gas production has important effect on oil recovery through expanding the pressure difference that drives the flow of oil in traditional old techniques. Anaerobic methane production from oxidation of oil has showed slight impact on MEOR technology because of its solubility. Carbon Dioxide has been found to be better agent

for MEOR because vaporization of light hydrocarbons occurs in gas phase while miscible carbon dioxide will be re3duced to liquid phase. Additional gasses and solvents can also dissolve carbonate rock, resulting in increased rock conductivity and porosity. [24].

1.3 Factors Effecting Bio Degradation

Different data types indicate that the presence of indigenous bacteria in oilfields can be restricted to a range of temperature between 80℃ and 90℃. Philippi (1977) noted that biodegradation of in situ oil has never been observed in reservoirs with a temperature above 82℃. Analysis of a series of 87 water samples from oil reservoirs in North America found that fatty acid concentrations in the reservoir were maximum at a temperature of 80℃ (Fisher 1987; Barth 1991). This suggested that maximum biodegradation occurs below 80℃ and above this temperature occurs thermal decarboxylation. In a microbiological analysis, it was not possible to extract hyperthermophilic bacteria from 100 oil field water samples with reservoir temperatures above 82 C (Bernard et al. 1992; Magot, unpubl.). Some reservoirs have isolated hyperthermophilic micro-organisms growing at extreme temperatures as as 103℃ but the writers also proposed that they are exogenous bacteria arising from treatments of seawater. (Stetter et al. 1993b; Grassia et al. 1996).

1.3.1 Salinity and pH

Often, salinity and pH of forming waters may restrict bacterial activity. The salinity varies from nearly fresh to salt-saturated water, and usually between 5 and 8 pH. However, at atmospheric pressure, however the pH measured may not directly represent the real in situ pH because it is affected by the high-pressure gas dissolution. Typically in situ pH is within the 3–7 scale. When developing cultural media, this physical feature must be taken into account or describe the possible

indigenous origin of bacteria retrieved from deep samples on the subsurface. Pressure inside oil reservoirs (up to 500 atm) is not known to prevent in situ bacteria from developing although it may affect their physiological or metabolic properties.

1.4 Environmental Factors that Affect Ammonia Oxidizing Archaea and Ammonia-Oxidizing Bacteria

1.4.1 Level of Ammonia

The concentration of ammonia in the atmosphere as a natural source (nitrogen source) of ammonia oxidizing archaea and ammonia-oxidizing bacteria strongly affects the development of these two forms of nitrogen-oxidizing microorganisms. ammonia oxidizing archaea has a greater resistance of ammonia than ammonia-oxidizing bacteria [47], leading to less concentration of ammonia oxidizing archaea inhibitors. Allowed access to a higher concentration of ammonia, ammonia oxidizing archaea can be reached early than ammonia-oxidizing bacteria in a suppressed situation. Sauder et al. [48] showed that the volume of ammonia oxidizing archaea amoA gene decreased with the rise in ammonia concentration in a metropolitan waste water treatment plant's revolving biological contactors, suggesting that ammonia oxidizing archaea was sufficient for low ammonia levels. According to Gao et al. [49], in high concentrations of ammonia ammonia-oxidizing bacteria was more productive than ammonia oxidizing archaea, and the greater the concentration of ammonia, the higher the abundance of ammonia-oxidizing bacteria was [54].

1.4.2 Organic Loading

Organic matter has an objective impact on the growth of microorganisms which oxidize ammonia. Ammonia-oxidizing bacteria is known as autotrophic microorganisms whereas ammonia oxidizing archaea is autotroph or mixotrophs, not clear. Inhibitory effect due to presence of organic substances on growth of ammonia oxidizing archaea strains has been observed [51,52]. More diverse metabolic pathway is present in ammonia oxidizing archaea relative to ammonia-oxidizing bacteria, ammonia oxidizing archaea show different metabolic features under various carbon conditions that results in difference between ammonia oxidizing archaea and ammonia-oxidizing bacteria's ammonia oxidation ability

1.4.3 Temperature

The temperature fluctuations effect the ammonia monoxygennsae activity of ammonia -oxidizing microbes [53]. At 0.2℃ significant ammonia-oxidations occurs in North Japan Sea deep water zone and at 74 degree Celsius in Yellowstone National Park hot springs [54]. It was observed that ammonia oxidation microbes behave as ammonia-oxidizing bacteria in summer and ammonia oxidizing archaea in winter in sediments of Shandong Peninsula's Rushan Bay [49]. Niu et al observed that ammonia-oxidizing bacteria in biologically activated carbon filtration system for drinking water purification decrease in winter reative to summer. Sims et al observed that ammonia-oxidizing bacteria is more prone to low temperatures than ammonia oxidizing archaea in waste water treatment wetlands.

1.4.4 Oxygen

Oxygen is required in nitrification reaction as substrate. The nitrification cycle can be influenced by concentration of oxygen they can alter the presence of nitrifying microbes (AOA>AOB>NOB). Ammonia oxidizing archaea are more than

ammonia-oxidizing acteria in hypoxic conditions such as deep oceans, heavy water and sediments due to high oxygen tolerance [55].

1.4.5 PH

Bioavailability of ammonia can be decreased by protonation of ammonia as pH decreases, which from the perspective of substrate use may be more desirable for ammonia oxidizing archaea production. Recent studies have shown that ammonia oxidizing archaea regulated the oxidation of ammonia in acidic soils, while ammonia-oxidizing acteria had trouble surviving at low pH values and was primarily responsible for nitrification in alkaline soils [56–57]. For low ammonium and/or low DO and/or low organic loading conditions ammonia oxidizing archaea will predominate over ammonia-oxidizing acteria. Even, when subjected to extreme high / low temperatures ammonia oxidizing archaea would be more successful than ammonia-oxidizing acteria. Therefore, Ammonia oxidizing archaea will prevail in salinity-containing wastewater as compared to ammonia-oxidizing acteria [58,59]. Molecular biology methods were used to analyze population features of ammonia oxidizers, and theoretical incubation of nitrification was implemented to consider the group behavior of the ammonia oxidizers. The goal was to establish the ammonia oxidizing archaea and ammonia-oxidizing acteria population structure and operation in water and soil samples collected from various regions. Study of pyrosequencing showed ammonia oxidizing archaea's variability was higher than ammonia-oxidizing acteria's. The majority of ammonia oxidizing archaea and ammonia-oxidizing acteria clustered respectively into Nitrosopumilus and Nitrosospira, respectively.

1.5 Problem Statement

Crude oil provided by primary oil recovery techniques account for 20-30% of total usable quantity. Petroleum companies are looking for new technology that can

extract oil from oil recovery reservoirs efficiently and that are also cost effective. by implementing enhanced oil recovery techniques remaining quantity of oil in existing areas can be extracted.

1.6 Aims and Objectives

1. To find physicochemical analysis of oil reservoir samples
2. To find prevalence of Nitrogen oxidization genes in different oil reservoir samples
3. To find correlation of Nitrogen oxidation gene prevalence with ammonia, nitrates and nitrites
4. Statistical analysis

Chapter 2

Literature Review

2.1 Microbial Processes for Recovering and Upgrading Petroleum

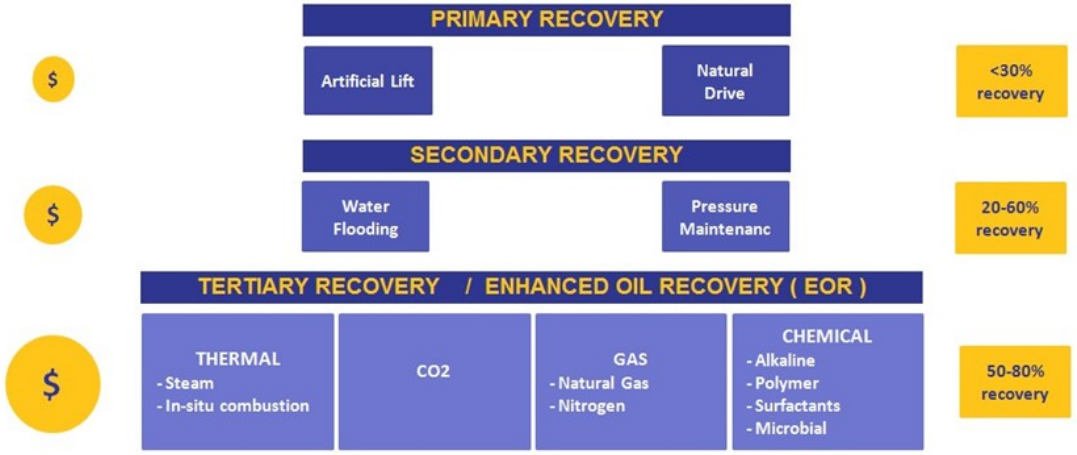

FIGURE 2.1: Microbial Processes for Recovering and Upgrading Petroleum

2.2 History

MEOR study was little known after Beckman's discovery but until Zobell and his researchers made a great contribution on laboratory study in 1947 [48]. After

that other contributions are also done by researchers to gain the study of MEOR [49,50]. European countries performed substantial MEOR investigation in 1960s and 1970s. Due to the petroleum crisis in the 1970s the MEOR research was improved and later become substantiated EOR processes maintained by research projects worldwide [1].

1990's era is well-known as an important year for MEOR expansion [49]. MEOR had developed a scientific and interdisciplinary method for enhanced oil recovery by the end of the 1990s. J.W.Beckman et al. studied and discovered that microbes can discharge trapped oil in permeable rock formations, bacterial formation of hydrogen sulfide in the waters of the Aspheron oil fields and also reported that Sulphate Reducing Bacteria (SRB) reside in samples from 67 wells located in California and Illinois. Others innovation established into novel information of improvement of oil creation consuming a bacterial network or MEOR. Meanwhile Bastin findings donated the information of biodeterioration of constituents or disintegration because of the occurrence and metabolic activities of microbes which are usually stated as Microbiologically Influenced Corrosion (MIC) [51-56].

Oil production is declining due to maturity of oil fields throughout the world. For example, all major oil fields in North Sea [51]. Increase in energy demand due to global population growth and unavailability of new resources are major factors for this downgrade. Therefore, it is necessary to search out new alternative technologies to increase oil recovery from existing reservoirs because fossil fuel still remains the key source of energy besides other resources such as solar energy, wind energy etc. Oil is required to produce fossil fuel energy, 30 to 40% oil is contributed by primary oil recovery while 15-20% is recovered by secondary method leaving behind 35-55% of oil as residual oil in reservoirs, during oil production process [54]. This residual oil is focus of several enhanced oil recovery technologies. This residual oil has production capacity of 2-4 trillion barrels approximately 67% of total oil reserves [88]. Oil companies are looking for cheap and efficient technologies that will raise the global oil production. Methods that are currently used in oil industries are Enhanced Oil Recovery (EOR) also called as tertiary recovery. EORs include Chemical flooding, miscible carbon dioxide injection and thermal

enhanced oil recovery methods that uses heat as main source for additional oil recovery [55]. Several companies are focusing on residual oil via EOR technologies [56]. Microbial Enhanced Oil Recovery (MEOR) is based on microorganisms which pull remaining oil from reservoirs and proved to be potential EOR methods [57-59]. Approximately 50% of residual oil can be extracted by this very low-cost technology [59].

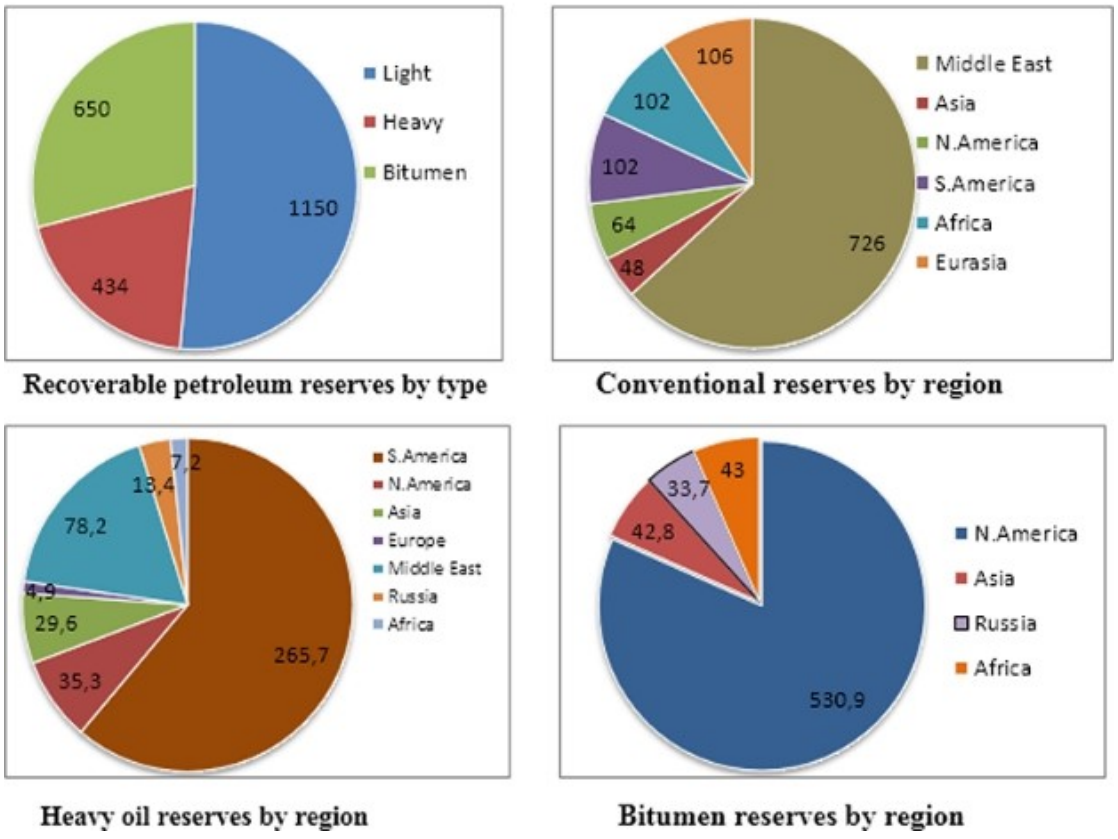

FIGURE 2.2: A breakdown of the worldwide recoverable petroleum reserves by type and region prepared by Meyer and Attanasi [7] and Schmitt [8].

2.3 Microbial Enhanced Oil Recovery (MEOR)

There are three stages by which oil is recovered named as primary secondary and tertiary oil recovery. While tertiary oil recovery technique used microorganisms or microbes to recover oil from oil wells. So microbial enhanced oil recovery is a tertiary oil retrieval practice. Primary recovery of oil recovers only 12-15% of oil in

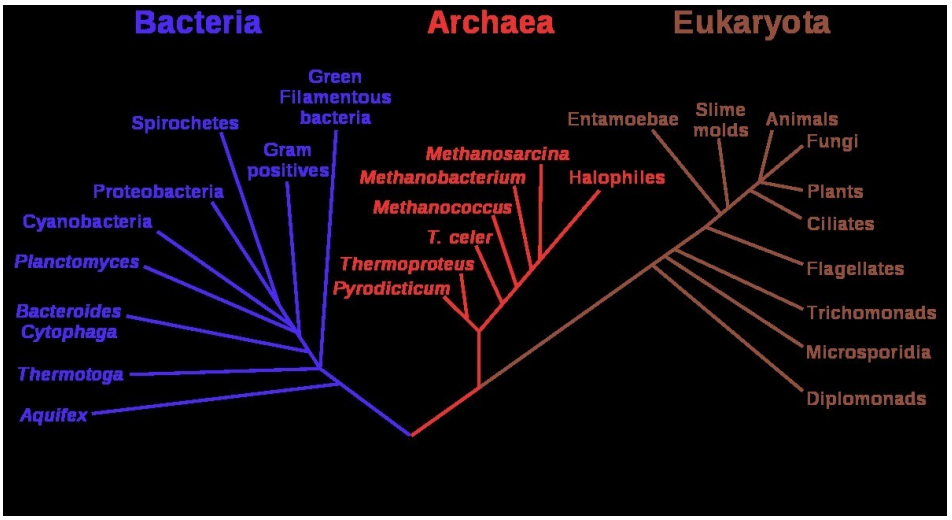

FIGURE 2.3: Microbial Enhanced Oil Recover

the wells without introducing other substances into the wells. While an additional 15-20% of oil from oil wells drives out by other substances and water flooding into the wells which is known as secondary oil recovery.

