CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Resistome Distribution Vs Total Bacterial Diversity in Five Major Glaciers of Pakistan

by

Farhana Khalil

A thesis submitted in partial fulfillment for the degree of Master of Science

in the

Faculty of Health and Life Sciences
Department of Bioinformatics and Biosciences

2020

I dedicate this thesis to my parents and my teachers.

CERTIFICATE OF APPROVAL

Resistome Distribution Vs Total Bacterial Diversity in Five Major Glaciers of Pakistan

by

Farhana Khalil

(MBS183003)

THESIS EXAMINING COMMITTEE

S. No.	Examiner	Name	Organization
(a)	External Examiner	Dr. Nadia Faqeer	UAA, Rawalpindi
(b)	Internal Examiner	Dr. Shaukat Iqbal Malik	CUST, Islamabad
(c)	Supervisor	Dr. Arshia Amin Butt	CUST, Islamabad

Dr. Arshia Amin Butt
Thesis Supervisor
October, 2020

Dr. Sahar Fazal
Head
Dept. of Biosciences & Bioinformatics
October, 2020

Dr. Muhammad Abdul Qadir
Dean
Faculty of Health & Life Sciences
October, 2020

Author's Declaration

I, **Farhana Khalil** hereby state that my MS thesis titled "**Resistome Distribution Vs Total Bacterial Diversity in Five Major Glaciers of Pakistan**" is my own work and has not been submitted previously by me for taking any degree from Capital University of Science and Technology, Islamabad or anywhere else in the country/abroad.

At any time if my statement is found to be incorrect even after my graduation, the University has the right to withdraw my MS Degree.

(Farhana Khalil)

Registration No: MBS183003

Plagiarism Undertaking

I solemnly declare that research work presented in this thesis titled "**Resistome Distribution Vs Total Bacterial Diversity in Five Major Glaciers of Pakistan**" is solely my research work with no significant contribution from any other person. Small contribution/help wherever taken has been dully acknowledged and that complete thesis has been written by me.

I understand the zero tolerance policy of the HEC and Capital University of Science and Technology towards plagiarism. Therefore, I as an author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/cited.

I undertake that if I am found guilty of any formal plagiarism in the above titled thesis even after award of MS Degree, the University reserves the right to withdraw/revoke my MS degree and that HEC and the University have the right to publish my name on the HEC/University website on which names of students are placed who submitted plagiarized work.

(Farhana Khalil)

Registration No: MBS183003

Acknowledgements

In the name of **Almighty Allah** the most gracious the most merciful, who blessed us with life, whose blessings are countless, whose mercy is everlasting, who is eternal, whose provisions are un-ending, and who loves us more than anyone loves us, whose worship is our faith. Simply I have no words to show my love my gratitude towards my Allah. I would also love to show acknowledgement towards the Holy Prophet Hazrat Muhammad (P.B.U.H) who is the last prophet of Allah. He who led us to the righteous path. It's a great honor for me to be the follower of him.

I would love to express my gratitude towards my research supervisor **Dr. Arshia Amin Butt** for her support and guidance throughout my research. Her patient behavior and motivation helped me a lot to overcome all the difficulties a faced during my research work. Her good command on the research problems helped me to reach at this stage. I cannot thank her for her support and guidance.

I would like express my gratitude to Dr. Sahar Fazal (Head Of the Department of Biosciences) for her support and guidance during my whole journey in this university. She is such a loveable and humble personality. I express my profound gratitude to my parents for their unconditional support and absolute trust in me allowed me to reach this stage. This accomplishment has not been possible without their support and prayers. I would like to express my heartiest gratitude to my brothers for their support. They helped me to fulfill my dreams.

In the end I would also thanks to my Beloved sister Memoona Khalil and to my friends especially Nazish Zareef , Asia Parveen , Rabiya Shahid , Saira Sadiq and all those who were directly or indirectly involved in enabling to complete this study.

Thanks to all

(Farhana Khalil)

Registration No: MBS183003

Abstract

"*Where there is water, there is life*" this reveals the existence of microbial community in active form. The northern glaciers of Pakistan are longest mid latitude and largest glaciers among world with an area estimated 15,000 km^2. The bacterial community colonizing glaciers in Pakistan are diverse depending upon the area and the type of glaciers studied. The present study aims to unveil the non culturable microbial diversity of the snow sampled from different glacierized area Kamri (Km), Burzil (Bz), Siachin (Sc), Baltoro (Bt), Control (C) in both the winter and in summer season through DNA Sequencing. This study adds to our understanding through Resistome Distribution Vs Total Bacterial Diversity in five major glaciers of Pakistan. In current study the glacial samples were collected from 5 accessible sites from Km, Bz, Sc and Bt Glaciers. There were about 10 samples collected from each site according to Quadrate method for selection Five samples were collected in winter season and five samples were collected in summer season. Two control samples were collected for comparison. 16S rRNA gene sequencing and various physicochemical parameters (pH, temperature, depth, EC, Sulphate, Nitrate, P, K, and Ca-Mg content) as well resistome and bacterial diversity were studied. This study reports metagenomic analysis done through Alpha and Beta diversity analysis to find out the diversity of bacterial communities and their correlation of environmental parameters with the antibiotic resistant gene. The most abundant group was *Proteobacteria* followed by *Actinobacteria*, *Firmicutes*, and then Bacteriodetes was revealed by the bacterial 16S rDNA sequence analysis. The *Proteobacteria* had the highest diversity in all the selected glaciers. In this study physicochemical characteristics of micro and macro nutrients were measured by using AAS method. The physicochemical features of the samples which were collected from the 5 glaciers and the parameters were depth, temperature, pH(power of hydrogen), EC(electrical conductivity), Sulphate, nitrate, phosphorous, Potassium, Calcium – Magnesium, pH ice and pH snow respectively. The PCA and CCA analysis was done in the beta diversity analysis to shows the correlation between the environmental aspects and the resistome gene. Different resistant

genes like Tetracycline, Sulfonamide, Microlids, Quinolone, Aminoglycoside and Beta Lactams were found in the sample.

Contents

List of Figures

List of Tables

Abbreviations

ARDB	Antibiotic Resistance Gene Database
ARG	Antibiotic Resistance Gene
Bt	Baltoro
Bz	Burzil
C	Control
CCA	Canonical Correlation Analysis
EC	Electrical Conductivity
Km	Kamri
PCA	Principle Component Analysis
Sc	Siachin

Chapter 1

Introduction

1.1 Background

Glaciers are formed by the accumulation of snow year by year or for hundreds to thousands of years, these glaciers contain microorganisms depending upon the climate. Seasonal temperature fluctuations, UV irradiation and high intensity light directly effect upon the microorganisms found in the glaciers that help out in their survival. The cryoconite holes in the glaciers having an important key role in the most biological active environment and adaptation to the cold. Little debris particles which absorb more solar rays than the surrounding ice form these [1], the microbial habitat for the microorganisms and these holes are enrich in organic matter and nutrients for the nourishment of microbes.

Regardless of the re-emergence of ancient bacteria, current natural communities face new experiences. Such threats are exacerbated by the re-emergence of pathogens stored in labs or by pathogens preserved in the glacial ice, which seem to be accessible at thawing caused by climate change. Here, we concentrate on the evidence of such a re-emergence in natural environments to examine the form of bacteria present for centuries in the absence of any natural host. We are aiming to explore resistome spreaded across the selective sites. connection between total resistome will be made with total bacterial classes present them.

1

1.2 Glaciers in Pakistan

In Pakistan more than 5000 glaciers were reported ranges from few tens of meter to 70 km long. Northern Pakistan's glaciers are some of Earth's largest and longest mid-latitude glaciers and are spread over an area of 16933 square kilometers. The largest glaciers are most frequently found in the mountains of Hindu Kush, Himalaya and Karakoram, covering an area of about 15,000 square kilometers. [2]. Siachin is 76 km in length, karakoarm is 55km in length the largest glaciers in this region, it is therefore also called Earth's third pole. HKKH distributed in Afghanistan, Bangladesh, Bhutan, China, India, Myanmar, Nepal and Pakistan over an area of more than 4.3 million square kilometers. Such mountain ranges include all the world's 14 peaks, over 8,000 m in height, and 108 peaks are more than 6,000 m high [3]. The glaciers are major drinking water supply basins, and
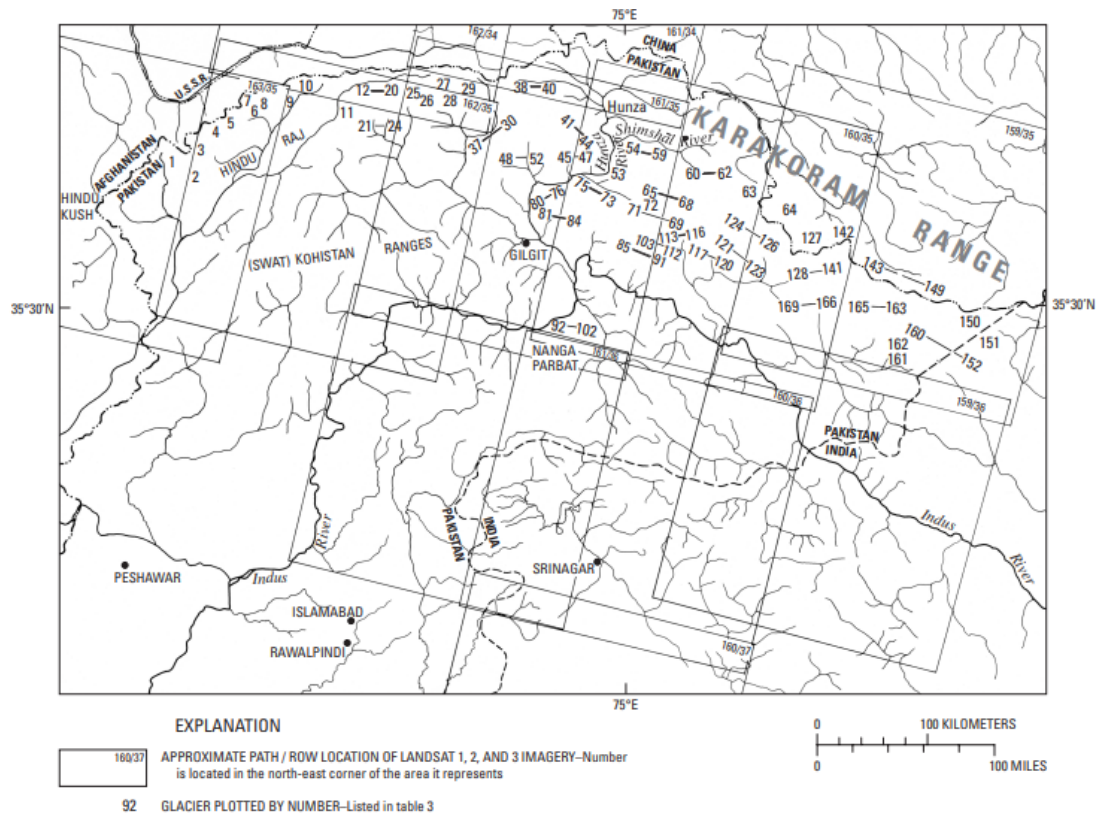
FIGURE 1.1: map showing the geographic distribution of glaciered region of Pakistan.

their daily melting of ice nourishes about sixty large and small country rivers.

Such reservoirs preserve and store water that is important for Pakistan's development and economy. Depletion in the glaciers would negatively impact irrigation, providing water sources, hydroelectric power and natural ecosystems. The effect would adversely affect Pakistan's economy. This reduction of glaciers and change in climate worldwide have triggered freshwater floods [3].

1.3   Microbes found in Glaciers

Microbes play a significant role in subglacial weathering [4], and make availability for the minerals other nutrients for lifeforms which is significant in climate change [5]. The previous studied shows that microbial community include bacteria, archaea, fungi and viruses also. Few microbial active organisms were identified in the extreme environment, glaciers having confined habitat and are highly selective which make the environment favorable for adaptation and speciation [6]. Microorganisms residing snow habitats are typically subjected to high light and ultraviolet irradiation as well as variations in periodic conditions. Glacier ice is a special habitat that chronologically retains microorganisms and historic seasonal changes for many of years.Almost 1 -10% of Earth terrestrial layer holds 77% of planets's fresh water which is mostly present in the Greenland and Antarctica's ice sheet of glacier ice region. Together, polar and temperate glaciers occupy an area of 15,861,766 square kilometers on other continents. There are more than 46,000 glaciers in China, and the bulk of other glaciers are above 5,000 meters in altitude. The ice depths of the glaciers ranges from few hundred meter to three to four kilometers, with a with a steady growth in depth and temperature. This ranges is from -50 ℃ on the upper layer to -6 ℃ to -10 ℃ in the lowest layer.

1.4   Surveillance of Microbes

Glaciers having an extreme cold habitat in which every type of microbes cannot survive only few can be viable that are tolerant against the low temperature and

thrive in the harsh conditions. All the microbes that grows at temperature less than 200C are called psychrophilic microbes [7]. These have the ability to cope up with low pH, high concentration of metals , low availabity of nutrients and no light mostly bacteria found in glaciers are anaerobic [8]. In glaciers bacteria are present on large scale , there are culturable and non-culturable bacteria presents only 1 % of the bacteria are culturable in the environment and ninety-nine percent of microbes are unculturable due to lack of suitable natural environment condition [9].

1.5   Resistome

The term Resistome was proposed to cover the both nonpathogenic as well as pathogenic bacteria consisting of all ARs and their precursors [10]. Resistome studies of soil, wastewater, and other environments have shown a great variety of ARs for major antibiotic classes. The resistance / tolerance mechanisms to antimicrobials and heavy metals can also encourage antibiotic resistance [14]. For a thorough resistome analysis of an area, therefore, the co-selection events need to be discussed. Events of co-selection occur by cross-resistance, or by co-resistance [5], Cross-resistance is associated with the acquisition of ARs without direct antibiotic exposure by employing mechanisms of resistance to heavy metals / biocides, like efflux pumps. Co-resistance results from the genetic association of genes that impart antimicrobial resistance, though they may be not related. These genes are also present in integrons, transposons or plasmids that confer multidrug resistance in a single horizontal gene transfer (HGT) event [14].

