Study of fungal diversity of Dal Lake, Kashmir with particular reference to pathogenic species

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By

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Certificate

Certified that the PhD thesis entitled **"Study of fungal diversity of Dal Lake, Kashmir with particular reference to pathogenic species"** is the original research work carried out by **Mr. Suhaib A. Bandh**, as whole time PhD scholar in Department of Environmental Science, University of Kashmir, Srinagar. This 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 this University. It is further certified that this thesis is fit for the submission of the degree of **Doctor of Philosophy in Environmental Science** and the candidate has fulfilled all the statutory requirements for the completion of the PhD Programme.

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truly brothers when needed. My sister has been my best friend all my life and I thank her for all her advice and support. These past several years have not been an easy ride, both academically and personally. I truly thank my parents for sticking by my side, even when I was irritable and depressed. I always fall short of words and felt impossible to describe their support in words. But, if I have to mention one thing about them, among many, then I would proudly mention that my parents are very simple and they taught me how to lead a simple life. I would simply say, “Amma, Abba you are great!”.

Suhaib A. Bandh

Abstract

Water from Dal Lake gives a unique opportunity to investigate the microbiology of a typical low altitude urban lake environment that remains completely influenced by different types of anthropogenic activities all through the year. Water samples collected from sixteen different sites of the lake were extensively studied in terms of the fungal component and some physical parameters like pH and temperature.

Fungal population was assessed in terms of colony forming units (cfu/ml) and total viable counts on Rose Bengal Streptomycin Agar (RBSA) and Potato Dextrose Agar (PDA). Fifty one (51) species of fungi belonging to two groups viz. the filamentous group (28 species) and the zoosporic group (23 species) were isolated and identified. The species were identified on the basis of morphological observation and culture using differential culture media like Czepek dox Agar (CZ) media, Czepek Yeast Agar (CYA) media, Malt Extract Agar (MEA) media, Potato Dextrose Agar (PDA) media, CHROM Agar *candida* and classical slide culture techniques besides the use of biochemical tests for the identification of yeast specimens and molecular approach. The molecular level identification was carried out by Polymerase chain reaction (PCR) using universal fungal primers (ITS1, ITS2, ITS3 and ITS4) often used for estimating fungal diversity in environmental samples and considered important in modern microbiological assays.

The fifty one (51) species of fungi belonged to five major phyla viz. Ascomycota (four major classes Eurotiomycetes, Sordariomycetes, Ascomycetes and Saccharomycetes), Oomycota (one class Oomycetes), Blastocladiomycota (one class Blastocladiomycetes), Zygomycota (one class Zygomycetes) and Besidiomycota (one class Tremellomycetes). The different classes of the fungi contributed a total of nine orders including: Saprolegniales, Pythiales, Eurotiales, Hypocreales, Tremellales, Mucorales, Blastocladiales, Saprolegniales and Pythiales. Of all the phyla Ascomycota contributed a maximum of seven (7) genera and twenty five (25) species followed by Oomycota contributing six (6) genera and nineteen (19) species. The

genus wise maximum relative abundance was shown by *Penicillium* (21.6%) with a contribution of eleven (11) species. The fungal species isolated belonged to nine families with maximum contribution of 19 species by family trichocomaceae and minimum by Nectriaceae, Hypocreaceae, Tremellaceae one species each. The developed colonies on the RBA plates enumerated by Qubeck Colony counter and the fungal load assessed in terms of colony forming units (cfu/ml) revealed that the concentration of fungal colonies was comparatively higher in all seasons of second year of study as compared to the seasons of first year of study with overall highest of 9.80×10^4 cfu/ml in summer season and lowest 0 cfu/ml in winter seasons. Out of a total of 6940 fungal colonies isolated during the study, 3157 colonies were contributed by 28 species of filamentous group and 3783 colonies were contributed by 23 species of zoosporic group. In the filamentous group the maximum contribution was from family Trichocomaceae (2517colonies, 79.72%) followed by Saccharomycetaceae, Mucoraceae, Nectriaceae, Tremellaceae and Hypocreaceae while as in case of zoosporic group Saprolegniaceae contributed with a maximum number of 2474 colonies (65.39%) followed by Blastocladiaceae and Pythiaceae.

The fungal population of the lake studied showed considerable seasonal variation with maximum number of fungal colonies from both the fungal groups in warm temperature seasons and the minimum in cold temperature seasons. The one way analysis of variance (ANOVA) carried out on the seasonal data showed that the colonial distribution of the fungal species was statistically significant ($p < 0.01$ for filamentous fungal group and $p < 0.05$ for zoosporic fungal group) in different seasons. Spatial variation of the fungal species showed that the highest number of 46 species was isolated at site 8 while as the lowest number of 32 species was isolated at site 3. The pH of the water samples collected from the lake during the study showed a negative correlation with the fungal load depicting while as the temperature showed a perfect positive correlation with the fungal load.

The distantly located sites showed the least similarity while as closely located sites showed maximum similarity. Occurrence of the fungal species showed a remarkable seasonal and temporal variation across the lake with *P. chrysogenum*, *S. parasitica*, *Dictyuchus* spp., *A. cornuta* and *P. funiculosum* as the five most dominant and prominent species. Other species were dominating the predefined categories of the lake like the littoral zones, the open water zones, the inlets and the outlets.

Diversity and evenness patterns calculated for the occurrence of different fungal species shows that, Shannon-Wiener (H) index was highest (3.69) at site 16 and lowest (3.21) at site 3. The evenness pattern for the said sites was also highest (0.93) at site 16 and lowest (0.76) at site 4. The one way Analysis of variance (ANOVA) carried out showed that the Dominance ($p < 0.01$), Diversity ($p < 0.01$) and Evenness ($p < 0.01$) varied significantly between different microhabitats of the lake. The one way analysis of variance (ANOVA) carried out pair wise between the different sites for both the filamentous and zoosporic fungal species isolated from different sites showed that 64% results were statistically significant with 54% as highly significant ($p < 0.01$) and 10% as significant ($p < 0.05$).

Furthermore the study also showed the occurrence of many pathogenic fungal species in the lake water samples. The prevalence of fungal infections investigated in 20% population (n=384) including both male and female from all age groups by interviewing and examining them for any sort of superficial fungal infection on their body showed the prevalence of a few cases of minor fungal skin infections, Onchomycosis and candidiasis. Of a total of 31 positive cases for different types of fungal diseases; 18 (4.68%) individuals were positive for fungal skin infections followed by 9 (2.34%) individuals positive for Onchomycosis and 4 (1.04%) female individuals positive for Candidiasis. The overall infection rate of the tested population was 8.07% (31 positive cases out of 384 individuals) with higher prevalence of fungal infections in males than females. The infection rate was higher in case of the working class population (first two age groups) compare to the non-working class (third age group). The prevalence of diseases also varied with the type of water being used for different purposes with a high prevalence of infection 6.77% seen in the people using lake water for different domestic purposes followed by the people using tap water with a positive prevalence of 1.30%.

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ABBREVIATIONS USED

Abbreviation		Full form
%O	:	Percentage Occurrence
%TC	:	% Total Count
°C	:	Degree Centigrade
°F	:	Degree Fahrenheit
ANOVA	:	Analysis of variance
APHA	:	American Public Health Association
BHN	:	Boathall Nallah
bp	:	Base pair
Ca	:	Calcium
CA	:	Citric acid;
CFU	:	Colony Forming Units
CPOM	:	Coarse Particulate Organic Matter
CYA	:	Czepek Yeast Agar
CZ	:	Czepek dox Agar
DLG	:	Dal Lock Gate
DM	:	D-Mannitol;
DNA	:	Deoxyribose Nucleic Acid
dNTP	:	deoxy Nucleotide Tri Phosphate
DOM	:	Dissolved Organic Matter
E	:	East
F_{cric}	:	F critical
FPOM	:	Fine Particulate Organic Matter
ft	:	Feet
G	:	Glucose
G37°C	:	Growth at 37°C
Ga	:	Galactose
GB	:	Gagribal
GCm	:	Growth on Cycloheximide medium;
Gl	:	Glycerol;
GTT	:	Germ Tube Test
H	:	Hydrolysis
HB	:	Hazratbal
IARI	:	India Agriculture Research Institute
ITS	:	Internal Transcribed Spacer
K	:	Potassium
L	:	Lactose
LA	:	Lactic acid;

LSU	:	large subunit
M	:	Maltose
MEA	:	Malt Extract Agar
Mg	:	Magnesium
$MgCl_2$	:	Magnesium Chloride
ml	:	Millilitre
N	:	North
Na	:	Sodium
NaCl	:	Sodium Chloride
NC	:	Near Centaur
NCI	:	Number of Cases of Isolation
NEO	:	Necrotising External Otitis
NL	:	Nageen lake
NO	:	Nishat Open
NS	:	Number of Species
NSS	:	Normal Saline Solution
OR	:	Occurrence Remarks
PCR	:	Polymerase Chain Reaction
PDA	:	Potato Dextrose Agar
PET	:	Poly Ethylene
PKB	:	Pokhribal nallah
PN	:	Potassium nitrate;
POM	:	Particulate Organic Matter
R	:	Raffinose;
RA	:	Relative Abundance
RBSA	:	Rose Bengal Streptomycin Agar
rDNA	:	Ribosomal DNA
RFLP	:	restriction fragment length polymorphisms
rpm	:	Revolutions per minute
rRNA	:	Ribosomal RNA
S	:	Sucrose
SA	:	Succinic acid
SS	:	Soluble Starch;
SSU	:	Small subunit
T	:	Trehalose
TBN	:	Tailbal nallah
TC	:	Total Count
TDS	:	Total Dissolve Solids
UV	:	Ultra Violet

V	:	Variable
WHO	:	World Health Organization
µl	:	Microlitre
µM	:	Micromoles

Chapter 1
Introduction

Water is a blessing of Allah and is very precious resource of this planet as it is an established source of life, without which, no life would be possible. Water is so important; it is everywhere, wherever we look on this planet. It is in the atmosphere as humidity, clouds or rain, it is in the Earth's crust, within the rock and soil fabric, it is on the soil as lakes, rivers, streams or oceans and it is in every living being, animal or plant. Over other planets and even galaxies, the constituent parts of water are searched as emblem of life. Water is considered as one of the nutrients, although it yields no calories, yet it enters into structural composition of cell and is an essential component of diet (Baloch *et al.,* 2000). It constitutes two-third of body cell matter and 90% of all body fluids, including the blood as well as the lymphatic and spinal fluids. It is necessary for all biological processes, and also contributes to the regulation of body temperature through perspiration (Buskirk *et al*., 1958). It is well known that human health and survival depends on use of uncontaminated and clean water for drinking and other domestic purposes.

1.1. Water: The Elixir of Life

Water, the magical substance from which all life springs forth, is essential to every life form on earth, without it the very existence of every life form on earth is impossible. Life originated in water and the ultimate basis of it, the protoplasm, is a colloidal solution of complex organic molecules in watery medium with 70-90% water. The role of water in the living organisms has not changed since life's first creation in salt water billions of years ago. Most of the biological phenomenons take place in water medium and water exists in nature at all levels of life. Water is essential at all levels of life, cellular to ecosystem. It is essential to the circulation of body fluids in plants and animals, and stands as the key substance for the existence and continuity of life through reproduction and 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. Ancient cultures and civilizations had great respect for water and worshipped this "life substance" which they often equated with life

force and surrounded with myths and legends. These people knew the vital importance of water.

As nature has an innate mechanism to maintain its purity after every natural use. It is unable to do this at the rate at which modern human beings add dirt to it. Nature does not know how to deal with several toxins and pollutants that are flowing from industrial and other waste sources. Today, people in the developed world take water largely for granted and treat it as a low cost commodity rather than the priceless elixir it is. Water has always been one of the most precious commodities and a source of civilization, without which life and civilizations could not have survived. Right from the beginning man has treated water as free gift from God and hence his birthright to use and squander it as he saw fit. Therefore, humans are bound to monitor the impact of this activity on natural freshwater continuously. In biological literature, this nine letter dirty word pollution has several connotations; to biologists, it can presage the development of conditions adverse to the continued existence of certain types of microbes inhabiting a water body. Human beings by their anthropogenic activity are making fresh water as dumping grounds for receiving solid and liquid waste from nearby human settlements. Eighty percent of water supply of cities finds its way to drainage system as domestic and industrial waste

1.2. Fungi

Fungi are ubiquitous and diverse eukaryotic, heterotrophic organisms, including both single-celled yeasts and multi-cellular filamentous fungi. They are accepted as a fifth kingdom comprising 700,000 named species with as many as 1.5 million species predicted (Hawksworth, 2001). Fungi living in water environments are represented by more than one thousand species. The members of Chytridiomycetes and Oomycetes are mostly aquatic and commonly known as water moulds. The Oomycetes are a class of fungal-like protists included an assemblage of lower eukaryotes often referred to as stramenopiles (Dick, 2001; Paliwal and Sati, 2009).The fungi in freshwater habitats comprise a diverse assemblage of true fungi and fungus-like straminopiles and protists. Fungi are found in all types of freshwater habitats, including rivers, streams, drainage ditches, marine swamps, bogs, lakes, ponds, temporary ponds, and wetlands. Representatives of all fungal classes can be found in or isolated from freshwater habitats. The groups are (1) Chytridiomycetes and Hyphochytridiomycetes (2) Peronosporomycetes (formerly Oomycetes) (3)

Ascomycetes and (4) mitosporic fungi. It is highly likely that yeasts play significant roles in freshwater habitats, but studies documenting their occurrence and importance are rare (Braun, 1856). Ingold (1940) isolated and described many filamentous fungi, whose spore morphologies were adapted to dispersal in running waters followed by attachment to plant detritus, such as leaves. They then became known as aquatic hyphomycetes or 'Ingoldian fungi'.

1.2.1. Evolution of fungi in aquatic habitats

An organism is always in the state of perfect balance with the environment. The environment refers to various conditions surrounding an organism which directly or indirectly influence the life and development of the organism and its population. In an ecosystem a basic functional unit called "Organism" interacts with the environment and other chain of organisms, various physico-chemical and climatic conditions. Water is an important natural resource for the survival of living beings. Water reservoirs, lakes, dams, ponds, rivers serve as natural habitat and help to maintain ecological balance of different kinds of microbes. In water bodies various organism act together and allow continuous recycling of each chemical element available in the system. In water bodies, it is the myriad of diverse organisms acting in conversion that allows continuous recycling of each chemical element available in the system. When this stops for some reason, pollution results and pollution pushes the environment out of balance and the scheme of nature is that it reacts to re-establish the balance (Dugan, 1974). The Kingdom Fungi was only recognized some 40 years ago, and its diversity is vastly understudied with only approximately 7% of total estimated species described (Mueller and Schmit, 2007). According to Shearer *et al*. (2007) there is an urgent need for better documentation of the numerous undescribed species, especially in aquatic habitats. Based on their evolutionary history, fungi in aquatic habitats are either primarily (Chytridiomycetes) or secondarily adapted to life in water (aquatic hyphomycetes, yeasts). In addition, primarily terrestrial forms (fungi imperfecti, endophytes) are found in lakes, suggesting that their occurrence is not necessarily restricted to a single habitat by stringent morphological or physiological adaptations. Like Oomycetes (fungus-like organisms belonging to the Kingdom Chromista), Chytridiomycetes (Kingdom Fungi) possess chemotactic, flagellated zoospores, which can disperse in the water column (Sparrow, 1968). They are located at the base of the fungal phylogenetic tree, which

means they had a window of 900 to 480 million years (Heckman *et al*., 2001) to potentially colonize and adapt to all aquatic habitats and niches. They may well be ubiquitous within freshwater ecosystems (Sparrow, 1960) but have often been overlooked (Powell, 1993; Canter-Lund and Lund, 1995) or misidentified, e.g. as flagellates (Lefèvre *et al*., 2007). In contrast, aquatic hyphomycetes and yeasts are secondary invaders of aquatic habitats and are polyphyletically scattered in the fungal tree. Aquatic hyphomycetes and other filamentous terrestrial forms generally need a solid substrate and use the water column only for dispersal of their propagules, whereas yeasts are potentially found everywhere including the pelagic zone.