An additional trapped oil from oil wells is extracted by the last phase of oil recovery which is tertiary oil recovery technique and it includes microbial enhanced oil recovery. As MEOR process has some advantages such as it forms an emulsion among two liquid states which decreases an interfacial stiffness and blocking the high porous zones. As bacteria are smaller in size and they have negative charge on their cell surface so in In situ microbial enhanced oil recovery initially bacteria which are injected with water into the wells move into the high porous regions and then they grow and block those high porous zones. Consequently, an additional effective retrieval of oil can be attained by this development as it increases the sweep efficiency [44,45].

Chemical enhanced oil recovery is an expensive technology therefore MEOR can substitute CEOR. Microbes are able to produce beneficial products by fermentation of inexpensive substrates or raw materials. Chemicals used in chemical enhanced oil recovery processes are very expensive so in microbial enhanced oil recovery selected microbial strains produces the substances which are similar to those chemicals used in CEOR procedures to rise the oil retrieval from washed-out and peripheral reservoirs. In addition to it microbial products are decomposable

and are little toxicant [6]. Microbial innovations are getting to be endorsed all around as valuable and eco-accommodating ways to deal with improve oil generation [46,47].

TABLE 2.1: MEOR Pilot Application and The Use of Functional Microbes in the Past 40 Years

Countries	Functional Microbes	EOR efficiency	Ref
USA	Bacillus, Clostridium, Pseudomonas, alkane degrading bacteria, Microtatobiotes and indigenous micro flora, etc.	+	[19-21]
Russia	Clostridium tyrobutiricum, indigenous micro flora, sewage bacteria, and mixed culture, etc.	+	[22]
China	Bacillus, Pseudomonas, Fusobacterium bacteroidete, bio-polymers and bio-surfactants, etc.	+	[23]
Australia	Microtatobiotes	+	[24]
Bulgaria	Hydrocarbon oxidizing bacteria	+	[25]
Canada	Leuconostoc mesenteroides	-	[5,26]
Czechoslovakia	Pseudomonas and SRB	+	[27]
Great Britain	Acid-producing bacteria,	+ -	[28]
Germany	Thermophilic Bacillus, Clostridium and indigenous micro flora, etc.	+	[29]
Hungary	Sewage mixed culture	+	[30]
Norway	Nitrate reducing bacteria	-	[3]
Poland	Mixed culture	+	[31]

Table 2.1 continued from previous page

Romania	Bacillus, Clostridium, Pseudomonas and mixed culture, etc.	+	[32,33]
Holland	Betacoccus dextranicus	-	[3]

2.4 A Short History and Early Development

MEOR study was little known after Beckman's discovery but until Zobell and his researchers made a great contributions on laboratory study in 1947 [82]. After that other contributions are also done by researchers to gain the study of MEOR [83]. European countries performed substantial MEOR investigation in 1960s and 1970s. Due to the petroleum crisis in the 1970s the MEOR research was improved and later become substantiated. EOR processes maintained by research projects world- wide. In 1970 a new stage of microbial technology development started in Russia mainly in two laboratories. The Department of Geological Microbiology at the Institute of Microbiology Academy of Sciences of the USSR headed by Kuznetsov. The Laboratory of Microbial Biochemistry and Physiology of Microorganism Academy of Sciences of the USSR organized by Ivanov. These research groups were united under the basis of the Institute of Microbiology, Russian Academy of Sciences in 1986. 1990's era is well-known as an important year for MEOR expansion . MEOR had developed a scientific and interdisciplinary method for enhanced oil recovery by the end of the 1990s [83]. J.W.Beckman et al. studied and discovered that microbes can discharge trapped oil in permeable rock formations, bacterial formation of hydrogen sulfide in the waters of the Aspheron oil fields and also reported that Sulphate Reducing Bacteria (SRB) reside in samples from 67 wells located in California and Illinois. Others innovation established into novel information of improvement of oil creation consuming a bacterial network or MEOR. Meanwhile Bastin findings donated the information of biodeterioration of constituents or disintegration because of the occurrence and

metabolic activities of microbes which are usually stated as Mi- crobiologically Influenced Corrosion(MIC) [84-85].

2.5 Mechanisms Involved in MEOR

Microbial enhanced oil recovery is advanced technology by which oil is recovered in trapped formations inside the reservoirs. In this technology nutrients and bacteria are added into oil reservoirs to proceed it. For MEOR technology growth of microbes, either indigenously [57,58] or exogenously to the reservoir is very significant as it produces valuable constituents [59-62]. Gases, Organic acids, Solvents, Polymers, Surfactants are constituents that play substantial part for the retrieval of remaining oil as they have beneficial effect on the formation water characteristics oil and gas mixtures as well as to transform the features of the reservoir medium [42].

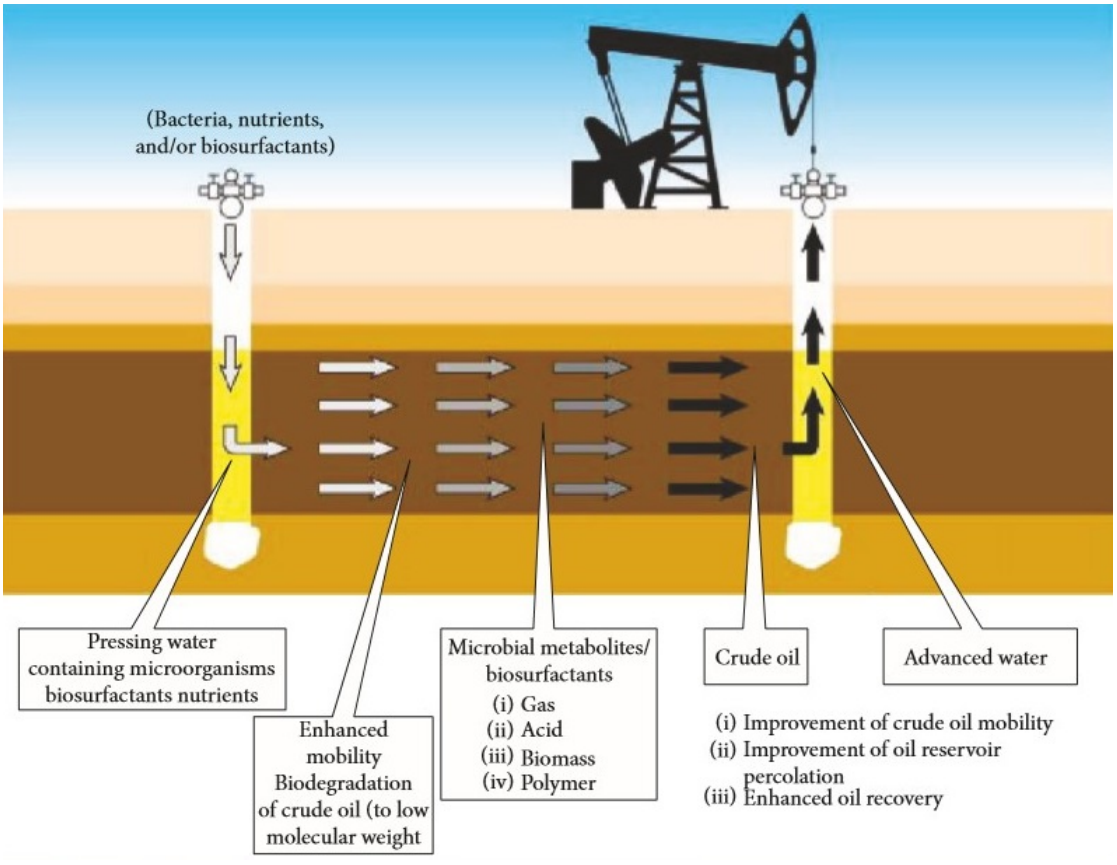

FIGURE 2.4: Enhanced Mobility Biodegradation of Crude Oil

2.6 Incomplete oxidization of Ammonia Oxidizing Bacteria

Nitrosomonas europaea can oxidize a wide range of hydrocarbons substrate through enzyme (AMO). Low sensitivity of AMO can leads to oxidation of hydrocarbon. this co-oxidation hinders AOB's by certain organic molecules e.g. *N. europaea* oxidize benzene to phenol that is more harmful than benzene. *N. europaea* do not co-oxidation materials that leads to accumulation of these in culture medium because HAO do not further oxidize them. Due to this energy loss occurs on AOB cells as AMO loses two electrons with each reaction. Recent studies suggest that AOA may have two distinct biological and physiological properties relative to AOB, which could be deemed beneficial in the attempt to mitigate nitrification-associated emissions. First, although inorganic ammonium added may be used, AOA prefers to primarily use ammonium extracted from mineralized organic matter in many soils, with AOB quickly oxidizing inorganic ammonium fertilizers applied at high concentrations. Second, while all ammonia oxidizers emit greenhouse gas nitrous oxide as a by-product of ammonia oxidation, the yield per ammonia oxidized is approximately half that of AOA. Therefore, the use of fertilization methods that promote AOA development and operation (e.g. the use of organic fertilizer) has the ability to substantially reduce nitrification-associated emissions. AOA has been shown to favor areas with low ammonium concentrations, whereas AOB supports areas with high ammonium concentrations.

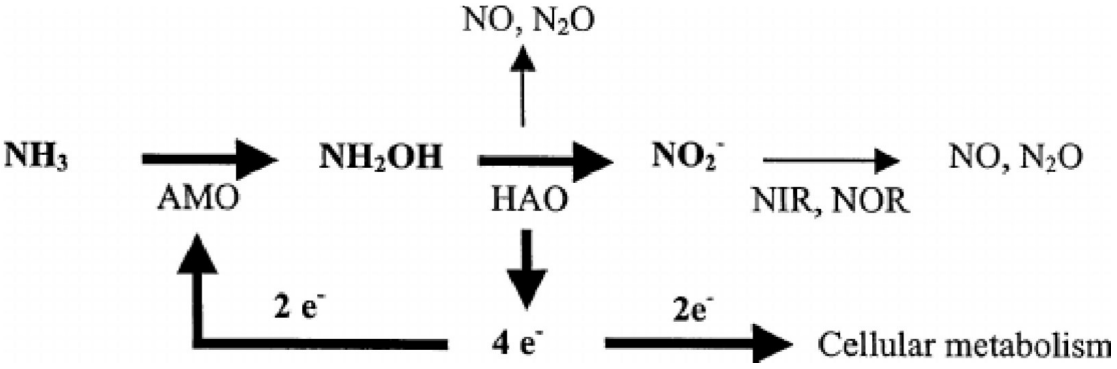

FIGURE 2.5: Incomplete oxidization of Ammonia Oxidizing Bacteria

2.7 Oil Contamination of Nitrogen Oxidizing Bacteria

Bacterial species that belong to genus Nitrobacter play a key role in nitrogen cycle by oxidizing nitrite to nitrate. Concentration of hydrocarbons negatively affect nitrite oxidation in Nitrobacter species however they are tolerant Nigerian crude oil. Nitrobacter's are more prone to hydrocarbons toxicity (numerous organic toxicants, such as trichloroethylene and toluene) than ammonia oxidizers.

2.8 Petroleum Bio Degradation Controls

Biological, molecular proof and low oil content of hydrocarbon degradation in Oil-Water Transition Zone (OWTZ) indicate that the oil depletion happens at the phase where biosphere enters the geosphere, because at the contact of oil and water, water is essential for life of microorganisms for electron donor and receiver. They use carbon from oil to generate energy and biomass. Important nutrients such as nitrogen and phosphorus are obtained from water. Methanogenic oxidation is primary mechanism for petroleum degradation in rivers where there is a low conc. of sulphate. Biostatic effects of oil reserves are due to harmful water-soluble hydrocarbons and other oil elements such as metals.

2.9 Anaerobic Hydrocarbon Metabolism

Anaerobic metabolism is a crucial mechanism for the biodegradation and bioremediation of petroleum hydrocarbons and, given the unique biochemistry currently being studied, is also essential for the production of biomimetic catalysts. Recent research on microbial consortia for oil industry, enrichment and microcosm cultures has shown that hydrocarbons such as toluene, alkyl benzenes like m-xylene, o-xylene, and p-xylene and trimethylbenzenes, benzene, naphthalene and

phenanthrene, methylnaphthalene and tetraline [95, 96], C6 n-alkanes , branched alkanes [93, 94] and hydrocarbon mixtures may be metabolized under anaerobic conditions. As microbial consortia have different properties such as they are able to degrade crude oil and can produce biosurfactants thus applying these diverse methods gives useful effects for enhanced oil recovery [14]. Various microbes used hydrocarbons as sole carbon sources and they havethe ability to degrade them [15]. Sabirova et al. reported that microbial n-alkane degradation has stimulating outcomes throughout the previous eras [18-20].

2.10 Metagenomics

Metagenomics has arisen as an important strategy for researching the abundance of both developed and uncultured microbes. Advances in Next Generation Sequences (NGS) enables the exploration of uncultured microbes for petroleum yield enhancement.

Metagenomics, is defined as a study of DNA from microbial communities in environmental samples without a specific need for cultivation A variety of statistical / computational metagenomic techniques and repositories have been developed to enable the processing of large data inflows. These have used culture-based [98], 16S rRNA gene-based culture-independent [97], and metagenomic [103, 101] methods. The previous 16S rRNA gene-based PhyloChip research, which analyzed the Alaska North Slope oil field samples described here, established species that could lead to the development of methane and hydrogen sulfide and the degradation of hydrocarbons [100]. Although a number of linear organisms were identified with a lack of cultivated representatives, the full diversity and functional capacity of these organisms remained uncertain. Previous metagenomical analyzes of the makeup of microbial species from other environments included the detection and processing of genomic DNA from coexisting populations [99,101].

Table below summarize the nitrogen fixation genes found in draft genomes from water samples collected from oil reservoirs Schrader Bluff formation (SB1 and

SB2), the Kuparuk formation (K3), or the Ivishak Formation (I2) of the Alaska North Slopea via metagenomic studies

TABLE 2.2: Draft Genomes Consisting Nitrogen Fixation Genes and Their Affiliation

Genome	Sample	Nitrogen fixation gene(s)	Affiliation
Methanosaeta_ harundinacea_ 57_489	SB1	*nifH (nitrogenase Fe alpha subunit), nifK (MoFe beta subunit), nifE (nitrogenase MoFe cofactor biosynthesis protein)*	Methanogenic Archaea
Methanocalculus_ 52_23	SB1	*nifH, nifK*	Methanogenic Archaea
Methanoculleus_ marisnigri_ 60_61_partial	SB1	*nifH, nifK*	Methanogenic Archaea
Desulfotomaculum _46_80	SB1	*nifH, nifK, nifE, nifB (nitrogenase cofactor biosynthesis protein)*	Bacteria
Methanobacteriales _53_19_partial	SB2	*nifH, nifK,*	Methanogenic Archaea
Methanoculleus _60_29	SB2	*nifH, nifK, nifE, anfO (nitrogenase iron-iron accessory protein)*	Methanogenic Archaea
Methanosaeta_ haundinacea_ 56_747	SB2	*nifH*	Methanogenic Archaea
Clostridia_ 45_118_partial	SB2	*nifH, nifK, nifE, nifB*	Bacteria
Methanothermobacter _50_10	I2	*nifH, nifK*	Methanogenic Archaea

Table 2.2 continued from previous page

Methanobacteriaceae _41_258_partial	I2	*nifH, nifK*	Methanogenic Archaea
Methanobacteria _50_154	K3	*nifH, nifK, nifE, glnB (nitrogen metabolism regulatory protein)*	Methanogenic Archaea

Chapter 3

Material and Methods

3.1 Sample Collection /Sampling Site Description

The water and soil samples were collected from deep oil well cavities of Punjab platform namely Nandupur (NP) (Nandpur gas field is located about 60km north-east of Multan city in Punjab province) Panjpir (PN) (Panjpir gas field located approximately 70 kilometers north-east of Multan in Punjab province) Bahu (BH) (Bahu gas field is located approximately 220km from Multan Punjab Province) chak Nourag (CN) Rajian (RJ) (Chak Nourag and Rajian oil field is located in Chakwal in Punjab Province) [49]. There were about 3 water samples 2 collected from each site (Nandpur Panjpir Bahu) at the depth of about 1717m-1884m.

