STUDY OF BACTERIAL DIVERSITY OF DAL LAKE, KASHMIR WITH PARTICULAR REFERENCE TO PATHOGENIC BACTERIA

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By

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Certificate

Certified that the Ph.D. thesis entitled **"Study of bacterial diversity of Dal Lake, Kashmir with particular reference to pathogenic bacteria" is** the original research work carried out by **Samira Saleem**, as whole time Ph. D.scholar in Department of Environmental Science, University of Kashmir, Srinagar. The work has been carried out under our joint supervision and has not been submitted to this University or to any other University so far and is submitted for the first time to the University of Kashmir. It is further certified that the thesis is fit for submission for the degree of ***Doctor of Philosophy in Environmental Science*** and the candidate has fulfilled all the statutory requirements for the completion of the Ph.D. Programme.

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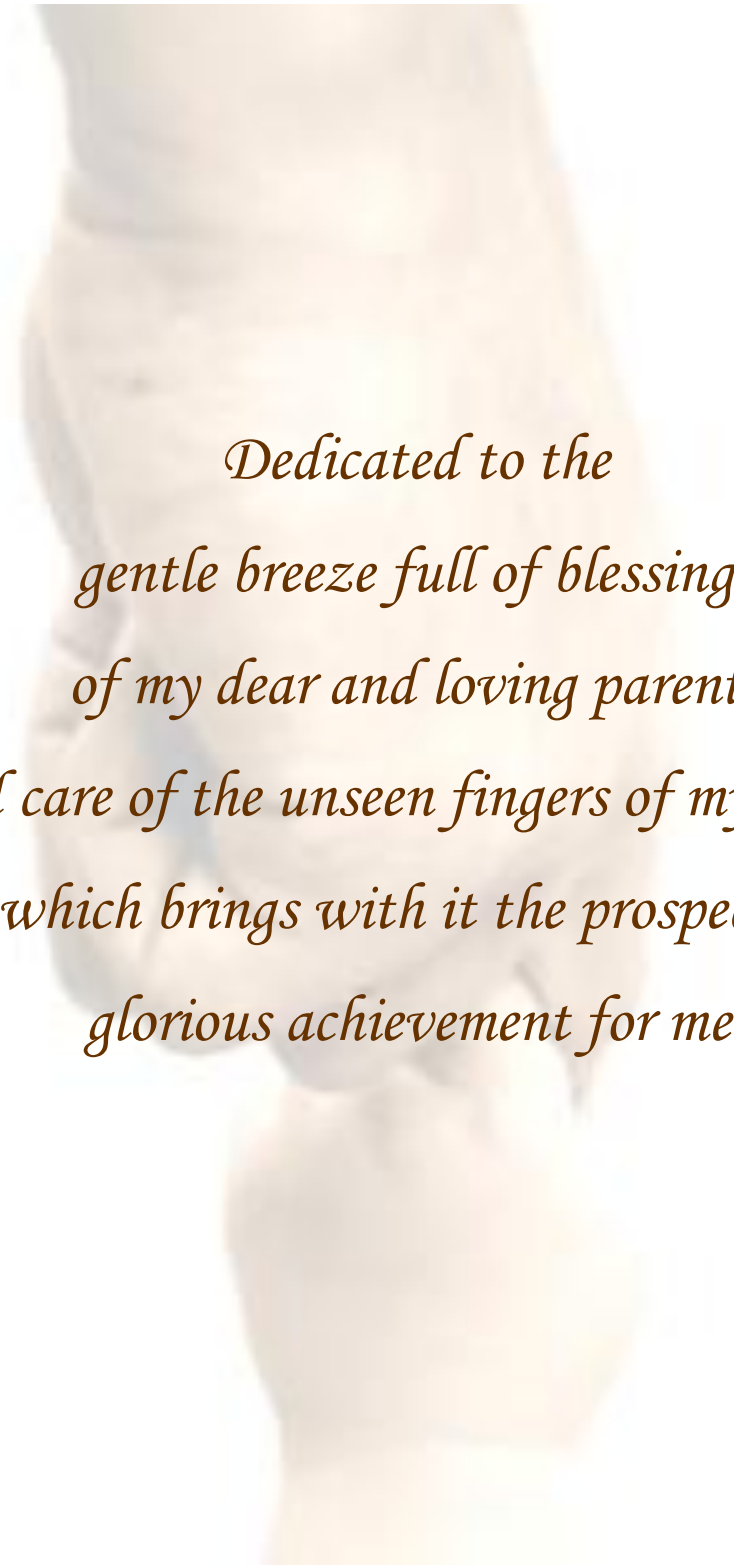

Dedicated to the
gentle breeze full of blessings
of my dear and loving parents
and care of the unseen fingers of my family
which brings with it the prospect of
glorious achievement for me.

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Samira Saleem

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Abbreviations used

ABBREVIATION	FULL FORM
ANOVA	Analysis of variance
APHA	American public health association
API	Analytical profile index
ARISA	Automated ribosomal intergenic spacer analysis
BIS	Bureau of Indian standard
BLAST	Basic local alignment search tool
BOD	Biological oxygen demand
C/P	Carbon-phosphorus ratio
Ca	Calcium
CARD-FISH	Catalysed reporter deposition fluorescent in situ hybridization
CFU	Colony forming units
COD	Chemical oxygen demand
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DOTUR	Distance based operational taxonomic unit and richness
EHEC	Enterohaemorrhagic *E. coli*
EMB	Eosin methylene blue agar
ETEC	Enterotoxigenic *E. coli*
F	Frequency
FAME	Fatty acid methyl ester
FC	Faecal coliform
Fig.	Figure
FISH	Fluorescent in situ hybridization
FS	Faecal streptococci
GI	Gastrointestinal
HPC	Heterotrophic plate count
Hrs	Hours
$Lb/inch^2$	Pound per inch square

m.a.s.l	Meters above sea level
Mg	Magnesium
Ml	Millilitre
MPN	Most probable number
N/P	Nitrogen-phosphorus ratio
NaCl	Sodium chloride
NCI	Number of cases of isolation
NSTC	National science of technology and council
OMZ	Oxygen minimum zone
ONPG	Ortho-nitrophenyl Beta-D-galactopyranoside
OR	Occurrence remarks
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PET	Poly-ethylene tetra phthalate
qPCR	Quantitative polymerase chain reaction
RF	Relative frequency
RNA	Ribonucleic acid
RPM	Revolution per minute
SPC	Standard plate count
Spp.	Species
SSCP	Single strand conformation polymorphism
STP	Sewage treatment plant
TC	Total count
TDA	Tryptophan deaminase
TP-RAPD	Two primer random amplified polymorphic DNA
TR-FLP	Terminal restriction fragment length polymorphism
TVC	Total viable count
UV	Ultra violet
VBNC	Viable but non culturable
WDS	Water treatment system
WHO	World health organization

Abstract

Water is a necessity for all living beings, without it there would be no life. Life originated in water and the ultimate basis of it, the protoplasm, is a colloidal solution of complex organic molecules in a watery medium (70 to 90% of water). Most of the biological phenomena take place in water medium. Moreover, wherever water exists in nature it always holds life. So the study of a water body is the study of life as well. Water is essential at all levels of life, cellular to ecosystem and it stands as the key substance for the existence and continuity of life through different cyclic process in nature; it plays the central role in mediating global scale ecosystem processes, linking atmosphere, lithosphere, and biosphere, by moving substances among them, and enabling chemical reactions to occur. Humans depend on this resource for all their needs of existence and survival. Nature has an innate mechanism to maintain its purity after every natural use, but unables to do so at the rate at which humans add dirt to it. Nature does not know how to deal with several toxins and pollutants that are flowing from industrial and other wastes. Therefore, humans are bound to monitor the impact of this activity on natural freshwaters continuously.

The study on Bacterial diversity of Dal lake with particular reference to Pathogenic bacteria was carried out from April 2010 to March 2012 .The study area

(Dal lake; latitude 34^0 07′ N, longitude 74^0 52′ E, altitude 1583 m) selected for this work is a multi-basined lake with many inlets and outlets, so an extensive network of sixteen sites with different altitudes and geographical co-ordinates viz., Hazratbal open, Hazratbal littoral, Nigeen open, Nigeen littoral, Gagribal open, Gagribal littoral, Nishat open, Near Centeur, Boathall nallah-I, Boathall nallah-II, Tailbal nallah-I, Tailbal nallah-II, Dal lock Gate-I, Dal lock gate-II, Pokhribal nallah-I and Pokhribal nallah-II were selected. Among the selected sites eight (8) sites were selected in the four basins, four (4) were selected from two inlets and four (4) were selected from two outlets. These sites selected included microhabitats from both littoral zones as well as limnetic zones. The water samples collected from sixteen sites were selected randomly from different basins, outlets and inlets of the lake, under consideration for exploring the bacterial diversity and were collected on seasonal basis in poly ethylene (PET) bottles, which were previously carefully cleaned and rinsed three to four times with distilled water. All the samples were collected from the surface and subsurface of lake water by plunging the open end of each sterile bottle before turning it upright to fill. During collection of samples, extreme care was exercised to avoid contamination of the parts of bottle and collected samples were processed for the analysis of bacterial community using the standard methodology. The glassware used and media prepared for the work was carefully sterilized using different standard techniques. The techniques used for the isolation of bacteria from the water samples included spread plate technique, pour plate technique and streak plate technique. The data on bacteriological analysis was analysed to measure the degree of contamination of water samples in Dal lake on seasonal basis from sixteen different microhabitats by plating the different dilutions on culture media that included general (Nutrient agar) as well as selective media like Eosin methylene blue agar, Cetrimide agar, etc. After incubation, the cultured bacterial colonies were enumerated in order to assess bacterial load in terms of colony forming unit (CFU/ml).The colonies were further characterized on the basis of macroscopic as well as microscopic character. The isolated colonies showed marked variation in their features and on these differences, a representative sample colony of each was coded as SMB-1 to SMB- 45.The coded colonies were streaked on selective media to obtain pure cultures and then for biochemical analysis, using Hi- media biochemical test strips, for further

identification. The biochemically identified bacteria (Bergey's manual specification) were subjected to molecular analysis by 16S rRNA gene using polymerase chain reaction which was carried out by means of universal bacterial primers 8F and 1492R which provides an accurate means to identify bacterial diversity and thereby studying the phylogenetic relationship between them. The sequences so obtained were confirmed and compared to known 16S rRNA sequences in gene bank (NCBI, Pune) by using BLAST algorithm and found to be 69% to 100% similar to the sequences of 16S rRNA gene of bacteria. Tests for the presence of total coliforms were carried out by multiple tube fermentation technique, which revealed higher level of total coliforms with their value ranging from 3 MPN/100ml.The highest number of these indicator organisms were observed at site 15 and 16 (Pokhribal outlet) in summer season and minimum at site 5 an 13 (inlet) in winter season. The category wise distribution of coliform count showed that 57.81% water samples lie in the category III followed by 39.07% in category II, 3.1% in category IV and 0% samples in category I. The perusal of data considers the water unfit for drinking purpose however, fit for recreational purposes. In order to gain insight into bacterial load, the culturable bacterial colonies were counted by Quebec colony counter and observed in terms of colony forming unit (CFU/ml) which reveals substantial number of heterotrophic bacteria. Total bacterial count ranged between 0.2×10^4 to 28.7×10^4 cfu/ml and the highest count was found at site 16 (Pokhribal outlet) in summer season and lowest count in winter season at site 6 (Boathall nallah). Out of 5941 colonies, 3123 colonies (52.56% occurrence) were isolated in summer season followed by 1502 colonies (25.28% occurrence) in autumn, 844 (14.87% occurrence) in spring and 432 (7.27% occurrence) in winter. The Gram's reaction revealed that out of 5941 colonies, 4146 (69.78%) were Gram negative and 1795 (30.21%) was Gram positive bacteria. Among 4146 Gram negative colonies, 4116 (69.33%) were bacilli and 30 (0.45%) were cocco- bacilli whereas among 1795 Gram positive colonies, 698 (11.72%) were bacilli and 1097 (18.49%) were cocci. The species identified biochemically belonged to family *Enterobacteriaceae* (23 spp.), *Bacillaceae* (5 spp.), *Enterococaceae* (5 spp.), *Vibrionaceae* (3 spp.), *Pseudomonadaceae* (2 spp.), *Micrococcaceae* (2 spp.), *Aeromonadaceae* (2 spp.), *Staphylococaceae* (1 spp.), *Moraxellaceae* (1 spp.) and *Alcaligenaceae* (1 spp.). The site wise abundance distribution and species

composition of different bacterial genera indicated that maximum number of species were isolated from family *Enterobacteriaceae* followed by *Bacillaceae* and *Enterococaceae.* The forty five (45) bacterial species were isolated during the course of study. The highest number of bacterial species occurred at site 1, 2, 9, 15 and 16 whereas lowest number of species (29) at site 6. The highest bacterial species were observed in summer months and lowest in winter month showing influence of temperature on bacterial colonies. Analysis of variance (ANOVA) showed that the observed distribution of the bacterial colonies in different seasons is statistically significant. Therefore, seasonal variation in occurrence of bacterial colonies was observed between different study sites of the lake. The bacterial flora in the lake consisted of diverse life forms ranging from Proteobacteria to Firmicutes and Actinobacteria which belonged to different selected micro habitats across the lake. The bacterial population showed a diverse seasonal and temporal variation on the basis of occurrence in different sites which were categorized into four groups named as open, littoral, outlet and inlet sites. *E.coli* was found to dominate different habitats of lake in all the seasons of the year. The ANOVA carried out between different sites for bacterial species isolated from different microhabitats showed that 71% results were statistically significant with 7% as highly significant ($p < 0.01$) and 27% as non significant ($p < 0.05$). The Bray Curtis cluster analysis of the study sites developed on the basis of presence and absence of a species at the respective sites showed similarity ranged from 31 to 87% with the least similarity of 31% between site 1 and 7 and maximum similarity of 87% between site 3 and 4. From the value of different indices computed for 16 sites for the occurrence of different bacterial species, the Shannon wiener index was highest at site 16 (3.68) and lowest at site 8 (3.26). The analysis of variance showed that highly dominance and diversity patterns varied significantly with highly even distribution of bacterial species in microhabitats of the lake. The data of correlation analysis between the pH and bacterial load at sixteen sites indicated that there was a negative correlation of pH with the bacterial load and positive correlation of temperature with bacterial load and the results were found to be statistically significant. For the purpose to study the impact of pathogenic bacteria isolated from Dal lake on humans, a random survey of the 20% of population i.e., 384 individuals (64 families) out of 1920 individuals (320 families) who were engaged in

one or the other activity related to lake water was carried out through questionnaire. The data of which reveals that the disease was more prevalent in males than in females under the age group of 20 years followed by age group of 21 to 40 years and then by above 40 years. The symptoms of gastrointestinal diseases were reported in 26 cases (6.77%) and other symptoms like high fever, chills, rigors, sweating and body aches in 17 cases (4.42%). The prevalence as per the source of water being used revealed that disease symptoms were more prevalent in individuals consuming lake water as compared to tap water. The results were statistically found to be significant. The most of the bacteria isolated were recognized as human pathogens, capable of initiating water borne infections, thus potentially water transmitted. The obtained data in the study reflect the importance of microbiological monitoring especially related to pathogenic bacteria.

Chapter: 1

INTRODUCTION

Microbial diversity is an unseen natural resource that deserves greater attention. Too small to be seen no longer means too small to be studied or valued. Microbial diversity covers the spectrum of variability among all types of microorganisms (bacteria, fungi and viruses) in the natural world and as altered by human intervention. They play many roles both on land and in water, being the first to colonize and ameliorate effects of naturally occurring and man-made disturbed environments (NSTC, 1994).

Microorganisms constitute huge and almost unexplained reservoir of resources likely to provide innovative applications useful to humans. Microorganisms numerically and biochemically dominate all inland water habitats. The micro-organisms encompass the diverse assemblages of organisms such as bacteria which exhibit widely different morphological, ecological and physiological characteristics.

Microorganisms are small and least known and this gap in knowledge is particularly apparent for bacteria and other prokaryotic organisms. Current evidence suggests that 300,000 to 1 million species of prokaryotes exist on earth, yet only 3,100 bacteria are described in Bergey's Manual (Tiedje, 1994; Holt, 1984). They are ubiquitous and live in most inhospitable sites across various ecosystems, in which the existence and continuance of other organisms may not be possible.

1.1. Microbes and the environment

Microorganisms are the largest reservoir of genetics and biochemical diversity. They have been evolving for the last 3.8 billion years and inhabit virtually every environment. Microbes drive the chemistry of life, do much of the biogeo-chemical cycling that keeps the world habitable and even affect the global climate. Microbes have developed a wealth of functions that enable their survival in virtually every environmental niche, often where no other life form exists (Zhou *et al.*, 2002).

There are probably million of species in the domains of bacteria and archea (the prokaryotes), but each follow its own act of rules at the same time governed by the community they belong. Accordingly their distribution and abundance in natural environments have been reported. One characteristic that has become clear is that prokaryotes diversity in aquatic environment in order of magnitude is less in sediments and soil (Lozupone and Knight, 2007).

The knowledge about the distribution and abundance of microbes in different habitat is highly rudimentary as there is practically no geographic limit for distribution of microbes, though the preference of species to occupy the habitat of their choice always exists. Unlike the higher plants and animals, many microbes have the ability to make their presence felt by much diverse type of habitats. Few microbes, especially bacteria, are however highly selective in their choice for a set of conditions. The knowledge on the diversity of microbes of various habitats is so limited that we are now unable to correctly quantify the density and diversity of microbes of a habitat (Fierier and Lennon, 2011).

Microorganisms have been overlooked in traditional ecological studies, owing to a lack of adequate methods to quantify and characterize free-living bacteria and minute eukaryotes. Their small size, the absence of diagnostic phenotypic characters, and the fact that many microorganisms are difficult to isolate and to rear in culture have limited the evaluation of their biodiversity (Dorigo *et al.*, 2005). The advent of novel techniques such as direct counting by epifluorescense microscopy, image analysis, electron microscopy, flow cytometry and most recently, molecular tools to identify as yet uncultivable organisms, has revolutionized the field of microbial ecology during the past few decades (Weisse, 2003; Dorigo *et al.*, 2005). Bacteria and small eukaryotes have been used as model organisms to address questions of general

ecological relevance. Microorganisms are sensitive indicators of environmental quality. Their responsiveness to environmental change and their rapid reproductive capacities result in population changes that alter relative number and type of microorganisms; thus indices of microbial diversity are a sensitive measure for the environmental state of a given habitat or ecosystem. By determining the diversity of microorganisms at selected sites using conventional and modern molecular approaches, one can establish baselines against which changes in environmental quality can be measured. Microbial diversity measurement can be a sensitive and suitable index of environmental status and trends. Measurement of microbial diversity could be predictive of changes in populations that have resulted in serious threats to human, plant and animal health such as the outbreak of *Cryptosporidium* infections in Milwaukee. They also would permit detection of impact from chemical pollution and recovery of impacted biological communities (Mackenzie *et al*., 1994).

Differences in microbial community structures reflect the abilities of microorganisms to respond to specific environmental factors and substrates. Different microbial communities perform different functions in the environment which not only facilitate the matter transfer but also help in the distribution of matter in different parts of the environment, for example all the microbes are more or less involved in degradation of organic matters in the environment, and some species perform the degradation process more effectively. It is more specific in nature when the organic matter is a synthetic one and there are only specific microorganisms that metabolically or co-metabolically degrade it. There are also specific species to perform other functions like nitrogen fixation and phosphorous solubilization.

1.2. Microbial diversity in fresh water habitats

It is generally accepted that life originated between 3.5 and 4 billion years ago in the aquatic environment, initially as self-replicating molecules (Alberts, 1962). The subsequent evolution of prokaryotes followed by eukaryotes led to the existence of microorganisms which are highly adapted to aquatic systems.

Bacteria represent the richest repertoire of molecular and chemical diversities in nature as they comprise the most diverse form of life. Some of these are key players in the biogeochemical processes that are crucial for entire ecosystems. Distribution of bacteria depends upon change in the temperature and other

physicochemical parameters (Alavandi, 1990). Bacteria not only maintain the pristine nature of the environment but serve as key factors for various environmental processes. Therefore, bacterial diversity constitutes the most extraordinary reservoir of life in the biosphere that people have only just begun to explore and understand (Huston, 1994). Thus the study of bacterial diversity is important to solve new and emergent problem. Though the negative effects of bacteria such as disease are well known, their often subtle functions explain why their biodiversity positively affects human.

The microbial world in the environment is highly diverse with a lot of variation in their morphology, physiology and metabolism. The metabolic diversity and genetic elasticity of the microbial groups has enabled them to show their presence in each and every corner of the earth ranging from a favorable mesophytic habitat to an extremely unfavorable deep vent. In environmental microbiology, microbes are categorized into two distinct domains as prokaryotes and eukaryotes, both of which are intricately involved in all environmental processes.

The amount of fresh water on earth is very small in comparison to the water of the oceans but the fresh water have much more rapid renewal time making it hither to sufficient, enough to satisfy the need of all plants and animals on the earth. On a volumetric basis, fresh water is concentrated in large, deep basins of several great lakes. Inland water covers less than 2% of the earth surface, approx. 2.5 million km^2. About 20 lakes are extremely deep and significant portion of the world's fresh water is contained in lakes. More than 90% of earth's fresh water remains as stagnant water in the lakes forming a characteristic ecosystem, the remaining 10% flows through the rivers and streams connecting lakes and rivers with the terrestrial habitat.

Lotic system such as streams and rivers within their drainage basis are central to surface water ecosystem. The lakes constitute the most part of lentic ecosystem. The dominant inflows from the lakes occur in the surface streams. Each lake receives drainage water from many rivers and streams thus becoming a sink for the flow of soluble organic nutrient and particulate matters. The rate of input of both particulate and especially dissolved organic matter to lakes from their catchment regulates the biotic activities and productivity of lakes. The effect, that terrestrial biota has on the quality and quantity of inorganic and organic loading to the lake can be profound.

Water laded with organic and inorganic loads flows from elevations to the recipient lake both in groundwater and surface streams. Chemical and biological reactions occur *en route* that selectively modify the quantity and quality of nutrient and organic substances entering lakes. An appreciable loading of nutrients is common from agricultural activities of human. These compounds reach the drainage basins and lake itself causes a significant change in total nutrient contaminant loading (Anonymous, 1994).

1.3. Freshwater bacterial groups and their habitats

Microscopic life dominates in many aquatic ecosystems, the bulk of the energy production and a significant fraction of the material transformations are carried out by microorganisms. Among the aquatic microbes, the bacteria are key players in the sequestration of inorganic compounds, remineralisation and dissipation of organic material. Surveys of 16S ribosomal RNA gene sequences from freshwater habitats provide evidence of a freshwater bacterial assemblage that is distinct from terrestrial communities. Many of the freshwater groups appear to have widespread geographic distributions. This suggests that freshwater habitats, despite their geographic isolation, harbour bacterial species drawn from a remarkably consistent pool and this may be a general characteristic of freshwater biology (Anonymous, 1994).

Major phyla of bacteria can be found in freshwater environments. Some of these groups are freshwater-specific taxa, whereas others are adapted to a broad range of habitats.

1.3.1. Proteobacteria

The Proteobacteria are often the dominant prokaryotes in aquatic systems. The Proteobacteria lineage contains phototrophs, chemolithotrophs and chemo-organotrophs, and their members can be found in both oxic and anoxic environments. This phylum consists of several evolutionarily distinct subdivisions viz, Alpha-proteobacteria, Gamma-proteobacteria, Beta-proteobacteria and Delta-proteobacteria.

1.3.2. Cyanobacteria

Cyanobacteria are, not the only phototrophic bacteria, but are generally the dominant bacterial phototrophs in the oxygenated portions of lakes. Many

Cyanobacteria are capable of nitrogen fixation and thus they can be key players in both the carbon and nitrogen cycles in some systems.

1.3.3. Actinobacteria

The Actinobacteria, formerly known as the high G+C gram-positive bacteria, are another group of bacteria that are commonly found in lakes with a wide range of water chemistry. Actinobacteria may comprise a large fraction (up to 60%) of the Bacterioplankton in some freshwater systems. Actinobacteria appear to be more tolerant of conditions with low concentrations of organic carbon.

1.3.4. Other freshwater phyla

Other bacterial groups such as Firmicutes and Verrucomicrobia known as low G+C gram positives have been recovered from both sediment and water column samples from oligotrophic and eutrophic lakes.

1.4. Temporal variation in bacterial communities

Lake bacterial communities harbor great genetic diversity, but they generally have low evenness when compared to other communities. That is, at any given time, communities tend to be dominated by only a few different taxa, with the majority of the species present showing very low abundance. Population dynamics of dominant strains show short-lived blooms at different times and different depths, resulting in a 'succession' of dominant community members. This 'bloom-and-bust' dynamic has led to the suggestion that lake bacterial dynamics are driven by a multitude of rapidly changing niches that are exploited by different species, which are from a large pool of dormant organisms. The activity of the dominant microbes is largely responsible for the creation of new ecological niches. These niches are rapidly filled by formerly dormant species, which then create new niches, and thus the functioning, diversity and dynamics of the system are inseparable phenomena. The rapid overturn and exploitation of niches can generate dramatic shifts in bacterial community composition over short periods of time, yet bacterial communities are not always changing rapidly. The pacing of change in bacterial communities appears to shift between long periods of stability and periods of rapid turnover in lakes. Data from many bacterial studies suggest that there is a strong seasonal component to the patterns of community change and thus it is reasonable to look seasonal events as primary sources of change in bacterial communities. Bacterial communities in

northern temperate lakes displayed seasonal dynamics over several years despite the fact that the identity of the important community members in these lakes differed in each year. The influence of these external events is different from the internal dynamics within the community that give rise to succession (Anonymous, 1994).

1.5. Spatial variation in bacterial communities

Spatial heterogeneity is important for the creation and maintenance of biological diversity. Spatial relationships can structure biological interactions and impose constraints upon the flow of matter and energy in ecosystems. Environmental changes occurring with depth are important sources of spatial variation for bacterial communities in lakes. One of the most important factors that change with depth is the presence or absence of available oxygen. The abundance and mean cell size of bacteria are greater in anoxic waters than in oxic waters, and anoxic bacterial communities are more productive overall than oxic communities. Bacterial community composition differs between the epilimnion and hypolimnion of lakes, particularly when the former is oxygenated and the latter is not. Bacterial communities are more similar in the oxic portions of different lakes than within the same lake across the oxycline. Given the wide range of environmental conditions represented by lakes, community composition exhibits such variation and lakes may be viewed as individual 'islands' where the communities and environments follow individual trajectories (Anonymous, 1994).

1.6. Impact on human health

Human and animal relationships with microorganisms are quite intimate and mostly to mutual advantage. However, a handful of microbial forms are causal agents of various diseases. In fact, appearance of disease is a manifestation of an ecological strategy for some microorganisms wherein human or animal body is used as a habitat for multiplication, persistence and transmission.

The bacteriology of water may be considered from two viewpoints, one of which is essentially that of natural history, the other that which involves its significance for man's welfare. The first deals with the distribution and physiology of bacteria that are indigenous to water, the true water bacteria. The second concerns the study of the occurrence and physiology of bacteria commonly found in water but not indigenous to that medium. These are organisms which have come from extraneous

sources such as soil, surface run off, rainfall, decomposing plant and animal structures, human activities and sewage. Bacterial waterborne pathogens and indicators fall into two groups:

- Native opportunistic pathogens such as *Aeromonas* species and *Myco-bacterium* species.
- Introduced pathogenic species that are not normally found in particular water system e.g. *Shigella* or other bacteria found only at relatively low concentration in natural waters and other environmental media's e.g. *Clostridium;* however, natural densities of pathogens are difficult to ascertain since most systems receive imports of bacteria through various sources.

Although some pathogenic bacteria exclusively inhabit humans but most also have environmental biotic reservoirs, and can be important in transmission of pathogens to other hosts. Aerobic gram negative bacteria such as *Pseudomonas, Enterobacter, Acinetobacter* and *Klebsiella* are frequently found in water sources, and are a common cause of Infection (Denton and Kerr, 1998; Hanberger, 1999). Large quantities of faecal coliforms in water are not harmful according to some authorities, but may indicate a higher risk of pathogens being present in the water. Some water borne pathogenic diseases that may coincide with faecal coliforms include infections (Domingo and Ashbolt, 2010).

The water related infectious diseases have been classified into four epidemiological categories, initially devised for classifying water-related infectious "diseases". The categories defined for water transmission of infectious agents were as waterborne (classic and other); water washed (intestinal and body surface); water-based (dependent on intermediate aquatic host; and water-related insect vectors (breed in near water). Waterborne diseases are those transmitted through the ingestion of contaminated water and water acts as the passive carrier of the infectious agent (Leclerc *et al*., 2012).The use of operative word such as "waterborne disease" is justified from reports of outbreaks or cases of disease associated with drinking water because traditional epidemiological investigation relies on the occurrence and prevalence of disease (Moe, 1997).

A wide variety of bacterial pathogens excreted in faeces are capable of initiat

-ing waterborne infections; although the potential for this is predicted from latency, survival, and infective dose data. The spread of waterborne infection by pathogenic agents depends on factors such as pathogen survival in water and the dose required for establishing infection in susceptible individuals. In addition to above, latency (the period between pathogen excretion and acquisition of actual infectious power) and pathogen ability for multiplication in the environment are factors influencing the infective dose. The minimum infective dose has been determined only for some bacteria, viruses, or protozoa that are excreted with faeces and thus potentially water transmitted (Moe, 1997).

The recognized waterborne bacterial pathogens include enteric and aquatic bacteria. The persistence of enteric bacteria, including *Salmonella* spp., *Shigella* spp., and *E. coli,* in the aquatic environment depends on various parameters. Although enteric bacteria are usually assumed to exist under starving conditions, there is evidence that some can grow in fresh water. Other bacterial infectious agents such as *Aeromonas* spp. and *P. aeruginosa* are indigenous aquatic organisms that can both survive and proliferate in drinking water (Craun, 1992).

Throughout the past half century the public health aspect of water bacteriology has received by far the greater share of attention, and it was not until lately that bacteria indigenous to water claimed the interest of any considerable number of investigators. In recent years rapid progress has been made in the latter field, particularly in the ecology of the bacterial flora of fresh-water lakes and sea water, including a large flora of sessile and other forms found on submerged surfaces (Prescott, 1946). Zutshi and Vass (1978), Yousuf *et al*. (1988) and Pandit *et al*. (2007) conducted studies on Kashmir lakes that mostly pertained to physicochemical parameters. But at the same time very little attention has been paid to study microbial flora of valley lakes including Dal lake. Hence, it was felt important to study the water quality of the lake visa-a-viz bacterial flora. However in 1989, Koul and Panhotra isolated strain of *Salmonella* spp. from Dal lake which was posed to be a health hazard. Later on, some preliminary investigation of total coliforms of Dal lake in relation to its water quality has been carried out by Kundangar *et al*. (2003).Very recently, Magray *et al.* (2011) isolated *E.coli* bacterial strain from the water samples of Dal lake, Srinagar and the strain was characterized by using 16S rRNA gene.

In this context present study was taken up and is aimed to get baseline data on the bacterial diversity and their variations in different microhabitats of Dal lake. The bacterial analysis i.e. Total viable Count (TVC), Total coliform counts (TC), and also different species of culturable bacteria will be evaluated by molecular characterization of 16S rRNA, which will lay down a set of criteria for an ideal diversity index and an attempt will be made to study the pathogenic bacteria and its impact on human.

1.7. Objectives

Hence, the objectives for the topic entitled "bacterial diversity of Dal lake, Kashmir with particular reference to pathogenic bacteria" are as:

- ✓ To enumerate the culturable bacterial flora of Dal lake, Kashmir.
- ✓ To study the seasonal fluctuations of bacteria.
- ✓ To identify the human pathogenic bacteria species.
- ✓ To study its impact on health of Dal lake dwellers by surveying the population of the area with help of questionnaire.

1.8. Study area

The Dal lake in Srinagar, (latitude 34^0 07′ N, longitude 74^0 52′ E, alt. 1583 m) the summer capital of state Jammu and Kashmir, considered as the symbol of Kashmir, represents an enchanting and unique ecosystem. The lake, the second largest in the state, is Himalayan urban lake that is integral to Kashmir tourism and recreation, though it sustains commercial operations of fisheries and water plant harvesting. It has been an epitome of the Kashmiri civilization from times immemorial.

According to Hassan (1833), in olden times at the site of present Dal lake was a plain desert which was known as Vitalini Marg. King Pravarsen closed the river Behat (now the river Jhelum) from Nowpora side and brought the river into city by way of the foot of Kohimaran (also known as Hari Parbat Hillock). After a great deal of time, the river Jhelum rose in space and as a result of the deluge, during the reign of King Durlab Vardhan (625-661 AD) desert became a lake. Consequently the Dal lake was divided into three parts viz. Bod Dal (in front of Hazratbal), Lokut Dal (expanse from Shankryacharya to Nishat Bagh) and Sodderkhun (situated in front of Hari Parbat). Hassan describes the water of the lake as sweet and delicious and it was so clear that one could see the fishes down below. Lawrence (1887), who was then Resettlement Commissioner of Kashmir, writes "Perhaps in the whole world there is no corner as pleasant as the Dal lake. The water of the Dal is clear and soft as silk, and the people say that the shawls of Kashmir owe much of their excellence to being washed in the soft waters of the lake". The lake up to the commencement of 16th century was in its pristine state. In 18th and 19th century, the city of Srinagar started expanding towards the lake resulting in far reaching changes in the lake environs. Human interference by way of settlement in the lake to facilitate pedestrian traffic and establishment of lake tourism by providing floating residences (house-boats) got accelerated.

The lake is in the foothill formations of the catchment of the Zabarwan mountain valley, a subsidiary of the Himalayan range, which surrounds it on three sides. It lies to the east and north of Srinagar city and is integral to the city. The Dal lake is a complex of four interconnected (with causeways) basins namely:

1. Gagribal, 2. Lokut Dal,
3. Bod Dal and 4. Nigeen

Lokut-dal and Bod-dal have an island each in the centre, known as *Rup Lank* (or *Char Chinari*) and *Sona Lank* respectively. Navigational channels provide the transportation links to all the four basins.

The lake's uniqueness lies in the fact that it dwells a population of more than 50,000 that live in the Lake's 111 hamlets, 320 families own Dunga Boat (Where they actually live); 758 own House Boats (For commercialization as tourist attraction); 3009 families in House (Hut); 5928 families in House (Pacca) and among them just 2.11 % fully aware about the problems of Dal lake.

Based on its thermal behavior, the lake has been type cast as 'warm monomictic' under the sub-tropical lake category. Spring sources are also mentioned as contributors to the flow though no specific data is available to quantify its contribution.

Sixteen (16) sites from this lake ecosystem viz., Hazratbal open, Hazratbal littoral, Nigeen open, Nigeen littoral, Gagribal open, Gagribal littoral, Nishat open, Near Centeur, Boathall nallah-I, Boathall nallah-II, Tailbal nallah-I, Tailbal nallah-II, Dal lock gate-I, Dal lock gate-II, Pokhribal nallah-I and Pokhribal nallah-II with 8 sites from the 4 basins, 4 sites from two inlets and 4 other sites from two outlets were selected for present study. The different altitude and geographical co-ordinates of the study sites are given in the Table 1; Plate 1, Plate 2 and Plate 3.

Table 1. Study sites with geographical coordinates

Site name	Representative site code	Altitude (m)	N	E
Hazratbal- I	1	1581	34°07′54.6″	74°52′21.6″
Hazratbal -II	2	1581	34°07′47.7″	74°50′38.0″
Tailbal nallah- I	3	1592	34°08′31.4″	74°51′40.0″
Tailbal nallah- II	4	1592	34°08′32.8″	74°51′40.4″
Boathall nallah- I	5	1601	34°08′45.0″	74°50′36.2″
Boathall nallah- II	6	1601	34°08′43.0″	74°50′35.0″
Nishat open	7	1580	34°07′26.9″	74°52′35.0 ″
Near Centaur	8	1581	34°07′25.1 ″	74°52′34.0 ″
Gagribal -I	9	1600	34°05′23.1″	74°51′02.9″
Gagribal -II	10	1600	34°05′31.2″	74°51′26.0″
Dal lock gate- I	11	1603	34°04′50.4″	74°49′48.2″
Dal lock gate -II	12	1603	34°04′50.1″	74°49′44.1″
Nigeen- I	13	1616	34°06′52.0″	74°49′54.7″
Nigeen -II	14	1592	34°06′54.1″	74°50′06.4″
Pokhribal nallah- I	15	1608	34°06′42.6″	74°49′45.2″
Pokhribal nallah -II	16	1608	34°06′45.7″	74°49′34.5″

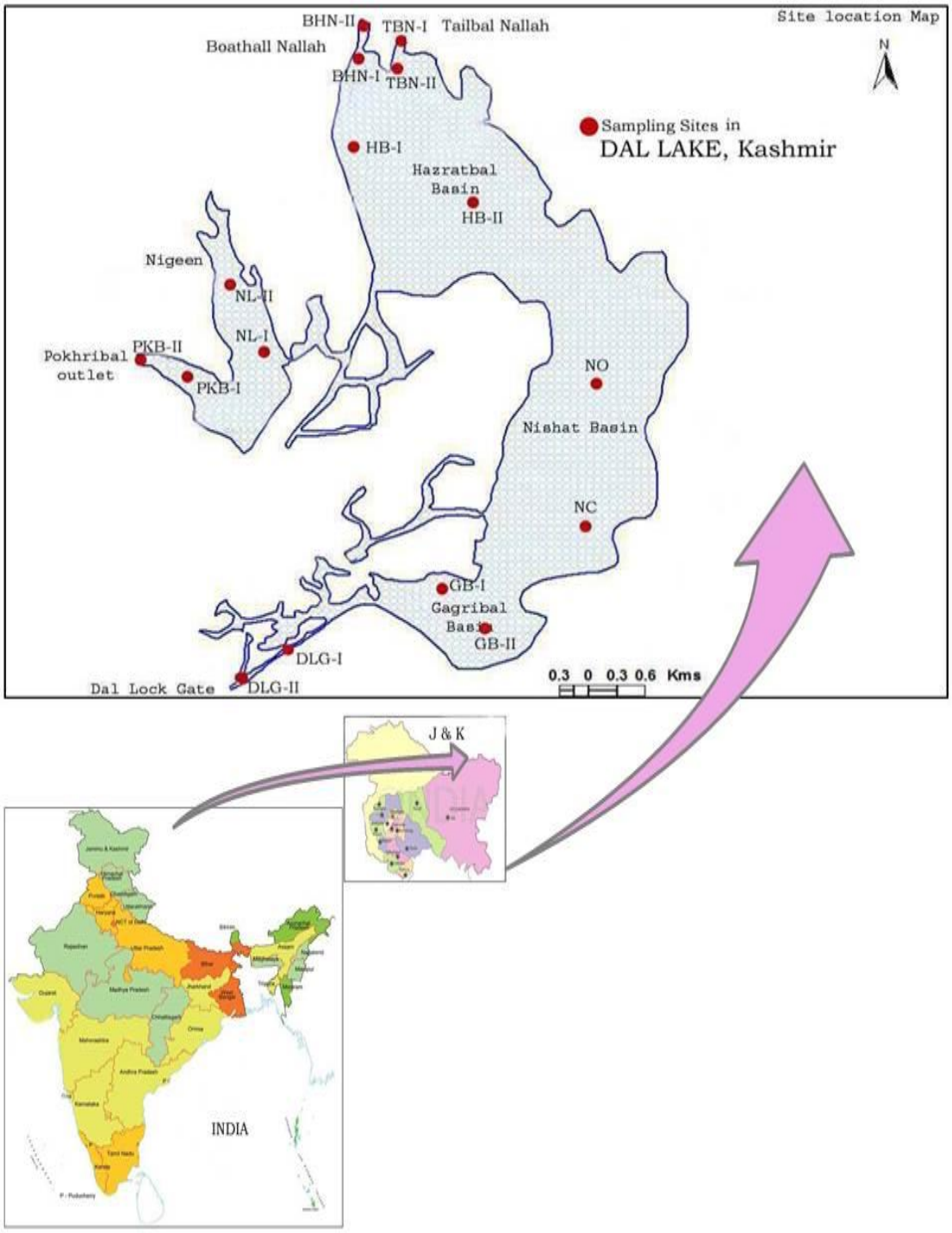


Plate 1. Map showing study sites of Dal lake

Site 1

Site 2

Site 3

Site 4

Site 5

Site 6

Site 7

Site 8

Plate 2. Study sites of Dal lake (Site 1-8)

Site 9

Site 10

Site 11

Site 12

Site 13

Site 14

Site 15

Site 16

Plate 3. Study sites of Dal lake (Site 9-16)

Chapter: 2

Review of Literature

Water is a necessity for all living beings, without it there would be no life. Life originated in water and the ultimate basis of it, the protoplasm, is a colloidal solution of complex organic molecules in a watery medium (70 to 90% of water). Most of the biological phenomena take place in water medium. Moreover, wherever water exists in nature it always holds life. So the study of a water body is the study of life as well. Water is essential at all levels of life, cellular to ecosystem and it stands as the key substance for the existence and continuity of life through different cyclic process in nature; it plays the central role in mediating global scale ecosystem processes, linking atmosphere, lithosphere, and biosphere, by moving substances among them, and enabling chemical reactions to occur. Humans depend on this resource for all their needs of existence and survival. Nature has an innate mechanism to maintain its purity after every natural use, but unables to do so at the rate at which humans add dirt to it. Nature does not know how to deal with several toxins and pollutants that are flowing from industrial and other wastes. Therefore, humans are bound to monitor the impact of this activity on natural freshwaters continuously.

Fresh water resources are most precious to earth as they are the basic ingredient to life. Increased demands on the resources have impacted heavily on

natural aquatic ecosystems. Fresh and pure water is limited in quantity indicates the need for comprehensive water management (WHO, 1992). So researches on the impacts of anthropogenial and technogenial factors on fresh water resources are imperative. Such studies provide us with information of our limits in nature (Ray, 1992).

As the supply of fresh water around the world continues to dwindle because of increased use and pollution, lakes of the world will undoubtedly be viewed as potential water reservoirs of convenience for human use (Odada *et al*., 2004). The author emphasized that basic research on the lakes of the world lags far behind similar to researches on the oceans. Lakes have a more complex and fragile ecosystem and they easily accumulate pollutants (Bhatt *et al*., 1999). Several characteristics of lakes make them ideal study sites to advance our basic understanding of ecosystem dynamics. Therefore, lakes in the world are in dire need of major new research initiatives. No serious research work has been undertaken for assessing the hydrobiology of these lakes and reservoirs. Only limited investigation has been carried out so far on the hydrobiology, flora and fauna of the lake in this region. Detailed investigations of anthropogenic influences on water quality and the bacterial diversity of this lake is very essential; these can help the conservation of diversity of aquatic systems, much needed for the sustainable development of the lake. Present study records the presence of bacterial diversity in this lake water and suggests measures for improving water quality of the lake.

Since very little published data is available on bacterial flora or pathogenicity in Dal lake, so for reviewing the literature on the subject all possible available data from different lakes or water bodies of the world was taken into consideration from 1989 onwards. The first study regarding the subject in case of Dal lake showed that the strains of *Salmonella typhimurium* were isolated from water samples which survived for longer time than in filterate at 4^0C and posed to be a health hazard as stated by Koul and Panhaotra (1989). The inadequate measures of water quality studied by Moe *et al*. (1991) have been used to monitor health effects associated with water supplies and the epidemiological and microbiological studies were used to establish relationship of prevalence of diarroheal diseases in population of Cebu, Philippines to bacterial indicators of drinking water quality and the study revealed that

contaminated water is a major source of exposure to faecal contamination and diarroheal pathogens. Although, the aquatic bacteria, representative of natural population, plays crucial roles in decomposition, food chains, and biogeochemical cycling, yet they are largely uncharacterized due to their small size, the limited range of morphologies, and the difficulties in obtaining pure cultures (Britschgi *et al*.,1991). It was stressed that the isolation of micro-organisms by growth on selective media has been universally applied for the detection of viable micro-organisms and the reliability of culture methods is of particular importance in testing for the presence of pathogenic micro-organisms (Mc Kay, 1992). He also stated that in an aquatic environment, pathogenic bacteria are capable to grow even under the conditions of starvation or exposure to sub-optimal temperature, salinity or toxic chemicals. The microbial indicators of faecal pollution help in monitoring the release and survival of genetically modified micro-organisms in water.

A new molecular biological approach, a simple and rapid method for the detection and identification, employs PCR of bacterial cell lysates with conserved primers located in the 16S rRNA sequence flanking a variable region, and analysis of the amplified product based on the principle of single-strand conformation polymorphism (Widjojoatmodjo *et al.*, 1994) and was applied to demonstrate the broad panel of gram-negative and gram-positive bacteria. Later, Kur *et al.* (1995) used this method for identification of *Serratia marcescens* in his epidemiological studies also. The recombinant DNA and molecular phylogenetic methods used by Hugenholtz and Pace (1996), have recently provided means for identifying the types of organisms that occur in microbial communities without the need for cultivation. The fresh lake waters of Antartica are characterized by short food chains but dominated by microbes. The wide range of trophic status was seen at the northern extremes of Antartica which reduces markedly further to south, but wide range of organisms do occur throughout the longitudinal range (Ellis-Evans, 1996). This information on seasonal and spatial patterns of microbial activity for freshwater lakes demonstrated rapid changes in community composition at times despite constant low temperatures. Atomic absorption spectrometry method used by Hassan *et al.* (1996) to monitor nutrients, heavy metal ions concentrations and selected bacterial community's counts along the eastern coast of the UAE, stated bacterial counts

displayed a distinct pattern with peaks fluctuated depending on several factors including the presence of nearby recreation and commercial areas, but were at no time consistently high.

The research work carried out by Kuhn (1997), for investigating *Aeromonas* populations in Swedish drinking water distribution systems, analyzed by the PhenePlate *Aeromonas* system, which is a highly discriminating biochemical fingerprinting method, the selected isolates from different phenotypes were further identified by the API 20 NE system and by gas-liquid chromatography analysis of fatty acid methyl esters (FAME), revealed that raw water contained very diverse *Aeromonas* populations which seemed to be remarkably stable within the studied water distribution systems and some potentially pathogenic *Aeromonas* strains could persist for several months in drinking water.

Sherr and Sherr (1999) discussed that the field of aquatic microbial ecology is flourishing. At the cell and molecular level, aquatic ecologists are unravelling the taxonomic composition of natural microbial assemblages and evaluating cell-specific activity *in situ*, and detection of changes in concentration of atmospheric oxygen has shown a strong seasonal signal of microbial production and respiration in the sea on a hemispheric scale at biosphere level. Future challenges include time, temperature and taxonomy.

The direct sequencing of amplified DNA from the 16S rRNA gene allows unambiguous, definitive identification and provides information on the taxonomic relatedness of new species, its use for species identification based on public 16S rRNA databases as proposed by Patel *et al*. (2000). Later, Schloter *et al*. (2000), employed high resolution molecular fingerprinting techniques like random amplification of polymorphic DNA, repetitive extragenic palindromic PCR and multilocus enzyme electrophoresis, in studying bacterial diversity below the species and subspecies level microdiversity. The observed bacterial microdiversity reflected the conditions of the habitat, ecological factors and influence of spatial separation on specific groupings of bacteria, which argue for the occurrence of isolated microevolution. In the same year Sabat *et al*. (2000) in their study designed a set of PCR primers targeting 16S rRNA gene sequences and PCR parameters to develop a robust and reliable protocol for selective amplification of *E.coli* 16S rRNA genes and

the method was capable of discriminating *E. coli* from other enteric bacteria, including *Shigella* which revealed to be effective for detecting *E. coli* DNA in heterogeneous DNA samples, such as those extracted from water. A number of *Pseudomonas aeruginosa, Aeromonas hydrophila* and *Staphylococcus* spp. in water and bottom sediments were isolated in the Czarna Hancza river in the region of Suwalki and Wigry national park were isolated by Niewolak and Opieka (2000). All these microorganisms were found in smaller quantities in water, and in larger quantities in the bottom sediments and suggested that number of *P.aeruginosa, A. hydrophila* and *Staphylococcus* spp. should be taken into account as well as the number of the indicators bacteria of a sanitary state (total coliforms, faecal coliforms and faecal streptococci) while estimating the usefulness of water in the Czarna Hancza river for recreation. This was followed by a report from Barrell *et al*. (2000) who reviewed the maintenance of the microbiological quality of water, as an important means of preventing waterborne disease through the legislative and other guidance for microbial standards in drinking and bathing waters and consider evidence for the relationship between the microbiological quality of water and risk to human health. Later, Spring *et al*. (2000), interpreted recent studies in which molecular methods were used to identify and characterize prokaryotes in lake sediments and related habitats. Studies based on the phylogenetic diversity of prokaryotes found in lacustrine habitats and the application of various cultivation independent methods for the characterization of distinct groups of sediment bacteria. Traditional and recently developed methods were described which could be used for linking the function of microbial populations with their identification.

The microbial organisms are pervasive, ubiquitous, and essential components of all ecosystems and the geochemical composition of Earth's biosphere has been molded largely by microbial activities through life's history, general principles, theory of microbial evolution and ecology. Until recently, investigators had no idea how cultivated microorganisms represented overall microbial diversity. Molecular phylogenetic surveys have revealed a vast array of new microbial groups which are widespread and abundant among contemporary microbiota and fall within novel divisions that branch deep within the tree of life. This more comprehensive picture will provide much better perspective on the natural history, ecology, and evolution of

extant microbial life as revealed by Delong and Pace (2001). A randomized, controlled trial was carried out by Hellard *et al*. (2001), in Melbourne Australia, to determine the contribution of drinking water to gastroenteritis. Families were randomly allocated to receive either real or sham WTUs installed in their kitchen. Pathogens were not more significantly common in the sham WTU group. No evidence of waterborne disease was found. The application of this methodology to other water supplies will provide a better understanding of the relationship between human health and water quality.

The pattern of bacterial community composition in freshwater habitats was identified by Zwart *et al*. (2002), who analyzed the available database of 16S rDNA sequences from freshwater plankton from Parker river, lake Soyang in Korea and lake IJssel. This habitat-specific clustering suggested that the clustered 16S rDNA sequences represent species or groups of species that are indigenous to freshwater, distinct from bacteria in neighbouring environments such as soil and sediments. Microbial communities in hydrothermally active sediments of the Guaymas lake, basin of Mexico studied by Teske *et al*. (2002) using 16S rRNA sequencing and carbon isotope analysis of bacterial and archeal lipids showed that the sediments harboured uncultured euryarchaeota of two distinct phylogenetic lineage and phylogenetically diverse bacterial population. The bacterial population displayed considerable overlap in the geothermal habitats and natural or anthropogenic hydrocarbon rich sites. Muylaert *et al*. (2002) monitored bacterial community composition in shallow eutrophic lakes using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified prokaryotic rDNA genes. Using this approach, they found no evidence for top-down regulation of bacterial community composition in the turbid lakes and suggested that in eutrophic shallow lakes, seasonality of bacterial community structure is dependent on the dominant substrate source as well as on the food web structure. Prokaryotes are small in size, a component in aquatic planktonic ecosystems and play a major role in biogeochemical processes as Cotner and Biddanda (2002) discussed that although the relative importance of prokaryotes to material and energy fluxes is maximized in oligotrophic ecosystems and decreases in high eutrophic ecosystems and concluded that competition with eukaryotic autotrophs for dissolved nutrients and competition with phagotrophic heterotrophs and physical

processes for organic carbon play important roles in determining the relative abundance and impact of prokaryotes in aquatic systems. Bronmark and Hansson (2002) found that biodiversity of lake and river ecosystems are currently threatened by a number of human disturbances.