Terrestrial fungi are often passively introduced into lakes in the form of high loads of fungal propagules via inflowing streams, rainwater, and wind (Smirnov, 1964). However, it is often unclear whether such fungi, lacking typical morphological adaptations, are terrestrial or truly aquatic. For example, species of *Aspergillus* and *Penicillium* are among the most common fungal isolates from terrestrial, freshwater, and marine environments, even from deep-sea sediments. Some are active and partially adapted to aquatic habitats. Conversely, truly aquatic fungi have been found active in several terrestrial habitats. Park (1972) provides a useful characterization of such fungi based on their activity, ranging from indwellers (with constant to no activity) to transients (no activity).

Not surprisingly, fungi are often found in disturbed areas with high anthropogenic loads from various sources like domestic, industrial, municipal and sewage treatment plants (Cooke, 1976; Weber *et al*., 2009); many fungal species can survive in oligotrophic environments, through scavenging nutrients from the substrate which they colonize, or the air or water in which they live. However, they also occur under ultra-oligotrophic conditions, such as in a water distillation apparatus (Wainwright, 2005).

1.2.2. Fungal occurrence in specific lake habitats

Lakes are highly structured into different zones, and each zone can harbor specific animal, plant and fungal communities. At the outset, and of particular relevance for fungi, it is important to distinguish between open-water and shallow habitats, highly structured lake zones that are dominated by macrophytes and periphyton, directly connected to terrestrial influences. Due to greater supply of organic matter, littoral zones are the hot spot for all kinds of fungi, whereas the

pelagic zones harbor only highly specialized species or serves as a medium for the dispersal of propagules.

1.2.2.1. Littoral zone

The littoral area in lakes is a transition zone between terrestrial and aquatic habitats. It is a biodiversity hot spot and home to many aquatic, aero-aquatic and terrestrial fungi as well as a place where many energy transformation processes take place. (Sparrow, 1968; Webster and Descals, 1981; Ali and Abdel-Raheem, 2003). These fungi grow on plant detritus in water and mud at low oxygen levels but need air exposure for reproduction. Over 600 fungal species have been recorded from the common reed *Phragmites australis* (Gessner and van Ryckegem, 2003), and other emergent macrophytes have also been a rich source of fungal taxa (Ellis and Ellis, 1985). Additionally, dead materials from macrophytes and allochthonous litter such as leaves, twigs, and wood accumulate in the littoral areas resulting in high abundance of aquatic hyphomycetes and other fungal groups (Nilsson, 1964; Casper, 1965), and there is a naturally high fungal diversity on all kinds of dead macrophytes and plant litter including their aufwuchs in lakes (Casper, 1965; Sparrow, 1968; El-Hissy *et al*., 1990; Czeczuga, 1991; Hyde and Goh, 1998; Wong *et al*., 1998; Cai *et al*., 2002; Luo *et al*., 2004; Orlowska *et al*., 2004; Czeczuga *et al*., 2005; Nechwatal *et al*., 2008). All these materials are readily colonized and degraded, often by zoosporic fungi (Sparrow, 1960 and 1968).

1.2.2.2. Pelagic zone

Most pelagic fungi are likely facultative parasites or saprotrophs (Sparrow, 1960) that quickly exploit any opportunity to scavenge nutrients. The dominant planktonic fungi are zoosporic chytrids which have adapted to the pelagic life by actively swimming between substrates. These fungi (van Hannen *et al*., 1999) have long been recognized as prominent members of plankton communities in lakes (Sparrow, 1960), their role as parasites and their influence on algal succession (Sparrow, 1960; Masters, 1976; van Donk and Bruning, 1992 and 1995; Canter-Lund and Lund, 1995; Ibelings *et al*., 2004; Sigee, 2005; Kagami *et al*., 2007) cannot be denied. Due to their short generation times, their appearance is sporadic and patchy (Sparrow, 1968; Masters, 1976) and strongly dependent on environmental factors such as substrate density, light, temperature, and oxygen (Masters, 1976; van Donk and Bruning, 1995; Holfeld, 1998; Ibelings *et al*., 2004). Under certain circumstances,

e.g. during an algal bloom, a mass development of one fungal species is observed and is often followed by its complete disappearance (Alster and Zohary, 2007). Filamentous fungi are thought to be absent in open water, which serves as an important dispersal medium for their propagules. The main representatives of higher fungi in open water are unicellular yeasts. In pelagic water, even in marine systems, there is a stable population of yeasts at relatively low densities (Ahearn *et al*., 1968). For many years it was assumed that yeasts are transients washed in from the phylloplane or the littoral zone, but there is now clear evidence for their more or less permanent residence in open water. In addition to *Cryptococcus* spp. several red and black yeast species occur in lake surface water (van Uden and Ahearn, 1963; Woollett and Hedrick, 1970; Slavikova *et al*., 1992; Rosa *et al*., 1995; Libkind *et al*., 2003; Lefèvre *et al*., 2007), and their production of photoprotective compounds is thought to be one of their adaptations as residents of surface waters (Libkind *et al*., 2006). Selected species can be used as bioindicators for several kinds of anthropogenic pollution in aquatic environments (Hagler, 2006).

1.2.2.3. Profundal zone and sediment

Sediments serve as seedbank for resting spores of not only aquatic fungi but also of various terrestrial species (Flegler *et al*., 1974). Fungi are generally thought to be aerophilic, but several species from all fungal phyla can withstand or remain active under anoxic conditions (Scupham *et al*., 2006). Many lake sediments connect with an aquifer and these aquifers seem to be a natural habitat for fungi (Ekendahl *et al*., 2003; Krauss *et al*., 2005), with an often astonishingly high diversity of yeasts and lower fungi (Brad *et al*., 2008). Hence, aquifers are potentially important for dispersal of fungal propagules between limnetic systems. Near the lake margin the fungal community in the sediment is enriched by littoral species (Collins and Willoughby, 1962; Sparrow, 1968; Ali and Abdel-Raheem, 2003).

1.2.2.4. Fungi in different lake ecotypes

Sparrow (1968) described bogs as one of the richest sources of bizarre types of chytrids. Peatlands also are a rich source for diverse fungal species (Thormann *et al*., 2007; Artz *et al*., 2007). From saline lakes, halotolerant black yeasts and other species could be isolated regularly (Mallea, 1992; Zalar *et al*., 1999; Butinar *et al*., 2005;

Zalar *et al*., 2005; Cantrell *et al*., 2006) with some of them now being considered indigenous (Gunde-Cimerman *et al*., 2000).

1.3. Ecological role of fungi in lake ecosystems

The ecological role of fungi in water bodies is structured by responses to the abiotic environment and interactions with the biotic environment, which can lead to very successful, permanent associations, as is the case with lichens. Obligate biotic interactions already occur in the basal fungal phylum of the Chytridiomycota, whose members often depend on living or dead host materials (www.int-res.com). Species often produce multinucleate hyphae and cell walls that comprise both of b-glucan and chitin (Tehler, 1988; Cavalier-Smith, 1998 and 2001; Kirk *et al*., 2004).

1.3.1. Decomposition process of particulate organic matter

In terrestrial ecosystems, fungi are widely accepted as the dominant decomposers of plant matter, especially of highly recalcitrant lignocellulosic fibers, primarily functioning as recyclers of organic material. This is also true for lotic systems, and fungi introduce allochthonous leaf litter compounds into the food web of streams (Gasith and Hasler, 1976; Preston *et al*., 2008). In oxygen-rich, wave-impacted zones, litter is colonized and conditioned by fungi and mechanically disintegrated by invertebrates (primarily by shredders and scrapers) and wave action (Baldy *et al*., 2002; Bohman 2005; Pabst *et al*., 2008). Fungal saprobes play a major role in freshwater ecosystems as decomposers of plant and animal tissues, and parasitic fungi occur on planktonic and benthic algae, aquatic macrophytes, fishes, amphibians, invertebrates, and animal-like protists. Watermolds contribute significantly in aquatic ecosystems and are seriously concerned with the utilization and degradation of complex organic matter such as animal and plant remains and recycling of nutrients (Wetzel and Likens, 2000; Mueller *et al*., 2004; Kiziewicz, 2005; Mazurkiewicz-Zapałowicz *et al*., 2008). The watermolds are primarily saprophytic in nature. A few species are parasitic on aquatic and terrestrial plants and on aquatic animals; they can be zoopathogenic or phytopathogenic (Kiziewicz, 2004; Rossetti, 2005; Feregendeza-Granez *et al*., 2007; Kiziewicz and Nalepa, 2008).

1.3.2. Inner pelagic recirculation of nutrients: fungi as part of the microbial loop

Fungi, as heterotrophic microbiota, are links in the microbial food web, transferring nutrients directly and indirectly to other trophic levels. The parasitic fungi support a direct link between large ‘inedible’ algae like *Asterionella* and filter-feeding

zooplankton like *Daphnia* (Kagami *et al*., 2004). Later terming it as 'mycoloop' (Kagami *et al*., 2007), Gleason *et al*. (2008) proposed the inclusion of (1) saprophytic fungi as a remineralization pathway in aquatic food webs based on all kinds of POM (dead phytoplankton, leaves, pollen grains, insects and crustacean exoskeletons, cadavers, and other detritus) that might be solubilized, and (2) the potential use of dissolved inorganic compounds (similar to bacteria) as seen in cell cultures. In contrast to bacteria, and due to their strong competition in the open water, fungi strictly depend on surfaces or steep gradients to settle e.g. lake snow aggregates. Fungi are not separated from, but are an integral part of the microbial loop.

1.4. Impacts on human health

Some fungal species are known to be pathogenic or allergic, thus implicating negative impacts on health. The consumption of fungi-contaminated drinking water has, as far as is known, not caused acute disease, at least in immuno-competent individuals (Hageskal *et al*., 2009). However, there is a risk of superficial or localised infection in healthy individuals and more severe and invasive infection in immuno-compromised patients. Some species also have the potential to cause allergic reaction and disease. Furthermore, investigation of health effects of fungal secondary metabolites is an active area of research since some of them are toxic and others are thought to cause taste and odour problems in tap water and other water resources.

There are a number of reasons to suggest that water should be considered as a potential transmission route for pathogenic or allergenic fungi. However, a number of other environmental sources exist.

1.4.1. Pathways of exposure

The four principal pathways by which people can be exposed to fungi of aquatic systems are:

- **Ingestion:** by drinking contaminated water directly;
- **Inhalation:** of aerosolised spores while showering or in the sauna;
- **Skin contact:** with contaminated water, while showering or bathing and
- **Introduction through mucous membranes:** such as the skin, eyes and oral cavity, while showering or bathing.

Aerosolisation of spores or fragments of hyphae from water has been particularly investigated as a pathway of exposure. There a number of infections

which are known to be caused by fungi, which can be classified according to the site of initial infection (Richardson, 2005)

- **Superficial mycoses**: infections of the skin, nails, hair and mucous membrane, such as topical candidiasis. Such infections are relatively common and easily treated.
- **Subcutaneous mycoses**: infections of the dermis, subcutaneous tissues and adjacent bones, usually arise from implantation of fungi in soil or decomposing vegetation and are most common in tropical and sub-tropical regions when skin is exposed to soil (e.g. when barefoot).
- **Systemic mycoses**: originate in an internal organ, often the lungs, and may spread to other organs (i.e. become invasive). These infections may be caused by true pathogens which can invade normal (i.e. immuno-competent) hosts, or by opportunistic pathogens which are less virulent and can only invade immuno-compromised hosts.

A limited number of species are responsible for such diseases; it is thought that of the 50,000 to 2,50,000 known species of fungi, 500 have been linked to disease in humans and 100 can cause disease in otherwise healthy individuals (Richardson and Warnock, 2003 and 2012). The most problematic species are *Candida* spp. (especially *C. albicans*), *Aspergillus* spp. (especially *A. fumigatus*) and *Cryptococcus neoformans* (Pfaller *et al*., 2006 and Paterson *et al*., 2009). Immuno-competent individuals with no underlying health condition may experience superficial or localised infections but with fewer complications and a much smaller risk of disseminated or invasive disease and death (Anaissie *et al*., 1989; Chen *et al*., 2001 and Walsh *et al*., 2004). It is worth mentioning that not all species of the same genus have the same degree of toxicity, pathogenicity or allergenicity (Hageskal *et al*., 2009). However, the genetic boundaries between species are not well defined and can be misleading (Paterson and Lima, 2005). Different species of different genera have been found pathogenic on different types of hosts with some of the following being worth mentioning.

1.4.2. *Aspergillus* spp.

Aspergillus spp. have been isolated from drinking water and other sources by many studies (Annaisie *et al*., 2002). Its different species are similar to

Legionella species, known water pathogens, in several aspects of their ecology, including amplification in water reservoirs. Infections caused by *Aspergillus* species are known as aspergillosis, a term which covers a range of invasive and non-invasive infections and allergic diseases. It is increasingly being recognised that water is an environmental source of *Aspergillus* spp. and has been identified as being the source of exposure (Annaisie *et al*., 2002; Warris *et al*., 2003).

- The skin and the digestive system have been identified as points of entry for *Aspergillus* spp. (as opposed to lungs which are the point of entry for airborne fungi);
- Invasive aspergillosis has been linked anecdotally with inhalation of contaminated surface water in patients who have suffered near drowning (Warris *et al*., 2001a & b).

The different species of genus *Aspergillus* like *Aspergillus terreus, Aspergillus ustus* and *Aspergillus fumigatus* are reported to cause onychomycosis, otitis media, primary cutaneous infection, endocarditis, pneumonia and disseminated infection (Hageskal *et al*., 2006; Panackal *et al*., 2006).

1.4.3. Other pathogenic taxa from water

Different species of genus *Candida* are frequent cause of infections, which can range from superficial candidiasis infections that are common and easily treated, to systemic candidiasis. Superficial infections can occur in the skin and mucous membranes, and can arise from the overgrowth of normal yeast flora. Systemic or invasive candidiasis includes disseminated candidiasis, candidemia (i.e. presence of *Candida* spp. in blood), endocarditis and meningitis. It has a mortality rate of 40-50% (De Rosa *et al*., 2009). The patients demonstrate a number of predisposing factors, including use of antibiotics, intensive care treatment, surgery, cancer and intravascular catheters (Douglas, 2003; Tortorano *et al*., 2004). *Candida* spp. have also been observed in drinking water however, it is not known whether this is a significant pathway for infection.

Infections caused by *Fusarium* spp. are increasing in frequency in immuno-compromised patients. They carry a high mortality rate; 79-87% of patients die within 90 days of being diagnosed. *Fusarium* species have been isolated from drinking water and have been identified as the environmental source of *Fusarium* infections (Kibbler

et al., 2003). *Acremonium* infections have also been observed in vulnerable individuals with chronic lymphocytic leukaemia (Herbrecht *et al*., 2002).

1.4.4. Allergies

Many species of fungi, including *A. fumigatus*, *A. niger*, *A. flavus*, *Penicillium* spp. and *Cladosporium* spp. also found in water, are known to be potential allergens (Paterson and Lima, 2005). Allergic symptoms may also arise in response to dead spores and other fungal debris that would not be culturable (Kauffman and van der Heide, 2003). Therefore, water that is found to be free of fungi from testing by culture may in fact still provoke allergic disease. There is strong evidence of a correlation between fungal exposure and severity of asthma (Hogaboam *et al*., 2005). Much of the evidence is related to associations between frequency of asthma attacks and numbers of airborne spores as, such spores may at times get aerosolised from a water source (Denning *et al*., 2006).

Allergic fungal rhinitis has also been reported, causing nasal obstruction and congestion. Symptoms are similar to allergic fungal sinusitis, which is caused by a wide range of fungal species, including *Alternaria* spp., *Aspergillus* spp., *Cladosporium* spp. and *Penicillium* spp., many of which have been isolated from drinking water (Ponikau *et al*., 1999). Fungi have been implicated in hypersensitivity pneumonitis or extrinsic allergic alveolitis (Seuri *et al*., 2000).