Table 3.1: Showing Detail of Water and Soil Samples

Sample #	**Well Name**	**GL (m)**	**Reservoir**
BH	Bahu-02	146	Samanasuk
NP	Nandpur-05	141	Lumshiwal
PN	Pinjpir-10	143	Samanasuk

Table 3.1 continued from previous page

CN	Chak Nourag- 5A	142	Lower Sakessar
RJ	Rajian-8	143	Nara Mughlan
Sample #	**Reservoir Depth (m)**	**Lithology**	**Reser.Tempe**
BH	1717-1733	Carbonate	56℃
NP	1792-1797	Sandstone	60℃
PN	1878-1884	Carbonate	59℃
CN	1000-2515	sandstone	61℃
RJ	2000-3550	Sand stone	60 ℃

Two Soil samples in duplicate from (CN, RJ) were collected at a depth of about 2515m [68]. A detailed description of the samples was provided in table 3.1. The samples were provided by Oil and Gas Development Company Ltd. Samples were collected in falcon tubes and were preserved in the refrigerator and then utilized for analysis [98].

3.2 Geographical Location of Sampling Site

3.2.1 Nandpur Gas Field

Nandpur gas field is located in Multan city of province Punjab approx. 60 Km north-east of Multan. The structure was discovered by OGDCL in 1984 and 1985. The Nandpur Field is a near symmetrical anticlinal structure, approximately 20 square kilometres in extent with gas reservoirs in carbonate and clastics of middle Jurassic and early Cretaceous age. Eleven wells have been drilled of which one well (Nandpur ♯8) has been abandoned. Gas from different reservoirs contains 10 percent to 46 percent Methane and 55 percent to 85 percent Nitrogen.

3.2.2 Panjpir Gas Field

Panjpir gas field is located approx. 70 Km north-east of Multan city. In 1984 and 1985 OGDCL discovered this field. Gas reserves, high in nitrogen content, have been discovered in the Lumshiwal carbonate and Samana Suk carbonate.

3.2.3 Bahu Gas Field

Bahu gas field is located approximately 220km from Multan, Punjab Province. The field was discovered in 2006 by OGDCL. Six wells have been drilled in the Bahu field. Gas reserves, high in nitrogen content, have been discovered in the Lumshiwal clastic/carbonate and Samana Suk carbonate. Table.8 a and b show detail of water samples.

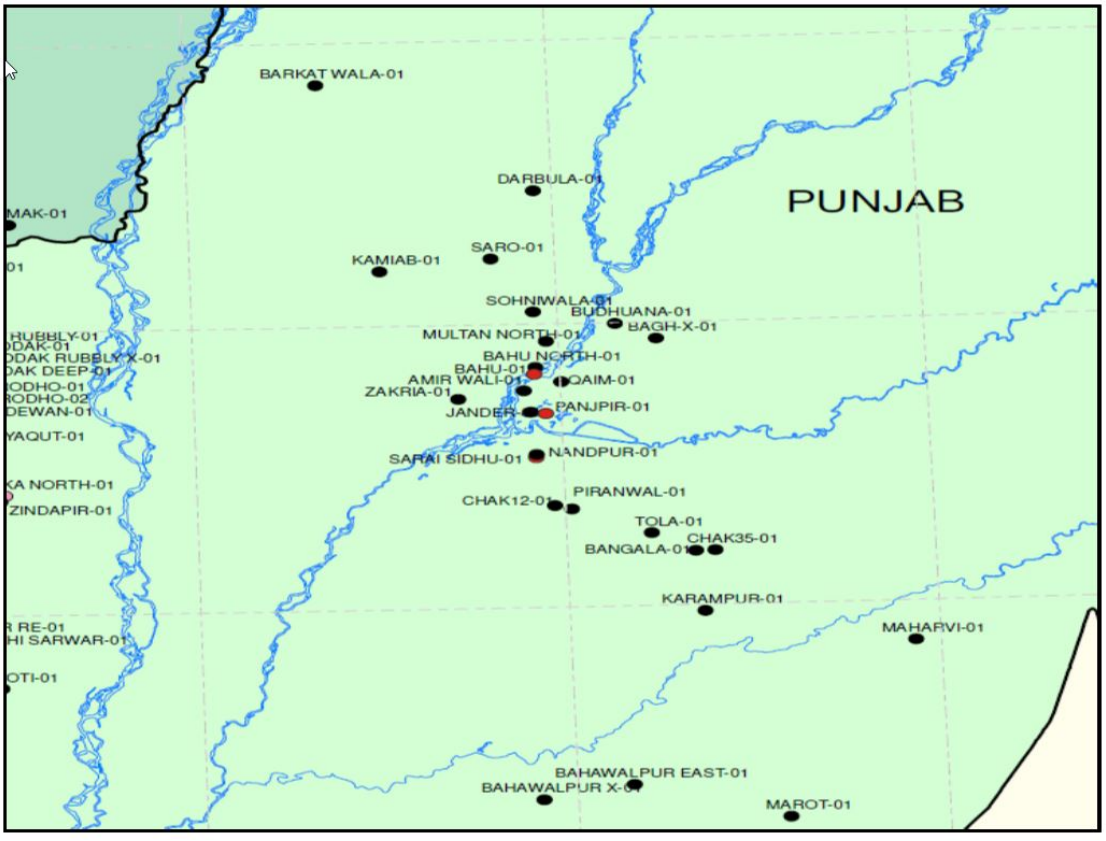

FIGURE 3.1: Site Map of Nandpur Penjpir and Bahu Gas Field

FIGURE 3.2: Site Map of Chak Naurang Oil Field

FIGURE 3.3: Site Map of Rajion Oil Field

TABLE 3.2: (A). Detail of water samples

Sample #	Well Name	GL (m)	Reservoir	Reservoir Depth (m)	Lithology	Reser. Tempe ℃
Bahu-2	Bahu-02	146	Samanasuk	1717-1733	Carbonate	56℃
NP-5	Nandpur-05	141	Lumshiwal	1792-1797	Sandstone	60℃
NP-10	Nandpur-10	143	Samanasuk	1878-1884	Carbonate	59℃

TABLE 3.3: (B). Detail of water samples

Sample #	Well Name	Gas Composition
Bahu-2	Bahu-02	C1 = 29%, C2+ = 0.9%, CO2 = 1.0%, N2 = 45%
NP-5	Nandpur-05	C1 = 37%, C2+ = 0.2%, CO2 = 2.0%, N2 = 60%
NP-10	Nandpur-10	C1 = 41%, C2+ = 0.15%, CO2 = 4.0%, N2 = 54%

3.3 Research Methodology

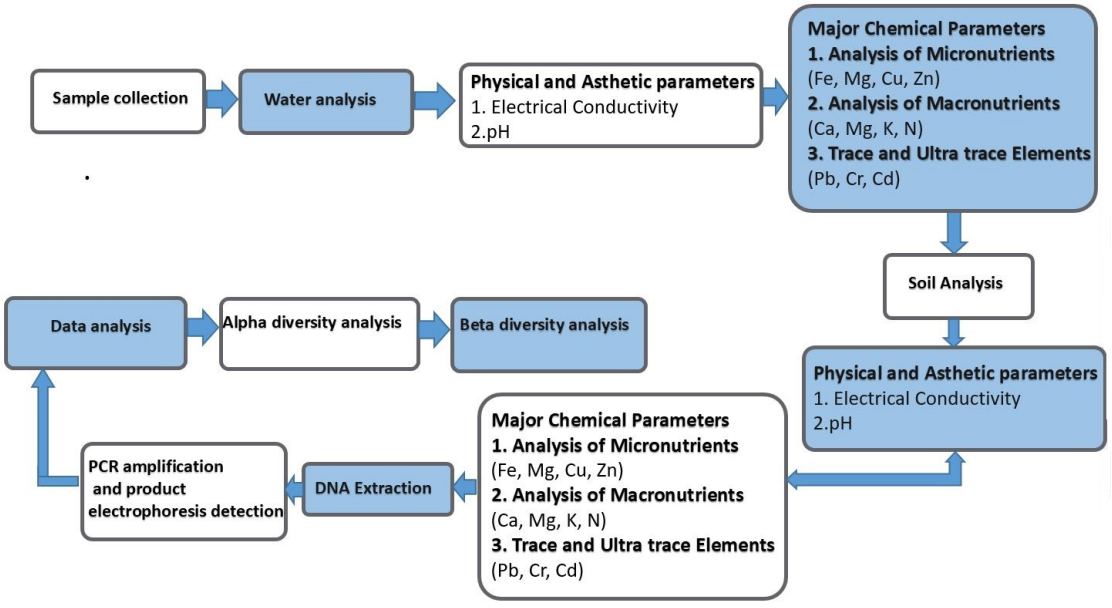

FIGURE 3.4: Methodology Overview

3.3.1 Soil Analysis

Soil Sample Drying

Soil samples were dried in wooden trays soil were dried in air. Soil can be dried by placing the trays in racks in hot air cabinet. The temperature for drying should be between below 35 ℃ and humanity content should be 30 to 60%. Excessive drying by oven should be avoided to ensure availability of the nutrients in sample [109]. Drying process had minimal effect on total N content but NH_4 and NO_3 content may vary with time and temperature. Drying at high temperature leads to the death of microorganisms present in soil samples

Soil Sample Preparation

Soil samples were grounded to fine powder using wooden pestle and mortar, samples were converted to fine state using 2-mm sieve so that the heterogeneity can be reduced and also to provide max. surface area for physiochemical reactions [99].

Soil Sample Digestion

1 gram of prepared dried soil sample was taken in 250 ml beaker and 15ml of aquaregia was added. This mixture was then digested at 70 ℃ up to the transparency of sample solution. The digested solution was filtered using filter paper No 42. Filtered solution was then diluted to 50ml using eionized water [111]. Sample solution was analyzed for con. of Pb, Cr, Cd, Ca, Fe, Na, Cu, Mn, Mg and K via flame atomic absorption spectrophotometer (Perkin Elmer Aanalyst 400)..

3.3.2 Chemical Analysis of Soil

The content of calcium, magnesium, potassium sulphate, phosphate, nitrate, nitrite, copper, iron, manganese, lead, chromium, calcium, and zinc from soil samples was determined by AAS Analyst 7000. Compressor was turned ON after fixing nitrous oxide and acetylene gas. Extra trapped liquid was removed. AAS and Extractor controls were turned ON. Slender tube and nebulizer were cleaned, the

acetylene gas pressure was set at 700 KPa that is equivalent to 100 psi and the valve was set to 11 psi for acetylene and 45 psi for air. The new sheet on Spectra AA software was opened, "Add Method" was used and desired element for analysis was selected. Type, Optical, SIPS parameters were selected accordingly.

Joined PC was used for the worksheet of ASS programming. Empty cathode was used for light embedded in light holder. The beam was adjusted to hit target zone of the arrangement cards for required light throughput. At that point the machine was turned off [61-63]. 10 ml graduated chamber containing deionized water was used to estimate the yearning rate. Calibration solutions were prepared along with analytical blank. Both were atomized and response was measured. Graph was plotted for each solution than sample solutions were atomized. The conc of various elements from sample solution was determined [78-79].

3.3.3 Water Analysis

Apparatus

Apparatus required for water analysis was pH meter containing combined electrodes, TFE beakers, stirrer with plastic coating and plastic wash bottle.

Reagents

Reagents required were Potassium Chloride 0.01m, 0.745g KCL was dissolved in DI water and solution was brought to 1 liter, buffer solutions for pH 4 and 7, boiled and cooled distill water that had conductivity less than 2 micromhos/cm

Procedure

PH meter was calibrated, 50ml water sample was taken in 100ml flask, combined electrodes were introduced in water sample about 3-cm deep. Readings were taken after 30 seconds. combined electrodes were removed from sample and rinsed with DI water; excess water was tried with tissue.

Electrical Conductivity

Apparatus

Conductivity meter, conductivity cell, thermometer, beakers.

Reagents

Potassium Chloride Solution 0.01N was taken, 2 to 3 grams of KCL was dried at 110 Degree Celsius for 2 hours. 0.745g KCL was dissolved in DI water and solution was brought to 1 liter. The solution was transferred to plastic flask.

Procedure

Conductivity meter was calibrated according to maker's instruction. Conductivity cells were rinsed thoroughly with distilled water. Excess water was dried carefully. Conductivity cell s were rinsed with measured solution for few times. 75 ml of sample was taken and conductivity cells were inserted, readings were taken.

3.3.4 Water Chemical Analysis

The content of calcium, magnesium, potassium sulphate, phosphate, nitrate, nitrite, copper, iron, manganese, lead, chromium, calcium, and zinc from water samples was determined by AAS Analyst 7000. Compressor was turned ON after fixing nitrous oxide and acetylene gas. Extra trapped liquid was removed [88-68]. AAS and Extractor controls were turned ON. Slender tube and nebulizer were cleaned, the acetylene gas pressure was set at 700 KPa that is equivalent to 100 psi and the valve was set to 11 psi for acetylene and 45 psi for air [78]. The new sheet on Spectra AA software was opened, "Add Method" was used and desired element for analysis was selected. Type, Optical, SIPS parameters were selected accordingly.

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3.4 DNA Extraction Amplification and Pyrosequencing

3.4.1 Experiment Process

3.4.1.1 DNA Extraction

The DNA extraction kit /CTAB method /SDS method was used for genomic DNA extraction. The integrity purity and conc. of DNA was checked by 1% agarose gel electrophoresis [50].

3.4.1.2 PCR Amplification and Product Electrophoresis Detection

Using genomic DNA as template according to the selection of sequencing region specific primers with barcode and Takara premier Taq version 2.0 (Takara Biotch. Co. Dalian China) were used for amplification by PCR.

3.4.1.3 Primer Corresponding Region:

Primer corresponding regions include

- 16S V4 primers (515f and 806r) that identify bacterial diversity
- 18S V4 primers (528f and 706r): identify the diversity of eukaryotes;

- ITS1 primers (its5-1737f and its2-2043r): identify the diversity of fungi;

In addition the amplification region also includes: 16S v3-v4 / 16S v4-v5; Archaea 16S v4-v5; 18S V5 and ITS2 Region; functional gene corresponding primers etc.

3.4.1.4 PCR Reaction Conditions

1. 94℃ 5min
2. 30 cycle of 94 x C 30s, 52 x C 30s, 72 x C 30s
3. 72℃ 10min
4. 4℃ Hold

Each sample was repeated three times and the PCR products were mixed PCR instrument: BioRad S1000 (CA)

3.4.1.5 PCR Reaction System

Table 3.4: Reagent Name and Dosage

Reagent Name	Dosage
2x Premix Taq	25 μl
Primer-F	10 mM
Primer-R	10 mM
DNA	60 ng
Nuclease-free water	Add to 50 μl

3.4.2 Electrophoresis Detection of PCR Products

The con. and length of PCR product were identified by 1% agrose gel electrophoresis. The length of the main band was within the normal range [60]. The length

of the main band was within the normal range. (for example, 16S v4:290-310bp / 16S v4-v5:400-450bp etc.) can be used for further experiments.

3.4.3 Pooling and Gel Cutting Purification

By comparing the conc. of PCR products by gene tools analysis software the volume of each sample was calculated with respect to principle of equal quality and then the PCR products were mixed the E.Z.N.A PCR Gel Extraction Kit was used to recover PCR mixed products. TE buffer was used to eluate the target DNA fragment [49].

3.4.4 Database Building and Sequencing

3.4.4.1 Database Building

Build the database according to the standard process of nebnext ultra DNA library prep kit for Illumina (New England Biolabs USA).

3.4.4.2 Sequencing

The amplified library was sequenced by PE250 using Illumina Nova 6000 platform (Guangdong Magigene Biotechnology Co. Ltd. Guangzhou China).

3.4.5 Analysis Process

3.4.5.1 Sequencing Data Processing

(1) Paid End Raw Reads Data Filtering: use fastp (an ultra-fast all in one fastq preprocessor version 0.14.1 https://github.com/opengene/fastp) to cut the sliding window quality (- w4-m20) of two end raw reads data respectively and use cut adapt software (https://github.com/marcelm/cutadapt/) to remove the

primer information at both ends of the sequence Primer obtained the paid end clean reads after quality control.