The patterns of change in bacterial communities indicated that seasonal forces were important in structuring the behavior of the bacterial communities and the changes were in community composition dramatic in summer as compared to spring and winter. Thus, seasonal forces may be important structuring elements of these systems as a whole even if they are uncoupled from the dynamics of the individual system components as revealed by Yannarell *et al*. (2003), using automated ribosomal intergenic spacer analysis (ARISA) to explore the patterns of change in lake bacterial communities in three temperate lakes over two consecutive years. The sequence analysis of the 16S rRNA gene as studied by Song *et al*. (2003) represents a highly accurate and versatile method for bacterial classification and identification, even when the species in question is notoriously difficult to identify by phenotypic means and also evaluated the utility of 16S ribosomal DNA sequencing as a means of identifying clinically important gram-positive anaerobic cocci. A comprehensive assessment of bacterial diversity and community composition in Arctic and Antarctic pack ice through cultivation and cultivation-independent molecular technique by sequencing 16S rRNA genes from pure cultures of bacteria was conducted by Brinkmeyer *et al*. (2003). At both poles, the alpha and gamma proteobacteria dominant taxonomic bacterial groups identified by the analysis of 16S rRNA gene which revealed a high incidence of closely overlapping 16S rRNA gene clone and isolate sequences. Zeigler (2003) studied thirty-two protein-encoding genes that are distributed widely among bacterial genomes for the potential usefulness of their DNA sequences in assigning bacterial strains to species. Pairwise comparisons of whole bacterial genomes were related at the genus or subgenus level and could be refined further by including two or three genes in the analysis. The proposal supported that sequence analysis of a small set of protein-encoding genes could assign novel strains or isolates to bacterial species. Humayoun *et al*. (2003) analyzed the variation with depth in the composition of members of the domain bacteria in samples from alkaline, hypersaline, and currently meromictic mono lake in California by DNA composition

was assessed by sequencing randomly selected cloned fragments of 16S rRNA genes retrieved samples. Elevated diversity in anoxic bottom water samples relative to oxic surface water samples suggested a greater opportunity for niche differentiation in bottom versus surface waters of this lake. The obtained data in the study on chemical and bacteriological examinations of surface and subsurface water layers of estuarine lake Gardno by Mudryk *et al*. (2003), indicated that there were substantial differences in chemical compound concentrations and bacterial number and activity was greater in surface water layers than in subsurface water. In the same year Ho *et al.* (2003) surveyed that, drinking water in Dongjiang river, China had been contaminated by organic and inorganic pollutants, whereas pathogens such as *Salmonella* spp.*, Vibro* spp. etc appeared occasionally in water samples. The analytical results revealed that currently most of the heavy metals, trace organics and microbes were removed by the drinking water treatment plants in Hong Kong. The bacterial biomass and functional diversity in marine and freshwater samples from Resolute Bay, Canada isolated by Tam *et al*. (2003), using fluorescent nucleic-acid staining and sole carbon source utilization. Viable microbial counts using the live/dead BacLight viability kit estimated viable marine bacterial numbers, which were lower than viable bacterial numbers in freshwater samples. ANOVA analysis and Shanon Weaver index was used to evaluate differences between fresh and marine biodiversity. Principal component analysis revealed differences in metabolic substrate utilization patterns and consequently the microbial diversity between water types and samples. Following these studies Byappanahalli *et al*. (2003) studied that Dunes Creek, a small lake Michigan coastal stream has chronically elevated *E. coli* levels along the bathing beach near its outfall. The study concluded that *E. coli* is ubiquitous and persistent throughout the Dunes Creek basin, *E. coli* occurrence and distribution in riparian sediments help account for the continuous loading of the bacteria in Dunes Creek, and ditching of the stream, increased drainage, and subsequent loss of wetlands may account for the chronically high *E. coli* levels observed. Zmyslowska and Golas (2003) carried out an investigation in the "Seven Islands" nature reserve of lake Oswin and discovered that the pollution degree indicator bacteria ranged very high which resulted in deteriorated sanitary state.This proved that human excretal contamination was prevailing in this lake.Water diseases result from ingestion of

water contaminated by faecal material or urine especially of mamalian origin that contain pathogenic microorganisms and the diseases like cholera, dysentery, typhoid and shigellosis are major killers world wide (Leonard *et al*., 2003).

Continued efforts in this direction lead Kemp and Aller (2004) to evaluate the substantial amount of information accumulated on bacterial diversity in a variety of environments and addressed several fundamental questions, focusing on aquatic systems but including other environments to provide a broader context like 16S rDNA libraries. Moore *et al*. (2004) developed methodologies having specific advantages and disadvantages for the analysis of microorganisms and microbial ecology, at the molecular level that had progressed phenomenally in recent years. However, the advances in PCR, cloning, gene probing, sequencing and fingerprinting have enabled techniques exploiting nucleic acids to be utilised extensively for the analysis of microorganisms. The phylogenetic relatedness of 16S rRNA genes from freshwater bacteria affiliated with the class Actinobacteria, analysed by Warnecke *et al*. (2004), by developing PCR assay to identify rare actinobacteria related inserts within 16S rRNA gene. It indicated that such bacteria are not inoculated to freshwaters from terrestrial sources, but are autochthonous components of freshwater microbial assemblages.

Allgaier and Grossart (2005) investigated the ecological role of specific bacteria and their spatial and temporal distribution by DGGE analysis and 16S rRNA genes for studying bacterial dynamics and community composition in the epilimnion of lake Stechlin and Grosse Fuchskuhle. This study revealed significant differences in community structure and seasonal dynamics of free-living and attached bacteria. Sequences of 16S rRNA genes suggested that members of Actinobacteria were most dominant bacteria studied in lakes. Pathogenic bacteria and enteric viruses can be introduced into the environment via human waste discharge. He and Jiang (2005) in their study revealed that methods for rapid detection and quantification of human viruses and faecal indicator bacteria in water are urgently needed to prevent human exposure to pathogens through drinking and recreational waters. Here they described the development of two real-time PCR methods to detect and quantify human adenoviruses and enterococci in environmental waters. Overall, the ability via real-time PCR to detect enterococci and adenoviruses rapidly and quantitatively in the

various environmental samples represents a considerable advancement and a great potential for environmental applications. Moeseneder *et al*. (2005) studied RNA has a much shorter half-life than DNA; it can be used to potentially describe active bacterial populations in complex environmental samples. Schloss and Handelsman (2005) in their study presented a method called DOTUR that addresses the challenge of assigning sequences to operational taxonomic units (OTUs) based on the genetic distances between sequences. Eva *et al*. (2005) investigated the distribution of different typical freshwater bacterial groups in diverse lakes in northern Europe using reverse line blot hybridization. Statistical evaluation of the data in relation to the characteristics of the lakes showed that pH, temperature, and the theoretical hydrological retention time of the lakes were most strongly related to variations in the distribution of bacterial taxa. Oliver *et al*. (2005) illustrated the methods and ability of cells to resuscitate from the VBNC state (viable but nonculturable) and return to an actively metabolizing and culturable form as well as the ability of these cells to retain virulence. The changing ecological and water quality of Manyas lake, resulting from increasing anthropogenic and human pollution on the natural variations of the water level was studied by Karafistan and Arik-Colakoglu (2005) and for this purpose, physical, chemical and microbiological aspects and associations between different parameters were searched by means of a water quality model whereas results obtained were used in the sustainable restoration of the lake. The distribution of different typical freshwater bacterial groups in diverse lakes in northern Europe was investigated by Eva *et al*. (2005) using reverse line blot hybridization. Statistical evaluation of the data in relation to the characteristics of the lakes showed that pH, temperature, and the theoretical hydrological retention time of the lakes were most strongly related to variations in the distribution of bacterial taxa.

Assessement on the biodiversity and distribution of Enterobacteriacae and a non fermenting group of bacteria in lake Baikal, the most potentially pathogenic bacteria was made by Coulter *et al*. (2006). These results showed that the bacteria of different genera are in abundance in littoral zones but their number is small compared to the total abundance and diversity of the lake which correlated with anthropogenic load and none were identified from the pelagic zone. Results of study conducted by Altug *et al*. (2006) on surface water of Sapanca lake, Turkey revealed that level of

indicator bacteria and percentage of potentially active bacteria was higher in samples of surface water. Temperature is a master variable controlling biochemical processes in organisms, as examined by Cotner *et al*. (2006) and its effects are manifested on many organizational levels in organisms and ecosystems. The effects of temperature on the biochemical composition and stoichiometry of a model heterotrophic bacterium, *E. coli*, led to increased cellular organic carbon and organic nitrogen with decreased phosphorus content, leading to increased C/P and N/P biomass ratios. To study growth rates of a non pathogenic strain of *E. coli* at different temperatures and for different times it was predicted that bacteria grew best at 37°C compared to the other temperatures, because the mean temperature of many mammals is 37°C and mammals often serve as a host to *E. coli* (Nguyen, 2006). The spatial and temporal variations of saprophytic bacteria and faecal indicators as well as the bacteria associated with nitrogen and carbon cycle in lake Bardawil waters was investigated by Sabae (2006) and data revealed that the faecal indicator bacteria were in acceptable levels according to the Egyptian and European guide standards. Agbogu *et al*. (2006) investigated the pollution level of surface waters in Zaria, Nigeria by performing bacteriological and physicochemical analysis in accordance with standard procedures. Positive correlation was found between faecal coliform counts with most of the physicochemical parameters. This study therefore stressed on the need to control the faecal pollution of the bodies of water. Bacterial community structure and seasonal dynamics were analyzed by denaturing gradient gel electrophoresis (DGGE) and clone libraries of 16S rRNA gene fragments for phylogenetic diversity of free living and particle associated bacterial communities in the epilimnion of lakes of the Mecklenburg lake in Germany by Allgaier and Grossart (2005). Later Shengkang *et al*. (2006) in their study investigated the bacterial community in a historic lake sediment core of Ardley Island, Antarctica by molecular approaches targeting the 16S rRNA gene fragments. Denaturing gradient gel electrophoresis (DGGE) analysis and sequencing of PCR amplified 16S rRNA gene fragments were performed to analyze the bacterial diversity over the entire column. The results demonstrated the presence of highly diverse bacterial community population in the Antarctic lake sediment core. The bacterial diversity and abundance in two moraine lakes and two glacial melt waters in the remote Mount Everest region through 16S rRNA gene clone library and

flow cytometry approaches by Liu *et al*. (2006). Low temperature at high altitude is considered to be critical for component dominancy. At the same altitude, nutrient availability played a role in regulating population structure.

The microbial ecology has undergone a profound change in the last two decades with regard to methods employed for the analysis of natural communities and Nocker *et al*. (2007) emphasized shifting from culturing to the analysis of signature molecules including molecular DNA based approaches that rely either on direct sequencing of DNA fragments or amplification of target sequences by use of the PCR, which can again be either cloned and sequenced or can be subjected to an increasing variety of genetic profiling methods like ARISA, TRFLP, denaturing gradient gel electrophoresis and denaturing high-performance liquid chromatography. It was reported by Lozupone and Knight in 2007 that the most comprehensive analysis of the environmental distribution of bacteria to date, based on 16S rRNA sequences compiled from diverse physical environments which were clustered on the basis of similarities in the phylogenetic lineages that they contain and found that, the major environmental determinant of microbial community composition is salinity rather than extremes of other physical and chemical factors. Saied (2007) studied first molecular genetics on the diversity of bacterial communities in Manzala lake, Egypt, determined by culture-independent 16S rRNA gene analysis amplified by polymerase chain reaction (PCR) from bulk DNA of each sample then cloned and sequenced. The study succeeded the determination of the actual composition of bacterial populations in the studied samples and the discovery of novel bacterial phylotypes, never recorded by traditional techniques. The bacteria in water samples from streams were cultured on Coliscan Easygel agar plates. Representative colonies were subjected to amplification and sequencing of 16S rRNA gene. PCR genotyping of isolates from the most prevalent genera and species as studied by Belt *et al*. (2007) indicated a high degree of diversity within stream for *E. coli* and *K. pneumoniae*. Skowronska (2007) demonstrated rapid and reliable definition of prokaryotes and quantification of population sizes, fluorescent in situ hybridization (FISH) with rRNA targeted oligonucleotide probes was used to study bacterioplankton composition in north Mamry lake, and followed the dynamics of two populations of common bacteria. Ksoll *et al*. (2007) investigated epilithic periphyton communities on the Minnesota

shoreline of lake Superior to determine if faecal coliforms and *E. coli* were present throughout the ice free season. Horizontal, fluorophore-enhanced repetitive-PCR DNA fingerprint analysis indicated that the source of the *E. coli* bacteria isolated from these periphyton communities could be identified when compared with a library of *E. coli* fingerprints from animal hosts and sewage. Results indicated that *E. coli* from periphyton released into waterways confounded the use of this bacterium as a reliable indicator of recent faecal pollution. Dorador *et al.* (2007) in their study reported that Rapel lake reservoir, eutrophic system in Chile, has undergone a series of anthropogenic impacts in recent decades and their results showed significant temporal variation in the physical and chemical composition of the water column, but no depth related differences. Another study analysed that nitrogen and phosphorus additions from anthropogenic sources can alter the nutrient pool of aquatic systems, both through increased nutrient concentrations and changes in stoichiometry and appeared important factors for structuring bacterial communities (Rubin and Leff, 2007). In 2007, Sabae and Rabeh evaluated the microbial quality of the river Nile water at Damietta branch. The faecal indicator counts revealed that their densities increased from up to down stream and pathogenic bacterial isolates representing eleven genera were identified to species level using environmental parameters such as temperature, transparency, depth and pH and API 20E strip system (BioMereux). The results of the investigation revealed that, the river Nile water at Damietta branch was subjected to sewage pollution during the study period. The microbiological contamination of waterways by pathogenic microbes has been, and is still, a persistent public safety concern in the United States and in most countries of the world as reported by Yan and Sadowsky (2007). As most enteric pathogens are transmitted through the faecal oral route, faecal pollution is generally regarded as the major contributor of pathogens to waterways and has been used successfully as the primary tool for microbiologically based risk assessment. The microbial pollution of Yellamallappachetty lake located near Krishnarajapuram was assessed by Usha *et al*. (2007) which was under severe stress due to agricultural run off, untreated sewage from city. As a result of discharge, socioeconomic and environmental services to surrounding community have seriously deteriorated causing harm to the lake water quality as well as exacerbating health risks to local people.

Shehata (2008) used PCR to obtain randomly amplified polymorphic DNA profiles for genetic fingerprinting of twelve different isolates of *Staphylococcus aureus* from Riyadh, Saudi Arabia and showed that a numerical analysis of the genomic profiles obtained demonstrated that it was possible to differentiate the *S. aureus* strains. The DNA fingerprint defined for each race of *S. Aureus* could be useful in epidermiological studies, medical diagnosis and the identification of new strains and their origins. By molecular techniques for community characterization by simultaneously use of molecular and non molecular methods within the same sampling program to compare old and new data by Knapp *et al*. (2008). These methods were used for characterizing microbial populations in lake Ontario, Canada. Results suggested that, although ssu-rRNA methods are fast, reproducible, and specifically detect "viable" organisms; their use is limited to non-eukaryotic populations unless new specific probes are developed. Logue *et al*. (2008) applied molecular techniques to study microbial communities in freshwater environments. He stressed that the field continues to mature and will most likely make substantial contributions in the future with additional efforts that include metagenomics and genomics. In 2008, Dimitriu *et al*. studied microbial community diversity and composition of meromictic Soap lake using culture dependent and culture independent approaches. Denaturing gradient gel electrophoresis of bacterial and archaeal 16S rRNA genes showed an increase in diversity with depth for both groups. Sugumar *et al*. (2008) investigated the prevalence and seasonal variation of bacterial indicators of faecal pollution in samples of water and beach sand from the four fish landing centres of Thoothukudi which showed maximum detectable level of Most Probable Number through out the year with no obvious seasonal variation, hence the coastal waters along Thoothukudi is polluted and presents a potential risk to public for recreational and fishing activities. In 2008, it was opined by Shade *et al*. that multiple forces make natural microbial community by gradients of physical and chemical parameters and defined spatial habitat heterogeneity as vertical temperature and dissolved oxygen (DO) gradients in the water column, and temporal habitat heterogeneity as variation throughout the open water and found stark differences in patterns of epilimnion and hypolimnion dynamics over time and space. The structure and diversity of bacterial communities associated with the oxygen minimum zone

(OMZ) of the South Pacific through phylogenetic analysis was also studied (Stevens and Ulloa, 2008) and observed vertical partitioning of the bacterial communities with main differences between the suboxic zone and the more oxygenated surface and deep oxycline waters.The bacterial richness in the oxygen minimum zone was higher than in the anoxic surface and deeper oxycline, as revealed by rarefaction analysis and the Chao1 richness estimator. In 2008, the molecular biodiversity within all three microbial domains (Bacteria, Archaea and Eukaryota) and the heterotrophic productivity in lake Mackenzie, in western Canadian Arctic was examined by Galand *et al.* and made comparative measurements in the freshwater (Mackenzie river) and marine (Beaufort sea) source waters. Bacterial and eukaryotic communities in the Stamukhi lake differed in composition and diversity from both marine and riverine environments, whereas the archaeal communities were similar in the lake and river. Tambe *et al*. in 2008 undertook a longitudinal study of the bacteriological quality of rural water supplies for a movement towards self help against diseases, such as diarrhoea, and improved water management through increased community participation. An increased awareness was observed through active participation of the people cutting across age groups and different socioeconomic strata of the society in village activities. Laboratory experiments were carried out by Bertoni *et al*. (2008) in one ultra oligotrophic pristine Andean lake, Argentina and in one subalpine lake that is now at the edge of the oligotrophic to mesotrophic condition (lake Maggiore, Italy) which was amended with phosphorus, organic carbon to test for short term changes in bacteria activity and community structure (CARD FISH). Results showed that bacterial production increased in the treatment in ultra oligotrophic lake. All results indicated the different response of bacterioplankton in systems at the edges of the oligotrophic range.

Jakee *et al*. (2009) stressed to understand the importance of water quality exposed to animals and human to the risk of diseases and they investigated water samples to detect the occurrence of coliforms by serotyping and screening for virulence genes and concluded that water may be an important reservoir for *E. coli* infection and the risks of contracting enterotoxigenic (ETEC) and or enterohemorrhagic *E. coli* (EHEC) infections from contaminated water. Studies on microbiological analysis of water samples originating from a small municipal lake

Syrenie Stawy conducted by Sliwa-Dominiak and Deptula (2009) and their results demonstrated high content of sanitary bacteria, which pointed to a significant contamination of the lake. High content of psychrophilic and mesophilic bacteria pointed to high amounts of organic substances in water. Luiza (2009) characterized bacterial spatio-temporal distribution in Conceicao lagoon, Brazil to determine the heterotrophic and photoautotrophic bacterial dominance in hypoxic and oxic stratified waters. Principal component analysis showed that salinity, temperature, and light were the abiotic factors that better explained the temporal variability of bacterial assemblages. An assessment of bacteriological quality of drinking water in Lahore, Pakistan was done by Anwar *et al*. (2009) and found large a number of infectious diseases are transmitted primarily through water supplies contaminated with human and animal excreta particularly faeces using Multiple Tube Method to determine Most Probable Number of total coliforms and faecal coliforms using standard procedure. Hacioglu and Dulger (2009) analysed water samples collected from three different sites of the Biga stream, Turkey for some physicochemical and microbiological parameters of the stream and concluded that there was a great potential risk of infection of waters from the Biga stream. Again in 2009, Novevska and Lokoska studied effect of climate change accompanied by irrational human's use of the local inhabitants on the water level of lake Prespa which decreased considerably and decreasing surface level posed sharp changes in its trophic state. McDonald *et al*. (2009) employed PCR and qPCR primers targeting the 16S rRNA gene to detect and quantify members of the genus *Fibrobacter* in lake water and the presence of these organisms may contribute to the primary degradation of plant and algal biomass in freshwater lake ecosystem. The heterotrophic microorganisms are widely recognized as crucial components of ecosystems. The composition of bacterial and fungal communities in a freshwater marsh was determined by DGGE which revealed diverse bacterial communities in four contrasting microhabitats as studied by Buesing *et al*. (2009). The bacterial diversity of lake Martel, Spain was studied by Rivas *et al*. (2009) and isolated samples in presence of low salt concentration and grouped on basis of Primers (TP)-RAPD patterns and classified into different genera and species on basis of their 16S rRNA gene sequences. The results obtained showed the complexity of bacterial populations in this lake. Tang *et al*. (2009) revealed that

organic aggregate associated bacteria play a pivotal role in microbial food webs and in the cycling of major elements, their community composition and diversity in shallow freshwater systems and eutrophic lake Taihu. Regional variability and diversity was studied by amplified ribosomal DNA restriction analysis and comparative analysis of large 16S ribosomal RNA clone libraries which demonstrated that microorganisms from terrestrial and sediment habitats are an important component. In 2009, Humbert *et al*. employed 16S rRNA sequencing approach to compare the structure and composition of the bacterial communities in deep subalpine lakes in France with those of communities in shallow tropical reservoirs in Burkina Faso. They found that their bacterial communities share the same composition in regard to the relative proportions of the different phyla, no significant differences in the richness and abundance diversity of the bacterial communities and the history of these ecosystems and regional environmental parameters have a greater impact on the relative abundances of the different OTUs in each bacterial community.

Abdo *et al*. (2010) investigated water samples collected during successive seasons from river Nile, Egypt and represented the effect of the factories effluent discharge on water quality by measuring its physical characteristics and chemical characteristics, which showed slight variations during different seasons. Additionally, the bacteriological analysis of water samples for total viable bacterial counts and the bacterial indicators of faecal pollution were assessed. Water samples which were collected from northern rivers were analysed by Kumar *et al*. (2010). The analysis showed permissible limits for physical and chemical parameters but all samples were positive for E. *coli*, which indicates faecal pollution of water. The result showed that Brahma Kund in Haridwar, a famous tourist places, is most polluted. Caporaso *et al*. (2010) reported that ongoing co-evolution of new sequencing platforms and new software tools allow data acquisition and analysis on an unprecedented scale. They demonstrated excellent consistency in taxonomic recovery and recapture diversity patterns that were previously reported from meta-analysis. The results thus open up the possibility of conducting large scale studies analyzing thousands of samples simultaneously to survey microbial communities at an unprecedented spatial and temporal resolution. The microorganisms play important roles in maintaining ecosystem functions and how microbial ecosystems respond to environmental

changes and human activities is emphasized by Dong *et al*. (2010). Microbial abundance and species diversity varied considerably along environmental gradients across the plateau. Studies on lake sediments and ice cores from the plateau revealed that bacterial abundance and diversity are positively correlated with dust particle concentration and temperature like warm and cold climate. Another study carried out by Cabral (2010) discussed that water is essential to life, but many people do not have access to clean and safe drinking water and many die of waterborne bacterial infections. He discussed indicators of feacal pollution should be used in current drinking water microbiological analysis. The physicochemical characteristics and seasonal variation of Yeoti lake were studied by Mane *et al*. (2010). The results revealed that there were significant seasonal variations in some physicochemical parameters and most of the parameters were in the normal range but higher MPN requires some treatment before using for drinking purpose. Obasohan *et al*. (2010) highlighted the historical perspective of the relationship between microbes and humans regarding the "ranging war" between them, arising from the reckless exploitation of the biosphere by humans and the resultant "revolt" by microbes in the form of various pathogenic diseases that now plague mankind. Rai *et al*. (2010) analyzed water samples from three sewage treatment plants which regularly discharge into the river Ganga. Physiochemical and microbiological parameters ranged above the permissible limits in water samples which indicated a serious health hazard posed by intense microbial and faecal pollution and recommended that an integrated approach of phytoremediation with aquatic macrophytes and ozonization of wastewater be adopted to curb the heavy metals and microbial pollution based on these recent research investigations. The physicochemical properties of the surface microlayer and its underlying water and compared to the composition and activity of their bacterial communities in lakes located across an altitude gradient were studied by Hortnagl *et al*. (2010). Activity was assessed at both the community level, by leucine bulk incorporation and by using micro autoradiography at the single-cell level. Catalyzed reporter deposition fluorescence in situ hybridization was used to quantitatively assess the structure of the bacterial assemblage. In the same year Sharma *et al*. (2010) conducted a study to investigate the water quality of seven important lakes in north India during different seasons. Bacteriological parameters

including physico-chemical parameters were assessed which exceeded the maximum permissible limits in all the lakes irrespective to different seasons which resulted from human activities in important north Indian lakes. Usharani *et al*. (2010) conducted study to find out the physico-chemical and bacteriological characteristics of Noyyal river and ground water quality of Perur, India. The river water and ground water samples were analyzed for various physico-chemical and bacteriological parameters which were greater than the permissible limit. Quality management should be properly maintained, so that the river water could be utilized for a wide range of application viz, irrigation, drinking etc. Venkatesharaju *et al*. (2010) studied Cauvery river of south India for physico-chemical and bacteriological parameters over a defined period. Over the years of time, the river has been subjected to human interference regularly and water quality was to be getting deteriorated profoundly generating serious threat to the biota of the river by altering the physico-chemical and biological concentration of the river system but the water was not polluted as all results are within permissible limit when compared with Bureau of Indian Standards (BIS) and National river water quality standards. Revetta *et al*. (2010) discussed that RNA-based studies provides an insights on active bacteria, biases associated with over representation of specific populations due to relative abundance of RNA transcripts could underestimate the importance of less active bacteria in any given environment. Another study carried out by Kormas *et al*.(2010) reported that sequence analysis of 16S rRNA gene clone libraries using DNA extracts as the PCR template has enhanced the understanding of the microbial composition and diversity of complex microbial networks, including oligotrophic environments such as water distribution systems (WDS) and found alpha, beta, and gamma-Proteobacteria to dominate chlorinated drinking water. Pradhan *et al*. (2010) studied bacterial diversity of two samples collected from the periphery of the Roopkund glacial lake and one sample from the surface of the Roopkund glacier in the Himalayan ranges by constructing three 16S rRNA gene clone libraries. Actinobacteria was the most predominant class in the three libraries. In the library from the glacial water, class Betaproteobacteria was most predominant. The rarefaction analysis in the samples collected from the periphery of the lake thus indicated a limited bacterial diversity

covered; at the same time, the coverage in the glacier sample indicated most of the diversity was covered.

In a recent study by Sadat *et al*. (2011) bacteriological quality of water collected from Yamoussoukro lake, was evaluated by heterotrophic plate count (HPC), total coliforms and faecal coliforms. Varying levels of bacteriological contamination result showed that the water quality has deteriorated in Yamoussoukro lake system. Kumar *et al*. (2011) studied that plankton diversity and physico-chemical parameters are an important criterion for evaluating the suitability of water for irrigation and drinking purposes. The water samples were analyzed for bacteriological and physico-chemical screening and revealed that the zooplankton population showed positive significant correlation with physico-chemical parameters like, temperature, alkalinity etc. whereas negatively correlated with rainfall and salinity. Humerah *et al*. (2011) designed study to ensure the access assessing microbial contamination, detection of indicator organisms performed by Most Probable Number technique and total bacterial count by pour plate method. The level of various heavy metals was monitored in water samples to assess the impact of toxic pollutants by atomic absorption spectroscopy. Some toxic chemical contaminants were estimated below the detection limit, while other several (essential) metal ions exceeded slightly in water samples. Zinger *et al*. (2011) discussed, about diverse microbial populations that ensure their functioning and sustainability. The advent of Sanger sequencing and now next-generation sequencing technologies has enabled the resolution of microbial communities. Together, these methods will gain an unprecedented understanding of microbial diversity in aquatic ecosystems. Water and sediment samples collected from Uppanar estuary were analysed for the distribution of total heterotrophic bacteria and pathogenic bacteria by Mahalakshmi *et al*. (2011). The population densities of pathogenic bacteria were detected as maximum values of *E.coli* followed by *V.cholerae* and *V. parahaemolyticus*. In 2011, Bahgat investigated effect of pollution on free living bacteria in Suez canal and West lagoon, lake Timsah. *Bacillus* was the most abundant genus due to organic, agricultural and municipal loads. Bacterial species richness differed among water depths and was higher in subsurface samples. In Suez canal more Gram negative populations were isolated. Shafi *et al*. (2011) in their research work determined the microbiological characteristics of waters of Sindh

river, Kashmir by assessing the bacterial and fungal flora at the different sites. The isolated strains were tested for sensitivity against some antibiotics. Almost all the drugs tested against except gentamycin and ofloxacin showed 100% susceptibility. A number of bacteria in surface waters by quantification of aerobic mesophilic bacteria by using the cultivation methods of the reservoir Celije and its tributaries were also isolated (Ciricl *et al*., 2011). However, all these methods detect only cultivable bacteria; these deficiencies were solved by using direct methods like quantification and epifluorescence microscopy after filtration of samples previously stained with acridine orange. Using the cultivation dependant and independant methods, the highest numbers of bacteria were recorded. Investigations regarding the status of physico-chemical chararacterstics and level of sewage pollution indicator bacteria and their variation at whole stretch of river Gomti were carried out (Anukool and Shivani, 2011); water samples were subjected to physico-chemical analysis like water temperature, total solids, total dissolved solids, total suspended solid, conductivity, pH, COD, BOD and DO. The bacteriological study of these samples included bacteriological parameters like total coliforms (TC), feacal coliforms (FC) and faecal streptococci (FS). The results were based on spatial variation, seasonal variation and temporal variation. Conclusions revealed that large number of drains in Lucknow city and industrial discharge is mainly responsible for pollution in river Gomti. Magray *et al*. (2011) isolated a bacterial strain from water sample, collected in Dal lake, Srinagar and the strain was characterized by using 16S ribosomal RNA gene and 16S-23S rRNA internal transcribed spacer region sequences. Phylogenetic analysis showed that 16S rRNA sequence of the isolate formed a monophyletic clade with genera *Escherichia*. The result of ribosomal database project's classifier tool revealed that the strain belonged to genus *Escherichia*. Further analysis of 16S-23S rRNA sequence of isolate confirms that the identified strain be assigned as the type strain of *E. coli* with 16S-23S rRNA sequence similarity. In 2011, Pindi and Yadav revealed that bacterial quality is probably the most important consideration in assessing drinking water. A polyphasic approach involving cultivation, direct viable counts, 16S rRNA based phylogenetic classification was applied for the characterization of the bacterial population in a public drinking water. Overall, the results suggested that these bacterial groups are amongst potentially active bacteria in drinking water. Revetta *et*

al. (2011) analyzed the bacterial composition of chlorinated drinking water using 16S rRNA gene clone libraries derived from DNA extracts and compared to clone libraries previously generated using RNA extracts from the same samples. Such differences between clone libraries demonstrated the necessity of generating both RNA and DNA derived clone libraries to compare these two different molecular approaches for community analyses. Llorens-Mares *et al*. (2011) analysed bacterial community composition in the slush layers of snow covered lake Redon in winter and spring and compared with bacteria from the lake water column, using 16S rRNA gene clone libraries and CARD-FISH counts. The set of biological data was related to changes in bacterial production and to other relevant environmental variables measured in situ. This strong bacterial composition switch was associated with consistent increases in bacterial abundance and production, and decreasing bacterial diversity. Shafi *et al*. (2011) carried out a preliminary microbiological study of river Sind, a glacier fed river of Sonamarg, Kashmir and revealed that bacterial and fungal flora showed variarions in relation to the conditions prevailing at different sites. Another study was carried out in same year by Saleem *et al*. on isolation, identification and seasonal distribution of bacteria in Dal lake, Kashmir who isolated eleven species of bacteria from surface water and found that seasonal fluctuation of bacteria was highest in spring season followed by summer, autumn and winter season.

Very recently Abraham *et al*. (2012) reported that poor sanitation, poor treatment of waste water, as well as catastrophic flood introduces pathogenic bacteria into rivers, infecting and killing many people. The goal of clean water for everyone has to be achieved with a still growing human population and their rapid concentration in large cities, often megacities. An overview about the current understanding of the fate and niches of pathogens in rivers, the multitude of microbial community interactions, and the impact of severe flooding, a prerequisite to control pathogens in polluted rivers. Again, in 2012 Smruti and Sanjeeda, isolated water borne bacterial pathogens from surface waters in Indore, India by bacteriological analysis of various water samples. In the bacteriological analysis, colliform group of bacteria were differentiated by the presumptive test, confirmed test and completed test. The water samples collected from different regions of Indore were found to have significant impurities, considerable deterioration and remarkable variation. The study

therefore, stressed for the need to control the faecal pollution of water bodies. In the same year, Paulse *et al*. isolated bacterial species present in Berg and Plankenburg rivers in South Africa from water samples. Various Enterobacteriacae species were present confirming the faecal contamination.This raises major health concern as human population densities along both rivers are high thus resulting in increased human exposure to these organisms. Later on, in the same year Shafi studied the bacterial flora of Manasbal lake, Kashmir and identified 19 pathogenic bacterial species by biochemical methods.

Chapter: 3

Material and Methods

The purpose of my study was to gain an insight of the bacterial diversity of Dal lake in relation to pathogenic bacteria by estimating the number and species of culturable bacteria in water samples and to assess if there is any impact on human. For this purpose, the protocol adopted is as under:

3.1. Cleaning of glassware

All the glassware used was cleaned with labolene to remove oils, grease and organic matter from it. Glassware was then rinsed under running tap water and then with distilled water. Subsequently, it was allowed to drain and dry in oven and then prepared for sterilization.

3.2. Sterilisation

Sterile environment was maintained while working in the laboratory by wiping the working benches and washing of hands with 90 % ethyl alcohol and by using sterile gloves. Various techniques were used for sterilisation of glassware, culture media, equipments and instruments etc., depending on the nature of material that needed to be sterilised.

3.2.1. High temperature

Heat sterilization was followed because it is the most common and reliable method for sterilization where the material to be sterilized is not modified by high temperature. High temperature was achieved by using either dry or moist heat sterilization.

i. Dry heat sterilization

Dry heat was used for the sterilization of the glassware, metal instruments and other items. A hot oven equipped with thermostat was used for dry heat sterilization. The time required for sterilization was about 12 to 16 hours at 120°C. However, even after this many bacteria in a desiccated vegetative state or as spores can survive; therefore moist heat was followed for further sterilization. The factors responsible for the death of bacteria in dry heat sterilization are desiccation and coagulation. The bacterial protoplasm contains approximately 85% moisture and when this moisture is reduced, various bacteria die.

Other methods of high temperature which were followed are:

ii. Red heat

The instruments were held on the flame till they became red hot e.g., inoculating wires, loops, tips of forceps, spatulas etc.

iii. Flaming

Burning instruments viz, spatulas, needles and inoculating loops were sterilized by exposing them to spirit lamp flame. Prior to flaming, it was first dipped into 70% ethyl alcohol.

iv. Moist heat sterilization

This method was used for sterilization of glassware and culture media.

Moist heat was usually provided by saturated pressure in an autoclave and was found to be the most effective and reliable method for sterilization (APHA, 1998). However, it was not used for culture media containing compounds hydrolyzed or reactive with other ingredients at higher temperature. The temperature and length of time for sterilization with steam are different from that of dry heat. Vegetative cells of most bacteria and fungi are killed at 60°C within 5 to 10 minutes. Yeasts and fungi are killed only above 80°C, while for bacterial spores about 15 minutes at 121°C and at 151 lb/inch^2 pressure are suggested. This temperature and pressure is also suitable for sterilization of media, hence this method was used.

3.2.2. Low temperature

This method is micro biostatic and was used to store media, solutions, reagents and cultures i.e. by using refrigerators and freezers. It does not sterilise but slow down the enzymatic activity and metabolism

3.2.3. Infra red radiations

U.V radiations were used for killing microbes in inoculation chamber (laminar air flow cabinet).

3.3. Collection of water samples

Water samples from 16 sites under reference were collected on seasonal basis during the period of study by collecting surface and subsurface water samples in sterile bottle. All the sampling and preservation procedures for water samples were performed according to standard method for the examination of water and waste water (APHA, 1995, 1998).

Sampling for bacteriological analysis was done aseptically with care, ensuring that there was no external contamination of the samples. For sampling, sterilised plastic polyethylene (PET) bottles were used, cleaned and rinsed carefully, given a final rinse with distilled water, and sterilized with 90% alcohol (APHA, 1995, 1998).

Bacteriological analysis was done as soon as possible after collection of water samples to minimize changes in bacterial population. Maximum gap between collection and examination of samples didn't exceed more than 24 hrs. If it was not possible to analyse immediately, the sample was refrigerated at or below 4^0C. This was done to minimize any changes in microbial populations.

3.4. Serial dilution technique

Since millions of bacteria can be found in a single drop of water, bacterial suspension was diluted serially seven folds so as to reduce the bacterial population by few cells/ml.

3.4.1. Procedure

i. Test tubes were arranged in the test tube stand and were labelled in a serial manner. All the tubes were filled with 9 ml sterile distilled water. However, first tube was filled with the sample solution.

ii. Samples were prepared by adding known amount of sample water to known volume of sterile distilled water and homogenized or mixed thoroughly. Then 10 ml of this suspension was poured in the first tube. This tube, thus contains concentrated bacterial suspension. It has maximum number of cells /ml.

iii. 1 ml of the sample suspension was taken from the first tube and added into second tube containing 9 ml of sterile water. This gives 1:10 or 10^{-1}.

iv. Then 1ml of this dilution was taken and added to next tube all ready containing 9ml of sterile water. This gives 1:100 dilutions or 10^{-2} dilution.

v. Once again 1 ml of this dilution was taken and added to 9 ml of sterile distilled water present in the next tube. This gives 1:1000 dilutions or 10^{-3} dilution.

vi. In this manner dilution upto 10^{-5}-10^{-7} dilutions were prepared.

vii. One ml of first dilution was taken and inoculated on a sterile Petri plate containing nutrient agar medium with it using spread plate technique.

viii. This was repeated with all dilutions individually and separately on Petri plates containing solidified nutrient medium.

ix. Plates containing overlapping or diffused colonies were discarded (usually more than 300 colonies).

x. Plates showing different types of distinct, individual well separated colonies were selected.

xi. Inoculum from any one colony was picked and mixed in 10 ml of sterile distilled water. This gives a second sample.

xii. The procedure was repeated from the beginning till all colonies of same colour, type etc was obtained. This gives pure culture of one bacterium.

xiii. The procedure was repeated with all different types of colonies to separate all bacteria present in the original sample.

xiv. The isolated bacteria were identified by standard identification methods using Bergey's manual of systematic bacteriology (Miles *et al*., 1938; Corry, 1982; Hammack *et al*., 1997).

3.5. Media

Culture media is an aqueous solution to which all the necessary nutrients are added. Nutrient medium designed by the Koch was based on the fact that microbes grow within the host so meat infusion extracts were chosen as basic nutrients. Basic medium contained 0.5% peptone and 0.3% meat extract. Depending upon the type and combination of nutrients, different categories of media were made like Nutrient broth, Peptone broth, Glucose phosphate broth, Lactose broth, Meat infusion broth, Brilliant green lactose bile broth, Nutrient agar, Semi solid agar, EMB agar, Simmon citrate agar, Gelatin agar, Urea agar, Triple sugar iron agar, Bromothymol blue agar, Endo

agar, Cetrimide agar, Deoxycholate citrate agar, Mac Conkey agar, Salmonella/Shigella agar, Thiosulphate citrate bile salt agar, Caprylate thallous agar, Plate count agar, Xylose lysine Deoxycholate agar and Blood agar. Solid media are useful for identifying bacteria by colony characteristics. Isolation of pure culture was done using solid media whereas liquid media yielded mixture of all types of bacteria present in the sample.

3.5.1. Media requirements

Bacteria display a wide range of nutritional and physical requirements for growth including:

1) Water.
2) A source of energy.
3) Sources of carbon, nitrogen, sulphur and phosphorus.
4) Minerals, e.g., Ca^{2+}, Mg^{2+}, Na.
5) Vitamins and other growth factors.

Bacteria may be grown in liquid, solid or semisolid media. Liquid media are utilized for growth of large numbers of organisms or for physiological or biochemical studies and assays. Some species, such as *Staphylococcus*, often demonstrate typical morphologies only when grown in liquid media. Solid media are useful for observations of characteristic colonies, for isolation of pure cultures and for short-term maintenance of cultures. Usually, the preparation of a solid medium for growth simply includes the addition of 1 to 2% agar to a solution of appropriate nutrients. Usually, bacteria are grown in complex media, because we simply do not know enough about the organism to define all of their requirements for growth and maintenance. Neither the chemical composition nor the concentration of substrates is defined. Media frequently contain nutrients in the form of extracts or enzymatic digests of meat, milk, plants or yeast. For fastidious organisms we must often use nutrient-rich medium such as brain-heart infusion broth or blood agar. There is no single medium or set of physical conditions that permits the cultivation of all bacteria, and many species are quite fastidious, requiring specific ranges of pH, osmotic strength, temperature and presence or absence of oxygen.

3.5.2. Media preparation

Preparation of media was done by mixing different individual components (Annexure I) but readymade media was also frequently used.

3.6. Agar plates / Broth preparation

When prepared for inoculation, a plate contains solid agar to provide a surface for growth, mixed with nutrient medium. Agar media was prepared either by mixing nutrients with individual components or by using a pre-mixed readymade powder. Either way, the dry components were mixed with distilled water and heated to melt the agar in flask or bottle and then sterilized in an autoclave at 121°C, 15lb/inch^2 for 15-20 minutes. The sterile media was poured into the plates using aseptic technique, preferably in a sterile cabinet (laminar flow hood).

3.6.1. Procedure

Ingredients of the Nutrient Agar were first dissolved in distilled water, except agar, pH was then adjusted at 7.2. Then agar powder was added followed by boiling in order to dissolve agar powder and finally sterilized in an autoclave at 121°C, 15lb/inch^2 for 15-20 minutes. Recommended volume (usually 15-20 ml) was poured into each plate in hood (recommended) or with very conscientious aseptic technique, at a bench. Plates were allowed to cool and any change in colour, consistency or growth in medium after incubation means some kind of contamination.

3.7. Agar tubes and Agar slant tubes for preservation

Agar medium was uniformly distributed after melting and dispensing in test tubes. For maintaining stocks of isolates slant tubes were helpful. The tubes were tightly capped for relatively long term storage of an isolate with low risk of contamination or drying out of the culture.

3.7.1. Culture preservation procedure

A sterile test tube was taken and 225 micro litre of sterile 80% glycerol was added into it and then 1.0 ml of the bacterial culture was added. Mixed well and tube was placed at -20^0 C. Regular periodic sub culturing of bacteria was done from stock cultures on fresh medium in order to maintain its viability.

3.8. Aseptic techniques

The media on which we culture desirable microorganisms will readily grow un-desirable contaminants, especially moulds and other types of fungi, and bacteria from skin and hair. It was therefore essential to protect cultures from contamination from airborne spores and living microorganisms, surface contaminants that may be on instruments or from skin contact. Sterile cabinet keeps airborne contaminants from

getting into the hood. A simple laminar flow hood protects exposed sterile surfaces that are placed inside.

3.9. Colony count

In order to gain insight into the bacterial load of Dal lake, colony count was done. After incubation, visibly distinguishable bacterial colonies were identified and the number on each plate was counted using Quebec colony counter.

Following formula was used to calculate CFU or SPC:

$$\text{CFU} = \frac{\textbf{Colonies counted}}{\textbf{ml of sample takem}} \text{x}100$$

3.10. Streak for isolation of pure cultures

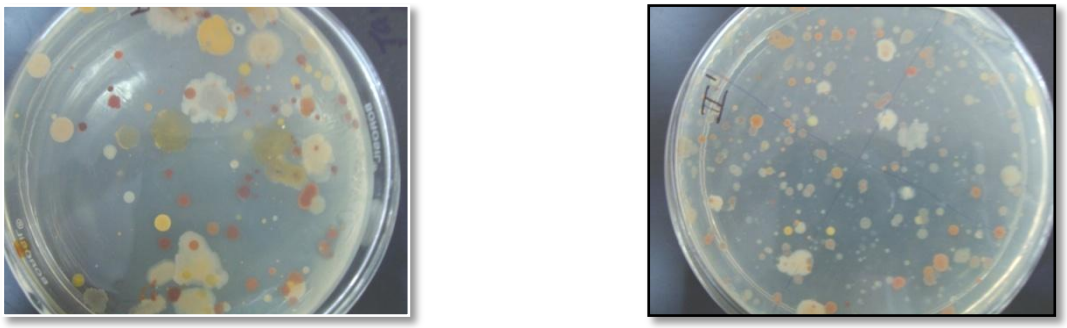
A representative sample colony of each visually differentiable bacterium was selected using a sterile inoculating loop. Each colony was transferred by streaking with inoculating loop in parallel lines over quadrants of plate. The plates were incubated at 37^{o}C for twenty-four hours (Leboffe and Pierce, 1996). At this point, initial observations about the shape, colour, size and other visual properties of each isolate were recorded (Plate 4).

3.11. Cultural characteristics

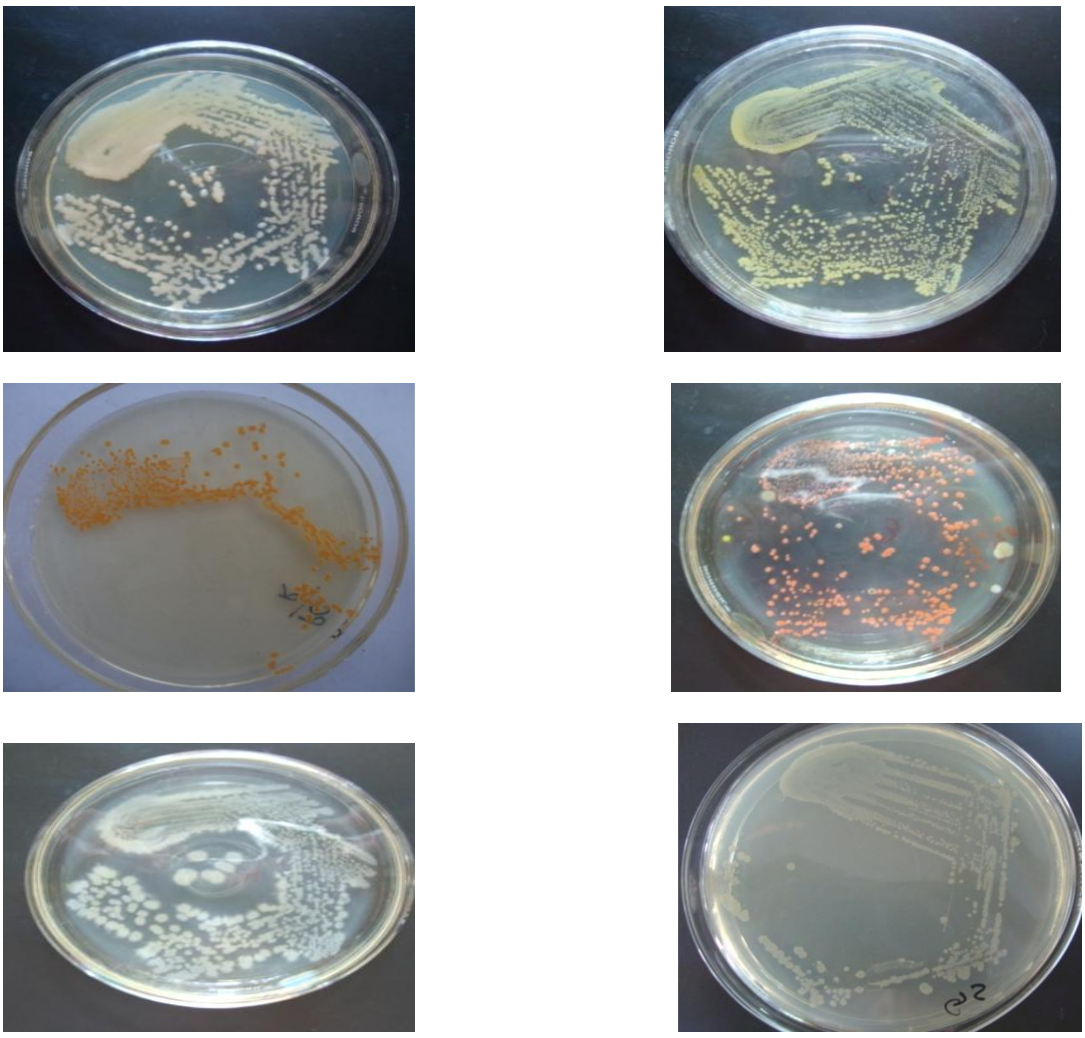
Colony size, colony shape, colony margin, colony elevation, colony surface, chromogenesis and haemolytic behaviour was recorded.

3.12. Gram's staining

An isolated colony from each streak plate was selected using a sterile inoculating loop and transferred to a clean slide with a drop of sterile water on it. The samples were air-dried onto the slides and heat-fixed by passing through a Bunsen burner flame several times. Each smear was covered first with crystal violet dye for one minute and washed with water. Next, the smear was overlaid with stabilized gram iodine for thirty seconds and again rinsed with water. The smear was next washed with gram decolourizer for ten seconds until no more violet dye was removed. Finally, gram saffranin was placed over the smear for one minute and rinsed with water. Slides were examined under 100 x oil immersion microscopy (Koneman *et al*., 1988). At this point, the morphology (rods or cocci) and grouping (clusters, chains, pairs, tetrads, etc.) of the cells was recorded as well as the gram stain results. Smears appearing to be predominantly purple were labelled as "gram-positive," and indicated the retention of



4. A. Spread plate technique



4. B. Streak plate technique

Plate 4. Bacterial cultures on nutrient agar using spread plate and streak plate techniques

crystal violet dye in a thick peptidoglycan layer of the cell wall. Predominantly pink smears were labelled "gram-negative"; crystal violet was not retained due to a thin layer of peptidoglycan, contained within the outer membrane of the cell wall of gram negative bacteria (Nester *et al*., 2007). Based on the gram stain and cell morphology results, diagnostic tests were selected on a sample-by-sample basis.

3.13. Spore staining

Some bacteria are known to form endospores within the cell in response to adverse conditions such as high temperature. The Schaeffer-Fulton procedure was performed to distinguish spore formers by the use of differential dyes. First, the samples in question were smeared on a slide and heat fixed. The primary stain, malachite green was applied over the smear, which was placed over a boiling water bath to steam. The smear was flooded with malachite green for fifteen minutes while heating. At the end of fifteen minutes, the slide was rinsed with water. The slide was then counterstained with saffranin for one minute and again rinsed with water. The slide was dried and microscopically examined; cells with green portions possessed stained endospores, while vegetative cells were pink (Spellman, 2000).

3.14. Biochemical properties for identification of isolates

Bacteria and other unicellular microbes lack extensive digestive system. Their nutrition is dependent on substances absorbed by the cell membrane. Since, macro molecules cannot pass through the membrane they have to be broken down into simpler substances by enzymatic activity outside the cell. The microbes therefore secrete extra cellular enzymes to degrade polysaccharides, proteins, lipids etc. The breakdown products are smaller in size, have low molecular weight and can be easily absorbed by the general body surface and later used for metabolic activity within the cell. All organisms have a preference for a particular substrate and secrete enzymes accordingly, therefore detection of exo-enzyme secretion is good method to identify and differentiate microbes. Presence or absence of enzyme activities is studied by providing the substrate in the medium. Digestion of the substrate is an indication of positive reaction. In some cases the reaction is clearly visible as change in either colour or property of medium, but in cases where the reaction is invisible, indicator dyes are introduced in the medium. The dyes change colour according to pH of medium. Any

change in biochemical composition of the medium is then reflected by change in colour of the dye (Edinger *et al*., 1985).

Therefore, several biochemical tests were performed to identify the pure isolates. These tests are grouped into four categories:

a. Test for utilization of specific substrate.
b. Test for enzymatic activity.
c. Test for specific breakdown of products.
d. Test for proteolytic activity.

3.14.1. Control test

One plate in each experiment was inoculated with stock culture known to give positive results and one with sterilized distilled water known to give negative results. These were incubated along with the experimental plates.

The various biochemical tests performed are as under:

3.14.2. Catalase test

Catalase, an enzyme which breaks down hydrogen peroxide, is frequently found in aerobic and facultative anaerobic bacteria because hydrogen peroxide is a potentially toxic by product of aerobic respiration. To test for the presence of catalase, isolated colonies were selected and transferred via a sterile inoculating loop to a clean slide. 3% Hydrogen peroxide (Hi-media) was applied drop wise to the bacterial colony on the slide. If the bacteria contained catalase, hydrogen peroxide was converted to water and oxygen gas, causing bubbles to appear on the slide. Catalase negative bacteria produced no reaction (Alexander and Strete, 2001).

3.14.3. Coagulase test

Bacteria especially *Staphylococcus aureus* has the property of clotting human or rabbit plasma. This is brought about by the enzyme coagulase which along with the coagulase reacting factor (CRF) present in plasma produces staphylothrombin which convert fibrinogen into fibrin. When a saline suspension of *S. aureus* is mixed on slide with a plasma drop, the cocci are clumped. This is due to precipitation of fibrin on cell surface by the activity of the bound coagulase or the clumping factor. The free and bound coagulase differs in several aspects. Free is a heat labile enzyme which is secreted free into the medium and required the co-operation of CRF for its action. Bound is a heat stable constituent of the cell wall and is independent of CRF. This

method was done by Slide method. A drop of saline was placed on a clean slide. A loop full of test colony was suspended in one drop of saline and a loop full of control organism in the other. The homogenous suspension was made with a sterile loop and a drop of rabbit or human plasma was added to one test. Clumping occurred (bound coagulase).

3.14.4. Aerobicity and Motility tests

The motility and aerobicity of bacteria can be determined using a single test medium. A semi-solid media containing beef extract, peptone, and 5% agar was used. The lower agar content allows bacteria with flagella for movement to demonstrate their motility by moving away from the stab line in an agar deep (Alexander and Strete, 2001). Motility was determined by examining the agar deep. Obligate aerobes require an atmosphere containing concentrations of oxygen similar to room air. Microaerophiles require oxygen, but less than that of room air. Anaerobes do not require oxygen for life, and can be either obligate anaerobes, which grow only in anaerobic environments, or facultative anaerobes, which grow in any oxygen environment (Burton and Engelkirk, 2000). In terms of test results, obligate aerobes appear only at the top of the deep, microaerophiles grow below the top of the stab line, obligate anaerobes grow only in the bottom portion of the stab line, and facultative aerobes grow evenly throughout the stab and on surface of the culture (Cullimore, 2000). Motility test medium was poured into deeps and allowed to solidify. Deeps were inoculated with a transfer needle and allowed to incubate at 37^{o}C for twenty-four hours before tests were read.

3.14.5. Urease test

This test detects the ability of an organism to split urea to ammonia by the action of enzyme urease. In case of positive reaction, the medium turns pink under alkaline conditions due to phenol red indicator in the medium. Orange- yellow colour indicates negative reaction.

3.14.6. Carbohydrate utilization test

Specific carbohydrates are added to basal media which contain phenol red as indicator. On fermentation of carbohydrate, acid is liberated which lowers down the pH of medium and this change of colour is indicated by yellow due to acid reaction. No

change in colour or red/pink indicated negative reaction. Sugars tested included: Glucose, Adonitol, Arabinose, Lactose, Sorbitol, Mannitol, Rhamnose, Sucrose, Lactose, Malonate, Trehalose and Raffinose.