1.5.1   Antibiotic Resistome

Resistance has contributed to inevitability to suggest the antibiotic resistome concept [15]. It consists of all AR genes in pathogens, antibiotic producers and benign bacteria in the environment. Antibiotic resistance at organism level can result from

the complex interplay between genes and their products resulting from exposure to toxic molecules [16].
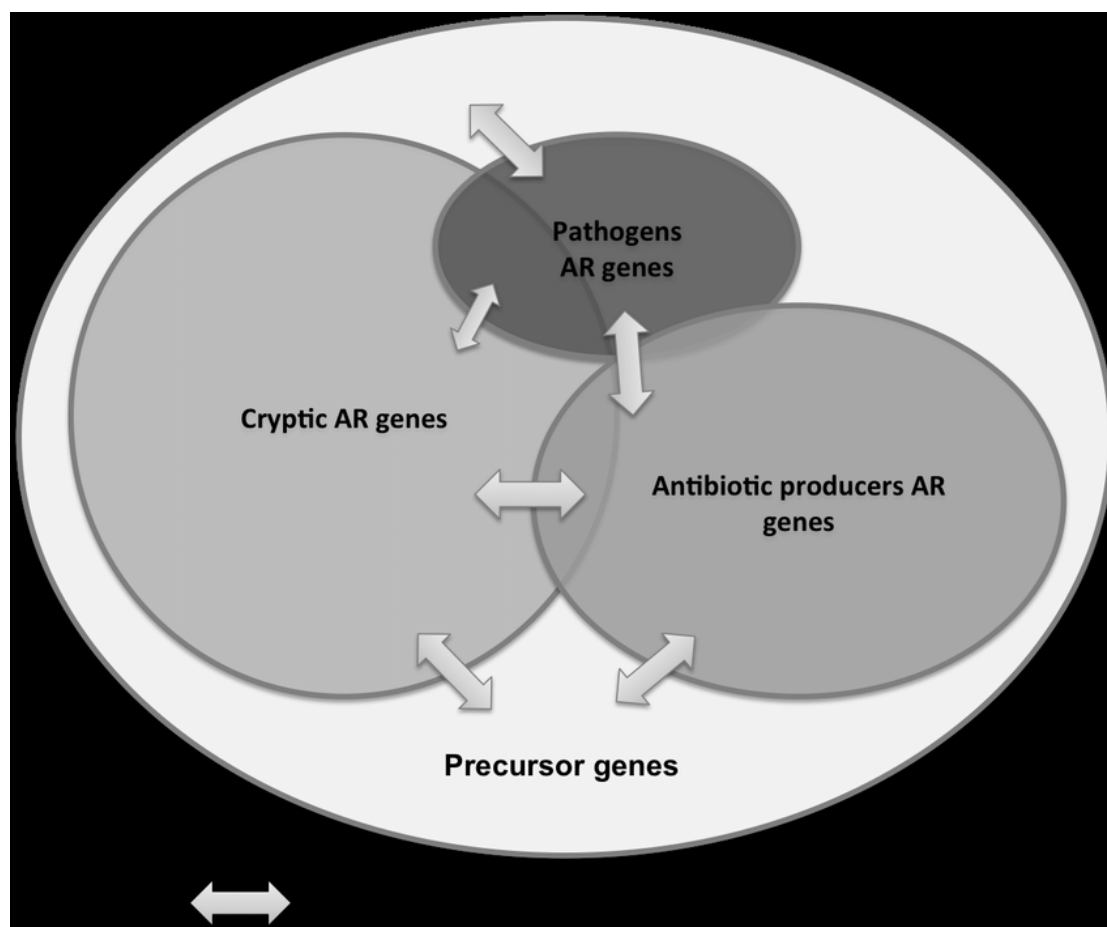
FIGURE 1.2: The antibiotic Resistome :It consists of genes encoding AR inter-connected to protein likely to evolve toward potent antibiotic resistance . This contain AR gene found in pathogenic Bacteria , AR genes which may be or not expressed but could confer resistance in another genetic context . Genes can ofcourse switch from one state to another through horizontal gene transfer (HGT) in another host.

Therefore, the resistome also includes the organisms' intrinsic systems biology which results in an organism's evasion of antibiotic activity.

1.5.2 Antibiotic Resistome to Bacteria

Some bacteria are destroyed by antibiotics, but certain bacteria manage to establish resistance and, in that case, antibiotics stop acting on them. The more widely

antibiotics are used, the greater the chance of resistance developing. Today, bacteria are immune to most known antibiotics, and infections such as these threaten life.

The key explanation for the incidence of bacterial resistance is unnecessary and unconscious use of antibiotics, taking them when not needed, discontinuing antibiotic therapy before completion of treatment, taking insufficient doses as required, and disregarding the appropriate intervals during therapy. Bacterial resistance is contagious, new bacterial generations inherit resistant genes.

People can pass their resistant bacteria to one another or, in other words, even though you have not used antibiotics before, you can infect other people with infectious bacteria [17]. Over time, bacteria can become resistant to several different forms of antibiotics, and so almost all antibiotics are super-resistant bacteria.

The implications of this trend are an increase in disease incidence, an increase in mortality from some infectious diseases and an increase in medical costs. Because of this trend new, more costly antibiotic are being used, but the bacteria are again becoming resistant to the antibiotics with their inappropriate use. Modern medicine desperately tries to win the war over infections [18].

The HGT is also responsible for the resistome , the transfer of the genes from one organism to another, the resistome consist of the genes and their products that contribute to AR genes. Due to HGT and co-optation of chemical mechanisms, the resistome is very redundant and interlocked. The notable extent of the microbial genome sequence revealed this phenomenon where the scars of HGT are identifiable in the presence of genes encoding elements required for HGT, which are distributed throughout the microbial genomes[79].

1.5.3   Mechanism of Antibiotic Resistome

Antimicrobials are substances which can destroy or inhibit the growth of microorganisms. The term antimicrobial refers to antibiotics, other chemicals and compounds which are used to destroy microorganisms.
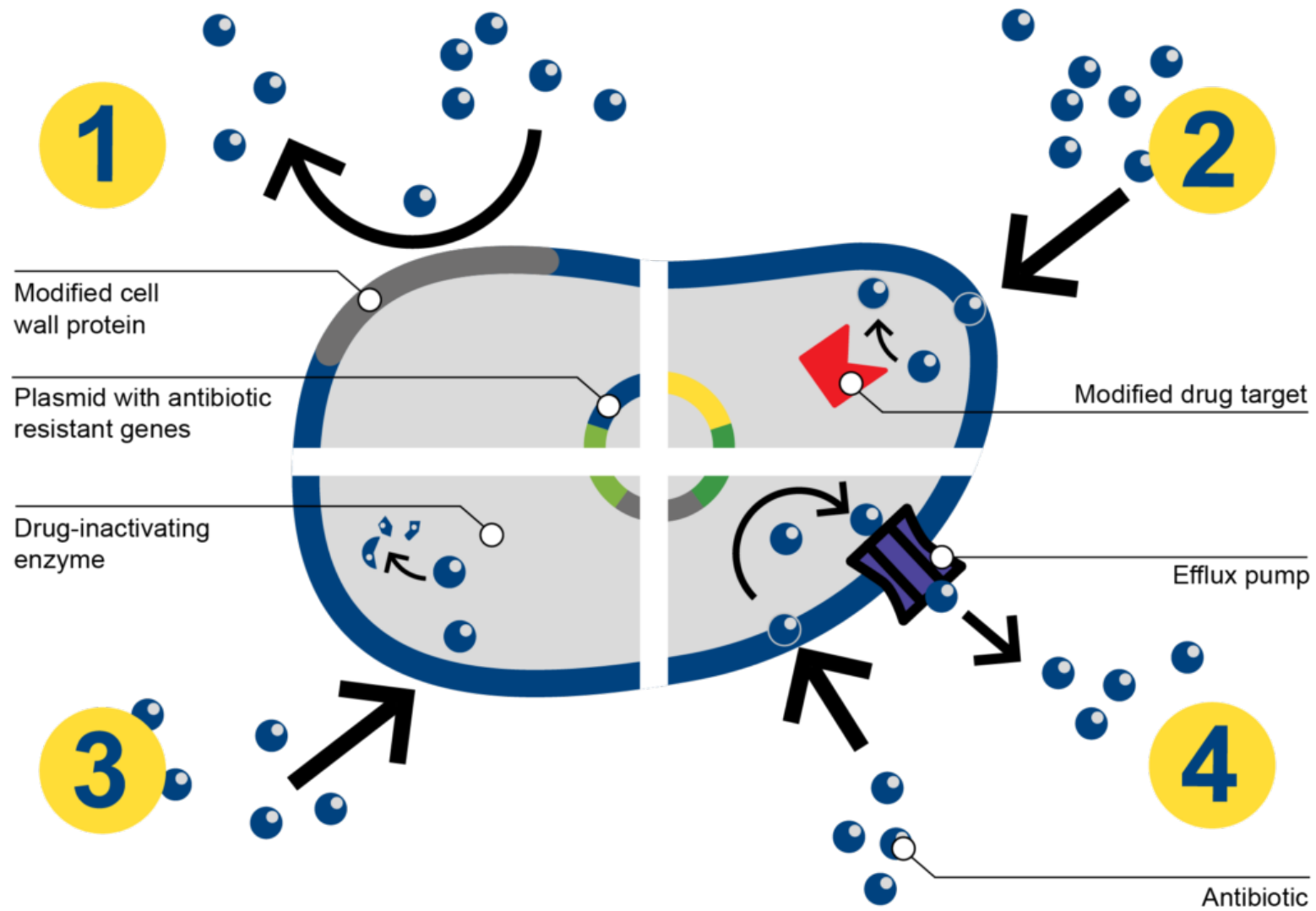
FIGURE 1.3: Mechanism of antibiotic resistance

The term "antibiotic" refers to substances which are generated naturally by some fungi and bacteria and which are created synthetically [19]. These may also be classified as bacteriostatic or bactericidal medicines. Bacteriostatic compounds, such as tetracyclines and sulfonamides, work by preventing replication of micro-organisms.

There are numerous mechanisms which use antibiotics to inhibit or destroy microorganisms. Because these processes are interrupted or made ineffective, understanding the different ways in which antibiotics operate is crucial. Therefore, while a good antibiotic is expected to kill the micro-organism it attacks, it should not necessarily harm human cells.

This should be taken into account, too. An important mechanism of action is performed by cell wall that is present in bacteria or prokaryotic cells but absent in humans or eukaryotic cells. The cell wall is a layer of peptidoglycan and inner cell membrane in bacteria classified as gram-positive.

Gram-negative bacteria also have an inner membrane and a peptidoglycan cell wall, but have an outer membrane as well. This outer cell membrane will often help to prevent the adverse antibiotic bacteria that damage the peptidoglycan cell wall [20].

1.6 Significance of Resistance Gene

Resistant genes in bacteria encode mechanisms of resistance against drugs which damage their survival. But that is not regarded their primary function; ARGs used to have regulatory functions, but they took on their roles of providing protection to their host over time, due to external pressures. Environmental bacteria may carry a collection of genes which, if ensnared by pathogenic bacteria, may be capable of being used as resistance genes.

These genes can encode a novel antibiotic resistance mechanism. Until the beginning it was thought that ARGs evolved to protect bacteria from harsh conditions

due to polluted climate, but then it was discovered that they existed long before antibiotics were used and that we only recently found out about them [21]. It is right to name ARGs "emerging contaminants of the environment" because they are omnipresent in different environmental compartments.

1.7   Problem Statement

The aim of the study is to analyze the resistome profiles against total bacteria profiles of glaciers and correlate them with environmental factors along with the classes of bacteria

1.8   Aims and Objectives

The aims and objectives of our study were

- To observe the diversity of bacteria in different region of glaciers metagenomically.

- To analyze the alpha and beta diversity "in site and between sites" from 20 selective areas.

- To find evidence of total resistance genes present (Resistome).

- To find in depth relationship of resistance genes with type of bacterial classes

- To find relationship of presence of certain bacteria with environmental factors.

Chapter 2

Literature Review

2.1 Historical Perspective

It was thought that there is no life in Antarctic desert region and no microbe's existence in Antarctic soil due to presence of extreme environment and absence of visible life [22]. However, it is found that there is abundance of microbial life exists in Antarctic desert soil and other extreme environments [23]. The microbes of these extreme environments are called Extremophiles. The word extremophile was first introduced by MaceLroy in 1974. These extremophiles are structurally adapted to face harsh conditions at molecular level [7].

Extreme environment that is considered extreme for one type of living organism may be vital for the existence of others. Extremophiles live in conditions that are usually toxic for other species. Extreme conditions include high temperature of 55 to 121 ℃ low temperature of -40 to 20 ℃ high salt concentration of 2-5 molar NaCl, high acidity like pH<4, and high alkalinity of pH >8 [8]. Extremophiles can tolerate high metal conc. low nutrient availability, low water availability, radiation, high pressure, low oxygen levels, toxic materials or environments such as rock life [8]. Almost all ecosystems contain more than one harsh condition therefore microbial life face many challenges for their survival such organisms that live in environments with extreme conditions are called "polyextremophiles".

One of the most common climates on Earth is low-temperature climate that include the polar zone, Europe high mountain glaciers, 95% of oceans, upperpart of the atmosphere and manmade refrigerators and freezers where temperature does not exceed five degree Celsius [23-28].

Psychrophilic organisms can survive below 20 degree Celsius temperature however species that grow at higher optimal temperatures and withstand low temperatures are called Psycrhotolerant [7]. In recent years, the terms eurypsychrophiles and stenopsychrophils have been proposed as more suitable for describing the temperature range that an organism can withstand [29].

2.1.1 Detection of Bacteria in Glaciers

While the presence of microorganisms found in the ice sheets of glaciers and in other habitats, the attention in microbiology of ice was not revived until Abyzov's groundbreaking research on the deep ice in Antarctica Microscopy technique was used to determine the sample of ice core from the upper layer to 3000 meter underground and some different shapes and size of cells were determined at comparatively low concentrations.

Additionally, for the first time the variations in cell numbers were related to the content of mineral particles and temperature shifts with a higher number of dust particles and cells that occur during colder periods. Later, a sys tematic analysis conducted on glacier samples of ice core ranges from some other geographic sites that ranges from 5 to 20,000 years ago, represents that different bacteria can be restored with success.

2.2 Glacial Microbial Flora

This Glacial microflora is made up of 90% by bacteria and 10% by fungi and archaea. Research about glaciers from several parts of world such as Alaska [31],

Tibet [32], China [33, 34], Canada [35], America [30], and New Zealand [36] suggested that the most dominant bacterial phyla is *Proteobacteria* that accounts for 65%, out of these Beta*Proteobacteria* are dominant class.

16S rRNA sequencing reveals that most of bacteria belong to gram-positive, non-spore forming bacteria that includes *Actinobacteria*, *Firmicutes*, and gram-negative bacteria and *Bacteroidetes*. Species of these phyla were consistent with colored and viscous colonies that helped them to protect from harsh environmental conditions.