(2) Paired End Clean Reads Splicing: for the data of two terminal sequencing according to the overlap relationship between PE reads usearch-fastq'mergepairs (V10 http://www.drive5.com/usearch/ preset parameters include the minimum overlap length set to 16bp the maximum mismatch allowed in the overlap area of splicing sequence 5bp etc.) should be used to filter the inconsistent tags and obtain the original ones Raw tags.

(3) Raw Tags Sequence Quality Filtering: use fastp (an ultra-fast all in one fastq preprocessor version 0.14.1 https://github.com/opengene/fastp) to cut the raw tags data with sliding window quality (- w4-m20) and get effective splicing fragments (clean tags).

3.4.5.2 OTU Clustering and Species Annotation

(1) OTU Clustering: OTU or operational taxonomic units is one of the most common terms in microbiology. The platform provides the following three methods and the default clustering method is uparse:

1. UPARSE (RC Edgar. highly accurate OTU sequences from microbial amplicon reads. Nature methods 2019 10(10): 996)
2. UNOISE3 (RC Edgar. UNOISE2: Improved error-correction for Illumina 16S and ITS amplicon read. bioRxiv 2016)
3. UCLUST (RC Edgar. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 2010 26(19):2460-2461)

(2) Representative Sequence Species Annotation: use usarch - sinax to compare the representative sequence of each OTU with Silva (16S) RDP (16S) greenenes (16S) Silva (18S) unite (ITS) database. Through the comparison the species annotation information (the default confidence threshold is 0.8 and the

default database is Silva (16S) Silva (18S) and unite (ITS)) so as to understand the origin of all sequence species. The taxonomic results from species annotation can be divided into seven levels that are Kingdome (L1) Phylum (L2) Class (L3) Order (L4) Family(L5) Genus(L6) Species(L7).

(3) Contaminated OTU Removal: the OTU and its tags annotated as chloroplast or mitochondria (16s amplicon) was removed that were unable to annotate to the boundary level the number of effective tags sequences were obtained for OTU table of taxonomy for each sample.

3.4.6 OTU Statistics

(1) OTU Table: Based on the above-mentioned OTU table after removing singleton OTU chimera and contaminated OTU count the number of reads and OTU contained in the sample or group.

(2) Pan'u Core Species Analysis: Use Qiime2 View to count the number of Union (Pan) and intersection (core) of the target classification level in different product numbers to evaluate whether the sample size is sufficient.

3.4.7 Species Community Analysis

(1) Community Structure of Species: Use Qiime2 View software to make statistics of common and endemic species community composition analysis and species abundance cluster analysis.

(2) Phylogenetic Analysis

1. Single Aample - Phylogenetic Analysis of each Classification Level:

a) Based on phylogenetic relationship and relative abundance of each OTU in sample Qiime2view was used to visualize the species annotation results.

b) LAN software was used in order to mine the species composition and abundance information in sample.

2. Significance Analysis of Community Structure Difference Among Groups: Anosim function, MRPP function, Adonis function and AMOVA of vegan and pegas package were used to analyze the significance of anosim, MRPP, Adonis and AMOVA.

3.4.7.1 Alpha Diversity Analysis

(1) Alpha Diversity Index Statistics (Default Parameter): Based on the OTU abundance table useQiime2View (https://view.qiime2.org) to calculate diversity indexes (richness Chao1 Shannon 2).

(2) Dilution Curve (Default Parameter): Based on OTU abundance table use Qiime2View (https://view.qiime2.org) to calculate the dilution curve of the above diversity indexes.

(3) Rank Independence Curve (Default Parameter): Based on OTU abundance table Qiime2View (https://view.qiime2.org) is used.

3.4.7.2 Beta Diversity Analysis

(1) PCA Analysis (Default Parameter): Based on OTU abundance table use the Qiime2View (https://view.qiime2.org) for analysis.

(2) PCoA Analysis (Default Parameter): Based on OTU abundance table Qiime2View (https://view.qiime2.org) is used to analyze with the above nine distance algorithms.

(3) Sample Distance Heatmap Analysis (Default Parameter): Use vegan package of R software to merge the above 9. Three distance algorithms and hclust function are used to cluster the heat map.

3.4.7.3 Correlation Analysis of Environmental Factors

Mantel UU Test Analysis: Based on OTU abundance table and environmental factor data, Mantel test analysis is carried out by using R software vegan, and the correlation between environmental factors and microbial community distribution is judged according to the R value and significance level P value.

CCA / RDA Analysis: Based on OTU abundance table and environmental factor data, first use R software to Line detrended correspondence analysis, i.e. DCA analysis, determines which is the most suitable linear model (RDA) or single peak model (CCA) according to the gradient value (CCA should be selected if the maximum value of axis length in the first four axes of DCA analysis result is greater than 4.0, and both RDA and CCA should be selected if it is between 3.0-4.0, and RDA is better than CCA if it is less than 3.0) , and then use for CCA or RDA analysis and mapping.

Chapter 4

Results and Discussion

4.1 Physical and Aesthetic Parameters

Table 4.1: Physical and Aesthetic Parameters of soil and water samples

Sample	Parameter								
	EC	pH	Cu	Fe	Mn	Zn	Ca	Mg	K
NP1	28500	6.6	1.21	8.8	0.89	1.43	1041	826	808
NP2	28900	6.8	1.25	8.6	0.69	1.45	1001	866	800
PN1	47600	6.6	2.11	32.9	1.42	2.29	566	488	208
PN2	51600	6.5	2.01	33.1	1.36	2.14	561	456	200
BH1	68100	6.3	1.50	9.80	1.0	1.11	1411	1098	860
BH2	68800	6.6	1.44	9.88	1.2	1.01	1402	1084	852
CN1	44600	8.1	4.58	34.16	0.95	2.21	29.45	4.55	3.05
CN2	43100	8.1	4.51	34.27	0.92	2.03	29.75	4.63	3.60
RJ1	34000	8.5	5.40	35.70	1.00	1.30	30.43	5.60	4.03
RJ2	33800	8.3	5.02	35.00	1.50	1.03	30.00	5.30	4.00
C1	24000	7.5	0.47	4.31	0.20	3.21	20.10	4.00	0.08
C2	20000	7.5	0.45	4.26	0.29	3.02	20.64	4.01	0.80
Samples	SO_4^{2-}	H_2S	PO_4^{3-}	NO^{3-}	NO^{2-}	Pb	Cr	Cd	
NP1	500	466	10.6	1.77	BDL	0.01	0.12	0.021	

Table 4.1 continued from previous page

NP_2	505	520	11.1	1.87	BDL	0.05	0.14	0.023	
PN1	81	67	0.51	3.47	BDL	0.11	0.004	0.013	
PN2	79	71	0.62	3.39	BDL	0.84	0.003	0.019	
BH1	33	34	0.03	1.88	BDL	0.01	0.064	0.010	
BH2	32	36	0.06	1.18	BDL	0.53	0.061	0.011	
CN1	402	403	12.46	0.64	BDL	68.44	0.656	0.019	
CN2	406	433	12.46	0.60	BDL	68.49	0.658	0.012	
RJ1	249	244	11.05	1.32	BDL	70.33	1.508	1.018	
RJ2	255	250	11.05	134	BDL	70.00	1.502	1.013	
C1	19.1	6.3	0.0001	2.37	0.0001	0.64	0.152	0.001	
C2	19.4	6.5	0.0001	2.43	0.0001	0.61	0.159	0.002	

BDL: Below Detection Limit, NP: Nanpur, PN: Penjpir,

BH: Bahu, CN: Chak Nourang, Rj: Rajion

4.2 Faith PD Values for Petroleum Reservoir Samples Against Control

TABLE 4.2: Faith PD values for petroleum reservoir samples against control

Ser no.	Sample	Faith_PD
1	NP1	25.28
2	NP2	24.09
3	PN1	26.19
4	PN2	26.13
5	BH1	23.22
6	BH2	23.95
7	CN1	24.61
8	CN2	24.29
9	Rj1	22.03

Table 4.2 continued from previous page

10	Rj2	23.67
11	C1	30.39
12	C2	30.09

Faith PD (Table 4.2; Figure 4.1) explains the distance between two samples. Null hypothesis here presents that either there is no difference between different samples or samples do not have any difference with the control. The observed values differ from the null distribution. However, because the observed PD is lower than the null PD, this means that less phylogenetic diversity was present in our samples than were expected. In the literature, you can find different explanations of the deviations from the null distribution (e.g. lower than null may indicate phylogenetic conservatism or selection pressures because of stressed environment).

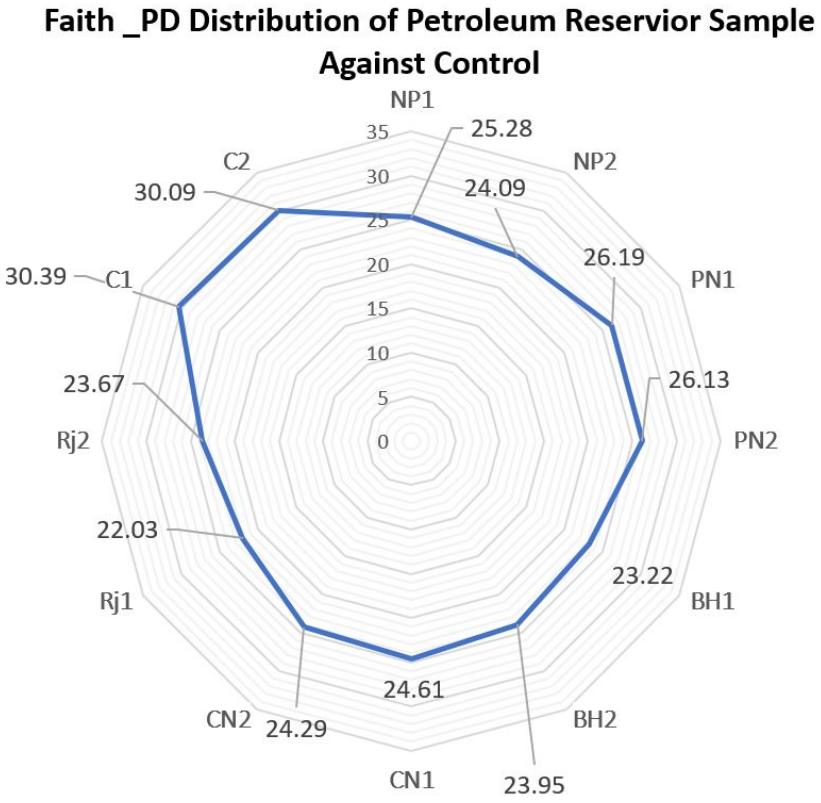

FIGURE 4.1: Faith PD values for petroleum reservoir samples and control. First of all, the observed PD values among different sites are varying but major difference is observed among samples and control samples, where PD higher for null distribution (control) is highest.

4.3 Distance Metrices between Different Sites

TABLE 4.3: Distance Matrices among sample sites and control

	NP1	NP2	PN1	PN2	BH1	BH2
NP1	0	0.500312	0.564456	0.570251	0.780778	0.465565
NP2	0.500312	0	0.582419	0.625978	0.783509	0.519435
PN1	0.564456	0.582419	0	0.379011	0.773225	0.42371
PN2	0.570251	0.625978	0.379011	0	0.772739	0.451802
BH1	0.780778	0.783509	0.773225	0.772739	0	0.70498
BH2	0.465565	0.519435	0.42371	0.451802	0.70498	0
CN1	0.437933	0.523432	0.433123	0.472681	0.693927	0.357003
CN2	0.688905	0.718332	0.77051	0.763775	0.768959	0.712936
Rj1	0.500512	0.525978	0.779011	0.672739	0.437933	0.437933
Rj2	0.560056	0.483509	0.713225	0.651802	0.523432	0.523432
C1	0.170251	0.119435	0.22371	0.272681	0.433123	0.433123
C2	0.180778	0.123432	0.233123	0.263775	0.472681	0.472681
	CN1	CN2	RJ1	RJ2	C1	C2
NP1	0.437933	0.688905	0.500512	0.560056	0.170251	0.180778
NP2	0.523432	0.718332	0.525978	0.483509	0.119435	0.123432
PN1	0.433123	0.77051	0.779011	0.713225	0.22371	0.233123
PN2	0.472681	0.763775	0.672739	0.651802	0.272681	0.263775
BH1	0.693927	0.768959	0.437933	0.523432	0.433123	0.472681
BH2	0.357003	0.712936	0.437933	0.523432	0.433123	0.472681
CN1	0	0.704428	0.437933	0.523432	0.433123	0.472681
CN2	0.704428	0	0.693927	0.523432	0.433123	0.472681
Rj1	0.437933	0.437933	0	0.433123	0.472681	0.693927
Rj2	0.523432	0.523432	0.433123	0	0.433123	0.472681
C1	0.433123	0.433123	0.472681	0.433123	0	0.433123
C2	0.472681	0.472681	0.693927	0.472681	0.433123	0

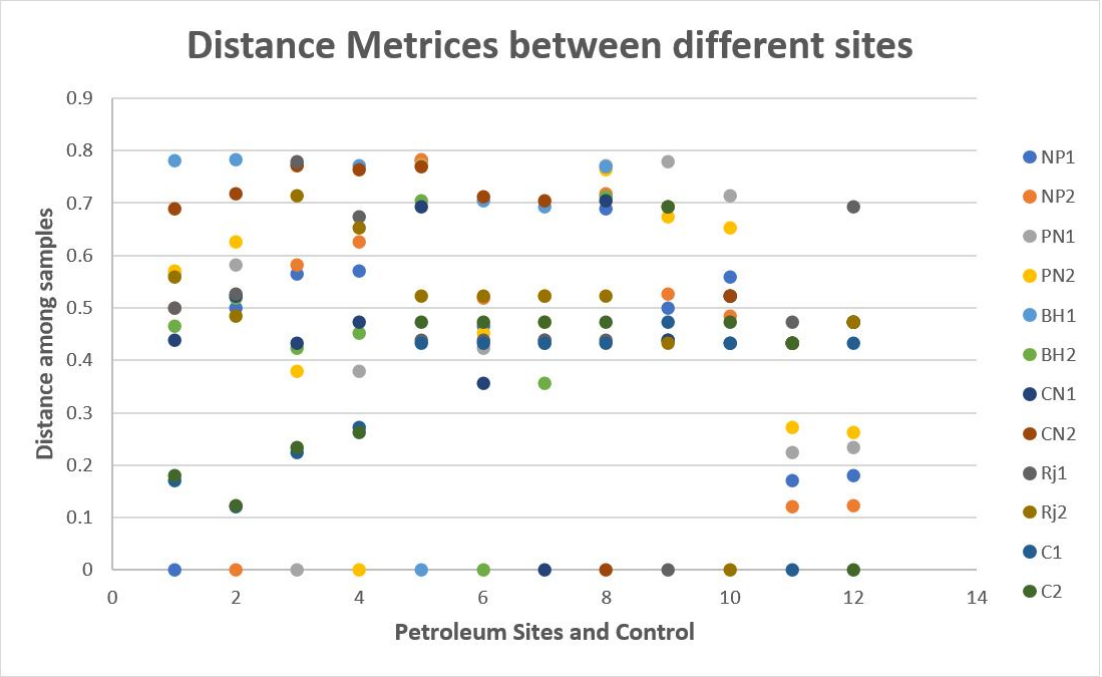

FIGURE 4.2: Distance metrices Xy Scatter chart showing distribution of OTUs in space. Color codes are presenting different samples and their overlapping OTUs among different sample sites. Overlapping positively showing homogeneity in the microflora of sites.