3.14.7. Hydrogen sulphide production test

All members of *Enterobacteriaceae* are capable of enzymatically liberating sulphur from sulphur containing amino acids or inorganic sulphur compounds .The H_2S released reacts with ferric ions or lead acetate to yield ferrous sulphide or lead sulphide, which are insoluble black precipitates. Blackening of medium indicates positive reaction.

3.14.8. Indole test

Some bacteria, particularly enteric pathogens such as *Escherchia coli*, are capable of utilizing the enzyme tryptophanase to metabolize the amino acid tryptophan to produce indole. Indole reacts with Kovac's reagent to produce a reddish pink colour. Kovac's reagent contains HCl and dimethylaminobenzaldehyde (DMABA) dissolved in amyl alcohol, which forms a layer on top of the inoculating medium. This causes the reddish pink colour to be very easily visible and distinguishable (Leboffe and Pierce, 1996). The samples were incubated for twenty-four hours at 37^{o}C; Kovac's reagent was added to the well and read for results.

3.14.9. Methyl red test

The methyl red test is used to determine organisms that ferment glucose to a stable acid end product, lowering the pH of the system. Media was inoculated and incubated at 30^{o}C for 24 hours to allow stable acids to be produced. After incubation methyl red indicator was added. Methyl red indicator is red at pH less than 4.4 and yellow at pH above 6.0, so a red colour was labelled positive, and a yellow colour as negative (Leboffe and Pierce, 1996).

3.14.10. Voges Proskauer

The Voges Proskauer reaction tests for the presence of acetoin (acetyl methyl carbinol) which is an intermediate in the butane diol fermentation (carbohydrate). This test must be done after only 24 hours of growth; if done later all the acetoin will be converted to butane diol for which there is no easy colour test. The Voges Proskauer reagents was added which reacts with the peptone of the agar and a positive test is the

production of a pinkish red colour. Negative reaction is indicated by slight copper colour.

3.14.11. Citrate test

The citrate tests the ability to grow on citrate as a sole carbon source in Simmon's media. The medium contains the dye Bromo thymol blue which is blue in the alkaline condition and yellow in the acid. At neutral pH the dye is half yellow and half green in colour. Citrate is supplied to the medium as sodium citrate, as the organism uses citrate, sodium ions remain in the medium making the medium basic and therefore a blue colour develops from green, as the citrate is utilized. This is a positive result.

3.14.12. Nitrate reduction test

The nitrate test is used to determine whether a bacterial sample has the ability to reduce nitrate to nitrite, free nitrogen gas, or ammonia. Though nitrites are colourless, they react with *N*-*N*-dimethyl-1-napthylamine to produce a pinkish red colour in an acidic environment. Zinc dust reacts with nitrates in an acidic environment to form a similar pinkish red colour (Gusberti and Syed, 1984). The bacteria was inoculated in the well, and incubated at 37^{o}C for twenty-four hours. Following the incubation, several drops of alpha-napthylamine as well as an equal amount of sulphanilamide reagent were added, mixed, and allowed to stand for 10 minutes. If a pinkish red colour developed, the bacterial species was known to reduce nitrate to nitrite.

3.14.13. Gelatin hydrolysis test

This test detects the ability of an organism to produce proteolytic enzymes using gelatin as substrate. For this test, gelatin agar was prepared. Medium was poured into culture tubes and allowed to solidify. Test organism was inoculated in gelatin agar and incubated at 37^{0} C for 2-7 days. Gelatin hydrolysis was tested by chilling the tubes with ice or putting in the freezer. Solidifying of gelatin is a negative test. Failure of solidification indicated secretion of gelatinase and hydrolysis of gelatin.

3.14.14. Beta galactosidase test

Two enzymes, permease and beta galactosidase are required for lactose fermentation. True non lactose fermenters are devoid of both enzymes; however some organisms may lack permease but possess the enzyme beta galactosidase. ONPG is

cleaved into galactose and o-nitrophenol, a yellow compound. ONPG is especially useful in identifying lactose fermentation. Development of yellow colour indicates positive reaction and no colour change indicates negative reaction.

3.14.15. Oxidase test

This test depends upon the presence in bacteria of certain oxidases that catalyze the transport of electrons between donors in the bacteria and a redox dye tetra methyl-p-phenylene diamine.The dye is reduced to deep purple colour.

3.14.16. Malonate test

This test medium contains Bromothymol blue as indicator. Sodium malonate is a carbon source and ammonium sulphate is nitrogen source. Bacteria which are able to utilize malonate release sodium hydroxide. The resulting alkaline condition causes the indicator to change from light green to blue. Malonate negative bacteria don't cause any change in the colour of the medium.

3.14.17. Phenyl alanine deamination

This test detects the ability of an organism to oxidatively deaminate phenyl alanine with production of phenyl pyruvic acid which reacts with ferric salts to give green colour. On adding TDA reagent to medium after incubation green colour indicates negative reaction.

3.14.18. PYR test

This test detects ability of gram positive bacteria to produce pyrrolidonyl aryl - amidase enzyme. It hydrolyses the substrate L-pyrrolidonyl beta-napthylamine to form free colourless alpha-pyrrolidone and free beta-napthylamine. Positive reaction is indicated by development of cherry red to red colour. No colour change indicates negative reaction.

3.14.19. Amino acid decarboxylation (Ornithine, Lysine and Arginine utilization).

The medium for this test contains bromocresol purple as pH indicator when carbohydrate present in the medium is utilized pH is lowered due to acid production changing the colour of medium to yellow. The acid production stimulates decarboxylase enzyme. The formation of amine due to this reaction increases the pH of the medium changing the colour of indicator from light purple to dark purple. Negative reaction indicated by yellow colour.

3.14.20. Esculin hydrolysis

Esculin is substituted glucoside that can be hydrolysed by certain bacteria to yield glucose and esculetin. The latter combines with ferric ion in the medium to form black coloured complex.

3.15. Hi-Media biochemical test kit

The Hi-Assorted biochemical test kit consists of a number of different tests compartmentally combined in to one or two convenient strip and was available commercially from Hi-Media (Annexure IV).

Biochemical test kit is a standardized colorimetric identification system utilizing seven conventional biochemical tests and five carbohydrate utilization tests. The tests are based on the principle of pH change and substrate utilization. On incubation organisms undergo metabolic changes which are indicated by a colour change in the media that can be either interrupted visually or after addition of the reagent. Three types of kits used were:

3.15.1. Biochemical test kit for Gram negative rods/ Enterobacteriaceae.

Kits were used for screening pathogenic organisms from water samples.

3.15.2. Biochemical test kit for Gram positive rods

Kits were used for screening gram positive rods.

3.15.3. Biochemical test kit for Gram positive cocci

Kits were used for identifying gram positive cocci.

3.16. Procedure for Hi-Media biochemical test kit

3.16.1. Preparation of Inoculum

The kits used for the identification purpose were not to be used directly. The organisms were first isolated and purified, and then only pure cultures were used. The organism identified were first cultured on Nutrient Agar and then a single well isolated colony was taken for preparing homogeneous suspension in 2-3ml of normal Saline/ nutrient broth. This inoculum was incubated at 35-37°C for 4-6 hours.

3.16.2. Inoculation of kit

The kit was opened aseptically by peeling off the sealing tape. Each well of the kit was then inoculated with the 50µl of the prepared inoculum by surface inoculation method.

3.16.3. Incubation

The kit was incubated at temperature of 35-37°C for 18-24 hours and then test results were read and recorded (Lindh, 1998).

3.17. Total coliforms

All natural waters contain bacteria. The aerobic gram negative rods of the genera *Pseudomonas, Alcaligenes* as well as others those are common in water. Many of these bacteria are capable of growing on a wide variety of single carbon sources. *Eshcerichia coli* which are in the large intestine of virtually all people have been used as the indicator of human faecal contamination of water. Tests for the presence of this bacteria (and closely related types generally known as coliforms) utilize either a multiple tube technique or direct plate count onto differential media.

Multiple Tube Techniques utilize selective and differential liquid media into which multiple aliquots of serial dilutions are inoculated. The advantage of this technique is that it will detect organisms a small titters, much less than one per ml which would otherwise require inoculation of large volumes. The multiple tube techniques yield the statistically derived Most Probable Numbers of organisms per aliquot (usually 100 ml) of water (APHA, 1998). In this experiment, three sets of tubes was inoculated with a tenfold difference in inoculum volume between each set: one set of three tubes with double strength lactose broth was inoculated with 10 ml sample in each tube, second set with single strength lactose broth was inoculated with 1 ml sample in each tube, and the third set with single strength lactose broth was inoculated with 0.1 ml sample in each tube. After appropriate incubation, the tubes were then examined for the diagnostic reaction: gas production for coliforms. Each set was scored for the number of positive tubes and the score of all three sets was then compared with the Most Probable Number Table (Mc Cardy, 1915) to determine the number of each group (coliforms) per 100 ml of water (Annexure II).

Coliforms are able to ferment lactose to acid and gas. The medium used in the multiple tube technique for coliforms was Lauryl SO_4 Lactose Broth and was placed in tubes containing little upside down tubes (Durham tubes). The gas production was seen in the Durham tubes: this is a positive Presumptive Test for coliforms. Lauryl SO_4. Lactose broth contains the detergent lauryl SO_4 which retards the growth of Gram

positive bacteria. It is possible that bacteria other than *E. coli* can give a positive Presumptive Test.

There are two different fermentation patterns: the Classical Mixed Acid fermenters and the Butane Diol fermenters both produce soluble end products and gas (CO_2 and H_2). The Classical Mixed Acid fermenters like *E. coli* ferment sugars to a variety of acids and only small amounts of soluble neutral end products. The Butane Diol fermenters like *E. aerogenes* ferment sugars to a small amounts of mixed acids and larger amounts of neutral end products: butane diol (2,3 butylene glycol), ethanol. Two points are important: classical mixed acid fermenters do not produce butane diol and butane diol fermenters produce very small amounts of acids. The American Public Health Association has developed Standard Methods to determine if *E. coli* is actually present.

The Presumptive test was then followed by the Confirmative Test, the Completed Test and the IMViC tests. The confirmative test involves streaking of a positive presumptive tube (gas production) onto eosin methylene blue Agar (EMB Agar). The agar contains lactose and the dyes eosin Y and methylene blue. When *E. coli* grows on EMB, it ferments so much acid that the two dyes precipitate out in the colony producing a metallic green sheen appearance. A positive confirmative test is then the presence of green sheen colonies on EMB streaked from a positive presumptive test. The completed test involves the inoculation of lactose broth with a green sheen colony from the confirmative test. A positive completed test was the production of acid and gas in this medium. This test detects only one biochemical trait: the fermentation of lactose. Thus, an additional biochemical test was performed from EMB colonies: the IMViC tests. The green sheen colony were inoculated into tryptone broth for the Indole test, MRVP broth for the methyl red and voges proskauer tests and a Simmons citrate slant for the citrate test.

E. coli (green sheen colonies) produces a ++-- IMViC. Thus it is positive in the indole and methyl red tests and negative in the voges proskauer and citrate tests. Another coliform (positive presumptive test), *E. aerogenes* produces pink colonies with a purple centre on EMB and a --++ IMViC.

3.18. 16S rRNA approach to study bacterial diversity

Ribosomes are complex structures found in all living cells which functions in protein synthesis machinery. Basically ribosome's consists of two subunits, each of which is composed of protein and a type of RNA, known as ribosomal RNA (rRNA). The objectives of this approach are to study the technique involved in sequencing of gene and to comprehend the importance of 16S rRNA in identification of bacteria. 16S ribosomal RNA sequencing is widely used in microbiology studies to identify the diversities in prokaryotic organisms as well as other organisms and thereby studying the phylogenetic relationships between them. The advantages of using ribosomal RNA in molecular techniques are:

1. RNA genes are highly conserved in nature.
2. Have conserved primer binding sites, and
3. Contain hypervariable regions that provide species specific signature sequences useful for bacterial identification.

3.18.1. Principle for bacterial DNA extraction

The principle for the extraction of bacterial DNA HiPurA bacterial and yeast genomic DNA miniprep Purification spin kit (Hi-Media, Lot MB505) used for the purpose was as follow:

Hi PurA genomic DNA kit provides fast and easy method for purification of total DNA for reliable application in PCR technique. The DNA purification procedure using the mini spin columns comprises of three steps viz, adsorption of DNA to the membrane, removal of residual contaminants and elution of pure genomic DNA. Its format allows rapid processing of multiple samples. The columns have a high binding capacity and high quality. DNA was obtained from various species of bacteria (Gram positive and Gram negative) bacterial cells were grown in a medium till they reach log phase and were harvested by centrifugation. After harvesting the bacterial (Gram positive) cell wall was degraded by lysozyme and proteinase K. For Gram negative bacteria, the lysozyme treatment was not required. Following lysis; the DNA was bound to the silica gel membrane of spin column to yield 20 micro grams of pure DNA. Two rapid wash steps removed trace amounts of salt and protein contaminants resulting in the elution of pure DNA in the elution buffer.

3.18.2. Enrichment and isolation of microorganisms

Lake water samples were collected in sterile bottles from defined sampling site. Enrichment of water samples was carried out using nutrient agar at pH 10.0 with 30 g l^{-1} sodium chloride. All flasks were incubated at 37°C on a rotary shaker (100 rpm) for 48h. After enrichment, the bacteria were isolated on respective media agar plates and incubated at 37°C for 24h. Well isolated and morphologically distinct colonies from these plates were transferred on the respective medium slants and maintained as stocks.

Table 2. Media composition enrichment medium and their composition

(Tambekar and Dhundale, 2012)

Chemical	Composition/litre
Glucose	10
Soluble Starch	10
Peptone	5
Yeast	1.5
Beef extract	1.5
KH_2PO_4	1
$MgSO_4.7H_2O$	0.2
Na_2CO_3	10
NaCl	30
Agar	20
DW	1000ml

3.18.3. Protocol for bacterial preparation

A. Gram negative bacterial preparation

a. Harvesting of cells

1.5 ml of an overnight bacterial broth culture was pelleted by centrifuging for 2 mins at 12000-16000 rpm.

b. Resuspending of cells

The pellet was re-suspended thoroughly in 180 micro litre of lysis solution.

c. Preparation for cell lysis

For 50 preparations, 27 mg of proteinase K was added to1.35 ml of molecular biology grade water. The proteinase K was re suspended to obtain a 20 mg/ml stock solution. 20 micro litre of the proteinase K solution (20mg/ml) was added to the sample. Mixed and incubated for 30 minutes at 55^0 C.

d. RNase A treatment

For RNA free genomic DNA is required, 20microlitre of RNase A solution was added, mixed and incubated for 5 minutes at room temperature (15-25^{0}C).

e. Cells lysis

200 micro litre of lysis solution was added, vortexed thoroughly (about15 sec) and incubated at 55^0 C for 10 minutes.

B. Gram positive bacterial preparation

45 mg/ml solution of lysozyme was prepared with the Gram Positive lysis solution as a diluent. 200 micro litre of lysozyme solution was required per isolation procedure. Extra solution was prepared to account for pipetting error.

a. Harvesting of cells

1.5 ml of bacterial broth culture was pelleted by centrifuging for 2 minutes at 12000-6000 rpm x g. The culture medium was removed completely and discarded.

b. Resuspending of cells

The pellet was resuspended thoroughly in 200 micro litre of lysozyme solution and incubated for 30 minutes at 37 ^{0} C.

c. Cell lysis

20 micro litre of the proteinase K solution (20mg/ml) was added to the sample.

d. RNase A treatment

For RNA –free genomic DNA, 20 micro litre of RNase solution was added, mixed and incubated for 5 minutes at room temperature (15-25^0 C) 200 micro litre of lysis solution was added. Then vortexes thoroughly for few seconds and incubated at 55 ^{0}C for 10 minutes.

3.18.4. DNA isolation protocol for Gram positive and Gram negative bacteria

a. Preparation for binding

200 microlitre ethanol (95-100%) was added to the lysate and mixed thoroughly by vortexing for few seconds.

b. Loading of lysate onto HiElute mini prep spin column

The lysate obtained was transferred from binding onto HiElute min prep spin column provided and centrifuged at > 6500x g (10,000rpm) for 1 minute.

c. Pre wash

500 micro litre of prewash solution was added to the Hi Elute mini prep spin

column and centrifuged at > 6,500 x g (10000 rpm) for 1 minute.

d. Wash

13ml wash solution was used for 50 preparations and concentrated to 52ml of (96-100) ethanol as specified in instruction kit. 500 micro litre of wash solution was added to the high elute mini prep spin column and centrifuged for 3 minutes at maximum speed 12,000-16,000 rpm × g. The high elute mini prep spin column was transferred to new collection tube and centrifuged again at same speed for the additional 1 minute to dry the column. The column must be free of ethanol before eluting the DNA.

e. DNA elution

The high elute mini prep spin column was transferred to new collection tube. 200 micro litre of the elution buffer was pipetted directly into the column without spilling to the sides and was incubated for 1 minute at room temperature. Then it was centrifuged at > 6,500 × g (10000rpm) for 1 minute to elute the DNA.

f. Storage of the elute with purified DNA

The elute contains pure genomic DNA. For short term storage (24-28 hrs) of the DNA, 2-8^0 C is recommended for long term storage upto 20 ^{0} C or lower temperature upto 80^0 C is recommended. Repeated freezing and thawing was avoided of the sample which may cause denaturing of DNA.

3.18.5. Polymerase chain reaction (PCR)

The forward (8F) and reverse (1492R) primers used in the PCR reactions performed in this study were developed by Liu *et al*. (1997) and Turner *et al*. (1999) to amplify the 16S rRNA gene. Amplifications were performed in 50µl PCR reaction tubes containing 5.2µl DNA, 5µl 10X thermophilic buffer, 2 µl $MgCl_2$ (25mM) 1.4 µl dNTP (10mM), 1.2µl (10µM) 8F, 1.2µl (10µM) 1492R, 32 µl ddH_2O and 2µl (10u/µl) *Taq* (Promega). Amplifications occurred in an Eppendorf master thermal cycler with the following program for 40 cycles: initial denaturation temperature 95° C for 5 min, melt temperature 94° C for 45 seconds, annealing temperature 52.5° C & 55° C for 45 sec, extending temperature of 72° C for 1 minute, final extension temperature 72^{o}C for 7 min, hold temperature 4 °C (Jasalavich *et al*., 2000). The bands were identified by gel electrophoresis on a 2% agarose gel. Molecular weights of each 16S rRNA fragment were determined using GelPro Express software and a standard 100 bp ladder.

3.18.6. Purity of DNA

The ratio of the absorptions at 260 nm and 280 nm (*A*260/ *A*280) provides an estimate of DNA purity with respect to contaminants that absorb UV light, such as protein. The *A*260/ *A*280 ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting *A*260/*A*280 ratio can vary greatly. Lower pH results in a lower *A*260/*A*280 ratio and reduced sensitivity to protein contamination. For accurate *A*260/ *A*280 values absorbance was measured in a slightly alkaline buffer.

3.19. Prevalence of bacterial symptoms

The prevalence of bacterial symptoms in people associated with the lake water was assessed by on-spot filling of a detailed questionnaire including the details about the medical history, medical records, disease symptoms if any, life style, personal hygiene, housing condition, type of water being used for different purposes like washing, drinking, bathing and occupation of the person interviewed was done. The questionnaire used is appended in Annexure III.

3.20. Statistical analysis

The whole data was fed into Microsoft Excel 2010. Statistical software viz. SPSS 16.0 for windows, Biodiversity pro, EcoSim700, Past and Primer were used for data analysis. One way analysis of variance (ANOVA) was carried out pair wise to determine the significance level of the data and the differences were considered significant when the p-value obtained was less than 0.05. Correlation of some physical parameters of water with the bacterial load at different sites was also determined using SPSS 16.0. Chi-square test was used for the analytic assessment of the bacterial symptom prevalence in the Dal inhabitants. Percentage (%) of symptom incidence to measure their prevalence was also assessed.

Chapter: 4

Results

Bacterial quality in the water samples collected from Dal lake, Kashmir on seasonal basis for a period of two years from sixteen different sites of the lake was assessed in terms of colony forming units (CFU/ml) and total viable counts on Nutrient agar. The samples were serially diluted seven folds using normal saline solution to get the desired number of colonies per plate so as to avoid any obfuscation in the results by the imbrications of different colonies.

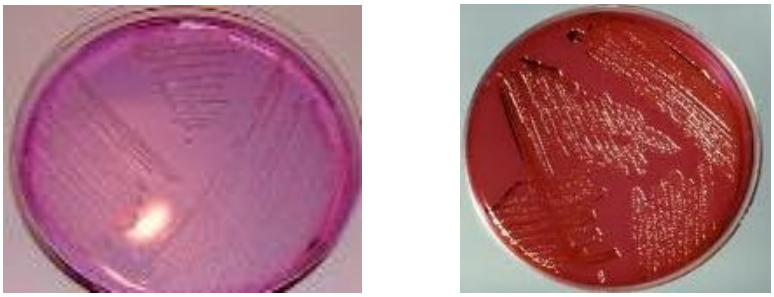
4.1. Identification

In all, the culturable aerobic bacterial population was assessed by using general and selective media like Nutrient agar, Semi solid agar, EMB agar, Simmon citrate agar, Gelatin agar, Urea agar, Triple sugar iron agar, Bromothymol blue agar, Endo agar, Cetrimide agar, Deoxycholate citrate agar, Mac Conkey agar, Salmonella/Shigella agar, Thiosulphate citrate bile salt agar, Caprylate thallous agar, Plate count agar, Xylose lysine Deoxycholate agar and Blood agar. Besides these, the biochemical tests and molecular tools for the identification of bacteria from the Dal lake water samples revealed forty five (45) species (Table 3) viz, *Citrobacter freundii* Werkman and Gillin., *Citrobacter diversus* Werkman and Gillin., *Enterobacter aerogenes* Hormaeche and Edwards., *Enterobacter agglomerans* Hormaeche and Edwards., *Enterobacter cloacae* Jordan., *Enterobacter intermedius* Hormaeche and Edwards., *Serratia marcescens* Bizio., *Serratia rubideae* Bizio., *Shigella flexneri*

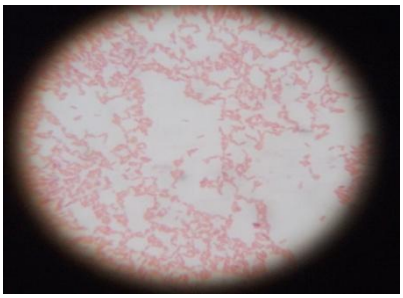
Castellani and Chalmers., *Shigella dysenteriae* Castellani and Chalmers., *Salmonella typhi* Lignieres., *Salmonella paratyphi* Lignieres., *Salmonella choleraesuis* Lignieres., *Proteus vulgaris* Hauser., *Proteus mirabilis* Hauser., *Klebsiella oxytoca* Flugge., *Klebsiella ozaenae* Trevisan., *Escherichia coli* Migula., *Staphylococcus aureus* Rosenbach., *Yersinia enterocolitica* Schleifstein and Coleman., *Cedacea davisae* Patrick and Grimont., *Cedacea lapagei* Patrick and Grimont., *Edwardseilla tarda* Sakazakii *et al., Acinetobacter* spp. Brinson and Prevot., *Kluyvera ascorbata* Farmer *et al., Enterococcus feacalis* Schleifer and Kilpper -Balz., *Enterococcus faecium* Schleifer and Kilpper-Balz., *Enterococcus durans* Schleifer and Kilpper-Balz., *Enterococcus hirae* Schleifer and Kilpper-Balz., *Enterococcus mundtii* Schleifer and Kilpper-Balz., *Vibrio cholerae* Pacini., *Vibrio parahaemolyticus* Pacini., *Vibrio vulnificus* Pacini., *Pseudomonas putida* Migula., *Pseudomonas alcaligenes* Migula., *Aeromonas hyrdophila* Stanier., *Aeromonas caviae* Stanier., *Bacillus sphaericus* Cohn., *Bacillus subtilis* Cohn., *Bacillus cereus* Cohn., *Bacillus alcalophillus* Cohn., *Bacillus brevis* Cohn., *Alcaligenes faecalis* Castellani and Chalmers., *Micrococcus luteus* Cohn and *Micrococcus roseus* Cohn were isolated and identified during the course of study and are depicted in Table 3 along with their Taxonomic classification (Plate 5-25).

4.1.1. Morphological identification

Identification of pure isolates was done by the method involving direct microscopic examination, study of cultural characteristics as well as biochemical properties. The observed macro-morphological features included study of colony characters in terms of size, shape, margin, elevation, surface, chromogenesis, motility and haemolytic behaviour. The colony characters observed on solid media varied with the type and other features on nutrient media used and the type of bacterial strain (Plate 26). The macros-copic as well as microscopic characters listed in (Table 4) revealed that the bacterial isolates exhibit great variety in colony characteristics. Cultural characteristics observed in terms of colony size varied from pinpoint to extremely large, colony shape circular and irregular, colony margin observed were entire to undulate, lobate, curled, colony elevation were flat, convex, raised, umbonate, colony texture smooth, moist, mucoid, wrinkled and sometimes rough, optical features like opacity, transparency were also noted.



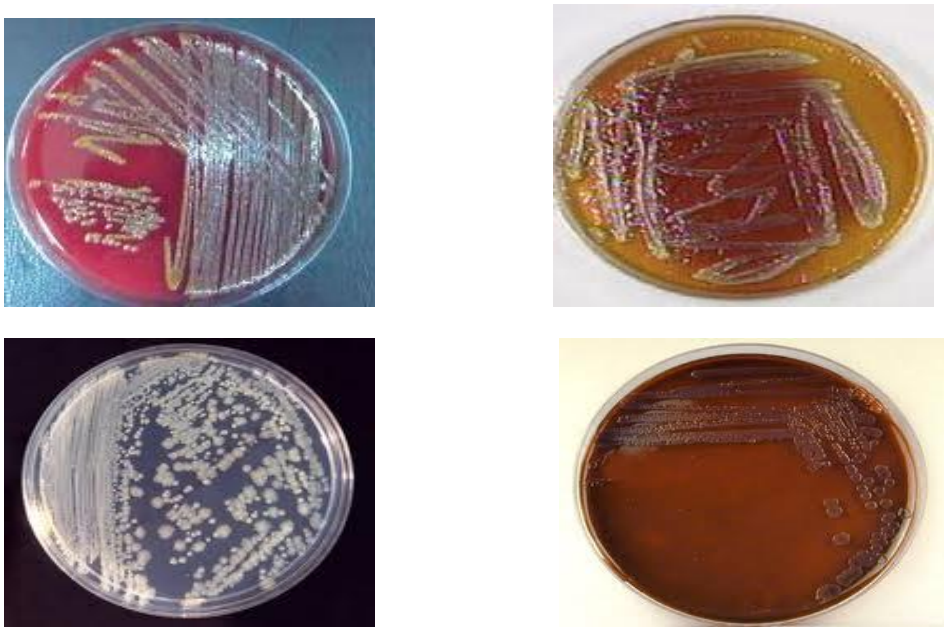
5. A. Culture plates of *Citrobacter* spp.



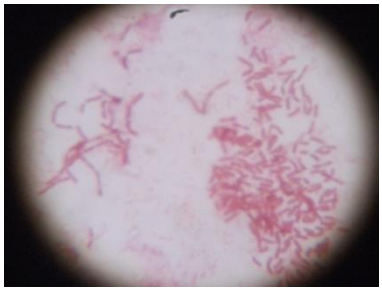
5. B. Gram negative bacilli of *Citrobacte*r spp.

5. C. Biochemical test results of *Citrobacte*r spp

Plate 5. Identification methods of *C. freundii and C. diversus*



6. A. Culture plates of *Enterobacter* spp.

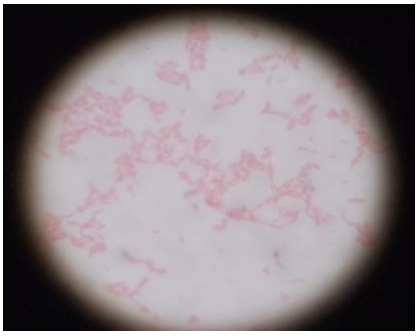


6. B.Gram negative baciili of *Enterobacter* spp.

6. C. Biochemical test results of *Enterobacter* spp.

Plate 6. Identification methods of *E. aerogenes, E. agglomerans, E. cloacae* and *E. Intermedius*

7. A. Culture plates of *Serratia* spp.

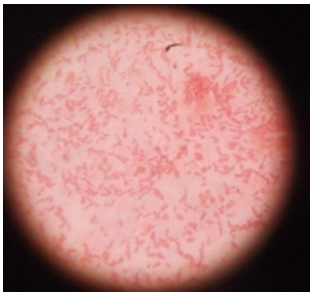


7. B. Gram negative bacilli of *Serratia* spp.

7. C. Biochemical test results of *Serratia* spp.

Plate 7. Identification *methods* of *S. marcescens and S. rubidaea*

8. A. Culture plates of *Shigella* spp.

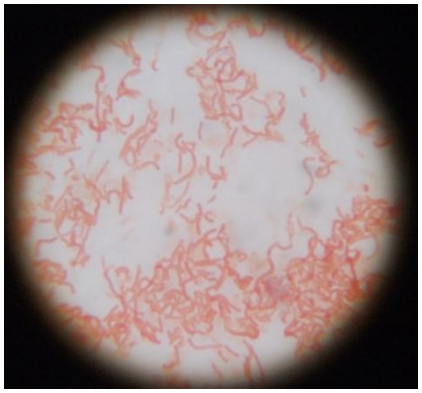


8. B. Gram negative bacilli of *Shigella* spp..

8. C. Biochemical test results of *Shigella* spp.

Plate 8. Identification methods of *S. flexneri* and *S. dysenteriae*

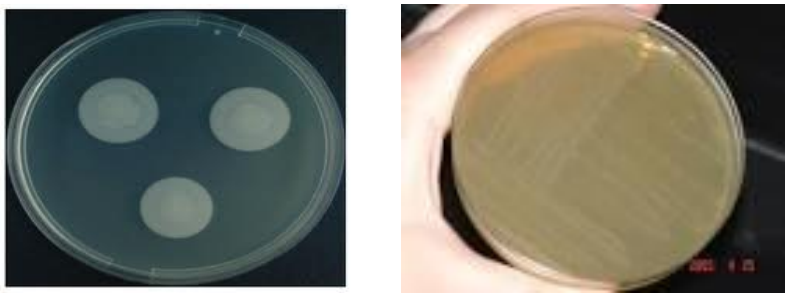
9. A. Culture plates of *Salmonella* spp.



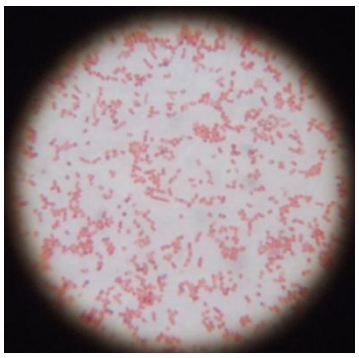
9. B. Gram negative bacilli of *Salmonella* spp.

9. C. Biochemical test results of *Salmonella* spp.

Plate 9. Identification methods of S. *typhi, S. paratyphi and S. choleraesuis*



10. A. Culture plates of *Proteus* spp.

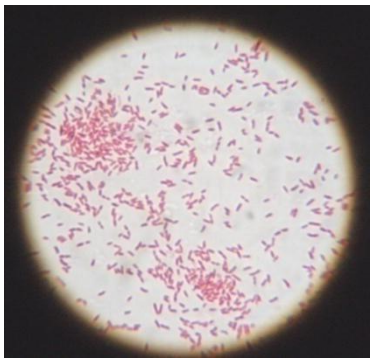


10. B. Gram negative bacilli of *Proteus* spp.

10. C. Biochemical test results of *Proteus* spp.

Plate 10. Identification methods of *P. mirabilis* and *P. vulgaris*

11. A. Culture plates of *Klebsiella* spp.

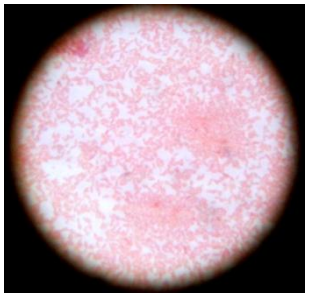


11. B. Gram negative bacilli of *Klebsiella* spp

11. C. Biochemical test results of *Klebsiella* spp

Plate 11. Identification methods of *K. oxytoca and K. ozaenae*

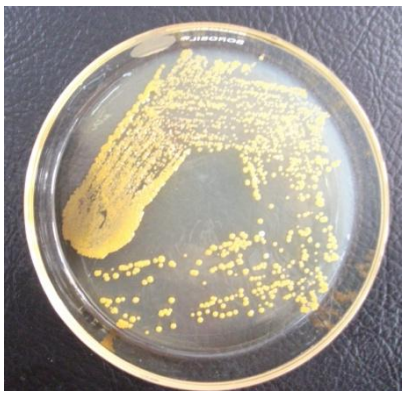
12. A. Culture plates of *Escherchia coli*



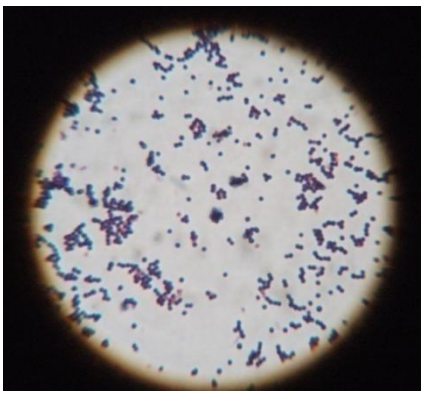
12. B. Gram negative bacilli of *Escherchia coli*

12. C. Biochemical test results of *Escherchia coli*

Plate 12. Identification methods of *Escherchia coli*



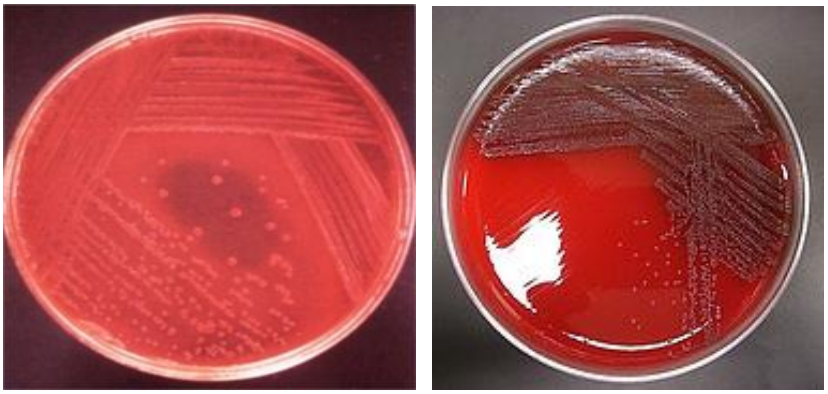
13. A. Culture plate of *Staphylococcus* spp.



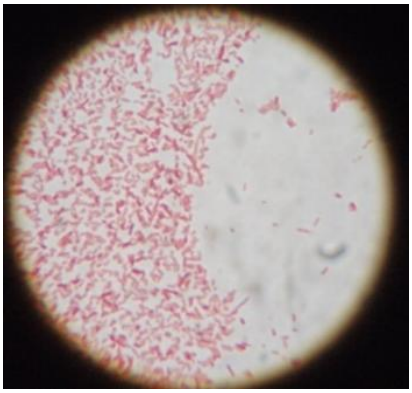
13. B. Gram positive coccus of *Staphylococcus* spp.

13. C. Biochemical test results *Staphylococcus aureus.*

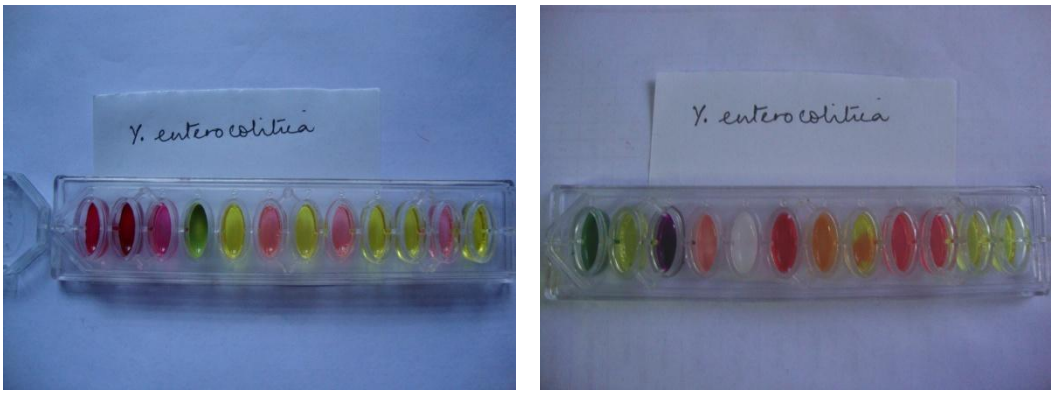
Plate 13. Identification methods of *Staphylococcus aureus*



14. A. Culture plates of *Yersinia enterocolitica.*



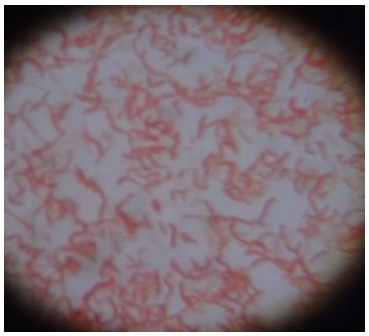
14. B. Gram negative bacilli *Yersinia enterocolitica.*



14. C. Biochemical test results of *Yersinia enterocolitica.*

Plate 14. Identification methods of *Yersinia enterocolitica.*

15. A. Culture plate of *Cedacea* spp.

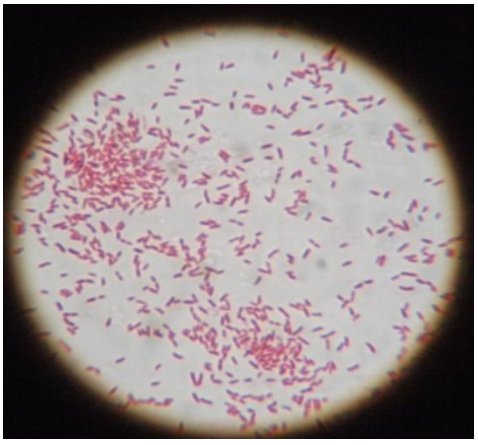


15. B. Gram negative bacilli of *Cedacea* spp.

15. C. Biochemical test results *Cedacea* spp.

Plate 15. Identification methods of *C. davisae* and *C. lapagei*

16. A. Culture plate of *Edwardseilla tarda.*



16. B. Gram negative bacilli of *Edwardseilla tarda.*

16. C. Biochemical test results of *Edwardseilla tarda.*

Plate 16. Identification methods of *Edwardseilla tarda.*

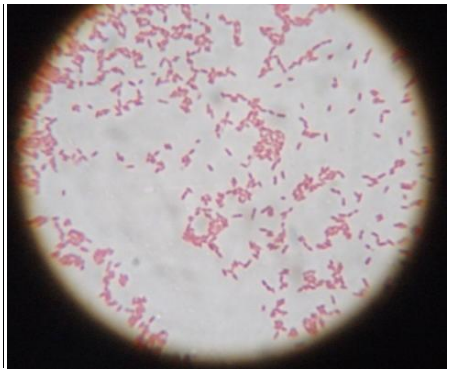
17. A. Culture plate of *Acinetobacter* spp.

17. B. Gram negative coccobacillus of *Acinetobacter* spp.

17. C. Biochemical test results *Acinetobacter* spp.

Plate 17. Identification methods of *Acinetobacter* spp.

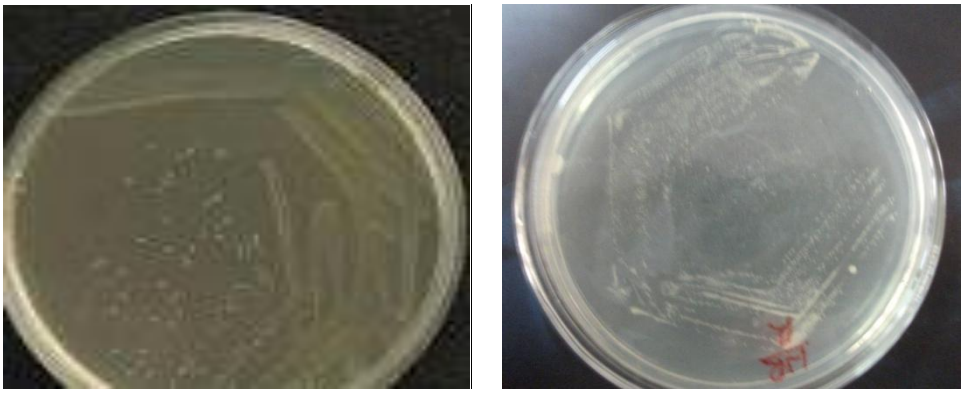
18. A. Culture plate of *Kluyvera ascorbata*



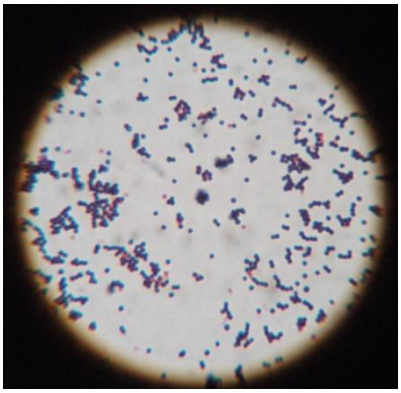
18. B. Gram negative bacilli of *Kluyvera ascorbata*

18. C. Biochemical test results *Kluyvera ascorbata*

Plate 18. Identification methods of *Kluyvera ascorbata*



19. A. Culture plates of *Enterococcus* spp.

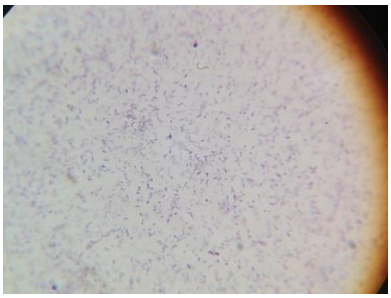


19. B. Gram positive coccus of *Enterococcus* spp.

19. C. Biochemical test results *Enterococcus* spp.

Plate 19. Identification methods of *E.faecalis, E.faecium, E.durans, E.hirae and E. mundtii*

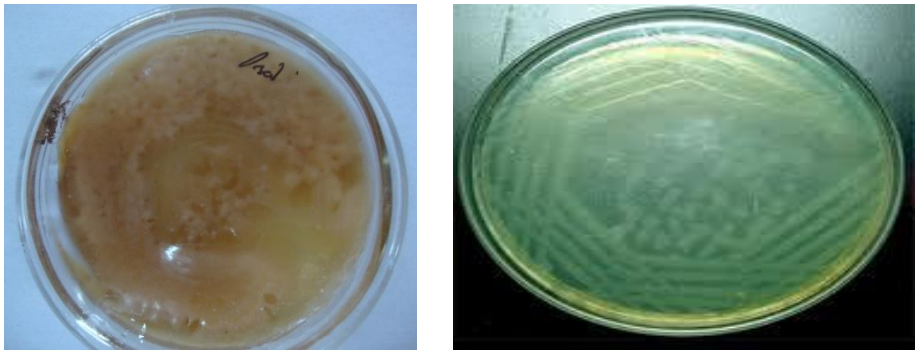
20. A. Culture plates of *Vibrio* spp.



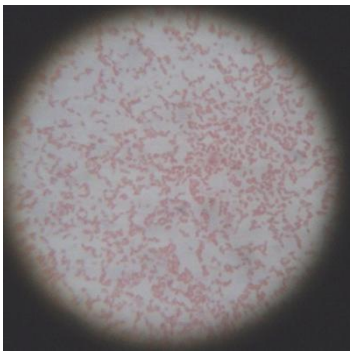
20. B. Gram negative bacilli of *Vibrio* spp.

20. C. Biochemical test results of *Vibrio* spp.

Plate 20. Identification methods of *V. cholerae, V. vulnificus and V. parhaemolyticus*



21. A. Culture plates of *Pseudomonas* spp.

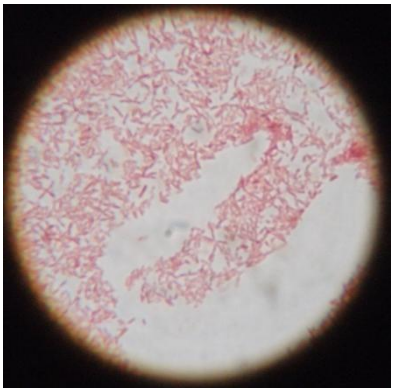


21. B. Gram negative bacilli of *Pseudomonas* spp.

21. C. Biochemical test results of *Pseudomonas* spp.

Plate 21. Identification methods of *P. putida and P. Alcaligenes*

22. A. Culture plates of *Aeromonas* spp.



22. B. Gram negative bacilli of *Aeromonas* spp.

22. C. Biochemical test results *Aeromonas* spp.

Plate 22. Identification methods of *A. caviae* and *A. hydrophila*.

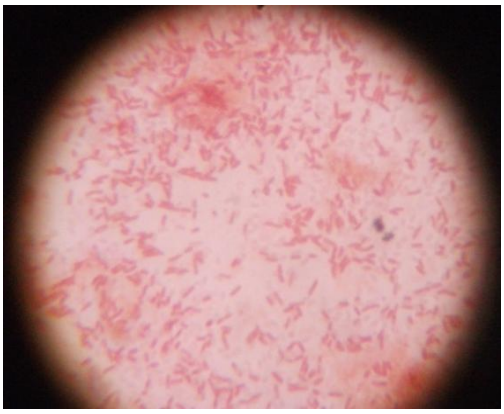
23. A. Culture plates of *Bacillus* spp.

23. B. Gram positive bacilli of *Bacillus* spp.

23. C. Biochemical test results *Bacillus* spp.

Plate 23. Identification methods of *B. cereus, B. subtilis, B. sphaericus, B.alcalophilus and B. brevis*.

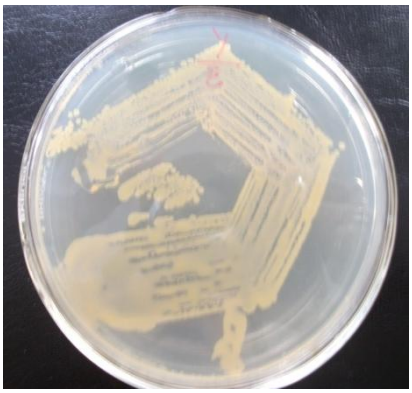
24. A. Culture plate of *Alcaligenes feacalis*



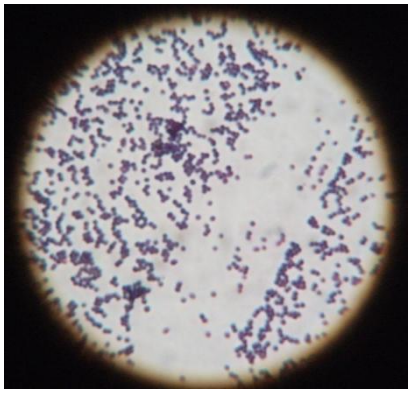
24. B. Gram negative bacilli of *Alcaligenes feacalis*

24. C. Biochemical test results *Alcaligenes feacalis*

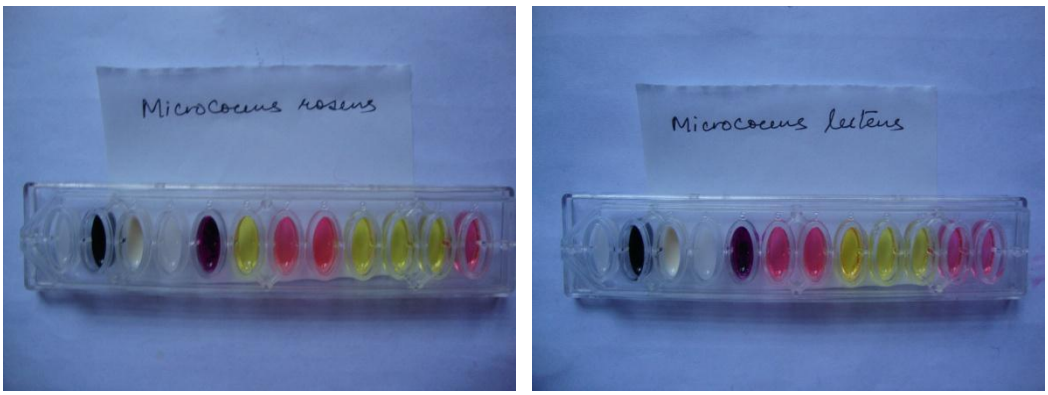
Plate 24. Identification methods of *Alcaligenes feacalis*



25. A. Culture plate of *Micrococcus* spp.



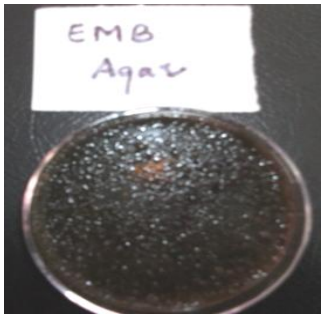
25. B. Gram positive coccus of *Micrococcus* spp.



25. C. Biochemical test results *Enterococcus* spp.

Plate 25. Identification methods of *M. luteus and M. roseus*

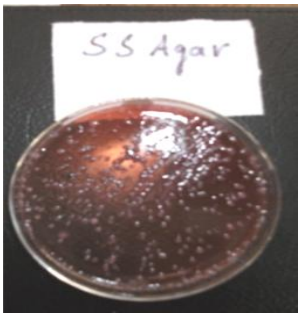
26. A. Pseudomonas agar



26. B. EMB agar

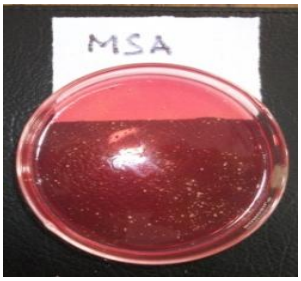
26. C. Cetrimide agar

26. D. Mac Conkey agar

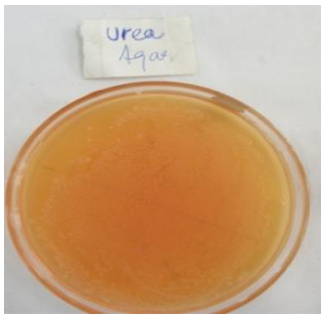


26. E. SS agar

26. F. Endo agar



26. G. MSA agar



26. H. Urea agar

Plate 26. Bacterial cultures on selective media

Table 3. Isolated bacterial species and their taxonomic classification

Isolate code	Kingdom	Phylum	Class	Order	Family	Genus	Species
SMBS-1	Monera (Prokaryotae)	Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Citrobacter*	*C. freundii*
SMBS-2		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Citrobacter*	*C. diversus*
SMBS-3		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Enterobacter*	*E. aerogenes*
SMBS-4		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Enterobacter*	*E. agglomerans*
SMBS-5		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Enterobacter*	*E. cloacae*
SMBS-6		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Enterobacter*	*E. intermedius*
SMBS-7		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Serratia*	*S. marcescens*
SMBS-8		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Serratia*	*S. rubidaea*
SMBS-9		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Shigella*	*S. flexneri*
SMBS10		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Shigella*	*S. dysenteriae*
SMB-11		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Salmonella*	*S. typhi*
SMB-12		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Salmonella*	*S. paratyphi*
SMB-13		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Salmonella*	*S. choleraesuis*
SMB-14		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Proteus*	*P. vulgaris*
SMB-15		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Proteus*	*P. mirabilis*
SMB-16		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Klebsiella*	*K. oxytoca*
SMB-17		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Klebsiella*	*K. ozaenae*
SMB-18		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Escherichia*	*E.coli*
SMB-19		Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	*S. aureus*
SMB-20		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Yersinia*	*Y. enterocolitica*
SMB-21		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Cedacea*	*C. davisae*
SMB-22		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Cedacea*	*C. lapagei*

Contd……

Table 3. contd.