1.4.5. Mycotoxin mediated health impacts

1.4.5.1. Mycotoxins and mycotoxin producers

Some fungal taxa, including *Penicillium* spp., *Aspergillus* spp., *Fusariam* spp., *Alternaria* spp. and *Claviceps* spp. produce mycotoxins during their metabolic processes and have been observed in water resources. Of the thousands of mycotoxins, aflatoxins and zearalenone are the most relevant and have been detected in drinking water (Paterson and Lima, 2005; Paterson *et al*., 2009).

1.4.5.2. Health effects of mycotoxins

The effects of mycotoxins can be mutagenic (cause mutations), teratogenic (disturb embryo development), oestrogenic (mimic the action of oestrogen) or carcinogenic (produce a cancer). The frequency of such impacts and their severity depends on the mycotoxin in question, its concentration, the exposure pathway and the duration of exposure. They can also damage major organs or systems such

as the nervous, endocrine or immune system (Paterson *et al*., 2009). However, no reports have been identified of disease attributed to mycotoxins produced in the water distribution system (Kelley *et al*., 1997; Paterson and Lima, 2005).

Aquatic habitats have disappeared at a rapid rate over the past 100 years, and many of them have been and continue to be altered by agrarian practices, land development and pollution. It is also imperative that cultures of freshwater fungi be isolated and maintained for future use in habitat restoration, experimental approaches to aquatic ecosystem function and phylogenetic studies.The relevance of fungi and their activities in water as indicator organisms, as human, animal and plant pathogens, as a source of food and energy for macrofauna, as agents in self-purification process and in the remineralisation of organic materials and formation of sediments emphasizes the importance of mycological studies of aquatic habitats. It is important that the species in existing aquatic habitats be documented so that the effect of habitat alterations on species composition and hence function can be detected. A detailed knowledge of the diversity and functioning of microorganisms dwelling in the freshwater ecosystem (like Dal Lake) will be crucially important in the sustainable management of the freshwater resources of Kashmir. Hence the present topic entitled "*Study of fungal diversity of Dal Lake, Kashmir with particular reference to pathogenic species*" was undertaken with the following objectives

- Isolation, identification, characterization and classification of fungal diversity of Dal Lake, Kashmir.
- Documentation of the fungal community characteristics (abundance, diversity and density).
- Identification of disease causing species of fungi.

The study focused on the unexplored and undocumented fungal diversity of this important water body of Kashmir valley.

1.4.6. Study Area

The Dal lake, in Srinagar, (latitude 34^{o} 07′ N; longitude 74^{o} 52′ E; altitude 1583 m) the summer capital of Jammu and Kashmir, represents an enchanting and unique ecosystem with respect to its species diversity, aesthetics and interaction with people living within it. The Himalayan lake is an 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) the 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 even see the fishes below. Perhaps in the whole world there is no corner as pleasant as the Dal lake (Lawrence, 1895). 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. During the British Raj, Srinagar was the summer capital. The British circumvented Dogra Maharaja's rule of not being allowed to own land and

build houses in Srinagar by commissioning houseboats to be built with lavish settings on the Dal lake to enjoy the decent climate of the Kashmir valley during summer months on the lake, amidst the back drop of the majestic snow covered Himalayan ranges. The houseboats, which they got built, are acclaimed as "each one a little piece of England afloat on Dal lake". Traditionally, for centuries, Kashmiri Hanji people have built, owned and maintained these houseboats. They live on the lake, cultivate floating gardens and market products and thus enjoy a unique life style in the Kashmir valley. The houseboats thus became an integral part of the celebrated beauty of the Dal lake and are infact still the best option of enjoyable accommodation in Srinagar.

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 lake is having a total catchment area of 337 km^2, out of which Teilbal-Dachigam is largest catchment (234 km^2), which is further divided into the Teilbal-Dara (89 km^2) and Dachigam National Wildlife Reserve (141 km^2) sub-catchments. The Teilbal-Khimber-Hadoora sub-catchment consists of the south facing slope and are mostly treeless (barren landscape) with soil characteristics being undifferentiated yellow grey podsolic to distinct podsolic (AHEC, 2000). During precipitation events it is a source of diffuse runoff laden with silt and nutrients. The Dal Lock Gate and Nallah Amir Khan outlets regulated by a weir system, flow almost year round, while the Brari-Numbal outflow carved out recently by the Authority discharges out water by gravity into River Jhelum. The main basin draining the 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 length of the lake is 7.44 kilometres (4.62 miles) with a width of 3.5 kilometres (2.2 miles). The average elevation of the lake is 1,583 metres (5,190 ft). The depth of water varies from 6 metres (20 ft) at its deepest in Nagin Lake to 2.5 metres (8.2 ft), the shallowest at Gagribal. It receives an average annual rainfall of 655 millimetres (25.8 inches) in the catchment that occurs during summer and also in the winter season. During the summer, snow melt from the higher ranges of the catchment results in large inflows into the lake. The lake experiences temperatures in the range of 11–1 °C (52–34 °F) during winter and 12–30 °C (54–86 °F) during the summer season (Sharad *et al*., 2007). The lake freezes when temperatures drop to about −11 °C (12.2 °F) during severe winter.

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 Boats (where they actually live); 758 own House Boats (for commercialization as tourist attraction); 3009 families in Hut house; 5928 families in Pacca House and among them just 2.11% fully aware about the problems of Dal lake (Anonymous, 2007). The lake is encircled by roads all along the periphery and the shore length about 15.5 kilometres (9.6 miles), is encompassed by boulevard road lined with Mughal gardens, parks, houseboats and hotels.

Based on its thermal behavior, the lake has been typified 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 their contribution. Further, the silt load has been estimated at 80,000 tonnes per year with 70% contribution from the Telabal Nallah, out of which the amount that settles in the lake is assessed to be 36,300 tonnes (ENEX, 1987).

Sixteen (16) sites from this lake ecosystem (Plate 1) viz. Hazratbal Open, Hazratbal littoral, Nageen Open, Nageen 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 geographical co-ordinates and altitude (positional error of ±6 meters) of the study sites is given in Table 1.

Table 1. Study sites with geographical co-ordinates

Site no.	Site name	Representative symbol	Altitude (m)	N	E
S1	Hazratbal I	(HBI)	1581	34°07′54.6″	74°52′21.6″
S2	Hazratbal II	(HBII)	1581	34°07′47.7″	74°50′38.0″
S3	Nageen I	(NL I)	1616	34°06′52.0″	74°49′54.7″
S4	Nageen II	(NL II)	1592	34°06′54.1″	74°50′06.4″
S5	Gagribal I	(GB I)	1600	34°05′23.1″	74°51′02.9″
S6	Gagribal II	(GB II)	1600	34°05′31.2″	74°51′26.0″
S7	Nishat Open	(NO)	1580	34°07′26.9 ″	74°52′35.0 ″
S8	Near Centaur	(NC)	1581	34°07′25.1 ″	74°52′34.0 ″
S9	Dal Lock Gate I	(DLG I)	1603	34°04′50.4″	74°49′48.2″
S10	Dal Lock Gate II	(DLG II)	1603	34°04′50.1″	74°49′44.1″
S11	Boathall nallah I	(BHN I)	1601	34°08′45.0″	74°50′36.2″
S12	Boathall nallah II	(BHN II)	1601	34°08′43.0″	74°50′35.0″
S13	Pokhribal nallah I	(PKB I)	1608	34°06′42.6″	74°49′45.2″
S14	Pokhribal nallah II	(PKB II)	1608	34°06′45.7″	74°49′34.5″
S15	Tailbal nallah I	(TBN I)	1592	34°08′31.4″	74°51′40.0″
S16	Tailbal nallah II	(TBN II)	1592	34°08′32.8″	74°51′40.4″

Chapter 2

Review of literature

Fungi are ubiquitous in aquatic ecosystems. They are important decomposers of plant litter in marshes and streams, and act as important intermediaries of carbon flow to higher trophic levels. Representatives of all major fungal phyla have been reported from aquatic habitats, although ascomycetes and their anamorphs (mostly hyphomycetes) dominate fungal communities in plant litter. Aquatic fungi possess the enzymatic capabilities to degrade the major plant constituents, with the possible exception of lignin. Fungi typically surpass bacteria in terms of both biomass and production associated with standing dead plant shoots in marshes and submerged leaf litter in streams.

The occurrence, distribution and seasonal periodicity of some species of aquatic fungi belonging to the orders Chytridiales, Lagenidiales, Blastocladiales, Saprolegniales and Peronosporales from two tanks of Kurukshetra, India, were studied by Gupta and Mehrotra (1989 a & b) describing them to be governed by temperature. While exploring the correlation of algae and fungi affecting the productivity of aquatic environments Zahid and Mehdi (1992) screened two hundred water samples from fifty eight localities of Karachi region and found that the dominating species of algae mostly belonged to the *Chlorophyceae* and *Cyanophyceae*. Among the fungi, species of *Alternaria*, *Aspergillus*, *Aureobasidium*, *Botrytis*, *Caetomium*, *Cladosporium*, *Fusarium*, *Penicillium* and *Trichodesmium* showed positive cellulolytic activity and were found to be responsible in the decomposition of algal cellulose. In 1993, Klich created an identification key of twenty-four species placed in Aspergillus section Versicolores by examining their macro-morphological and micro-morphological characteristics. A cluster analysis of the data indicated that five of the species, *A. silvaticus, A. speluneus, A. floriformis, A. pseudodeflectus*, and *A. pulvinus* were only weakly aligned with other members of the section.

CHROM agar Candida is a novel, differential culture medium that is claimed to facilitate the isolation and presumptive identification of some clinically important yeast species. Odds and Bernaerts (1994) evaluated the use of this medium with 726 yeast isolates, including 82 isolated directly on the medium from clinical material and after two days of incubation at 37°C the *Candida albicans* isolates gave distinctive green colonies, *C. tropicalis* developed distinctive dark blue-gray colonies with a halo

of dark brownish purple in the surrounding agar. *C. krusei* isolates also formed highly characteristic rough, spreading colonies with pale pink centres and a white edge that was otherwise encountered only rarely with isolates of *C. norvegensis*. *Trichosporon* spp. formed small, pale colonies that became larger and characteristically rough with prolonged incubation. Most of the other 310 yeasts studied formed colonies with a color that ranged from white to pink to purple with a brownish tint. The specificity and sensitivity of this medium for the presumptive identification of *C. albicans, C. krusei,* and *C. tropicalis* exceeded 99% for all three species. A blinded reading test involving four personnel and 57 yeast isolates representing nine clinically important species confirmed that colonial appearance after 48 h of incubation on CHROM agar Candida afforded the correct presumptive recognition of *C. albicans, C. tropicalis, C. krusei,* and *Trichosporon* spp. It was inferred that CHROM agar *Candida* was a useful isolation medium capable of the presumptive identification of the yeast species most commonly isolated from clinical material and facilitating recognition of mixed yeast cultures.

Wong *et al*. (1998) worked on the role of fungi in freshwater ecosystems and observed that there are more than 600 species of freshwater fungi with a greater number known from temperate regions compared to tropical regions. The species occurring in lentic habitats mostly differ from those occurring in lotic habitats. Although freshwater fungi are thought to have evolved from terrestrial ancestors, many species are clearly adapted to life in freshwater as their propagules have specialised aquatic dispersal abilities and are involved in the decay of wood and leafy material and also cause diseases of plants and animals.

El-Nagdy and Nassar (2000) reported 21 species belonging to 10 genera of zoosporic fungi from 23 surface water samples collected from ponds of accumulated rainfall in the Riyadh region (Saudi Arabia) using sesame seeds as baits at 20±2 °C. The most common genera found were *Allomyces, Aqualinderella, Dictyuchus* and *Pythium* while as the remaining six genera were less frequent with four species namely; *Allomyces macrogynus*, *Aqualinderella fermentans*, *Pythium rostratum* and *Pythium ultimum* were recorded for the first time in Saudi Arabia. Additionally fifty-one species and one variety of terrestrial fungi belonging to 21 genera were also identified on glucose and cellulose Czapek's Dox agar incubated at 28ºC, with most common species as *Aspergillus flavus, A. fumigates, A. niger, A. flavus* var.

columnaris, *Acremonium stictum*, *Cladosporium herbarum*, *Fusarium oxysporum* and *Penicillium corylophilum*. 17 species of aquatic fungi were identified by El-Hissy *et al*. (2000) in water samples collected from Aswan High Dam Lake. They collected vertical samples on monthly basis to determine fluctuations of these fungi and their correlation with the physicochemical characteristics of the collected water samples from the lake. Surface water samples yielded the highest number of aquatic fungal genera and species (8 genera, 13 species), while water samples collected from near the bottom were poor (3-4 genera or species) and this reduction in fungal taxa, correlated markedly with the reduction in the amount of dissolved oxygen and organic matter. The most common genera found were *Achlya*, *Aqualinderella*, *Pythium* and *Saprolegnia* with moderate occurrence, whereas *Allomyces*, *Aphanomyces*, *Dictyuchus* and *Pythiopsis* were of rare occurrence and irregularly distributed in vertical strata.

Boysen *et al*. (2000) re-evaluated the taxonomic identity of 52 livestock feed isolates from Sweden, previously identified by morphology as *P. roqueforti*, by comparing the sequences of the ribosomal internal transcribed spacer region. Identities were confirmed with random amplified polymorphic DNA analysis and secondary metabolite profiles. Specific oligonucleotide probes were developed by Lindsley *et al*. (2001) to identify medically important fungi that displayed yeast-like morphology *in-vivo*. Universal fungal primers ITS1 and ITS4, directed to the conserved regions of ribosomal DNA, were used to amplify DNA from *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis*, *Paracoccidioides brasiliensis*, *Penicillium marneffei*, *Sporothrix schenckii*, *Cryptococcus neoformans*, five *Candida* species, and *Pneumocystis carinii*. Specific oligonucleotide probes to identify these fungi, as well as a probe to detect all dimorphic, systemic pathogens, were developed. The probes developed for yeast-like pathogens were found to be highly specific and proved useful in differentiating these organisms in the clinical setting.

While working on isolation and identification of native isolates of *Penicillium chrysogenum* from different samples comprising of fruits, vegetables, bread and grains from market around Faisalabad (Pakistan) Rafi and Rahman (2002) adopted morphological features and slide culture method for their identification. Most of the cultural characteristics of *Penicillium chrysogenum* isolates were observed on

Sabouraud's glucose agar medium and Czapek yeast autolysate agar medium. Furthermore, a study of fungal colonization on two timbers (*Dipterocarpus alatus* and *Xylia dolabriformis*) was done by Sivichai *et al*. (2002) in a freshwater stream at KhaoYai National Park, Thailand. Data on sequence of fungal sporulation, the frequency of occurrence of the fungi and percentage cover on the test blocks was presented and 73 species of fungi (48 species on *D. Alatus* and 47 species on *X. dolabriformis*) categorised into 3 groups with clear difference in the dominant species on each timber was observed. Kane *et al*. (2002) submerged blocks of *Fagus sylvatica* and *Pinus sylvestris* in the River Severn, England, for 92 weeks, incubated the recovered test blocks in moist chambers and recorded 35 anamorphic species of sporulating fungi; 13 ascomycota and 2 basidiomycota showing a succession of sporulating fungi on the wood over a 3 month incubation period in the laboratory. The most common fungi on beech were *Camposporium pellucidum, Dictyochaeta parva*. The results were comparable with other studies on lignicolous aquatic fungi from temperate and tropical locations indicating that different fungal communities occur in different geographical locations. Microbial growth in moisture-damaged buildings has been associated with respiratory health effects, and the spores of the mycotoxin producing fungus *Aspergillus versicolor* are frequently present in the indoor air. To characterize the potential of these spores to cause harmful respiratory effects, mice were exposed via intra-tracheal instillation to a single dose of the spores of *A. versicolor*, isolated from the indoor air of a moisture-damaged building. Inflammation and toxicity in lungs were evaluated 24 h later by assessment of biochemical markers and histopathology. The exposure to the spores increased transiently pro-inflammatory cytokine levels (tumor necrosis factor [TNF] α and interleukin [IL]-6) in broncho-alveolar lavage fluid (BALF) with the cytokine responses as dose and time dependent. Moreover, the spores of *A. versicolor* recruited inflammatory cells into airways: Neutrophils peaked transiently at 24 h, macrophages at 3 days, and lymphocytes at 7 days after the dosing. The spores of *A. versicolor* caused acute inflammation in mouse lungs indicating that they have potential to provoke adverse health effects in the persons exposed to them (Jussila *et al*., 2002). A 3-year prospective study of the air, environmental surfaces and water distribution system of a hospital carried out by Anaissie *et al*. (2002) showed significantly higher concentrations of airborne *Aspergillus* propagules in bathrooms, where water use was

highest (2.95 cfu/m^3) than in patient rooms (0.78 cfu/m^3) and in hallways (0.61 cfu/m^3). Water from tanks yielded higher counts of colony-forming units than did municipal water. Isolates of *Aspergillus fumigates* recovered from a patient with aspergillosis was genotypically identical to an isolate recovered from the shower wall in the patient's room. In addition to the air, hospital water systems also appeared to be a source of Nosocomial aspergillosis, thought to be caused primarily by the presence of *Aspergillus* in air and is a life-threatening infection in immunocompromised patients.