4.4 Species Community and Phylogenetic Analysis

4.4.1 Phylogenetic Abundance of Archaea

TABLE 4.4: Showing Phylogenetic Abundance of Archaea and Number of OUT's Present

Classification		Samples	Number of OTU's present in samples
Kingdom	Archaea	NP1	0
Phylum	Euryarchaeota	PN1	0
Class	Halobacteria	BH1	5
Order	Halobacteriale	CN1	0
Family	Halobacteriaceae	RJ1	0
Genus	Halorubrum	C1	0

Table 4.4 continued from previous page

Specie			
Classification		Samples	Number of OTU's present in samples
Kingdom	Archaea	NP1	31
Phylum	Euryarchaeota	PN1	20
Class	Methanobacteria	BH1	16
Order	Methanobacteriales	CN1	0
Family	Methanobacteriaceae	RJ1	31
Genus	Methanobacterium	C1	0
Specie	uncultured Methanobacteriaceae archaeon		
Classification		Samples	Number of OTU's present in samples
Kingdom	Archaea	NP1	0
Phylum	Euryarchaeota	PN1	4
Class	Methanomicrobia	BH1	0
Order	Methanosarcinales	CN1	0
Family	Methanosarcinaceae	RJ1	0
Genus	Methanosarcina	C1	0
Specie			
Classification		Samples	Number of OTU's present in samples
Kingdom	Archaea	NP1	0
Phylum	Euryarchaeota	PN1	13
Class	Thermoplasmata	BH1	0
Order	Thermoplasmatales	CN1	0
Family	Thermoplasmatales Incertae Sedis	RJ1	10
Genus	Methanomassiliicoccus	C1	0

Table 4.4 continued from previous page

Specie			
Classification		Samples	Number of OTU's present in samples
Kingdom	Archaea	NP1	0
Phylum	Euryarchaeota	PN1	0
Class	Thermoplasmata	BH1	6
Order	Thermoplasmatales	CN1	0
Family	Thermoplasmatales Incertae Sedis	RJ1	0
Genus	Methanomassiliicoccus	C1	0
Specie	uncultured archaeon		
Classification		Samples	Number of OTU's present in samples
Kingdom	Archaea	NP1	0
Phylum	Thaumarchaeota	PN1	10
Class	Soil Crenarchaeotic Group (SCG)	BH1	0
Order		CN1	0
Family		RJ1	10
Genus		C1	482
Specie			
Classification		Samples	Number of OTU's present in samples
Kingdom	Archaea	NP1	0
Phylum	Thaumarchaeota	PN1	0
Class	Soil Crenarchaeotic Group (SCG)	BH1	0
Order	Unknown Order	CN1	0
Family	Unknown Family	RJ1	0

Table 4.4 continued from previous page

Genus	Candidatus Nitrososphaera	C1	25
Specie	uncultured euryarchaeote		

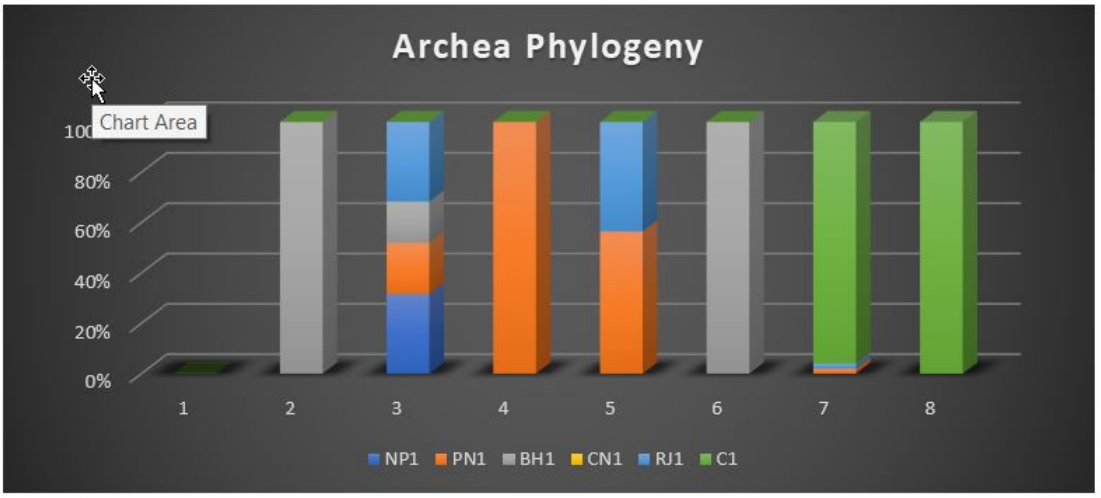

FIGURE 4.3: showing taxonomic abundance of different archaeal OTU'S among the samples.

Table 4.4 shows taxonomy of archaea in five different fields and in control sample, above results depicted that there are number of archaea's present in the oil fields. Phylum Euryarchaeota (Family Halobacteriaceae) shows its abundance in BH1 field, 5 OTUs' were present. Family Mthanobacteriaceae shows its abundance in NP1 (31 OTU's), PN1 (20 OTU's), BH1 (16 OTU's) and RJ1(31 OTU's). Family Methanosarcinaceae is only found in PN1 (4 OTU's). Family Thermoplasmalates Incertae Sedis in BH1 (6 OTU's). Most of the archea isolated from oil fields belong to Euryarchaeota phylum.

The most diverse group of microorganisms in archaea is related to phylum Euyarchaeota that has adapted most extreme environments. This phylum includes thermophiles, mesophiles, and psychrophiles. Some microbes including both aerobes and anaerobes live at extreme temperatures from 41 to 122 degree Celsius. Acidophiles members can live in highly acidic and halophytes in high salty environments. These microbes are different from others on the bases of ribosomal

RNA and unique DNA polymerases [106-107]. Members of this phylum has diverse appearance and metabolic properties. They are in rods or cocci shapes, either Gram-positive or Gram-negative. They can be methanogens, halophytes and sulphate reducers. They are found in oceans. Although marine members of this phyla are difficult to culture but genomic sequence studies suggest that they are motile heterotrophs [106],[111].

Euryarchaeota are also habitants of several moderate environments like water springs, marshlands, soil and rhizospheres. They are also known as highly adaptable e.g. heliobacteria's order can live and grow in high salt concentrations to slow salt concentrations such as in sea water. Members of this phyla lack defense mechanisms against oxidative stress (ROS) [109-111]. Phylum Thaumarchaeota is identified in PN1(10 OTU's), RJ1 (10 OTU's) and C1 (482 OTU's) of Soil Crenarchetic Group, 25 OTU's of genus Candidatus Nitrososphaera were also identified in C1. Thaumarchaeota members are ammonia-oxidizing organisms that live in soil, marine and hot springs habitats [112-122]. They are autotrophs and fix cabondioxide and few of them are dependent on other bacteria or small amounts of organic matter. Members of this phyla are also capable of oxidizing methane. Marine Thaumarchaea members produce nitrous oxide that is greenhouse gas and has role in climate change.

4.4.2 Phylogenetic Abundance of Bacteria

Vast majority of bacterial phylum's have been identified from the oil field samples, includes phylum *Acidobacteria, Actinobacteria, Armatimonadetes, Bacteriodetes, Chlamydiae, Chlorobi, Chloroflexi, Cyanobacteria, Deferribacteres, Deferribacteres, Deinococcus, Elusimicrobia, Firmicutes, Fusobacteria, Gemmatimonadetes, Ignavibacteriae, Latescibacteria, Nitospirae, Percubacteria, Planctomycetes, Proteobacteria, Saccharibacteria, and Verrucomicrobia. Among all above mention phylums the dominating one are Acidobacteria, Actinobacteria, Bacteriodetes, Firmicutes, Planctomycetes, chloroflexi* and *Protobacteria*. Detail of these phylums were given in table 4.5.

TABLE 4.5: Showing Abundant Bacterial Phylum's OTU's

Phylum	Total OTU's among different samples					
	NP1	PN1	BH1	CN1	RJ1	C1
Acidobacteria	1280	13,343	2178	157	2404	763
Actinobacteria	3552	211	2866	3732	3808	3314
Bacteriodete	159	260	311	6938	480	448
Firmicutes	162	188	272	156	146	118
Planctomycetes	193	216	200	210	211	316
Protobacteria	17337	15552	20090	31296	31097	15573
Chloroflexi	4375	4212	373	17	4050	568

Acidiobacteria is abundant phylum in natural ecosystem. NP1 shows 1280, PN1 13343, BH1 2178, CN1 157, RJ1 2404, C1 763 OTU's of Acidobacteria. It is observed that exopolysaccharide producing bacteria has longer viability in soil. Due to high synthesis of exopolysaccharide they are dominant in acidic and chemically polluted environments with heavy metals, petroleum compounds. These bacteria are considered to be important contributors for ecosystem [99],[11],[121].

It is clear that Actinobacteria are nitrogen fixing bacteria. Actinobacters that have characteristics of fungi as well has role in recycling biomaterials are widely distributed in terrestrial and aquatic environments. In NP1 3552, PN1 211, BH1 2866, CN1 3732, RJ1 3808, C1 3314 OTU's of Actinobacteria were identified. More than 10,000 bioactive metabolites are found to be produced by these bacteria that are useful in natural products with potential applications [98]. For Example, streptomyces species are industrially important microorganism due to several useful bioactive natural products.

The species of Bacteroidetes play important role in protein metabolism by proteolytic activity. Some species can be utilized as source of urea as nitrogen cycle. In NP1 159, PN1 260, BH1 211, CN1 6938, RJ1 480, C1 448 OTU's of Bacteroidetes were identified. Significant abundance of phylum chlorofexi were also observed, in

NP1 4375, PN1 4212, BH1 373, CN1 17, RJ1 4050, C1 568 OTU's of Chloroexi are present [120].

The Firmicutes have mostly Gram-positive members that produce endospores that are resistant to desiccation due to which they can survive in extreme conditions. They are found in various environments. In NP1 162, PN1 188, BH1 272, CN1 156, RJ1 146, C1 118 OTU's of Firmicutes were identified.

Proteobacteria are ubiquitous in oil reservoirs over all temperature ranges. Species of Proteobacteria are al Gram-negative and they have outer membrane composed of lipopolysaccharides In NP1 17337, PN1 15552, BH1 20090, CN1 31296, RJ1 31097, C1 15573 OTU's of Protobacteria were identified. Planctomycetes are also present in NP1 193 , PN1 216 , BH1 200 , CN1 210 , RJ1 211, C1 316 OTU's represent significant abundance of these bacteria.

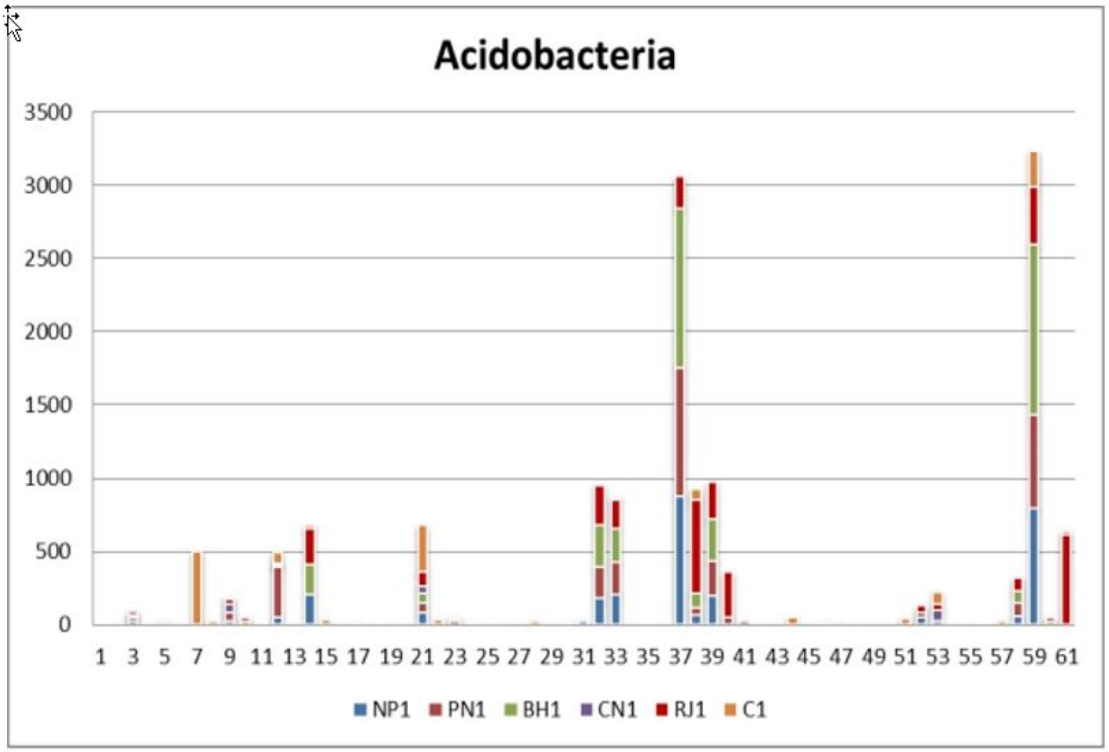

FIGURE 4.4: shows OTU's of acidobacteria among different samples, higher number of OTU's were observed in PN1 sample

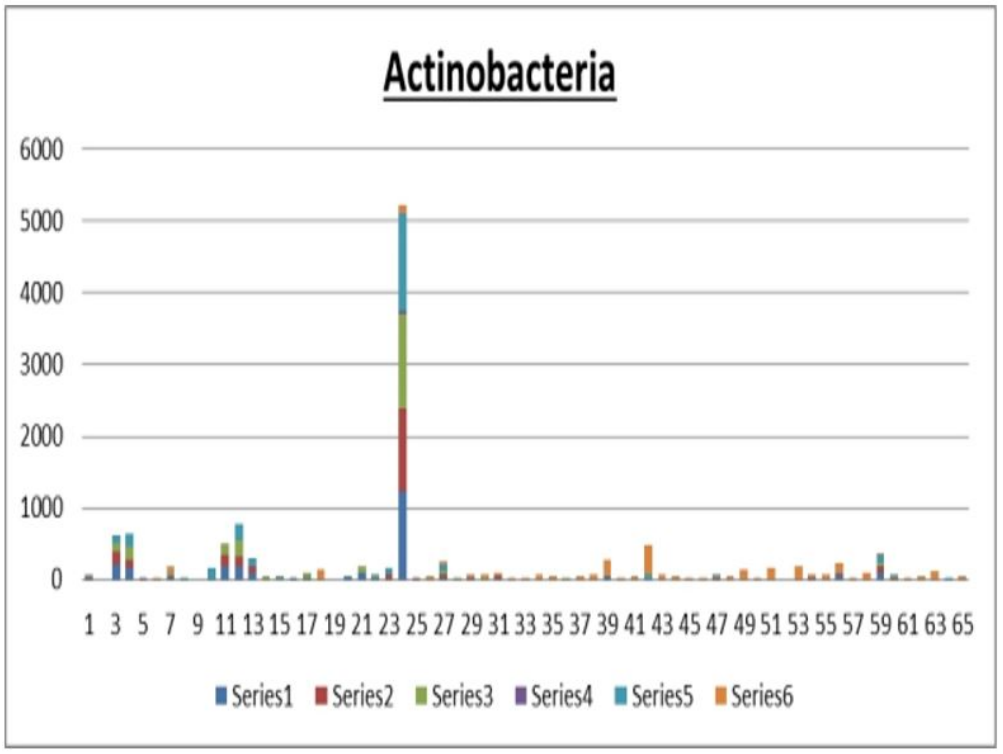

FIGURE 4.5: shows OTU's of actinobacteria among different samples, higher number of OTU's were observed in RJ1 sample.