Isolate Code	Kingdom	Phylum	Class	Order	Family	Genus	Species
SMB-23	Monera (Prokaryotae)	Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Edwardseilla*	*E. tarda*
SMB-24		Proteobacteria	Gamma Proteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	-
SMB-25		Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Kluyvera*	*K. ascorbata*
SMB-26		Firmicutes	Bacilli	Lactobacillales	Enterococaceae	*Enterococcus*	*E. faecalis*
SMB-27		Firmicutes	Bacilli	Lactobacillales	Enterococaceae	*Enterococcus*	*E. faecium*
SMB-28		Firmicutes	Bacilli	Lactobacillales	Enterococaceae	*Enterococcus*	*E. durans*
SMB-29		Firmicutes	Bacilli	Lactobacillales	Enterococaceae	*Enterococcus*	*E. hirae*
SMB-30		Firmicutes	Bacilli	Lactobacillales	Enterococaceae	*Enterococcus*	*E. mundtii*
SMB-31		Proteobacteria	Gamma Proteobacteria	Vibrionales	Vibrionaceae	*Vibrio*	*V. cholerae*
SMB-32		Proteobacteria	Gamma Proteobacteria	Vibrionales	Vibrionaceae	*Vibrio*	*V. parahaemolyticus*
SMB-33		Proteobacteria	Gamma Proteobacteria	Vibrionales	Vibrionaceae	*Vibrio*	*V. vulnificus*
SMB-34		Proteobacteria	Gamma Proteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	*P. putida*
SMB-35		Proteobacteria	Gamma Proteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	*P. alcaligenes*
SMB-36		Proteobacteria	Gamma Proteobacteria	Aeromonadales	Aeromonadaceae	*Aeromonas*	*A. hydrophila*
SMB-37		Proteobacteria	Gamma Proteobacteria	Aeromonadales	Aeromonadaceae	*Aeromonas*	*A. caviae*
SMB-38		Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*B. sphaericus*
SMB-39		Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*B. subtilis*
SMB-40		Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*B. cereus*
SMB-41		Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*B. alcalophilus*
SMB-42		Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*B. brevis*
SMB-43		Proteobacteria	Beta Proteobacteria	Burkholderales	Alcaligenaceae	*Alcaligenes*	*A. faecalis*
SMB-44		Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	*Micrococcus*	*M. luteus*
SMB-45		Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	*Micrococcus*	*M. roseus*

Table 4. Morphological characteristics of bacterial colonies

Isolate code	Total colonies	Macroscopic						Microscopic		Motility
		Shape	Size	Margin	Elevation	Colour	Texture	G. Reaction	S. forming	
SMB -1	145	Circular	Small	Entire	Convex	Purple with green sheen	Smooth	Negative / Bacillus	-	M
SMB-2	156	Circular	Medium	Entire	Convex	Transparent/Grey	Smooth/Moist	Negative / Bacillus	-	M
SMB-3	176	Circular	Small	Entire	Convex	Shiny/Opaque	Smooth	Negative/ Bacillus	-	M
SMB-4	167	Circular	Small	Entire	Convex	Yellow/Translucent	Smooth	Negative/ Bacillus	-	M
SMB-5	172	Irregular	Small	Undulate	Flat	Non pigmented	Moist	Negative/ Bacillus	-	M
SMB-6	46	Irregular	Small	Undulate	Flat	Pigmented	Smooth	Negative/ Bacillus	-	M
SMB-7	171	Irregular	Medium	Undulate	Raised	Red	Moist/Smooth	Negative/ Bacillus	-	M
SMB-8	29	Round	Small	Entire	Raised	Shiny/Opaque	Smooth	Negative/ Bacillus	-	M
SMB-9	217	Circular	Pinpoint	Entire	Raised	Shiny/Pigmented	Moist	Negative/ Bacillus	-	NM
SMB-10	242	Circular	Pinpoint	Entire	Raised	Shiny/Pigmented	Moist	Negative/ Bacillus	-	NM
SMB-11	201	Circular	Small	Entire	Raised	Shiny/White	Moist	Negative/ Bacillus	-	M
SMB-12	49	Circular	Small	Entire	Raised	Shiny/White	Moist	Negative/ Bacillus	-	M
SMB-13	50	Circular	Small	Entire	Raised	Shiny/White	Moist	Negative/ Bacillus	-	M
SMB-14	151	Circular	Pinpoint	Lobate	Flat	Shiny/Pigmented	Smooth	Negative/ Bacillus	-	M
SMB-15	151	Circular	Pinpoint	Undulate	Raised	Shiny/Pigmented	Moist	Negative/ Bacillus	-	M
SMB-16	113	Circular	Small	Undulate	Raised	Shiny/White	Moist	Negative/ Bacillus	-	NM
SMB-17	24	Round	Medium	Irregular	Convex	Glistening	Moist	Negative/ Bacillus	-	NM
SMB-18	364	Circular	Small	Entire	Convex	Black centred colony	Smooth	Negative/ Bacillus	-	M
SMB-19	157	Round	Small/Pinhead	Entire	Convex	Golden Brown	Smooth	Positive / Coccus	-	NM
SMB-20	198	Round	Small	Irregular	Undulate	Grey/White opaque	Rough	Negative/ bacillus	-	NM
SMB-21	39	Circular	Medium	Irregular	Convex	Non pigmented	Smooth	Negative/ bacillus	-	M
SMB-22	50	Circular	Medium	Irregular	Convex	Non pigmented	Smooth	Negative / Bacillus	-	M

Contd……..

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Table 4. contd.

Isolate code	Total colonies	Macroscopic						Microscopic		Motility
		Shape	Size	Margin	Elevation	Colour	Texture	G. Reaction	S. forming	
SMB-23	39	Circular	Medium	Entire	Convex	Back centred colony	Smooth	Negative / Bacillus	-	M
SMB-24	30	Circular	Medium	Irregular	Convex	Non pigmented	Mucoid	Negative/ Coccobacillus	-	M
SMB-25	46	Circular	Large	Irregular	Convex	Grey	Smooth	Negative / Bacillus	-	M
SMB-26	305	Circular	Pinpoint	Entire	Convex/Raised	Non pigmented	Shiny	Positive / Coccus	-	NM
SMB-27	85	Circular	Pinpoint	Entire	Convex/Raised	Non pigmented	Shiny	Positive / Coccus	-	NM
SMB-28	39	Circular	Pinpoint	Entire	Convex/Raised	Non pigmented	Shiny	Positive / Coccus	-	NM
SMB-29	40	Circular	Pinpoint	Entire	Convex/Raised	Non pigmented	Shiny	Positive / Coccus	-	NM
SMB-30	36	Circular	Pinpoint	Entire	Convex/Raised	Non pigmented	Shiny	Positive / Coccus	-	NM
SMB-31	167	Circular	Pinpoint	Entire	Convex/Raised	Non pigmented	Shiny	Negative / Bacillus	-	NM
SMB-32	149	Circular	Pinpoint	Entire	Convex/Raised	Non pigmented	Shiny	Negative / Bacillus	-	NM
SMB-33	133	Circular	Pinpoint	Entire	Convex/Raised	Non pigmented	Shiny	Negative / Bacillus	-	NM
SMB-34	144	Circular	Pinpoint	Entire	Convex/Raised	Non pigmented	Shiny	Negative / Bacillus	-	M
SMB-35	172	Circular	Pinpoint	Entire	Convex/Raised	Non pigmented	Shiny	Negative / Bacillus	-	M
SMB-36	155	Circular	Medium	Irregular	F/lat/Convex	Colourless	Smooth	Negative / Bacillus	-	M
SMB-37	37	Circular	Medium	Irregular	F/lat/Convex	Colourless	Smooth	Negative / Bacillus	-	M
SMB-38	43	Round	Medium	Irregular	Flat	White	Dry	Positive / Bacillus	+	M
SMB-39	172	Round	Medium	Irregular	Flat	Off White	Dry	Positive / Bacillus	+	M
SMB-40	177	Round	Large	Undulate	Flat	Opaque	Smooth	Positive / Bacillus	+	M
SMB-41	143	Round	Large	Undulate	Flat	Opaque	Smooth	Positive / Bacillus	+	M
SMB-42	163	Round	Large	Undulate	Flat	Opaque	Smooth	Positive / Bacillus	+	M
SMB-43	202	Round	Medium	Irregular	Flat	Translucent	Wrinkled/Moist	Negative / Bacillus	-	M
SMB-44	144	Circular	Pinhead	Entire	Convex	Beige to Yellow/Shiny	Smooth	Positive / Coccus	-	NM
SMB-45	261	Round	Small	Entire	Convex	Yellow	Smooth	Positive / Coccus	-	NM

(S. forming=spore forming; M=Motile; NM=Non-motile)

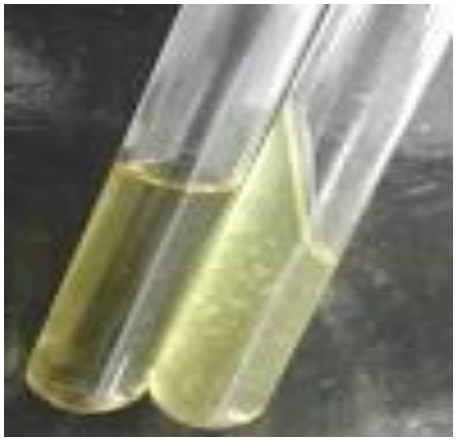
Chromogenesis observed were from transparent to coloured like *S. marcescens* showed red colonies, *S. aureus* showed golden yellow colonies etc. (Table 4) Moreover, bacteria were inoculated into semi solid nutrient agar and nutrient broth to study the motility and oxygen requirement of pure isolate respectively. Depending upon growth in semisolid agar they were motile (diffused cloud of growth away from the line of inoculation) non motile (confined to the line of inoculation). Depending upon growth they were grouped into aerobes, micro aerophile and facultative anaerobes. Haemolytic behaviour was studied on blood agar (10%) and on observation they were grouped into haemolytic (alpha, beta or gamma haemolysis) and non haemolytic bacteria (Plate 27-28). The bacterial isolates employing direct microscopic technique was observed in terms of Gram staining reaction for shapes, size and grouping pattern (arrangement) of bacteria, presence and absence of spores. These variations in the micro and macro morphological features in different bacteria helped us in preliminary identification of bacterial species.

4.1.2. Biochemical identification

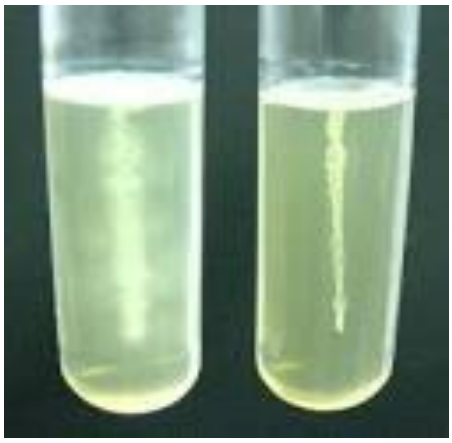
The biochemical and the physiological properties were studied in detail in order to detect enzyme activity and pH change by providing the substrate in the medium in order to identify and differentiate the isolated bacteria. The biochemical tests conducted for the identification of bacterial isolates included different tests for different bacterial species (Plate 29). The application of biochemical test strip was based on the earlier noticed results of microscopic examination like Gram's nature and shape in (Table 5) which revealed that out of 5941 bacterial isolates 4146 (69.78%) were Gram negative isolates and 1795 (30.21 %) were Gram positive isolates. Among 1795 Gram positive isolates, 698 (11.72 %) were Gram positive bacilli and 1097 (18.49%) were Gram positive cocci; whereas among 4146 Gram negative isolates, 4116 (69.33%) were bacilli and 30 (0.45%) were cocco- bacilli (Fig.1and 2). The biochemical tests conducted for gram negative bacilli species included indole, methyl red, voges proskauer, citrate utilization, lysine decarboxylation, ornithine decarboxylation, urease, tryptophan deaminase, nitrate reduction, H_2S production and fermentation tests like glucose, adonitol, lactose, mannitol, rhamnose, sucrose, arabinose and sorbitol (Table 6). The species identified by means of biochemical tests included *C. freundii, C. diversus, E. aerogenes, E. agglomerans, E. cloacae, E. intermedius, S. marcescens, S.*

27. A. Positive catalase test

27. B. Positive coagulase test



27 .C. Positive gelatin hydrolysis test



27. D. Motility test

Plate 27. Catalase, gelatin hydrolysis and motility tests

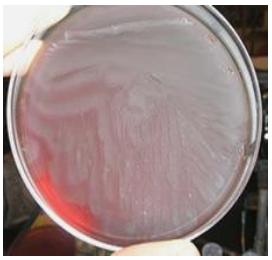
28. A. *Bacillus* spp.
(Beta haemolysis)

28. B. *E coli*
(Beta haemolysis)

28. C. *Enterococcus* spp.
(Gamma haemolysis)

28. D .*Klebsiella* spp.
(Gamma haemolysis)

28. E. *Micrococcus* spp.
(Non haemolytic)



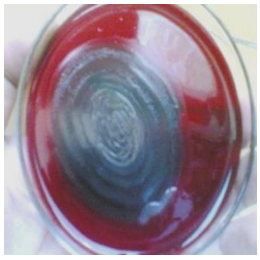
28. F. *Proteus* spp.
(Beta haemolysis)

28. G. *Pseudomonas* spp.
(Beta haemolysis)

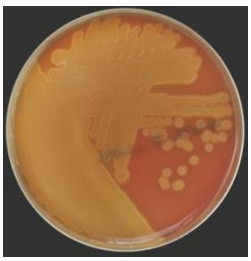
28. H. *Salmonella* spp.
(Non haemolytic)

28. I. *Serratia* spp.
(Beta haemolysis)

28. J. *S. aureus*
(Beta haemolysis)

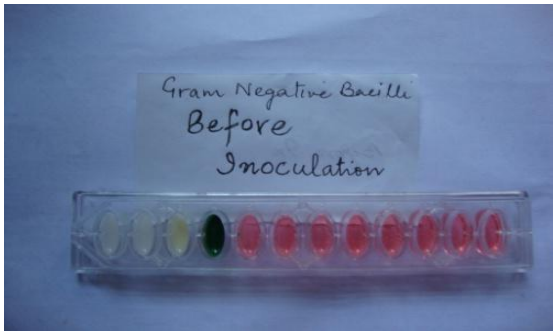


28. K. *Proteus* spp.
(Beta haemolysis)



28. L. *A. faecalis*
(Alpha haemolysis)

Plate 28. Haemolysis of bacterial species on blood agar



29. A. Kit for Gram negative bacilli

29. B. Kit for Gram negative bacilli

29. C. Kit for Gram negative bacilli

29. D. Kit for Gram negative bacilli

Plate 29. Uninoculated Hi-Media biochemical test kits for bacterial identification

Table 5. Percentage occurrence of isolated colonies as per Gram's reaction

Site	Gram positive						Gram negative						G. Total
	Bacilli	% O	Cocci	% O	Total	% O	Bacilli	% O	Cocci-bacilli	% O	Total	% O	
1	31	0.52	45	0.76	76	1.28	161	2.70	1	0.01	162	2.72	238
2	23	0.40	42	0.71	65	1.11	115	1.93	1	0.01	116	1.95	181
3	33	0.55	40	0.67	73	1.23	149	2.50	0	0	149	2.5	222
4	25	0.40	39	0.66	64	1.08	151	2.54	0	0	151	2.54	215
5	27	0.45	44	0.74	71	1.20	144	2.42	0	0	144	2.42	215
6	14	0.23	22	0.37	36	0.60	102	1.71	0	0	102	1.71	138
7	46	0.77	56	0.94	102	1.71	277	4.70	0	0	277	4.66	379
8	16	0.27	34	0.57	50	0.84	111	1.90	0	0	111	1.86	161
9	51	0.86	74	1.25	125	2.10	264	4.44	4	0.06	268	4.51	393
10	76	1.28	101	1.70	177	2.98	410	6.90	1	0.01	411	6.91	588
11	103	1.73	146	2.46	249	4.20	559	9.41	5	0.08	564	9.49	813
12	59	1.00	78	1.32	137	2.30	346	5.82	0	0	346	5.82	483
13	11	0.19	28	0.47	39	0.65	105	1.80	1	0.01	106	1.78	145
14	16	0.26	17	0.30	33	0.55	51	0.85	0	0	51	0.85	84
15	73	1.23	132	2.22	205	3.45	508	8.55	7	0.11	515	8.66	720
16	94	1.58	199	3.35	293	4.93	663	11.16	10	0.16	673	11.32	966
Total	698	11.72	1097	18.49	1795	30.21	4116	69.33	30	0.45	4146	69.78	5941

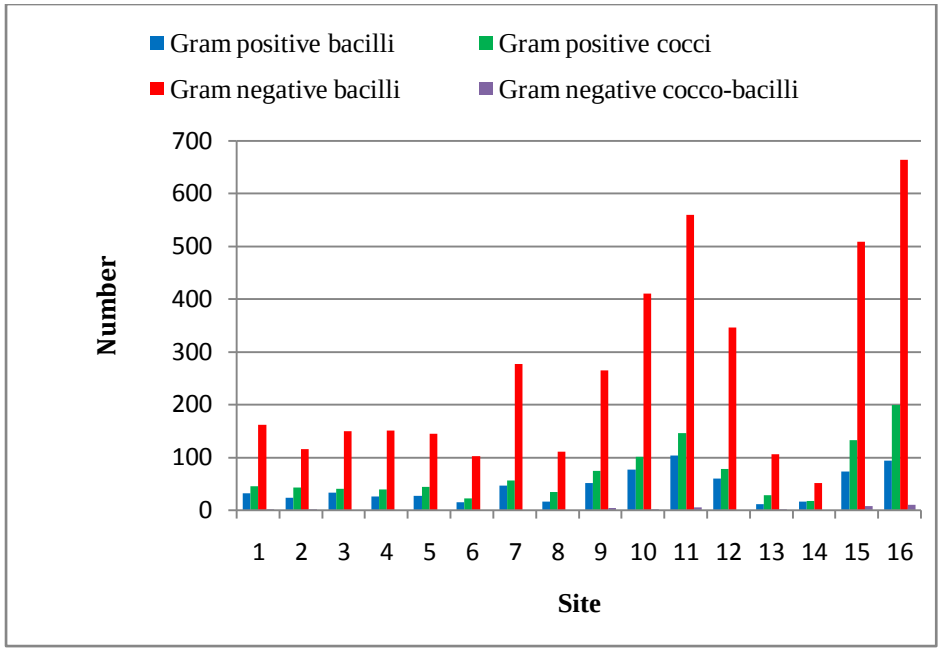


Fig. 1. Occurrence of isolated colonies as per Gram's reaction

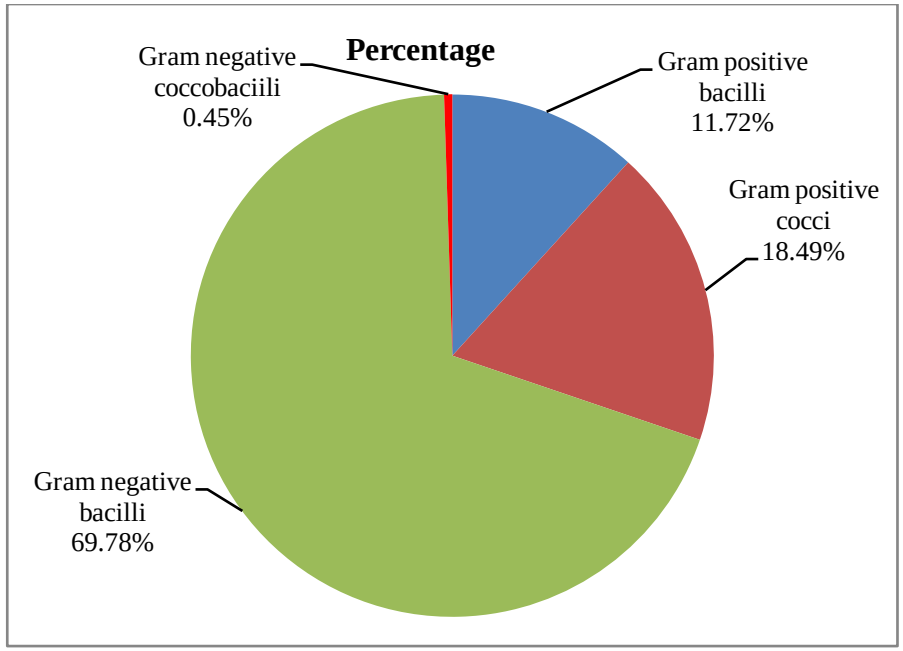


Fig.2. Percentage occurrence of Gram positive and negative bacteria

rubideae, S. flexneri, S. dysenteriae, S. typhi, S. paratyphi, S. choleraesuis, P. vulgaris, P. mirabilis, K. oxytoca, K. ozaenae, E.coli, Y. enterocolitica, C. davisae, C. lapagei, E. tarda, K. ascorbata, V. cholerae, V. parahaemolyticus, V. vulnificus, P. putida, P. alcaligenes, A. hyrdophila, A. caviae and *A. faecalis.* For gram negative cocco-bacilli, *Acinetobacter* spp., tests conducted were Catalase, oxidase, glucose, xylose, mannitol, sucrose, galactose, lactose, Esculin, hydrogen sulphide, citrate, methyl red and voges Proskauer (Table 7).Similarly the tests conducted for gram positive bacilli included maloanate, voges proskauer, citrate, beta-galactosidase, nitrate, catalase, arginine, sucrose, mannitol, glucose, arabinose and trehalose. The species identified by means of biochemical tests included *B. sphaericus, B. subtilis, B. cereus, B. alcalophillus, B. brevis* (Table 8). For gram positive cocci, tests conducted were voges proskauer, esculin hydrolysis, PYR (pyrrolidonyl aryl-amidase), beta-galactosidase test (ortho-nitrophenyl-b-d-galactopyranoside), arginine dihydrolase, glucose, lactose, arabinose, sucrose, sorbitol, mannitol and raffinose. The species identified by means of biochemical tests included *S. aureus, E. faecalis, E. faecium, E. durans, E. hirae, E. mundtii, M. luteus* and *M. roseus* (Table 9).

Table 6. Biochemical identification of isolated Gram negative rods

Isolate code	Indole	Methyl red	Voges Proskauer	Citrate	Glucose	Adonitol	Arabinose	Lactose	Sorbitol	Mannitol	Rhamnose	Sucrose	Lysine	Ornithine	Urease	TDA	Nitrate reduction	H_2S	Species identified
SMB-1	-	+	-	+	+	-	+	V	+	+	+	V	-	V	V	-	+	V	*C. freundii*
SMB-2	+	+	-	+	+	+	+	V	+	+	+	V	-	+	V	-	+	-	*C. diversus*
SMB-3	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	*E. aerogenes*
SMB-4	-	V	+	+	+	-	+	V	-	+	+	+	-	V	-	+	+	-	*E. agglomerans*
SMB-5	-	-	+	+	+	-	+	+	+	+	+	+	-	+	V	-	+	-	*E. cloacae*
SMB-6	+	+	+	+	+	-	+	+	+	+	+	+	-	+	-	-	+	-	*E. intermedius*
SMB-7	-	V	+	+	+	V	-	-	+	+	-	+	+	+	V	-	+	-	*S. marcescens*
SMB-8	-	V	+	+	+	+	+	+	-	+	-	+	V	-	-	-	+	-	*S. rubidaea*
SMB-9	V	+	-	-	+	-	V	-	V	+	-	-	-	-	-	-	+	-	*S. flexneri*
SMB-10	V	+	-	-	+	-	V	-	V	+	-	-	-	-	-	-	+	-	*S. dysenteriae*
SMB-11	-	+	-	-	+	-	-	-	+	+	-	-	+	-	-	-	+	+	*S. typhi*
SMB-12	-	+	-	-	+	-	-	-	+	+	-	-	+	-	-	-	+	+	*S. paratyphi*
SMB-13	-	+	-	+	+	-	+	-	+	+	+	-	+	+	-	-	+	+	*S. choleraesuis*

Contd……..

Table 6 contd.

Isolate code	Indole	Methyl red	Voges Proskauer	Citrate	Glucose	Adonitol	Arabinose	Lactose	Sorbitol	Mannitol	Rhamnose	Sucrose	Lysine	Ornithine	Urease	TDA	Nitrate reduction	H_2S	Species identified
SMB-14	+	+	-	V	+	-	-	-	-	-	-	+	-	-	+	+	+	+	*P. vulgaris*
SMB-15	-	+	V	V	+	-	-	-	-	-	-	V	-	+	+	+	+	+	*P. mirabilis*
SMB-16	-	+	-	V	+	+	+	V	V	+	V	V	V	-	-	-	V	-	*K. ozaenae*
SMB-17	+	V	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	*K. oxytoca*
SMB-18	+	+	-	-	+	-	+	+	+	+	V	V	+	V	-	-	+	-	*E. Coli*
SMB-20	V	+	-	-	+	-	+	-	+	+	-	+	-	+	V	-	+	-	*Y. enterocolitica*
SMB-21	-	+	V	+	+	-	-	V	-	+	-	+	-	+	-	-	+	-	*C. davisae*
SMB-22	-	V	V	+	+	-	-	V	-	+	-	-	-	-	-	-	+	-	*C. lapagei*
SMB-23	+	ND	-	-	+	-	-	-	-	-	-	-	+	+	-	ND	+	+	*E. tarda*
SMB-25	+	+	-	+	+	-	+	+	V	+	+	+	+	+	-	-	+	-	*K. ascorbata*
SMB-31	ND	ND	ND	-	-	+	-	-	-	ND	ND	ND	+	+	+	-	-	+	*V. cholerae*
SMB-32	ND	ND	ND	-	+	-	V	-	-	ND	ND	ND	+	+	V	-	+	-	*V. parahaemolyticus*
SMB-33	ND	ND	ND	V	+	-	-	V	-	V	-	-	+	V	-	V	+	-	*V. vulnificus*
SMB-34	ND	ND	ND	+	+	ND	V	V	ND	ND	ND	ND	-	-	V	-	+	-	*P. putida*
SMB-35	ND	ND	ND	V	-	ND	ND	-	ND	ND	ND	ND	-	-	-	-	V	-	*P alcaligenes*
SMB-36	ND	ND	ND	V	+	-	+	V	-	ND	ND	ND	V	-	-	-	+	+	*A .hydrophila*
SMB-37	ND	ND	ND	V	+	-	+	V	-	ND	ND	ND	-	-	-	-	+	-	*A. caviae*
SMB-43	ND	ND	ND	+	-	-	-	-	-	-	-	-	V	+	-	+	+	-	*A. faecalis*

Table 7. Biochemical identification of isolated Gram negative coccobacilli

Isolate code	Catalase	Oxidase	Glucose	Xylose	Mannitol	Sucrose	Galactose	Mannose	Rhamnose	Lactose	Esculin	H2S	Citrate	MR	VP	Species identified
SMB-24	+	-	+	+	-	-	ND	+	-	+	-	-	-	+	-	*Acinetobacter* spp.

Table 8. Biochemical identification of isolated Gram positive rods

Isolate code	Malonate	VP	Citrate	ONPG	Nitrate reduction	Catalase	Arginine	Sucrose	Mannitol	Glucose	Arabinose	Trehalose	Species identified
SMB-38	-	-	V	-	-	+	-	-	-	-	-	-	*B. sphaericus*
SMB-39	-	+	+	V	+	+	-	-	-	+	+	-	*B. subtilis*
SMB-40	-	+	+	-	+	+	-	-	-	+	-	+	*B. cereus*
SMB-41	-	-	-	-	-	+	-	+	+	+	+	-	*B. alcalophillus*
SMB-42	-	V	V	-	V	+	-	-	V	V	-	-	*B. brevis*

Table 9. Biochemical identification of isolated Gram negative cocci

Isolate code	VP	Esculin hydrolysis	PYR	ONPG	Arginine dihydrolase	Glucose	Lactose	Arabinose	Sucrose	Sorbitol	Mannitol	Raffinose	Species identified
SMB-26	+	+	+	+	+	+	+	-	+	+	+	-	*E. faecalis*
SMB-27	+	+	-	+	+	+	+	+	+	V	+	V	*E. faecium*
SMB-28	+	+	+	-	+	+	+	-	-	-	-	-	*E. durans*
SMB-29	+	+	+	+	+	+	+	-	+	-	-	+	*E. hirae*
SMB-30	+	+	+	+	+	+	+	+	+	V	+	+	*E. mundtii*
SMB-31	+	-	ND	-	+	+	+	-	+	+	+	-	*S. aureus*
SMB-44	-	+	ND	-	+	-	-	+	+	+	-	ND	*M. luteus*
SMB-45	-	+	ND	-	+	+	-	-	+	+	+	-	*M. roseus*

(+ = Positive (>90%), - = negative (>90%), V = (11 – 89 % positive), ND =No data available]
{TDA=Tryptophan-deaminase; H_2S=Hydrogen sulphide; VP=Voges Proskauer; PYR= Pyrrolidonyl-aryl-amidase; ONPG=Ortho-nitrophenyl-b-D-galactopyranoside (Beta-galactosidase); MR=Methyl red.)

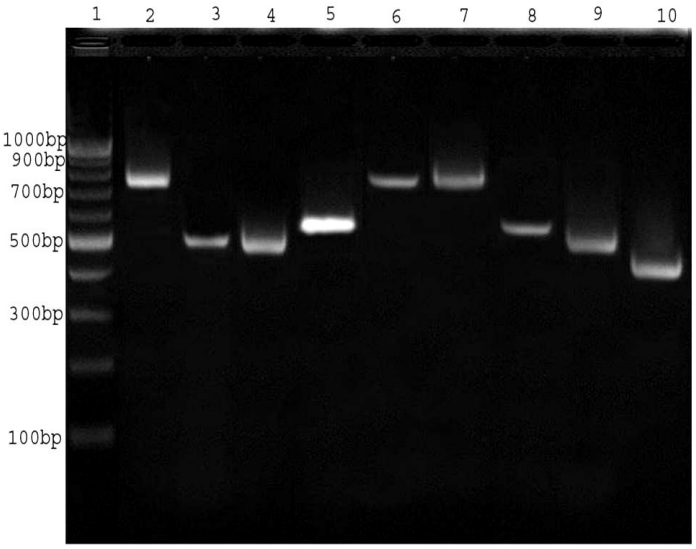
4.1.3. Molecular identification

The isolated bacterium species was keyed out according to Bergey's manual specifications by examining their macroscopic (colonial), microscopic and biochemical characteristics on nutrient agar and different selective culture media like Eosin methylene blue agar, Simmon citrate agar and Cetrimide agar etc. Finally

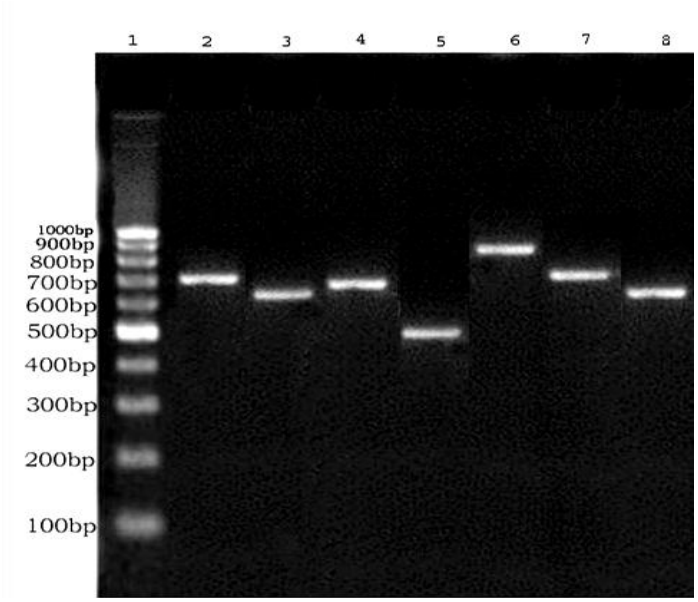
genome analysis on the basis of 16S rRNA using polymerase chain reaction (PCR) was carried out by universal bacterial primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and1492R (5'-GGTTACCTTGTTACGACTT-3') which provide an accurate means to determine the phylogenetic affinity of any microbial isolate to record environmental biodiversity. The observations were compared with the available data in specified data banks to find out the genus, species and strain status.

DNA from all isolates was successfully extracted and amplified using universal primer pair 8F and 1492R. The PCR products obtained were sequenced and then identified by using the BLAST search program. All the sequences were found to be similar to sequences of primer regions of the respective bacteria. The banding pattern produced by each species on a 2% agarose gel can be seen in Plate 30-32). Agarose gels were successful in providing enough band separation to identify the presence of largely different sized segments specific to each bacterium. On the basis of BLAST search, the species identified (Table 10) were *C. freundii* with gene bank accession number (KC109319), with a fragment size of 771 bp and percent identity of 99; *C. diversus* (Acc. No.AG549509) with a fragment size of 513 bp and percent identity of 100; *E. aerogenes (*Acc. No. AF227845) with a fragment size of 1418 bp and percent identity of 97; *E. agglomerans* (Acc. No.AY914094) with a fragment size of 723 bp and percent identity of 98; *E. cloacae* (Acc. No.KC109315) with a fragment size of 486 bp and percent identity of 99; *E. intermedius* (Acc. No.AF310217) with a fragment size of 1453 bp and percent identity of 98; *S. marcescens* (Acc. No. JX 315621) with a fragment size of 837 bp and percent identity of 100; *S. rubidaea* (Acc. No.JN089365) with a fragment size of 666 bp and percent identity of 100; *S. flexneri* (Acc. No. JX436483) with a fragment size of 847 bp and percent identity of 100; *S. dysenteriae* (Acc. No. JX827386) with a fragment size of 606 bp and percent identity of 98; *S. typhi* (Acc. No.JX407094) with a fragment size of 1289 bp and percent identity of 97; *S. paratyphi* (Acc. No. JQ410232) with a fragment size of 590 bp and percent identity of 97; *S. choleraesuis* (Acc. No.DQ344535) with a fragment size of 416 bp and percent identity of 98; *P. vulgaris* (Acc. No.NR025336) with a fragment size of 1495 bp and percent identity of 99; *P. mirabilis* (Acc. No .DQ364576) with a fragment size of 434 bp and percent identity of 98; *K. oxytoca* (Acc. No. AY914090) with a fragment size of 711 bp and

percent identity of 100; *K. ozaenae* (Acc. No.AF130982) with a fragment size of 1436 bp and percent identity of 98; *E.coli* (Acc. No.JQ965012) with a fragment size of 516 bp and percent identity of 100; *S. aureus* (Acc. No.FM992870) with a fragment size of 491 bp and percent identity of 97; *Y. enterocolitica* (Acc. No.FN561632) with a fragment size of 985 bp and percent identity of 98, *C. davisae* (Acc. No. JQ396389) with a fragment size of 1476 bp and percent identity of 100; C. *lapagei* (Acc. No.AB273742) with a fragment size of 1400 bp and percent identity of 100; E. *tarda* (Acc. No. JX393017) with a fragment size of 1449 bp and percent identity of 96; *Acinetobacter* spp. (Acc. No.GU826603) with a fragment size of 771 bp and percent identity of 69; *K. ascorbata* (Acc. No.JX267094) with a fragment size of 674 bp and percent identity of 97; *E. faecalis* (Acc. No NR040789) with a fragment size of 1517 bp and percent identity of 100; *E. faecium* (Acc. No.JF302668) with a fragment size of 641 bp and percent identity of 98; *E. duran* (Acc. No.HQ615677) with a fragment size of 659 bp and percent identity of 98; *E. hirae* (Acc. No.AJ554205) with a fragment size of 1007 bp and percent identity of 98; *E. mundtii* (Acc. No.JN995580) with a fragment size of 1473 bp and percent identity of 98; *V. cholerae* (Acc. No.AJ554204) with a fragment size of 762 bp and percent identity of 99; *V. parahaemolyticus* (Acc. No.HM022726) with a fragment size of 1285 bp and percent identity of 96; *V. vulnificus* (Acc. No.KC291508) with a fragment size of 1420 bp and percent identity of 96; *P. putida* (Acc. No.KC128883) with a fragment size of 844 bp and percent identity of 98; *P. alcaligenes* (Acc. No.NR043419) with a fragment size of 1530 bp and percent identity of 99; *A. hyrdophila* (Acc. No.JX416386) with a fragment size of 954 bp and percent identity of 100; *A. caviae* (Acc. No.JN703730) with a fragment size of 499 bp and percent identity of 98; *B. sphaericus* (Acc. No.NR043334) with a fragment size of 1478 bp and percent identity of 97; *B. subtilis* (Acc. No.HE605037) with a fragment size of 1460 bp and percent identity of 100; *B. cereus* (Acc. No.AY753909) with a fragment size of 801 bp and percent identity of 100; *B. alcalophillus* (Acc. No.NR036889) with a fragment size of 1505 bp and percent identity of 98; *B. brevis* (Acc. No.JX286686) with a fragment size of 1106 bp and percent identity of 99; *A. faecalis* (Acc. No.JF297973) with a fragment size of

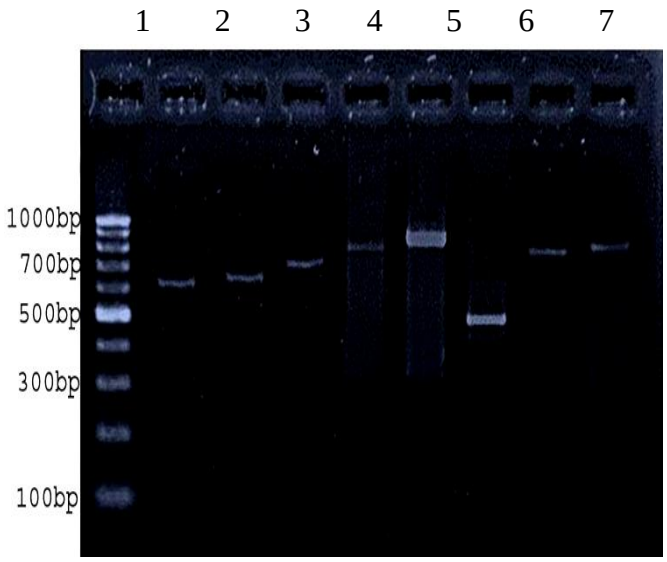


30. A. *Well no. 1= 100bp ladder; 2= C. freundii; 3= C. diversus; 4= E. cloacae; 5= S. dysenteriae; 6= S. flexneri; 7= S. marcescens; 8= S. paratyphi; 9= E.coli; 10= P. Mirabilis*

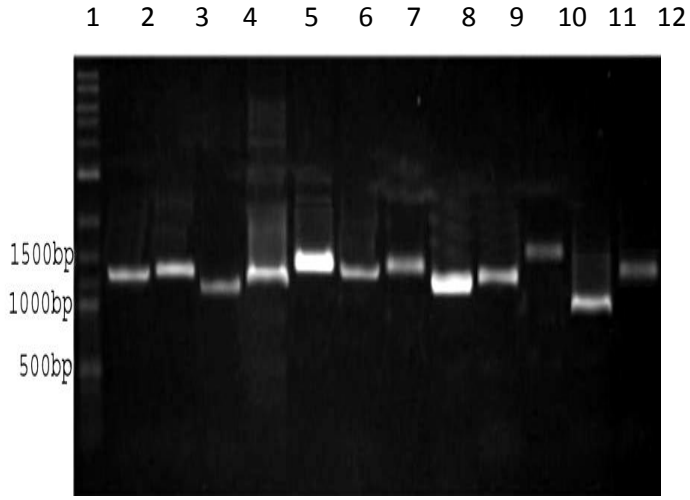


30. B. *Well no. 1= 100bp ladder; 2= E. agglomerans; 3= S. rubideae; 4= K. oxytoca; 5= S. aureus; 6= Y. enterocolitica; 7= Acinetobacter* spp*; 8= K. ascorbata*

Plate 30. Banding pattern of bacterial species on 2% agarose gel using 8F and 1492R universal bacterial primers

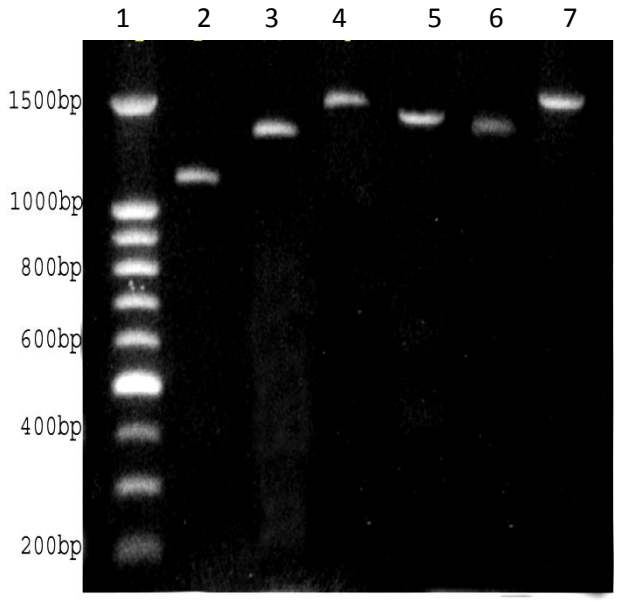


31. A. *Well no. 1= 100bp ladder; 2= E. faecium; 3= E. durans; 4= V. cholerae; 5= P. putida; 6= A. hydrophila; 7= A. caviae; 8= B. cereus; 9=M. Luteus*

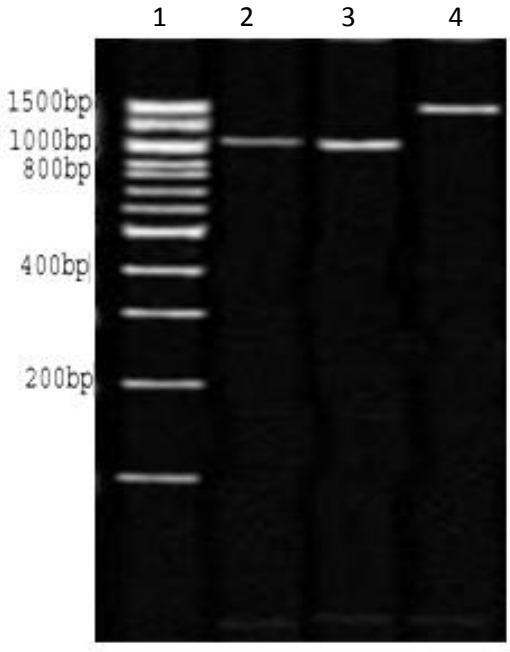


31. B. *Well no. 1= 100bp ladder; 2= E. aerogenes; 3= E. intermedius; 4= S. typhi; 5= S. choleraesuis; 6= P. vulgaris; 7= K. ozaenae; 8= C. davisae; 9=C. lapagei; 10= E. tarda;11=E. faecalis; 12=E. hirae; 13=E. mundtii*

Plate 31. Banding pattern of bacterial species on 2% agarose gel using 8F and 1492R universal bacterial primers



32. A. *Well no. 1= 100bp ladder; 2= V. parahaemolyticus; 3= V. vulnificus; 4= P.alcaligenes; 5= B. sphaericus; 6= B. subtilis; 7= B. alcalophilus*



32. B.. Well no. 1= 100bp ladder; 2=*B. brevis*; 3=*A. faecalis*; 4=*M. roseus*

Plate 32. Banding pattern of bacterial species on 2% agarose gel using 8F and 1492R universal bacterial primers

1078 bp and percent identity of 98; *M. luteus* (Acc. No.HE648128) with a fragment size of 885 bp and percent identity of 98; *M. roseus* (Acc. No.X877561) with a fragment size of 1481 bp and percent identity of 100.

Table 10. Result of BLAST search for identification of isolated bacterial species

Isolate code	Identified bacteria	% Similarity	Gen. Bank Acc. No	Fragment size
SMB -1	*C. freundii*	99%	KC 109319	771 bp
SMB-2	*C. diversus*	100%	AJ549509	513 bp
SMB-3	*E. aerogenes*	97%	AF227845	1,418 bp
SMB-4	*E.agglomerans*	98%	AY914094	723 bp
SMB-5	*E. cloacae*	99%	KC109315	486 bp
SMB-6	*E. intermedius*	98%	AF 310217	1453 bp
SMB-7	*S. marcescens*	100%	JX 315621	837 bp
SMB-8	*S. rubidea*	100%	JN 089365	666 bp
SMB-9	*S. flexneri*	100	JX 436483	847 bp
SMB-10	*S. dysenteriae*	98%	JX827386	606 bp
SMB-11	*S. typhi*	97%	JX 407094	1289 bp
SMB-12	*S. paratyphi*	97%	JQ 410232	590 bp
SMB-13	*S. choleraesuis*	98%	DQ 344535	1416 bp
SMB-14	*P. vulgaris*	99%	NR 025336	1495 bp
SMB-15	*P. mirabilis*	98%	DQ 364576	434 bp
SMB-16	*K. oxytoca*	100%	AY914090	711 bp
SMB-17	*K. ozaenae*	98%	AF 130982	1436 bp
SMB-18	*E.coli*	100%	JQ 965012	516 bp
SMB-19	*S. aureus*	97%	FM992870	491 bp
SMB- 20	*Y. enterocolitica*	98%	FN561632	985 bp
SMB-21	*C. davisae*	100%	JQ 396389	1476 bp
SMB-22	*C. lapagei*	100%	AB 273742	1400 bp
SMB-23	*E. tarda*	96%	JX 393017	1449 bp
SMB-24	*Acinetobacter* spp.	69%	GU 826603	771 bp
SMB-25	*K. ascorbata*	97%	JX 267094	674 bp
SMB-26	*E. faecalis*	100%	NR040789	1517 bp
SMB-27	*E. faecium*	98%	JF302668	641 bp
SMB-28	*E. durans*	98%	HQ 615677	659bp
SMB-29	*E. hirae*	98%	AJ554205	1007bp
SMB-30	*E. mundtii*	98%	JN995580	1474 bp
SMB-31	*V. cholerae*	99%	AJ554204	762bp
SMB-32	*V. parahaemolyticus*	96%	HM 022726	1285 bp
SMB-33	*V. vulnificus*	96%	KC 291508	1420 bp
SMB-34	*P. putida*	98%	KC 128883	844 bp
SMB-35	*P. alcaligenes*	99%	NR043419	1530 bp
SMB-36	*A. hydrophila*	100%	JX416386	954 bp
SMB-37	*A. caviae*	98 %	JN 703730	499 bp

Contd……

Table 10 contd.

Isolate code	Identified bacteria	% Similarity	Gen. Bank Acc. No	Fragment size
SMB-38	*B. sphaericus*	97%	NR043334	1478 bp
SMB-39	*B. subtilis*	100%	HE 605037	1460 bp
SMB-40	*B. cereus*	100%	AY753909	801 bp
.**SMB-41**	*B. alcalophilus*	98%	NR036889	1505 bp
SMB-42	*B. brevis*	99%	JX286686	1106 bp
SMB-43	*A. faecalis*	98%	JF297973	1078 bp
SMB-44	*M. lutes*	98%	HE648128	885 bp
SMB-45	*M. roseus*	100%	X 877561	1481 bp

4.2. General distribution

Table 11 and Fig. 3 depicts overall systematic diversity of the isolated taxa from 16 sites of Dal lake. Forty five (45) species of bacteria isolated and identified during the period of study belong to major kingdom Monera with three major phyla namely Proteobacteria, Firmicutes and Actinobacteria. The Proteobacteria contributing thirty two (32) species, Firmicutes eleven (11) species and Actinobacteria two (2) species. Phylum Proteobacteria consists of class Beta proteobacteria and Gamma proteobacteria. Phylum Firmicutes and Actinobacteria consist of class Bacilli. All the taxonomic classes contribute with one (1) order Viz: Bacilli; Beta proteobacteria except Gamma proteobacteria contributes with four (4) orders, viz: Enterobacteriales, Pseudomonadales, Vibrionales and Aeromonadales. Phylum Firmicutes contributes with two (2) orders viz: Bacillales and Lactobacillales. Order Enterobacteriales consists of twelve (12) genera viz: *Citrobacter* contributing two (2) species, *Enterobacter* of four (4) species, *Serratia* of two (2) species, *Shigella* of two (2) species, *Salmonella* of three (3) species, *Proteus* two (2) species, *Klebsiella* two (2) species, *E.coli* one (1) species, *Yersinia* one (1) species, *Cedacea* two (2) species, *Edwardseilla* one (1) species, *Kluyvera* one (1) species; order Pseudomonadales contributing three (3) species; order Vibrionales contributing three (3) species; order Aeromonadales contributing two (2) species; order Burkholderales contributing one (1) species. Bacillales contributing six (6) species; order Lactobacillales contributing five (5) species and order Actinomycetales two (2) species.

Table 11. Systematic diversity of isolated taxa of bacteria

Kingdom	Phylum	Class	Number of taxa		
			Order	Genera	Species
Monera (Procaryotae)	Proteobacteria	Gamma Proteobacteria	Enterobacteriales	12	23
			Pseudomonadales	2	3
			Vibrionales	1	3
			Aeromonadales	1	2
		Beta Proteobacteria	Burkholderales	1	1
	Firmicutes	Bacilli	Bacillales	1	6
			Lactobacillales	1	5
	Actinobacteria	-	Actinomycetales	1	2

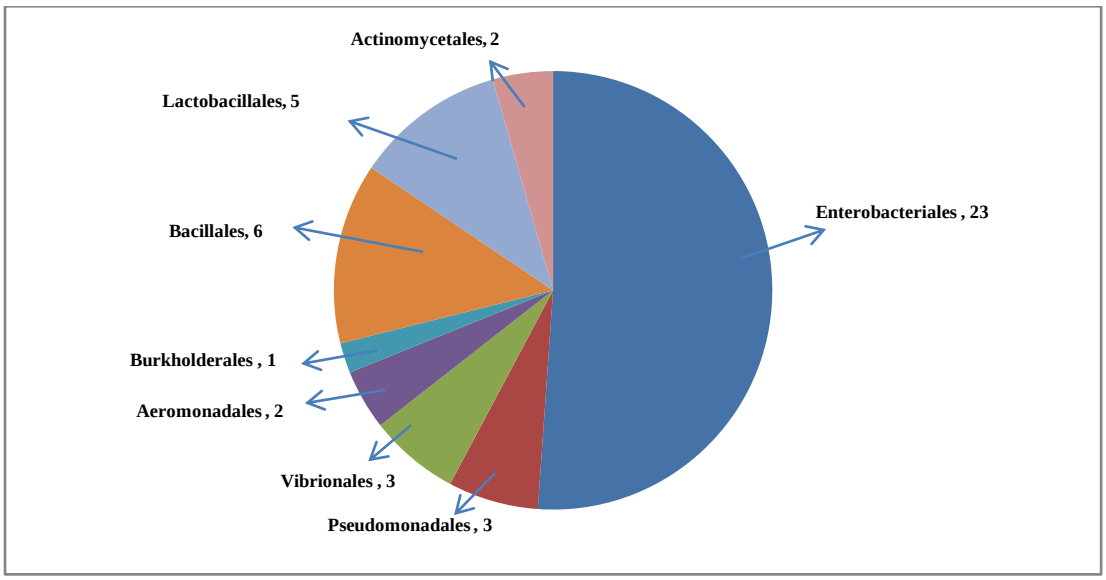


Fig.3. Graphical representation of systemic diversity of isolated taxa

In all, the genus *Enterococcus* and *Bacillus* contributes five species with a relative abundance of 11.11% respectively followed by genus *Enterobacter* with four species (8.88%), genus *Salmonella* and *Vibrio* with three species (6.66%) respectively, genus *Serratia, Shigella, Proteus, Klebsiella, Cedacea, Pseudomonas* and *Aeromonas*, genus *Micrococcus* with two species (4.44%) respectively, genus *E.coli, Staphylococcus, Yersinia, Edwardseilla, Acinetobacter, Kluyvera and Alcaligenes*, one species (2.22%) respectively (Table 12 and Fig. 4).

Table 12. Relative abundance distribution of each genus

Genera	Number of species	Relative abundance %
Citrobacter	2	4.44
Enterobacter	4	8.88
Serratia	2	4.44
Shigella	2	4.44
Salmonella	3	6.66
Proteus	2	4.44
Klebsiella	2	4.44
Escherichia	1	2.22
Staphylococcus	1	2.22
Yersinia	1	2.22
Cedacea	2	4.44
Edwardseilla	1	2.22
Acinetobacter	1	2.22
Kluyvera	1	2.22
Enterococcus	5	11.11
Vibrio	3	6.66
Pseudomonas	2	4.44
Aeromonas	2	4.44
Bacillus	5	11.11
Alcaligenes	1	2.22
Micrococcus	2	4.44

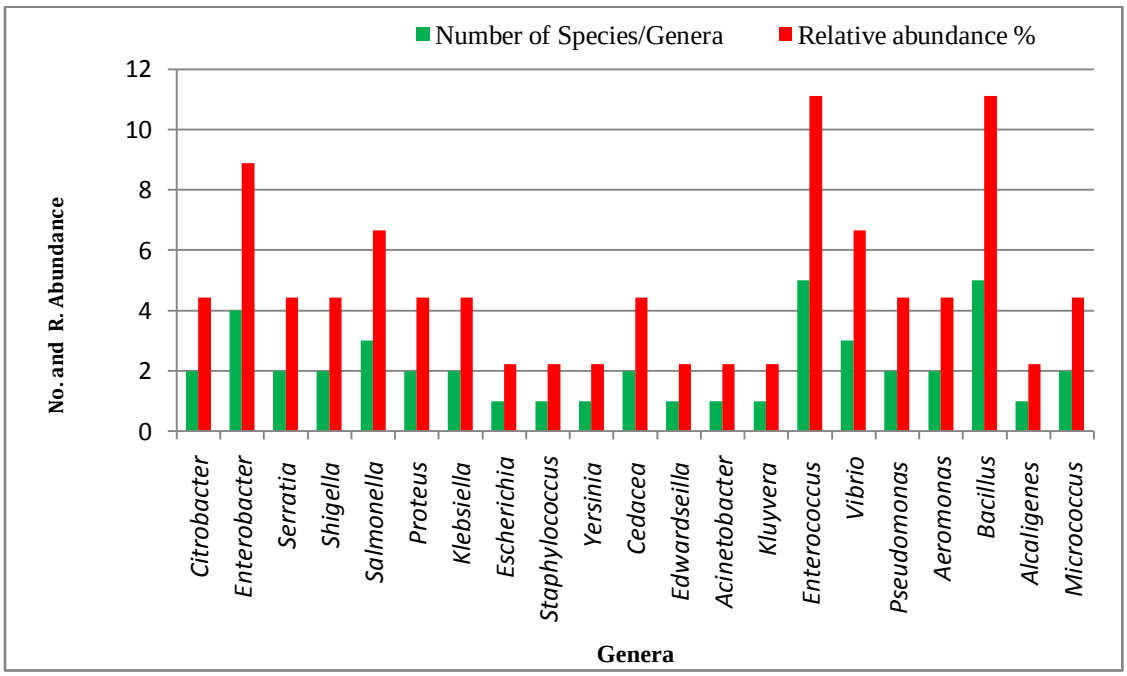


Fig. 4. Percentage relative abundance distribution of each genus

The bacterial species isolated belong to eleven families shown in (Table 13 and Fig. 5) with maximum contribution of twenty three (23) species by family Enterobacteriaceae followed by five species each of family Bacillaceae and Enterococaceae, three (3) species of family Vibrionaceae, two (2) species each of family Pseudomonadaceae, Micrococcaceae and Aeromonadaceae and one (1) species each of family Staphylococaceae, Moraxellaceae and Alcaligenaceae.