Aquatic fungi from four brackish water lakes – Edku, Burullus, Manzala and Qarun located in the city of Egypt studied by Mahmoud and Zeid (2002) reported the recovery of 29 fungal species belonging to 19 genera of aquatic fungi from the water samples collected, with the most frequent occurrence of *Chytridium conferrop, Allomyces throughout* and *Rhizoclosmatium globosum*, and a moderate occurrence of *Thraustochytrium amoeboidum* and *Leptolegniella exoosporus*. The study was further extended to test the ability of six selected aquatic fungi (*Brevilegniella keratinophila, Blastocladiella* c*ystogena, Chytridium conferrop, Entophlyctis variabilis,* S*chizochytrium mangrovei* and *Thraustochytrium rosii*) to uptake the radionuclide from their culture medium as a step to biologically treat the waste water or solution with radio-cesium and radio-cobalt which finally showed that 57% of Cs-137 and 35% of Co-60 could be removed from liquid waste by the selected aquatic fungi. Meanwhile Cai *et al*. (2002) recovered 64 species of higher fungi on submerged wood, bamboo and tree roots in lake Fuxian, China with a frequent occurrence of *Aniptodera chesapeakensis, Dictyosporium heptasporum,* and *Savoryella lignicola* on the wood samples. The occurrence of *Halosphaeria etorquens* and *Halosphaeria cucullata* which were previously recorded from marine habitats was interesting, while as *Lulworthia* was the first record of this genus from a lake. *Pseudohalonectria fuxianii* sp. novo was described, illustrated and compared with similar species in the genus and was declared as the first report of aquatic fungal communities in a lake from mainland China by comparing the data with previous studies. The identification of microfungi by morphology is generally regarded to be very difficult, as traits used for delimitation of species within a genus often show minute differences that can only be reliably evaluated by experienced mycologists. Different strains of one species slightly vary in their morphology. This is often used as an argument by investigators

with rather limited experience that identification on a morphological basis is not reliable and leads to inconsistent results. The development of sensitive methods and techniques contributing to improved identification concerning (i) isolation from mixed cultures, (ii) inoculation and incubation, (iii) usage of media (iv) identification and usage of determination keys, (v) preparation of microscopic slides, (vi) microscopy and (vii) storage of isolates, are very much needed. The techniques compiled here can improve routine determination work considerably and lead to much more accuracy and consistency of results (Fischer and Dott, 2002).

With the aim of surveying the quantitative and qualitative fungal composition of the air and water of the Lake Terkos Istanbul, Turkey Asan *et al*. (2003) conducted a study on the isolation and identification of airborne and waterborne fungi from different parts of the lake by collecting water samples at five different stations on monthly basis for a year. Airborne fungal spore levels were estimated by exposing a petri-dish containing Rose-bengal streptomycin agar medium to air for 15 minutes to air. They reported the recovery of a total of 2372 fungal colonies (1032 from air and 1340 water) on 216 petri plates. 20 mold species belonging to 9 genera with *Scopulariopsis brevicaulis* (22%), *Penicillium expansum* (13.4%) and *Cladosporium herbarum* (12.9%) were the most abundant species. *Cladosporium herbarum* (29.7%) and *sphaerospermum* (27%) were very common in air samples. Isolation of *Aspergillus niger* and *Cladosporium variabile* were rare in the atmospheric and water environment and there was a positive correlation between total colony forming units and a number of environmental factors. The incidence of mold infections in patients with hematologic malignancies continues to increase despite the widespread use of air filtration systems, suggesting the presence of other hostile sources for these molds. Water and other environmental sources are considered to harbour these pathogenic molds. In order to assess their presence in water, air and other environmental sources from a bone marrow transplantation unit with optimal air precautions Anaissie *et al*. (2003) recovered molds (*Aspergillus* species, others) in70% of 398 water samples, 22% of 1311 swabs, plumbing structures and environmental surfaces and 83% of 274 indoor air samples. Microscopic examination of the water plumbing lines revealed hyphal forms compatible with molds. In the study four main findings suggested that indoor airborne molds were aerosolized from the water: (1) higher mean airborne concentrations of molds in bathrooms than in patient rooms and hallways, (2) a strong

type and rank correlation between molds isolated from hospital water and those recovered from indoor hospital, (3) lack of seasonal correlation between the airborne mold concentration in outdoor and indoor air and (4) molecular relatedness between a clinical strain and a water-related strain. It was concluded that hospital water distribution systems serve as potential indoor reservoirs of *Aspergillus* and other pathogenic molds leading to potential exposure for patients.

Luo *et al*. **(**2004) collected 100 submerged woody substrates and grasses from Lake Dianchi, a highly polluted lake in Kunming, China after every three months and examined them for freshwater fungi to investigate their seasonal variation. In the study a total of 97 fungal species including 56 ascomycetes and 41 anamorphic fungi were recorded with a variation in the diversity indices (H´) at the four seasonal collections which were insignificant. Furthermore, the fungal community on woody substrates from this lake was compared with that obtained from lake Fuxian during the same season displaying apparent differences in diversity with higher in lake Fuxian (H´ = 3.808) than in lake Dianchi (H´ = 3.368), which they related to riparian vegetation and pollution. The effects of pollution on freshwater fungal communities was later discussed and elaborated. The same year, a closed lake located in central California called Mono Lake, sometimes compared to the Dead Sea because of its exceptionally higher concentrations of dissolved carbonates, sulfates and chlorides was investigated by Steiman *et al*. (2004) for the mycobiota living in such extreme conditions along with the analysis of soil, tufa, water and sediment samples for their mineral and salt composition. The results showed that soil, tufa, water and sediment samples were rich enough in various chemical elements. Water samples were found sterile in the conditions chosen for fungal isolation, while sediment, soil and tufa samples led to the isolation of a total of 67 fungal species belonging to various taxonomic groups displaying no clear effects of the chemical parameters of the samples analysed on fungal life apart from the pH which showed some correlation.

As fungal infections in the eggs of freshwater fish are very prevalent and are considered as a problematic disease, Chukanhom and Hatai (2004) examined the same in common carp (*Cyprinus carpio*) eggs at three fish farms in Khon Kaen province, northeast Thailand with an attempt to isolate the pathogenic fungi from their eggs. They successfully isolated 19 stocks of fungi from farm A, 2 fungi from farm B and 2 fungi from farm C. Three of them were identified as *Saprolegnia diclina*, *Achlya*

klebsiana, Allomyces arbuscula which grew well at 25°–30°C and pH 6–7, at 30°–35°C and pH 6–7, and at 30°–40°C and pH 6–8 respectively. *Saprolegnia diclina* grew in a medium containing NaCl up to a concentration of 3.0%, whereas *A. klebsiana* and *Al. Arbuscula* grew poorly in 1.0% NaCl. Artificial infection to platyfish (*Xiphophorus maculatus*) was also made using the above mentioned fungal species in which injured fishes were exposed to 104 spores/ml of *S. diclina* and *A. klebsiana* showing 100% mortality rate. Czeczuga and Muszynska (2004) investigated zoosporic fungi on spore baits of 24 taxa of cryptogams from three water bodies and isolated a total of 61 zoosporic fungal taxa, with predominance of the *Chytridiales* (27) and *Peronosporales* (14) species. The largest number of species (46) was recorded in the stagnant water at Dojlidy pond and lower number of species (31) from Suprasl River and (34) from Jaroszowka spring. In addition to this five species of *Pythium* were also recorded for the first time from Poland. In Tutong river and its tributary, the Sungai Kelakas investigation of fungi on decaying wood lead to the recovery of 66 taxa including two new genera and two new species with nearly half of these (48%) isolates being new records for Brunei. Although common taxa were generally not unique to any particular site, however species distributions were correlated to the salinity gradient which showed consonance with previous studies depicting that some marine fungi are tolerant to less saline conditions while as some freshwater species are tolerant to more saline conditions with some species as potentially brackish water species. The ratio of ascomycetes to anamorphic fungi was higher in marine than freshwater sites (Fryar *et al*., 2004).

Aquatic fungi developing on the seeds of some plants like *Cannabis sativa* (hemp-seeds), *Fagopyrum esculentum* (buckwheat-seeds) and on *Vicia sativa* (vetch-seeds) in various types of water bodies were investigated by Kiziewicz (2005) by using baiting method which lead to the isolation of 74 aquatic fungal species and showed the presence of various mould species belonging to Blastocladiales (5), Chytridiales (6), Monoblepharidales (1), Hyphochytriales (1), Lagenidiales (5), Leptomitales (4), Peronosporales (14), Saprolegniales (37) and Plasmodiophorales (1) in water. The most common species encountered in the water bodies were *Achlya americana, Achlya polyandra, Aphanomyces laevis*, *Pythium rostratum, Saprolegnia ferax* and *Saprolegnia parasitica*. Some phytopathogens like *Achlya racemosa*, *Phytophthora infestans*, *Pythium butleri*, *Pythium debaryanum* and *Pythium*

myriotylum were also reported. Another study conducted by McClenny (2005) proved that although molecular and immunologic tests promise better, faster laboratory diagnosis of diseases like aspergillosis, still microscopy and culture remain commonly used and essential tools. However, some procedural changes like use of Blankophor or Calcofluor for microscopic examinations; improving recognition of morphologic characteristics of opportunistic fungi in stained smears of specimens; maximizing the growth rate and production of conidia by *Aspergillus* spp. in culture; and recognizing atypical variants of common aspergilli, as well as adequate training of laboratory professionals, can enhance the value of these traditional tools and techniques. While working on the incidence and distribution of terrestrial mycobiota in 60 samples of accumulated rainfall, collected from different sites in eastern and western regions of Saudi Arabia, Nasser (2005) reported the presence of 45 species belonging to 21 genera of terrestrial fungi on glucose Czapek's Dox agar (GCDA) and cellulose Czapek´s Dox agar (CCDA) media at 28ºC with CCDA medium giving higher species diversity (38 species belonging to 20 genera) compared to GCDA (35 species belonging to 16 genera). The majority of the identified species were similar on both media, although some species were recovered only once on one of both media. The most prevalent taxa of mycobiota on both the growth media were *Aspergillus* and *Penicillium* contributing the broadest spectra of the isolated terrestrial fungal species whereas several genera had only one species.

Amal *et al*. (2006) during their work on the water and floating organic matter taken from a dam near Rabat, Morocco found a novel species of zoosporic fungi namely *Achlya abortispora* having distinguishing characteristics like production of long fusiform sporangia with achlyoid and aplanoid discharge of zoospores; smooth-walled spherical to club-shaped oogonia, usually lateral, but at times intercalary, containing 1 to 20 oospheres; antheridial branches usually coiling and wrapping around the oogonia; which was later described and compared with other species of the genus. Furthermore morphologic features of the oomycete and the sequence of the ITS region of its rDNA, as well as their comparison with related species were also done. Zoosporic fungi are common inhabitants of aquatic environments ,in this connection Marano and Steciow (2006) quantified zoosporic fungi in terms of frequency of occurrence and abundance in three lotic environments of the Rio de La Plata system, Argentina, using the baiting technique and recovered eight genera of

fungi (*Achlya*, *Aphanomyces*, *Dictyuchus*, *Olpidiopsis*, *Phytophthora*, *Pythium*, *Rhizophlyctis* and *Saprolegnia*) with their patterns of frequency and abundance distribution showing certain similarities in the streams analysed. Another study conducted by Suhail *et al*. (2006) on the mycoflora of Indus river bed at Kotri by using soil dilution and soil plate method, reported the recovery of 73 strains of fungi including 10 species of *Penicillium* viz., *P. caseicolum* (1.81%), *P. commune* (1.81%), *P. chrysogenum* (14.73%), *P. funiculosum* (28.36%), *P. lilacinum* (4.33%), *P.notatum* (12.53%), *P. sclerotiorum* (2.52%), *P. tardum* (26.47%), *P. vinaceum* (5.51%*)* and *P. roseopurpureum* (1.89%) with greater incidences of species isolation on soil plate method than on dilution plate method.

After random collection of 84 polluted water samples from different polluted sites of the water drainages along the Niles Delta in Lower Egypt Ali (2007) isolated and identified 34 species in addition to 5 unidentified species belonging to 10 genera of zoosporic fungi by Baiting sesame seeds at 20±2°C for their recovery. The genera *Pythium* and *Saprolegnia* (8 and 7 zoosporic fungal species respectively) showed the broadest spectra of species diversity. *Saprolegnia delica* and *Dictyuchus carpophorus* were most dominant with high occurrence especially in the hyper-polluted water samples with heavy metals, thus considered as indicators for the response of the structure and function of microbial communities for water pollution. Water samples with high concentrations of heavy metals were the poorest in the species diversity of zoosporic fungi. Despite that, fungal species belonging to the family *Saprolegniaceae* flourished in hyper-polluted water samples whilst those belonging to the family *Pythiaceae* predominated in more diluted water samples. Also, the prevalent species; *S. delica* and *D. Carpophorus* were not affected by heavy metals concentrations being as indicators for water pollution with the heavy metals. Water samples with high organic matter content and low total soluble salts were rich in zoosporic fungal species. Macro-morphological features like the colony diameter, colour (conidia and reverse), exudates, colony texture and micro-morphological features like conidial heads, stipes, colour and length, vesicles shape and serration, metula covering, conidia size, shape and roughness using differential media is the most reliable and sensitive assay to identify more medically important *Aspergillus* species. The same was used by Diba *et al*. (2007) to identify *Aspergillus* isolates in the level of species using the differential culture media. A total of 205 *Aspergillus* isolates were studied which

included; 153(75%) environmental Aspergilli and 52 (25%) clinical isolates. Within 11 *Aspergillus* species identified *A. flavus* (55%), *A. niger* (31.7%) and *A. fumigatus* (8.7%) were most common from all of the specimens.