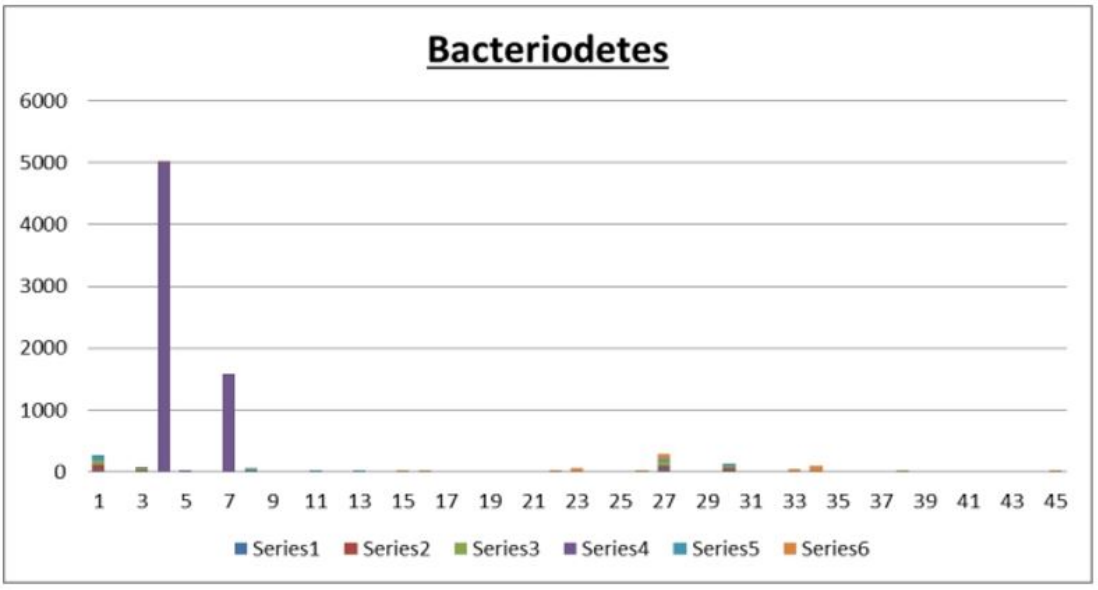

FIGURE 4.6: shows OTU's of bacteriodetes among different samples, higher number of OTU's were observed in CN1 sample.

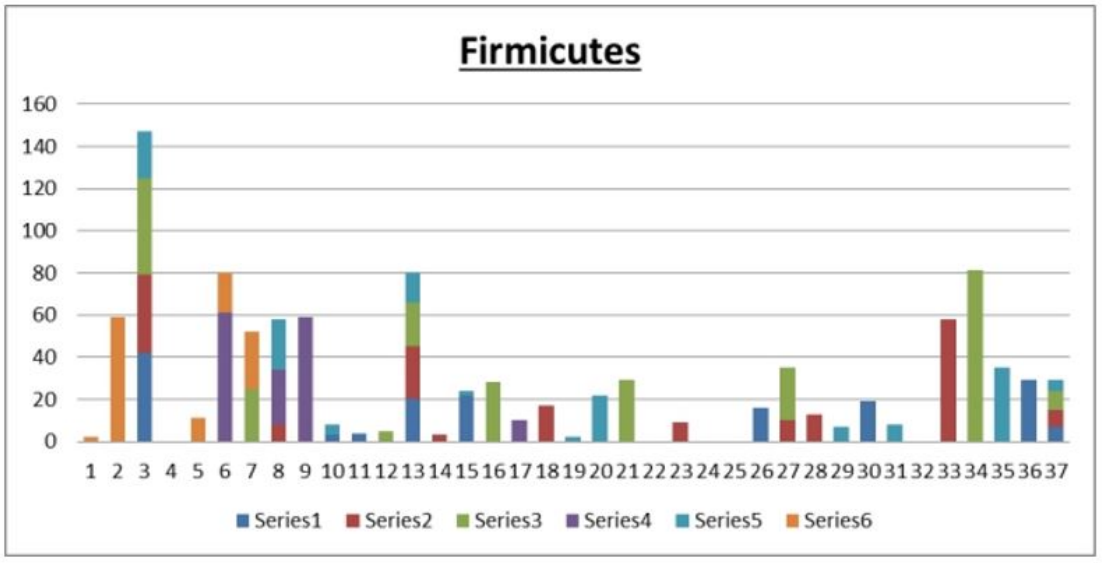

FIGURE 4.7: Shows OTU's of firmicutes among different samples, higher number of OTU's were observed in BH1 sample.

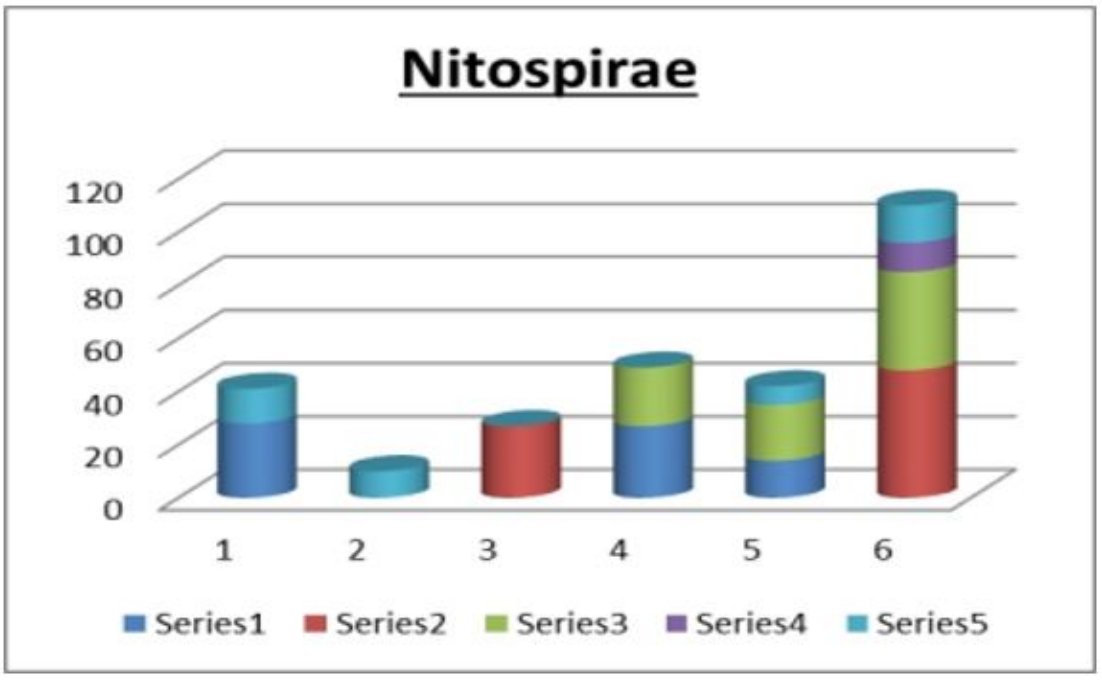

FIGURE 4.8: shows OTU's of nitospirae among different samples.

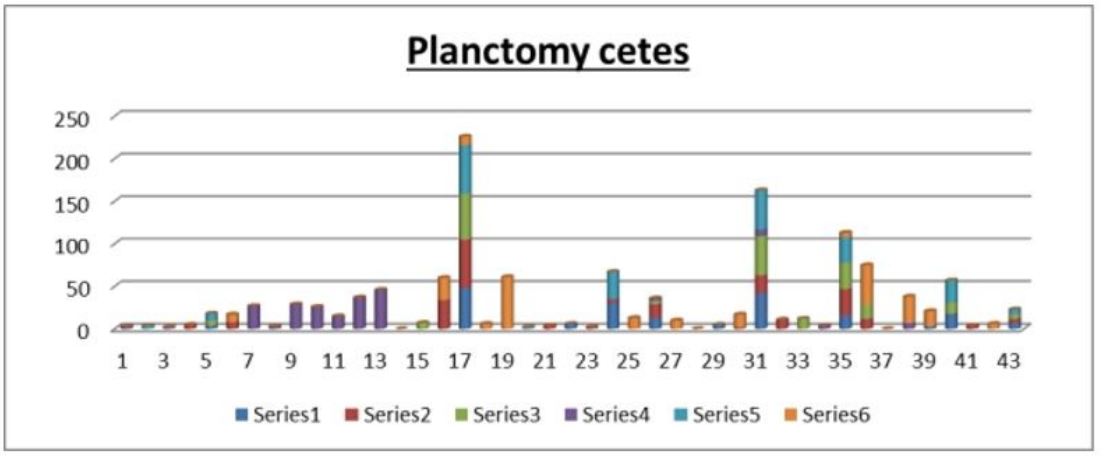

FIGURE 4.9: shows OTU's of planctomycetes among different samples, higher number of OTU's were observed in C1 sample.

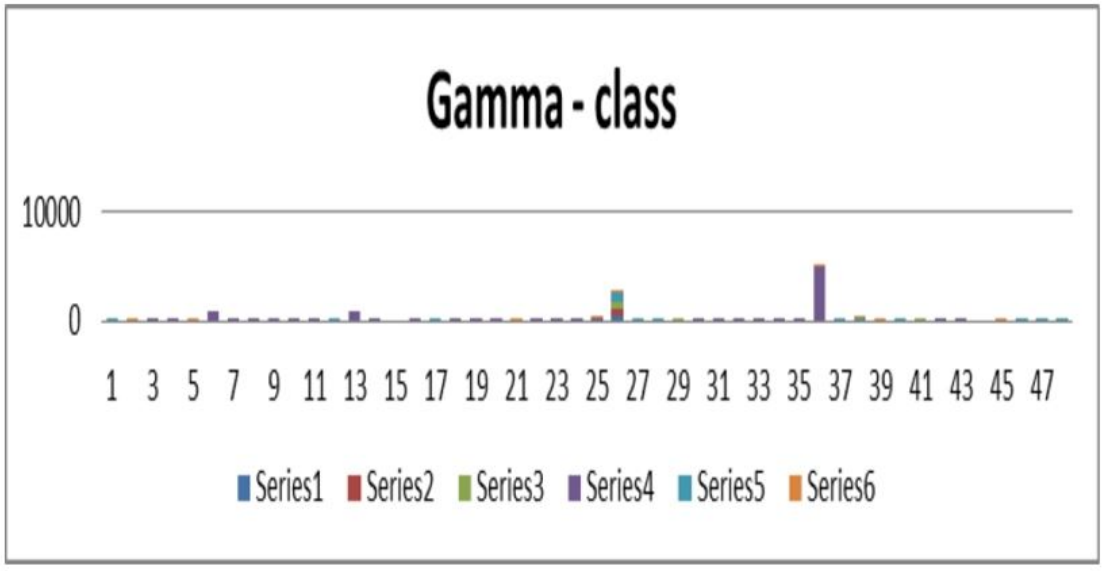

FIGURE 4.10: shows OTU's of Gamma-class among different samples.

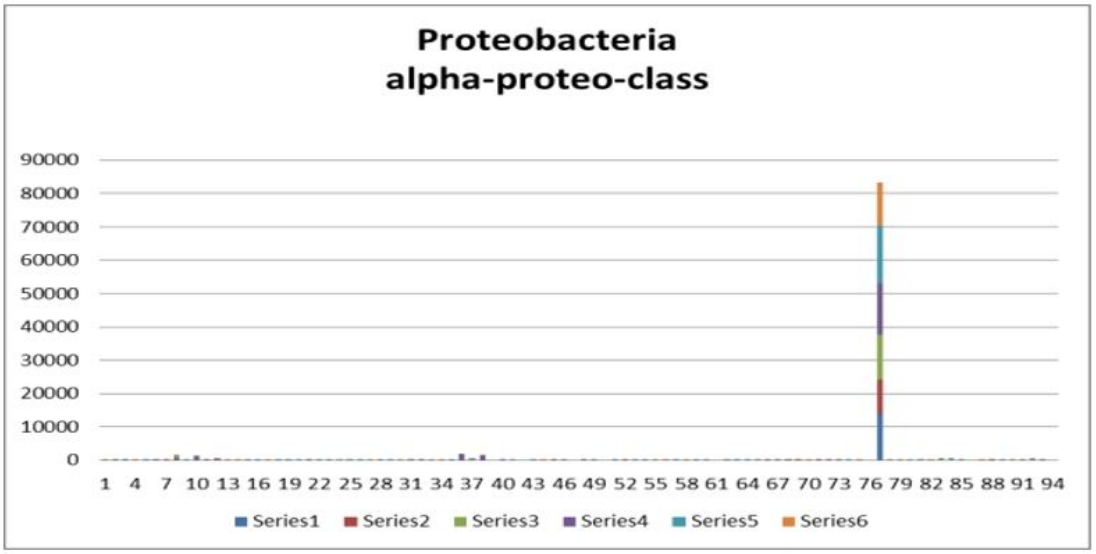

FIGURE 4.11: shows OTU's of alpha-proto-class among different samples.

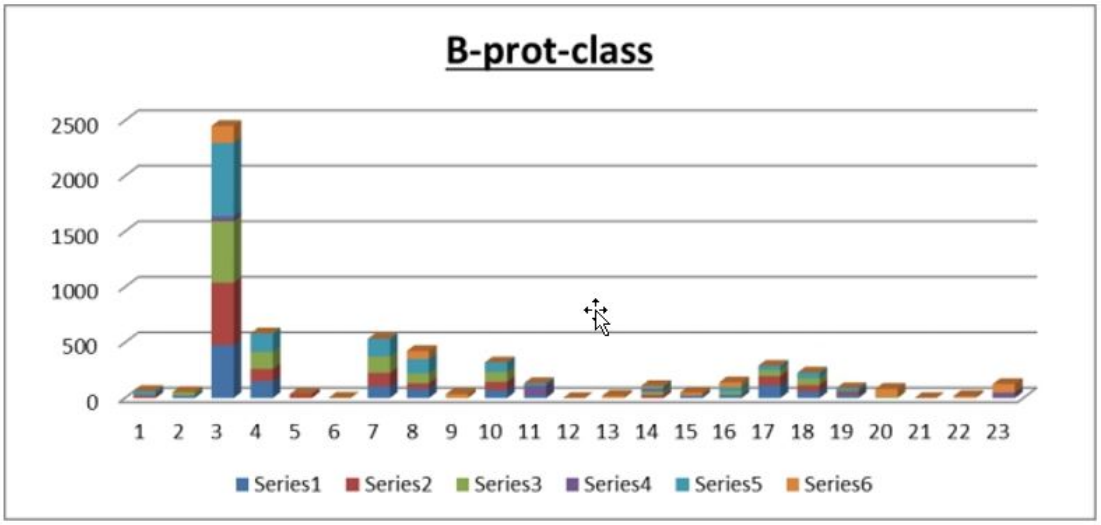

FIGURE 4.12: shows OTU's of B-prot-class among different samples.

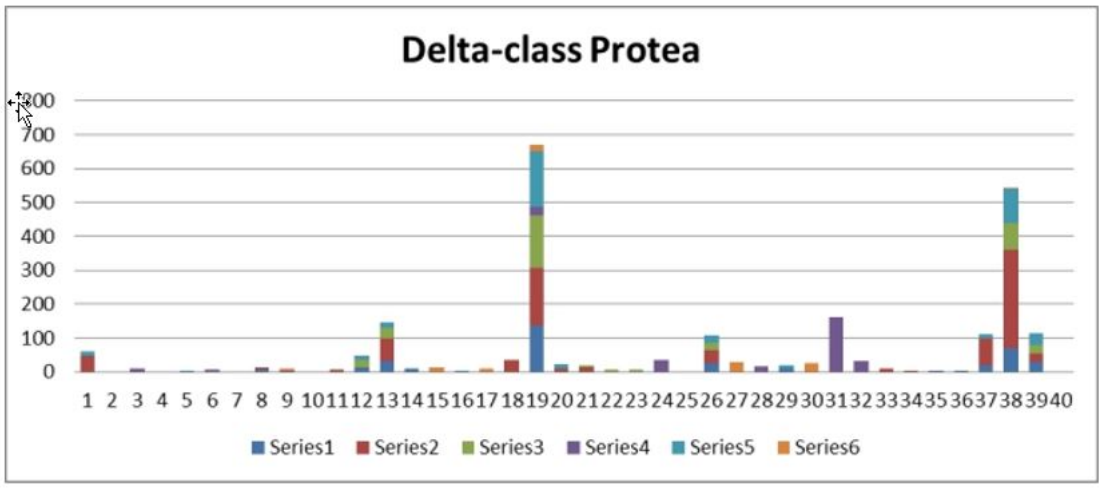

FIGURE 4.13: shows OTU's of Delta-class protea among different samples.