Table 13. Family wise distribution of species

Name of family	Number of species
Enterobacteriaceae	23
Staphylocoaceae	1
Moraxellaceae	1
Enterococaceae	5
Vibrionaceae	3
Pseudomonadaceae	2
Aeromonadaceae	2
Bacillaceae	5
Alcaligenaceae	1
Micrococcaceae	2
Total	**45**

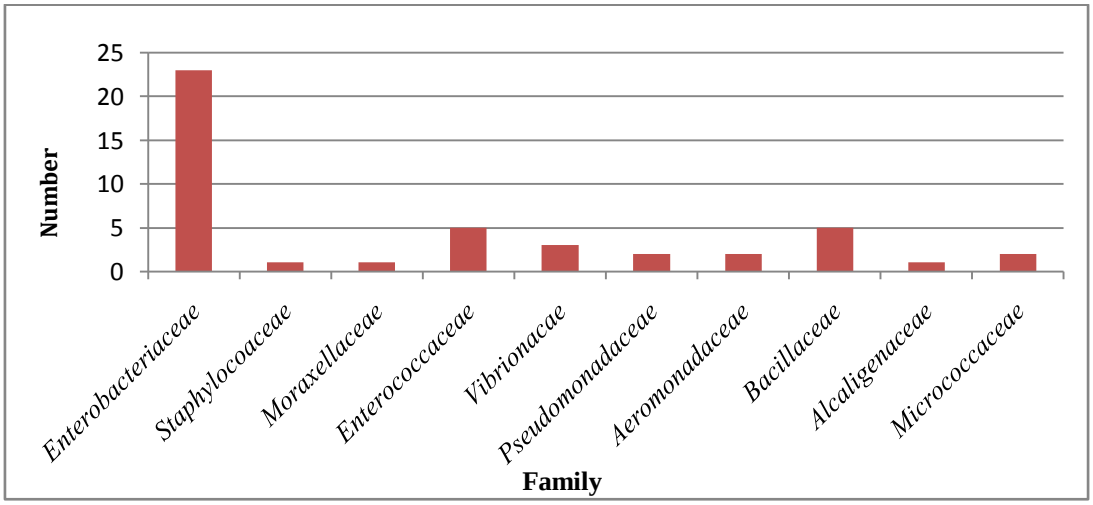


Fig.5. Family wise distribution of species

4.3. Colonial distribution

The bacterial colonies were isolated by serial dilution technique using normal saline solution (0.9% NaCl) on nutrient agar and were incubated at 37°C for 24 hours. The developed colonies were enumerated on plates by Quebec colony counter and the bacterial load was assessed in terms of colony forming units (CFU/ml). Bacterial count of the water samples of lake water revealed substantial number of heterotrophic bacteria as shown in (Table 14). The overall bacterial load in summer season was found to be higher than in winter season thereby showing an increase in bacterial count in the successive years of study. Moreover data revealed that the bacterial colonies were comparatively higher in all seasons of the second year of study as compared to the first year of study. Total bacterial count on nutrient agar for 16 sites ranged between 0.2 x 10^4 CFU/ml to 28.7x 10^4 CFU/ml and the highest count was found at site 16 (Pokhribal outlet) in summer season and lowest count was found at site 6 (Boathall nallah inlet) in winter season. Season wise, the bacterial load ranged between 0.3x10^4 CFU/ml at site 5 to 6.0 x10^4 CFU/ml at site 12 in spring 2010, 0.8 x 10^4 CFU/ml at site 4, 5 and 13 to 9.4x10^4 CFU/ml at site 15 in spring 2011, 2.0x10^4 CFU/ml at site 6 and 11 to 14.9x10^4 CFU/ml at site 16 in summer 2010, 2.4x10^4 CFU/ml at site 14 to 28.7x10^4 CFU/ml at site 16 in summer 2011, 0.4x10^4 CFU/ml at site 14 to 13.6x10^4 CFU/ml at site 16 in autumn 2010, 1.5x10^4 CFU/ml at site 6 to 21.0x 10^4 at site 16 in autumn 2011, 0.2 x10^4 CFU/ml at site 6, 13 and 14 to 2.7 x10^4 CFU/ml at site 15 in winter 2011, 0.3 x 10^4 CFU/ml at site 14 to 4.8 x 10^4 CFU/ml at site 15 in winter 2012.

Table 14. Site wise colony forming units (CFU/ml) of bacteria

Site	Spring		Summer		Autumn		Winter	
	2010	2011	2010	2011	2010	2011	2011	2012
1	1.3×10^4	2.7×10^4	3.7×10^4	7.1×10^4	2.0×10^4	4.1×10^4	1.3×10^4	1.6×10^4
2	1.2×10^4	1.2×10^4	3.5×10^4	5.2×10^4	1.9×10^4	3.0×10^4	0.8×10^4	1.3×10^4
3	0.9×10^4	2.2×10^4	4.3×10^4	5.0×10^4	3.3×10^4	4.5×10^4	0.9×10^4	1.1×10^4
4	0.7×10^4	0.8×10^4	6.3×10^4	6.4×10^4	2.8×10^4	3.4×10^4	0.4×10^4	0.7×10^4
5	0.3×10^4	0.8×10^4	3.6×10^4	10.0×10^4	2.0×10^4	3.4×10^4	0.4×10^4	1.0×10^4
6	0.4×10^4	2.6×10^4	2.0×10^4	5.6×10^4	0.6×10^4	1.5×10^4	0.2×10^4	0.9×10^4
7	2.1×10^4	2.7×10^4	9.3×10^4	10.8×10^4	4.1×10^4	5.2×10^4	1.6×10^4	2.1×10^4
8	0.9×10^4	1.5×10^4	3.5×10^4	4.3×10^4	1.9×10^4	2.7×10^4	0.5×10^4	0.8×10^4
9	3.2×10^4	4.2×10^4	7.9×10^4	13.1×10^4	3.5×10^4	4.4×10^4	1.0×10^4	2.0×10^4
10	2.4×10^4	3.1×10^4	12.1×10^4	3.0×10^4	3.3×10^4	6.0×10^4	0.6×10^4	1.3×10^4
11	5.9×10^4	6.0×10^4	20.0×10^4	3.0×10^4	7.2×10^4	8.4×10^4	1.7×10^4	2.1×10^4
12	6.0×10^4	7.4×10^4	9.2×10^4	11.6×10^4	4.3×10^4	5.5×10^4	2.0×10^4	2.3×10^4
13	0.4×10^4	0.8×10^4	3.0×10^4	6.0×10^4	1.4×10^4	2.2×10^4	0.2×10^4	0.5×10^4
14	0.4×10^4	1.1×10^4	2.0×10^4	2.4×10^4	0.4×10^4	1.6×10^4	0.2×10^4	0.3×10^4
15	3.3×10^4	9.4×10^4	11.0×10^4	19.8×10^4	8.0×10^4	13.0×10^4	2.7×10^4	4.8×10^4
16	4.4×10^4	8.1×10^4	14.9×10^4	28.7×10^4	13.6×10^4	21.0×10^4	1.8×10^4	4.1×10^4

4.4. Most Probable Number

For different sites under reference, the total coliform count in terms of Most Probable Number (MPN/100ml) ranged from 3 to 1100. The lowest count (3) was observed at sites 5 (Boat hall inlet) and 13 (Nigeen) in winter of 2012 whereas the highest coliform count ranged upto 1100 at sites 15 and 16 (Pokhribal outlet) in summer of 2010 and 2011 (Table 15). The distribution of coliform count (Table 16) categorized the lake water into four categories with MPN range of 0 (zero) for category I, 3-50 MPN/100ml for category II, 51-400 MPN/100ml for category III and 401-1100 MPN/100ml for category IV which shows that the maximum (57.81%) water samples lie in category III, followed by 39.07% samples in category II, 3.12% samples in category IV and 0% sample lying in category I. The perusal of the data indicates that none of the samples was fit for drinking purpose having an excellent water quality. Most of the water samples obtained from the lake were fit for bathing and swimming with a good or fair quality. However, some areas of the lake were having very much poor water quality, hence unfit for any type of use.

Table 15. MPN index (MPN/100ml) at different sites and seasons

Site code	Spring		Summer		Autumn		Winter	
	2010	2011	2010	2011	2010	2011	2011	2012
1	20	28	43	75	39	23	14	9
2	21	39	64	93	43	28	21	11
3	23	43	75	120	93	64	39	20
4	28	39	64	93	75	64	43	21
5	7	14	39	120	94	39	14	3
6	9	15	43	120	75	43	15	4
7	64	93	150	240	210	460	120	43
8	75	120	210	460	150	240	150	64
9	64	93	120	460	93	150	39	64
10	75	120	210	460	150	240	43	75
11	75	150	150	460	93	120	43	64
12	93	210	210	460	150	240	64	75
13	7	14	39	75	43	28	14	3
14	9	15	43	93	64	39	15	4
15	240	460	1100	1100	240	460	150	210
16	240	460	1100	1100	240	460	150	210

Table 16. Category wise distribution of coliform count (MPN/100ml)

Categories	MPN range	% age	Usage	Grade	Reference
Category I	0	0	Drinking	Excellent	WHO (1998)
Category II	3-50	39.07	Bathing , swimming	Good	(Pandey and Sharma, 1995)
Category III	51–400	57.81		Fair	
Category IV	401-1100	3.12	Unfit	Poor	

The family wise and species wise Total count (TC), Percentage total count (%TC), Occurrence remarks (OR) and Number of cases of isolation (NCI) shown in (Table 17) reveal that the different species of family Enterobacteriaceae isolated in a low to high frequency contributed to a total count of 2987 colonies constituting 50.27%. Family Bacillaceae isolated in a high frequency contributed a total of 698 colonies constituting 11.74%. Family Enterococcaceae isolated in a high frequency contributed a total of 505 colonies constituting 8.50 % of this group. Family Vibrionaceae isolated in a high frequency contributed a total of 449 colonies constituting 7.55% of this group. Family Pseudomonadaceae isolated in a high frequency contributed a total of 316 colonies constituting 5.31% of this group. Family Micrococcaceae isolated in a high frequency contributed a total of 405 colonies constituting 6.81% of this group. Family Alcaligenaceae isolated in a high frequency contributed a total of 202 colonies constituting 3.40% of this group. Family Aeromonadaceae isolated in a high frequency contributed a total of 192 colonies constituting 3.23% of this group. Family Staphylococcaceae isolated in a high frequency contributed a total of 157 colonies constituting 2.64% of this group. Family Moraxellaceae isolated in a low frequency contributed a total of 30 colonies constituting 0.50% of this group.

Table 17. Total count, percentage total count, occurrence and number of cases of isolation as per families and species isolated

Family / Species	TC	% TC	NCI	OR
Enterobacteriaceae	**2987**	**50.27**		**H**
Citrobacter freundii	145	2.44	16	**H**
Citrobacter diversus	156	2.62	16	**H**
Enterobacter aerogenes	176	2.96	15	**H**
Enterobacter agglomerans	167	2.81	16	**H**

Contd……..

Table 17 contd.

Family / Species	TC	% TC	NCI	OR
Enterobacter cloacae	172	2.89	16	**H**
Enterobacter intermedius	46	0.77	10	**L**
Serratia marcescens	171	2.87	16	**H**
Serratia rubidaea	29	0.48	8	**L**
Shigella flexneri	217	3.65	16	**H**
Shigella dysenteriae	242	4.07	16	**H**
Salmonella typhi	201	3.38	16	**H**
Salmonella paratyphi	49	0.82	10	**L**
Salmonella choleraesuis	50	0.84	11	**L**
Proteus vulgaris	151	2.54	16	**H**
Proteus mirabilis	151	2.54	16	**H**
Klebsiella oxytoca	113	1.90	14	**M**
Klebsiella ozaenae	24	0.40	6	**L**
Escherichia coli	364	6.12	16	**H**
Yersinia enterocolitica	189	3.18	16	**H**
Cedacea davisae	39	0.65	10	**L**
Cedacea lapagei	50	0.84	12	**L**
Edwardseilla tarda	39	0.65	11	**L**
Kluyvera ascorbata	46	0.77	11	**L**
Staphylococcaceae	**157**	2.64		**H**
Staphylococcus aureus	157	2.64	16	**H**
Moraxellaceae	**30**	0.50		**L**
Acinetobacter spp.	30	0.50	8	**L**
Vibrionaceae	**449**	**7.55**		**H**
Vibrio cholerae	167	2.81	16	**H**
Vibrio parahaemolyticus	149	2.50	16	**H**
Vibrio vulnificus	133	2.23	16	**H**
Pseudomonadaceae	316	5.31		**H**
Pseudomonas putida	144	2.42	16	**H**
Pseudomonas alcaligenes	172	2.89	16	**H**
Micrococcaceae	**405**	**6.81**		**H**
Micrococcus luteus	144	2.42	16	**H**
Micrococcus roseus	261	4.39	16	**H**
Bacillaceae	**698**	**11.74**		**H**
Bacillus sphaericus	43	0.72	11	**L**
Bacillus subtilis	172	2.89	16	**H**
Bacillus cereus	177	2.97	16	**H**
Bacillus alcalophilus	143	2.40	16	**H**
Bacillus brevis	163	2.74	16	**H**

Contd……..

Table 17 contd.

Family / Species	TC	% TC	NCI	OR
Alcaligenaceae	**202**	**3.40**		**H**
Alcaligenes feacalis	202	3.40	16	**H**
Enterococaceae	**505**	**8.50**		**H**
Enterococcus fecium	85	1.43	14	**M**
Enterococcus durans	39	0.65	10	**L**
Enterococcus hirae	40	0.67	10	**L**
Enterococcus mundtii	36	0.60	9	**L**
Aeromonadaceae	**192**	**3.23**		**H**
Aeromonas hydrophila	155	2.60	16	**H**
Aeromonas caviae	37	0.62	9	**L**
Total Colonies: 5941				

(TC=Total count, % TC=Percentage total count, OR=Occurrence, NCI=No of cases of isolation)

4.5. Species diversity pattern

The site wise colony count of the bacterial species listed in (Table 18) shows that a total of 5941 colonies, with a contribution of forty five (45) species, were cultured from the lake water samples. The maximum number of 966 colonies (16.25%) was obtained at site 16 followed by 813 colonies (13.68%) at site 11, 720 colonies (12.11%) at site 15, 588 colonies (9.89%) at site 10, 483 colonies (8.12%) at site 12, 393 colonies (6.61%) at site 9, 379 colonies (6.37%) at site 7, 238 (4.00%) colonies at site 1, 222 (3.73%) colonies at site 3, 215 (3.61%) colonies at site 4 and 5, 181 (3.04%) colonies at site 2, 161 (2.70%) colonies at site 8, 145 (2.44%) colonies at site 13, 138 (2.32%) colonies at site 6, 84 (1.41%) colonies at site 14.

The species wise percentage occurrence of different bacterial species isolated from the water samples from different sites shown in (Table 19) explains that percentage occurrence of *K. ozaenae* ranged between a maximum of 41.6 % at site 16 to a minimum of 4.16% at sites 1 and 5; *E. durans* ranged between a maximum of 38.4% at site 16 to a minimum of 2.56% at sites 1, 2, 5 and 7; *Acinetobacter* spp. ranged between a maximum of 33.3% at site 16 to a minimum of 1.11% at site 9; *S. paratyphi* ranged between a maximum of 32.65% at site 16 to a minimum of 2.04% at site 2; *E. hirae* ranged between a maximum of 32.5% at site 16 to a minimum of 2.5% at sites 2, 5, 7 and 13; *S. choleraesuis* ranged between a maximum of 32% at site 16 to a minimum of 2% at site 13; *E. intermedius* ranged between a maximum of

30.43% at site 16 to a minimum of 2.17% at site 2; *C. davisae* ranged between a maximum of 28.2% at site 16 to a minimum of 2.56% at sites 5 and 13; *E. mundtii* ranged between a maximum of 27.77% at site 16 to a minimum of 2.77% at sites 1, 2, 5 and 14; *S. rubideae* ranged between a maximum of 27.58% at site 16 to a minimum of 3.44% at sites 1, 2, 4 and 5; *B. sphaericus* ranged between a maximum of 23.25% at site 11 to a minimum of 2.32% at sites 5 and 13; *E. tarda* ranged between a maximum of 23% at site 16 to a minimum of 2.56% at sites 2 and 5; *K. oxytoca* ranged between a maximum of 22.12% at site 16 to a minimum of 0.88% at sites 13 and 14; *A. caviae* ranged between a maximum of 18.9% at sites 15 and 16 to a minimum of 2.7% at sites 2, 5 and 13; *V. vulnificus* ranged between a maximum of 18.7% at site 11 to a minimum of 0.75% at site 1; *P. putida* ranged between a maximum of 18.05% at sites 11 and 16 to a minimum of 1.3% at site 13; *C. lapagei* ranged between a maximum of 18% at site 16 to a minimum of 1% at sites 2 and 4; *V. cholerae* ranged between a maximum of 17.96% at site 16 to a minimum of 1.19% at site 14; *S. typhi* ranged between a maximum of 17.9% at site 16 to a minimum of 0.99% at site 14; *B. brevis* ranged between a maximum of 17.79% at site 11 to a minimum of 1.22% at sites 6, 8 and 13; *E. feacium* ranged between a maximum of 17.6% at site 11 to a minimum of 1.17% at sites 8 and 14; *S. dysenteriae* ranged between a maximum of 17.3% at site 16 to a minimum of 1.23% at sites 6 and 14. *Y. enterocolitica* ranged between a maximum of 16.93% at site 11 to a minimum of 1.58% at sites 2, 5 and 14; *V. parahaemolyticus* ranged between a maximum of 16.77% at site 11 to a minimum of 0.67% at sites 1 and 14; *P. vulgaris* ranged between a maximum of 16.55% at site 11 to a minimum of 1.32% at site 14; *M. roseus* ranged between a maximum of 16.47% at site 16 to a minimum of 0.76% at site 14; *S. marcescens* ranged between a maximum of 16.37% at site 16 to a minimum of 1.16% at sites 6 and 14; *E. agglomerans* ranged between a maximum of 16.16% at site 11 to a minimum of 1.79% at sites 2, 13 and 14; *K. ascorbata* ranged between a maximum of 15.21% at sites 10, 15 and 16 to a minimum of 2.17% at site 3; *B. cereus* ranged between a maximum of 15.25% at site 11 to a minimum of 1.69% at site 6; *E. aerogenes* ranged between a maximum of 15.34% at site 11 to a minimum of 1.7% at site 13; *C. diversus* ranged between a maximum of 15.38% at site 16 to a minimum of 1.92% at sites 2 and 13; *S. flexneri* ranged between a maximum of 15.66% at site 16

to a minimum of 2.3% at site 5; *P. alcaligenes* ranged between a maximum of 15.69% at site 11 to a minimum of 1.16% at site 13. *P; mirabilis* ranged between a maximum of 15.89% at sites 15 and 16 to a minimum of 0.66% at site 14; *M. luteus* ranged between a maximum of 15.97% at site 16 to a minimum of 0.69% at sites 13 and 14; *A. faecalis* ranged between a maximum of 14.85% at site 15 to a minimum of 0.99% at site 14. *E.coli* ranged between a maximum of 14.83% at site 16 to a minimum of 1.37% at site 14. *B. alcalophillus* ranged between a maximum of 14.68% at site 16 to a minimum of 0.69% at sites 13 and 14; S. *aureus* ranged between a maximum of 14.6% at site 11 to a minimum of 1.27% at site 13; *C. freundii* ranged between a maximum of 14.48% at sites 16 and 11 to a minimum of 1.37% at site 14; *A. hyrdophila* ranged between a maximum of 14.19% at site 16 to a minimum of 0.64 at site 13; *B. subtilis* ranged between a maximum of 13.95% at site 10 to a minimum of 1.74% at sites 13 and 14; *E. cloacae* ranged between a maximum of 13.9% at site 11 to a minimum of 0.58% at site 14; *E. faecalis* ranged between a maximum of 13.44% at site 11 to a minimum of 1.63% at sites 6 and 16.

4.6. Seasonal variation of bacterial population

Table 20 shows the results of bacteriological examination of lake water during four seasons of the two study years at 16 selected sites. The total colony count 5941 (99.89%) was observed in all the four seasons at different sites. The highest number of bacterial colonies 3123 (52.56%) was observed in summer season followed by 1502 colonies (25.28%) in autumn, 884 colonies (14.87%) in spring and the lowest number of 432 colonies (7.27%) in winter season (Fig. 6 and 7). The occurrence of bacterial population was found to be statistically significant by the analysis of variance (ANOVA) carried out for different seasons ($p< 0.01$). Highest number of colonies 966 (16.25%) was observed at site 16 followed by 813 (13.68%) colonies at site 11; 720 (12.11%) colonies at site 15, 588 (9.89%) colonies at site 10, 483 (13.68%) colonies at site 12; 393 (13.68%) colonies at site 9; 379 (13.68%) colonies at site 7; 238 (4%) colonies at site 1; 222 (3.73%) colonies at site 3; 215 (3.61%) colonies at site 4 and 5; 181 (3.04%) colonies at site 2; 145 (2.44%) colonies at site 13; 138 (2.32%) colonies at site 6; 84 (1.41%) colonies at site 14.

Showing considerable seasonal variations in all the selected sites, highest number of bacterial colonies 3123 (52.56%) was obtained in summer season,

Table18. Colony count of isolated bacteria at different sites

Name of species	Sites																Total
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	
C. freundii	4	3	5	6	6	3	14	4	11	10	21	17	4	2	14	21	**145**
C. diversus	7	3	7	6	5	6	12	6	10	9	22	15	3	4	17	24	**156**
E. aerogenes	6	6	7	7	5	9	13	4	9	16	27	20	3	0	20	24	**176**
E. agglomerans	7	3	6	6	5	4	12	5	13	15	27	16	3	3	19	23	**167**
E. cloacae	7	4	9	7	5	7	14	7	8	20	24	16	8	1	16	19	**172**
E. intermedius	3	1	0	0	3	0	2	0	3	7	0	4	2	0	7	14	**46**
S. marcescens	5	5	6	6	7	2	13	5	10	19	27	12	4	2	20	28	**171**
S. rubideae	1	1	0	1	1	0	0	0	3	7	0	0	0	0	7	8	**29**
S. flexneri	6	8	8	8	5	7	17	8	14	20	28	19	6	4	25	34	**217**
S. dysenteriae	6	5	8	11	11	3	13	10	17	22	35	20	6	3	30	42	**242**
S. typhi	13	8	7	9	9	3	14	8	14	18	17	17	3	2	23	36	**201**
S. paratyphi	3	1	4	2	2	0	0	0	3	7	0	0	3	0	8	16	**49**
S. choleraesuis	3	1	2	2	2	0	2	0	4	8	0	0	1	0	9	16	**50**
P. vulgaris	8	5	5	6	3	7	9	4	13	15	25	12	4	2	16	17	**151**
P. mirabilis	5	4	6	4	4	3	11	5	10	13	20	14	3	1	24	24	**151**
K. oxytoca	6	4	3	0	3	2	8	0	8	9	16	10	1	1	17	25	**113**
K. ozaenae	1	2	0	0	1	0	0	0	3	0	0	0	0	0	7	10	**24**
E.coli	17	12	12	14	24	11	19	14	23	33	39	27	19	5	41	54	**364**
S. aureus	7	7	10	8	4	8	11	5	11	9	23	14	2	5	12	21	**157**
Y. enterocolitica	5	3	9	7	3	5	11	6	9	22	32	17	5	3	21	31	**189**
C. davisae	3	1	0	0	1	0	2	0	4	2	6	0	1	0	8	11	**39**
C. lapagei	3	2	0	2	1	0	3	0	5	5	7	6	1	0	6	9	**50**
E. tarda	4	1	3	0	1	0	2	0	5	3	5	0	2	1	6	9	**39**

Contd…..

Table 18 contd.

Name of species	Sites																Total
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	
Acinetobacter spp.	1	1	0	0	0	0	0	0	4	1	5	0	1	0	7	10	**30**
K. ascorbata	5	2	1	3	0	0	2	0	4	7	5	3	0	0	7	7	**46**
E. faecalis	9	12	10	11	16	5	16	13	16	24	41	25	11	7	39	50	**305**
E. faecium	8	5	5	3	2	0	0	1	7	9	15	4	4	1	7	14	**85**
E. durans	1	1	0	0	1	0	1	2	3	5	0	4	0	0	6	15	**39**
E. hirae	2	1	0	0	1	0	1	0	5	7	4	0	1	0	5	13	**40**
E. mundtii	1	1	0	0	1	0	0	0	3	7	5	0	0	1	7	10	**36**
V. cholerae	6	3	7	5	10	5	13	3	9	20	17	15	6	2	16	30	**167**
V. parahaemolyticus	1	2	8	6	3	3	12	3	10	18	25	14	3	1	19	21	**149**
V. vulnificus	1	2	5	6	4	3	12	2	7	16	25	15	4	2	14	15	**133**
P. putida	5	4	4	5	4	3	9	4	7	10	26	15	2	1	19	26	**144**
P. alcaligenes	3	6	7	8	5	6	14	4	8	18	27	14	2	4	24	22	**172**
A. hydrophila	8	5	6	8	5	5	13	4	11	13	20	11	1	5	18	22	**155**
A. caviae	2	1	0	0	1	0	0	0	3	6	9	0	1	0	7	7	**37**
B. sphaericus	4	2	0	0	1	0	2	0	4	6	10	0	1	2	6	5	**43**
B. subtilis	8	9	9	6	7	4	11	4	13	24	20	14	3	3	15	22	**172**
B. cereus	8	6	9	6	5	3	9	4	11	21	27	14	4	7	17	26	**177**
B. alcalophilus	7	3	5	5	8	5	13	6	13	10	17	13	1	1	15	21	**143**
B. brevis	4	3	10	8	6	2	11	2	10	15	29	18	2	3	20	20	**163**
A. faecalis	8	8	7	6	5	5	11	5	10	23	32	17	5	2	30	28	**202**
M. luteus	7	6	4	4	8	4	13	5	10	12	18	12	1	1	16	23	**144**
M. roseus	9	8	11	13	11	5	14	8	15	27	35	19	8	2	33	43	**261**
Total	**238**	**181**	**222**	**215**	**215**	**138**	**379**	**161**	**393**	**588**	**813**	**483**	**145**	**84**	**720**	**966**	**5941**

Table 19. Percentage occurrence of isolated bacteria at different sites

Name of species	Sites															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
C. freundii	2.75	2.06	3.44	4.13	4.13	2.06	9.65	2.75	7.58	6.89	14.48	9.98	2.75	1.37	9.65	14.48
C. diversus	4.48	1.92	4.48	3.84	3.2	3.84	7.69	3.84	6.41	5.76	14.1	9.61	1.92	2.56	10.89	15.38
E. aerogenes	3.4	3.4	3.97	3.97	2.84	5.11	7.38	2.27	5.11	9.09	15.34	11.36	1.7	0	11.36	13.63
E. agglomerans	4.19	1.79	3.59	3.59	2.99	2.39	7.18	2.99	7.78	8.98	16.16	9.58	1.79	1.79	11.37	13.77
E. cloacae	4.06	2.32	5.23	4.06	2.9	4.06	8.13	4.06	4.65	11.62	13.9	9.3	4.65	0.58	9.3	11.04
E. intermedius	6.52	2.17	0	0	6.52	0	4.34	0	6.52	15.21	0	8.69	4.34	0	15.21	30.43
S. marcescens	2.92	2.92	3.5	3.5	4.09	1.16	7.6	2.92	5.84	11.11	15.78	7.01	2.33	1.16	11.6	16.37
S. rubideae	3.44	3.44	0	3.44	3.44	0	0	0	10.34	24.13	0	0	0	0	24.13	27.58
S. flexneri	2.76	3.68	3.68	3.68	2.3	3.22	7.83	3.68	6.45	9.21	12.9	8.75	2.76	1.84	11.52	15.66
S. dysenteriae	2.47	2.06	3.3	4.5	4.5	1.23	5.37	4.13	7.02	9.09	14.46	8.2	2.47	1.23	12.3	17.3
S. typhi	6.46	3.98	3.48	4.47	4.47	1.49	6.9	3.98	6.9	8.95	8.45	8.45	1.49	0.99	11.44	17.9
S. paratyphi	6.12	2.04	8.16	4.08	4.08	0	0	0	6.12	14.2	0	0	6.12	0	16.32	32.65
S. choleraesuis	6	2	4	4	4	0	4	0	8	16	0	0	2	0	18	32
P. vulgaris	5.29	3.31	3.31	3.97	1.98	4.63	5.96	2.64	8.6	9.93	16.55	7.94	2.64	1.32	10.59	11.25
P. mirabilis	3.31	2.64	3.97	2.64	2.64	1.98	7.28	3.31	6.62	8.6	13.24	9.27	1.98	0.66	15.89	15.89
K. oxytoca	5.3	3.53	2.65	0	2.65	1.76	7.07	0	7.07	7.96	14.15	8.84	0.88	0.88	15.04	22.12
K. ozaenae	4.16	8.33	0	0	4.16	0	0	0	12.5	0	0	0	0	0	29.16	41.6
E.coli	4.67	3.29	3.29	3.84	6.59	3.02	5.21	3.84	6.31	9.06	10.71	7.41	5.21	1.37	11.26	14.83
S. aureus	4.45	4.45	6.36	5.05	2.54	5.09	7	3.18	7	5.73	14.6	8.91	1.27	3.18	7.64	13.3
Y. enterocolitica	2.64	1.58	4.76	3.7	1.58	2.64	5.82	3.17	4.76	11.64	16.93	8.99	2.64	1.58	11.11	16.4
C. davisae	7.69	2.56	0	0	2.56	0	5.12	0	10.25	5.12	15.3	0	2.56	0	20.5	28.2
C. lapagei	6	1	0	1	2	0	6	0	10	10	14	12	2	0	12	18
E. tarda	10.25	2.56	0	0	2.56	0	5.12	0	12.8	7.69	12.8	0	5.12	2.56	15.38	23

Contd……

Table 19 contd.

Name of species	Sites															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Acinetobacter spp.	3.33	3.33	0	0	0	0	0	0	1.11	3.33	16.6	0	3.33	0	23.3	33.3
K. ascorbata	10.8	4.34	2.17	6.52	0	0	4.34	0	8.69	15.21	10.86	6.52	0	0	15.21	15.21
E. faecalis	2.95	3.93	3.27	3.6	5.24	1.63	5.24	4.26	5.24	7.86	13.44	8.19	3.6	2.29	12.78	1.63
E. faecium	9.41	5.88	5.88	3.52	2.35	0	0	1.17	8.23	10.5	17.6	4.7	4.7	1.17	8.23	16.49
E. durans	2.56	2.56	0	0	2.56	0	2.56	5.12	7.69	12.8	0	10.25	0	0	15.38	38.4
E. hirae	5	2.5	0	0	2.5	0	2.5	0	12.5	17.5	10	0	2.5	0	12.5	32.5
E. mundtii	2.77	2.77	0	0	2.77	0	0	0	8.33	19.44	13.88	0	0	2.77	19.44	27.77
V. cholerae	3.59	1.79	4.19	2.99	5.98	2.99	7.78	1.79	5.38	11.97	10.17	8.98	3.59	1.19	9.58	17.96
V. parahaemolyticus	0.67	1.34	5.36	4.02	2.01	2.01	8.05	2.01	6.71	12.08	16.77	9.39	2.01	0.67	12.7	14.09
V. vulnificus	0.75	1.5	3.75	4.51	3	2.25	9.02	1.5	5.26	12.03	18.7	11.27	3	1.5	10.5	11.27
P. putida	3.47	2.77	2.77	3.47	2.77	2.08	6.25	2.77	4.86	6.94	18.05	10.4	1.38	0.69	13.19	18.05
P. alcaligenes	1.74	3.48	4.06	4.65	2.9	3.48	8.13	2.32	4.65	10.46	15.69	8.13	1.16	2.32	13.95	12.79
A. hydrophila	5.16	3.22	3.87	5.16	3.22	3.22	8.38	2.5	7.09	8.38	12.9	7.09	0.64	3.22	11.6	14.19
A. caviae	5.4	2.7	0	0	2.7	0	0	0	8.1	16.2	24.3	0	2.7	0	18.9	18.9
B. sphaericus	9.3	4.65	0	0	2.32	0	4.65	0	9.3	13.95	23.25	0	2.32	4.65	13.95	11.62
B. subtilis	4.65	5.23	5.23	3.48	4.06	2.32	6.39	2.32	7.55	13.95	11.62	8.13	1.74	1.74	8.72	12.79
B. cereus	4.51	3.38	5.08	3.38	2.82	1.69	5.08	2.25	6.21	11.86	15.25	7.9	2.25	3.95	9.6	14.68
B. alcalophilus	4.89	2.09	3.49	3.49	5.59	3.49	9.09	4.19	9.09	6.99	11.88	9.09	0.69	0.69	10.48	14.68
B. brevis	2.45	1.84	6.13	4.9	3.68	1.22	6.74	1.22	6.13	9.2	17.79	11.04	1.22	1.84	12.26	12.26
A. faecalis	3.96	3.96	3.46	2.97	2.47	2.47	5.44	2.47	4.95	11.38	15.84	8.41	2.47	0.99	14.85	13.86
M. luteus	4.86	4.16	2.77	2.77	5.55	2.77	9.02	3.47	6.94	8.33	12.5	8.33	0.69	0.69	11.11	15.97
M. roseus	3.44	3.06	4.21	4.98	4.21	1.91	5.36	3.06	5.74	10.34	13.4	7.27	3.06	0.76	12.64	16.47

followed by 1502 colonies (25.28%) in autumn season, 884 colonies (14.87%) in spring season and the least number of 432 colonies (7.27%) in winter season. Analysis of variance (ANOVA) showed that the observed distribution of the bacterial colonies in different seasons are statistically significant ($F=11.42$, $F_{crit}= 2.75$, $p<0.01$). Visible from the tables the seasonal variation of occurrence of bacterial colonies was also observed between different study sites of the lake.

Table 20. Total colony count and percentage occurrence of bacterial colonies in different seasons (2010-2012) at different sites of Dal lake

Site	Spring	Summer	Autumn	Winter	Total	% Occurrence
1	40	108	61	29	238	4%
2	24	87	49	21	181	3.04%
3	31	93	78	20	222	3.73%
4	15	127	72	11	215	3.61%
5	11	136	54	14	215	3.61%
6	30	76	21	11	138	2.32%
7	48	201	93	37	379	6.37%
8	24	78	46	13	161	2.70%
9	74	210	79	30	393	6.61%
10	55	421	93	19	588	9.89%
11	119	500	156	38	813	13.68%
12	134	208	98	43	483	8.12%
13	12	90	36	7	145	2.44%
14	15	44	20	5	84	1.41%
15	127	308	210	75	720	12.11%
16	125	436	346	59	966	16.25%
Total	884	3123	1502	432	5941	99.89%
% O	14.87%	52.56%	25.28%	7.27%	99.98%	

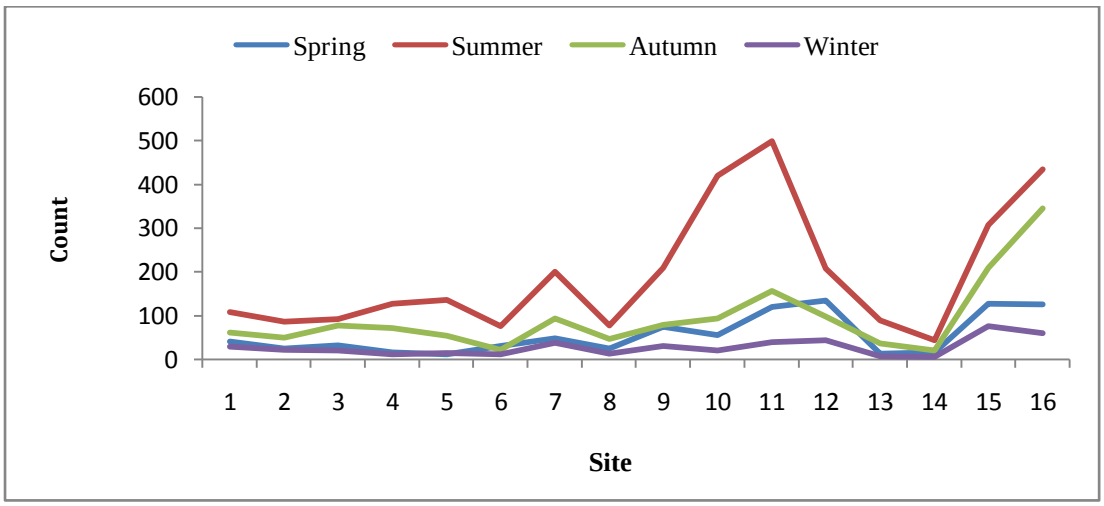


Fig.6. Variation of total colony count in different seasons

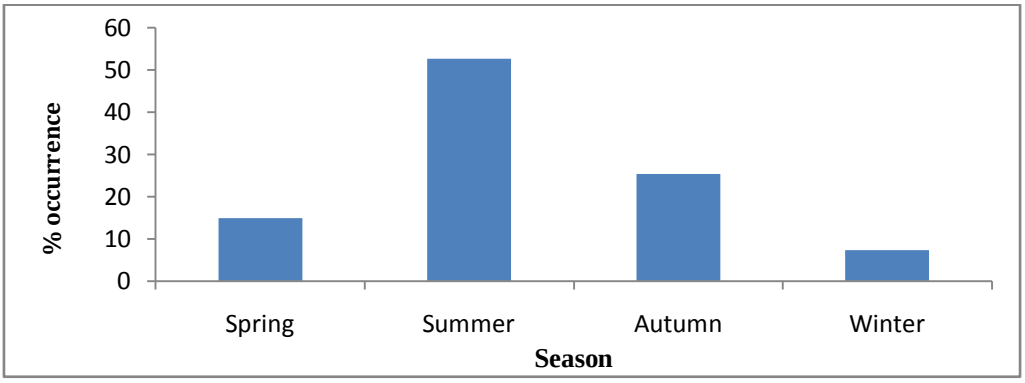


Fig.7. Percentage occurrence of total colony count in different seasons

4.7. Seasonal variation in species composition at different sites

Results of seasonal variation of isolated bacterial species at different sites are depicted in Table 21. The overall highest bacterial species (45) was observed in both the summer season and lowest number of species (1) was observed in winter season. During spring 2010, highest number of 35 species were recorded at site 16 whereas a maximum of 45 species were recorded at site 15 in spring 2011.In summer 2010, 45 species were recorded at site 16 and 45 species were also recorded at site 1, 2, 9 and 15 in summer 2011. In autumn 2010, 44 species were recorded at site 15 and 45 species at site 15 and 16. In winter 2010, 16 species were isolated at site 7 and 24 species at site 15 in winter 2012 (Fig.8, 9, 10 and 11).

Table 21. Site and Season wise variation in species composition

Sites	Spring		Summer		Autumn		Winter	
	2010	2011	2010	2011	2010	2011	2011	2012
1	11	24	31	45	20	32	13	15
2	12	12	27	45	17	25	8	10
3	9	18	31	33	28	30	9	11
4	7	8	31	32	28	27	4	7
5	2	7	19	42	14	19	4	8
6	4	23	19	29	6	11	2	5
7	19	23	36	38	28	30	16	16
8	9	11	27	25	15	25	5	8
9	23	27	44	45	30	34	10	19
10	21	26	41	44	25	29	6	13
11	30	29	39	39	29	30	14	16
12	28	29	33	34	29	32	14	17
13	4	8	23	39	11	18	1	4
14	4	10	19	21	4	13	2	3
15	17	45	40	45	44	45	15	24
16	35	43	45	44	41	45	14	21

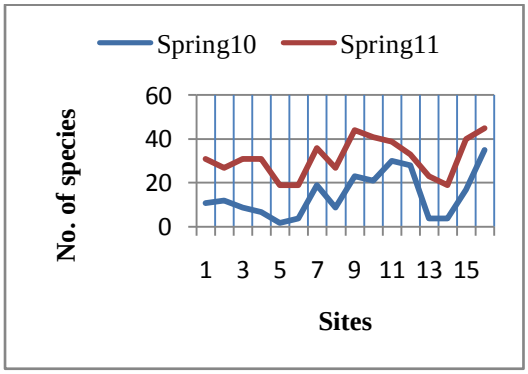


Fig. 8. Variation in species composition in spring 2010-2011

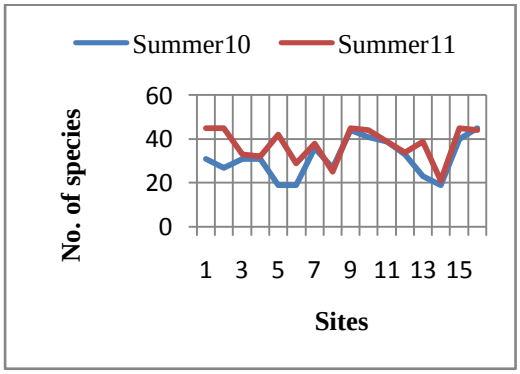


Fig. 9. Variation in species composition in summer 2010-2011

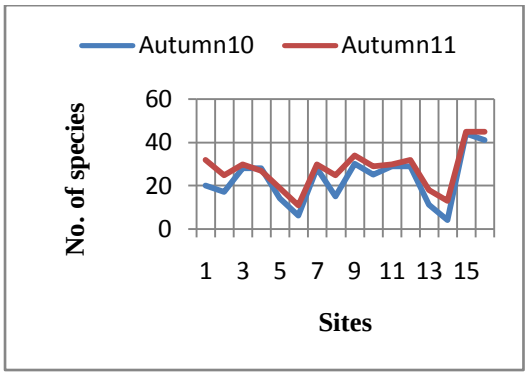


Fig. 10. Variation in species composition in autumn 2010-2011

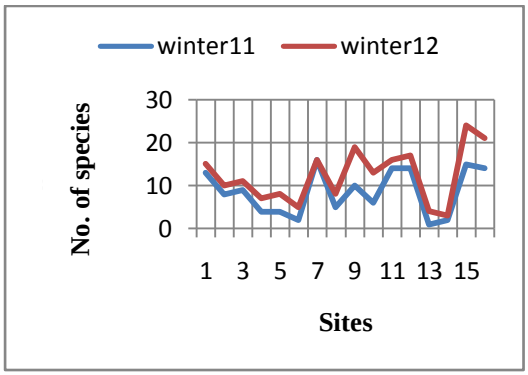


Fig. 11. Variation in species composition in winter 2011-2012

4.8. Seasonal occurrence of bacterial species at different sites

The occurrence value, percent frequency (% F) and percent relative frequency (% RF) was calculated on the basis of presence and absence of a particular species at a particular site out of eight seasons of two successive years. Table 22 depicts the variation in occurrence value of different species between a maximum of eight thereby indicating its occurrence in all the four seasons of two years and minimum of zero indicating its absence in all the seasons. The occurrence value of *B. sphaericus* ranged between 0 and 4, *E. intermedius*, *S. rubideae*, *K. ozaenae*, *C. lapagei*, *K. ascorbata*, *E. durans*, *E. mundtii* and *A. caviae* ranged between 0 and 5, *S. paratyphi*, *S. choleraesuis*, *C. davisae*, *E. tarda*, *Acinetobacter* spp., *E. faecium* and *E. hirae* ranged between 0-6, *E. aerogenes* and *K. oxytoca* ranged between 0 and 8, *V. vulnificus* and *V. parahaemolyticus* ranged between 1 and 6, *E. cloacae*, *S. marcescens*, *P. mirabilis*, *P. putida*, *A. hyrdophila*, *B. alcalophillus* and *M luteus* ranged between 1 and 8, *C. freundii*, *S. dysenteriae*, *S. typhi*, *P. vulgaris*, *S. aureus*,

Table 22. Seasonal occurrence of isolated bacterial species during 2010-2011 and 2011-2012

Name of species	Sites															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
C. freundii	3	3	4	4	4	3	8	3	7	5	8	8	4	2	7	8
C. diversus	6	3	5	4	3	4	6	5	7	5	6	6	3	3	7	7
E. aerogenes	4	6	5	5	3	6	6	4	5	4	6	8	3	0	8	8
E. agglomerans	6	3	4	4	4	3	7	4	8	6	6	7	3	3	8	7
E. cloacae	7	4	6	4	3	6	7	5	5	6	8	7	5	1	7	7
E. intermedius	2	1	0	0	1	0	2	0	2	2	0	3	2	0	5	5
S. marcescens	4	5	4	4	4	2	6	4	7	5	6	8	2	1	7	7
S. rubideae	1	1	0	1	1	0	0	0	2	3	0	0	0	0	5	4
S. flexneri	5	6	5	6	3	3	8	6	7	4	6	7	4	4	7	8
S. dysenteriae	4	3	5	7	4	2	5	6	7	6	7	8	4	3	7	7
S. typhi	7	5	4	6	4	3	5	5	7	8	5	7	3	2	6	7
S. paratyphi	2	1	3	2	1	0	0	0	2	2	0	0	3	0	5	6
S. choleraesuis	3	1	2	2	1	0	2	0	3	2	0	0	1	0	5	6
P. vulgaris	7	5	5	4	2	6	6	3	7	7	8	7	3	2	6	8
P. mirabilis	4	4	6	2	3	3	5	4	7	5	7	7	2	1	8	8
K. oxytoca	6	4	3	0	2	1	6	0	5	6	6	5	1	1	8	7
K. ozaenae	1	2	0	0	1	0	0	0	2	0	0	0	0	0	5	4
E.coli	8	7	8	8	8	6	8	8	8	8	8	8	8	5	7	8
S. aureus	5	7	8	6	2	5	6	5	8	6	8	7	2	3	6	8
Y. enterocolitica	4	3	8	5	2	3	7	5	7	7	8	7	4	3	7	6

Contd……..

Table 22 contd.

Name of species	Sites															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
C. davisae	3	1	0	0	1	0	2	0	2	1	2	0	1	0	5	6
C. lapagei	3	2	0	2	1	0	3	0	4	3	2	3	1	0	4	5
E. tarda	3	1	0	0	1	0	2	0	3	2	2	0	2	1	4	6
Acinetobacter spp.	1	1	0	0	0	0	0	0	3	1	3	0	1	0	5	6
K. ascorbata	3	2	1	3	0	0	2	0	2	2	2	2	0	0	5	5
E. faecalis	8	7	8	7	6	3	8	8	8	8	8	8	7	6	8	8
E. faecium	6	5	4	3	1	0	0	1	6	5	4	2	3	1	5	6
E. durans	1	1	0	0	1	0	1	2	2	2	0	2	0	0	5	5
E. hirae	2	1	0	0	1	0	1	0	4	3	2	0	1	0	4	6
E. mundtii	1	1	0	0	1	0	0	0	2	1	2	0	0	1	5	5
V. cholerae	4	2	4	4	4	3	6	2	3	4	6	6	3	2	6	6
V. parahaemolyticus	1	2	5	4	2	2	6	3	4	6	6	6	2	1	6	6
V. vulnificus	1	2	4	4	2	3	6	2	4	4	6	6	3	2	6	5
P. putida	4	4	4	3	2	2	6	4	6	6	7	7	1	1	6	8
P. alcaligenes	2	4	5	5	4	4	6	4	6	7	8	7	2	4	7	6
A. hydrophila	8	5	4	4	4	4	7	4	6	6	7	7	1	4	7	8
A. caviae	2	1	0	0	1	0	0	0	2	2	2	0	1	0	5	5
B. sphaericus	4	2	0	0	1	0	2	0	3	2	2	0	1	2	4	3
B. subtilis	7	7	8	4	3	3	7	4	7	6	6	7	3	3	7	6
B. cereus	6	5	7	4	3	2	6	4	6	6	8	8	3	6	6	6
B. alcalophilus	7	3	4	3	5	4	7	4	7	5	8	6	1	1	7	6
B. brevis	4	3	8	4	3	2	6	2	7	6	8	8	2	3	7	6
A. faecalis	7	8	6	4	3	4	7	4	6	8	8	7	4	2	6	8
M. luteus	6	5	4	4	4	3	6	4	7	5	6	6	1	1	6	8
M. roseus	8	7	8	8	5	4	8	8	7	7	8	8	7	2	8	8

Y. enterocolitica, *P. alcaligenes*, *B. cereus*, *B. brevis*, *A. feacalis* and *M. roseus* ranged between 2 and 8, *C. diversus* ranged between 3 and 7, *E. agglomerans*, *S. flexneri*, *E. faecalis* and *B. subtilis* ranged between 3 and 8, *E.coli* ranged between 5 and 8, *V. cholerae* ranged between 2 and 6.

4.9. Spatial variation of bacterial species

Spatial variation of the bacterial species is evident from the revealed data (Table 23 and Fig. 12) as the highest number of 45 species occurred at sites 1, 2, 9, 15 and 16 followed by 44 species at site 10; 43 species at site 5; 40 species at site 13; 39 species at site 11; 38 species at site 7; 34 species at sites 4 and 12; 33 species at site 3; 32 species at site 14; 30 species at site 8 and lowest number of species (29) at site 6.

Table 23. Spatial variation of species

Site	Number of species isolated
1	45
2	45
9	45
15	45
16	45
7	44
5	43
13	40
11	39
10	38
4	34
12	34
3	33
14	32
8	30
6	29

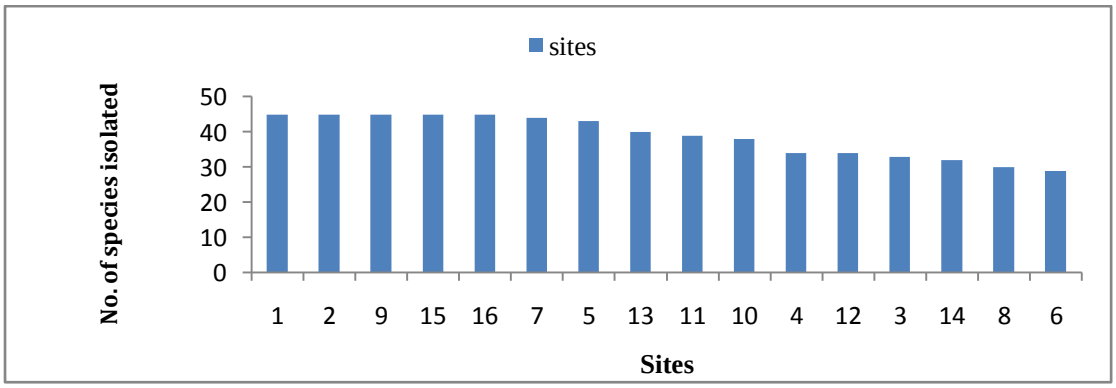


Fig.12. Spatial variation of species at different sites

4.10. Abundance distribution of bacterial genera at different sites

The site wise abundance distribution and species contribution of different bacterial genera is shown in Table 24. At sites 1, 2, 9, 15 and 16, out of total 45 species, the maximum number of species was five (5) with a relative abundance of (11.11%) contributed by genus *Bacillus* and *Enterococcus* followed by four species (8.88%) by genus *Enterobacter*, three species (6.66%) by genus *Salmonella* and *Vibrio*, two species (4.44%) by *Citrobacter, Serratia, Shigella, Proteus, Klebsiella, Cedacea, Micrococcus, Pseudomonas* and *Aeromonas* and one species (2.22%) by *E.coli, Staphylococcus, Yersinia, Edwardseilla, Acinetobacter, Kluyvera* and *Alcaligenes*. At site 6, out of total 29 species, the maximum number of species four (4) with a relative abundance of 13.79 % was contributed by genus *Bacillus* followed by three species (10.34 %) by genus *Enterobacter, Vibrio* two species (6.89 %) by genus *Citrobacter, Shigella, Proteus, Micrococcus* and *Pseudomonas*; one species (3.44%) by *Salmonella, Serratia, Klebsiella, E.coli, Staphylococcus, Yersinia, Enterococcus, Aeromonas, Alcaligenes* and *Micrococcus* and no contribution by genus *Cedacea, Edwardseilla, Acinetobacter* and *Kluyvera.*

4.11. Seasonal distribution of bacterial species at different sites

The various bacterial species isolated from different sampling sites of the lake depicted in the (Table 25) reveals variation in occurrence of the bacterial species in different seasons. From genus *Citrobacter,* two species were present at all sampling sites in summer followed by its presence in autumn, spring and winter. From genus *Enterobacter,* all the three species were found to be present in summer season followed by autumn, spring and winter at all sites. Furthermore, from genus *Serratia, S. marcescens* was dominant at all the sites as compared to *S. rubideae* which was isolated from few sampling sites. From genus *Shigella,* all the species were found to be dominant with its presence in all sites in summer followed by autumn and spring. Similarly, from genus *Salmonella, S. typhi* was isolated from all sampling sites in summer besides its presence in autumn followed by spring and winter, however, other two species were isolated from few sites in all seasons of the year. From genus *Proteus* and *Klebsiella*, all the four species were found to be dominant at different sites in different seasons except *Klebsiella oxytoca* which was reported to be absent in winter season. From genus *Escherichia, Bacillus* and *Staphylococcus*, all the seven

Table 24. Abundance distribution of different bacterial genera at different sites

Site		*Citrobacter*	*Enterobacter*	*Serratia*	*Shigella*	*Salmonella*	*Proteus*	*Klebsiella*	*Escherichia*	*Staphylococcus*	*Yersinia*	*Cedacea*	*Edwardseilla*	*Acinetobacter*	*Kluyvera*	*Enterococcus*	*Vibrio*	*Pseudomonas*	*Aeromonas*	*Bacillus*	*Alcaligenes*	*Micrococcus*	Total
1	NS	2	4	2	2	3	2	2	1	1	1	2	1	1	1	5	3	2	2	5	1	2	45
	RA %	4.44	8.88	4.44	4.44	6.66	4.44	4.44	2.22	2.22	2.22	4.44	2.22	2.22	2.22	11.11	6.66	4.44	4.44	11.11	2.22	4.44	99.92
2	NS	2	4	2	2	3	2	2	1	1	1	2	1	1	1	5	3	2	2	5	1	2	45
	RA %	4.44	8.88	4.44	4.44	6.66	4.44	4.44	2.22	2.22	2.22	4.44	2.22	2.22	2.22	11.11	6.66	4.44	4.44	11.11	2.22	4.44	99.92
3	NS	2	3	1	2	3	2	1	1	1	1	0	0	0	1	2	3	2	1	4	1	2	33
	RA %	6.06	9.09	3.03	6.06	9.09	6.06	3.03	3.03	3.03	3.03	0	0	0	3.03	6.03	9.09	6.06	3.03	12.12	3.03	6.06	99.96
4	NS	2	3	2	2	3	2	0	1	1	1	1	0	0	1	2	3	2	1	4	1	2	34
	RA %	4.65	6.97	5.88	5.88	8.82	5.88	0	2.94	2.94	2.94	2.94	0	0	2.94	5.88	8.82	5.88	2.94	11.76	2.94	5.88	96.88
5	NS	2	4	2	2	3	2	2	1	1	1	2	1	0	0	5	3	2	2	5	1	2	43
	RA %	4.50	9.30	4.50	4.50	6.80	4.50	4.50	4.50	2.30	2.30	4.50	2.30	0.00	0.00	11.30	6.80	4.60	4.50	11.30	2.30	4.60	99.90
6	NS	2	3	1	2	1	2	1	1	1	1	0	0	0	0	1	3	2	1	4	1	2	29
	RA %	6.89	10.34	3.44	6.89	3.44	6.89	3.44	3.44	3.44	3.44	0	0	0	0	3.44	10.34	6.89	3.44	13.79	3.44	6.88	99.87
7	NS	2	4	1	2	2	2	1	1	1	1	2	1	0	1	3	3	2	1	4	1	2	37
	RA %	5.40	10.81	2.70	5.40	5.40	5.40	2.70	2.70	2.70	2.70	5.40	2.70	0	2.70	8.10	8.10	5.40	2.70	10.81	2.70	5.40	99.92
8	NS	2	3	1	2	1	2	0	1	1	1	0	0	0	0	3	3	2	1	4	1	2	30
	RA %	6.66	10.00	3.33	6.66	3.33	6.66	0	3.33	3.33	3.33	0	0	0	0	10.00	10.00	6.66	3.33	13.30	3.33	6.66	99.91

Contd……..