As *Aspergillus* infections have grown up in the last few years, most of the studies are focused on *Aspergillus fumigatus*, the most prevalent species in the genus, but in certain localities and hospitals, *Aspergillus flavus* is more common in air than *A. fumigatus,* for unclear reasons. After *A. fumigatus, A. flavus* is the second leading causative agent of invasive aspergillosis and is the most common cause of superficial infection. The flavus complex currently includes 23 species or varieties, including two sexual species, *Petromyces alliaceus* and *P. albertensis*. However, Hedayati *et al*. (2007) showed that experimental invasive infections in mice by *A. flavus* were 100-fold more virulent than *A. fumigatus* in terms of inoculum required. Working on the occurrence of fungi in water distribution systems, Loniewska *et al*. in the same year demonstrated that contamination of water distribution systems with fungi is determined by the number and species composition of the mycoflora of waters supplying a given water treatment plant (WTP). Moulds, including species pathogenic and potentially pathogenic to humans and warm-blooded animals, constituted the mycoflora. Shearer *et al*. (2007) conducted a study and estimated fungal biodiversity on submerged substrates for a large number and variety of aquatic habitats including freshwater, brackish water, marine water habitats and reported 3,000 fungal species and 138 saprolegnialean species on the basis of presence or absence of data which belonged to various taxonomic groups like Ascomycetes (exclusive of yeasts), Basidiomycetes, Chytridiomycetes, and the non-fungal Saprolegniales in the Class Oomycetes with the greatest number of taxa comprising the Ascomycetes, including mitosporic taxa, and Chytridiomycetes. The greatest biodiversity for all groups occurred in temperate areas, followed by Asian tropical areas. Some species overlap was also reported among terrestrial and freshwater taxa but little species overlap among freshwater and marine taxa.

Gomes *et al*. (2008) worked on the isolation of filamentous fungi from sand and water of "Bairro Novo" and "Casa Caiada" beaches, Brazil using sand suspension and water sample. During their study they isolated, identified and classified 57 species belonging to 20 genera with *Aspergillus* and *Penicillium* the most frequent genera in both sand and water, with a total of 11 and 19 species, respectively. A two year study

conducted by Javorekova and Felsociova (2008) to evaluate microbial quality of surface water of the Zitava river, its branches and adjacent wetlands in the Zitavskyluh Nature Reserve showed the presence of coliform bacteria, myxobacteria, actinomycetes and soil microscopic fungi in the plankton and benthos areas of flowing water, stagnant water and wetlands. The most numerous communities in the area of benthos were coliform bacteria and microscopic fungi. From microscopic fungi, species of the genera *Aspergillus*, *Penicillium*, *Trichoderma*, *Cladosporium* and *Fusarium* occurred in particular with the dominance of species of the genus *Trichoderma* in the area of wetlands and species of the genera *Penicillium* and *Aspergillus* in the Zitava River. Kiziewicz and Nalepa (2008) while working on the determination of fungi in the water and associated with the benthic amphipod *Diporeia* spp., in Lake Michigan in an area where the *Diporeia* population was in a severe state of decline no fungi were found associated with living, freshly-dead, or dried *Diporeia* cultured separately from the Lake water. When dead *Diporeia* and other organic substrates (snake skin and hemp seeds) were used to grow fungi in Lake Michigan water, a rich and diverse fungal and water mold community with a total of 31 species, the most common genera being *Achlya*, *Aphanomyces*, *Myzocytium* and *Pythium* were found showing a homogeneous distribution of fungi in the water i.e., a few differences were found in species richness between near shore and offshore waters, and between near-surface and near-bottom waters. However, Mbata (2008) worked on the isolation of filamentous fungi from hyper saline environment (Dead Sea), reporting species belonging to six different genera namely; *Gymnascella* spp. 1 (1.5%), *Eurotium* spp. 6 (8.8%) *Chactomium globosum* 14 (20.6%), *Aspergillus versicolor* 30 (44.1%), *Hortaea werneckii* 9 (13.2%), *Aureobasidium pullulans* 8 (11.8%) with *Aspergillus versicolor* and *Chaetomium globosum* as the most predominant. Additionally he along with his co-workers worked on the isolation and characterization of filamentous fungi from the Yardenit-Baptismal site on the Jordan River by investigating 126 water samples and showed that filamentous fungi were isolated from 61.9% samples and yeasts from 4.0% with *Aspergillus* spp., (isolated from 41 samples) the most predominant genera followed by *Penicillium* spp., (from 33 samples) and *Candida* spp. (from 4 samples) suggesting that the river may be a potential transmission route of fungi and may pose a health hazard mainly for the immune-depressed individual. Using direct plating techniques and serial dilution

technique Nazim *et al*. (2008) isolated 9 species of fungi belonging to 4 genera from water and 8 species of fungi belonging to 4 genera from juices with highest number of fungi isolated by serial dilution technique followed by direct plating method. The occurrence of *Aspergillus niger* was found to be dominant in drinking water as well as in juice samples followed by *A. clavatus* and *A. ustus*.

Abdel-Azeem *et al*. (2009) in a study identified fifteen species of aquatic-derived fungi with the dominant taxa as *Aspergillus niger, A. flavus, A. terrus, Mucor racemosus* and *Penicillium chrysogenum*. Eight taxa of aquatic fungi were also reported with the most dominant being *Achlya prolifera, Saprolegnia diclina* and *Pythium intermedium*. Counts of total coliform reached up to 154700 CFU/100ml in wells. This was followed by Devi *et al*. (2009) who investigated the impact of environmental conditions on diversity of aquatic fungi by assessing the seasonal variations in physico-chemical factors of the water of Tunga river, with respect to aquatic and aero-aquatic fungal diversity. During the study, 12 aquatic fungal species and 14 aero-aquatic species, belonging to classes *Chytridiomycetes, Oomycetes,* P*hycomycetes*, *Ascomycetes*, *Zygomycetes* and *Deuteromycetes* were isolated and identified. Maximum fungal distribution was recorded during the monsoon period followed by pre-monsoon periods in all the four stations. But comparatively less percentage of fungal incidence was recorded during post monsoon period. This investigation revealed that the difference in the percent occurrence, distribution of aquatic fungi and aero-aquatic species proved to depend on the physico-chemical factors of the water and also on seasonal variations. Kiziewicz (2009) conducted a study with the main purpose to determine the diversity and compare the frequency of occurrence of fungi discovered in three different water bodies situated in Bialystok. 24 species of aquatic fungi belonging to thirteen genera were isolated from the reservoirs with *Saprolegnia, Achlya* and *Pythium* as the most commonly encountered fungal genera in the research habitats. Moreover, Kowalik and Krasny (2009) carried out a study on plants in garden ponds; white water lily (*Nymphaea alba*), yellow iris (*Iris pseudoacorus*), common cattail (*Typhalati folia*), soft rush (*Juncus effusus*) and calamus (*Acorus calamus*); frequently isolating the following fungal species as *Alternaria alternata, Aspergillus versicolor, Sordaria fimicola, Penicillium expansum, Epicoccum purpurascens, Phialophora cyclaminis, Mortierella isabellina, Botrytis cinerea, Chaetomium elatum, Phialophora richardsiae* and *Penicillium*

verrucosum v. *corymbiferum* from the affected tissues of the water plants with White water lily being the most affected plant.

A review of fungi in drinking water was presented with emphasis on methods of isolation, detection, problems, avoidance or removal, and methods to detect biochemicals such as mycotoxins (Paterson *et al*., 2009). According to this review the occurrence of filamentous fungi in drinking water has been known for many years however, the quantity of scientific literature on the subject is only beginning to increase. Filamentous fungi have been considered only as annoying contaminants of agar plates used to count bacteria from water. There are various potential issues associated with the fungi-in-drinking-water phenomenon, including blockage of water pipes, organoleptic problems such as odour and taste, biofilm formation, spread of pathogenic fungi, and mycotoxins production. In the same year Paliwal and Sati, carried out a study on Watermolds- the members of Chytridiomycetes and Oomycetes fungi, which possess the ability of colonizing a variety of substances. Kosi river water was assessed with reference to watermolds diversity, pH, water temperature and total organic matter at 3 study sites and it was reported that a total of 16 fungal species belonging to 7 genera of watermolds were present in the water. Maximum number of fungal species was recorded during spring and rainy season, while minimum number of watermolds was observed during winter season. Also during this study, sterile species of watermolds showed dominance followed by eccentric species. They also reported that the interaction of physicochemical factors greatly influenced the diversity of watermolds. Sati and Arya (2009) analyzed the relative effect of some physico-chemical parameters of water to the occurrence of water borne conidial fungi in a high altitudinal fresh water stream and reported a total of 30 species belonging to 21 genera. There was marked seasonal fluctuation in the occurrence of the species and the maximum number of species was found during spring to early summer and winter, while there was a decline in the number of species during late summer, rainy and early autumn seasons. Species richness was correlated with seven different water quality variables measured for each sample period, viz., temperature, pH, dissolved oxygen, organic and inorganic content, phosphate and sulphate concentration. The data were analyzed statistically for correlation and these factors were found to be significant for the occurrence of water borne conidial fungi.

In the recent past, Adam *et al*. (2010) carried out a study with the purpose of devising an algorithm utilizing chromogenic media for the detection and presumptive identification of *Candida* species in a clinical microbiology laboratory. They succeeded in established the use of chromogenic media for the rapid presumptive identification of *C. albicans*, *C. tropicalis*, *C. krusei*, and *C. glabrata* in the routine microbiology laboratory. Polymerase Chain Reaction (PCR)-based, single sequence repeats (SSR) micro satellites analysis has been used successfully in the analysis of DNA relatedness of species of fungi, bacteria, plants and animals. SSR micro satellites analysis present in nuclear and organelles DNA can be used as molecular markers and has wide ranging applications in the field of genetics including kinship and population studies. These Inter simple sequence repeats (ISSR) represent genome region between micro satellite loci. Sequences amplified by ISSR-PCR can be used for delimiting species. Dendograms which evaluate the likeness between different isolates has also been used (Hatti *et al*., 2010).

Jebaraj *et al*. (2010) performed phylogenetic analyses of small subunit ribosomal RNA gene (18S rDNA) sequences obtained from fungal isolates and environmental samples of the Arabian Sea and demonstrated that fungal diversity in anoxic marine habitats was severely underestimated. Community similarity analyses suggested distinctively different structures of fungal communities from permanently oxic sites, seasonally anoxic sites and permanently anoxic sites. Most of the 26 cultures obtained in this study were closely related to well-described Dikarya, indicating that standard cultivation mainly produced more of what is already known. This study also demonstrated the importance of a multiple primer approach in combination with cultivation to obtain deeper insights into the true fungal diversity in any environmental sample and to enable adequate inter-sample comparisons of fungal communities.

The examination of geothermal sites near neutral and alkalescent thermal spring in Tengchong Rehai National Park for thermophillic fungi through cultivation dependent methods by collecting soil samples and plating them on agar media to obtain pure cultures. The cultures were then subjected to internal transcribed spacer (ITS) sequencing combined with morphological analysis for identification of thermophilic fungi to the species level was employed. In total, 102 strains were isolated and identified as *Rhizomucor miehei*, *Chaetomium* sp., *Talaromyces*

thermophilus, *Talaromyces byssochlamydoides*, *Thermomyces lanuginosus*, *Scytalidium thermophilum*, *Malbranchea flava*, *Myceliophthora* sp., *Myceliophthora* sp., *Myceliophthora* sp., and *Coprinopsis* sp. Two species, *T. lanuginosus* and *S. thermophilum* were the dominant species, representing 34.78% and 28.26% of the sample, respectively. These results indicated a greater diversity of thermophilic fungi in neutral and alkaline geothermal sites than acidic sites around hot springs as was reported in previous studies. Most of these strains thrived at alkaline growth conditions (Pan *et al*., 2010). Pearman *et al*. 2010 also conducted a study on the species composition and diversity of wood inhabiting fungi and compared three aquatic habitats; a marine, an estuarine and a freshwater site, in order to determine whether the species present in these areas were cosmopolitan, or if there was a distinct separation between the sites correlated with the change in water conditions. Both molecular and morphological analysis was used for identification purposes and in all 35 species were observed, three of them *Fusarium* sp., *Orbilia* sp. and *Neonectria lucida*, were found at more than one site. Seventeen species were observed in both the freshwater and estuarine sites (Kinness Burn and Guardbridge, respectively), but only four were present at Kinkell Rocks, the marine site. The Shannon Wiener Diversity Index was highest for the freshwater Kinness Burn site (2.8) and lowest for the marine Kinkell Rocks (1.33). The Sorensen's Index of Similarity confirmed that there were no species in common between the marine site, and either of the other sites. The Index between the estuarine and freshwater sites (0.18) suggested that there was some overlap in species composition, but there was still a wide variation in species identity between the two sites. Thus the diversity of the mycota inhabiting the aquatic environments decreased in more saline habitats.

A study on the riverine ecosystem of Tunga River showed that the microflora is dominated by Algae, Bacteria and fungi with fungi playing an important role and vitally link the food web primarily as saprophytes and secondarily as pathogens (Prasad and Rajanaika, 2010). Tunga River ecosystem consists of a diversified flora of aquatic phycomycetes, in which majority of them are Chytrids and Oomycetes. These fungi are zoosporic in nature and some of them mainly predate on Ichthyofauna and other riverine fauna. Aquatic phycomycetes are the primitive fungi and they are at the bottom of evolutionary series and they have their significance as pathogens, combating them in the riverine ecosystem has become a mammoth's task. During

monsoon and winter the fisher man's face severe loss in fish productivity majority of them are due to fungal infections on fishes and on their eggs. Sridhar and Sudheep (2010) conducted a study on the diurnal changes in species and spore richness of freshwater hyphomycetes in relation to physicochemical features in two streams of the southwest India and recorded drift spores belonging to 16−19 species with *Anguillo sporalongissima*, *Flagello sporacurvula*, *Lunulo sporacurvula* and *Triscelophorus monosporus* as dominant in both streams. Total species showed two peaks at 12 hr intervals (9 am and 9 pm) in both streams. The species richness, diversity and evenness were higher during the night than day in the Konaje stream, while it was reverse in the Mallapura stream. The species richness of both streams had no significant correlation with water quality. The total spores differed significantly between streams and was higher during the night than day in both streams, possibly due to lack of leaf shredding invertebrates. Among the five dominant species, peak spore production of *Lunulo sporacurvula* in both streams coincided with the peaks of total spores. The spore richness in the Konaje stream had no significant correlation with water parameters, but showed significant negative correlation with temperature in the Mallapura stream. This study revealed contrasting results in diurnal fluctuation of spores of freshwater hyphomycetes compared to temperate region. Singh *et al*. (2010) studied the diversity of culturable fungi in deep-sea sediments of the Central Indian Basin reporting a total of 16 filamentous fungi and 12 yeasts isolated from 20 sediment cores which were identified by 18S and ITS sequencing of SSU rDNA. Most of the filamentous fungi were Ascomycota, while two were Basidiomycota. Microscopic identification of sporulating cultures mostly matched either with 18S or ITS sequences but seldom with both the sequences. Phylogenetic analysis of ascomycetes using 18S sequence data grouped them into 7 clusters belonging to *Aspergillus* sp., *Sagenomella* sp., *Exophiala* sp., *Capronia* sp., *Cladosporium* sp., *Acremonium* sp. and *Tritirachium* sp. ITS sequence data grouped isolates into 6 clusters belonging to *Aspergillus* sp., uncultured member of Hypocreaceae, *Exophiala* sp., uncultured soil fungus, Hypocreales and *Trichothecium* sp. The two basidiomycete isolates were a *Tilletiopsis* sp. evident from 18S as well as ITS sequence data. In contrast, most of the yeast isolates belonged to Basidiomycota and only one isolate belonged to the phylum Ascomycota. Sequences of 18S as well as

ITS gave matching identification of most of the yeasts. This study reported on the presence of terrestrial fungi as a component of culturable fungi in deep-sea sediments.

Working on the isolation and identification of various filamentous fungal species from the Dal Lake water Bandh *et al*. (2011a & b) recently isolated various species of genus *Penicillium* and *Aspergillus* showing a great deal of seasonal variation with a highest percentage occurrence in summer season followed by spring, autumn and winter seasons. The species isolated from the water samples were identified by using the traditional methods of morphological observation and culture by studying various micro and macro-morphological features of the isolated species. Moreover in Shafi *et al*. (2011) carried out a preliminary microbiological study on the river Sindh, a glacier fed river of the valley and isolated various species of fungi belonging to *Aspergillus*, *Penicillium* and *Candida* by the simple method of direct culture of the water samples collected.