TABLE 2.1: List of Polar and Non Polar Microbes with their habitat

Major zone˜	Microbes˜	Location˜	Ref˜
Polar zone˜	*Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Cyanobacteria*, Acido, *bacteria*, *Verrucomicrobia*, ˜and *Planctomycetes*˜	Alaska˜	[31]
	Actinobacteria, *Proteobacteria*, *Firmicutes*, *Cytophaga* - *Flavobacteria – Bacteroides*	Canada˜	[35]
	Arthrobacter, *Microbacterium*, *Paenibacillus*, *Sphingomonas*	Greenland˜	[36]
Non Polar ˜zone˜	*Proteobacteria*, *Actinobacteria* and *Bacteroidetes*	Tibetan plateau	[32]
	Friedmanniella, *Microbacterium*, *Micrococcus*, *Mycobacteria*, *Norcardia*, *Norcardioides*, *Planococcus* *Staphylococcus*.˜ Ice	America˜	[30]

2.2.1 Glacial Ecosystem

Glacier covers 10% of the Earth's atmosphere and about 80-85% of the Earth's temperature is less than five degree Celsius that includes Alps, Himalayas, saeas, polar regions, mesosphere and stratosphere [29]. Some of these low temperature ecosystems are deep sea and oceans with temperatures ranging from -1 to 4 ℃ accompanied by ecosystems like cold deserts, lakes, caves, sea ice and glaciers. This shows that the low temperature is the most common extreme condition that is widely dispersed on Earth. Microorganisms have adapted mechanisms to low temperature to face various stress conditions [37] such as desiccation, radiation, low concentration of nutrients, high osmotic pressure and intense pH [38]. There are different glaciers found in Pakistan having diverse bacterial diversity.
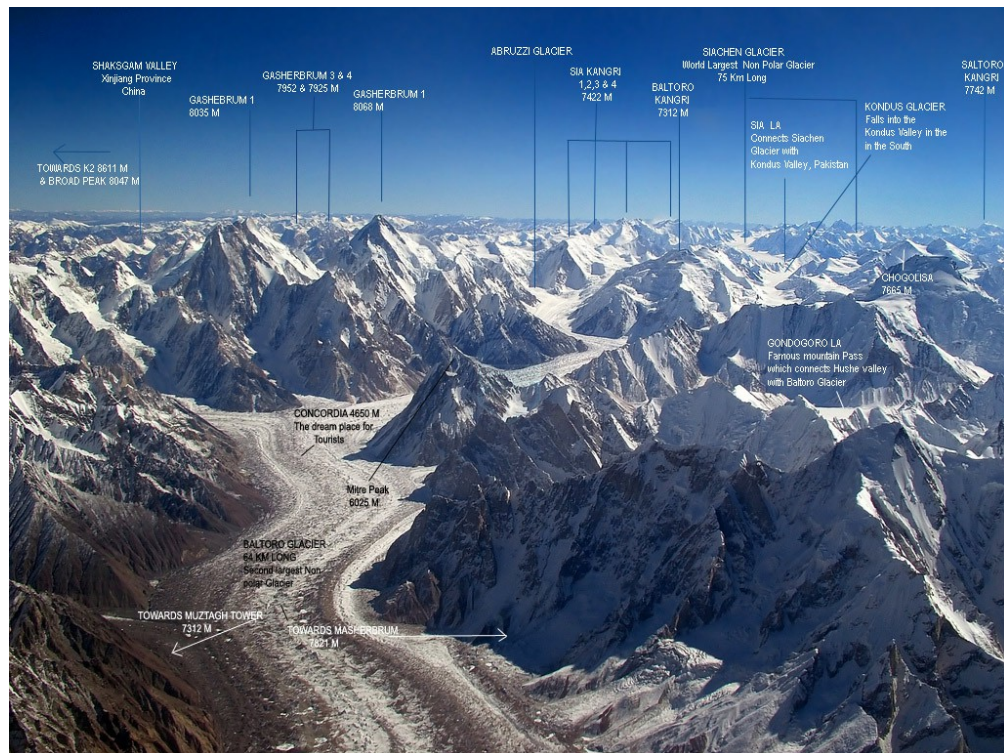
FIGURE 2.1: Siachin and baltoro glacier shown in the map.

Other low temperature habitats include cold water lakes, clouds, man-made freezers and refrigerators, surface of animals and plants that live in cold areas where temperature remain below 5 degree Celsius. Cold and low temperature environments are less explored by scientist that increase their interest due to possibility of discovering new species [39].

2.2.2 Role of Glaciers in the Environment

Glaciers occur in areas where snow formation supports climatic and topographic conditions. Their position can be seen on a global as well as regional scale, as shown in Figure 2.1
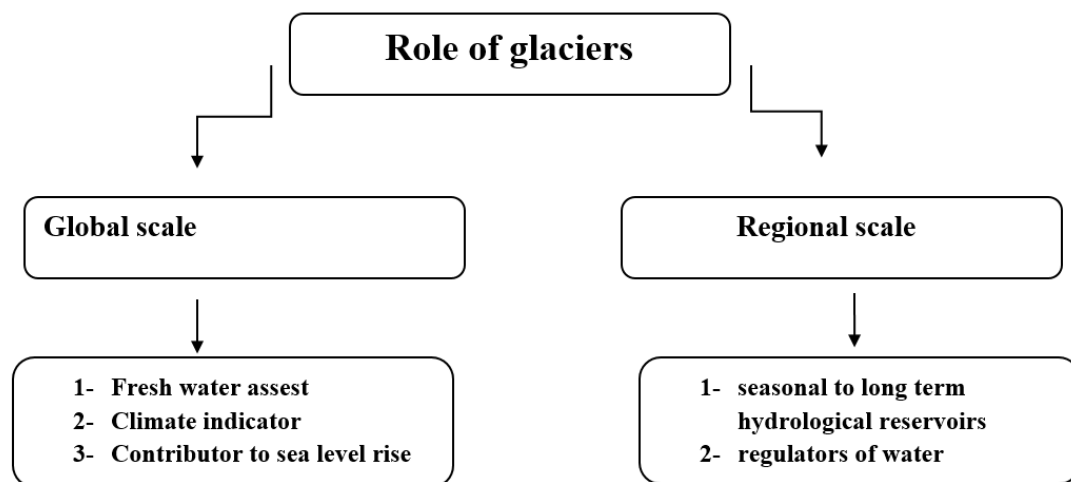
FIGURE 2.2: Role of Glacier in the Environment

2.2.3 Physiochemical Characteristics of Glacier Microbes

Low temperature has various detrimental effects on cell integrity, water availability, solute absorption, membrane stability, enzyme activity and other biochemical processes [37]. Therefore, a microbe can't tolerate cold condition without proper tools and accessories. As such, psychrophiles have developed strategies to effectively combat additional cold-related stress factors such as desiccation, heat, excessive UV, high or low pH, high osmotic pressure and low nutrient availability. Psychrophilic bacteria have evolved various survival techniques to deal with such extreme environments and develop metabolically active enzymes and proteins at these low temperatures. Psychrophils also alter their structural proteins and components which protect and maintain the structure of the cell from adverse effects of low temperature. Psychrophilic proteins have low bonding interactions on the cell surface compared to mesophilic proteins, such as ionic, hydrophobic, and hydrogen bonds. These improvements have made proteins versatile to operate

efficiently in these low temperature environments. Furthermore, the unsaturated fatty acids are found in membranes that preserve for fluidity and carry the frozen state. A number of cold-shock and antifreeze proteins also synthesize psychrophils. Environment made these protein suitable for the preservation and regulation of enzyme production within the cell and at freezing temperature. The bacteria capable of these remarkable modifications of phenotypic and genotypic traits to suit low-temperature conditions lead to abundant life in all psychriphilic habitats, including the polar, non-polar and deep oceans

2.3   Characteristics of Psychrophilic Bacteria

Bacteria found in glaciers are mostly cold adapted, psychrophilic and cold tolerant, there is a diversity of bacteria that is present in the glacier region and faces a huge number of challenges, to adapt the environment and to be viable in the cold environment. Bacteria in the region having low temperature, due to which the physical and biological processes slow down the activity, they also have to come up with the low availability of the nutrients, high salinity, low water activity, dryness and even high UV irradiations and oxidative stress at high altitudes with high pressure.

2.3.1   Up-regulated Functions for Growth of Psychrophilic Bacteria

Cell barriers (cell envelope biogenesis, membrane biogenesis, membrane-transfer proteins, membrane fluidity maintenance), metabolism (specific metabolic processes, transport of nutrients, energy metabolism), cell defense (production and ingestion of cryoprotective compounds, antioxidant activities), protein synthesis, and folding (transcription, translation, RNA helicases, chaperones; cold shock, cold acclimation and heat shock proteins; adaptation of protein structures to ensure increased flexibility at low temperatures) are widely used for bacteria growth

at low temperatures. The Down-regulated genes are linked to flagellar motility, proteins and structures of the outer membrane (flagella, chemotaxis), and pathways that contain reactive oxygen. It is interesting to remember that certain bacteria will only reproduce at temperatures below -6 ℃This bacterium will develop over an extremely large range of temperatures from -15 to 37 ℃ and affects the metabolism of peptidoglycans. In addition, the cell membrane, when grown at -15 ℃ varies from that of cells grown at 25 ℃ by the accumulation of high content of calcium carbonate (due to biomineralization) and choline, which may reflect cryoprotective mechanisms at subzero temperature

2.4   Bacterial Diversity

Glacier snow in the Tibetan plateau has vast diversity of bacterial genera that are also found commonly in all other glaciers [43]. Several psychrophilic, psychrotolerant and eukaryotic bacteria has been found with seasonal fluctuation [41,42]. Both culture dependent and metagenomic approach has revealed that there is high diversity and abundance of bacteria in Arctic snow [44,45].

Biogeochemical cycling at low temperatures of dry regions polar snow in microorganisms that supports the bacterial and fungal diversity in alpine tundra soil [46,47]. Permafrost soil is an intense niche for microbial population due to the presence of sub-zero temperature, gamma radiation freezing stress and a broad variety of antibiotics [48-49]. The viable fractional ranges dominated by grampositive (*Actinobacteria* and *Firmicutes*) in different permafrost regions while Gamma-*Proteobacteria* (especially *Xanthomonadaceae*) were dominated among Gram negative bacteria. Alpine permafrost information is scarce, aboundance of gram-positive *Arthrobacter* has been reported in permafrost of China [33], however *Proteobacteria* is found dominated via culture dependent approach [51]. Many newly adapted cold bacteria have been detected, including Sphingomonas glacialis, Pedobacter and Rhodotorula glacialis yeast [32, 52]. Lands of cold environments prevail in methanotrophic bacterial communities [53].

Such micro-organisms were researched using culturally dependent, as well as autonomous methods [54]. Arctic lakes were investigated for microbial diversity, and numerous species of bacteria were identified [55]. The abundance of *Actinobacteria* and *β-Proteobacteria* was also recorded from high mountain lakes, as well as from various layers such as surface layers and underlying water from various alpine lakes, followed by *Actinobacteria.*

Psychrophilic methanotrophs were also recorded from Arctic soil, and *Proteobacteria* with an abundance of 73 per cent were also revealed from the Himalayan mountains using molecular methods. Viable bacteria consisting of identical and equivalent gram-positive bacteria (48 percent) and negative bacteria of 51 percent. All these isolates had been shown to produce specific hydrolytic enzymes. An altered image has been provided by the culture based and metagenomic research. For example, *Actinobacteria* dominated the soils of Dry Valley, Antarctica, although most culture-dependent research was dominated by Streptomyces (> 80 per cent).

The microbial community of the deep sea and sea ice have many adaptation strategies to survive in these climates, consisting of piezophilic, psychrophilic, and psychropiezophilic microorganisms. These are distinct from microorganisms in the polar zone, the majority being primary producers, i.e. 98 percent. Cloud is the most suitable environment for microbial development, at below zero temperature the cloud droplet remains liquid.

2.5 Community Structure of Bacteria on Glaciers

The evolutionary study published in 1918 on microorganisms that colonize frozen ecosystems such as glaciers, the diversity of microbes in Vostok Lake, Antarctica's subglacial lake was observed by researchers. *Actinobacteria* was found to be dominant phylum followed by *BetaProteobacteria* in Arctic permafrost soil. The ancient permafrost is dominated by *Actinobacteria* that are non-spore forming

because of their metabolic activity at low temperatures. Cold environments typically contain bacterial phylum of *Proteobacteria*, bacteroids, and *Actinobacteria*, while Archaea is understated, several bacterial clusters were successfully identified in glacial cryoconite holes in Antarctica and the Arctic during the bacterial population comparison study, showing that specific micro-organisms are present in these harsh conditions. Phylum's of bacteria such as *Proteobacteria, Bacteroidetes, Actinobacteria, Cyanobacteria, Firmicutes* and *Fusobacteria* were very higher in soil, while small numbers of *Bacteroidetes, Actinobacteria* and *Verrucomicrobia* and few freshwater *Proteobacteria* and *Cyanobacteria* were identified. This clearly suggested that large microbial organisms occupy cold habitats occur. These particular microorganisms affect the glacial environment's dynamics and play a part in soil development and other biogeochemical processes.

2.6   Effect of Global Warming on Glaciers

The absorption and release of infrared radiation by atmospheric gasses warms the atmosphere and surface of a earth because of the greenhouse effects. The key natural greenhouse gasses on Earth are water vapor, which produces approximately 36–70% of the greenhouse effect Carbon Dioxide, 9–26%; methane, 4–9%; and ozone, which produces 3–7%. Since 1750, atmospheric CO_2 and CH4 concentrations have risen by 31 per cent and 149 per cent respectively above pre-industrial levels. This is significantly higher than in the last 650,000 years, at any time. Around three quarters of the anthropogenic CO_2 emissions to the atmosphere over the last 20 years are attributed to the combustion of fossil fuel.

The remaining portion of the anthropogenic emissions are mainly due to changes in land-use, especially deforestation. The current concentration of CO_2 in the atmosphere is about 383 ppm by volume. Future rates of CO_2 are projected to increase due to increased burning of fossil fuels, deforestation and changes in land use. The rate of increase may depend on unpredictable cultural, sociological, technical and natural changes but will eventually be constrained by fossil fuel

availability. Fossil fuel reserves are enough to achieve this amount if heavy use is made of shale, tar sands, or methane clathrates. Some of the major factors relates to water evaporation. CO_2 released into the atmosphere allows the atmosphere to warm and the earth's surface to warm.

The heating is allowing more water to evaporate into the atmosphere. Because water vapor functions as a greenhouse gas itself, this causes much further heating; the heating allows further water to evaporate, and so on. The increased CO_2 in the atmosphere warms the surface of the Planet and contributes to the melting of ice close to the poles. When the ice melts, land takes its place, or open water. In average, both land and open water are less reflective than ice, and thus absorb more solar radiation. It induces more heat, resulting in more melting and this process continues. The average global temperature for 2004 has risen to 14.60 Celsius due to global warming, making it the fourth warmest year ever. After record keeping started in 1880, October and November 2004 were the hottest of all months on record. The second warmest was February 2004, and the third warmest was March, April and December of these three [57].

2.7   Antibiotic Resistome

The antibiotic resistant is a set of all genes that directly or indirectly contribute to the resistance. The term "antibiotic" refers to substances produced naturally by certain fungi and bacteria and that are generated synthetically. Antibiotic resistance genes are transmitted biologically from the microbes to the microbes, especially in microenvironments where dense microbial communities are often subjected to excessive antibiotic use. Microbe resistance may develop through the following approaches: reduction of the membrane's permeability to antibiotics, rapid release of the antibiotic, deactivation of the antibiotic, or modification of cellular targets. Hence the resistome is a complex array of genes and their roles that act directly or indirectly to inhibit antibiotic activity. The resistome includes all antibiotic resistance genes in pathogens, antibiotic generators and benign bacteria

in the environment. In addition, because resistance develops from precursor genes that encode metabolic or 'housekeeping' functions, the resistome contains these genes, which we called protoresistance elements [21]. Antibiotic resistance at the level of the organism may emerge from the dynamic interplay between genes and their products resulting from exposure to toxic molecules [59]. Often environmental changes, such as pH, light, and radiation, cause resistance in bacterial species to grow [60].

2.7.1 Antibiotic Resistance in Environment

The sources of antibiotic resistance and the distribution are wide. The application of antibiotics has been that day by day in the biotic sector; not only is human health directly affected, but there are also horrible effects on animals and agriculture. Antibiotic resistant may be found in soils, surface waters, and in animal and human waste streams. Effective inspection and detailed assessment of the sources of contaminants will aid in reducing antimicrobial resistance. Lack of awareness and overuse of antimicrobials are two major obstacles in handling bio-contaminants for the ecosystem.

Antimicrobial resistance is a very complicated phenomenon; the survival of resistant microorganisms depends on various abiotic and biotic factors, such as edaphic influences, solar radiation, temperature, rainfall and residue retention capacity. There are several biotic factors but the most important factors are the rate of bacterial proliferation, die off, exchange of resistance genes and dispersion.

2.8 Metagenomic Studies

Microbial genomics research uses metagenomics as a powerful technique in extraction and sequencing of DNA directly from a microbial population present in an ecological system [61]. It is difficult to study microbial species worldwide due to its high diversity but the NGS technology along with metagenomic approach is

increasingly growing. These techniques enable us to examine microbes without there cultivation.