Table 24 contd.

Site		*Citrobacter*	*Enterobacter*	*Serratia*	*Shigella*	*Salmonella*	*Proteus*	*Klebsiella*	*Escherichia*	*Staphylococcus*	*Yersinia*	*Cedacea*	*Edwardseilla*	*Acinetobacter*	*Kluyvera*	*Enterococcus*	*Vibrio*	*Pseudomonas*	*Aeromonas*	*Bacillus*	*Alcaligenes*	*Micrococcus*	Total
9	NS	2	4	2	2	3	2	2	1	1	1	2	1	1	1	5	3	2	2	5	1	2	45
	RA %	4.44	8.88	4.44	4.44	6.66	4.44	4.44	2.22	2.22	2.22	4.44	2.22	2.22	2.22	11.11	6.66	4.44	4.44	11.11	2.22	4.44	99.92
10	NS	2	4	2	2	3	2	1	1	1	1	2	2	1	5	3	2	0	2	5	1	2	44
	RA %	4.54	9.09	4.54	4.54	6.81	4.54	2.27	2.27	2.27	2.27	4.54	4.54	2.27	11.36	6.81	4.54	0	4.54	11.36	2.27	4.54	99.91
11	NS	2	3	1	2	1	2	1	1	1	1	2	1	1	1	4	3	2	2	5	1	2	39
	RA %	5.12	7.69	2.56	5.12	2.56	5.12	2.56	2.56	2.56	2.56	5.12	2.56	2.56	2.56	10.25	7.69	5.12	5.12	12.80	2.56	5.12	99.87
12	NS	2	4	1	2	1	2	1	1	1	1	1	0	0	1	3	3	2	1	4	1	2	34
	RA %	5.88	11.76	2.94	5.88	2.94	5.88	2.94	2.94	2.94	2.94	2.94	0	0	2.94	8.82	8.82	5.88	2.94	11.76	2.94	5.88	99.96
13	NS	2	4	1	2	3	2	1	1	1	1	2	1	1	0	3	3	2	2	5	1	2	40
	RA %	5.00	10.00	2.50	5.00	7.50	5.00	2.50	2.50	2.50	2.50	5.00	2.50	2.50	0.00	7.50	7.50	5.00	5.00	12.50	2.50	5.00	100
14	NS	2	2	1	2	1	2	1	1	1	1	0	1	0	0	3	3	2	1	5	1	2	32
	RA %	6.25	6.25	3.12	6.25	3.12	6.25	3.12	3.12	3.12	3.12	0	3.12	0	0	9.37	9.37	6.25	3.12	15.62	3.12	6.24	96.81
15	NS	2	4	2	2	3	2	2	1	1	1	2	1	1	1	5	3	2	2	5	1	2	45
	RA %	4.44	8.88	4.44	4.44	6.66	4.44	4.44	2.22	2.22	2.22	4.44	2.22	2.22	2.22	11.11	6.66	4.44	4.44	11.11	2.22	4.44	99.92
16	NS	2	4	2	2	3	2	2	1	1	1	2	1	1	1	5	3	2	2	5	1	2	45
	RA %	4.44	8.88	4.44	4.44	6.66	4.44	4.44	2.22	2.22	2.22	4.44	2.22	2.22	2.22	11.11	6.66	4.44	4.44	11.11	2.22	4.44	99.92

(NS= Number of species; RA= Relative abundance)

Table 25. Seasonal distribution of bacterial species at different sites

Species	Spring	Summer	Autumn	Winter
C. freundii	2,6,7,8,9,10,11,12,13,15,16	1 to16	1,2,3,4,5,7,9,10,11,12,13,14,15	5,7,9,10,11,12,15,16
C. diversus	1,3,6,7,8,9,10,11,12,14,15,16	1 to 16	1,2,3,4,5,6,7,8,9,10,11,12,13,15,16	1,7,8,9,15,16
E. aerogenes	2,3,4,6,7,8,9,10,11,12,15,16	1 to 16	1,2,3,4,5,6,7,8,9,10,11,12,13,15,16	2,6,7,12,15,16
E. agglomerans	1,2,5,6,7,8,9,10,11,12,13,14,15,16	1 to 16	1,2,3,4,5,6,7,8,9,10,11,12,13,15,16	1,7,9,12,15,16
E. cloacae	1,2,3,5,6,7,9,10,11,12,13,15,16	1 to 16	1,2,3,4,5,7,8,9,10,11,12,13,15,16	1,3,6,7,8,10,11,12,13,15,16
E. intermedius	15,16	1,2,5,7,9,10,12,13,15,16	1,12,13,15,16	-
S. marcescens	2,6,7,8,9,10,11,12,15,16	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15	1,2,3,4,5,7,8,9,10,11,12,14,15,16	2,5,7,9,12,15,16
S .rubideae	15,16	1,2,5,9,10,14,15	4,15,16	10
S. flexneri	1,2,3,4,6,7,9,11,12,13,14.15,16	1 to 16	1,2,3,4,5,7,8,9,10,11,12,13,14,15,16	2,4,7,8,9,12,16
S. dysenteriae	1,2,3,4,8,9,10,11,12,14,15,16	1 to 16	1,3,4,5,6,7,8,9,10,11,12,13,14,15,16	4,7,8,9,10,11,12,15,16
S. typhi	1,2,3,4,6,7,8,9,10,11,12,13,15,16	1 to 16	1,2,3,4,5,7,8,9,10,11,12,15,16	1,2,4,5,9,10,12,15,16
S. paratyphi	15,16	1,2,3,4,5,9,10,13,15,16	3,4,13,15,16	16
S. choleraesuis	1,15,16	1,2,3,5,7,9,10,13,15,16	4,9,15,16	16
P. vulgaris	1,2,3,6,7,9,10,11,12,15,16	1 to 16	1 to 16	1,6,9,10,11,12,16
P. mirabilis	2,3,6,8,9,10,11,12,15,16	1 to 16	1,2,3,5,6,7,8,9,10,11,12,13,15,16	3,7,9,11,12,15,16
K. oxytoca	1,2,7,9,10,11,12,15,16	1,2,3,5,6,7,9,10,11,12,13,14,15,16	1,3,7,9,10,11,12,15,16	1,2,15,16
K. ozaenae	15,16	1,2,5,9,15,16	2,15,16	-
E.coli	1 to 16	1 to 16	1,2,3,4,5,6,7,8,9,10,11,12,13,16,16	1 TO 16
S. aureus	2,3,4,6,7,9,10,11,12,14,15,16	1 to 16	1,2,3,4,5,6,7,8,9,10,11,12,14,15,16	1,2,3,6,7,8,9,11,12,15,16
Y. enterocolitica	1,3,4,6,7,8,9,10,11,12,15,16	1,2,5,7,9,10,11,13,15,16	2,3,4,5,7,8,9,10,11,12,13,14,15,16	3,7,9,10,11,12,15
C. davisae	1,15,16	1,2,4,5,7,9,10,11,12,13,14,15	15,16	-
C. lapagei	15,16	1,2,5,7,9,10,11,13,14,15,16	1,7,9,10,12,15,16	-

Contd…….

Table 25 contd.

Species	Spring	Summer	Autumn	Winter
E. tarda	1,15,16	1,2,9,10,11,13,15,16	9,13,15,16	-
***Acinetobacter* spp.**	15,16	1,2,3,4,7,9,10,11,12,15,16	9,11,15,16	-
K. ascorbata	15,16	1 to 16	1,4,12,15,16	-
E. faecalis	1,2,3,4,5,7,8,9,10,11,12,13,14,15,16	1,2,3,4,5,8,9,10,11,12,13,14,15,16	1 to 16	1,2,3,4,5,7,8,9,10,11,12,13,14,15,16
E. faecium	1,3,4,9,10,11,13,15,16,	1,2,5,7,9,10,12,15,16	1,2,3,9,11,13,15,16	1,2,9,10,15
E. durans	15,16	1,2,5,7,9,10,12,15,16	8,15,16	-
E. hirae	15,16	1,2,5,7,9,10,11,13,15,16	1,9,10,15,16	-
E. mundtii	15,16	1,2,5,9,10,11,15,16	14,15,16	14
V. cholerae	6,7,10,11,12,15,16	1 to 16	1,3,4,5,7,9,10,11,12,13,15,16	-
V. parahaemolyticus	3,6,7,8,10,11,12,15,16	1 to 16	2,3,4,5,7,8,9,10,11,12,15,16	-
V. vulnificus	7,11,12,14,15,16	1 to 16	2,3,4,5,6,7,8,9,10,11,12,13,15,16	-
P. putida	7,9,10,11,12,15,16,	1 to 16	1,2,3,4,6,7,8,9,10,11,12,15,16	1,7,11,12,15,16
P. alcaligenes	4,5,6,7,8,9,10,11,12,15,16	1 to 16	1,2,3,4,5,7,8,9,10,11,12,13,14,15,16	2,3,4,5,7,10,11,12,15,16
A. hydrophila	-	1 to 16	9,13,15,16	1,5,6,7,9,11,12,15,16
A. caviae	15,16	1,2,5,9,10,11,15,16	1,9,15,16	-
B. sphaericus	1,15	1,2,5,7,9,10,11,13,14,15,16	1,2,3,4,5,7,8,9,10,11,12,13,14,15,16	1,14
B. subtilis	1,2,3,6,7,9,10,11,12,14,15,16	1 to 16	1,2,3,4,7,8,9,10,11,12,13,14,15,16	1,2,3,7,9,12,15
B. cereus	1,3,5,7,9,10,11,12,14,15,16	1 to 16	1,2,3,4,5,6,7,8,9,10,11,12,15,16	1,2,3,7,8,11,12,14,15
B. alcalophilus	1,7,9,10,11,12,14,15,16	1,2,3,4,5,6,7,8,9,10,11,12,13,15,16	1,2,3,4,5,6,7,8,9,10,11,12,15,16	1,5,7,8,9,11,15
B. brevis	1,3,5,7,9,10,11,12,15,16	1,2,3,4,5,6,7,9,10,11,12,13,14,15,16	1,2,3,4,5,6,7,8,9,10,11,12,14,15,16	3,7,9,11,12,15
A. faecalis	1,2,3,4,5,6,7,9,10,11,12,14,15,16	1 to 16	1,2,3,4,7,8,9,10,11,12,13,15,16	1,2,3,5,6,7,9,10,11,12,15,16
M. luteus	1,2,6,7,8,9,10,11,12,15,16	1 to16P	1,2,3,4,5,6,7,8,9,10,11,12,15,16	1,5,9,10,15,16
M. roseus	1,2,3,4,6,7,8,9,10,11,12,13,15,16	1 to 16	1 to 16	1,2,3,4,5,7,8,9,10,11,12,13,15,16

species were present at all the sampling sites in all the seasons. Among the genus *Cedacea,* two species were equally dominant at all sampling stations in various seasons except in winter season. From family Enterobacteriaceae species like *E. tarda, Acinetobacter* and *K. ascorbata* showed presence at various sites except in winter. Among this *Y. enterocolitica* was found to be more dominant at all study sites in all seasons. From genus *Enterococcus, E .faecalis* and *E. faecium* was present in all study sites in all seasons, except for *E. durans* and *E. hirae* which were absent in winter season. From genus *Vibrio,* all the three species were reported to be present at all sampling sites in summer and showed no presence in winter season. From genus *Pseudomonas,* both the species showed highest presence in summer at all the sites as compared to other seasons. Likewise, for genus *Aeromonas, A. hyrdophila and A. caviae* were present at different sites in different seasons but showed no presence in spring and winter season respectively.

4.12. Comparison of different microhabitats

The Bray Curtis cluster analysis diagram of the study sites developed on the basis of presence and absence of a species at the respective sites. Fig. 13 reveals that similarity ranged from 31 to 87% with the least similarity of 31% between site 1 and 7 and maximum similarity of 87% between site 3 and 4. The similarity indices given in Table 26 calculated on the basis of species composition reveal that there is a greater similarity in the species composition of the surveyed sites of the Lake.

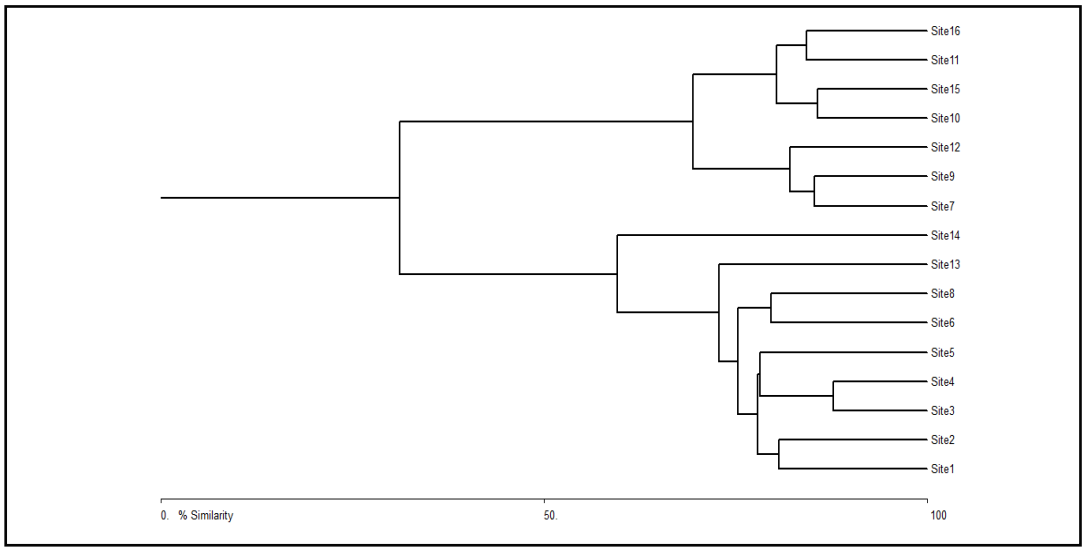


Fig.13. Bray Curtis similarity diagram of different microhabitats

Table 26. Similarity indices of different study sites

Study site group		Similarity index
I	**II**	**Bray curtis**
3	4	87.72
10	15	85.62
7	9	85.23
11	16	84.20
7	12	82.07
1	2	80.66
10	11	80.30
6	8	79.59
3	5	78.18
1	3	77.84
1	6	75.29
1	13	72.80
7	10	69.43
1	14	59.53
1	7	31.14

The bacterial flora in the lake consists of diverse pattern of life ranging from Proteobacteria to Firmicutes and to Actinobacteria which belonged to different habitats across the lake. The sites were categorized into four groups namely as open water, littoral, outlet and inlet sites. The bacterial population in the lake showed a diverse seasonal and temporal variation which were recorded on the basis of overall analysis of occurrence. *E.coli, E. faecalis, M. roseus, S. dysenteriae, S. flexneri, A. feacalis, S. typhi, Y. enterocolitica, E. aerogenes and B. cereus* were found to be dominant species as per their highest order of occurrence across all categories of the lake. *E.coli, E. feacalis, E. feacium, S. typhi, M. roseus, P. vulgaris, A. hyrdophila, B. subtilis, B. cereus and B. alcalophillus* are the most dominant representatives of the open water sites. *E.coli, E. faecalis, B. subtilis, S. flexneri, S. dysenteriae, S. typhi, A. faecalis, M. roseus, S. aureus and E. aerogenes* were found to be dominating at littoral sites. At inlet sites the dominant species recorded were *E.coli, M. roseus, S. aureus, E. faecalis, E. cloacae, B. cereus, S. flexneri, S. dysenteriae, B. brevis* and *B. alcalophillus.* and at out let sites, the most dominant representative of bacterial species reported were *E.coli, E. faecalis, S. dysenteriae, M. roseus, Y. enterocolitica, A. feacalis, B. brevis, S. flexneri, E. aerogenes* and *E. agglomerans.* It is quite evident

from the results cited in the Table 27 that different species were dominating different habitats of the lake.

Table 27. Most dominant species as per the percentage occurrence Across the microhabitats.

Across the lake			
E.coli *E. faecalis* *M. roseus* *S. dysenteriae* *S. flexneri* *A. faecalis* *S. typhi* *Y. enterocolitica* *B. cereus* *E. aerogenes*			
Across the major habitats			
Open water site	**Littoral site**	**Inlet site**	**Outlet site**
E.coli	*E.coli*	*E.coli*	*E.coli*
E .faecalis	*E. faecalis*	*M. roseus*	*M. roseus*
S .typhi	*B. subtilis*	*S. aureus*	*S. dysenteriae*
M. roseus	*S. flexneri*	*E .faecalis*	*E .feacalis*
E. faecium	*S. dysenteriae*	*E. cloacae*	*Y. enterocolitica*
P .vulgaris	*S .typhi*	*B. cereus*	*A. faecalis*
A. hydrophila	*A. faecalis*	*S .flexneri*	*B. brevis*
B. subtilis	*M. roseus*	*S. dysenteriae*	*S .flexneri*
B. cereus	*S. aureus*	*B .brevis*	*E. aerogenes*
B. alcalophillus	*E. aerogenes*	*B. alcalophillus*	*E. agglomerans*

Contd……..

Table 27 contd.

Site 1	Site 2	Site 3	Site 4
E.coli	*E.coli*	*E.coli*	*E.coli*
E. faecalis	*E. faecalis*	*S. faecalis*	*M. roseus*
S. typhi	*B. subtilis*	*S. aureus*	*S. dysenteriae*
M. roseus	*S. typhi*	*E .faecalis*	*E. faecalis*
E. faecium	*S. flexneri*	*B .brevis*	*S. typhi*
P. vulgaris	*A. faecalis*	*E. cloacae*	*S. flexneri*
A. hydrophila	*M. roseus*	*Y. enterocolitica*	*S. aureus*
B. subtilis	*S .aureus*	*B. subtilis*	*P. alcaligenes*
B .cereus	*E. aerogenes*	*B. cereus*	*A. hydrophila*
A. faecalis	*P. alcaligenes*	*S. flexneri*	*B. brevis*
Site 5	**Site 6**	**Site 7**	**Site 8**
E.coli	*E.coli*	*E.coli*	*E.coli*
E. faecalis	*E. aerogenes*	*S. flexneri*	*E. faecalis*
S. dysenteriae	*S. aureus*	*E. faecalis*	*S. dysenteriae*
M. roseus	*E. cloacae*	*C. freundii*	*S. flexneri*
V. cholerae	*S. flexneri*	*E. cloacae*	*S. typhi*
S .typhi	*P. vulgaris*	*S. typhi*	*M. roseus*
B. alcalophillus	*C. diversus*	*P. alcaligenes*	*E. cloacae*
M. luteus	*P. alcaligenes*	*M. roseus*	*C. diversus*
S. marcescens	*Y. enterocolitica*	*E. aerogenes*	*Y. enterocolitica*
B. subtilis	*E. faecalis*	*S. marcescens*	*B. alcalophillus*
Site 9	**Site 10**	**Site 11**	**Site 12**
E.coli	*E.coli*	*E. faecalis*	*E.coli*
S. dysenteriae	*M. roseus*	*E.coli*	*E. faecalis*
E. faecalis	*E. faecalis*	*S. dysenteriae*	*E. aerogenes*
M. roseus	*B. subtilis*	*M. roseus*	*S. dysenteriae*
S. flexneri	*A. faecalis*	*Y. enterocolitica*	*S. flexneri*
S. typhi	*S. dysenteriae*	*A. faecalis*	*M. roseus*
E. agglomerans	*Y. enterocolitica*	*B. brevis*	*B. brevis*
P. vulgaris	*B. cereus*	*S. flexneri*	*C. freundii*
B. subtilis	*E. cloacae*	*E. aerogenes*	*S. typhi*
B. alcalophillus	*S. flexneri*	*E. agglomerans*	*Y. enterocolitica*
Site 13	**Site 14**	**Site 15**	**Site 16**
E.coli	*E.coli*	*E.coli*	*E.coli*
E. faecalis	*E. faecalis*	*E. faecalis*	*E. faecalis*
E. cloacae	*B. cereus*	*M. roseus*	*M. roseus*
M. roseus	*S. aureus*	*S. dysenteriae*	*S. dysenteriae*
S. flexneri	*A. hydrophila*	*A. faecalis*	*S. typhi*
S. dysenteriae	*C. diversus*	*S. flexneri*	*S. flexneri*
V. cholerae	*S. flexneri*	*P. mirabilis*	*Y. enterocolitica*
Y. enterocolitica	*P. alcaligenes*	*P. alcaligenes*	*V. cholerae*
A. faecalis	*E. agglomerans*	*S. typhi*	*S. marcescens*
C. freundii	*S. dysenteriae*	*Y. enterocolitica*	*A. faecalis*

The most common species with their seasonal occurrence in all the sites within the categorized groups listed in the Table 28 depicts that 26 bacterial species were found to be common in all the groups. At open water sites viz. *C. freundii, C. diversus, E. aerogenes, E. agglomerans, E. cloacae, E. intermedius, S. marcesens, S. flexneri, S. dysenteriae, S. typhi, S. choleraesuis, P. vulgaris, P. mirabilis, K. oxytoca, E.coli, S. aureus, Y. enterocolitica, C. davisae, C. lapagei, E. tarda, E. faecalis, E. hirae, V. cholerae, V. parahaemolyticus, V. vulnificus, P. putida and M. roseus;* at littoral sites viz. *C. freundii, C. diversus, E. agglomerans, E. cloacae, S. marcescens, S. flexneri, S. dysenteriae, S. typhi, P. vulgaris, P. mirabilis, E.coli, S. aureus, Y. enterocolitica, E. faecalis, E. faecium, V. cholerae, V. parahaemolyticus, V. vulnificus, P. putida, P. alcaligenes, A. hyrdophila, B. subtilis, B. cereus, B. alcalophillus, M. luteus* and *M . roseus*; at inlet sites viz. *C. freundii, C. diversus, E. aerogenes, E. agglomerans, E. cloacae, S. marcescens, S. flexneri, S. dysenteriae, S. typhi, P. vulgaris, P. mirabilis, E.coli, S. aureus, Y. enterocolitica, E. faecalis, V. cholerae, V. parahaemolyticus, V. vulnificus, P. putida, P. alcaligenes, A. hyrdophila, A. caviae, B. sphaericus, B. subtilis, B. cereus, B. alcalophillus and M. roseus;* at outlet sites viz. *C. freundii, C. diversus, E. aerogenes, E. agglomerans, E. cloacae, S. marcescens, S. flexneri, S. dysenteriae, S. typhi, P. vulgaris, P. mirabilis, K. oxytoca, E.coli, S. aureus, Y. enterocolitica, C. lapagei, K. ascorbata, E. faecalis, E. faecium, V. cholerae, V. parahaemolyticus, V. vulnificus, P. putida, P. alcaligenes, A. hyrdophila, B. subtilis* and *M. roseus* were found to be common.

A comparative analysis of four basins of Dal lake in terms of bacterial load shown in Fig. 14 indicated heavy bacterial load in Gagribal followed by Nishat, Hazratbal and Nigeen basins. Similarly analysis for outlet sites shown in Fig. 15 depicts heavy bacterial load in Pokhribal as compared to Dal lock gate and for inlet sites shown in Fig. 16 heavy bacterial load in Tailbal Nallah as compared to Boathall Nallah.

4.13. Statistical appraisal of the data

The analysis of variance (ANOVA) presented in Table 30 carried out between the different sites for bacterial species isolated from different microhabitats showed that 71% results were statistically significant ($p < 0.05$) with 7% as highly significant ($p<0.01$).

Table 28. Most common species as per their occurrence across the major habitats.

Open water site	Littoral site	Inlet site	Outlet site
C. freundii	*C. freundii*	*C .freundii*	*C. freundii*
C .diversus	*C. diversus*	*C. diversus*	*C. diversus*
E. aerogenes	*E. agglomerans*	*E. aerogenes*	*E. aerogenes*
E. agglomerans	*E. cloacae*	*E. agglomerans*	*E. agglomerans*
E. cloacae	*S. flexneri*	*E. cloacae*	*E. cloacae*
E. intermedius	*S. marcescens*	*S. marcescens*	*S. marcescens*
S .marcescens	*S. dysenteriae*	*S. flexneri*	*S. flexneri*
S. flexneri	*S. typhi*	*S. dysenteriae*	*S. dysenteriae*
S .dysenteriae	*P. vulgaris*	*S. typhi*	*S. typhi*
S .typhi	*P. mirabilis*	*P. vulgaris*	*P. vulgaris*
S. choleraesuis	*E. coli*	*P. mirabilis*	*P. mirabilis*
P. vulgaris	*S. aureus*	*E.coli*	*K. oxytoca*
P. mirabilis	*Y. enterocolitica*	*S. aureus*	*E.coli*
K. oxytoca	*E. faecalis*	*Y. enterocolitica*	*S. aureus*
E.coli	*E. faecium*	*E. faecalis*	*Y. enterocolitica*
S. aureus	*V. cholerae*	*V. cholerae*	*C. lapagei*
Y. enterocolitica	*V. parahaemolyticus*	*V. parahaemolyticus*	*K. ascorbata*
C. davisae	*V. vulnificus*	*V. vulnificus*	*E. faecalis*
C. lapagei	*P. putida*	*P. putida*	*E. faecium*
E. tarda	*P. alcaligenes*	*P. alcaligenes*	*V. cholerae*
E. faecalis	*A. hydrophila*	*A .hydrophila*	*V. parahaemolyticus*
E. hirae	*B. subtilis*	*A. caviae*	*V. vulnificus*
V. cholerae	*B. cereus*	*B. sphaericus*	*P. putida*
V. parahaemolyticus	*B. alcalophillus*	*B. subtilis*	*P. alcaligenes*
V. vulnificus	*M. luteus*	*B. cereus*	*A. hydrophila*
P .putida	*M. roseus*	*B. alcalophillus*	*B. subtilis*

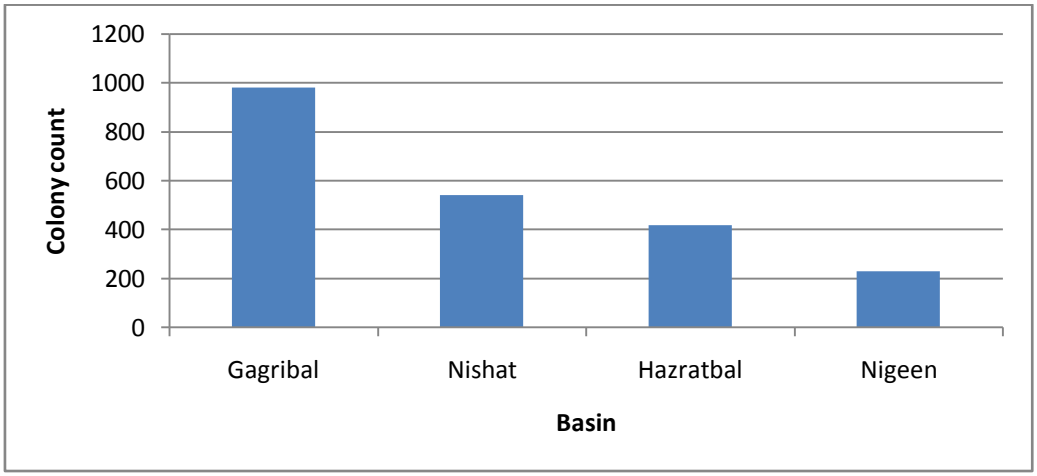


Fig. 14. Comparative analysis of bacterial load at four basins

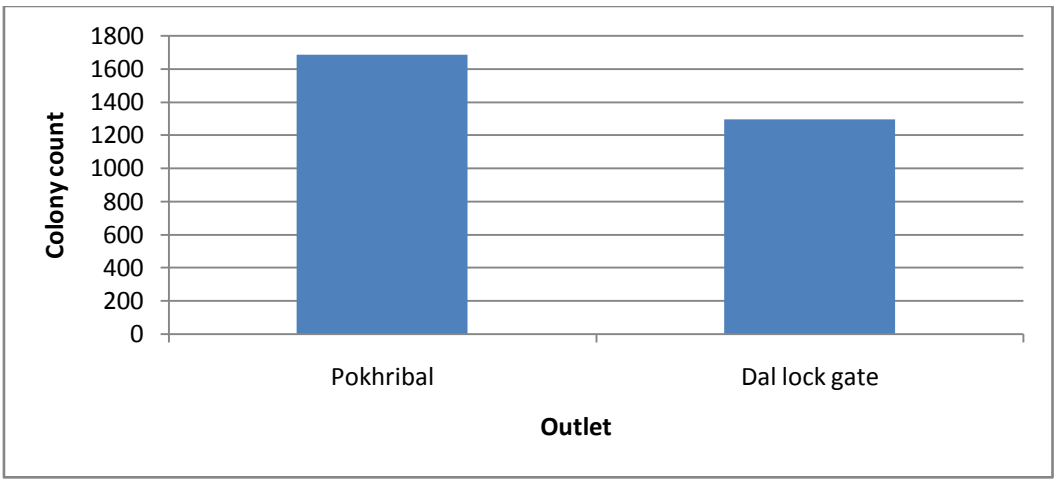


Fig. 15. Comparative analysis of bacterial load at outlet

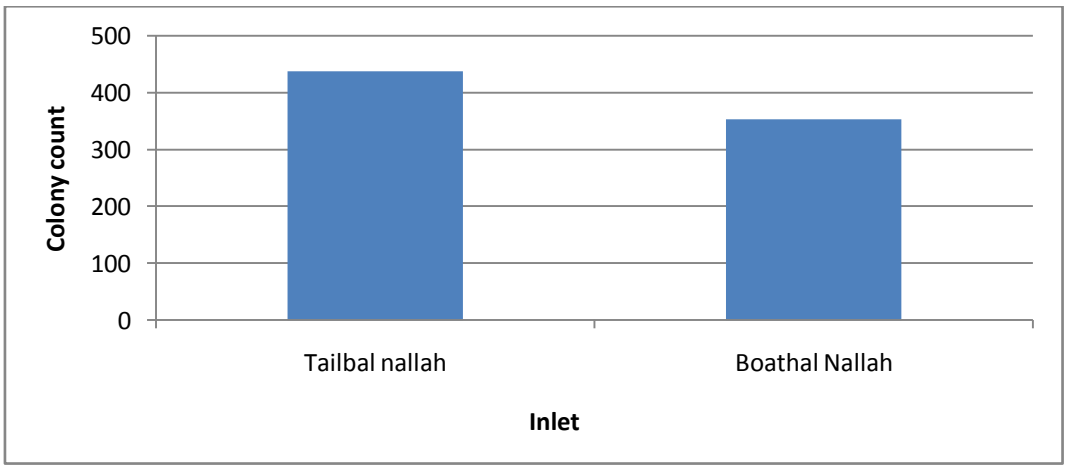


Fig. 16. Comparative analysis of bacterial load at inlet

4.14. Diversity and evenness patterns

From the values of different indices computed for sixteen sites for the occurrence of different bacterial species presented in Table 29 shows that Shannon-Wiener (H) index is highest (3.68) at site 16 followed closely by the species rich site 9 (3.67), site 15 (3.65), site 10 (3.62), site 1 (3.61) and lowest at site 6, 14 (3.27) and 8 (3.26). The one way Analysis of variance (ANOVA) carried out showed that the Dominance ($F = 50.57$ $F_{crit} = 4.17$, $p < 0.01$), Diversity ($F = 17.73$, $F_{crit} = 4.17$, $p < 0.01$) and Evenness ($F = 41.28$, $F_{crit} = 4.17$, $p < 0.01$) varied significantly between different microhabitats of the lake. The evenness pattern calculated was also highest (0.92) at site 3 and 12 and lowest (0.73) at site 5 and 13.

Table 29. Diversity and evenness pattern

Sites	Dominance (D)	Shannon (H)	Evenness (E)
S1	0.030	3.61	0.82
S2	0.033	3.56	0.78
S3	0.033	3.44	0.92
S4	0.035	3.41	0.89
S5	0.041	3.44	0.73
S6	0.041	3.27	0.90
S7	0.032	3.47	0.85
S8	0.043	3.26	0.87
S9	0.028	3.67	0.87
S10	0.029	3.62	0.85
S11	0.031	3.53	0.87
S12	0.033	3.44	0.92
S13	0.046	3.37	0.73
S14	0.044	3.27	0.82
S15	0.029	3.65	0.86
S16	0.028	3.68	0.88

Table 30. Detailed result of ANOVA test carried out pair wise between the bacterial species at sixteen sites

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16
S1		3.71	0.15	0.46	0.38	11.55	9.72	5.66	16.82	39.63	49.02	18.62	8.62	36.66	56.09	86.76
		NS	NS	NS	NS	*	*	**	*	*	*	*	*	*	*	*
S2			1.95	1.12	0.89	2.42	20.19	0.42	33.91	55.43	60.14	29.22	1.43	17.28	71.93	102.6
			NS	NS	NS	NS	*	NS	*	*	*	*	NS	*	*	*
S3				0.08	0.06	7.71	10.98	3.52	18.29	41.1	50.4	19.93	5.72	26.08	57.56	88.22
				NS	NS	*	*	NS	*	*	*	*	**	*	*	*
S4					0	5.82	12.25	2.43	20.05	42.9	51.85	21.27	4.24	21.48	59.4	90.09
					NS	*	*	NS	*	*	*	*	**	*	*	*
S5						4.63	10.94	1.99	17.2	39.7	49.96	19.74	3.45	16.18	55.98	86.47
						**	*	NS	*	*	*	*	NS	*	*	*
S6							30.05	0.56	49.37	67.95	68.69	38.23	0.05	5.44	84.04	114.31
							*	NS	*	*	*	*	NS	**	*	*
S7								22.47	0.07	10.5	24.11	2.52	26.44	50.76	22.32	48.01
								*	NS	*	*	NS	*	*	*	*
S8									35.9	57.66	62.37	31.46	0.23	8.25	74.17	104.86
									*	*	*	*	NS	*	*	*
S9										10.73	24.51	2.2	42.27	86.92	23.28	50.05
										*	*	NS	*	*	*	*
S10											5.71	2.06	62.94	91.98	2.78	17.37
											**	NS	*	*	NS	*
S11												12.11	65.9	82.84	0.87	1.97
												*	*	*	NS	NS
S12													35.14	55	8.79	27.91
													*	*	*	*
S13														5.52	79.31	109.86
														**	*	*
S14															106.11	134.61
															*	*
S15																6.52
																**
S16																

Pair of variables marked with the asterisks co-vary non randomly (* = P< 0.01, ** = P<0.05, NS = Non significant)

4.15. Relation of water temperature and pH with bacterial load

The pH values of the collected water samples fluctuated between 7.2-9.3 in spring, 7.3-8.6 in summer, 6.6-8.8 in autumn and 7.5-8.4 in winter at the different sites of the lake, showing a lot of variation from one site to another in different seasons of the study. The data of correlation analysis between the pH and bacterial load shown in Figures 17-32 of the sixteen sites respectively indicated that in the entire sites and seasons there was a negative correlation of pH with the bacterial load indicating that with increasing pH the bacterial load got decreased. Similarly the water temperature fluctuated a great deal from one season to another in different sites of the lake with maximum temperature in summer seasons and minimum in winter seasons. The temperature of water at the different sites fluctuated between 14-22°C in spring, 16-25.8°C in summer, 12.5-19.4°C in autumn and 3.2-6.4°C in winter. Here the correlation analysis between water temperature and bacterial load shown in Figures 33-48 of the sixteen sites respectively showed that there was a strong positive correlation of bacterial load with temperature all through the sites and seasons indicating that with increasing water temperature the bacterial load got increased and the results were significant as shown in Table 31.

Table 31. Correlation of bacterial load at different sites with pH and temperature of water.

Sites	pH		Temperature	
	r	p value	r	p value
S1	-0.031*	0.942	0.429	0.28
S2	-0.385	0.34	0.454	0.25
S3	-0.624	0.09	0.847**	0.008
S4	0.882**	0.004	0.647	0.08
S5	-0.544	0.16	0.288	0.48
S6	-0.183	0.66	0.578	0.13
S7	-0.215	0.6	0.571	0.13
S8	-0.047	0.92	0.544	0.16
S9	-0.252	0.54	0.897**	0.003
S10	-0.051	0.89	0.861**	0.006
S11	-0.265	0.52	.990**	0
S12	-0.478	0.23	0.956**	0
S13	-0.648	0.08	0.672	0.06
S14	-0.021	0.96	.759**	0.02
S15	-0.622	0.1	0.651	0.08
S16	-0.157	0.71	.909**	0.002

(=P value< 0.01;**=p value <0.05)*

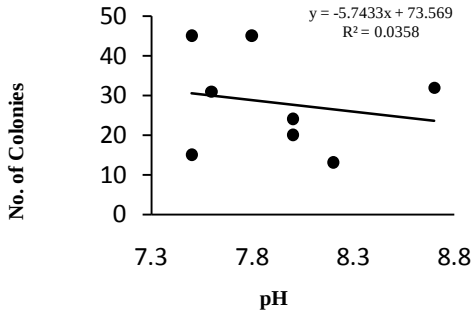


Fig.17. Correlation of pH with bacterial load at Site 1

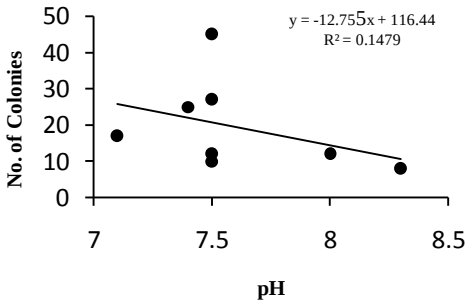


Fig.18. Correlation of pH with bacterial load at Site 2

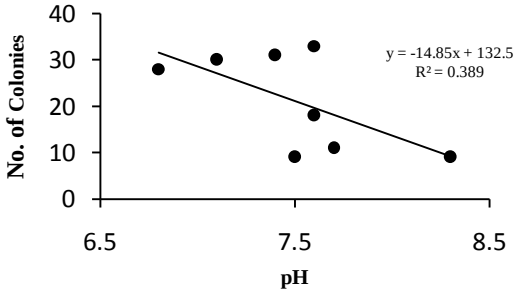


Fig.19. Correlation of pH with bacterial load at Site 3

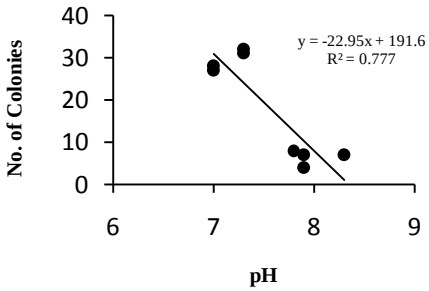


Fig.20. Correlation of pH with bacterial load at Site 4

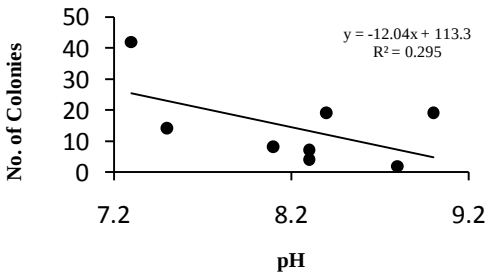


Fig.21. Correlation of pH with bacterial load at Site 5

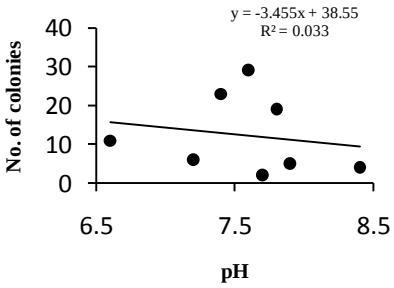


Fig.22. Correlation of pH with bacterial load at Site 6

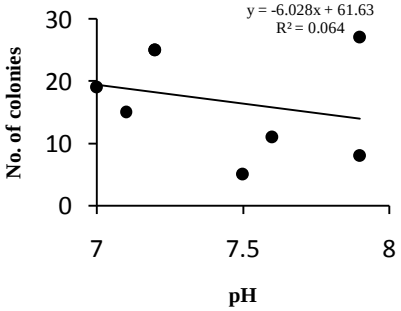


Fig.23. Correlation of pH with bacterial load at Site 7

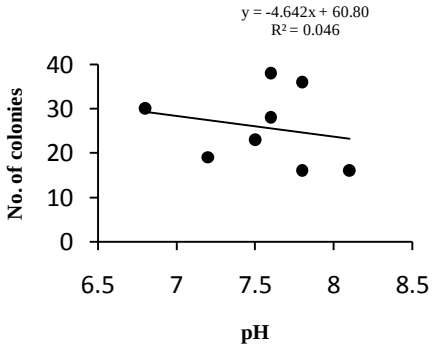


Fig.24. Correlation of pH with bacterial load at Site 8

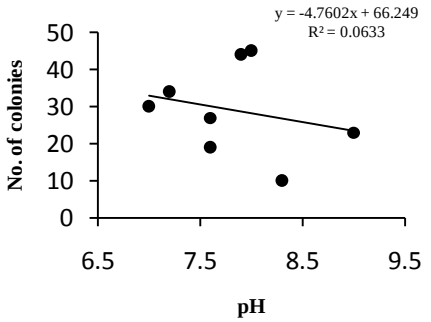


Fig.25. Correlation of pH with bacterial load at Site 9

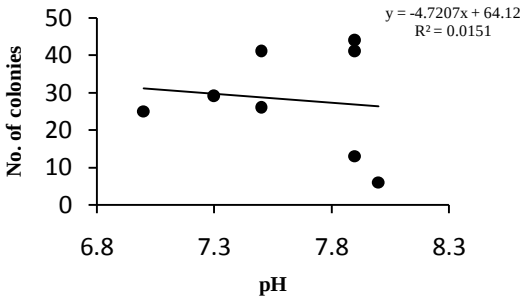


Fig.26. Correlation of pH with bacterial load at Site 10

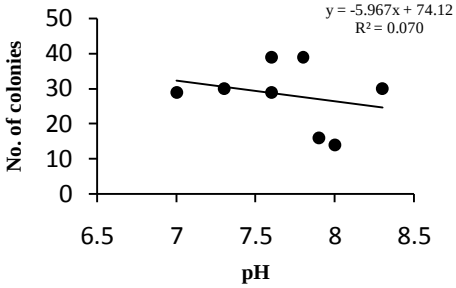


Fig.27. Correlation of pH with bacterial load at Site 11

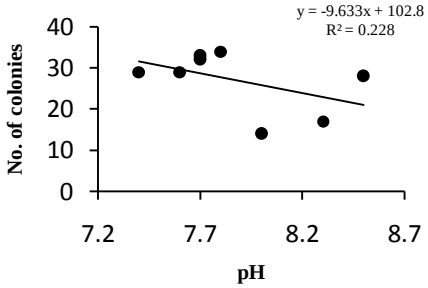


Fig.28. Correlation of pH with bacterial load at Site 12

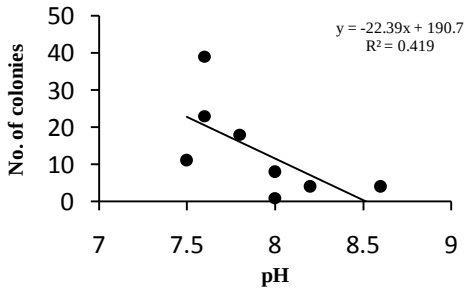


Fig.29. Correlation of pH with bacterial load at Site 13

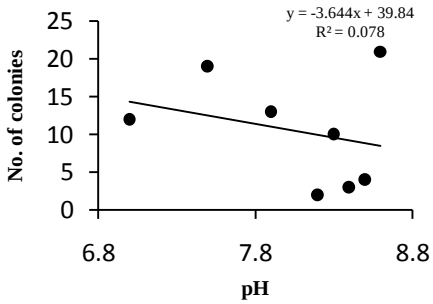


Fig.30. Correlation of pH with bacterial load at Site 14

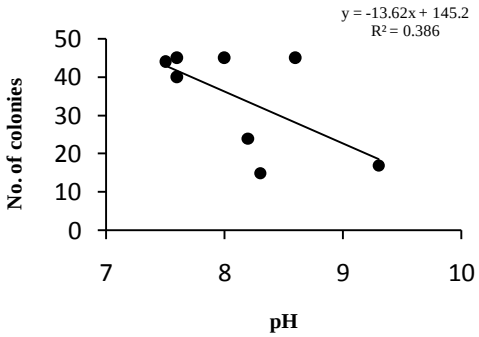


Fig.31. Correlation of pH with bacterial load at Site 15

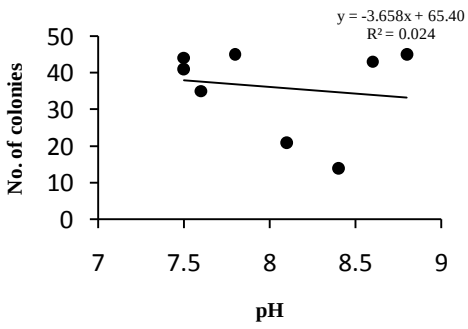


Fig.32. Correlation of pH with bacterial load at Site 16

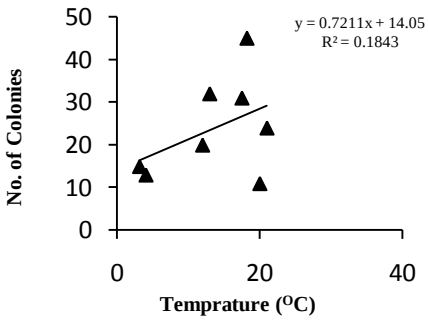


Fig.33. Correlation of temperature with bacterial load at site 1

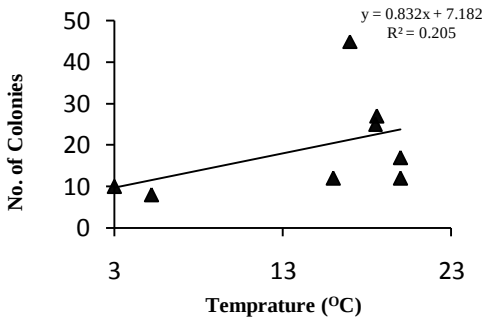


Fig.34. Correlation of temperature with bacterial load at site 2

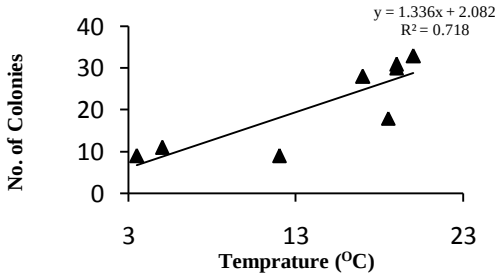


Fig.35. Correlation of temperature with bacterial load at site 3

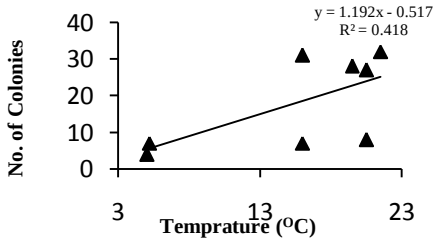


Fig.36. Correlation of temperature with bacterial load at site 4

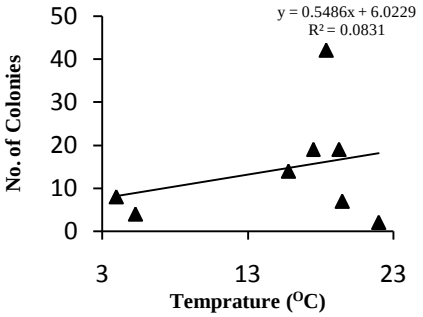


Fig.37. Correlation of temperature with bacterial load at site 5

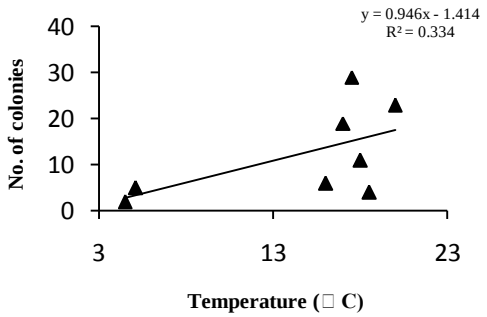


Fig.38. Correlation of temperature with bacterial load at site 6

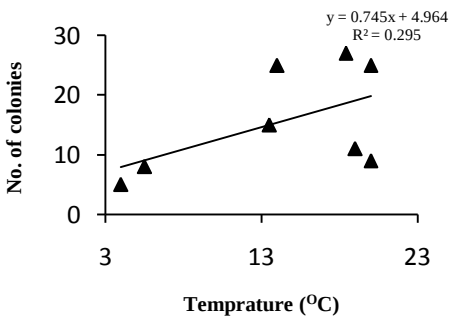


Fig.39. Correlation of temperature with bacterial load at site 7

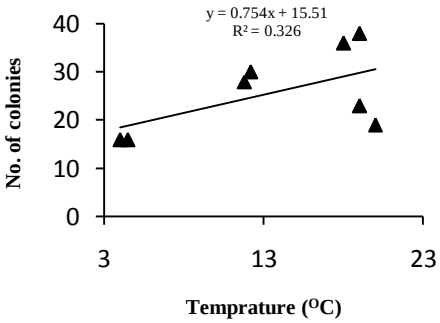


Fig.40. Correlation of temperature with bacterial load at site 8

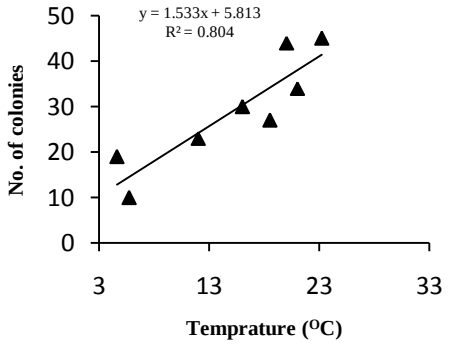


Fig.41. Correlation of temperature with bacterial load at site 9

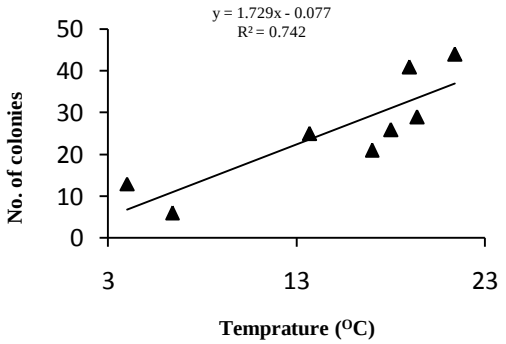


Fig.42. Correlation of temperature with bacterial load at site 10

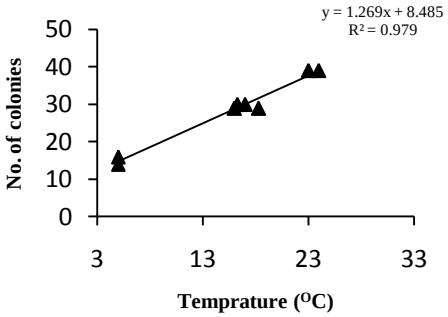


Fig.43. Correlation of temperature with bacterial load at site 11

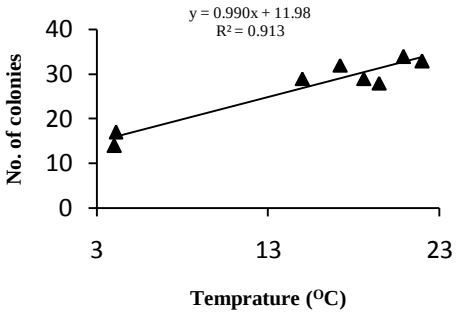


Fig.44. Correlation of temperature with bacterial load at site 12

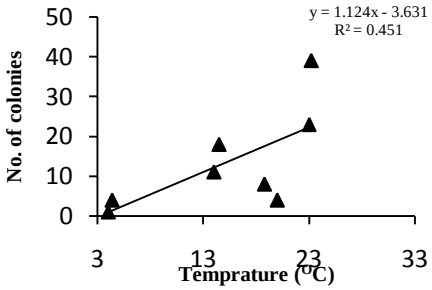


Fig.45. Correlation of temperature with bacterial load at site 13

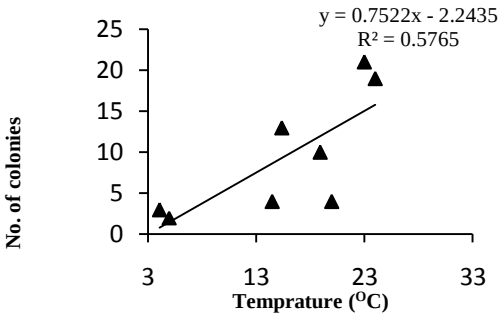


Fig.46. Correlation of temperature with bacterial load at site 14

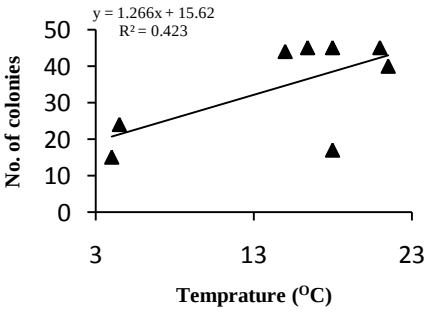


Fig.47. Correlation of temperature with bacterial load at site 15

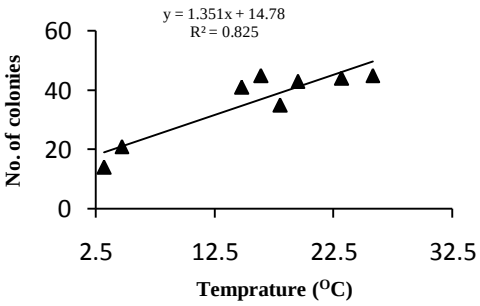


Fig.48. Correlation of temperature with bacterial load at site 16

4.16. Symptom incidence in Dal inhabitants

For the purpose of study, out of 320 families having a population of 1920 individuals, 384 individuals, which is 20 % of the population (n= 384; comprising of 211 (54.95%) males and 173 (45.05%) females, (Table 32) who are actively engaged in one or other activity related to lake water like rowing of shikaras ,extraction of nadru, washing of clothes and for other daily uses, were randomly selected for the purpose of study of prevalence of the symptoms due to pathogenic bacteria isolated in the Dal lake through questionnaire. The population selected for the purpose was interviewed as per the questionnaire (Annexure III) which was recorded on spot. For the sake of clear understanding, the recorded data was analyzed and the observations are as under:-

4.16.1. Age wise incidence of symptoms

Among the 384 individuals, 43 individuals (11.19%) of the study population comprising of 23 males (5.99%) and 20 females (5.20%) reported the symptoms. With the view of maintaining age stratification (Table 32 and 34) study population was divided into three age groups of upto 20 years (first age group), 21-40 years (second age group) and 41 years and above (third age group). In the first age group, out of 172 individuals, 27 individuals (15.7%) comprising of 16 males (9.30%) and 11 females (6.39%) reported symptoms; In the second age group, out of 139 individuals, 5 individuals (3.6%) comprising of 2 males (1.43%) and 3 females (2.15%) reported symptoms and in the third age group, out of 73 individuals, 11 individuals (15.06%) comprising of 5 males (6.85%) and 6 females (8.22%) reported symptoms. Statistically analyzing the data of prevalence of symptoms in the associated population by applying Chi-square test showed it was significant.