The aim of the study conducted by Seydametova *et al*. (2011) was to examine the fungal isolates from soils of Pahang state. Twenty fungal cultures were examined for characters of morphology using macro-morphological characters like colony diameter, obverse and reverse colony colour, the presence or absence of exudates and microscopic characteristics of the fungal isolates. The colony appearance of each fungal isolate was characterized on different agar media like CZ, CYA, YES, MEA. The macro- and micro-morphological characters of isolated cultures appeared to be distinctive. Based on colony macro-morphology, as well as the structure of conidiophores, it was revealed that fungal isolates belonged to *Penicillium* genus. During a survey for freshwater hyphomycetes on submerged plant debris in Brazil, Barbosa and Gusmao (2011) collected six rare species of freshwater hyphomycetes like *Brachydesmiella anthostomelloidea*, *Camposporidium cristatum*, *Dactylaria hyalotunicata*, *Lauriomyces sakaeratensis*, *Pleurophragmium malaysianum* and *Pyricularia rabaulensis*. Additionally, 37 new records for Western hemisphere, Neotropics, South America, Brazil and Bahia State were listed thus contributed to knowledge about the geographic distribution of freshwater hyphomycetes and reflected the lack of studies of these fungi in tropical regions.

Chen *et al*. (2011) in their study established a novel rapid and efficient DNA extraction method based on alkaline lysis, which could deal with a large number of filamentous fungal isolates in the same batch. The filamentous fungal genomic DNA

required only 20 min to prepare and could be directly used as a template for PCR amplification. The amplified internal transcribed spacer regions were easy to identify by analysis. The extracted DNA also can be used to amplify other protein-coding genes for fungal identification. Thus, this method can be used for rapid systematic identification of filamentous fungal isolates. Jadhav *et al*. (2011) carried out a study which dealt with five species of aquatic fungi belonging to five genera of freshwater hyphomycetes found in foam samples collected from Trambkeshwar stream of Nasik District. The foam spora of this region represented a mixture of both tropical and temperate species. Patil and Borse (2011) conducted a similar study on foam samples collected from the river Tapti and Panzara of North Maharashtra and reported conidia of water-borne hyphomycetes. In all, five hyphomycetous taxa assignable to five genera were identified. The foam spora of North Maharashtra represented a mixture of both tropical and temperate fungi. Three species *viz. Anguillospora crassa* Ingold, *Lunulospora curvula* Ingold and *Tetracladium marchalianum* de Wildman were reported for the first time from North Maharashtra region while two species *viz. Campylospora filicladia* Nawawi, *Clavariopsis azalanii* Nawawi, were reported for the first time from Maharashtra State.

Saju (2011) investigated the fungal diversity of Dumara tarai Talab ponds of Raipur cityreporting an overall total of 91 fungal species (362 colonies) belonging to 45 genera with maximum numbers isolated during winter season, moderate in rainy season and minimum is summer season. Maximum numbers of fungal species (32) were isolated in December month and minimum (05 fungal species) in March. All these fungi were highly adaptable to the pond water environment. In the same year Siqueira *et al*. (2011) conducted a study on the presence of filamentous fungi in drinking water. The problems associated with fungi include blockage of water pipes, organoleptic deterioration, pathogenic fungi and mycotoxins. This study updated the topic and introduced novel methods on fungal biofilm analysis, particularly from work based in Brazil. Further recommendations for standard methodology were also provided. A study was carried out by Sharma and Parveen (2011) on the quantitative and qualitative fungal composition of Dudhawa Dam water located in Dhamtari district of Chhattisgarh in India. A variety of fungal strains were isolated and identified from the water. Out of a total 193 fungal colonies, 35 fungal species belonging to 16 fungal genera were isolated. *Aspergillus niger* was most frequent

species with 91.67%, *Neoarachnotheca keratinophila* (75%) followed by *A. flavus* and *A. fumigates* with 66.67% frequency. It was also found that maximum percentage contribution was observed for *Aspergillus fumigatus* (19.68%), which was followed by *Aspergillus niger* with (18.65%) contribution. In another study carried out by Sridhar and Sudheep (2011) on filamentous fungal association with mechanically "hard" and "soft" woody litter naturally deposited in a stream of the Western Ghats of India. From three stream locations, hardwood and softwood sections were assessed for the occurrence of lignicolous and Ingoldian fungi. In hardwood sections, 17 lignicolous fungi and ten Ingoldian fungi as core-group taxa were recovered. In softwood, ten lignicolous fungi and 26 Ingoldian fungi as core-group taxa were recovered. The Shannon diversity of lignicolous fungi was higher in hardwood than softwood, whereas it was opposite for Ingoldian fungi. The overall fungal diversity was higher in softwood than hardwood. Zhengping *et al*. (2011) conducted a research to detect *Penicillium griseofulvum*, a dominant species related to heavy metal pollution, which was screened from marine contaminated sediments by Molecular methods. Based on differences in Internal Transcribed Spacer (ITS) sequences of *Penicillium* genus and specific Isoamyl Alcohol Oxidase (IAO) sequences, species-specific primers AS1/RS4 and IAO1/IAO2 of *Penicillium griseofulvum* were designed and synthesized which were then employed in optimized PCR systems. The detection sensitivities were compared through ordinary PCR and nested-PCR using two pairs of primers, respectively. Both primer pairs could exclusively amplify destined DNA fragment from contaminated environmental samples. Despite the difference in detection sensitivity, it is feasible that the species-specific primers could be used as probes for the detection of environmental pollution dominant species, *Penicillium griseofulvum*, since the frequency of occurrence and amount of this strain could preferably indicate the pollution degree.

Very recently Bandh *et al*. (2012a) conducted a qualitative survey of filamentous fungi in Dal Lake, Kashmir by collecting water samples obtained seasonally at sixteen different sites of Dal Lake, Kashmir which were serially diluted five folds followed by spread plate technique for the isolation of filamentous fungi, spreading 0.1 ml inoculum from the serial dilution tubes on the Petri dishes containing Rose-Bengal Streptomycin Agar medium. During the study a total of twenty three (23) species of fungi namely *Penicillium caseicolum, P. commune, P. chrysogenum,*

P. funiculosum, P. lilacinum, P. olivicolor, P. dimorphosporum, Penicillium spp. I, *Penicillium* spp. II, *Penicillium* spp. III, *Penicillium* spp. IV, *Aspergillus flavus, A. fumigatus, A. japonicus, A. niger, A. terreus, A. versicolor, A. wentii, Aspergillus* spp. *Fusarium* spp. *Rhizopus* spp. *Acremonium* spp. and *Mucor* spp. belonging to five genera were recovered. *Penicillium* and *Aspergillus* were the most dominant genera with a total of 11 and 8 species respectively. The most prevalent species was *P. chrysogenum* with its occurrence at all the sampling stations and a highest total of seventeen species was recorded at the Pokhribal outlet.

Some fungal species are known to be pathogenic or allergic, thus implicating negative impacts on health (Hageskal *et al*., 2009). There is a risk of superficial or localised infection in healthy individuals and severe invasive infection in immuno-compromised patients. Some fungal species like *Achlya debaryana, Saprolegnia parasitica, Achlya klebsiana, Aphanomyces laevis, Dictyuchus monosporus* and *Brevilegnia subclavata* have been found pathogenic to fishes (Prasad and Rajanaika, 2010; Osman *et al*., 2010; Kiziewicz, 2004; Czeczuga *et al*., 2002; Smith, 1940; Khulbe, 1989), *Pythium debaryanum, Acremonium strictum, Penicillium funiculosum, Allomyces arbusculus* have been reported to be the plant pathogens (Hendrix and Campbell, 1973; Lim and Rohrbach, 1980; NBRC). However, a wide range of these fungal species like *Aspergillus fumigatus, A. terreus, A. flavus, A. niger, A. versicolor, A. wentii, A. japonicas, Candida albicans, Candida glabrata, C. krusei, Cryptococcus neoformans, Fusarium* spp., *Rhizopus* spp., *Mucor* spp., *Penicillium lilacinum* and *P. olvicolor* are pathogenic to humans as these species of fungi cause a number of deadly infections in the human beings including invasive aspergillosis, onychomycosis, pulmonary aspergillosis, necrotising external otitis (NEO), invasive mycoses, candidiasis, respiratory infections, ocular infections, cutaneous infections and chronic farmer's lung (FL) disease (Hinson *et al*., 1952; Sakai *et al*., 1992; Weitzman and Summerbell, 1995; Nakagawa-Yoshida *et al*., 1997; Richardson 1998; Summerbell, 1998; Latge, 1999; de Hoog *et al*., 2000; Gottlieb and Atkins, 2001; Crowe *et al*., 2003; Vazquez and Sobel, 2003; Pommerville, 2004; Morgan *et al*., 2005; Hogan, 2006; Armstrong, 2007; Pfaller and Diekema, 2007; Behnsen *et al*., 2008; Willey *et al*., 2008; Dagenais and Keller, 2009; Khan *et al*., 2010; Moreno and Arenas, 2010; Chaudhuri *et al*., 2011; Halsey *et al*., 2011; Khokhar *et al*., 2011). Mycotoxins such as

sterigmatocystin produced by many species may cause DNA damage *in vitro* and are potentially carcinogenic (Morgan *et al*., 2005; Moreno and Arenas, 2010).

Chapter 3

Material and Methods

Water samples from sixteen sites selected randomly from different basins, outlets and inlets of Dal Lake, Kashmir under consideration for exploring the fungal diversity were collected on seasonal basis in Poly Ethylene (PET) bottles, which were previously carefully cleaned, rinsed three to four times with distilled water and sterilized (APHA, 1998). All the samples were collected just below the surface 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 fungal community using the standard methodology (APHA, 1998). The steps involved in the protocol included:

3.1. Cleaning of glassware

All the glassware used was cleaned with labolene to remove oils, greases and organic matter from it, followed by running tap water and then with distilled water. Subsequently, it was allowed to drain and dry in an oven at 110°C and then prepared for sterilization.

3.1.1. Sterilization

Sterilization of glassware, culture media, suspending fluids, reagent containers and equipments was carried out to make them free from all kinds of living organisms including bacteria, fungi, and viruses etc. Heat sterilization, the most common and reliable method was followed where the material to be sterilized was not having any danger of damage by high temperature. High temperature was achieved by using either dry sterilization or moist heat sterilization.

3.1.2. Dry heat sterilization

A hot oven equipped with thermostat was used for dry heat sterilization of the glassware, metal instruments and other items. 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.

3.1.3. Moist heat sterilization

Moist heat provided by saturated pressure in an autoclave- the most effective and reliable method for sterilization (APHA, 1998) was used for sterilization of glassware and culture media. However, it was not used for materials damaged by moisture or 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. Since the 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 15 lb/inch^2 pressure are suggested. This temperature and pressure is also suitable for sterilization of media, hence this method was used.

Other methods of sterilization followed were:

3.1.4. Flaming

Objects like inoculating wires, inoculating loops, forceps, spatulas, needles and spreaders were dipped into 70% ethyl alcohol followed by exposing them to spirit lamp flame till they turn red hot.

3.1.5. Radiations

Ultra violet (UV) radiation treatment was used to kill the microbes in inoculation chamber (Laminar air flow cabinet).

3.2. Culture media

As cultural methods depend on properly prepared media, use of best available material and techniques in media preparation, storage, and application is very important. For quality control, commercially prepared media were used on a first-in, first-out basis. Media that got caked, discolored, or showed other signs of deterioration were discarded. If expiration date was given by manufacturer, unused media after that date was also discarded. Opened bottles of media were used within six months to protect them from moisture and opened bottles were stored in a desiccator.

3.2.1. Preparation of media

Media was prepared in containers that were at least twice the volume of the medium being prepared. Scorching or boil-over was avoided by using a boiling water bath for small batches of media and by continually attending to larger volumes heated on a hot plate or gas burner. Preferably hot plate-magnetic stirrer combinations were used. Water volumes and media were measured with graduate measuring cylinders or pipets conforming to APHA standards. pH of a portion of each medium after sterilization and cooling was checked and recorded. Minor adjustments in pH (<0.5 pH units) with 1N NaOH or HCl solution to the pH specified in formulation were made. Prepared media was examined for unusual color, darkening or precipitation. Variations in sterilization time and temperature as possible causes for problems were also observed. If any of the above problems occurred, the media was discarded.

3.2.2. Media sterilization

Sterilization of media was done by autoclaving at 121°C for the minimum time specified. A double-walled autoclave permits maintenance of full pressure and temperature in the jacket and reduces chances of heat damage. Following manufacturer's directions for sterilization of specific media the media and other materials were sterilized. The required exposure time varied with form and type of material, type of media, presence of carbohydrates and volume. Sterilized media was removed from the autoclave as soon as chamber pressure reached zero. Effectiveness of sterilization was checked weekly by placing *Bacillus stearothermophilus* spore suspensions or strips (commercially available) inside glassware. Sterilized at 121°C for 15 min. they were placed in trypticase soy broth tubes and incubated at 55°C for

48h. If growth of the autoclaved spores occurred after incubation at 55°C, sterilization was taken as inadequate.

3.2.3. Dispensing of media

About 15 to 20 ml of the medium was dispensed into sterilized petri plates in a laminar flow cabinet and allowed to solidify followed by overnight incubation to check the contamination, if any, inside the media.

3.3. Serial dilution of samples

Before inoculation, the lake water samples were diluted serially to different levels, in order to get the desired number and density of fungal colonies. The original samples were diluted five folds (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) using normal saline solution (NSS) prepared by dissolving 0.86g of sodium chloride (NaCl) in 1litre of distilled water.

- In the first dilution 1ml of original sample was diluted by 9ml of normal saline solution.
- In the second dilution, 1ml from the first dilution was further diluted with 9ml of normal saline solution and the same procedure was continued until it reached the fifth dilution.

3.4. Inoculation

Fungal colony count in different samples was estimated by inoculating Rose-Bengal streptomycin agar medium plates with 0.1ml of suitable dilutions by plate count technique which allows the microbial colonies to grow over the surface of the medium and eventually making counting easier.

3.5. Incubation

After inoculation, the culture plates were incubated upside down (inverted position) at a temperature of 30-37°C for seven days in an incubator to allow the growth of fungal colonies (APHA, 1998).

3.6. Culture techniques

3.6.1. Spread plate technique

After pouring 15-20 ml of sterilized media into sterile 100×15 mm or 90×15mm petri dishes to get solidified the plates were pre-dried overnight with lids on. 0.1 ml inoculum from different dilution was pipetted onto the surface of the pre-dried agar plates. Using a sterile bent glass rod, the inoculum was distributed over the surface of medium by rotating the dish by hand or on a turntable. Inoculum was

allowed to absorb completely into the medium before incubating. The plates were then incubated at 20-25°C for 5 to 7 days for noting down the growth of fungal colonies (APHA, 1998).

3.6.2. Pour plate technique

Different inoculum volumes from the decimal dilutions of the sample were delivered onto the sterile petri dishes before adding 15-20 ml of melted culture media into each dish by gently lifting the cover just high enough to pour and mix carefully and thoroughly by rotating the dish first in one direction and then in opposite direction or by rotating and tilting. While pouring the media care was taken not to splash the mixture over the edges of the petri dishes. After solidification of the inoculum-media mixture the plates were inverted and incubated in dark at 20-25°C for 5 to 7 days to note down the growth of fungal colonies (APHA, 1998).

3.6.3. Streak plate technique

The isolation method most commonly used to get pure cultures was the streak plate method. A sterile inoculating loop was dipped into a mixed culture that contained more than one type of colonies (microbes) and was streaked in a zigzag pattern over the surface of the nutrient medium. As the path was traced, fungal spores were rubbed off the loop into the medium in paths fewer and fewer cells. The last cell to be rubbed off the loop was far enough apart to grow into isolated colonies. These colonies were picked up by an inoculating needle and transferred to a test tube of nutrient medium to form a pure culture containing only one type of organism (Tortora *et al*., 1995).