Metagenomics provides knowledge of microbial diversity and functional ability of microbes living at different locations such as soil, human intestine, seas, and snow etc. metagenomic approach helped to assess the biotechnological ability as well as helps to assess the number of genes present, and the microbes' biochemical and metabolic characteristics hence metagenomic is most popular technique now days for analysis of microbial populations [62, 63].

2.8.1 Alpha Diversity

Alpha diversity can be described as the diversity within a given region or ecosystem normally represented by the number of species in that ecosystems.

2.8.2 Beta Diversity

A comparison of ecosystem diversity, typically measured as change in the amount of species between the ecosystem.

2.9 Glacial Metagenomics

Ice on glaciers contain wide variety of several micro-habitants where microbial life exists that are difficult to culture under laboratory conditions. Therefore, it is difficult to assess microbial gene pool and genetic potential of glacial microbes through cultivation dependent approach. The composition of entire glacial microbial communities can be determined by PCR-based analysis of the 16S rRNA or 18S rRNA gene using directly isolated glacial DNA as the starting material. It is feasible to classify trapped, proliferating, or dead species that are alive, frozen, or concealed through ice, water, or sediment. Metagenomic sequencing of whole genome and its bioinformatic analysis will not only describe the composition of the

population but also provide details about the content of the whole gene. DNA libraries constructed from glacial DNA allow to identify potentially new biocatalysts and compounds. Escherichia coli is widely used as a host for the above approach to the libraries and screens. Inclusion bodies are made from mesophilic host *E. Coli* can be introduced to acquire soluble and active enzymes by genes that encode cold-activated proteins for incubation temperatures below 30 degrees. Additionally, a psychrophilic host will be used in the future to screen metagenomic libraries from cold environments. Glacial metagenomics research may provide details on the microorganisms in cold and frozen places on Earth.

All the microbes especially bacterial microoraganisms found in the glaciers accommodate their self according to the environmental conditions. These microorganisms have the ability to survive in the extremely harsh conditions. The most dominant group of bacteria found from the glacier ecosystem was *Cyanobacteria* and *Proteobacteria*. In the glacier ecosystem the bacteria are present on larger scale that are viable in cold temperature. The main goal of the study is to unveil the bacterial community through the DNA sequencing and then to analyze the antibiotic resistome profile against the total bacteria found in the glaciers through the metagenomics analysis, then to correlate these resistome profile with the environmental factors along different classes of the bacteria.

Chapter 3

Material and Methods

3.1 Sample Collection /Sampling Site Description

The glacial samples were collected from 5 accessible sites from Kamri (Km), Burzil (Bz), Siachin (Sc) and Baltoro (Bt) Glaciers. There were about 10 samples collected from each site according to Quadrate method for selection.

Five samples were collected in winter season and five samples were collected in summer season. Two control samples were collected for comparison. Samples were collected in falcon tubes and were preserved in the refrigerator and then utilized for analysis.

TABLE 3.1: List of sample and their sites

Code	Sampling site
Km	Kamri
Bz	Burzil
Sc	Siachin
Bt	Baltoro
C	Control

3.2 Research Methodology

Overview of methodology adopted for project is summarized in figure 3.1 blow.
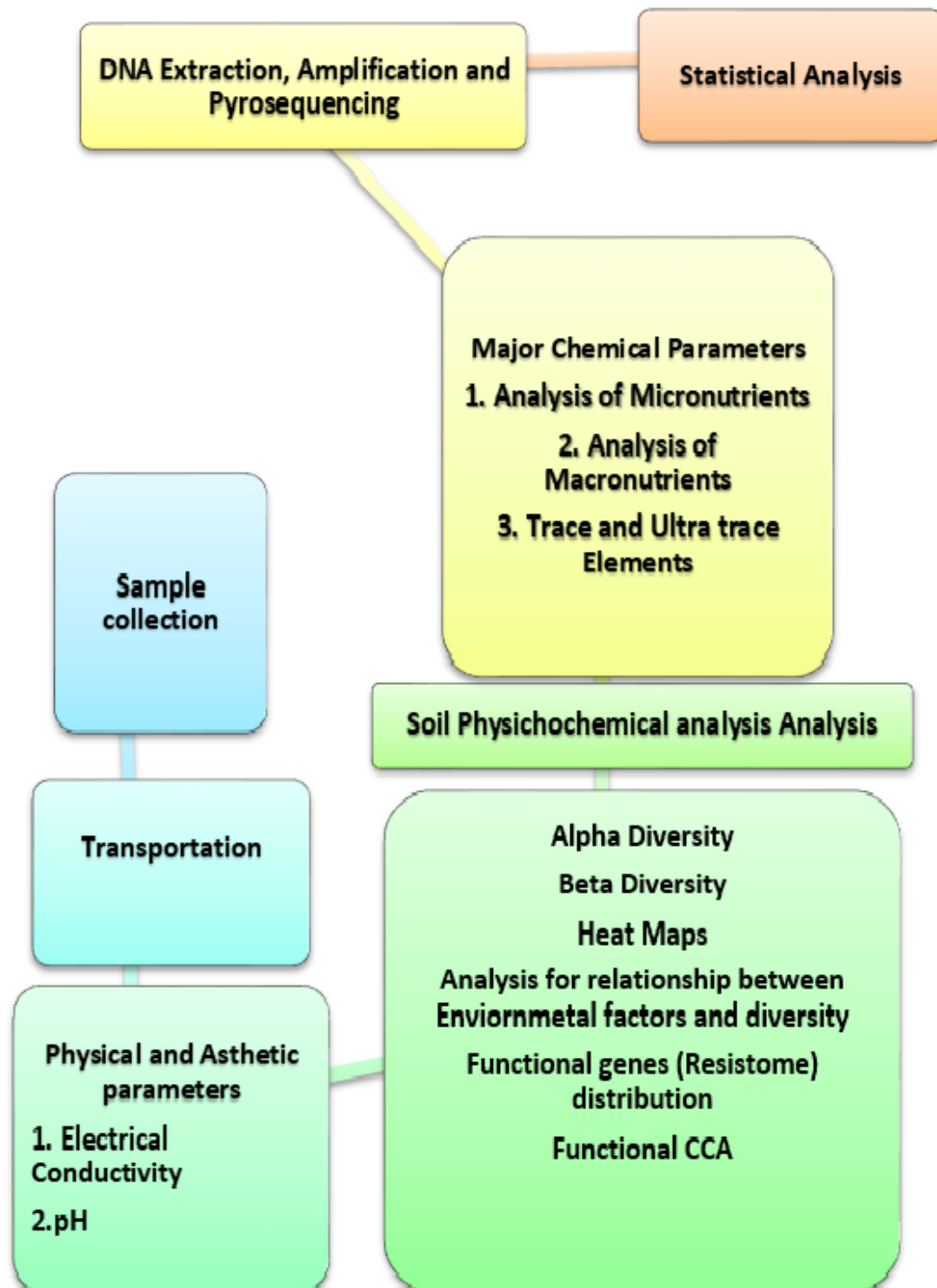
FIGURE 3.1: Methodology Overview

3.3 Soil Analysis

3.3.1 Geographical Location of Sampling site

Site Map of the selected Glaciers , these snips was taken from the Google Map.
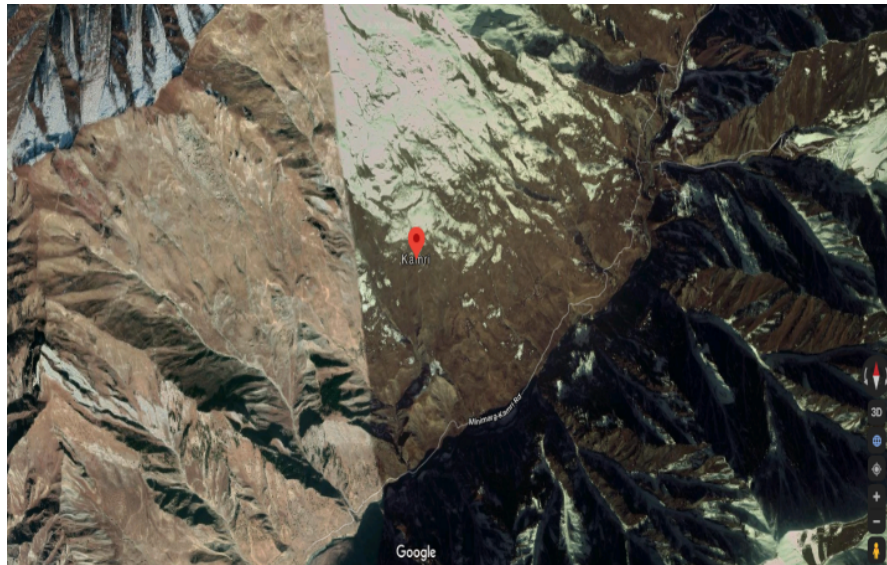
FIGURE 3.2: Site Map of Kamri (Km) Glacier

FIGURE 3.3: Site Map of Burzil (Bz) Glacier
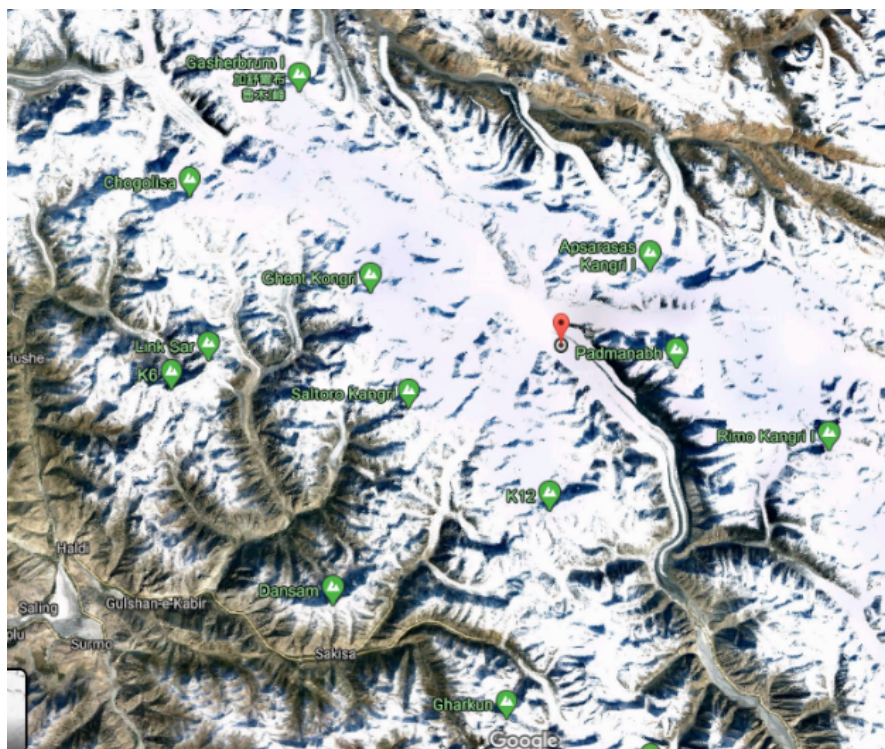
FIGURE 3.4: Site Map of Siachin (Sc) Glacier

FIGURE 3.5: Site Map of Baltoro (Bt) Glacier

3.3.2 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 degree Celsius and humanity content should be 30 to 60%. Excessive oven-drying should be avoided to ensure the nutrients in the sample are accessible. Dry process had limited impact on total nitrogen content, but with time and temperature, NH4 and NO3 content may differ. Drying at high temperature leads to the death of microorganisms present in soil samples

3.3.3 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 physicochemical reactions.

3.3.4 Soil Sample Digestion

1 gram of prepared dried soil sample was taken in 250 ml beaker and 15ml of aqua Regia was added. This mixture was then digested at 70 degree Celsius 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 deionized water. Sample solution was analyzed for conc. of Pb, Cr, Cd, Ca, Fe, Na, Cu, Mn, Mg and K via flame atomic absorption spectrophotometer (Perkin Elmer Analyst 7000).

3.3.5 Chemical Analysis of Soil

AAS Analyst 7000 was used to calculated the content of calcium , magnesium, potassium sulfate, phosphate, nitrate , nitrite, copper , iron , manganese, lead , chromium, calcium and zinc from the soil samples. 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 ASS programming worksheet. Empty cathode was used for light embedded in holder light. For the necessary light throughput, the beam was calibrated to reach target zone of the arrangement cards. The machine was then switched off. To estimate the yearning factor, a graduated 10 ml chamber containing deionized water was used. 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.

3.3.6   Water Analysis

3.3.6.1   Apparatus

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

3.3.6.2   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.

3.3.6.3   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.

3.3.7 Electrical Conductivity

3.3.7.1 Apparatus

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.

3.3.7.2 Reagents

Potassium chloride solution (KCl) to be taken 0.01N. Dried around 2-3 g KCl during 2 hours in an oven at 110 oC. Dissolved 0.7456 g of KCl in DI water and gave volume of 1-L. The solution was transferred to a plastic flask.

3.3.7.3 Procedure

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

3.3.8 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. 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 holder light. For the necessary light throughput, the beam was calibrated to reach target zone of the arrangement cards. The machine was then switched off. To estimate the yearning factor, a graduated 10 ml chamber containing deionized water was used. Calibration solutions were prepared along with analytical blank. Both were atomized and response was measured. Graph was plotted for for each solution than sample solutions were atomized. The concentration of various elements from sample solution was determined.

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.

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

- 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; description

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 System

TABLE 3.2: Reagent name and dosage

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

3.4.1.5 PCR Reaction Conditions

PCR reaction cycles were performed as follows:

1. After 5min of initial denaturation at 94℃ followed by 30 cycle of 9℃ for 30sec, 52℃ for 30sec, 72℃ for 30sec

2. Extension step at 72℃ for 10min

3. Hold at 4℃.

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

3.4.2   Electrophoresis Detection of PCR Products

The concentration and length of PCR product were identified by 1% agarose gel electrophoresis. The length of the main band was within the normal range. 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.

3.4.4   Database Building and Sequencing

Build the database according to the standard process of nebnext ultra-DNA library prep kit for Illumina (New England Biolabs USA). The amplified library was sequenced by PE250 using Illumina Nova 6000 platform (Guangdong Magigene Biotechnology Co. Ltd. Guangzhou China).

3.5   Analysis Process

3.5.1   Sequencing Data Processing

3.5.1.1   Paired 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.

3.5.1.2 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.

3.5.1.3 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).

3.5.2 OTU Clustering and Species Annotation

3.5.2.1 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)

3.5.2.2 Representative Sequence Species Annotation

Use usarch - sinax to compare the representative sequence of each OTU with Silva (16S) RDP (16S) greengenes (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).

3.5.2.3 Contaminated OUT 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 OUT table of taxonomy for each sample.

3.5.3 OTU Statistics

3.5.3.1 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.

3.5.3.2 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.

3.5.4 Species Community Analysis

3.5.4.1 Community Structure of Species

Use Qiime2 View software to make statistics of common and endemic species community composition analysis and species abundance cluster analysis.

3.5.4.2 Phylogenetic Analysis

Single sample - phylogenetic analysis of each classification level, Based on phylogenetic relationship and relative abundance of each OTU in sample Qiime2view was used to visualize the species annotation results

3.5.5 Alpha Diversity Analysis

3.5.5.1 Alpha Diversity Index Statistics, Dilution Curve and Rank Independence Curve (Default Parameter)

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

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

(3). Based on OTU abundance table Qiime2View (https://view.qiime2.org) is used.

3.5.6 Beta Diversity Analysis

3.5.6.1 PCA Analysis (Default Parameter)

Based on OTU abundance table use the Qiime2View (https://view.qiime2.org) for analysis.

3.5.6.2   PCoA Analysis (Default Parameter)

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

3.5.7   Correlation Analysis of Environmental Factors

Based on OTU abundance table and environmental factor data the association between community structure and environmental factors will be studied.