4.16.2. Sex wise prevalence of infection

Data analysis of the studied population who reported the symptoms (Table 33) reveals that out of 43 individuals , 26 individuals (6.77%) had symptoms of diarrhea, bloody stools, nausea, vomiting and fever; and 17 individuals (4.42%) reported symptoms of high fever ,chills, rigors, sweating and body aches.

Out of 26 individuals, 19 received medical treatments from health institutions, 7 received treatment from local medical shops.

Out of 17 individuals, 10 received medical treatments from health institutions,

7 received treatment from local medical shops.

Statistically analyzing the data by applying Chi-square test showed that the results were insignificant.

4.16.3. Prevalence of infection on the basis of type of water used for different purposes

The prevalence of symptoms also varied with the type of water being used for different purposes (Table 35) among 384 individuals, 264 (68.75%) were using lake water and 120 (31.25%) were using the tap water .Higher prevalence of symptoms 13.25% (35 out of 264 individuals) was seen among the individuals using lake water for different domestic purposes followed by the positive prevalence of 6.66% (8 among 120 individuals).

Statistical analysis of the data by applying Chi-square test showed that the results were nearly significant.

Table 32. Sex and age distribution of population (n=384).

Age group	Male	Female	Total
< 20 years	87	85	172
21 - 40 years	71	68	139
> 40 years	53	20	73
Total	**211**	**173**	**384**

Table 33. Prevalence of various symptoms in the study population.

Symptoms	No of cases	Percentage	$\chi^2_{(df)}$	p value
Loose motions, bloody stools, nausea, vomiting and fever	26	6.77	13.14 (2)	0.001
High fever, Rigors , chills, Sweating and body aches	17	4.42		
Total	**43**	**11.19**		

Table 34. Sex and age wise symptoms in study population.

Age group	No of persons interviewed	No of Cases with symptoms	Male percentage	Female percentage	χ^2(df)	p value
< 20 years	172	27 (15.7%)	16 (9.30%)	11 (6.39%)	1.01 (2)	0.6
21 - 40 years	139	5 (3.6 %)	2 (1.43%)	3 (2.15%)		
> 40 years	73	11 (15.06%)	5 (6.85%)	6 (8.22%)		
Total	**384**	**43 (11.19%)**	**23 (5.99%)**	**20 (5.20%)**		

Table 35. Prevalence of symptoms with respect to water usage in study population.

Type of water	No of persons interviewed	No of Cases with symptoms	Percent prevalence	χ^2(df)	p value
Lake water	264	35	13.25	3.6 (1)	0.058
Tap water	120	8	6.66		

Chapter: 5

Discussion

Dal lake, a catchment of the Zabarwan mountain valley, a subsidiary of the Himalayan range, remains completely influenced by different types of anthropogenic activities all through the year, and provides a unique opportunity to investigate its microbial diversity, as Dal lake ecosystem is of particular interest because of its voluminous nature, diversity of microhabitats within the lake, influence of variable climatic conditions and the variations in water temperature. Water samples collected from different microhabitats of the lake were extensively studied in terms of microbial component and some physiochemical parameters like pH and temperature. Although till now a lot of work has been carried out on the biodiversity components of the various lakes but the present findings are the attempts offered for the first time when bacteria from the Dal lake have been specifically targeted for culture and molecular identification and as such, this is the initial detailed study of known heterotrophic bacteria in the lake water. World over, many lakes and other lotic and lentic water bodies in different environmental setups have been increasingly analyzed with regards to microbes, with some of these environments as potential sources of microbes that have been removed from the global gene pool for evolutionarily significant periods of time and allowed the study of processes of microbial speciation (Vincent, 2000). The lakes with of urban

environmental setups offer a potentially good habitat for microorganisms due to the presence of nutrients and minerals (Sharp *et al*., 1999). Generally, anthropogenic activities such as urban development, the releases of untreated sewage water, runoff associated with human and animal waste from impervious surfaces, groundwater drainage, faulty sewage pipes are believed to have significant effects on microbial communities (Mallin *et al.*, 2000; NRDC, 2006), hence characterization of microbial communities is therefore important to determine what kind of microorganisms are associated with specific water and habitat properties as well as to gain understanding of the ecological function of the microbes.

Microorganisms are widely distributed in nature in their abundance and diversity and may be used as an indicator for the suitability of water (Okpokwasili and Akujobi, 1996). According to Bonde (1977), bacteriological examination of water is a powerful and foremost tool in order to foreclose the presence of bacteria that might constitute a health hazard. Bacteria that are used commonly as indicator of water include coliforms. Naturally, waters contain a large number and variety of bacteria, which does not necessarily make such water potable. The use of bacteria as water quality indicators can be viewed in two ways: first, the presence of such bacteria can be taken as an indication of faecal contamination of the water and thus as a signal to determine why such contamination is present, how serious it is and what steps can be taken to eliminate it; second, their presence can be taken as an indication of the potential danger of health risks that they possess. The higher the level of indicator bacteria and the level of faecal contamination, greater is the risk of water-borne diseases (Pipes, 1981). A wide range of pathogenic microorganisms can be transmitted to humans via water contaminated with faecal material. These include entero-pathogenic agents such as *Salmonella*, *Shigella* spp. etc as well as opportunistic pathogens like *P. aeruginosa*, *Klebsiella* spp., *V. parahaemolyticus* and *A. hyrdophila* (Hodegkiss, 1988). It is not practicable to test water for all these organisms. This is because the isolation and identification of many of these is extremely complicated and seldom quantitative (Cairneross *et al*., 1980; World Health Organization, 1983). An indirect approach is based on assumption that the estimation of groups of normal enteric organism will indicate the level of faecal contamination of the water (WHO, 1983). Concurrently, contamination of water by enteric pathogens

has increased worldwide (Craun, 1986; Pathak *et al*., 1991; Islam *et al*., 2001). Kistemann *et al*. (2002) observed that in the case of rainfall, the microbial loads of running water may suddenly increase and reach water bodies very quickly. The observations indicate that the bacterial contamination increases from inlet to outlet having highest percentage occurrence at site Pokhribal outlet. The heavy bacterial load in the littoral zones and the extreme exit points of the lake was due to increased anthropogenic and socio-cultural activities. Rapid increase in human population and tourist influx in the surrounding vicinity of the lake may also add strains in the runoff to an extent resulting in the degradation of its quality. Mc Lellan *et al*. (2001) stated that faecal pollution indicator organisms can be used for a number of conditions related to the health of aquatic ecosystems and to the potential for health effects among individuals using aquatic environments. The presence of such indicator organisms may provide indication of water-borne problems and is a direct threat to human and animal health. Our studies on bacterial diversity in the water of the Dal lake in relation to pollution have clearly revealed that there is significant presence of bacterial indicators of faecal pollution in all stretches, the situation of Dal lake is not very serious but alarming. Presence of bacterial indicators of faecal contamination in different latitudes of Dal lake clearly reveals the bacteriological status of the water at that site. For this reason, monitoring of bacterial contamination in the inlet of lake should be an essential component of the protection strategy in lake area. The base line data generated on bacteriological water quality of the lake may serve as bio-monitoring standard and comparisons for other lakes including Anchar, Manasbal and Wular etc and may be useful for all scientists, decision makers and resource managers working with environmental planning and management of such areas. For the purpose of my study sixteen (16) sites from this lake ecosystem (8 sites from the 4 basins, 4 sites from two inlets and 4 other sites from two outlets) were selected and samples collected on seasonal basis during 2010-2011 and 2011-2012 for isolation and identification of bacteria from the water samples of the lake which offered evidence to the range of microbial diversity in the lake, and further supports the likelihood of a complex ecosystem within the lake and to determine if lake could be the source of water-borne bacterial contamination to human populations in the area.

5.1. Physico-chemical Parameters

Environmental factors affect the abundance, species, stability, productivity, and physiological condition of aquatic microbial populations. The microbial ecology of Dal lake is partly elucidated by relating abundances of bacteria to some physico-chemical properties of the water.

Physico-chemical tests were conducted to determine if these parameters of the lake could support water-borne bacterial communities. Present results were compared with those found in previous environmental studies (Westerfield, 1996; Wolf, 1996). All values were within physiologic ranges, with our results which determined survival of bacterial pathogens or their growth in the lake water.

Temperature (°C) ranged from 3.2°C to 25.8°C, correlates with seasonal changes. The broad range of temperatures measured suggested that water temperature may be an important indicator of the abundance of bacterial growth in the lake water. At lower temperatures, the heterotrophic plate counts for the water samples were consistently lower than the counts at higher temperatures. However, additional bacteria may survive at lower temperatures in viable, but non-culturable states (Roszak and Colwell, 1987; Davies *et al*. 1995). Now it is quite clear from the study that bacteria are directly related to the ambient water temperature, with increase in water temperature from spring onwards there is corresponding increase in bacterial count indicating that it governs the bacterial dynamics in the lake which is in conformation with other works (Sastry *et al*., 1970; Verma and Paul, 1996; Hadas 1988, 2000). According to Wetzel (1975) bacterial biomass is generally lower during winter than summer in temperate lakes, which can be correlated with low winter temperature and reduced loading of particulate and dissolved organic substrates from autochthonous (phytoplankton, littoral plants) and allochthonous sources.

The pH of the water is the most important single chemical parameter of natural water, because it dictates the rate and extent of all chemical and biological reactions (APHA, 1995). The pH of the lake water ranged from a slightly acidic pH of 6.6 to a basic pH of 9.3. This is within the optimum pH range for growth of most biota (EPA, 1974; Baron and Finegold, 1990; APHA, 1995) including bacteria. The pH variations during the sampling period can be attributed to differences in rainfall amounts. In addition to this, the pH of the lake water may be affected due to

photosynthetic activity (Gamila *et al*., 2000) in different seasons or at different times of the day. This variation could also be due to increased amounts of faecal wastes from the humans, or other domestic animals, or chemical runoff from fertilizers in use during the sampling period. The composition of bacterial community has been shown to vary with agricultural growing practice (Mc Caig *et al*., 1999; Buckley and Schmidt, 2001) temperature (Ward *et al*.,1998) nutrient status (Broughton and Gross, 2000) contamination with faecal matter (Muller *et al*., 2001) and other environmental variables. In the present study, pH was found alkaline to slightly acidic for all the sites.

5.2. Bacterial count in relation to different seasons

Heterotrophic plate counts were used to quantify normal bacterial flora that could be cultivated on standard media. Nutrient agar was used because it is a medium often used in laboratory as an all-purpose growth media for bacterial isolates. During this study, heterotrophic bacterial load ranged between $0.2x10^4$ to $28.7x10^4$ cfu/ml revealing impacts of human activity as bacterial population was estimated in higher concentration from water samples collected from the lake. Garnier *et al*. (1991, 1992) and Hassan *et al*. (2006) in their study reported more or less similar results showing the load of heterotrophic bacteria in river Buriganga. In our study, the possibility of the fluctuation in total average culturable aerobic bacterial count might be due to increased eutrophication of water body. The distribution and seasonal variation of the total bacterial counts, as described in results, the number of bacteria ranged high during summer and autumn and lowest during winter reflecting the effect of high content of organic matter due to flourishing of phytoplankton which increases active multiplication of the bacteria. This agrees with previously reported results by Daboor (2001); Othman *et al*. (2008). The highest number of bacterial count were recorded during summer and autumn which might be attributed to high level of suspended solids and nutrients in the drainage water which effect the survival of the aquatic micro flora (Hader *et al*., 1998). The value obtained in the study are similar to those in earlier reports for microbial population in some polluted waters that are exposed to human, agricultural and industrial wastes (Lateef, 2004; Toroglu *et al*., 2006).

5.3. Total coliform (TC)

Total coliform (TC) is one of the important microbiological parameters of water quality. The highest total coliform number was determined as 1100 MPN per 100 ml in the lake .This high value in coliform count has probably arisen from untreated wastewater discharges from households, other domestic sources and sewage. The study also revealed that total coliform was significantly different at all the sites in particular basin. The possible reason being, that the sites in each basin are more prone to direct impact from human interventions from within and outside activities and also from point and non point sources of sewage. The number of bacteria increases with increasing productivity and concentration of inorganic and organic compound in the lake (Wetzel 1975). Kundangar *et al.* (2003) attributed increase in total coliform count of Dal lake to environmental variables such as low dissolved oxygen at inshore sites. According to, Rai and Hill (1978) total coliforms indicate degree of pollution and the annual average densities of total coliforms portray the differences between clean and polluted water. Coliform group of bacteria in general was found to be universal indicator of faecal pollution (Godfree *et al*., 1997). Moreover, greater tourist influx resulting in the increased movement of house boats in the Dal lake, where there is no proper disposal system for the night soil resulting in deterioration of water quality coupled with outburst of bacterial counts. Similar observation has been recorded in house boats by ENEX (1978). Seyfried (1995), while investigating the effect of various site characteristics on bacterial levels, found a positive correlation between the bacterial number, and number of boats. The results of the present study draw support from the findings of Mihindu and Oppenheimer, (1992) who had worked on faecal pollution which he confirmed by the presence of coliforms in the water samples during the different seasons especially in winter which may arise from animal dungs carried by run-off to the lake. The high MPN values in most of water samples collected can be attributed to the agricultural runoff and sewage drained into the lake from the catchment area. The results are in conformation with the results of Geldrich (1972) who observed an increase in the total coliform count of water bodies arise due to increased use of animal wastes as manure in the agricultural fields. The classified indicator for water analysis is *E. coli* and its presence suggests enteric pathogens (Nwadiaro, 1982). There is a direct relationship between the numbers of *E. coli* and the extent of faecal pollution. The higher the

number, the more polluted is the sample (Akoleowo, 2002). The occurrence of coliforms in the sample is also confirmed by the local study conducted by Latief *et al.*, 2003 reporting high coliform count in fifteen springs of Kashmir valley.

5.4. MPN (Most probable number)

The MPN of total coliforms ranged from 3 MPN per 100ml to1100 MPN per 100ml. Generally, the data of the present study showed marked increase in the indicator bacteria, and our results are in accordance with report by Toroglu and Toroglu (2009) who pointed that the microbial index is high in Golbasi lake, indicating dense bacterial population which contained coliform bacteria with high MPN (>1100). The highest bacterial indicators were recorded at outlet (site 15 and 16). This, as also reported by Shaaban- Dessouki *et al*. (1993), might be explained by the effect of domestic and agricultural wastes discharge from the urbanized surrounding area. The MPN index as proposed by McCardy (1915) observed for water samples revealed that the maximum samples were crossing the permissible limits set by WHO (2003) indicating gross pollution of the lake and its transition to eutrophic status. Poor sanitary practices could be one of the main causes of indicator bacteria from faeces being introduced into lake water (Ologe, 1989).

5.5. Culture Results and Identification

The analysis of culturable, aerobic bacteria of Dal lake was undertaken by using spread-plate technique, general media and selective media to enrich and promote their growth, by studying macro-morphological features of the colonies, by studying micro-morphological characteristics of Gram' reaction and by using Hi-Media assorted biochemical kit panel for their biochemical results displayed in accordance to Bergey's manual of systematic bacteriology (Krieg and Holt, 1984). The spread plate technique used was in confirmation to Collins and Lyne (1976) and Singleton (1977). The use of these techniques for the study of various macro and microscopic features of bacterial species is confirmed by some studies conducted by Saleem *et al*. (2011) and Shafi (2012). In a recent survey published in Nature microbiology, the insights that have arisen from culture independent analyses have been phenomenal, but to truly be able to verify these insights, the organism must be grown in the laboratory. Despite the revolutionary advances made through the application of Metagenomic approach, the importance of studying bacteria in pure

culture should not be forgotten. The negligence of basic principles is the fact that bacterial culturing has somewhat fallen by the way side in research laboratories, having been superseded by 16S rRNA analysis (Anonymous, 2013). Although preliminary studies for presence or absence of bacteria has been done before but no detailed study had been carried out to isolate and identify aerobic, culturable bacteria of Dal lake by using different culture techniques. In this study, besides, using general purpose media and specific selective media to increase the chances for isolation of different bacteria were used as per established protocol for cultivation of each bacterium. A spread plate technique is to inoculate water directly to general purpose media such as Nutrient agar and selective media like eosin methylene blue agar, (EMB) for cultivation of *E. coli,* SS agar for cultivation *of Salmonella*, *Shigella* spp., and *Enterococcus* spp. etc. The above technique employed was confirmed by Collin *et al*. (1976) and Westerfield (1996). Similarly, Warren (1998) used AER for the isolation of *A. hyrdophila* (Atlas, 1995), Inositol Brilliant green bile salts agar (IBB) for the isolation *of Klebsiella* spp. Huq *et al.* (1991) and SS agar for the isolation *of Salmonella* and *Shigella* spp. This method along with Gram's reaction lead to primary identification of forty five colonies on the basis of micro-morphological characteristics; being either Gram positive and negative bacilli or cocci. The dominance of Gram negative bacilli with a percentage occurrence of 69.78% in the lake waters is a cause of concern, as these gram negative bacteria are mostly of pathogenic in nature, although some of them are beneficial in the aquatic environments and can be attributed to the human activities taking place in the catchment area. The abundance of the gram negative bacteria observed during the study at the different sites may be attributed to the increased addition of the excretory substances to the water. Gram negative bacteria having a reservoir in the intestines of man and other warm blooded animals are excreted in faeces and are known to survive in the environment but do not reproduce (Feachem *et al*., 1983). However, in tropical environments there are evidences that the enteric bacteria can survive as well as multiply (Rivera *et al*., 1988). These revelations are also confirmed by a study carried out by Gandotra (2009) in river Tawi in Jammu city confirming the dominance of Gram negative bacilli in the river water samples. The results of biochemical tests by

using Hi-media assorted biochemical kit panel revealed that 45 strains have been identified.

In the present research work, the bacterial species identified by means of biochemical tests were further confirmed by PCR (Mullis and Faloona, 1987), a molecular technique using 16S rRNA approach. Since, in modern era, the most frequently used molecular method for bacterial identification is 16S rRNA gene analysis. This technique takes advantage of the conserved nature of the 16S rRNA gene. This gene does not code for a protein but for a structural RNA part of the ribosome. Ribosome play an essential role in protein synthesis, this gene is ubiquitous in bacteria, highly conserved and it almost never horizontally transferred making it ideal for phylogeny reconstruction and identification (Miller *et al*., 2005). More highly conserved regions in the ribosomal RNA gene sequence allow for the creation of "universal" primers for the amplification of this gene from DNA extracted from natural environments. Genome analysis on the basis of 16S rRNA using polymerase chain reaction (PCR) was confirmed out by using universal bacterial primers 8F (5'-AGAGTTTGATCCTGGCTCAG- 3') and 1492R (5'-GGTTACCTTGTTACGACTT -3') designed by Liu *et al*, (1997); Turner *et al*. (1999) which provides an accurate means to determine the phylogenetic affinity of almost any microbial isolate to record environmental biodiversity. These primers flank a divergent region of the 16S r RNA gene in all bacteria used and allow amplification of >1500 base pair fragment (Turner *et al.*, 1999). The use of these primers to study the bacterial diversity of different ecosystems is confirmed by many other studies who worked time to time (Lane *et al.*, 1991; Newton *et al*., 2006; Julia *et al.*, 2008; Ichijo *et al*., 2008; Sah *et al.*, 2009). Nowadays, classification methods for 16S rRNA gene analysis are becoming increasingly popular especially for environmental studies. Ribosomal RNA databases play a key role in this process by providing analysis tools, a standard taxonomy, and high quality sequences that can be used as references in the study of environmental sequences (Persing *et al*., 1993).

According to previous studies conducted by Westerfield, (1996) and Warren, (1998) these bacteria should be considered as a part of the normal lake flora, though both have a definite potential to cause human disease. The above findings were in accordance to our obtained results. The water samples of Dal lake harboured bacterial

communities mainly containing Beta and Gamma-Proteobacteria, Actinobacteria and Bacilli. The large proportion of Gamma-Proteobacteria in the lake is not surprising since these bacterial groups are frequently found in eutrophic water environments (Muyzer *et al*., 1993; Manz *et al*., 1994; Bond *et al*., 1995; Snaidr *et al*., 1997). The different genera identified like *Pseudomonas* spp. are able to degrade a wide range of toxic organic pollutants under denitrifying conditions (Song *et al*., 2000, 2001; Mechichi *et al.*, 2002). *Pseudomonas* spp. has also been reported in soda lake water which probably originates from wastewaters (Jones et al., 1998). The family Bacillaceae constitute a heterogeneous group of organisms able to survive through adverse environmental conditions; they have been associated with highly alkaline environments (Ventosa et al., 1998; Takami and Horikoshi 2000). The isolation of different bacterial species in the present study corroborates well with the studies conducted by Glockner *et al.* (2000); Zwart *et al*. (2002) and Warnecke *et al*. (2004) that identified thirteen fresh water specific bacteria in Crystal Bog lake, which included members of Actinobacteria, Alpha, Beta proteobacteria and Verrucomicrobia. Therefore in order, to better define the bacterial populations that inhabit waters from the Dal lake, we used a culture-dependent strategy based on the construction of 16S rRNA libraries by amplifying 16S rRNA sequences from total DNA using PCR, followed by cloning of the produced amplicons. The pitfalls associated with this strategy have been extensively reviewed elsewhere (Von Wintzingerode *et al*., 1997). Several previous studies have used clone libraries to characterize the bacterial community composition of freshwater ecosystems (Hiorns *et al*., 1997; Belkova *et al*., 2003; Boucher *et al*., 2006). Each of these studies explored the bacterial community composition of different lakes. The lakes ranged in trophic status from oligotrophic to eutrophic, in latitude from arctic to temperate, and samples were collected over time scales ranging from a single season (three months) to over two years. However, several general trends were identified, despite the vast differences in locale and date. In spite of the extremely broad types of habitat represented in the lakes in these studies, two dominant phyla identified across each lake system were Proteobacteria and Firmicutes (Hiorns *et al*., 1997; Bel'kova *et al*., 2003; Boucher *et al*., 2006). The results obtained in the present study also follow this trend, with the same two phyla contributing to the majority of both phyla.

Acinetobacter spp. is naturally found in soil, water, and sewage and these organisms may function as opportunistic pathogens, infecting the respiratory tract, urinary tract and wounds (Holt *et al*., 1994). *E. coli* are a normal component in the intestinal micro flora of warm-blooded organisms, including humans. However, while most strains of *E. coli* inhabiting human and animal intestines are harmless, commensally bacteria, a small percentage can cause disease in humans. *E. coli* bacterium is used as the indicator for faecal contamination in water (Hartl and Dykhuizen, 1984). Magray *et al.* (2011) isolated *E.coli* from water samples of Dal lake and this correlates with our study. Two species of *Klebsiella* were identified namely *Klebsiella ozaenae* and *K. oxytoca.* The most common clinical syndromes are pneumonia, abdominal infection, UTI, wound infection, soft tissue infection, and bacteremia (Kasper and Harrison, 2005). *Kluyvera ascorbata naturally* occurs in soil and water and certain strains have been characterized as significantly enhancing plant growth in heavy-metal contaminated soils (Ma, 2001). It has also been known to cause infections among humans in a various organs, excluding the brain. *K. ascorbata* opportunistic infection is rare, but very serious. (West, 1999). Three different species of *Salmonella* which differed in one or other biochemical tests in the study were isolated. *Salmonella* spp. is of concern because of its pathogenicity that is of worldwide importance and transmitted mainly through water and food. The common environmental reservoir of *Salmonella* spp. is the faecal material of infected persons, although they can be also found in asymptomatic persons. However the host range of *Salmonella* spp. extends also to animals such as horses and sheep, and contaminated food (Baer *et al*., 1971). The occurrence of *Salmonella* spp. in Dal lake water is confirmed by number of studies revealing the incidence and survival of *Salmonella* in lakes, rivers, coastal water and beach sediments (Polo *et al*., 1998). In these environments some, but not all, strains of *Salmonella* are pathogenic. *Salmonella* infection can be severe with diarrhoea, septicaemia, and bowel bleeding as seen with *S. typhi* and *S. paratyphi* infection (Bean *et al*., 1997). *Salmonella* species, including *S. Choleraesuis,* are confirmed pathogenic water-borne bacteria and are the cause of potentially serious infectious diseases (Kelly *et al*., 1985; APHA, 1992; Hui *et al*., 1994). Studies undertaken to investigate the survival of enteric pathogens in aquatic environment have shown that *Salmonella* are frequently isolated from polluted water and can

persist in high nutrient environment and warm temperature. They have been isolated from sewage polluted surface water, ground water runoff from agricultural land and leaking domestic drains (Angulo *et al*., 1997). Koul and Panhaotra (1989) have also isolated *Salmonella* species from Dal lake which confirms our results of the study. *Enterobacter* spp**.** viz *E. cloacae, E. aerogenes, E. agglomerans* and *E. intermedius* were identified. They are distributed worldwide and may be found in water, soil, plants and animals. *Enterobacter* species are common causes of nosocomial infections in humans (Percival *et al*., 2004). Two species namely *A. hyrdophila* and *A. caviae* were isolated during this study. *Aeromonas* spp. are widespread in the environment and are commonly found in fresh- and marine waters, soil, and agricultural product (Percival *et al*., 2004) as well as in sea shore sand (Ghinsberg *et al*.,1995). *Aeromonas* spp. pathogenesis primarily results in gastrointestinal distress. While the disease conditions are usually self-limiting, they have been shown to produce severe, life-threatening cholera-like symptoms (Joseph, 1996). *Aeromonas* spp. has been shown to cause gastroenteritis (Reina and Lopez, 1996), but generally is not considered to be pathogenic to humans (Parras *et al.,* 1993; Abbott *et al.,* 1994; Janda *et al.,* 1994; Hanninen *et al.,* 1995; Utsalo *et al*.,1995; Reina and Lopez, 1996) These bacteria are considered normal inhabitants of environmental waters (Graevenitz, 1985; Joseph and Carnahan, 2000) and isolation of both from lake water was consistent with results cited from studies by Westerfield (1996) and Warren (1998). Ingestion of contaminated water can also lead to human infections by *A. hyrdophila* and has been associated with diarrheal disease in humans, as well as with septicaemia and osteomyelitis (Graevenitz, 1985; Auerbach *et al*., 1987; Baron and Finegold, 1990). *Y. enterocolitica* has been previously isolated from lakes and streams, though few cases have been linked to ingestion of contaminated drinking water (Mandell *et al.,* 1979; Kelly *et al*., 1985). Raw sewage and human faecal material would be the most likely contaminating source of lake waters and it would probably be isolated only in contaminated sites which results from untreated sewage being released into the lake (Kelly *et al.,* 1985; APHA, 1992; Ervin, 1998). *Y. enterocolitica* classified as human pathogen as described by Romalde (1993) is known to cause severe gastroenteritis, fever, vomiting, abdominal pain, systemic disorders ,pneumonia, Pericarditis and Reiter's syndrome (Lecomte *et al*.,1989; Bottone, 1992). Several

medically important species exist including *V. cholerae, V. alginolyticus, V. parahaemolyticus* and *V. vulnificus* (Ghinsberg *et al*., 1995). *V. cholerae* is the causal agent of cholera, therefore, the most important to public health. Infection results from the colonization of the epithelium of the small intestine and subsequent production of an enterotoxin called cholera toxin (Taylor *et al*., 1987). Following infection, *V. cholerae* causes watery diarrhoea and a sudden onset of effortless vomiting, which combine to cause potentially deadly rapid and severe dehydration (Percival *et al*., 2004). The primary reservoir of *V. cholerae* in the environment is the faecal material of human carriers (Percival *et al*., 2004) although *Vibrio* spp. is known to be natural inhabitants of fresh waters worldwide (Oliver, 1995; Montanari *et al*., 1999). Infections by *Vibrio* spp. result from contact of wounds with contaminated water or ingestion of contaminated, raw food (Klontz *et al*., 1988; Hlady and Klontz, 1996; Patel *et al*., 2002). *V. cholerae* (Baumann and Schubert, 1984; Farmer and Hickman Brenner, 1994) is the only pathogenic species of significance from freshwater environments. There are a number of pathogenic species, including *V. cholerae, V. parahaemolyticus* and *V. vulnificus.* Non-toxigenic contamination of water due to poor sanitation is largely responsible for disease transmission. *Vibrio cholerae* has killed millions of people during numerous cholera epidemics (Shimada *et al*., 1993). *Vibrio* spp. has been found in association with a wide range of aquatic life (Islam *et al*., 1989). Two species of *Serratia* were isolated namely *S. marcescens* and *S. rubidaea and* are considered to be ubiquitous in the environment. They can be found in surface and groundwater, soils and other decaying matter. *Serratia* spp. is an opportunistic pathogen, usually causing infections in immune compromised hosts (Grimont and Grimont, 1992). *Serratia* spp. bacteremia has been reported in community-acquired infections (Wong *et al*., 1991; Heltberg *et al*., 1993; Vandenbroucke-Grauls *et al*., 1993), and is associated with urinary tract infection, pneumonia, meningitis, intra-abdominal infection, skin and soft-tissue wound infection (Pegues *et al*., 1991; Theccanat *et al*., 1991; Wong *et al*., 1991). Fatalities due to *Serratia* spp. infection have been reported (Wong *et al*., 1991). *P. putida* and *P. alcaligenes* compose a substantial portion of the micro flora of free-living saprophytes in soils and water, and are associated with plants and animals as pathogens, and all the two species isolated are denitrifiers (Palleroni, 1992). *P.*

aeruginosa is an opportunistic pathogen most commonly associated with community-acquired urinary tract infections (Vigg and Jad, 1991). It is a normal component of human skin, gastrointestinal and respiratory tracts, ubiquitous in nature, as reported by Morrison and Wenzel, (1984). It was first documented to be pathogenic in an endocarditis infection (Aragone *et al*., 1992), but documented infections are rare. A survey undertaken within portable supplies and surface water have shown *Pseudomonas* to constitute around 2-3 % of the total heterotrophic plate count and is responsible for 10% nosocomial infections. A large number of *Pseudomonas* spp. is often isolated from environmental water samples. They have included species like *P. aeruginosa, P. alcaligenes, P. putida* and *P. echinoids*. Recreational and occupational infections are associated with *pseudomonas,* which include Jacuzzi or whirlpool rashes (Bert *et al*., 1998). *Pseudomonas* spp. is always found in water that has been contaminated with faecal material such as surface waters. It is excreted in the faeces of many healthy people (Pollack, 2000). Five distinct species of *Enterococcus* were recognized viz *E. faecalis, E. faecium, E. durans, E. hirae and E. mundtii. Enterococci* are found in the gastrointestinal tract of warm-blooded mammals. These bacteria can be shed through faecal matter into soil, water, and food. They have been isolated from plants, soil, and water as faecal contaminants (De Vaux *et al*., 1998). In water, the predominating species are *E. faecalis,* E. *faecium, E. durans and E. hirae,* have a broad distribution in the environment (Devriese *et al*., 1995). *E. faecalis* is part of the normal intestinal flora of humans, but can be pathogenic to other organ systems. *Enterococci* are the second most frequently reported pathogens (Tailor *et al*., 1993), and *E. faecalis* is the pathogen responsible for most enterococcal infections. It has also been implicated as an important cause of endocarditis, bacteraemia, urinary tract infections, intra abdominal infections, and rare cases of meningitis (Kaye, 1982; Vigg and Jad, 1991; Devriese *et al*., 1992; Tailor *et al*., 1993). *E. faecalis* is one of the currently preferred indicators of human faecal contamination of recreational waters. *Enterococcus* group provide supplementary data on bacteriological quality of water and augment the faecal contamination of water (Laukova and Juris, 1997). Results of the study suggest contamination is widespread during the entire study period. This report was in accordance with Rajurkar *et al*., (2003). Two *Shigella species* were identified namely *Shigella flexneri* and *S.*

dysenteriae. Shigella species viz *S. flexneri*, *S. boydii*, and *S. sonnei*, including *S. dysenteriae*, which produces Shiga toxin, represents the most mildly pathogenic species. Species of the genus *Shigella* are among the bacterial pathogens most frequently isolated from patients with diarrhoea. *Shigella* is responsible for 5-15% of diarrheal case including 1.1 million fatal ones (Kotloff *et al*., 1999). Shigellosis remains a common gastrointestinal disease in developing and industrialized countries (Mead *et al*., 1999). All *Shigella* spp. are pathogenic, and since humans are their primary natural host, their common reservoir is the faecal material of infected persons (Baer *et al*., 1971). *Shigella* spp. cause gastrointestinal illness by invading the colonic epithelium, causing bacillary dysentery (shigellosis). *Shigella dysenteriae* is responsible for many cases of haemolytic uraemic syndrome (Struelens *et al*., 1985; Jennison and Verma, 2004). Hendricks (1972) reported that Shigella flexneri was able to multiply in river water. Sultanov and Solodovnikov (1977) considered that the dysentery was due to widespread use of polluted surface water for domestic purpose. *S. aureus* has been found in air, dust, soil and water. The major concern with *S. aureus* is water transmission in contact with cuts and scratches on the skin, infecting eyes and nose during bathing or in polluted water used for drinking water. *S. aureus* in the open environment and water supplies are generally classified as opportunistic pathogens, however they may play role in disease transmission like genitourinary, pneumonia, osteomylitis, toxic shock syndrome etc (Percival *et al*., 2004). Two species of *Citrobacter* were identified, namely *C. diversus* and *C. freundii*, are found in nutrient-rich waters, soil, decaying plant material as well as in drinking-water containing relatively high concentrations of nutrients. It causes infections of the urinary tract and infant meningitis (Drelichman and Band, 1985; Badger *et al*., 1999).The presence of *C. freundii* and *C. diversus* in the water shows it is not fit for consumption. Members of the genus *Citrobacter* are common causes of bladder, kidney and other body infections (Stanier *et al*., 1987). *Proteus* spp. viz *P. vulgaris and P. mirabilis* were identified. They are pathogenic to humans, causing chronic urinary tract infections, bacteraemia, pneumonia, focal lesions and wound infections. They exhibit characteristic swarming motility and are part of the normal flora of the gastrointestinal tract. These species only become pathogenic if present outside the G.I tract. *Proteus* species can easily adhere to the kidney urothelium, which facilitates the

upper urinary tract infection. *Proteus* also hydrolyzes urea, which alters the pH of urine and may lead to the formation of kidney stones. *P. mirabilis and P. vulgaris* are usually found to inhabit the intestinal tract of animals, however it can be found in water, soil, faecal matter, and raw meat. *P. mirabilis* is the most frequently isolated member of the *Proteus* genus and is found to be most often associated with infection (Matsen *et al*., 1972). *Cedacea* spp., which is the member of *Enterobacteriaceae* family, is frequently isolated from sputum, but their clinical importance is not clear. *C. davisae and C. lapagei* (Aguilera *et al*., 1995; Dalamaga *et al*., 2008a) were reported to cause bacteremia, ulcer, abscess, wound and ophthalmic infections and *C. lapagei* was reported to cause pneumonia (Farmer, 1982; Dalamaga *et al*., 2008b; Yetkin *et al*., 2010). Brenner *et al.,* (1993) in his study isolated different *Cedacea species* from fresh water environment by using different media. Our research work draws support from the above confirmed study. *E. tarda* is predominantly found in freshwater environments colonizing in the gut of fish. It can also be found in the intestinal tract of birds, reptiles, and mammals. It can be found as part of the human intestinal flora as well, although this seems to be rare, and is usually due to an infection or prolonged contact with contaminated water (Janda *et al.,* 1991). This microorganism is an opportunistic pathogen, and is able to live inside and outside the host. One of the most common diseases among freshwater fish is hemorrhagic septicemia, also known as edwardsiellosis, which usually results in the death of the fish (Yousuf, 2006). This disease is usually prevalent in flounder fish. A few known epidemics of edwardsiellosis have been recorded, and can be devastating to the fish population, but if the infection is detected early, the epidemic may be easily avoided (Yousuf, 2006). In humans, *E. tarda* can also cause colitis and dysentery-like diseases (Janda *et al*., 1991) as well as gastroenteritis (Clarridge, 1980), infections of wounds, gas gangrene associated with trauma to mucosal surfaces, and systemic disease such as septicemia and meningitis (Janda and Abbott, 1993). Five species were identified viz *B. cereus, B. subtilis; B. alcalophilus, B. sphaericus* and *B. brevis*. Many species of this genus exhibit a wide range of physiologic abilities that allow them to live in many natural environment (Ahn *et al*., 2001) *Bacillus spp* spore formation occurs when nutrients are scarce within the environment and germinate into vegetative cells once they are available (Wijnands *et al*., 2006). They are distributed widely in nature

and are commonly found in the soil as a saprophytic organism (Vilain *et al.*, 2006). In addition, *B. cereus* is an opportunistic human pathogen and is occasionally associated with infections, causing periodontal diseases and other more serious infections (Hoffmaster *et al.*, 2006). Likely strains of this species have been isolated from alkaline waste water (Ntougias *et al*., 2006). Similar observation was reported by (Park *et al*., 2003) in Korea who isolated *Bacillus* spp. and identified from rotating biological contactor based on their biochemical properties. Also Joshi *et al*. (2007) in India identified *B. brevis* from fresh water lake and studied its biochemical characteristics. Many studies have suggested that strains of the genus *Bacillus* are more phenotypically heterogeneous than most other bacterial genera. There is a diverse group of *Bacillus* spp. living in highly alkaline terrestrial and aquatic environments (Berber and Nya, 2005). The presence of *Bacillus* spp. is further confirmed by Ostensvik *et al.* (2004) who in his study reported B*acillus* spp. from Norwegian surface waters run off which were subjected to variable degree of faecal pollution from agricultural fields. *A. faecalis* is known to inhabit soil and water environments (Winn *et al.*, 1988). *A. faecalis* also inhabits the alimentary canal of humans (Austin *et al*., 1981).Two species of *Micrococcus* viz *M. luteus* and *M. roseus* has been isolated from human skin, animal and dairy products, and beer. It can be found in many other places in the environment, like freshwater, dust, and soil. *M. roseus* can grow well in environments with little water or high salt concentrations (Liu, 2011).

Part of my research involved the acquisition of data to evaluate whether the water-borne pathogens of the lake are a cause for concern. Bogomolni *et al.* (2008) reported that the majority of bacteria isolated in his study were recognized as human pathogens or potential human pathogens. All pathogens found in water were recognized by the Biological Safety Association (BSA) as human pathogens i.e. *Acinetobacter* spp., *C. freundii, E. cloacae* and P. *alcaligenes*. Other isolates recovered that are known to cause infection in humans from handling fish include *A. hyrdophila, E. tarda, V. cholerae,* and *V. Parahaemolyticus* (Harper, 2002) and many of these isolates (*Pseudomonas* spp., *A. hyrdophila* and *E. tarda* had been isolated also in our study. Ramya *et al*. (2012) has isolated six species of pathogenic bacteria

viz *K. ozaena*e, *S. rubidaea, E. agglomerans, S. Arizona, E. sakazakii* and *E. cloacae* in Dalvayi lake, Mysore and our study draws support to the above confirmed study.

The bacteriological results of this study indicate that waters of all selected sites of Dal lake were unfit for primary contact by humans throughout the entire year. Many of the pathogens isolated have been known to cause septicaemia, gastroenteritis, pneumonia, and meningitis (Ellison and Mostow, 1984; Sirinavin *et al*., 1984; Pryor *et al*., 1987; Baddouir and Baselski, 1988; San Joaquin and Pickett, 1988; Willoughby *et al*., 1989; Golik *et al.*, 1990; Ong *et al*., 1991; Goncalves *et al*., 1992).

5.6. Symptom incidence pattern in the Dal inhabitants

Twenty percent population (divided into three age groups from both male and female sections including children as well as adults) of Dal inhabitants living in close proximity of the lake water involved in such acts which made them to come in contacts with the lake during their day to day activities, interviewed as per questionnaire in the study, showed a relatively less prevalence of the water borne bacterial diseases. The lesser prevalence of these water borne bacterial infections in the population could be directly correlated to the fact that the people usually avoid coming in contact with this water as they normally don't use the same for any domestic purpose; bathing and drinking the lake water is far beyond their normal policy. However, a few case reporting of symptoms of gastrointestinal disturbances could be correlated to the presence of bacterial pathogens like *E.coli, Enterobacter* spp., *Shigella* spp. etc. in the lake water which are the causative agents of such infections as we are familiar with the fact that majority of bacterial infections of the GI tract are caused by water borne bacteria. Favouring the statement Burke *et al*. (1984) and Moyer (1987) reported that *Aeromonas* spp. are opportunistic pathogens known to cause disease symptoms like Gastroenteritis. The occurrence of these infections in a few individuals is nothing strange as the species of bacteria can cause a variety of opportunistic infections which pose a variety of health risks like gastro intestinal and respiratory infections (Henrickson *et al*., 2001). Water contaminated with faecal pollution shows a positive impact on health as is clearly evident from the study on an investigation carried out by Qureshi *et al*. (2011) into the prevalence of water borne diseases in relation to microbial estimation of water in the community

residing near river Ravi, Lahore. In present study it was observed that the prevalence of symptoms of gastrointestinal disturbances is higher for younger individuals and elderly people as the same has been reported by NRDC (2006).The organisms are the main cause of gastrointestinal diseases and has major health impacts (Murray *et al*.,1997).The report of Esrey *et al.* (1990,1991) who carried out 142 studies on six of the major water borne diseases and estimated that in developing countries there were 875 million cases in mid 1980's.

Chapter: 6

Summary Conclusions and Recommendations

The study on Bacterial diversity of Dal lake with particular reference to Pathogenic bacteria was carried out from April 2010 to March 2012 which is summarized as under:

- The study area (Dal lake; latitude 34^0 07′ N, longitude 74^0 52′ E, altitude 1583 m) selected for this work is a multi-basined lake with many inlets and outlets, so an extensive network of sixteen sites with different altitudes and geographical co-ordinates viz., Hazratbal open, Hazratbal littoral, Nigeen open, Nigeen littoral, Gagribal open, Gagribal littoral, Nishat open, Near Centeur, Boathall nallah-I, Boathall nallah-II, Tailbal nallah-I, Tailbal nallah-II, Dal Lock Gate-I, Dal lock gate-II, Pokhribal nallah-I and Pokhribal nallah-II were selected. Among the selected sites eight (8) sites were selected in the four basins, four (4) were selected from two inlets and four (4) were selected from two outlets. These sites selected included microhabitats from both littoral zones as well as limnetic zones.
- The water samples collected from sixteen sites were selected randomly from different basins, outlets and inlets of the lake, under consideration for exploring the bacterial diversity and were collected on seasonal basis in poly ethylene (PET) bottles, which were previously carefully cleaned and rinsed three to four times with distilled water. All the samples were collected from the surface and subsurface of lake water by plunging the open end of each sterile bottle before turning it upright to fill. During collection of samples, extreme care was exercised to avoid contamination of the parts of bottle and collected samples were processed for the analysis of bacterial community using the standard methodology.
- The glassware used and media prepared for the work was carefully sterilized using different standard techniques. The techniques used for the isolation of bacteria from the water samples included spread plate technique, pour plate technique and streak plate technique.
- The data on bacteriological analysis was analysed to measure the degree of contamination of water samples in Dal lake on seasonal basis from sixteen different microhabitats by plating the different dilutions on culture media that included general (Nutrient agar) as well as selective media like EMB agar,

Cetrimide agar, etc. After incubation, the cultured bacterial colonies were enumerated in order to assess bacterial load in terms of colony forming unit (cfu/ml).

- The colonies were further characterized on the basis of macroscopic as well as microscopic character.
- The isolated colonies showed marked variation in their features and on these differences, a representative sample colony of each was coded as SMB-1 to SMB- 45.
- The coded colonies were streaked on selective media to obtain pure cultures and then for biochemical analysis, using Hi- media biochemical test strips, for further identification.
- The biochemically identified bacteria (Bergey's manual specification) were subjected to molecular analysis by 16S rRNA gene using polymerase chain reaction which was carried out by means of universal bacterial primers 8F and 1492R which provides an accurate means to identify bacterial diversity and thereby studying the phylogenetic relationship between them
- The sequences so obtained were confirmed and compared to known 16S rRNA sequences in gene bank (NCBI, Pune) by using BLAST algorithm and found to be 69% to 100% similar to the sequences of 16S rRNA gene of bacteria.
- Tests for the presence of total coliforms were carried out by multiple tube fermentation technique, which revealed higher level of total coliforms with their value ranging from 3 MPN /100ml.The highest number of these indicator organisms were observed at site 15 and 16 (Pokhribal outlet) in summer season and minimum at site 5 an 13 (inlet) in winter season.
- The category wise distribution of coliform count showed that 57.81% water samples lie in the category III followed by 39.07% in category II, 3.1% in category IV and 0% samples in category I. The perusal of data considers the water unfit for drinking purpose however, fit for recreational purposess
- In order to gain insight into bacterial load, the culturable bacterial colonies were counted by Quebec colony counter and observed in terms of colony forming unit (CFU/ml) which reveals substantial number of heterotrophic

bacteria. Total bacterial count ranged between 0.2×10^4 to 28.7×10^4 cfu/ml and the highest count was found at site 16 (Pokhribal outlet) in summer season and lowest count in winter season at site 6 (Boathall nallah).

- Out of 5941 colonies, 3123 colonies (52.56% occurrence) were isolated in summer season followed by 1502 colonies (25.28% occurrence) in autumn, 844 (14.87% occurrence) in spring and 432 (7.27% occurrence) in winter.
- The Gram's reaction revealed that out of 5941 colonies, 4146 (69.78%) were Gram negative and 1795 (30.21%) was Gram positive bacteria. Among 4146 Gram negative colonies, 4116 (69.33%) were bacilli and 30 (0.45%) were cocco- bacilli whereas among 1795 Gram positive colonies, 698 (11.72%) were bacilli and 1097 (18.49%) were cocci.
- The species identified biochemically belonged to family *Enterobacteriaceae* (23 species), *Bacillaceae* (5 species), *Enterococaceae* (5 species), *Vibrionaceae* (3species), *Pseudomonadaceae* (2 species), *Micrococcaceae* (2 species), *Aeromonadaceae* (2 species), *Staphylococaceae* (1 species), *Moraxellaceae* (1species) and *Alcaligenaceae* (1 species).
- The site wise abundance distribution and species composition of different bacterial genera indicated that maximum number of species were isolated from family *Enterobacteriaceae* followed by *Bacillacae* and *Enterococaceae.*
- The forty five (45) bacterial species were isolated during the course of study. The highest number of bacterial species occurred at site 1, 2, 9, 15 and 16 whereas lowest number of species (29) at site 6.
- The highest bacterial species were observed in summer months and lowest in winter month showing influence of temperature on bacterial colonies.
- Analysis of variance (ANOVA) showed that the observed distribution of the bacterial colonies in different seasons is statistically significant. Therefore, seasonal variation in occurrence of bacterial colonies was observed between different study sites of the lake.
- The bacterial flora in the lake consisted of diverse life forms ranging from Proteobacteria to Firmicutes and Actinobacteria which belonged to different selected micro habitats across the lake.
- The bacterial population showed a diverse seasonal and temporal variation on

the basis of occurrence in different sites which were categorized into four groups named as open, littoral, outlet and inlet sites.

- *E.coli* was found to dominate different habitats of lake in all the seasons of the year. The ANOVA carried out between different sites for bacterial species isolated from different microhabitats showed that 71% results were statistically significant with 7% as highly significant ($p < 0.01$) and 27% as non significant ($p < 0.05$).
- The Bray Curtis cluster analysis of the study sites developed on the basis of presence and absence of a species at the respective sites showed similarity ranged from 31 to 87% with the least similarity of 31% between site 1 and 7 and maximum similarity of 87% between site 3 and 4.
- From the value of different indices computed for 16 sites for the occurrence of different bacterial species, the Shannon wiener index was highest at site 16 (3.68) and lowest at site 8 (3.26).
- The analysis of variance showed that highly dominance and diversity patterns varied significantly with highly even distribution of bacterial species in microhabitats of the lake.
- The data of correlation analysis between the pH and bacterial load at sixteen sites indicated that there was a negative correlation of pH with the bacterial load and positive correlation of temperature with bacterial load and the results were found to be statistically significant.
- For the purpose to study the impact of pathogenic bacteria isolated from Dal lake on humans, a random survey of the 20% of population i.e., 384 individuals (64 families) out of 1920 individuals (320 families) who were engaged in one or the other activity related to lake water was carried out through questionnaire. The data of which reveals that:
    I. The disease was more prevalent in males than in females under the age group of 20 years followed by age group of 21 to 40 years and then by above 40 years.
    II. The symptoms of gastrointestinal diseases were reported in 26 cases (6.77%) and other symptoms like high fever, chills, rigors, sweating and body aches in 17 cases (4.42%).

III. The prevalence as per the source of water being used, revealed that disease symptoms were more prevalent in individuals consuming lake water as compared to tap water. The results were statistically found to be significant.

- The most of the bacteria isolated were recognized as human pathogens, capable of initiating water borne infections, thus potentially water transmitted.
- The obtained data in the study reflect the importance of microbiological monitoring especially related to pathogenic bacteria.