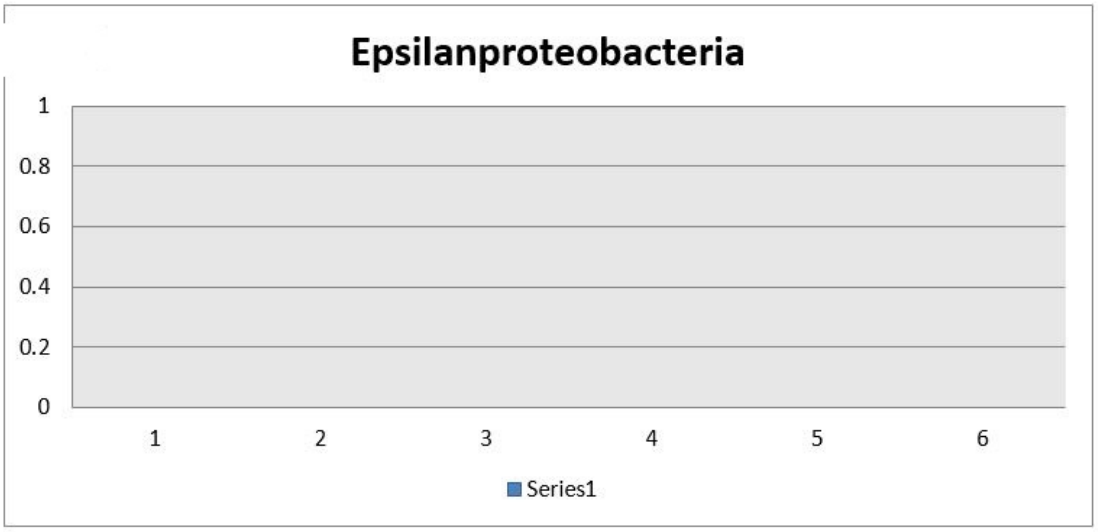

FIGURE 4.14: Epsilanproteobacteria

4.5 Phylogenetic Abundance Ammonia Oxidizing Bacteria

Table 4.6: Abundance of Ammonia-oxidizing Bacteria

Classification		Samples	Number of OTU's present in per sample
Kingdom	*Bacteria*	NP1	28
Phylum	*Nitrospirae*	PN1	0
Class	*Nitrospira*	BH1	0
Order	*Nitrospirales*	CN1	27
Family	*0319-6A21*	RJ1	14
Genus		C1	0
Specie			
Classification		Samples	Number of OTU's present in per sample
Kingdom	*Bacteria*	NP1	0
Phylum	*Nitrospirae*	PN1	0
Class	*Nitrospira*	BH1	27
Order	*Nitrospirales*	CN1	0
Family	*0319-6A21*	RJ1	0
Genus	*uncultured bacterium*	C1	48
Specie	*uncultured bacterium*		
Classification		Samples	Number of OTU's present in per sample
Kingdom	*Bacteria*	NP1	0
Phylum	*Nitrospirae*	PN1	0
Class	*Nitrospira*	BH1	0
Order	*Nitrospirales*	CN1	22

Table 4.6 continued from previous page

Family	*Nitrospiraceae*	RJ1	21
Genus	*Nitrospira*	C1	37
Specie	*uncultured Nitrospira sp.*		
Classification		Samples	Number of OTU's present in per sample
Kingdom	*Bacteria*	NP1	0
Phylum	*Nitrospirae*	PN1	0
Class	*Nitrospira*	BH1	0
Order	*Nitrospirales*	CN1	0
Family	*Nitrospiraceae*	RJ1	0
Genus	*Nitrospira*	C1	11
Specie	*uncultured Nitrospiraceae bacterium*		
Classification		Samples	Number of OTU's present in per sample
Kingdom	*Bacteria*	NP1	13
Phylum	*Nitrospirae*	PN1	10
Class	*Nitrospira*	BH1	0
Order	*Nitrospirales*	CN1	0
Family	*Nitrospiraceae*	RJ1	17
Genus	*Nitrospira*	C1	14
Specie			
Classification		Samples	Number of OTU's present in per sample
Kingdom	*Bacteria*	NP1	0
Phylum	*Proteobacteria*	PN1	26
Class	*Betaproteobacteria*	BH1	32
Order	*Nitrosomonadales*	CN1	19

Table 4.6 continued from previous page

Family	*Nitrosomonadaceae*	RJ1	22
Genus	*uncultured*	C1	10
Specie			
Classification		Samples	Number of OTU's present in per sample
Kingdom	*Bacteria*	NP1	23
Phylum	*Proteobacteria*	PN1	0
Class	*Betaproteobacteria*	BH1	0
Order	*Nitrosomonadales*	CN1	0
Family	*Nitrosomonadaceae*	RJ1	0
Genus	*uncultured*	C1	26
Specie	*uncultured Burkholderiales bacterium*		
Classification		Samples	Number of OTU's present in per sample
Kingdom	*Bacteria*	NP1	30
Phylum	*Proteobacteria*	PN1	0
Class	*Betaproteobacteria*	BH1	26
Order	*Nitrosomonadales*	CN1	0
Family	*Nitrosomonadaceae*	RJ1	42
Genus	*uncultured*	C1	44
Specie	*uncultured Oxalobacteraceae bacterium*		

4.6 Alpha Diversity Analysis

4.6.1 Alpha Box Plot of NO_{2-}

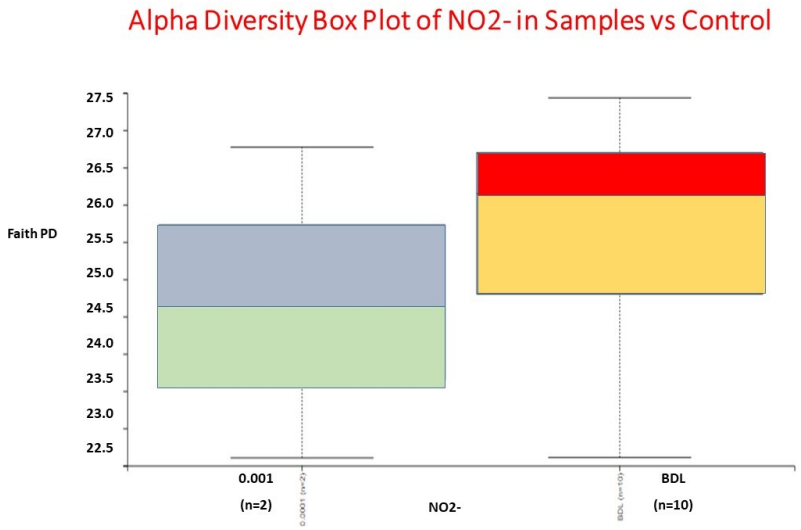

FIGURE 4.15: Box plot of sample sites and control against NO_2^- concentration

Figure 4.15 reveals that in all 10 samples NO_2^- was present in below detectable level against control where it was present in low range i.e., 0.001 as is shown in meta data table (Write table no) . It divides whole experimental samples into clear two categories of control vs petroleum soils. There is no significant difference among all five soil types if distribution of taxa (OTUs) is directly compared with effect of NO_2^- concentration. Tables below are also showing similar results.

TABLE 4.7: Alpha Diversity Plot NO_2 Gene

Group 1	Group 2	H	p-value	q-value
0.0001 (n=2)	BDL (n=10)	0.185915	0.666338	0.666338

TABLE 4.8: Kruskal-Wallis (All Groups)

	Result
H	0.18591549295774581
p-value	0.6663380847253242

TABLE 4.9: Kruskal-Wallis results for Faith pd and NO_2 concentration

id	NO^{-2}	faith-pd
types	categorical	numeric
NP1	BDL	27.43249114
NP2	BDL	26.708537037
PN1	BDL	26.364818734
PN2	BDL	22.607914096
BH1	BDL	22.607914096
BH2	BDL	26.658818552
CN1	BDL	25.928865265
CN2	BDL	24.428165446
RJ1	BDL	26.905348133
RJ2	BDL	25.928865265
C1	0.0001	22.60314447
C2	0.0001	26.770058245

Whole data is divided into two groups. Group 1 has 2 controls and group 2 has all sample sites soils. 0.6 p and q value is also indicator or no significant effect of OTUs distribution by NO_2^- concentration.

4.7 Beta Diversity Analysis

4.7.0.1 PCO-A Electrical Conductivity pH, Cu, Fe

Figure 4.17 and 4.18 The X-axis of the graph represents the variable physicochemical characteristics of Cu, Fe, $SO_4{}^{-2}$, H_2S, $PO_4{}^{-3}$, $NO_3{}^{-2}$ for effect of Electrical conductivity and PH respectively. while y-axis shows the values of pielou-e

Black dots are representing Sampling sites and size of dots is representing the concentration of OTUs. Graphs show positive effect of variables EC, pH, Cu ,

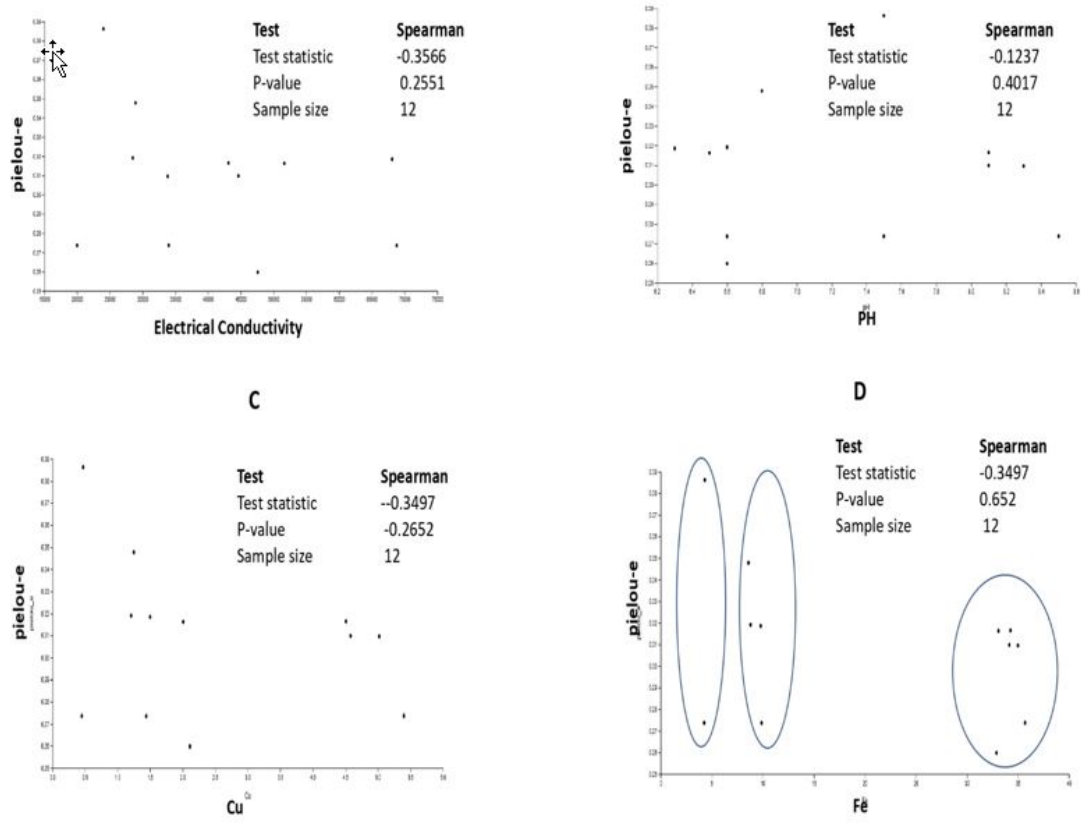

FIGURE 4.16: PCO-A Electrical Conductivity (a)

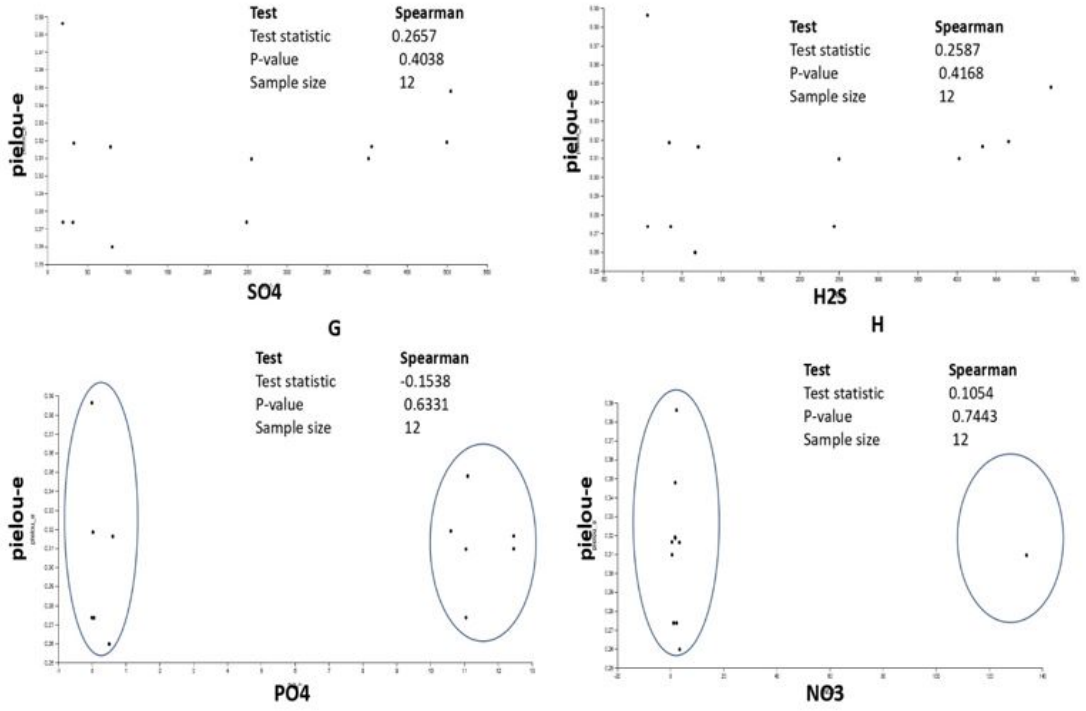

FIGURE 4.17: PCO-A Electrical Conductivity (b)

SO_4^{-2}, H_2S, respectively. But Nitrate and Fe showed clustering of sampling sites based on their effects. Spearman showed P-value 0.2551 for electrical conductivity that shows significant effect on distribution of OTUs.

P-value showed by spearman for PH depicted value of 0.4017 that has significant effect on distribution of OTUs.

P-value for 12 sample size showd value of 0.2652 for Cu element that represents positive effect of Cu on distribution of OTUs.

P-value for Fe is 0.652 does not represent significance effect of Fe on distribution of OTUs. Spearman showed P-value of SO_4^{-2} (0.4038) that represent significant effect of SO_4^{-2} on distribution of OTUs.

P-value of H_2S by spearman 0.4168 showed significant effect of H_2S on distribution of OTUs.

P-value of PO_4^{-3} and NO_3^{-2} by spearman 0.6331 and 0.7443respectively showed no significant effect of PO_4^{-3} and NO_3^{-2} on distribution of OTUs.

4.7.0.2 CCA (Canonical Correlation Analysis

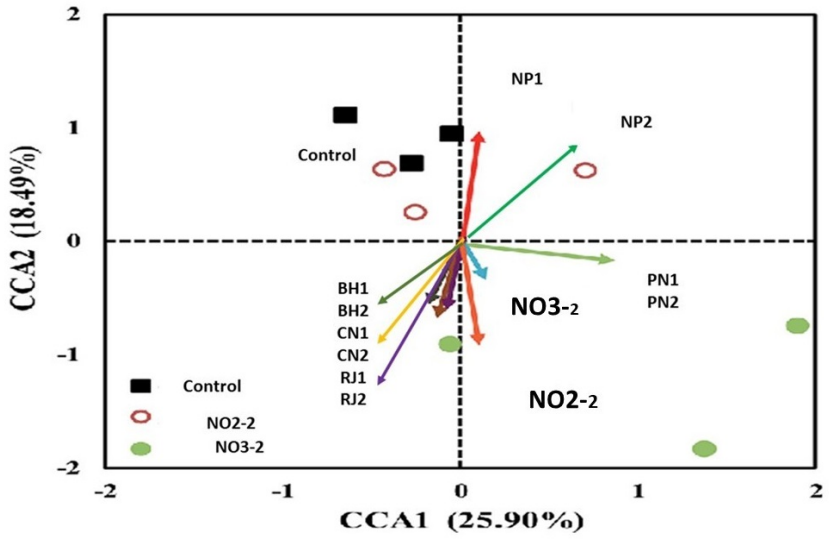

FIGURE 4.18: Canonical Correlation Analysis (CCA) was plotted at CC1 with 25.90% coordinates and at CCA2 with 18.49% coordinates.