3.6   Analysis of Antibiotic Resistance Genes

ARG-related genes were filtered using in-house command lines via lists with gene names extracted from the Antibiotic Resistance Genes Database (ARDB). All the specific genes were conferred for successful classification in the UniProt and KEGG libraries, and genes with one nucleotide (SNP) mutations were not considered. These genes have been identified using the BacMet database that uses the following keywords for each function: protein binding, chaperone, Effux pump, enzyme, membrane protein, membrane transporter, and regulator. The organization of ARs was focused on classifying the broad antibiotic classes, such as the beta-lactam class. The table without any filter was assumed to be a global annotation, and the table with ARG filters was considered to be a particular annotation.

Chapter 4

Result and Discussion

The glacial samples from Pakistan were collected from five accessible sites which are Kamri (Km), Burzil (Bz), Siachin (Sc) and Baltoro(Bt) Glaciers. There were about 10 samples collected from each site according to Quadrate method for selection, five samples were collected in winter(w) season and five samples were collected in summer(s) season. Control(C) samples were collected for comparison. Samples were collected in falcon tubes and were preserved in the refrigerator and then utilized for analysis.

4.1   Physical and Aesthetic Parameters

The physicochemical features of the samples which were collected from the 5 glaciers and the parameters are depth, temperature, pH(power of hydrogen), EC(electrical conductivity), Sulphate, nitrate, phosphorous, Potassium, Calcium – Magnesium, and pH ice and pH snow respectively, have been taken to define the physiochemical properties of the samples which are taken from these glaciers, and which are represented in the following table 4.2. Table 4.2 shows the physical and aesthetic parameters of different glacier samples, From the table it is noted that temperature in winter is highest in Siachin (-50C), where as in summer it is -20C, and in summer the pH of Siachin is maximum (8.229).

TABLE 4.1: Physical and Aesthetic Parameters of glacier samples.

Sample	KMs	BZs	SCs	BTs	Cs	KMw	BZw	SCw	BTw	Cw
Depth (cm)	302	262	264	300	5	158	140	120	130	5
Temp.(℃)	-4	-4	-20	-5	27	-10	-20	-50	-20	5
pH	7.371	7.621	8.229	6.49	8.81	7.210	6.231	6.121	6.91	8.51
EC uS/cm	114±0	242± 0.02	349± 0.05	349± 0.05	49± 0.05	437± 0.03	67± 0.03	730± 0.01	730± 0.01	30± 0.01
Sulphate %	0.039%	0.411%	0.004%	0.004%	0.025%	0.041%	0.045%	0.045%	0.045%	0.005%
Nitrate %	0.00095%	0.0015%	0.0087%	0.0025%	0.0055%	0.0076%	0.0716%	0.0078%	0.0088%	0.008%
P %	0.026%	0.0005%	0.0031%	0.0009%	0.0001%	0.0037%	0.0064%	0.0052%	0.0022%	0.0002%
K mg|kg	240±0.03	244± 0.02	246 ± 0.06	344± 0.02	144± 0.02	250 ± 0.03	260 ± 0.05	264 ± 0.03	284 ± 0.01	164 ± 0.01
Ca-Mg	3.671%	4%	4.333%	5.333%	1.300%	4.671%	5.41%	5.671%	5.771%	1.671%
pHsnow, pHice	8.18±0.04 (Ice)	7.20± 0.03 (ice)	8.08± 0.06 (ice)	8.08± 0.06	-	0.178± 0.02 (snow)	0.164± 0.01 (snow)	0.170± 0.01 (snow)	0.170± 0.01	-

From the table it is noted that temperature in winter is highest in Siachin (-50C), where as in summer it is -20C, and in summer the pH of Siachin is maximum (8.229), Where as in winter the pH of Baltoro has the maximum (6.91), from the sample the EC was highest in Siachin and Baltoro in summer and winter both.

The nutrients concentration found highest in the Burzil in summer which is (0.411%) same as in the nitrate, phosphorous and in the potassium respectively, maximum concentration of sulpate (0.411%) is observed in Burzil in summer and minimum value (0.004%) is observed in Baltoro in summer, maximum concentration of nitrate (0.0087%) is observed in Siachin in summer and minimum value.

(0.00095%) in Kamri in summer, phosphorus maximum concentration (0.0261%) is observed in Kamri in summer, minimum concentration (0.0001%) is observed in control in summer, maximum concentration of potassium (344%) is observed in Baltoro in summer, minimum concentration of potassium (144) is observed in control in summer, concentration of calcium and magnesium is maximum (5.771%) in Baltoro in winter, while minimum (1.300%) in control in summer.

We can found that mostly minimum values of nutrients were observed in control sample the reason behind that control sample is not in a stressed environment so most minimum values observed in control sample.

4.2 Alpha Diversity Analysis

4.2.1 Demultiplexed Sequence Counts Summary

Table 4.3 shows per- sample Demultiplexing sequence obtained from the samples Demultiplexed sequence is applied on the sequence forward reads as well as reverse reads and it is observed that minimum sequence count for forward read is 238 nts, in forward reads its median is 238nt, and in reverse reads its 250 nts respectively. Per sample sequence counts of 10 samples shows that higher number of sequence count is observed in Reverse reads sample.

TABLE 4.2: Per- sample Demultiplexing sequence obtained after data analysis from the samples.

Forward Reads		Reverse Reads	
Total Sequences Sampled	10000	Total Sequences Sampled	10000
2%	238 nts	2%	250 nts
9%	238 nts	9%	250 nts
25%	238 nts	25%	250 nts
60% (Median)	238 nts	60% (Median)	250 nts
75%	238 nts	75%	250 nts
91%	238 nts	91%	250 nts
98%	238 nts	98%	250 nts

4.2.2 Forward and Reverse Reads of the Sequence

Figure 4.1 shows the forward and reverse reads of the sequence obtained from the glacier samples. The soil of the glaciers was tested and in forward reads the graph was generated using random sampling of 10000 out of 695238 sequences without replacement.
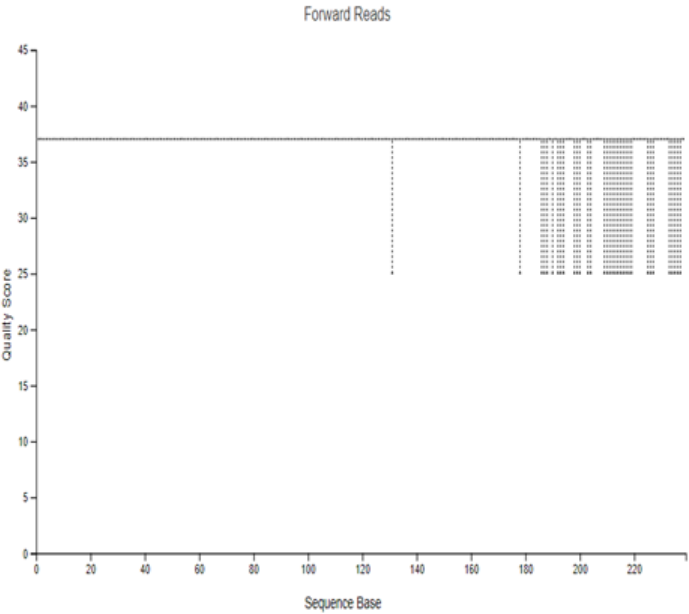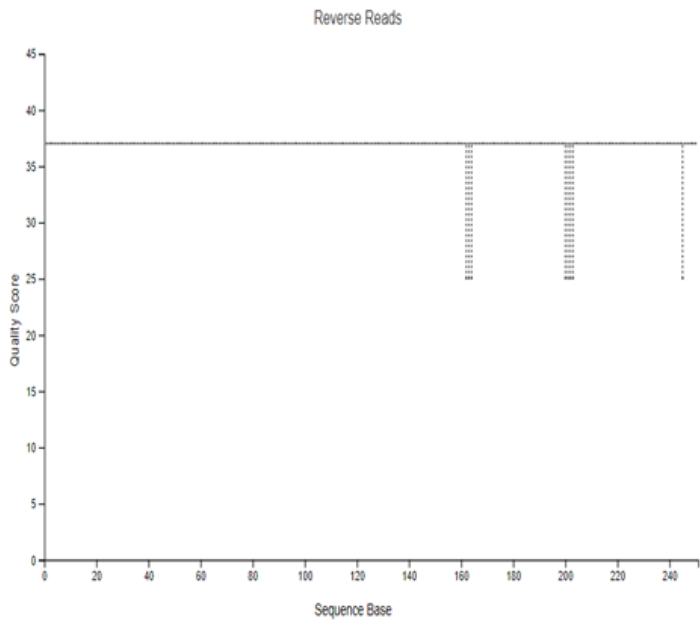
FIGURE 4.1: shows the forward and reverse reads of the sequence obtained from the glacier samples.

The minimum sequence length identified during subsampling was 238 bases. Outliers are ignored for better result and symmetry. Where as in Reverse reads the graph was generated using random sampling of 10000 out of 695238 sequences without replacement. The minimum sequence length identified during subsampling was 250bases.Outliers is ignored for better result and symmetry respectively.

4.2.3 Parametric Box Plot

TABLE 4.3: shows Parametric summary taken for box plot.

Parametric seven number summary			Parametric seven number summary		
Box plot feature	Percentile	Quality score	Box plot\ feature	Percentile	Quality score
Not shown in box plot	2nd	..	Not shown in box plot	2nd	..
Lower whisker	9th	..	Lower whisker	9th	..
Bottom of box	25th	..	Bottom of box	25th	..

	Parametric seven number summary			Parametric seven number summary	
Mode of box	50th (median)	..	Mode of box	50th (median)	..
Top of box	75th	..	Top of box	75th	..
Upper whisker	91st	..	Upper whisker	91st	..
Not shown in box plot	99th	..	Not shown in box plot	99th	..

The median is minimally influenced by outliers than the mean, and is generally the chosen central tendency indicator when the distribution is not symmetrical. Demultiplexing is the step involved in processing the information in order to know which sequences came from which samples after they had all are sequenced together. By applying the box plot on the obtained result on the samples taken from the 5 glaciers, Figure 4.2 shows parametric Box plot applied on the sequence depth (parameter) on the samples. This describes that the given samples have the maximum or prominent sequence depth at 1800 and 2500 approximately.
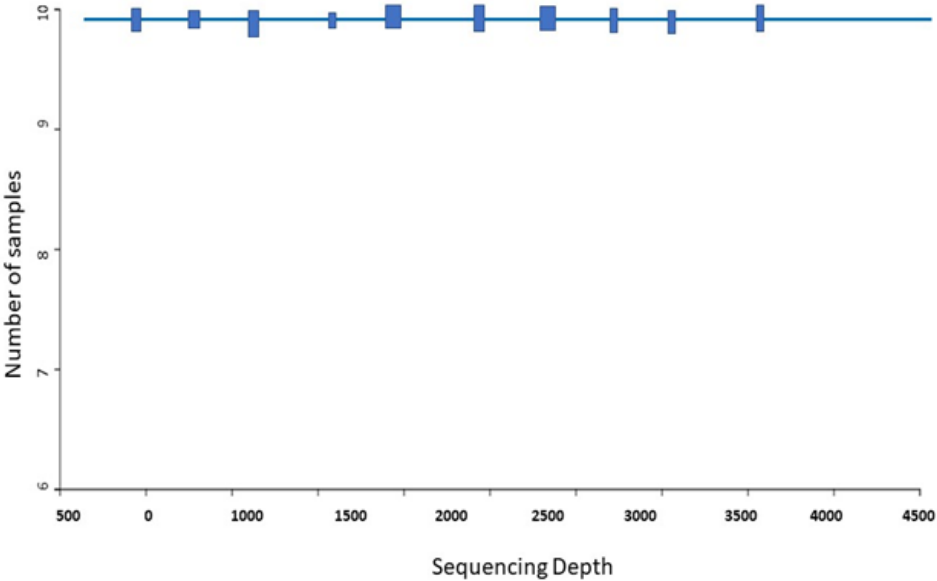