Conclusions

From the foregoing account the following conclusions can be drawn:

- Bacteriological analysis of water is a powerful and foremost tool to foreclose its bacterial diversity in particular reference to health hazard associated with pathogenic bacteria.
- Microscopy provided a useful tool in determining shape and arrangement of bacteria.
- A general purpose medium, Nutrient agar is useful in studying of macro morphological features of bacterial colonies. Other selective media like EMB agar, Cetrimide agar etc helped us to observe macro morphological colonies of specific cultivable bacteria.
- The use of general purpose media helped us to assess bacterial load in terms of colony forming units on culture plates (CFU/ml).
- The bacterial count of water samples of lake water revealed substantial number of heterotrophic bacteria in the successive years of study.
- Total coliforms exceeded the limit set by WHO (1998) indicates, recent contamination of the water sources with faecal matter.
- The MPN index revealed the high coliform count, hence cannot be used as potable water but is fit for recreational purposes. Since, the MPN count was found quite high at most sites in the lake, danger of spreading to human population is possible.
- The high count of sewage pollution indicator bacteria revealed that the microbiological quality of lake water was very poor, unsafe and not acceptable for drinking purpose.
- Dal lake harbour large collection of bacterial species belonging to two main groups on the basis of Gram reaction viz Gram positive and Gram negative bacteria.
- The combined morphological and biochemical testing approach helped us to identify bacteria on the basis of various colony characteristics and enzymatic profile which were found to be specific for each bacterial species.
- The molecular identification approaches based on Polymerase chain reaction,

amplification of genomic DNA followed by sequencing of resulting amplicons is the most promising technique for their identification. The identification and taxonomic analyses of bacterial pathogens are increasingly becoming dependant on modern molecular techniques, based on PCR amplification of conserved regions of the genome and sequencing of the resulting PCR products.

- The universal bacterial primers viz 8F (5′ AGAGTTTGATCCTGG CTCAG-3′) and 1492 R (5′- GGTTACCTTGTTACGACTT-3′) provided an accurate means to determine phylogenetic affinity of microbial isolate to record environmental biodiversity. The use of these primers helped in genome analysis on the basis of 16S rRNA quite effectively.
- The Dal lake is not only rich in terms of the diversity of all other biodiversity components like phytoplankton, zooplanktons and macrophytes but also in terms of the bacterial diversity which was assessed for the first time with various isolation approaches (like direct plating and dilution methods) and identification approaches (like morphological approach, biochemical approach and molecular approach) in this study.
- The bacterial flora in the Dal lake, showed diverse pattern of life ranging from Proteobacteria to Firmicutes and to Actinobacteria.
- The majority of species belonged to Enterobacteriaceae with 23 species.
- The family Enterobacteriaceae was found to be highly dominant with total percentage colony count of 50.27 and among the species isolated *E.coli* was highly dominant, a matter of concern to human health.
- Forty five bacterial species were isolated from all selected sites of Dal lake. Most of the bacterial species isolated and characterized were Gram negative bacilli with a percentage occurrence of 69.33.
- Dal lake provides a unique opportunity to investigate the microbiology of urban lake environment that remains completely influenced by different type of anthropogenic influenced by different type of anthropogenic activities all through the year.
- The variation in the environmental setup (including the human interference, catchment area conditions, catchment area activities, commercial activities and

other related activities) of various microhabitats selected for the present study with respect to the bacterial diversity in a vast habitat like Dal lake is having a profound influence on percentage of occurrence, diversity, density, evenness, dominance and species variability of the bacterial species.

- The highest bacterial count was isolated from Pokhribal I and II (site 15 and 16) and the lowest at Nigeen I and II (site 13 and 14), therefore, selection of areas for sampling resulted in detection of high bacteria count in more polluted sites of Dal lake.
- There is a direct relationship between the concentration of bacteria and the temperature in the lake water environment. It appears that seasonally induced changes act as important restructuring forces for lake bacterial communities.
- The correlation of pH was found to be inversely proportional to bacterial load as it dictates the rate and extent of all chemical and biological reactions.
- The occurrence of bacterial species showed a remarkable seasonal and temporal variation across the lake with *E.coli* as the most dominant and prominent species across the lake.
- Species like *E. coli, E. faecalis, S. typhi, M. roseus, P. vulgaris* and *A. hyrdophila* are a few dominant species occurring at the open water sites however *E.coli, B. subtilis, S. flexneri, S. dysenteriae, S. typhi* and *S. aureus* are the dominant representative of the littoral sites.
- In case of outlet sites the most prominent species included *E.coli, M. roseus, E. faecalis, B. brevis, E. aerogenes* and *S. flexneri* while as for inlet sites they included *E.coli, M. roseus, S. aureus, E. cloacae, B. cereus* and *B. alcalophillus*.
- The Dal lake is a species rich habitat with many overlapping bacterial species dominating at different sites.
- The analysis of variance between different sites for bacterial species showed that 71% results were statistically significant with 7% as highly significant and 27% as non significant.
- A number of pathogenic bacteria species like *V. cholerae, S. typhi, P. vulgaris, Y. enterocolitica and P. alcaligenes* etc. are present in the lake water, thus posing a health risk to human beings who are actively involved in the works

related to the lake water like rowing of shikaras, extraction of "Nadru" from the lake, women washing the clothes and other daily use items in the lake water.

- The prevalence of symptoms in the selected population varies with the type of water being used for different purposes. Higher prevalence of infection 13.25% (35 positive cases among 264 individuals studied) was seen in the people using lake water for different domestic purposes followed by the people using Tap water with a positive prevalence of 6.66% (8 positive cases among 120 individuals studied).
- Owing to awareness of impact of pathogenic bacteria, majority of the people are not using the water of this lake for drinking, washing, bathing and other domestic purposes, thus indicating not emerging as a serious threat of human bacterial infections, but where and when the individuals use it for various purposes, symptoms like loose motion, bloody stools, nausea, vomiting, fever, rigors, chills, sweating and body aches are being reported.

Recommendations

On the basis of the present study following suggestions/ recommendations are being made for follow up action.

- The surface water represents a significant, but understudied reservoir of environmental pathogens. Taken together, the data highlight the need to address the pathogen ecology in surface waters, as the increased presence and survival of pathogen indicators might signal a significant public health concern.
- Regular studies on the bacterial component of the freshwater lakes of Kashmir valley like all other components of biodiversity should be undertaken to know the dynamic bacterial prosperity of these water bodies. So, that the bacteria which could essentially be used for lake restoration does not skip away from our attention.
- Although the lake does have the potential of undergoing self purification but the recent study appoints the importance of regular monitoring of water to implement suitable remedial measures like proper disposal of sewage and human faecal wastes of the population living within and outside the system by setting up of sewage treatment plants before it drains into the lakes.
- The number of the indicator bacterial count detected was above the permissible limits for drinking purposes in all samples. Data suggested the importance of greater attention of:
    - ✓ At improving personal, household and community hygiene,
    - ✓ Implementation of sanitation control program and
    - ✓ Create awareness about water contamination
- The obtained data in this study appoint the importance of intensive and continuous microbiological monitoring of total coliforms, effective treatment and reinforce the need to implement environment protection programs especially related to bacterial species in order to meet the recommended standard of drinking water for its safe use.
- Severe nature of microbial pollution in the lake and the need to establish benchmarks for the restoration of lake water quality to improve the ecological profile of the lake and enhancing health indices of local population should be

envisaged and should be undertaken on top priority for Dal lake and possibly other lakes of Kashmir .

- Constant surveillance of water bodies with respect to true microbiological monitoring to suggest proper management actions that could be applied in order to improve the quality of lake water and reduce public health risk.
- From the aesthetic perspective also, it is important to render the lake sanitized as this forms an attractive tourist destination in Kashmir since Mughal era. However, strict eco-tourist norms should to be adhered to.
- Considering the ecological and eco-tourist importance of the lake and health hazards caused by the bacteria, it is suggested that the sewage water should not be let into the lake even after the treatment. Instead, the treated sewage water can be diverted for use in agriculture in vicinal locations where water is scarce.
- The bacterial biodiversity present in existing aquatic habitats should be acknowledged so that the effects of habitat shift on species composition and functions could be detected.
- Bacterial studies of aquatic habitats should be given due emphasis and preference due to the significance of bacteria and their activities in water as indicator organisms, as human, animal and plant pathogens, as driving force in self-purification process, in the remineralisation of organic materials and as a detector of influence of catchment area upon the lake.
- Use of specifically designed primers is recommended to know the phylogenetic relationship of the unknown microbial cultures.
- Metagenomic approach should be adopted in order to reveal total bacterial community composition especially uncultivable bacteria. Efforts on the extraction, identification and characterization of enzymes from the isolated bacterial species from the lake should be carried out in profundity as this particular facet has immense commercial and industrial applications.
- Research in the area of applied microbiology should carry on with an interdisciplinary approach where traditional microbiologists team up with molecular biologists, animal scientists, biologists and environmentalists.

These common themes identify research and management needs for the lake basins. First, there is a need to understand the natural history and ecology of pathogens and indicators in order to better detect and manage risks to human health. Secondly, a comprehensive strategy to monitor waterborne microbial pathogens is required. This strategy should use consistent methods across the basin, use the latest technologies, be maintained so that long-term data are collected, and be readily accessible.

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Annexure

Annexure-I

Composition of media used for bacterial cultures

S. No	Nutrient Medium	Type	Composition		pH
			Chemical	Quantity(g/l)	
01	Nutrient Broth	General purpose media for bacteria	Peptone NaCl Beef extract DW	5.0 3.0 3.0 1000 ml	7.0
02	Nutrient Agar	General purpose media for bacteria	Peptone NaCl Beef extract Agar DW	5.0 3.0 3.0 15.0 1000ml	6.8-7.2
03	Eosin Methylene Blue Agar (E.M.B)	Selective media for coliform bacteria	Peptone Lactose Sucrose K_2HPO_4 Eosin Y Methylene Blue Agar DW	10 5.0 5.0 2.0 0.4 0.06 15.0 1000ml	7.2
04	Simmon's Citrate Agar	Test for utilization of specific substrate	$MgSO_4$ $NH_4H_2PO_4$ K_2HPO_4 Sodium Citrate NaCl Bromothymol Blue Agar DW	0.2 1.0 1.0 2.0 5.0 0.08 15g 1000ml	6.9

Contd.....

Annexure-I

S. No	Nutrient Medium	Type	Composition		pH
			Chemical	Quantity(g/l)	
05	Nutrient Gelatin	Test for proteolytic activity	Nutrient broth in DW KH_2PO_4 K_2HPO_4 Gelatin Glucose	11g/1000ml 0.5 1.5 4.0 0.05	7.0
06	Peptone broth	Test for indole activity	Peptone DW	4.0 1000ml	7.2
07	Glucose phosphate broth	Indole reaction	Glucose Protease peptone KH_2PO_4 DW	5.0 5.0 5.0 1000ml	7-7.2
08	Urea agar	Urease activity	Peptone Glucose NaCl K_2HPO_4 Phenol Red aqueous sol	1.0 1.0 5.0 5.0 6ml 1:5	6.8-6.9
09	Triple Sugar Iron Agar	Test for H_2S production	Protease peptone Peptone Trypticase NaCl Lactose Sucrose Dextrose Ferrous ammonium sulphate Sod.thiosulphate Phenol red Agar DW Beef extract Yeast extract	5.0 15.0 10.0 5.0 10.0 10.0 10.0 0.2 0.2 0.025 13.0 1000ml 3.0 3.0	7.4

Contd.....

Annexure-I

S. No	Nutrient Medium	Type	Composition		pH
			Chemical	**Quantity(g/l)**	
10	Bromothymol blue agar	Selective media for *E.coli*	Peptone Oxoid yeast extract NaCl Agar DW autoclave and add: 1ml of 5%maranil sol. 2ml of 50% sod thiosulphate. 10ml of 1% Bromothymol blue. 5ml of 33% lactose sol. 1.2 ml of 33% glucose sol.	10.0 5.0 5.0 20 1000ml	7
11	Lactose broth	Presumptive test for coliforms.	Beef extract Peptone Lactose DW	6.0 10 10 1000ml	7
12	Endo agar	Confirmed test	Peptone Lactose K_2HPO_4 Na2 SO3 Basic Fuchsin Agar DW	10 10 3.5 5.0 0.5 15 1000ml	7
13	Cetrimide agar	Selective media for *P. aeruginosa*	Peptone $MgCl_2$ Pot. Sulphate Cetrimide Agar DW	20 1.4 10 0.3 13.6 1000ml	7

Contd.....

Annexure-I

S. No	Nutrient Medium	Type	Composition		pH
			Chemical	Quantity(g/l)	
14	Brilliant green lactose bile broth	Confirmed test	Lactose Brilliant green Agar DW	10 13.3 15 1000ml	7.2
15	Deoxycholate citrate agar	Enrichment medium for *Proteus*	Peptone Lactose Sod. Deoxycholate NaCl K_2HPO_4 Ferric citrate Sod. Citrate Neutral red Agar DW	10 10 1.0 5.0 2.0 1.0 1.0 0.03 15 1000ml	7.0
16	Mac Conkey agar	General purpose medium	Peptone NaCl Sod. Taurocholate DW 40 % NaOH Agar	20 5.0 5.0 1000ml 2-3 drops 20	7.4-8.0
17	SS agar	Medium for *Salmonella/ Shigella*	Beef extract Lactose Bile salt Sodium citrate Sodium thiosulphate Ferric citrate Agar DW	5.0 10 8.5 8.5 8.5 1.0 13.5 1000ml	7

Contd.....

Annexure-I

S. No	Nutrient Medium	Type	Composition		pH
			Chemical	Quantity(g/l)	
18	Thiosulphate citrate bile salt agar (TCBS)	Medium for *Vibrio*	Yeast extract Peptone Sucrose Sod.thiosulphate Sodium citrate Ox gall NaCl Thymol blue Bromothymol blue Agar DW Ferric citrate	5.0 10 20 10 3.0 5.0 10 0.04 0.04 15 1000ml 1.0	8.6
19	Caprylate thallous agar	Medium for *Serratia*	$FeSo_4.7\ H_2O$ $ZnSO_4.4\ H_2O$ $Co(NO_3)_2.6H_2O$ H_3BO_3 H_3PO_4 $MnSO_4.4H_2O$ $CuSO_4.5H_2O$ DW	0.05 0.02 0.003 0.006 1.96 0.02 0.02 1000 ml	7.4
20	Peptone water	Motility test	Peptone NaCl DW	10 5.0 1000ml	7
21	Plate count agar	Medium for *Staphylococci*	Tryptone Yeast extract Glucose Agar DW	5.0 2.5 1.0 15 1000ml	7

Contd.....

Annexure-I

S. No	Nutrient Medium	Type	Composition		pH
			Chemical	Quantity(g/l)	
22	Blood agar (nutrient agar +10% blood)	Haemolysis	Tryptone Beef extract NaCl Agar DW	10 3.0 5.0 15 1000ml	7.4
23	Meat infusion broth	General purpose medium	Lean meat Peptone NaCl DW	500 10 5.0 1000ml	7.4
24	Semisolid agar	Motility test	Agar	0.2-0.5 % in 1 litre DW	
25	Xylose lysine Deoxycholate agar	Enteric pathogens	Yeast Extract Lactose Sucrose Xylose L-Lysine hydrochloride Sodium Chloride Ferric Ammonium Citrate Sodium Thiosulfate Sodium Deoxycholate Phenol Red Agar	3.0 7.5 7.5 3.5 5.0 5.0 0.8 6.8 2.5 0.08 15	7.4

(Kanika Sharma, 2005)

Annexure-II

MPN table for three tube dilution series using inoculum 0.1, 1 and 10 ml (Mc Cardy, 1915)

Combination of positives			MPN index per ml	95 % confidence limit	
				Lower	Upper
0	0	0	3	0	9.5
0	0	1	3	0.15	9.6
0	1	0	3	0.15	11
0	1	1	6	1.2	18
0	2	0	6	1.2	18
0	3	0	9	3.6	38
1	0	0	4	0.17	18
1	0	1	7	1.3	18
1	0	2	11	3.6	38
1	1	0	7	1.3	20
1	1	1	11	3.6	38
1	2	0	11	3.6	42
1	2	1	15	4.5	42
1	3	0	16	4.5	42
2	0	0	9	1.4	38
2	0	1	14	3.6	42
2	0	2	20	4.5	42
2	1	0	15	3.7	42
2	1	1	20	4.5	42
2	1	2	27	8.7	94
2	2	0	21	4.5	42
2	2	1	28	8.7	94
2	2	2	35	8.7	94
2	3	0	29	8.7	94
2	3	1	36	8.7	94
3	0	0	23	4.6	94
3	0	1	38	8.7	110
3	0	2	64	17	180
3	1	0	43	9	180
3	1	1	75	17	200
3	1	2	120	37	420
3	1	3	160	40	420
3	2	0	93	18	420
3	2	1	150	37	420
3	2	2	210	40	430
3	2	3	290	90	1000
3	3	0	240	42	1000
3	3	1	460	90	2000
3	3	2	1100	180	4100
3	3	3	>1100	420	4100

Annexure–III

Questionnaire

DEPARTMENT OF ENVIRONMENTAL SCIENCE,UNIVERSITY OF KASHMIR		
QUESTIONAIRE FOR SURVEILLANCE OF WATER BORNE DISEASE		
SERIAL No:	LOCATION:	DATE:
GENERAL INFORMATION		
NAME:		
PARENTAGE:		
ADDRESS:		
OCCUPATION		
AGE:	SEX: M F	MARITAL STATUS M UM
SOURCE OF WATER SUPPLY:		
1. PIPED WATER Y N		
2. LAKE Y N		
3. OTHER		
DO YOU CONSUME BOILED OR UNBOILED WATER:		
CLINICAL INFORMATION		
HAVE YOU EVER BEEN ILL : Y N		
WHAT TYPE OF SYMPTOMS YOU HAD:		
LOOSE MOTIONS	VOMITING	CHILLS
NAUSEA	BLOODY DIARRHOEA	BODY ACHES
ABDOMINAL CRAMPS	FEVER	FATIGUE
SWEATS	HEADACHE	MUSCLE ACHES
OTHERS:		
DID YOU SEE A DOCTOR: Y N		
DID YOU GOT TREATED AT HOME: Y N		
DID YOU GOT HOSPITILISED: Y N		
DAYS OF HOSPITILISATION:		
WHERE WAS TESTING DONE:		
DID ANY OTHER FAMILY MEMBER HAD SIMILAR SYMPTOMS: Y N		
IF YES:THEN		
I. NAME:		
II. PARENTAGE:		
III. ADDRESS:		
IV. AGE:		
V. RELATIONSHIP TO YOU:		
DID YOU ATTENDED ANY LARGE GATHERING e.g. wedding, party, funeral etc PRIOR TO ILLNESS: Y N		
DID YOU TRAVELLEDANY WHERE PRIOR TO ILLNESS: Y N		

Signature of scholar…….

Annexure IV

Interpretation chart of biochemical tests for Gram negative bacilli

No	Test	Reagent added	Principle	Original colour of medium	Positive reaction	Negative reaction
1	Indole	Kovac's reagent	Detects the deamination of tryptophan	Colouless	Reddish pink	Colouless
2	Methyl red	Methyl red reagent	Detects acid production	Colouless	Red	Yellow orange
3	Voges Proskauer	Baritt reagent A and B	Detects acetoin production	Colouless	Reddish pink	Slight copper
4	Citrate	-	Detects citrate as carbon source	Green	Blue	Green
5	Glucose	-	Glucose utilization	Pinkish red	Yellow	Red/Pink
6	Adonitol	-	Adonitol utilization	Pinkish red	Yellow	Red/Pink
7	Arabinose	-	Arabinose utilization	Pinkish red	Yellow	Red/Pink
8	Lactose	-	Lactose utilization	Pinkish red	Yellow	Red/Pink
9	Sorbitol	-	Sorbitol utilization	Pinkish red	Yellow	Red/Pink
10	Mannitol	-	Mannitol utilization	Pinkish red	Yellow	Red/Pink
11	Rhamnose	-	Rhamnose utilization	Pinkish red	Yellow	Red/Pink
12	Sucrose	-	Sucrose utilization	Pinkish red	Yellow	Red/Pink

Interpretation chart of biochemical tests for Gram negative bacilli

No	Test	Reagent added	Principle	Original colour of medium	Positive reaction	Negative reaction
1	Citrate	-	Detects citrate as carbon source	Yellow green	Blue	Yellow green
2	Lysine decarboxylase	-	Detects lysine decarboxylation	Olive green	Purple	Yellow
3	Ornithine decarboxylase	-	Detects Ornithine	Olive green	Purple	Yellow
4	Urease	-	Detects urease	Orange yellow	Pink	Orange yellow
5	Phenylalanine deamination	TDA reagent	Detects phenylalanine	Colourless	Green	Colourless
6	Nitrate	Sulphanilic acid and Dimethyl napthylamine	Detects nitrate reduction	Colourless	Pinkish red	Colourless
7	H_2S production	-	Detects H_2S	Orange yellow	Black	Orange yellow
8	Glucose	-	Glucose utilization	Red	Yellow	Red/Pink
9	Adonitol	-	Adonitol utilization	Red	Yellow	Red/Pink
10	Lactose	-	Lactose utilization	Red	Yellow	Red/Pink
11	Arabinose	-	Arabinose utilization	Red	Yellow	Red/Pink
12	Sorbitol	-	Sorbitol utilization	Red	Yellow	Red/Pink

Contd……..

Annexure IV Contd.

Interpretation chart of biochemical tests for Gram positive Bacilli

No	Test	Reagent added	Principle	Original colour of medium	Positive reaction	Negative reaction
1	Maloanate	-	Detects maloanate	Light green	Blue	Light green
2	Voges Proskauer	Baritt A and B	Detects acetoin	Colourless	Pink red	Slight copper
3	Citrate	-	Detects citrate	Green	Blue	Green
4	ONPG	-	Detects beta - galactosidase	Colourless	Yellow	Colourless
5	Nitrate	Sulphanilic acid and Dimethyl napthylamine	Detects nitrate	Colourless	Pink red	Colourless
6	Catalase	2% H_2O_2	Detects catalase)	effervescence	effervescence	
7	Arginine	-	Detects arginine	Light purple	Dark purple	Yellow
8	Sucrose	-	Detects sucrose	Pink red	Yellow	Red/pink
9	Mannitol	-	Detects mannitol	Pink red	Yellow	Red/pink
10	Glucose	-	Detects glucose	Pink red	Yellow	Red/pink
11	Arabinose	-	Detects arabinose	Pink red	Yellow	Red/pink
12	Trehalose	-	Detects trehalose	Pink red	Yellow	Red/pink

Interpretation chart of biochemical tests for Gram positive cocci

No	Test	Reagent added	Principle	Original colour of medium	Positive reaction	Negative reaction
1	Voges Proskauer	Baritt reagent A and B	Detects acetoin	Colourless	Pink red	Slight copper
2	Esculin hydrolysis	-	Detects esculin	Cream	Black	Cream
3	PYR	PYR reagent	Detects PYR enzyme	Cream	Cherry red	Cream
4	ONPG	-	Detects beta - galactosidase	Colourless	Yellow	Colourless
5	Arginine	-	Detects arginine	Light purple	Dark purple	Yellow
6	Glucose	-	Detects glucose	Pink red	Yellow	Pink/Red
7	Lactose	-	Lactose utilization	Pink red	Yellow	Pink/Red
8	Arabinose	-	Detects arabinose	Pink red	Yellow	Pink/Red
9	Sucrose	-	Detects sucrose	Pink red	Yellow	Pink/Red
10	Sorbitol	-	Sorbitol utilization	Pink red	Yellow	Pink/Red
11	Mannitol	-	Detects mannitol	Pink red	Yellow	Pink/Red
12	Raffinose	-	Detects raffinose	Pink red	Yellow	Pink/Red

Appendix

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Isolation, identification and seasonal distribution of bacteria in Dal Lake, Kashmir

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ABSTRACT

This research work determined the bacteriological characteristics of the waters of Dal Lake, Kashmir. Bacteriological studies were carried out from April 2010 to March 2011 at eight different sites. Out of total 894 bacterial colonies isolated, 11 isolates of bacteria namely *Klebsiella pneumoniae, Citrobacter freundii, Enterobacter aerogenes, Escherichia coli, Vibrio cholerae, Serratia marcesens, Pseudomonas aeruginosa, Staphylococcus aureus, Micrococcus luteus, Bacillus* spp, and *Salmonella* spp. were studied for isolation, identification and seasonal distribution in Dal lake waters.. Out of these species, *E. coli* was most abundant (15.77%) followed by *Enterobacter aerogenes* (12.19%), *Bacillus spp* (11.96%), *Staphylococcus aureus* (10.85%), *Micrococcus luteus* (10.17%), *Pseudomonas aeruginosa* (8.27%), *Klebsiella pneumoniae* (6.71%), *Vibrio cholerae* (6.59%), *Salmonella* spp (6.15%), *Serratia marcesens* (5.92%) and *Citrobacter freundii* (5.59%). Highest number of colonies 155(17.33%) were obtained from site PKB followed by DLG- 136 (15.21%), GB-126 (14.09%), NL-116 (12.97%), TBN-113 (12.63%), BHN-107 (11.96%), HB-74 (8.27%), BD-67 (7.49%).

Keywords: Dal Lake, Bacterial colonies, Seasonal distribution, Colony count

1. Introduction

Dal Lake has been one of the most beautiful water bodies in the past. However, once beautiful water body has been the worst victim of tremendous anthropogenic pressures. The uniqueness of the lake has deteriorated as it is getting enriched with pollutants. Due to ecological stress from human activities the lake system is not only shrinking in surface area but its water quality has also deteriorated as large quantity of untreated sewage and garbage are received by the lake from human settlements as well as business settlements. Atlas and Bartha (1993) considered that bacteria play an important role in global ecosystems which are major factors in controlling the quality of water and are fate determinators of pollution released to environment. The cycling of nutrients such as carbon, nitrogen and sulphur is completed by bacteria. Decomposers form an especially important part of fresh water and convert detritus and organic material into needed nutrients and are exchanged between various parts of the ecosystems. Clark and Pagel (1977) considered bacteria as a reliable indicator of contamination. The usefulness of monitoring the microbial community is due, in part to its ability to respond quickly to environmental conditions and major role it plays in biogeochemical cycling process (Griffiths and Babick, 1983). Microorganisms often play a major role in determining the extent of the pollution (Higgins and Burns; 1975). In general, it is important to evaluate the changes in microbial diversity, community composition in aquatic ecosystem because these factors are the foundation of biogeochemical cycles

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(Sekiguchi et al., 2002). In order to gain an insight into the possible reasons responsible for microbial pollution in the Dal lake ecosystem, the present study was undertaken.

2. Materials and Method

2.1 Location and Site Description

The Dal lake, located at 34^0 07 N ,74^0 52'E, 1584 m a.s.l in Srinagar, Jammu and Kashmir, India- a multi basined lake with Hazratbal ,Bod Dal Gagribal,and Nagin as its four basins having two main inlets as Boat Hall Nallah and Tailbal Nallah and two main outlets as Dal Lock Gate and Pokhribal Nallah was taken up for the current study Eight(8) sites were selected for the present study with four (4)sites in the four basins, two(2) sites in the two inlets and two more sites in the two outlets. The selected sites are HB (Hazratbal), NL (Nagin Lake), GB (Gagribal), and BD (Bod Dal), TBN (Tailbal Nallah), BHN (Boat hall Nallah), DLG (Dal Lock Gate) and PKB (Pokhribal Nallah)

2.2 Collection of Water Samples

The water samples were collected on seasonal basis for a period of 12 months between April 2010-March 2011, from the eight (8) different study sites of the lake in white plastic containers, which were previously rinsed with distilled water and sterilized with70% alcohol. At the lake, the containers were rinsed thrice with the lake water before being used to collect the samples.

2.3 Isolation of Bacteria

Water samples obtained from different sites were serially diluted ten folds and then spread plate technique was followed for isolation of waterborne bacteria in the study, spreading 0.1ml inoculums from the serial dilution tubes on the Petri dishes containing Nutrient agar medium. The pH of the isolation medium was adjusted to 7.2 before sterilization. Two different techniques viz Serial dilution plate (Clesceri et al., 1998) and Spread plate (Sharp and Lyles, 1969) were used for enumeration and isolation of bacteria the plates were incubated at a temperature of 37 °C for a period of 24-48 hours. Growing colonies were counted on the digital Quebec colony counter to determine the number of colony forming units (cfu/ml) of the water samples. Colonies were transferred to Petri dishes containing selective culture media like, EMB agar, Simmons Citrate agar, Cetrimide agar, Pseudomonas agar, Mac Conkey agar, SS agar, Blood agar, KF agar and other culture media like Wilson and Blair media, TCBS agar for identification purpose. Then every colony was transferred to nutrient agar for preservation of stock cultures. For provisional identification of bacteria important Gram staining, Endospore staining, Capsule staining, Motility test were done. In order to study the morphology of bacteria, cells were heat killed and fixed on the slide. The fixed bacteria were stained and studied for size, shape, arrangement, spore formation and capsulation etc. Hanging drop method was performed to study motility of bacteria.

2.4 Identification of Bacteria

In order to identify bacteria, it is important to observe the cultural characteristics of isolates in terms of growth on differential medias and colony characteristic. The colony characteristics include; colony size, shape, margin, elevation, surface, chromogenesis and hemolytic behavior and the most important biochemical behavior of bacteria for utilization of specific

substrate and enzymatic activity. These included carbohydrate fermentation, arginine hydrolysis, catalase test, gelatin hydrolysis, IMViC test, phenylalanine deaminase test, citrate utilization test, urease test etc. The isolates of gram positive bacteria were identified with the help of Bergey's manual of Systematic bacteriology (Sneath et al., 1986). On the other hand, enteric bacteria were identified by using Bergey's manual of systematic bacteriology (Krieg and Holt, 1984).

3. Results and discussions

During the study period, bacterial count of the water samples of the Dal Lake revealed substantial number of coliform bacteria. A total of 894 bacterial colonies were isolated and purified to determine the individual and total % occurrence (table 1 and 2).The study revealed that the most dominant species of bacteria in Dal lake water is *E. coli* 15.77% followed by *E. aerogenes* with 12.19%, *Bacillus* spp with 11.96%, *S. aureus* with 10.85%, *M. luteus* with 10.17%, *P. aeruginosa* with 8.27%, *K. pneumoniae* with 6.71%, *V. cholerae* with 6.59%, *Salmonella spp* with 6.15%, *S. marcesens* with 5.92% and *C. freundii* with 5.59%. The results demonstrated that the Dal Lake is considerably polluted with bacterial population. Earlier Garnier et al. (1992) and Hasan et al. (2006) have also reported more or less similar results showing the load of heterotrophic bacteria in the river and sewage lagoon of Buriganga River. The results are in confirmation with the results of Kumar et al. (2011) who found the presence of 17 bacterial isolates from Three Ponds of Karwar District, Karnataka.

Table 1: Colony count of different bacteria in different seasons at different sites of Dal Lake, Kashmir

S. No	Name of bacteria	PKB				TBN			
		Spr	Sum	Aut	Win	Spr	Sum	Aut	Win
1.	*Klebsiella pneumoniae*	3	0	5	1	2	1	3	1
2.	*Citrobacter freundii*	1	1	3	0	3	1	2	1
3.	*Vibrio cholera*	6	4	0	1	1	0	0	2
4.	*Staphylococcus aureus*	9	12	6	10	4	8	5	8
5.	*Enterobacter aerogenes*	2	10	3	0	6	9	2	1
6.	*Serratia marcesens*	1	2	1	0	0	1	0	1
7.	*Pseudomonas aeruginosa*	5	4	3	1	3	2	3	2
8.	*Escherichia coli*	13	2	5	8	8	1	6	4
9.	*Micrococcus luteus*	4	1	3	0	2	0	2	1
10.	*Bacillus spp*	4	0	8	3	1	1	9	3
11.	*Salmonella spp*	1	2	0	7	1	1	0	1
S. No	Name of bacteria	DLG				GB			
		Spr	Sum	Aut	Win	Spr	Sum	Aut	Win
1.	*Klebsiella pneumoniae*	2	2	1	1	1	8	1	0
2.	*Citrobacter freundii*	4	5	3	2	3	2	1	1
3.	*Vibrio cholera*	1	8	0	0	5	1	2	1
4.	*Staphylococcus aureus*	0	0	5	2	2	0	2	1
5.	*Enterobacter aerogenes*	6	0	8	3	8	6	4	4
6.	*Serratia marcesens*	8	0	0	2	4	2	0	1
7.	*Pseudomonas aeruginosa*	3	0	4	1	5	1	3	O
9.	*Micrococcus luteus*	3	0	8	6	7	4	5	3

10.	*Bacillus spp*	3	5	11	1	2	8	7	0
11.	*Salmonella spp*	0	8	1	5	1	3	0	6

Table 2: Colony count of different bacteria in different seasons at different sites of Dal Lake, Kashmir - Continued

S. No	Name of bacteria	NL				BHN			
		Spr	Sum	Aut	Win	Spr	Sum	Aut	Win
1.	*Klebsiella pneumoniae*	2	6	4	1	1	2	0	1
2.	*Citrobacter freundii*	1	1	1	3	0	0	0	1
3.	*Vibrio cholerae*	4	0	2	2	2	3	1	1
4.	*Staphylococcus aureus*	1	3	2	0	1	2	3	2
5.	*Enterobacter aerogenes*	6	4	2	1	2	6	0	0
6.	*Serratia marcesens*	2	1	1	2	6	1	6	3
7.	*Pseudomonas aeruginosa*	3	0	1	4	2	4	7	1
8.	*Escherichia coli*	7	2	4	3	4	3	8	2
9.	*Micrococcus luteus*	6	1	0	7	7	2	4	4
10.	*Bacillus spp*	1	7	6	6	1	5	2	2
11.	*Salmonella spp*	2	2	0	2	2	1	1	1
S. No	Name of bacteria	HB				BD			
		Spr	Sum	Aut	Win	Spr	Sum	Aut	Win
1.	*Klebsiella pneumoniae*	2	2	0	1	1	2	1	2
2.	*Citrobacter freundii*	1	1	1	1	0	2	1	3
3.	*Vibrio cholerae*	3	1	0	2	1	0	1	4
4.	*Staphylococcus aureus*	2	1	1	1	1	1	0	2
5.	*Enterobacter aerogenes*	6	0	0	3	1	1	2	1
6.	*Serratia marcesens*	2	0	2	1	1	0	2	0
7.	*Pseudomonas aeruginosa*	1	2	2	0	1	2	4	0
8.	*Escherichia coli*	7	3	5	4	3	1	3	6
9.	*Micrococcus luteus*	3	2	2	1	2	1	2	2
10.	*Bacillus spp*	2	4	0	0	1	2	1	1
11.	*Salmonella spp*	1	1	0	0	4	0	0	1

B = Hazratbal; NL = Nigeen Lake; GB = Gagribal; BD = Bod Dal; TBN = Tailbal Nallah; BHN = Boathall Nallah; DLG = Dal Lock Gate; PKB = Pokhribal Nallah
Spr = spring season; Sum =summer season; Aut = autumn season; Win = winter season

Table 2: Colony count and Percentage occurrence of different bacteria at different sites of Dal Lake, Kashmir

Name of bacteria	TC								GT	%Occurrence
	PKB	TBN	DLG	GB	NL	BHN	HB	BD		
K. pneumoniae	9(15%)	7(11.66%)	6(10%)	10(16.66%)	13(21.67%)	4(6.67%)	5(8.33%)	6(10%)	60	6.71
C. freundii	5(10%)	7(14%)	14(28%)	7(14%)	6(12%)	1(2%)	4(8%)	6(12%)	50	5.59
V. cholerae	11(18.64%)	3(5.08%)	9(15.25%)	9(15.25%)	8(13.55%)	7(11.86%)	6(10.17%)	6(10.17%)	59	6.59
S. aureus	37(38.14%)	25(25.77%)	7(7.21%)	5(5.15%)	6(6.18%)	8(8.24%)	5(5.15%)	4(4.12%)	97	10.85
E. aerogenes	15(13.76%)	18(16.51%)	17(15.59%)	24(22.01%)	13(11.92%)	8(7.33%)	9(8.25%)	5(4.58%)	109	12.19
S. marcesens	4(7.54%)	2(3.77%)	10(18.86%)	7(13.20%)	6(11.32%)	16(30.18%)	5(9.4%)	3(5.66%)	53	5.92
P.aerugin osa	13(17.56%)	10(13.51%)	8(10.81%)	9(12.16%)	8(10.81%)	14(18.91%)	5(6.75%)	7(9.45%)	74	8.27
E. coli	28(19.85%)	19(13.47%)	12(8.51%)	17(12.05%)	16(11.34%)	17(12.05%)	19(13.47%)	13(9.21%)	141	15.77
M. luteus	8(8.79%)	5(5.49%)	19(20.87%)	13(14.28%)	14(15.38%)	17(18.68%)	8(8.79%)	7(7.69%)	91	10.17
Bacillus sp.	15(14.01%)	14(13.08%)	20(18.69%)	17(15.88%)	20(18.69%)	10(9.34%)	6(5.60%)	5(4.6%)	107	11.96
Salmonella sp.	10(18.18%)	3(5.45%)	14(25.45%)	10(18.18%)	6(10.90%)	5(9.09%)	2(3.63%)	5(9.09%)	55	6.15

TC = Total Count; GT = Grand Total

The maximum % of bacterial colonies were at the site PKB (17.33%) followed by DLG (15.21%), GB (14.09%), NL (12.97%), TBN (12.63%), BHN (11.96%), HB (8.27%) and BD

(7.49%). The results present in table 3 revealed that the highest number of bacterial colonies 265 (29.6%) was found in spring season followed by 224 (25.05%) in autumn 221 (24.72%) in summer season and 184 (20.58%) in winter season.

Table 3: Total Colony count and Percentage Occurrence of bacterial colonies in different seasons at different sites of Dal Lake, Kashmir

Sites	Total number of colonies in different seasons				
	Spr	**Sum**	**Aut**	**Win**	**Total**
PKB	49	38	37	31	155
TBN	31	25	32	25	113
DLG	32	36	44	24	136
GB	44	37	26	19	126
NL	35	27	23	31	116
BHN	28	29	32	18	107
HB	30	17	13	14	74
BD	16	12	17	22	67
Total	265(29.6%)	221(24.72%)	224(25.05%)	184(20.58%)	894

The seasonal variation found in the study is in more or less conformation with the results of Ajibade and Ayodele (2006). Microorganisms in air and in the soil can have access to the water bodies and contamination may take place either more or less continually or at regular intervals under certain unusual condition as during or immediately after heavy rains. According to Bonde (1977) bacteriological analysis is a powerful tool in order to foreclose the presence of microorganis that might constitute health hazard. Zaky et al. (2006) studied Manzala lake Egypt reporting that it suffers from water pollution induced by drainage, sewage and has increased bacterial content and same was reported in the current study. Out of the 894 bacterial isolates obtained in the present study some were randomly selected and purified for detailed bacterial study. Among these some were gram positive and some gram negative. Among gram positive strain were member of genus *Bacillus, M. luteus* and *S. aureus.* The results clearly indicated that among the gram positive bacteria *bacillus* was dominant genus. Enteric bacteria were gram negative which belonged to *E. coli* followed by *E. aerogenes*. *E. coli* was found to be the dominant group among the gram negative bacteria.
Based on biochemical tests, *K. pneumoniae* showed positive reaction to Voges Proskauer test, Citrate test, Glucose fermentation, Adonitol test Arabinose test, Lactose fermentation, Sorbitol test, Mannitol test and Sucrose fermentation whereas *C. freundii* displayed positive to Methyl red test, Citrate test, Glucose Arabinose, Lactose, Sorbitol, and Sucrose fermentation test. *E. aerogenes* responded to some of the biochemical tests like VP test, Citrate as well as the Carbohydrate fermentation test. *E. coli* showed positive response to Indole test, Methyl red test and the entire Carbohydrate test except, Adonitol fermentation test. *Vibrio* showed positive response to Oxidase, Catalase, Indole, Lysine decarboxylase and Ornithine deaminase test. *S. aureus* showed Glucose fermentation Catalase and Coagulase positive reactions. *S. marcesens* responded positive to Lysine and Citrate and also showed Indole reaction negative. *P. aeruginosa* displayed fruity odour, greenish pigment, positive reaction towards Oxidase and beta haemolysis. *Micrococcus* showed positive reaction towards Catalase and Oxidation of glucose. *Salmonella* spp showed positive reaction towards Lysine, Hydrogen sulphide, Indole and Citrate tests. Based on the microscopic studied it was

conformed that majority of the above identified coliform bacteria were found to be gram negative.

4. Conclusion

The preliminary identification revealed that highly polluted surface waters are abundant in various types of bacteria among which, *E. coli* as well as *K. pneumoniae*, *C. freundii*, Vibrio cholerae, *E. aerogenes*, *Salmonella* spp and *P. aeruginosa* were predominant. Moreover, Gram-positive bacteria includes *Bacillus* spp, *S. aureus* and *M. luteus* which are washed out from the soil and get their entry into the water bodies during heavy rain falls also belong to the allochthonous bacteria. The results present in table 3 revealed that the highest number of bacterial colonies 265(29.6%) was found in spring season followed by 224(25.05%) in autumn 221(24.72%) in summer season and 184(20.58%) in winter season.Therefore, in spring season, seasonal fluctuation of bacteria was highest and in winter season it showed lowest seasonal fluctuation. Municipal wastes and faecal matter are the main source of pathogenic bacteria. The role of air in water contamination is significant in densely populated areas of cities. The developmental activities and occupancy in the area is exerting pressure on the water body. Surface water in urban water bodies almost always contains some degree of contamination. This is due to exposure to animals, humans, aquatic life, etc. In addition to this, variety of other human activities resulted in increasing the bacterial concentration of lake water.

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Water quality assessment of Dal Lake, Kashmir using the coliforms as indicator bacteria

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ABSTRACT

Polluted water contains pathogenic bacteria that are usually involved in disease transmission and are referred to as "indicator bacteria". The most common indicators used are the coliform bacteria. In present study an assessment of this indicator group of bacteria was carried out in Dal Lake at sixteen (16) different sites. The seasonal and spatial variation of the total coliform outbursts showed the influence of water temperature, sewage influx, intervention of human activities from within and outside the lake. Coliforms were enumerated using multiple-tube fermentation (MTF) technique with lactose broth as the presumptive medium, Brilliant Green Bile broth for completed test and EMB medium as the confirmatory medium. All the samples tested were positive with respect to the coliform occurrence, though the count was variable. The coliform count at all sites ranged between a MPN/100ml value of 3 to 1100 and the overall coliform load observed in the water samples was lowest in winter season compared to the summer season. It was further observed that none of the samples was fit for drinking purpose with respect to this particular parameter as it does not meet requirements of World Health Organization (WHO) standards. 89.07% of the water samples obtained from the lake were having a good or fair quality and 10.93% were having poor quality hence unfit for any use.

Key Words: Dal Lake; coliforms; indicator bacteria; water quality

INTRODUCTION

There has been growing concern about the needs to protect the environment from various forms of pollution caused by growing population, industrialization and by modern agricultural methods (Hunt & Wilson 1986). Water and land based anthropogenic activities within the system and in the catchment including the release of nutrients, organic matter, toxic chemicals and water borne pathogens have a negative effect on water quality. Bacterial contamination in particular accelerates when human activities are augmented, jeopardizing the safe use of water for drinking and recreational purposes. Bacteria often play a vital role in determining the extent of pollution (Higgins & Burns 1975) and the presence of faecal coliform is considered as presumptive evidence of faecal pollution (Mara 1978). The density of coliform bacteria in water is a significant criterion of degree of pollution in aquatic ecosystems (Odum 1985). It is well established that a large number of infectious diseases are transmitted primarily through water supplies contaminated with human and animal excreta particularly faeces (WHO 1993). Outbreaks of water borne diseases continue to occur throughout the world but especially serious in developing countries (Jones *et al.* 2007). The human pathogens that present serious risk of disease whenever present in drinking water include *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica*, *Campylobacter* spp. and so on (Geldreich 1992; Pommervilli 2007). But it is not practicable to monitor drinking water for every possible pathogen. Therefore, normal intestinal organisms including coliform group of organisms (Covert *et al*., 1989) are used as indicator of faecal pollution (WHO 1984; Cartwright *et al.* 1993). They are considered as suitable indicators because they are easy to detect and enumerate in water. Considering the reality about the coliforms, present work was undertaken to determine total coliform count to the context of biological pollution level to reveal the overall status of water quality in Dal Lake.

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MATERIAL AND METHODS

Location and site description

Dal Lake, lying between geographical coordinates 34° 07′ N, 74° 52′ E, 1584 m a.s.l in Srinagar, Jammu and Kashmir, a multi-basined lake with Hazratbal, Bod Dal, Gagribal and Nageen as its four basins, having two main inlets as Boathall Nallah and Tailbal Nallah and two main outlets as Dal Lock Gate and Pokhribal Nallah, was taken up for the current study. Sixteen (16) sites viz., Hazratbal Open, Hazratbal littoral, Nageen Open, Nageen near Houseboats, Gagribal Open, Gagribal near Houseboats, Nishat Open, Near Centeur, Boathall Nallah-I, Boathall Nallah-II, Tailbal Nallah-I, Tailbal Nallah-II, Dal Lock Gate-I, Dal Lock Gate-II, Pokhribal Nallah-I and Pokhribal Nallah-II with 8 sites from the 4 basins, 4 sites from two inlets and 4 other sites from two outletswere selected.

Collection of water samples

Water samples were collected on seasonal basis in white plastic containers, which were previously sterilized with 70% alcohol and rinsed with distilled water. At the lake, the containers were rinsed thrice with the lake water before being used to collect the samples. The samples were transferred immediately to the laboratory for analysis within 24 hours (APHA 1998).

Multiple tube fermentation technique

The technique used for enumerating coliforms was multiple-tube fermentation (MTF) technique (Rompre *et al*. 2002). The method consisted of inoculating a series of tubes with appropriate decimal dilutions of the water sample. Production of gas, acid formation or abundant growth in the test tubes after 48 h of incubation at 35°C constituted a positive presumptive reaction. Lactose broth was used as presumptive media (Collins & Lyne 1976; Bakare *et al*. 2003) and all tubes with a positive presumptive reaction were subsequently subjected to a confirmation test. The formation of gas in a brilliant green lactose bile broth fermentation tube (Coyne & Howell 1994) at any time within 48 h at 35°C constitutes a positive confirmation test. The results of the MTF technique were expressed interms of the most probable number (MPN) of microorganisms present. This number is a statistical estimate of the mean number of coliforms in the sample.

RESULTS

The data revealed that all the samples collected for different sites of the lake were positive with respect to the coliform occurrence, though the count was variable. All the samples tested showed coliform counts above the permissible limits. The coliform count at all the sixteen sites (Table 1) ranged between a MPN/100ml of 3 to 1100. The overall coliform count in the open water sites of different lake basins ranged between a minimum of 3 MPN/100ml to a maximum of 1100 MPN/100ml. While drawing a comparison between the different open water sites of these basins highest count of these indicator organisms was observed in Nigeen followed by the Gagribal basin, Hazratbal basin and Nishat basin. In the littoral sites of these basins again a highest count of coliform bacteria was found in the Nigeen lake followed by the Gagribal basin, Hazratbal basin and Nishat basin Here it ranged between a minimum of 4 MPN/ 100ml to a maximum of 1100 MPN/100ml. However, the count was higher in the group of littoral sites and lower in the group of open water sites as in case of the littoral sites 11 (34.37%) observation showed the coliform count above 200 MPN/100ml while as in case of the open water sites the observation with such higher coliform count was only 7 (21.87%). The coliform count of all the inlet sites with 9 (28.12%) observations having the MPN count >200 was comparatively higher than those of the outlet sites with 96.87% (31) observation having the MPN/100ml of coliform bacteria <100. In both inlets the coliform count was higher towards the outer ends (TBN2 and BHN2) as compared to the end connected with the lake (TBN1 and BHN1). But in case of the outlet sites the count was higher towards the extreme exit points (DLG2 and PKB1) compared to the near outlets (DLG1 and PKB2). The data further reveals a great deal of seasonal and spatial variation in coliform bacterial count in the lake water as the overall coliform load observed was lowest in winter season compared to summer season. Furthermore the load was higher in the second year of study compared to the first year of study throughout the lake.

The category wise distribution of coliform count (Table 2) into four categories with MPN range of 0 (zero) for category I, 4-50 MPN/100ml for category II, 51-400 MPN/100ml for category III and 401-1100 MPN/100ml for category IV shows that 50% water samples lie in category III, followed by 39.06% samples in category II, 10.93% samples in category IV and 0% sample in category I. The perusal of the data indicates that none of the samples was fit for drinking purpose with an excellent water quality with respect to this particular parameter.

Table 1: MPN index of water samples collected from Dal Lake

S.No	Site code	Spring		Summer		Autumn		Winter	
		2010	**2011**	**2010**	**2011**	**2010**	**2011**	**2011**	**2012**
Open sites	HB1	7	14	39	120	94	39	14	3
	NO	7	14	39	75	43	28	14	3
	GB1	75	150	150	460	93	120	43	64
	NL1	240	460	1100	1100	240	460	150	210
Littoral sites	HB2	9	15	43	120	75	43	15	4
	NC	9	15	43	93	64	39	15	4
	GB2	93	210	210	460	150	240	64	75
	NL2	240	460	1100	1100	240	460	150	210
Inlet sites	TBN1	64	93	150	240	210	460	120	43
	BHN1	64	93	120	460	93	150	39	64
	TBN2	75	120	210	460	150	240	150	64
	BHN2	75	120	210	460	150	240	43	75
Outlet sites	DLG1	20	28	43	75	39	23	14	9
	PKB2	28	39	64	93	75	64	43	21
	DLG2	21	39	64	93	43	28	21	11
	PKB1	23	43	75	120	93	64	39	20

Table 2: Category wise distribution of coliform count (MPN/100ml)

Categories	MPN range	% age	Usage	Grade
Category I	0	0	Drinking	Excellent
Category II	4-50	39.06	Bathing , swimming	Good
Category III	51-400	50		Fair
Category IV	401-1100	10.93	Unfit	Poor

DISCUSSION

The principal coliforms are *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella* spp. and *Citrobacter* spp. *E.coli* is abundantly found in the gastro intestinal tracts of humans, birds and animals, but rarely found in water or soil that has not been subjected to faecal pollution. Thus presence of coliforms in Dal lake indicates the extent of faecal pollution in it (Godfree *et al*. 1997; Mossel 1958). The presence of classified indicator (*E. coli*) in water suggests the presence enteric pathogens (Nwadiaro 1982) and there is a direct relationship between the numbers of *E. coli* and the extent of faecal pollution. The higher its number, the more polluted is the sample (Akoleowo 2002). The count of total coliform bacteria was significantly higher at all littoral sites in all basins and the possible reason for this was that these sites in each basin are more prone to direct impact from human activities and also from point and non-point sources of sewage. As human activities increase so does the bacterial contamination as found in all the sites which are directly susceptible to interventions taking place within and outside the lake. Kundangar *et al.* (2003) also has attributed increase in total coliform count of Dal Lake to environmental variables such as low dissolved oxygen and human influence at inshore sites. The polluted nature of these sites is further clarified by the findings of various studies (Rai & Hill 1978; Ramadhan 1971; Clark & Pagel 1977) reporting that the total coliforms indicate degree of pollution and are a reliable indicator of contamination or pollution. Seyfried (1995) while investigating the effect of various site characteristics on bacterial levels, found a positive correlation between the bacterial number, number of boats and the amount of organic carbon in the sediments. The occurrence of coliforms in the sample is also confirmed by a local study conducted by Latief *et al*. (2003) reporting high coliform count in fifteen springs of Kashmir valley.

The coliform count in the lake water showed considerable seasonal and spatial variation with highest coliform load in summer season and lowest in winter season that could be related directly to the water temperature. With the increase in water temperature from spring onwards there was a corresponding increase in coliform count indicating that it governs the coliform dynamics in the lake, which is in conformation with various other studies (Sastry *et al*. 1970; Hadas 1988; Verma & Paul 1996; Hadas *et al*. 2000). The present observation is also favoured by the study carried out by Wetzel (1975) reporting that the lower bacterial biomass during winter and higher during summer in temperate lakes can be correlated to low winter temperature and reduced loading of particulate and dissolved organic substrates from allochthonous sources and the vice versa in summer. Moreover the higher load of coliform bacteria in the summer seasons could also be attributed to tourist influx resulting in the increased movement of house boats in the Lake, where there is no proper disposal system for the night soil, thus resulting is water quality deterioration coupled with the outburst of bacterial counts.

The MPN index observed for water samples revealed that the maximum samples were crossing the permissible limits set by WHO (2003) indicating gross pollution of the lake and its transition to eutrophic status. Water source used for drinking or cleaning purpose should not contain any organism of faecal origin (Sabongari 1982; Fonseca *et al*. 2000). The World Health Organization (WHO 1984) suggested that treated water entering the distribution system should contain no coliform organisms. Thus in accordance to the WHO limits and the work of Pandey and Sharma, 1999 it was observed that most of the water samples obtained from the lake were fit for bathing and swimming with a good or fair quality while as some areas of the lake were having very much poor quality, hence unfit for any use.

CONCLUSION

The density of total coliform bacteria in the lake water indicates that the lake water is deteriorated and is not fit for drinking purposes. It is further visible that the heavy influence of human activities has resulted in elevated levels of total coliforms as compared to natural conditions. Inadequate sanitary system, poor land use pattern in the immediate catchment and the discharge of waste water continues to jeopardize the water quality of the lake for human use. Therefore, control must be implemented to minimize bacterial transport to such natural systems.

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