CCA showing 3 dimensional coordinates with one controlled direction. CCA dimension 1 showing Sulphur percentage in PN1 and PN2. Red dots are presenting $SO_4{}^{-2}$ black are depicting control. Arrows shows presence of OTUs of a specific sampling site.

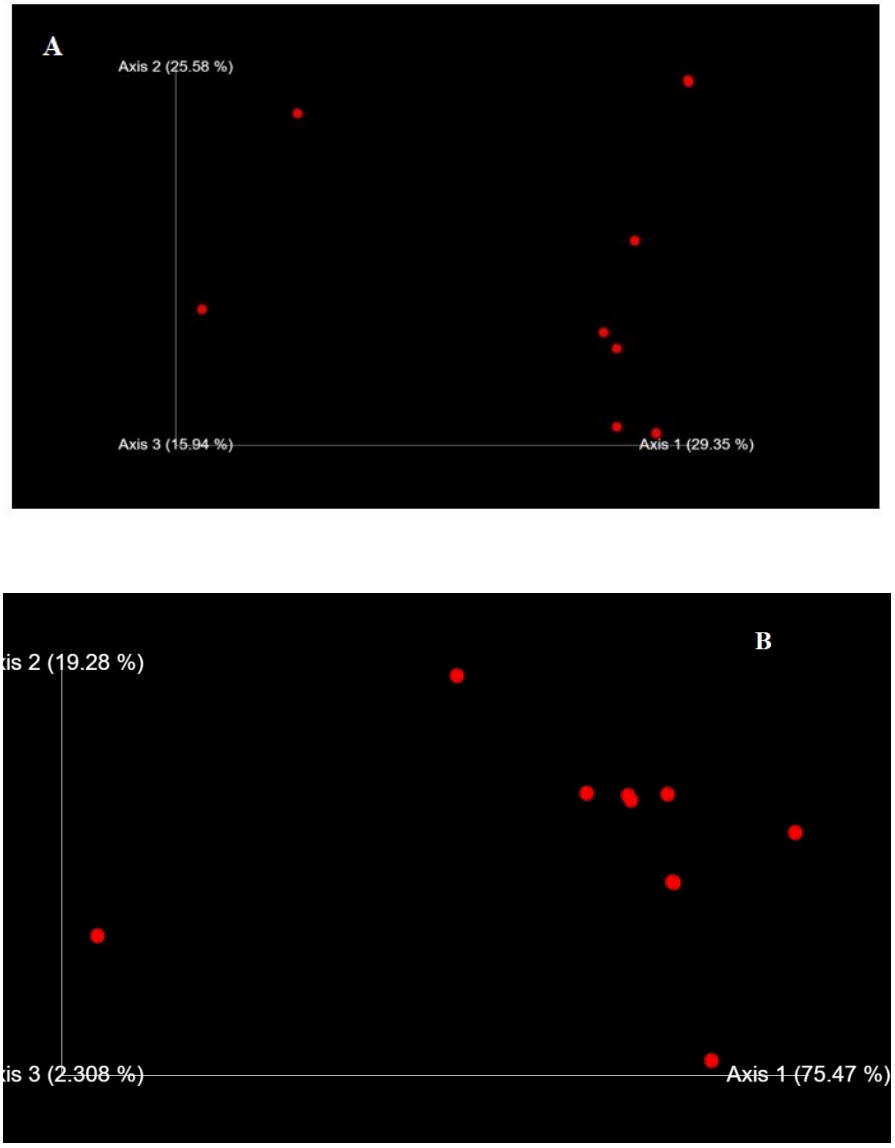

FIGURE 4.19: (A) is showing weighted unifrac emperor plot with Axis 1, Axis 2 and Axis 3 at 29.35%, 25.58 % and 15.94% respectively and (B) is showing unweighted unifrac emperor plot with Axis 1,2 and 3 at 75.47%, 19.28% and 2.3 % respectively

Figure 4.19 is representing Emperor plot which has the ability to visualize gradients, visualize different principal coordinates axes. Our data is presented in the

form of parallel coordinates and is showing taxa as red dots. Environmental samples dynamics are adjusted in the varying sizes. In figure above all spheres are of almost same size that showed the effects of metabolites equally on distribution of OTUs in three-dimensional space.

Table 4.10: Demultiplexed sequence counts summary

Minimum:	84502
Median:	87306.5
Mean:	86934.5
Maximum:	88981
Total:	1043214

Table 4.11: Shows per-sample sequence counts of 12 samples.

Sample name	Sequence count
CN2	88981
C1	88680
C2	88039
RJ1	88039
BH2	88039
NP1	87438
RJ2	87175
CN1	87175
NP2	85700
BH1	84723
PN2	84723
PN1	84502

In figure 4.20 graph depicted the per sample sequence counts higher number of sequence count is observed in CN2 samples shows that this sample contains maximum numbers of different microbial and archaeal species as compared to other samples.

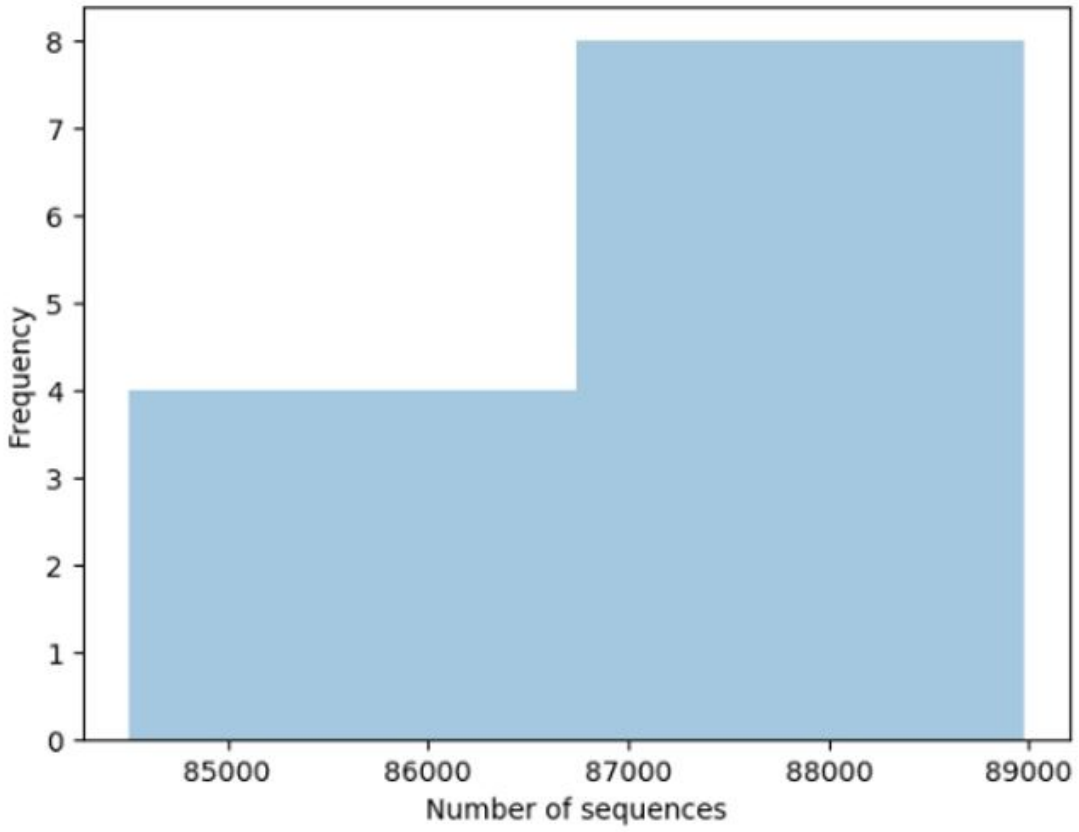

FIGURE 4.20: Shows per-sample sequence counts of 12 samples.

4.8 Discussion

Taxonomy of archaea in five different fields and in control sample, above results depicted that there are number of archaea's present in the oil fields. Phylum *Euryarchaeota* (Family *Halobacteriaceae*) shows its abundance in BH1 field, 5 OTUs' were present. Family *Mthanobacteriaceae* shows its abundance in NP1 (31 OTU's), PN1 (20 OTU's), BH1 (16 OTU's) and RJ1(31 OTU's) [104]. Family *Methanosarcinaceae* is only found in PN1 (4 OTU's). Family *Thermoplasmalates* Incertae Sedis in BH1 (6 OTU's). Most of the archea isolated from oil fields belong to *Euryarchaeota* phylum [103-105].

The most diverse group of microorganisms in *archaea* is related to phylum *Euyarchaeota* that has adapted most extreme environments. This phylum includes thermophiles, mesophiles, and psychrophiles. Some microbes including both aerobes and anaerobes live at extreme temperatures from 41 to 122 degree Celsius.

Acidophiles members can live in highly acidic and halophytes in high salty environments. These microbes are different from others on the bases of ribosomal RNA and unique DNA polymerases [106-107]. Members of this phylum has diverse appearance and metabolic properties. They are in rods or cocci shapes, either Gram-positive or Gram-negative. They can be methanogens, halophytes and sulphate reducers. They are found in oceans. Although marine members of this phyla are difficult to culture but genomic sequence studies suggest that they are motile heterotrophs [106],[111]. *Euryarchaeota* are also habitants of several moderate environments like water springs, marshlands, soil and rhizospheres. They are also known as highly adaptable e.g halobacterials order can live and grow in high salt concentrations to slow salt concentrations such as in sea water. Members of this phyla lack defence mechanisums against oxidative stress (ROS) [109-111].

Phylum Thaumarchaeota is indentified in PN1(10 OTU's), RJ1 (10 OTU's) and C1 (482 OTU's) of Soil Crenarchetic Group, 25 OTU's of genus Candidatus Nitrososphaera were also identified in C1. Thaumarchaeota members are ammonia-oxidizing organisms that live in soil, marine and hot springs habitats [112-122]. They are autotrophs and fix cabondioxide and few of them are dependent on other bacteria or small amounts of organic matter. Members of this phyla are also capable of oxidizing methane. Marine Thaumarchaea members produce nitrous oxide that is greenhouse gas and has role in climate change.

Among all phylum's the bacterial phylum's dominating one is *Acidobacteria, Actinobacteria, Bacteriodetes, Firmicutes, Planctomycetes, Protobacteria,* and *Chloroflexi*. Detail of these phylums. *Acidiobacteria* is abundant phylum in natural ecosystem. NP1 shows 1280, PN1 13343, BH1 2178, CN1 157, RJ1 2404, C1 763 OTU's of *Acidobacteria*. It is observed that exopolysaccharide producing bacteria has longer viability in soil. Due to high synthesis of exopolysaccharide they are dominant in acidic and chemically polluted environments with heavy metals, petroleum compounds. These bacteria are considered to be important contributors for ecosystem [99],[11],[121]. It is clear that *Actinobacteria* are nitrogen fixing

bacteria. Actinobacters that have characteristics of fungi as well has role in recycling biomaterials are widely distributed in terrestrial and aquatic environments. In NP1 3552, PN1 211, BH1 2866, CN1 3732, RJ1 3808, C1 3314 OTU's of Actinobacteria were identified. More than 10,000 bioactive metabolites are found to be produced by these bacteria that are useful in natural products with potential applications [98]. For Example, *streptomyces* species are industrially important microorganism due to several useful bioactive natural products.

The species of *Bacteroidetes* play important role in protein metabolism by proteolytic activity. Some species can be utilized as source of urea as nitrogen cycle. In NP1 159, PN1 260, BH1 211, CN1 6938, RJ1 480, C1 448 OTU's of *Bacteroidetes* were identified. Significant abundance of phylum *chlorofexi* were also observed, in NP1 4375, PN1 4212, BH1 373, CN1 17, RJ1 4050, C1 568 OTU's of *Chloroflexi* are present [120].

The *Firmicutes* have mostly Gram-positive members that produce endospores that are resistant to desiccation due to which they can survive in extreme conditions. They are found in various environments. In NP1 162, PN1 188, BH1 272, CN1 156, RJ1 146, C1 118 OTU's of *Firmicutes* were identified. Proteobacteria are ubiquitous in oil reservoirs over all temperature ranges. Species of Proteobacteria are al Gram-negative. In NP1 17337, PN1 15552, BH1 20090, CN1 31296, RJ1 31097, C1 15573 OTU's of Protobacteria were identified. Planctomycetes are also present in NP1 193, PN1 216, BH1 200, CN1 210, RJ1 211, C1 316 OTU's represent significant abundance of these bacteria [121].

Phylum Proteobateria, Class Betaproteobacteria include order Nitrosomonadales showed 83 OTUs in NP1, 26 OTUs in PN1, 58 OTUs in BH1, 19 OTUs IN CN1, 64 OTUs in RJ1 and 80 OTUs in C1. Members of this class are Gram-negative. Morphologically they are either rods, spirillae or curved rods. They are chemolitho autotrophs and some are also methylotrophs and heterotrophs. They play significant role in nitrogen cycle in terrestrial, freshwater and marine environment and are significantly important for economic and environment, that leads to loss of ammonium-based fertilizers, nitrous oxide production and nitrate

pollution. Betaproteobacteria also showed 53 OTUs in NP1, 26 OTUs in PN1, 58 OTUs in BH1, 19 OTUs in CN1, 64 OTUs in RJ1 and 80 OTUs in C1. This class is comprised of 75 genera and 400 species of bacteria.

Betaproteobacteria are photo-heterotrophic as they drive energy and electrons form organocorbon sources. They are also autotropic that drives energy from light and electrons from reduced inorganic ions such as nitrite, ammonium, thiosulfate or sulfide. Betaproteobacteria maintain soil pH, use nitrate as their terminal electron acceptor and they can be used in industries to remove nitrate from wastewater.

Burkholderiales (order) comprises the families Burkholderiacae (type family) consist of several morphologies that include rods, curved rods, cocci, spirillae and multicellular tablets Burkholderia are heterotrophs, photohetertrophs and facultative autotrophs, commonly found in soil and groundwater. They are also found at high temperatures upto 70 degree Celsius of Artic Soil. Oxalobacteraceae family of Burkholderiales are Gram-negative, this family includes aerobes, strict anaerobes and nitrogen fixing bacterial members.

Nitrospira is nitrite-oxidizing bacteria that are chemolithautotrophic found in freshwater as well as in saltwater showed 41 OTUs in NP1, 10 OTUs in PN1, 27 OTUs in BH1, 52 OTUs in RJ1, 47 OTUs in CN1 and 11O OTUs in C1. These bacteria use inorganic carbon (like HCO^{3-} and CO_2) and pyruvate in aerobic conditions. Members of this group are important for marine ecosystem as well as for wastewater treatment plants and laboratory scale reactors as they are main nitrite oxidizers.

Chapter 5

Conclusion and Recommendations

The basic task of Microbial Enhanced Oil Recovery technology is to solve the problem and issues of microorganisms that are based on the findings of field trials, the diversity and distribution of microorganisms that are related to MEOR mechanism and their function on the MEOR effect by the use of nutrients injected into the reservoir. In general, biotechnology and microbiology, which have an impact on the advancements of oil and gas field, to resolve the several issues involved in the EOR or IOR.

However, most of these innovations hinder the further production of oilfields due to their economic limits. From many years the Microbial Enhanced Recovery (MEOR) of Petroleum technique has been recommended as a cheap and effective solution for enhanced oil recovery. Microbes are rich in diversity within the specified reservoirs. Activated microbes may generate multi-functions during oil displacement. Microbes could freely move inside the porous medium Over nearly a century, MEOR's potential developments have been surpassed by others on the grounds of their economic efficiency and environmental protection. Gradually, tertiary oil recovery technology is becoming an effective technique, particularly for the exploitation of high-water and heavy oil reservoirs. In order to overcome its

shortcomings by promoting more industrial applications, the implementation of MEOR could, in the future, concentrate on the following aspects: 1. Development of a practical microbial library: The nature and distribution of indigenous microorganisms under target oil reservoirs should be investigated and evaluated using molecular cloning technology. Microbial candidates could then be isolated and classified on the basis of their different oil-displacement functions. 2. Specific analysis of the MEOR system: a mechanism research will rely on one or more specific microorganisms. Such cells could be chosen from a generic microbial collection that could have different oil-displacement roles. Consequently, their MEOR functions are examined, including structural gene expression, functional enzyme development and essential biochemical pathways, etc.

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