FIGURE 4.2: shows parametric Box plot applied on the sequence depth (parameter) on the samples.
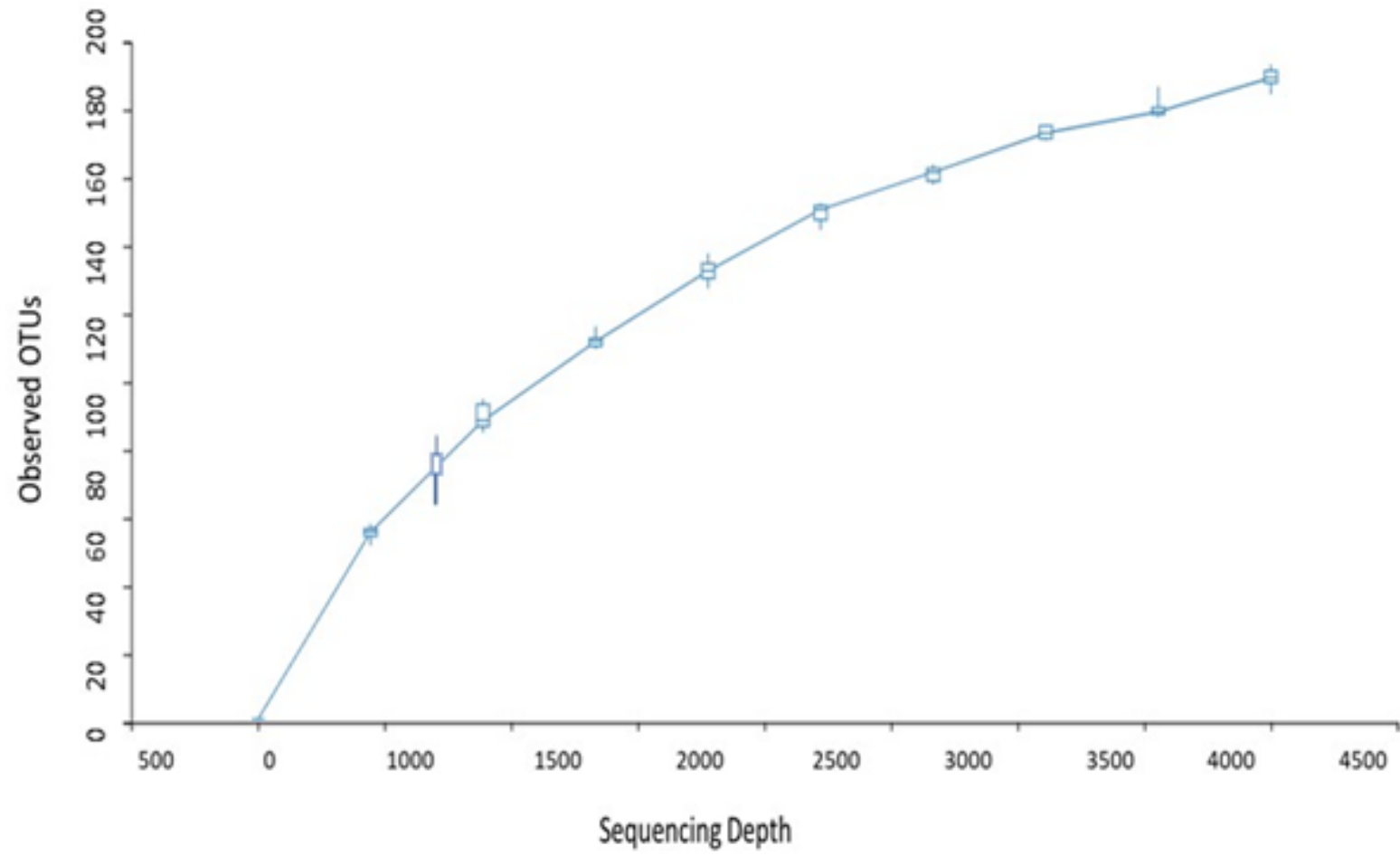
FIGURE 4.3: describes the Observed operational taxonomic unit (OTU)
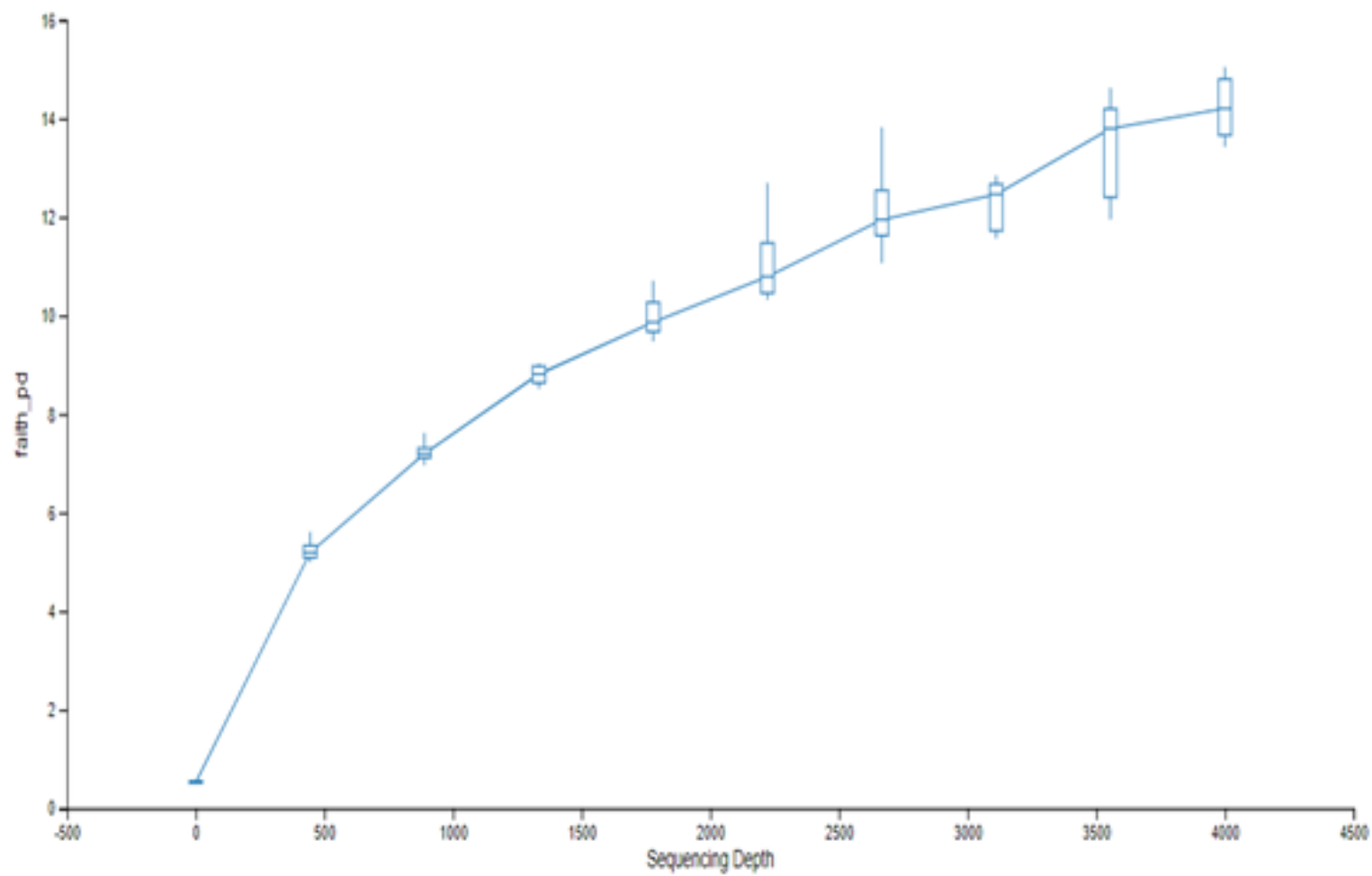
FIGURE 4.4: shows the Phylogenetic diversity against the sequence depth (Physiochemical parameter).

Figure 4.3 (OTU) which is used to classify groups of closely related individuals against the sequence depth ((Physiochemical parameter). It is the isotherm which means there is a linear increase in observed OTUs with increase in sequence depth, which means higher or larger the sequence depth more and greater the similarities. It is the regular linear increase in observed Phylogenetic diversity with increase in sequence depth, which means higher or larger the sequence depth more and greater the similarities. Resistance Genes (Heat Map):
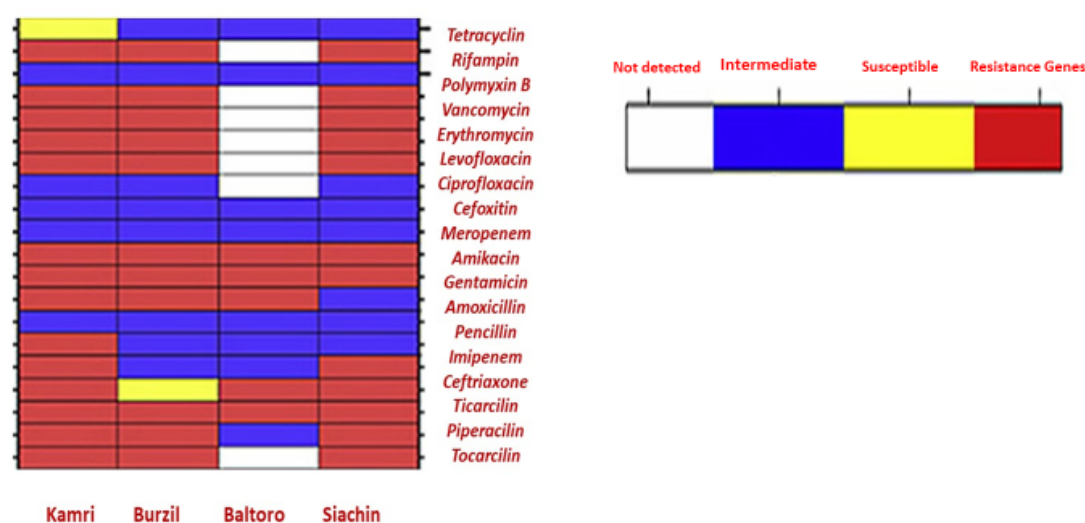
FIGURE 4.5: Total Abundance of Antibiotic Resistance Gene Sequences in Glacial Soils

Figure 4.5 shows the diversity abundance of the antibiotic markers gene sequences in the glacial soil. Highest number of resistance gene found in the Kamri region while Ticarcilin, Amikacin and Gentamicin were present in all the four region as antibiotic markers . In the Baltoro region some of the antibiotic marker were not detected , lowest number of antibiotic resistance markers/genes were recorded from this region.

4.2.4   Shanon Index Against Sequence Depth

The Shannon index is in the range of 2.2 to 2.6. Generally, its value between 1.5 and 3.5 in most ecological studies. The Shannon index rises as the community 's richness as well as its evenness rise. This describes the index for a species' Beta
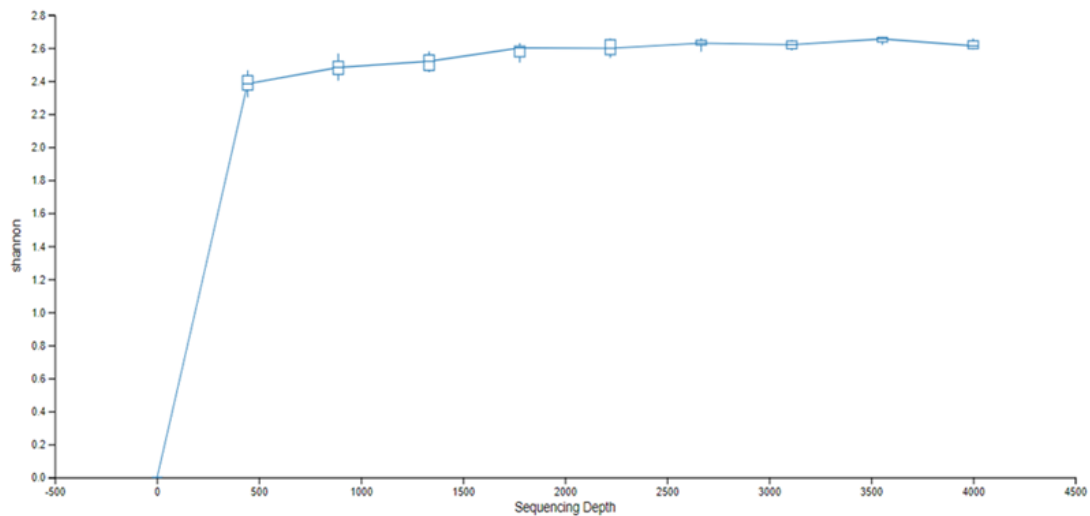
FIGURE 4.6: shows the Shannon index against the sequence depth of Physio-chemical parameters.

diversity in a population, since it is the combination of all the pairs collected from the various samples.

4.2.5 Phylogenetic Diversity (Heat Map)

Figure 4.6 shows the diversity abundance in different samples. Heat map shows that highest bacterial diversity is observed in Kamri in summer (KMs), while lowest diversity is present in Burzil in winter (BZw).

Proteobacteria display higher OTUs accompanied by *Actinobacteria*, *Firmicutes*, and then *Bacteriodetes* in the heat map correlated with the relative percentage of each OTU in the samples. The *Proteobacteria* having the highest diversity in all the selected glaciers. Values are the taxa 's relative abundance in percentage of total sequences and figure shows taxa with an abundance of ¿ 0.01 per cent. It is important to remember that values are rounded to one digit; thus, the abundance in one sample of a taxon with a value of 0.0 that differ between 0.00 and 0.04 percent.

Values are the taxa 's relative abundance in percentage of total sequences and fig-ure shows taxa with an abundance of ¿ 0.01 per cent. It is important to remember
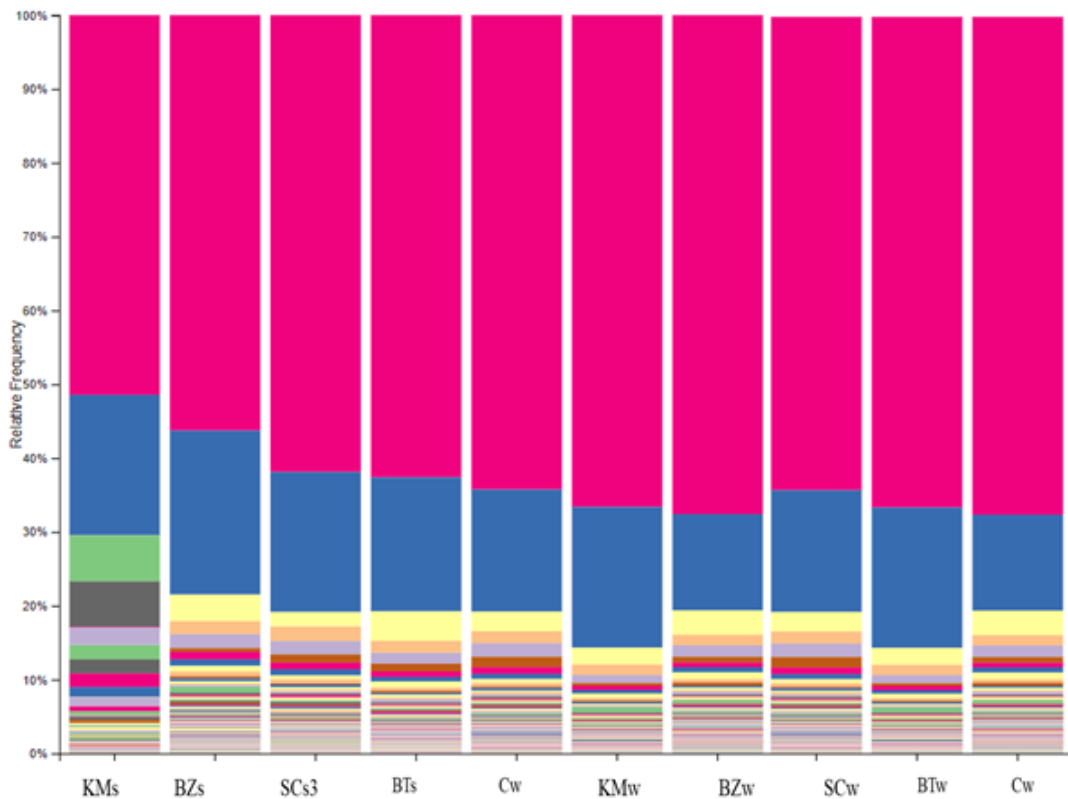
FIGURE 4.7: The diversity abundance in different samples.
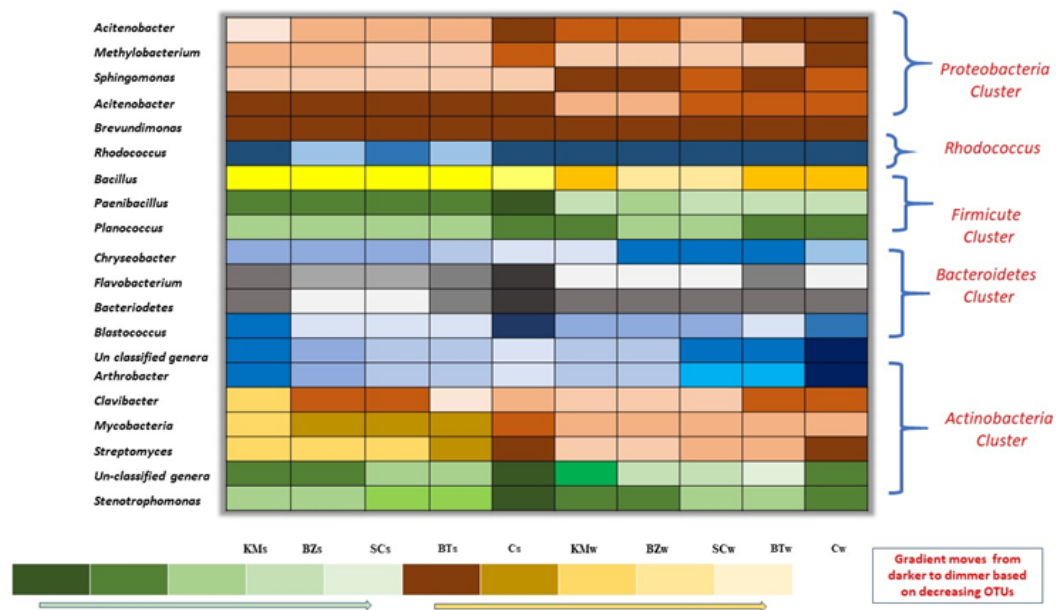
FIGURE 4.8: Distribution of aligned and assigned 97 per cent clustered OTUs to bacterial genera.

that values are rounded to one digit; thus, the abundance in one sample of a taxon with a value of 0.0 that differ between 0.00 and 0.04 percent.

Approximately 40% of the 5 detected bacterial groups were found in a majority of glaciers. Approximately 60% of the resistance bacteria group were detected and are found only in a rest of glaciers, particularly *Proteobacteria, Rhoddococcus, Firmicute, Bacteriodetes, Actinobacteria* clusters. These data will be used to study and understand the pool of resistance bacteria present in the 5 glaciers samples of Pakistan and to understand the links between samples(glaciers) and environmental bacteria with respect to these resistance genes.