3.6.4. Enrichment culture technique

Microbes if present in small numbers can be missed, especially if other organisms of competitive nature are present in large numbers, it is necessary to use an enrichment culture. The medium which is usually liquid and provides nutrients and environmental conditions that favor the growth of a particular microbe but not suitable for other types of microbes. In this sense, it is also a selective medium, but it is designed to increase very small numbers of the desired types of microbe to detectable levels (Tortora *et al*., 1995).

3.7. Enumeration of colonies

Colonies that developed on agar plates were counted with digital Qubec-colony counter. Plates with colony numbers lying between 10-100 were selected for

counting and the counts were expressed as cfu/ml of water sample calculated by using the following formula

$$\text{cfu/ml} = \frac{\text{number of colonies} \times \text{d}}{\text{volume inoculated}}$$

Where, d = dilution factor

cfu= colony forming units

3.8. Point inoculation method

Three-point inoculation on 100×15 mm or 90×15 mm Petri dish an accepted standard technique for cultivation and morphological identification of *Penicillium*, *Aspergillus* and other related genera was followed. The three-point inoculation was done by using glass Petri dishes inoculated with very low quantities of conidia using glass needles. Petri dishes were incubated at different temperatures upside down for 7 days to prevent spread of conidia all over the plate and growth of the colonies.

3.9. Slide culture technique

The desired agar medium (10 ml) was poured into a 60mm petri dish, allowed to solidify and cut with a sterile stainless steel spatula into blocks approximately 5 to 8 mm^2. One block was aseptically removed and placed on the cover glass. Inoculation of the agar block on one or more sides with fungal hyphae or conidia was followed by placement of a second sterile cover glass on top of it. After this petri dish lid was replaced, the completed modified slide culture was incubated at the desired temperature until adequate growth and conidiogenesis had occurred. Each cover glass was used to prepare a semi-permanent mount on a standard microscope slide. The top cover glass was lifted off with forceps and wetted on the specimen side with a drop of ethanol (70 to 90%). One drop of fungus mounting medium (lactophenol cotton blue) was applied to the specimen, and the cover glass was lowered gently onto the slide, specimen side down. The bottom cover glass was lifted and similarly mounted on a second slide (Riddle, 1950; Harris, 1986).

3.10. Identification

The identification was achieved by placing a drop of the stain on clean slide with the aid of a mounting needle, where a small portion of the mycelium from the fungal cultures was removed and placed in the stain. The mycelium was spread very well on the slide with the aid of the needle. A cover slip was gently applied with little pressure to eliminate air bubbles. The slide was then mounted and observed with 10x,

40x and 100x objective lenses respectively. The species encountered were identified in accordance with various mycological texts (Raper and Fennell 1965; Cheesbrough, 2000). Fungal morphology was characterized by using a semiautomatic image analysis system consisting of an Olympus microscope (Olympus, New Hyde Park, NY, U.S.A.) operated as phase contrast, a charge coupled device (CCD) camera (Sony, Cambridge, U.K.) a PC with a frame-grabber, and the image analysis software (SIS, Olympus, Germany). Sample preparation and measurement was done as described by Papagianni *et al*. (1998 and 1999). A magnification of 100x was applied for measurement of mycelial particles to estimate the individual mycelia and other micro-morphological features.

3.11. Baiting

For the isolation of zoosporic fungi the baiting technique was used (Sparrow, 1960; Stevens, 1974). Water samples of each site were put together (100 ml) and four aliquots of 25 ml were placed in 9 cm Petri dishes with five to ten sterile baits (sesame and mustard seeds). Water cultures were incubated at room temperature (20-25°C) for 4-7 days. Generic identification of grown up zoosporic fungi was made according to Coker (1923), Johnson (1956), Sparrow (1960), Scott (1961) and Karling (1977).

Because of the few methods developed to quantify zoosporic fungal abundance (Zhang *et al*., 1998), we established two different ways of calculating the relative abundance of a genus: in the first, each genus that appeared in a dish was counted as a colony and rated to the total number of isolations for each site and in the second one, each bait colonized by a genus was recorded as an isolation and then the relative abundance (%) was calculated (Yanna *et al*., 2001). Presence–absence (occurrence) of a genus in each Petri dish was recorded.

3.12. DNA extraction

3.12.1. Growth and harvesting cells from liquid cultures

The isolated fungal colonies were grown in Potato Dextrose Broth and Sabouraud Dextrose Broth. To 100ml of the medium (Potato Dextrose Broth/ Sabouraud Dextrose Broth) 10 µl of Tween-80 was added before autoclaving (at 121^{O}C for 15 minutes) as Tween-80 keeps the fungal cells dispersed and helps in weighing the culture. After inoculation of fungal cultures, incubation at 30^{O}C,

preferably on a shaker was done. Since wet fresh fungal tissue, contain more cells per weight and result in higher yields, therefore they were preferably used.

Fungal walls consist of complex fibrillar material embedded in polysaccharide that enables the proteins present to make the wall a functional unit. The wall may be highly protected or relatively susceptible to the environment, because of various constituents in the matrix. Therefore extraction of DNA from fungal cultures is difficult and the yield varies with different species.

3.12.2. Manual disruption with mortar and pestle

It is advisable to use mortar and pestle with rough grinding surfaces (rather than smooth agate mortar and pestle) as fungal tissue is a hard and sticky mass, difficult to grind. Therefore, the weighed fungal tissue was ground to a fine powder in a mortar and pestle using liquid nitrogen. Then the liquid nitrogen was allowed to evaporate and the samples were not allowed to thaw. So we proceeded immediately to the DNA isolation protocol.

3.12.3. DNA isolation protocol

1. To a maximum of 100-150mg of fungal tissue previously disrupted by grinding in liquid nitrogen, 400 µl of Lysis Buffer and 20 µl of RNase A (20mg/ml) were added and vortexed vigorously.
2. The mixture was incubated for 10 minutes at 65^{O}C and mixed 2-3 times by inverting the tube.
3. Then 130 µl of Precipitation Buffer was added to the lysate, mixed and incubated for 5 minutes on ice.
4. Then it was Centrifuged for 5 minutes at 20,000 x g (=14,000 rpm).
5. The lysate was loaded in the HiShredder placed in a 2 ml collection tube followed by centrifugation for 2 minutes at 20,000 x g (=14,000 rpm).
6. The flow through fraction from the above step was transferred to a new 2 ml collection tube without disturbing the cell debris pellet.
7. To the cleared lysate was added 1.5 volumes of the diluted Binding Buffer and mixed by pipetting (675 µl of diluted binding buffer was added to 450 µl of lysate). Sometimes precipitate was formed after the addition of ethanol but it never affected the DNA isolation procedure.
8. 650 µl of the mixture from the above step was loaded to the HiElute Miniprep Spin Column sitting in a 2 ml collection tube and centrifugation for 1 minute

at 6000 x g (=8000 rpm) was done and the flow through fraction was discarded.

9. The above step was repeated with the remaining sample and the flow through fraction liquid and the 2 ml collection tube were discarded.
10. HiElute Miniprep Spin Column was placed again in a new 2 ml collection tube, followed by adding 500 µl of diluted wash buffer and centrifugation for 1 minute at 6000 x g (=8000 rpm). The flow through fraction was discarded and 2 ml collection tube was reused in the new step.
11. Another 500 µl of wash buffer was added to the column and centrifugation for 2 minutes at 20,000 x g (=14,000 rpm) was done to dry the membrane.
12. 100 µl of the elution buffer was added directly into the column without spilling the sides followed by incubation for 1 minute at room temperature. To elute the DNA centrifugation at 6500 x g (=10,000 rpm) was done. This step was repeated with another 100 µl of elution buffer for high yield of DNA. To increase the elution efficiency, incubation for 5 minutes at room temperature (15-25^{O}C) after adding the elution buffer, was also done. DNA elution was also performed in single step by the addition of 200 µl of Elution buffer at a time.
13. The Eluent containing pure genomic DNA was stored for further use at 2-8^{O}C as recommended. For long-term storage the DNA was stored either at -20^{O}C or lower temperature (-80^{O}C). Repeated freezing and thawing of the samples which may cause denaturing of DNA was avoided.

3.13. Purity of DNA

The ratio of the absorption 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.

3.14. Polymerase chain reaction (PCR)

The forward (ITS-1) and reverse (ITS-4) primers were used in the PCR reactions performed in this study as developed by White *et al*. (1990) to amplify the ITS region of the rRNA operon. Amplification was performed in 50µl PCR reaction

tubes containing 6µl genomic DNA, 5µl 10x thermophilic buffer, 2µl $MgCl_2$, 1.4µl dNTP (10mM), 1.2µl (10µM) ITS1, 1.2µl (10µM) ITS4, 6µl (10u/µl) *Taq* (Promega) and 32 µl ddH_2O. Amplifications was carried out in an Eppendorf Thermal Cycler with the following program for 40 cycles: initial denaturation temperature 94°C for 5 minutes, melt temperature 94°C for 50 seconds, annealing temperature 54.5°C for 45 seconds, extending temperature of 72°C for 45 seconds, final extension temperature 72°C for 10 minutes and hold temperature of 4°C. The ITS bands were identified by gel electrophoresis on a 2% agarose gel. PCR products obtained were sequenced and the sequences obtained were BLAST searched to conform the percent similarity to the ITSI, 5.8S rRNA gene and ITSII regions of the respective fungi in the Gene Bank. Molecular weights of each ITS fragment was determined using a 100 bp ladder.

3.15. Physiological profiling

Physiological profiling of a few yeast species was carried out by the biochemical tests included Physiological tests, Fermentation tests and Assimilation tests.

3.15.1. Preparation of microplates

Specified media were prepared and sterilized by heating or filtration prior to their addition to sterile microplates. The wells of the assimilation and growth microplate wells (96 wells, flat bottom) were filled with 100 µl of the media.

3.15.2. Inoculation and incubation of microplates

50µl of inoculum (MacFarland standard No. 2 diluted by a factor of 10) was introduced into each well using a multi-channel (8 or 12 channels) pipette. Loose cellophane was placed on the microplates to avoid desiccation of the wells. The microplates were incubated without agitation at 25°C for 3 to 10 days.

3.15.3. Test reading

Microplates were properly shaken (with a microplate shaker) just before automatic reading using a microplate reader. Absorbance values at 405nm were transferred by cable (RS-232 through a serial port) to the computer and transformed by the BioloMICS software into negative, weak or positive results. The results of every test were transformed independently.

3.16. Media used

Eight different media with some general purpose media and some specific media were used for the present work whose composition is depicted in Table 2.

Table 2. Composition of media used

Aureomycin Rose Bengal Glucose Peptone Agar		
Glucose	:	10 g
Peptone	:	5 g
KH_2PO_4	:	1 g
$MgSO_4.7H_2O$	:	0.5 g
Agar	:	20 g
Distilled water	:	1 litre
Rose Bengal	:	0.035 g
Aureomycin HCl	:	70 mg
Streptomycin Terramycin Malt Extract Agar		
Malt Extract	:	30 g
Peptone	:	5 g
Agar	:	15 g
Distilled water	:	1 litre
Streptomycin	:	70 mg
Terramycin	:	70 mg
Potato Dextrose Agar		
Potato extract	:	4 g
Dextrose	:	20 g
Agar	:	15 g
Distilled water	:	1 litre
Sabouraud's Dextrose Agar		
Peptone	:	10 g
Dextrose	:	40 g
Agar	:	15 g
Distilled water	:	1 litre

Corn Meal Agar		
Corn Meal extract	:	2 g
Agar	:	15 g
Distilled water	:	1 litre
Malt Extract Agar (MEA)		
Powdered malt extract	:	2 g
Peptone	:	10g
Glucose	:	20g
Agar	:	20 g
Distilled water	:	1 litre
Czapek Dox Agar		
Czapek concentration	:	10.0ml
K_2HPO_4	:	1g
Sucrose	:	30g
Agar	:	17.5g
Distilled water	:	1litre
Czapek Yeast Agar		
Czapek concentration	:	10.0ml
K_2HPO_4	:	1g
Sucrose	:	30g
Powdered yeast extract	:	5g
Agar	:	17.5g
Distilled water	:	1litre

3.17. Prevalence of fungal disease

The prevalence of fungal disease 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 and examined was done. These persons were interviewed and examined for any sort of superficial fungal infection on their body. The questionnaire used is given as Annexure I

3.18. 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. Descriptive analysis of the data was carried out by the Box and Whisker models. In addition to this some diversity and similarity indices were calculated using the software. Correlation of some physical parameters of water with the fungal load at different sites was also determined using SPSS 16.0. Chi-square test was used for the analytic assessment of the fungal disease prevalence in the Dal inhabitants. Percentage (%) of disease incidence to measure their prevalence was also assessed.

Chapter 4

Results

Fungal population in the water samples collected from Dal lake on seasonal basis from sixteen different sites of the Lake was assessed in terms of colony forming units (CFU/ml) and total viable counts on Rose Bengal Streptomycin Agar (RBSA) and Potato Dextrose Agar (PDA). The samples were serially diluted five folds using normal saline solution to get the desired number of colonies per plate so as to avoid any ambiguity in the results by the overlapping of different colonies. In all, the fungal population assessed from the lake water samples belonged to two groups of fungi viz. the filamentous fungi and the zoosporic fungi with twenty eight (28) species belonging to the filamentous group and the rest twenty three (23) species belonging to the zoosporic group.

4.1. Identification

A total of fifty one (51) species (Table 3) of fungi viz. *Penicillium chrysogenum* Thom., *Penicillium funiculosum* Thom., *Penicillium caseicolum* Bain., *Penicillium lilacinum* Thom., *Penicillium olivicolor* Pitt., *Penicillium commune* Thom., *Penicillium dimorphosporum* Swart., *Penicillium* spp. I., *Penicillium* spp. II., *Penicillium* spp. III., *Penicillium* spp. IV., *Aspergillus niger* Van Tieghem.,

Aspergillus fumigatus Fresenius., *Aspergillus japonicus* Saito., *Aspergillus versicolor* gr., *Aspergillus wentii* gr., *Aspergillus terreus* Thom., *Aspergillus oryzae* (Ahlburg) E. Cohn, *Mucor* spp., *Rhizopus stolonifer* (Ehrenb. Fr.) Vuill., *Fusarium oxysporum* (Schlecht.) Emend, *Acremonium strictum* W. Gams, *Candida albicans, Candida krusei, Candida parapsilosis, Candida glabrata, Cryptococcus neoformans, Allomyces moniliformis* Coker and Braxton., *Allomyces anomalus* Emerson., *Allomyces arbuscula* Butler., *Allomyces* spp., *Achyla klebsiana* Pieters., *Achyla apiculata* de Bary., *Achyla flagellate* coker., *Achyla cornuta* Archer., *Achyla conspicua* Coker., *Brevilegnia indica* Prabuji and Sinha., *Brevilegnia linearis* Coker and Braxton., *Brevilegnia diclina* Harvey., *Brevilegnia subclavata* Couch., *Dictyuchus monosporus* Leitgeb., *Dictyuchus* spp., *Aphanomyces laevis* de Barey., *Aphanomyces cladogamous* Dreschler., *Aphanomyces* spp., *Pythium proliferum* de Bery., *Pythium elongatum* Matthews., *Pythium debaryanum* Hesse., *Pythium* spp. and *Saprolegnia parasitica* Coker were isolated and identified during the course of study.

The species were identified on the basis of morphological observation and culture using differential culture media like Czepek dox agar (CZ) media, Czepek Yeast Agar (CYA) media, Malt Extract Agar (MEA) media, Potato Dextrose Agar (PDA) media, CHROM Agar *candida* and classical slide culture techniques besides the use of biochemical tests for the identification of yeast specimens and molecular approach.