	KMs	BZs	SCs	BTs	Cs	KMw	BZw	SCw	BTw	Cw
Unassigned	11.33	6.72	14.22	11.56	12.55	11.45	12.34	16.75	15.45	22.34
Proteobacteria	60.33	83.56	76.77	78.66	80.43	45.67	40.78	38.67	30.11	89.97
Firmicutes	2.75	1.61	1.55	1.33	65.54	3.33	1.72	2.11	1.56	70.45
Bacteriodetes	18.73	10.64	5.44	7.43	11.32	2.34	4.53	1.34	3.45	20.98
Actinobacteria	4.12	4.54	3.21	2.34	7.89	20.98	33.45	29.98	30.78	8.90
Acidobacteria	1.34	1.54	2.43	3.00	1.91	2.34	3.32	4.56	3.99	0.02

FIGURE 4.9: shows the dominancy of *Proteobacteria* diversity abundance in terms of clusters in different samples.

It shows that the proteobacter cluster is dominant in all the samples obtained from selected glaciers of Pakistan . In summers most of the *Proteobacteria* found from the Burzil glacier , in winters highest numbers of *Proteobacteria* found from the controlled sample.

4.3 Beta Diversity Analysis

4.3.1 Canonical Correspondence Analysis

Canonical Correspondence Analysis (CCA) based on chi-squared distance showing the correlation between parameters of the environment and genes and genes resistant. Every row is significantly correlated ($p < 0.05$) and reflects the value of ambient parameters. The circles represent the genes that represent antibiotic resistance.
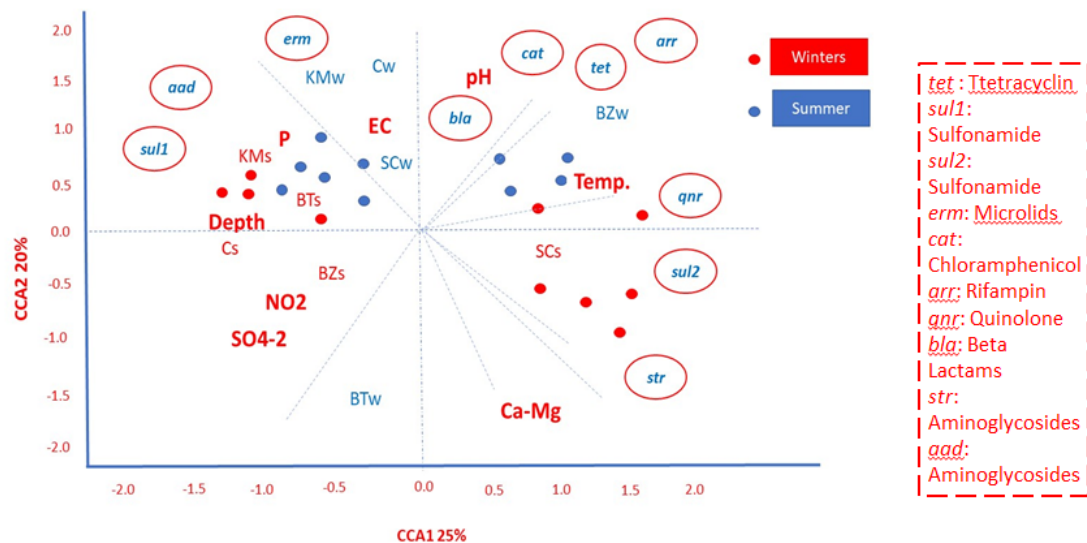
FIGURE 4.10: Canonical Correlation Analysis (CCA) was plotted at CC1 with 25% coordinates and at CCA2 with 20% coordinates.

Results from this study suggest that the value of a resistant in depth (comprises all antibiotic resistance genes, including resistance elements present in both pathogenic bacteria and antibiotic-producing bacteria), investigation to access the genetic capacity that microbial communities have acquired to thrive in these cold, remote and seemingly pristine environments. The PH of physiochemical aspects resistance genes are present in Burzil in winter which can resist Tetracycline, Chloramphenicol, Rifampin and beta lactams. The Temperature of physiochemical aspects resistance genes is present in Burzil in winter which can resist quinolone.

The Calcium- Magnesium aspects of physiochemical aspects resistance genes are present in Siachin in summer which can resist sulphonamide, and aminoglycosides. The Electricity conductivity of physiochemical aspects resistance genes is present in Siachin, Kamri in winter which can resist microlids. The Depth and Phosphorous of physiochemical aspects resistance genes are present in Kamri and Baltoro in summer which can resist aminoglycosides and sulphonamide. From these analysis it is observed that maximum resistivity offered in Burzil glacier in winter to Tetracycline, Chloramphenicol, Rifampin and beta lactams in PH(physiochemical aspect) and in Temperature(physiochemical aspect) resistivity is offered to quinolone in winter.

4.3.2 Principle Component Analysis
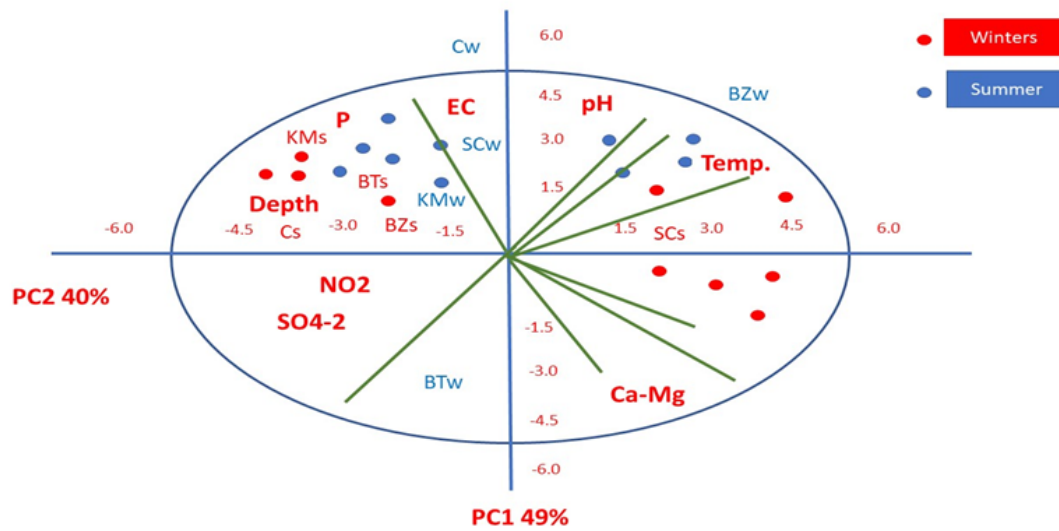
FIGURE 4.11: Principle Component Analysis was plotted at PC1 with 49% coordinates and at PC2 with 40% coordinates.

Principle Component Analysis is based on physicochemical characteristics and phyla distribution in samples. So from these analyses it is observed that maximum distributions of phyla in the samples are present in Siachin and Kamri in winter, and Baltoro, Burzil and Kamri in summer among electricity conductivity, phosphorous and depth (physiochemical aspects).

4.4 Discussion

In the recent study , the samples were taken from the different glaciers present in Pakistan , the sample were taken during winter and summer as well so that complete conditions on these glaciers were easily be recorded as shown in (figure 4.2.) and identify the different physiochemical aspects to know the in-depth climate condition and environment condition of these glaciers. The samples were collected during winter and summer to analyse the proper distribution of bacteria around the whole year on these glaciers. The physiochemical features of the samples which were collected from the 5 glaciers and the parameters are depth,

temperature, pH(power of hydrogen), EC(electrical conductivity), Sulphate, nitrate, phosphorous, Potassium, Calcium – Magnesium, and pH ice and pH snow respectively, shows it is noted that temperature in winter is highest in Siachin (-50C), where as in summer it is -20C, and in summer the pH of Siachin is maximum (8.229), where as in winter the pH of Baltoro has the maximum (6.91), from the sample the EC was highest in Siachin and Baltoro in summer and winter both , the nutrients concentration found highest in the Burzil in summer which is 0.411% same as in the nitrate, phosphorous and in the potassium respectively, maximum concentration of sulpate 0.411% is observed in Burzil in summer and minimum value 0.004% is observed in Baltoro in summer, maximum concentration of nitrate 0.0087% is observed in Siachin in summer and minimum value 0.00095% in Kamri in summer, phosphorus maximum concentration 0.0261% is observed in Kamri in summer, minimum concentration 0.0001% is observed in control in summer, maximum concentration of potassium 344% is observed in Baltoro in summer, minimum concentration of potassium 144 is observed in control in summer, concentration of calcium and magnesium is maximum 5.771% in Baltoro in winter, while minimum 1.300% in control in summer.

We can find that mostly minimum values of nutrients were observed in control sample the reason behind that control sample is not in a stressed environment so most minimum values observed in control sample. The surroundings of the four glaciers study areas are almost similar. We investigated snow sample bacterial communities from Pakistan glaciers (Km, Bz, Sc and Bt) and identified OTUs, which were mostly associated with Burkholderiales (*Proteobacteria*), Xanthomonadales (*Proteobacteria*), Cytophagales (Bacteroidetes) and Bacillales (*Firmicutes*). *Cyanobacteria* abundance was found in all samples and contrasted with other reports of snow bacteria from both polar and other mountain regions [66] ; [67]; [69]. Yoshimura and others (1997), recorded low *Cyanobacteria* abundance in the snow but more diversity on the glaciers[65]. For the understanding the bacterial diversity of the antibiotic resistome producing the antibiotic resistant genes from the selected four sites of the glaciers used as a indicator for the anthropological impact on the natural environment.

The soil of these glaciers was tested and in forward reads the graph was generated using random sampling of 10000 out of 695238 sequences without replacement. The minimum sequence length identified during subsampling was 238 bases.Outliers are ignored for better result and symmetry. Where as in Reverse reads the graph was generated using random sampling of 10000 out of 695238 sequences without replacement.The minimum sequence length identified during subsampling was 250bases.Outliers is ignored for better result and symmetry respectively (figure 4.3).

Demultiplexed sequence is applied on the sequence forward reads as well as reverse reads and it is observed that in forward reads its median is 238nt, and in reverse reads its 250 nt respectively. The median is minimum affected by outliers than the mean, and it is usually the preferred measure of central tendency when the distribution is not symmetrical.(figure 4.4).By applying the box plot on the obtained result on the samples taken from the 5 glaciers for better visualization of the obtained results.(figure4.6) and its observed that the result is significant at the sequence depth between 1800 to 2500. OTU is performed on the given result which is an isotherm means that there is a linear increase in observed OTUs with increase in sequence depth, which means higher or larger the sequence depth more and greater the similarities, which means more depth more similarities and less depth less similarities(figure 4.7).

To know the phylogenetic distribution about these resistant genes applied the phylogenetic distribution (figure 4.8), and then on the basis of phylogenetic distribution it is applied the Shannon index is in the range of 2.2 to 2.6. In prokaryotic species, Pyrosequencing of DNA isolated from glacial ice of Northern Schneeferner, Germany, assessed phylogenetic abundance as being the predominant phylogenetic classes of *Proteobacteria* (mostly Beta*Proteobacteria*), Bacteroidetes and *Actinobacteria*[67]. For most ecological research its importance is usually between 1.5 and 3.5. The Shannon index rises as the community 's richness as well as its evenness rise. It explains that in a population the index for a species' Beta diversity as it is the combination of all the pairs collected from the various samples.

Approximately 40% of the 5 detected bacterial groups were found in a majority of glaciers. Approximately 60% of the resistance bacteria group were detected and are found only in a rest of glaciers, particularly *Proteobacteria*, *Rhoddococcus*, *Firmicute*, *Bacteriodetes*, *Actinobacteria* clusters. Among all the antibiotic resistance genes analyzed, 5 resistant bacteria phyla were detected in glaciers samples. In 2009 Xinag et al reported that *Proteobacteria*, *Cyanobacteria*, *Firmicutes*, and Bacteroidetes were the most commonly found phyla in glaciers[70]. The most frequently encountered genes were *Proteobacteria* phylum in all the sample sites. For better visualization and distribution of phylum among the glaciers pie chart are made and it is observed that physiochemical aspects such as EC(electrical conductivity and P (phosphates) are most abundant in all the antimicrobial activity in mostly all the seasons[68](figure 13,14).

The dominancy of *Proteobacteria* cluster is dominant in all the phylum of bacteria , the highest number of species of Protobacteria was in Burzil summer and control winter. *Actinobacteria* in baltoro winter and most species of *Firmicutes* in the Control winters. The least number of species found in the Kamri summer and baltoro winter.This shows that *Proteobacteria* can easily be survived in the harsh cold environment they have the genome variability that help in the growth. CCA analysis and PCA analysis studied for the distribution of bacterial phyla from the selected sites of the glaciers according to the physiochemical parameters. The CCA analysis revealed the coorelation between the environmental factors, the resistome gene and the genera that have the greater impact on the environment. From the results the maximum resistivity was found in the Burzil Glacier. In winters Tetracyclin, Choloramphenicol, Rifampin, and Beta Lactams having resistivity to pH(physiochemical aspect), and in Temperature Quinoline in winters offered the maximum resistivity in the Burzil Glacier.

These data were used to study and understand the pool of resistance bacteria present in the 5 glaciers samples of Pakistan and to understand the links between samples(glaciers) and environmental bacteria with respect to these resistance genes. Because as these regions contains bacteria resistant genes so people from those areas will not recovered if giving these resistance antibiotics, so have

to give other antibiotic in order to overcome these microbial activities as these bacteria pool are resistant. In the previous findings the Antarctic surface snow samples were totally free from the resistance genes [64], here we detected different antibacterial resistant genes from the sample like tetracyclin, beta lactams, sulfomamide and others.

Chapter 5

Conclusions and Recommendations

Our study of the bacterial communities of Pakistan's selected glaciers showed that communities rich in the microbial population and bacterial diversity were dominated by similar phyla, particularly Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes. This result supports the argument that the composition of snow bacterial communities is driven primarily by bacteria present in the atmosphere above the region in which snowfall occurred and viable to the harsh cold environment in which they are fully involved. Bacterial isolates were mainly halotolerant and halophilic in nature. Nearly 60 percent of isolates with a wider temperature range for bacteria 's viability and growth. This study revealed the bacterial diversity of the glaciers having the high richness ecology and greater potential to produce the metabolites in the environment. Prevalent bacteria are strongly influenced by the type of physiochemical characteristics which was present there.

Since the samples were opportunistically obtained from four different expeditions with various ecological environments, this is the first comparison between bacterial communities with the antibiotic resistome from different areas investigated using the same analytical approach allowing for a novel analysis of the key factors that

form the snow bacterial culture. A warning bell may be the widespread presence of antibiotic resistance genes, suggesting a source of new genes that could further intensify the increase in antimicrobial resistance that could have clinical significance in glacier climate. In all the selected region the antibiotic markers were observed especially in the kamri region where most of the resistance genes were present but in the Baltoro region some of the genes were not detected. 59 Human activity can also be responsible for the introduction into the atmosphere of antibiotic-resistant bacteria, we can say that the antibacterial resistant bacteria may be introduced in the community because of the human activities.

Biologically AR genes are transmitted from one organism to another, especially in the micro environment where diverse microbial communities are exposed to an extensive antibiotic usage. Current studies, however found AR genes geographically isolated from certain area in the natural world. We examine the prevalence of antibiotic resistance genes in 10 glacier samples collected from 5 accessible sites from Kamri, Burzil, Siachin and Baltoro Glaciers, to assess the level of these genes in environments. A widespread distribution of antibiotic resistance genes in samples from various glaciers samples were observed namely Tetracycline (most of the bacteria were either intermediately or completely resistant to tetracycline), Sulfonamide, Microlids, Quinolone, Aminoglycoside and Beta Lactams were reported from the sample. Results shows the regional geographical distribution of AR genes, with the most feasible modes of transmission through airborne bacteria and migrating birds.

Bibliography

[1]. Ambrosini, R., et al., Diversity and assembling processes of bacterial communities in cryoconite holes of a Karakoram glacier. Microbial ecology, 2017. 73(4): pp. 827-837.

[2]. Williams, R.S., J.G. Ferrigno, and W. Manley, Glaciers of Asia. US geological survey professional paper, 2010: pp. 349-350.

[3]. Abbas, Q., et al., Floristic inventory and ethnobotanical study of the Naltar valley (Karakoram Range), Gilgit, Pakistan. Pak J Bot, 2013. 45: pp. 269-277.

[4]. Kohshima, S., A novel cold-tolerant insect found in a Himalayan glacier. Nature, 1984. 310(5974): pp. 225-227.

[5]. Montross, S.N., et al., A microbial driver of chemical weathering in glaciated systems. Geology, 2013. 41(2): pp. 215-218.

[6]. Garcia-Lopez, E., A.M. Moreno, and C. Cid, Microbial Community Structure and Metabolic Networks in Polar Glaciers, in Metagenomics-Basics, Methods and Applications. 2019. 53(543): pp 501-505.

[7]. Gomes, J. and W. Steiner, The biocatalytic potential of extremophiles and extremozymes. Food technology and Biotechnology, 2004. 42(4): pp. 223-225.

[8]. Rothschild, L.J. and R.L. Mancinelli, Life in extreme environments. Nature, 2001. 409(6823): pp. 1092-1101.

[9]. Rafiq, M., Culture Dependent and Metagenomic study of Microbial Diversity of Glaciers in HKKH (Hindu Kush, Karakoram and Himalaya) mountain range., Quaid-i-Azam University Islamabad, Pakistan, 2016. 45(321): pp. 101-106

[10]. D'Costa, V.M., et al., Sampling the antibiotic resistome. Science, 2006. 311(5759): pp. 374-377.

[11]. D'Costa, V.M., E. Griffiths, and G.D. Wright, Expanding the soil antibiotic resistome: exploring environmental diversity. Current opinion in microbiology, 2007. 10(5): pp. 481-489.

[12]. Wang, F., et al., Influence of soil characteristics and proximity to Antarctic research stations on abundance of antibiotic resistance genes in soils. Environmental science & technology, 2016. 50(23): pp. 12621-12629.

[13]. Fitzpatrick, D. and F. Walsh, Antibiotic resistance genes across a wide variety of metagenomes. FEMS microbiology ecology, 2016. 92(2): pp. 168-170.

[14]. Wales, A.D. and R.H. Davies, Co-selection of resistance to antibiotics, biocides and heavy metals, and its relevance to foodborne pathogens. Antibiotics, 2015. 4(4): pp. 567-604.

[15]. Wright, G.D., The antibiotic resistome: the nexus of chemical and genetic diversity. Nature Reviews Microbiology, 2007. 5(3): pp. 175-186.

[16]. Tamae, C., et al., Determination of antibiotic hypersensitivity among 4,000 single-gene-knockout mutants of Escherichia coli. Journal of bacteriology, 2008. 190(17): pp. 5981-5988.

[17]. Balabanova, B., Potential environmental, ecological and health effects of soil antibiotics and ARGs, in Antibiotics and Antibiotics Resistance Genes in Soils. 2017, Springer. pp. 341-365.

[18]. Martínez, J.L., F. Baquero, and D.I. Andersson, Predicting antibiotic resistance. Nature Reviews Microbiology, 2007. 5(12): pp. 958-965.

[19]. Martínez, J.L., Antibiotics and antibiotic resistance genes in natural environments. Science, 2008. 321(5887): pp. 365-367.

[20]. Ehret, G.B., et al., Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk. Nature, 2011. 478(7367): pp. 103-104.

[21]. D'Costa, V.M., et al., Antibiotic resistance is ancient. Nature, 2011. 477(7365): pp. 457-458.

[22]. Allen, A.P. and J.F. Gillooly, Assessing latitudinal gradients in speciation rates and biodiversity at the global scale. Ecology letters, 2006. 9(8): pp. 947-954.

[23]. Cowan, D.A., et al., Diverse hypolithic refuge communities in the McMurdo Dry Valleys. Antarctic Science, 2010. 22(6): pp. 714-720.

[24]. Cavicchioli, R., Extremophiles and the search for extraterrestrial life. Astrobiology, 2002. 2(3): pp. 281-292.

[25]. Deming, J.W., Psychrophiles and polar regions. Current opinion in microbiology, 2002. 5(3): pp. 301-309.

[26]. Margesin, R., G. Zacke, and F. Schinner, Characterization of heterotrophic microorganisms in alpine glacier cryoconite. Arctic, Antarctic, and Alpine Research, 2002. 34(1): pp. 88-93.

[27]. Feller, G. and C. Gerday, Psychrophilic enzymes: hot topics in cold adaptation. Nature reviews microbiology, 2003. 1(3): pp. 200-208.

[28]. Georlette, D., et al., Some like it cold: biocatalysis at low temperatures. FEMS microbiology reviews, 2004. 28(1): pp. 25-42.

[29]. Margesin, R. and V. Miteva, Diversity and ecology of psychrophilic microorganisms. Research in microbiology, 2011. 162(3): pp. 346-361.

[30]. Christner, B.C., et al., Recovery and identification of viable bacteria immured in glacial ice. Icarus, 2000. 144(2): pp. 479-485.

[31]. Segawa, T., et al., Altitudinal changes in a bacterial community on Gulkana Glacier in Alaska. Microbes and environments, 2009: pp. 1006140196-1006140196.

[32]. Zhang, S., et al., Abundance and community of snow bacteria from three glaciers in the Tibetan Plateau. Journal of Environmental Sciences, 2010. 22(9): pp. 1418-1424.

[33]. Bai, Y., et al., Phylogenetic diversity of culturable bacteria from alpine permafrost in the Tianshan Mountains, northwestern China. Research in microbiology, 2006. 157(8): pp. 741-751.

[34]. Xiang, S.-R., et al., Dominant bacteria and biomass in the Kuytun 51 Glacier. Appl. Environ. Microbiol., 2009. 75(22): pp. 7287-7290.

[35]. Cheng, S.M. and J.M. Foght, Cultivation-independent and-dependent characterization of bacteria resident beneath John Evans Glacier. FEMS microbiology ecology, 2007. 59(2): pp. 318-330.

[36]. Foght, J., et al., Culturable bacteria in subglacial sediments and ice from two southern hemisphere glaciers. Microbial Ecology, 2004. 47(4): pp. 329-340.

[37]. Piette, F., C. Struvay, and G. Feller, The protein folding challenge in psychrophiles: facts and current issues. Environmental Microbiology, 2011. 13(8): pp. 1924-1933.

[38]. Morgan-Kiss, R.M., et al., Adaptation and acclimation of photosynthetic microorganisms to permanently cold environments. Microbiol. Mol. Biol. Rev., 2006. 70(1): pp. 222-252.

[39]. Lee, Y.M., et al., Polar and Alpine Microbial Collection (PAMC): a culture collection dedicated to polar and alpine microorganisms. Polar biology, 2012. 35(9): pp. 1433-1438.

[40]. Amato, P., et al., Bacterial characterization of the snow cover at Spitzberg, Svalbard. FEMS microbiology Ecology, 2007. 59(2): pp. 255-264.

[41]. Larose, C., et al., Microbial sequences retrieved from environmental samples from seasonal Arctic snow and meltwater from Svalbard, Norway. Extremophiles, 2010. 14(2): pp. 205-212.

[42]. Liu, Y., et al., Seasonal variation of snow microbial community structure in the East Rongbuk glacier, Mt. Everest. Chinese Science Bulletin, 2006. 51(12): pp. 1476-1486.

[43]. Liu, Y., et al., Culturable bacteria in glacial meltwater at 6,350 m on the East Rongbuk Glacier, Mount Everest. Extremophiles, 2009. 13(1): pp. 89-99.

[44]. Miteva, V., et al., Comparison of the microbial diversity at different depths of the GISP2 Greenland ice core in relationship to deposition climates. Environmental microbiology, 2009. 11(3): pp. 640-656.

[45]. Liu, Y., et al., Bacterial diversity in the snow over Tibetan Plateau Glaciers. Extremophiles, 2009. 13(3): pp. 411-423.

[46]. Amoroso, A., et al., Microorganisms in dry polar snow are involved in the exchanges of reactive nitrogen species with the atmosphere. Environmental science & technology, 2010. 44(2): pp. 714-719.

[47]. Zinger, L., et al., Microbial diversity in alpine tundra soils correlates with snow cover dynamics. The ISME journal, 2009. 3(7): pp. 850-859.

[48]. Zakhia, F., et al., Cyanobacteria in cold ecosystems, in Psychrophiles: from biodiversity to biotechnology, 2008. 41(6): pp. 121-135.

[49]. Vincent, W.F., Cold tolerance in cyanobacteria and life in the cryosphere, in Algae and cyanobacteria in extreme environments. 2007. 10(2): pp. 287-301.

[50]. Yang, D., et al., Diversity and distribution of the prokaryotic community in near-surface permafrost sediments in the Tianshan Mountains, China. Canadian journal of microbiology, 2008. 54(4): pp. 270-280.

[51]. Zhang, G., et al., Diversity and distribution of alkaliphilic psychrotolerant bacteria in the Qinghai–Tibet Plateau permafrost region. Extremophiles, 2007. 11(3): pp. 415-424.

[52]. Margesin, R. and J.W. Fell, Mrakiella cryoconiti gen. nov., sp. nov., a psychrophilic, anamorphic, basidiomycetous yeast from alpine and arctic habitats. International Journal of Systematic and Evolutionary Microbiology, 2008. 58(12): pp. 2977-2982.

[53]. Trotsenko, Y.A. and V.N. Khmelenina, Aerobic methanotrophic bacteria of cold ecosystems. FEMS Microbiology Ecology, 2005. 53(1): pp. 15-26.

[54]. Steven, B., T.D. Niederberger, and L.G. Whyte, Bacterial and archaeal diversity in permafrost, in Permafrost soils. 2009. 24(9). pp. 59-72.

[55]. Adams, H.E., B.C. Crump, and G.W. Kling, Temperature controls on aquatic bacterial production and community dynamics in arctic lakes and streams. Environmental microbiology, 2010. 12(5): pp. 1319-1333.

[56]. Männistö, M.K., M. Tiirola, and M.M. Häggblom, Bacterial communities in Arctic fjelds of Finnish Lapland are stable but highly pH-dependent. FEMS microbiology ecology, 2007. 59(2): pp. 452-465.

[57]. Jilani, R., M. Haq, and A. Naseer. A study of Glaciers in Northern Pakistan. in The first joint PI symposium of ALOS data nodes for ALOS science program in Kyoto Secretariat, Japan Aerospace Exploration Agency (JAEA), Kyoto International Conference Centre, Main Hall. 2007. 30(14): pp. 1571-1591

[58]. Baker-Austin, C., et al., Co-selection of antibiotic and metal resistance. Trends in microbiology, 2006. 14(4): pp. 176-182.

[59]. Breidenstein, E.B., et al., Complex ciprofloxacin resistome revealed by screening a Pseudomonas aeruginosa mutant library for altered susceptibility. Antimicrobial agents and chemotherapy, 2008. 52(12): pp. 4486-4491.

[60]. Ali, J., Q.A. Rafiq, and E. Ratcliffe, Antimicrobial resistance mechanisms and potential synthetic treatments. Future science OA, 2018. 4(4): pp. O290.

[61]. Handelsman, J., et al., Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. Chemistry & biology, 1998. 5(10): pp. R245-R249.

[62]. Simon, C., et al., Phylogenetic diversity and metabolic potential revealed in a glacier ice metagenome. Appl. Environ. Microbiol., 2009. 75(23): pp. 7519-7526.

[63]. Von Mering, C., et al., Quantitative phylogenetic assessment of microbial communities in diverse environments. science, 2007. 315(5815): pp. 1126-1130.

[64]. Ushida, K., et al., Application of real-time PCR array to the multiple detection of antibiotic resistant genes in glacier ice samples. The Journal of General and Applied Microbiology, 2010. 56(1): pp. 43-52.

[65]. Yoshimura, Y., S. Kohshima, and S. Ohtani, A community of snow algae on a Himalayan glacier: change of algal biomass and community structure with altitude. Arctic and Alpine Research, 1997. 29(1): pp. 126-137.

[66]. Hauptmann, A.L., et al., Bacterial diversity in snow on North Pole ice floes. Extremophiles, 2014. 18(6): pp. 945-951..

[67] Michaud, L., et al., Snow surface microbiome on the High Antarctic Plateau (DOME C). PLoS One, 2014. 9(8): pp. e104505.

[68]. Azzoni, R.S., et al., Geomorphology of Mount Ararat/Ağri Daği (Ağri Daği Milli Parki, Eastern Anatolia, Turkey). Journal of Maps, 2017. 13(2): pp. 182-190.

[69]. Cameron, K.A., et al., Diversity and potential sources of microbiota associated with snow on western portions of the G reenland I ce S heet. Environmental Microbiology, 2015. 17(3): pp. 594-609.

[70]. Xiang, S.R., et al., Changes in diversity and biomass of bacteria along a shallow snow pit from Kuytun 51 Glacier, Tianshan Mountains, China. Journal of Geophysical Research: Biogeosciences, 2009. 114(G4): pp 12-19.

[71]. Miteva, V., Bacteria in snow and glacier ice, in Psychrophiles: from biodiversity to biotechnology. 2008, Springer.55(7): pp. 31-50.

[72]. Price, P.B., A habitat for psychrophiles in deep Antarctic ice. Proceedings of the National Academy of Sciences, 2000. 97(3): pp. 1247-1251.

[73]. Siegert, M.J., et al., Physical, chemical and biological processes in Lake Vostok and other Antarctic subglacial lakes. Nature, 2001. 414(6864): pp. 603-609.

[74]. Yergeau, E., et al., The functional potential of high Arctic permafrost revealed by metagenomic sequencing, qPCR and microarray analyses. The ISME journal, 2010. 4(9): pp. 1206-1214.

[75]. Johnson, S.S., et al., Ancient bacteria show evidence of DNA repair. Proceedings of the National Academy of Sciences, 2007. 104(36): pp. 14401-14405..

[76]. Cameron, K.A., A.J. Hodson, and A.M. Osborn, Structure and diversity of bacterial, eukaryotic and archaeal communities in glacial cryoconite holes from the Arctic and the Antarctic. FEMS microbiology ecology, 2012. 82(2): pp. 254-267.

[77]. Møller, A.K., et al., Bacterial community structure in High-Arctic snow and freshwater as revealed by pyrosequencing of 16S rRNA genes and cultivation. Polar Research, 2013. 32(1): pp. 17390.

[78]. Cheng, S.M. and J.M. Foght, Cultivation-independent and -dependent characterization of bacteria resident beneath John Evans Glacier. FEMS microbiology ecology, 2007. 59(2): pp. 318-330.

[79]. Wright, G. D. (2007). The antibiotic resistome: the nexus of chemical and genetic diversity. Nature Reviews Microbiology, 5(3): pp. 175-186.