4.1.1. Morphological Identification

The morphological identification of the fungal species was based on the observation of some macro-morphological features like Conidium (front) color, reverse color of colonies, colony diameter and some micro-morphological features like conidia length, conidia width, conidia shape, conidia ornamentation, stipe length, stipe width, stipe ornamentation, phialide shape and branching pattern. The color of colonies observed varied with the type of media and type of fungal strain as well. The macroscopic characteristics as presented in Table 4 reveal that the most common conidium color of the fungal colonies was green and reverse color was white to cream. However, some more colors like yellow, brown and yellow orange were also shown by the conidia and reverse side of the isolated fungal strains (Plate 2, 3, 4, 5 & 6). The colony diameter shown by different species ranged between a minimum of 19mm and a maximum of 50mm. The microscopic features of these fungal species (Table 5) reveal that conidia length, conidia width, stipe length and stipe width varied between 2.5 to 3.5 µm, 2.2 to 3 µm, 28 to 350 µm and 2 to 3.5 µm respectively (Plate 7 & 8).

The conidia shape shown by the strains was globose, ellipsoid, subglobose and pyriform with ornamentation of Conidia and stipe as smooth, coarsely roughned and finely roughened. Moreover, the phialide shape was ampulliform and cylindrical, branching pattern was Mono-verticillate, Bi-verticillate, Ter-verticillate and Quarte-verticillate. These variations in the morphological features helped in identifying the fungal species.

4.1.2. Biochemical Identification

The biochemical tests conducted for the identification of the yeast specimens included three types of tests viz. Physiological tests including Germ Tube Test, Hydrolysis, Growth on Cycloheximide medium and Growth at 37^{O}C; Fermentation tests including Glucose, Trehalose, Lactose, Galactose, Maltose, Sucrose fermentation (Table 6) and Assimilation tests including Glucose, Trehalose, Lactose, Galactose, Maltose, Sucrose, Raffinose, D-Manitol, Soluble starch, Glycerol, Lactic acid, Potassium nitrate, Citric acid and Succinic acid assimilation with the species showing positive, negative and variable results for the respective tests for which they are positive, negative and variable (Table 7). The species identified by means of the biochemical tests included *C. glabrata*, *C. albicans*, *C. krusei*, *C. parapsilosis* and *C. neoformans*. Additional confirmation of the results was made by the growth of these species on *Candida* specific CHROM Agar (Plate 9) for the observation of color of the moist colonies, with the colonies of *C. albicans* showing green color, *C. glabrata* showing pink colonies, *C. krusei* showing whitish pink colonies with a wrinkled surface and *C. parapsilosis* showing cream color colonies.

Table 6. Physiological and fermentation test reaction of yeast species

Species	Physiological tests				Fermentation tests					
	GTT	H	GCm	G37^{O}C	G	T	L	M	S	Ga
C. glabrata	-	-	-	+	+	v	-	-	-	-
C. albicans	+	-	+	+	+	v	-	+	-	v
C. krusei	-	v	-	+	+	-	-	-	-	-
C. parapsilosis	-	-	-	+	+	-	-	-	-	v
C. neoformans	-	+	-	+	-	-	-	-	-	-

GTT=Germ Tube Test; H=Hydrolysis; GCm=Growth on Cycloheximide medium; G37^{O}C =Growth at 37^{O}C; G=Glucose; T=Trehalose; L=Lactose; Ga=Galactose; M=Maltose; S=Sucrose; v=variable; (+)=positive for the test; (-)=no reaction

Table. 7 Assimilation test reaction of yeast species

Species	Assimilation tests													
	G	T	L	Ga	M	S	R	DM	SS	Gl	LA	PN	CA	SA
C. glabrata	+	+	-	-	-	-	-	-	-	v	v	-	-	-
C. albicans	+	+	-	+	+	+	-	+	+	v	v	-	v	v
C. krusei	+	-	-	-	-	-	-	-	-	+	+	-	v	+
C. parapsilosis	+	+	-	+	+	+	-	+	-	+	v	-	v	v
C. neoformans	+	+	-	+	+	+	+	+	v	v	v	-	v	v

G=Glucose; T=Trehalose; L=Lactose; Ga=Galactose; M=Maltose; S=Sucrose; R=Raffinose; DM=D-Manitol; SS=Soluble Starch; Gl=Glycerol; LA=Lactic acid; PN=Potassium nitrate; CA=Citric acid; SA=Succinic acid; v=variable; (+)=positive for the test; (-)=no reaction

4.1.3. Molecular Identification

The molecular level identification of various species was done by using Polymerase chain reaction (PCR), targeting internal transcribed spacer (ITS) region by universal fungal primers (ITS1-TCCGTAGGTGAACCTGCGG, ITS2-GCTGCGTTCTTCATCGATGC, ITS3-GCATCGATGAAGAACGCAGC and ITS4-TCCTCCGCTTATTGATATGC) often needed for estimating fungal diversity in environmental samples and considered important in modern microbiological assays. DNA from all the unknown cultures was successfully extracted by using a HiPur A^{TM} SP Fungal DNA Mini kit (Himedia) and the purity of DNA (Plate 10) was measured by the absorbance ratio (*A260/A280*) which ranged between 1.7-1.9 for all the samples. Amplification of the extracted DNA was carried out by using primer pair ITS1 and ITS4 and the PCR products obtained were sequenced. The sequences obtained were BLAST searched to conform the percent similarity to the Gene Bank and all the sequences were found to be 90 to100% similar to the sequences of the ITSI, 5.8S rRNA gene, and ITSII regions of the respective fungi. The banding pattern produced by each species on 2% agarose gel can be seen in Plate 11 & 12. Agarose gel was successful in providing enough band clarity to identify the presence of largely different sized ITS regions. On the basis of BLAST search results (Table 8) the species identified were *Penicillium chrysogenum* with gene bank accession number FJ499458, fragment size of 539 bp and 100% similarity with the gene bank. *Penicillium funiculosum* (Acc. no. JQ670958) with a fragment size of 507bp and 99% similarity. *Aspergillus niger* (Acc. no. JQ867187) with a fragment size of 595bp and 100% similarity. *Aspergillus fumigatus* (Acc. no. FJ499462) with a fragment size of 452bp and 100% similarity. *Aspergillus terreus* (Acc. no. JQ889709) with a fragment size of 608bp and 98% similarity. *Aspergillus flavus* (Acc. no. HQ395774) with a

fragment size of 596bp and 100% similarity. *Penicillium commune* (Acc. no. FJ499450) with a fragment size of 521bp and 98% similarity. *Penicillium dimorphosporum* (Acc. no. AF081804) with a fragment size of 739bp and 98% similarity. *Aspergillus japonicas* (Acc. no. JQ886415) with a fragment size of 552bp and 98% similarity. *Aspergillus versicolor* (Acc. no. JQ724473) with a fragment size of 565bp and 98% similarity. *Achlya klebsiana* (Acc. no. AF218156) with a fragment size of 590bp and 98% similarity. *Achlya flagellate* (Acc. no. AF218143) with a fragment size of 680bp and 100% similarity. *Achlya apiculata* (Acc. no. AJ238656) with a fragment size of 730bp and 100% similarity. *Allomyces moniliformis* (Acc. no. HQ888725) with a fragment size of 567bp and 100% similarity. *Allomyces anomalus* (Acc. no. JN943676) with a fragment size of 623bp and 100% similarity.

Table 8. Molecular details of some fungal species isolated from Dal lake

Isolate Code	Name	% Similarity	Gen Bank Acc. no.	Fragment size
CORD 1	*Penicillium chrysogenum*	100%	FJ499458	539 bp
CORD 2	*Penicillium funiculosum*	99%	JQ670958	507 bp
CORD 3	*Aspergillus niger*	100%	JQ867187	595 bp
CORD 4	*Aspergillus fumigatus*	100%	FJ499462	452 bp
CORD 5	*Aspergillus terreus*	98%	JQ889709	608 bp
CORD 6	*Aspergillus flavus*	100%	HQ395774	596 bp
CORD 7	*Penicillium commune*	98%	FJ499450	521 bp
CORD 8	*Penicillium dimorphosporum*	98%	AF081804	739 bp
CORD 9	*Aspergillus japonicus*	98%	JQ886415	552 bp
CORD10	*Aspergillus versicolor*	98%	JQ724473	565 bp
CORD11	*Achlya klebsiana*	98%	AF218156	590bp
CORD12	*Achlya flagellata*	100%	AF218143	680bp
CORD13	*Achlya apiculata*	100%	AJ238656	730bp
CORD14	*Allomyces moniliformis*	100%	HQ888725	567bp
CORD15	*Allomyces anomalus*	100%	JN943676	623bp
CORD16	*Dictyuchus monosporus*	100%	FJ746662	643 bp
CORD 17	*Aphanomyces laevis*	99%	HQ643122	695 bp
CORD 18	*Aphanomyces cladogamus*	99%	HQ643114	696 bp
CORD 19	*Candida albicans*	98%	JQ901930	498 bp
CORD 20	*Saprolegnia parasitica*	99%	AY455776	742 bp
CORD 21	*Pythium debaryanum*	99%	HQ643519	944 bp

Dictyuchus monosporus (Acc. no. FJ746662) with a fragment size of 643bp and 100% similarity. *Aphanomyces laevis* (Acc. no. HQ643122) with a fragment size of 695bp and 99% similarity. *Aphanomyces cladogamus* (Acc. no. HQ643114) with a fragment size of 696bp and 99% similarity. *Candida albicans* (Acc. no. JQ901930) with a fragment size of 498bp and 98% similarity. *Saprolegnia parasitica* (Acc. no. AY455776) with a fragment size of 742bp and 99% similarity. *Pythium debaryanum* (Acc. no. HQ643519) with a fragment size of 944bp and 99% similarity.

4.2. General distribution

The fifty one (51) species of fungi isolated and identified from the lake water during the course of study belonged to five major phyla viz. Ascomycota contributing twenty five (25) species, Oomycota contributing nineteen (19) species, Blastocladiomycota contributing four (4) species, Zygomycota contributing two (2) species and Besidiomycota contributing only one (1) species. Phylum Ascomycota consisted of four major classes Eurotiomycetes, Sordariomycetes, Ascomycetes and Saccharomycetes; phylum Besidiomycota consisted of one class Tremellomycetes, Blastocladiomycota of one class Blastocladiomycetes and Oomycota of one class Oomycetes, and Zygomycota of one class Zygomycetes (Table 9). All the taxonomic classes contributed with one order each except Oomycetes which contributed with two orders viz. Saprolegniales and Pythiales. Order Eurotiales consisted of two (2) genera viz. *Penicillium* contributing ten (10) species and *Aspergillus* contributing eight (8) species; order Hypocreales consisted of three (3) genera viz. *Fusarium* contributing one (1) species, *Acremonium* contributing one (1) species and *Penicillium* contributing one (1) species; order Saccharomycetes consisted of one (1) genus *Candida* contributing four (4) species; order Tremellales consisted of one (1) genus *Cryptococcus* contributing only one (1) species; order Mucorales consisted of two (2) genera viz. *Mucor* contributing one (1) species and *Rhizopus* contributing one (1) species; order Blastocladiales consisted of one (1) genus *Allomyces* contributing four (4) species; order Sparolegniales consisted of five (5) genera viz. *Achyla*, *Brevilegnia*, *Dictyuchus*, *Aphanomyces* and *Saprolegnia* contributing five (5), four (4), two (2), three (3) and one (1) species respectively. The rest order Pythiales consisted of one (1) genus contributing four (4) species.

Table 9. Systematic diversity of the isolated taxa of fungi

Kingdom	Phylum	Class	Number of taxa		
			Order	Genera	Species
Fungi	Ascomycota	Eurotiomycetes	1	2	17
		Sordariomycetes	1	3	3
		Ascomycetes	1	1	1
		Saccharomycetes	1	1	4
	Basidiomycota	Tremellomycetes	1	1	1
	Blastocladiomycota	Blastocladiomycetes	1	1	4
	Zygomycota	Zygomycetes	1	2	2
Chromista	Oomycota	Oomycetes	2	6	19

In all the genus *Penicillium* contributed a maximum of eleven species with a relative abundance of 21.6% followed by genus *Aspergillus* eight species (15.7%), *Achyla* five species (9.8%), *Candida*, *Allomyces*, *Brevilegnia* and *Pythium* four species (8%) each, *Aphanomyces* three species (5.9%), *Dictyuchus* two species (3%), *Mucor*, *Rhizopus*, *Fusarium*, *Acremonium*, *Cryptococcus* and *Saprolegnia* one species (2%) each (Table 10 and Fig. 1). The fungal species isolated belonged to nine families (Table 11 and Fig. 2) with maximum contribution of nineteen species by family trichocomaceae followed by family saprolegniaceae with a contribution of fifteen species, four species each by saccharomycetaceae, blastocladiaceae and pythiaceae, two species by family mucoraceae and one species each by families nectriaceae, hypocreaceae and tremellaceae.

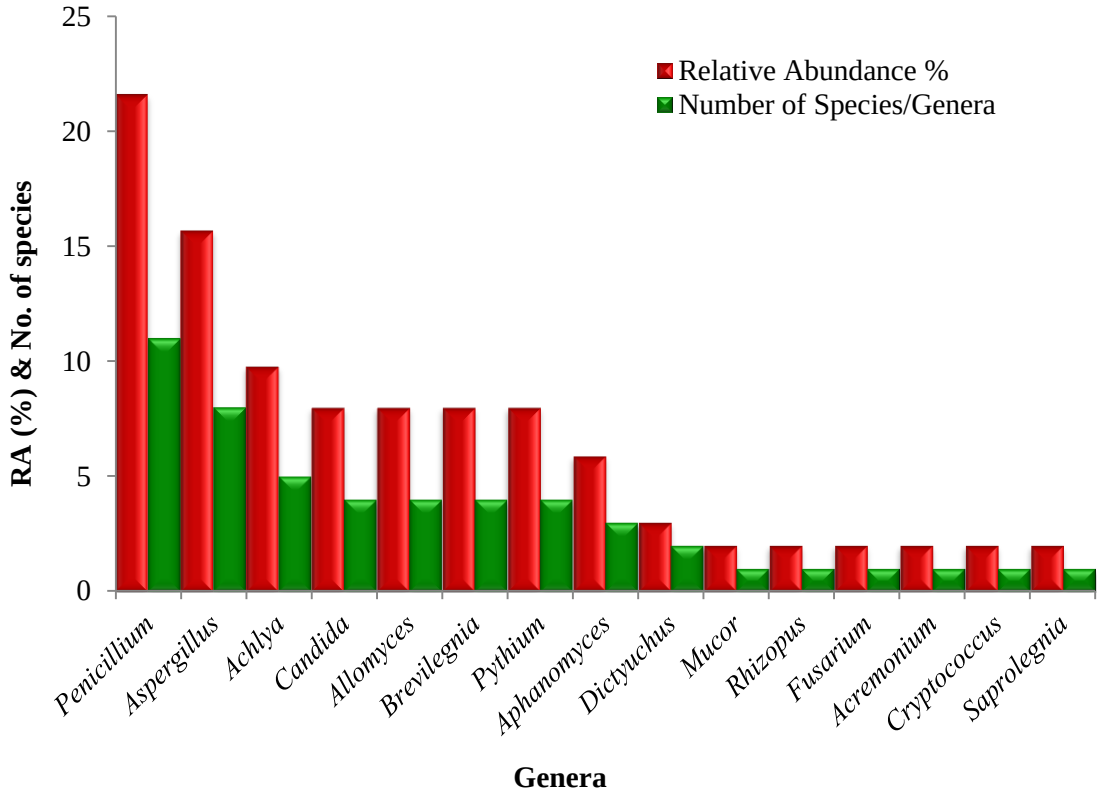


Fig. 1. Graphical representation of generic relative abundance

Table 10. Relative abundance amongst the isolated genera

Genera	Number of species	Relative Abundance (%)
Penicillium	11	21.6
Aspergillus	8	15.7
Achlya	5	9.8
Candida	4	8
Allomyces	4	8
Brevilegnia	4	8
Pythium	4	8
Aphanomyces	3	5.9
Dictyuchus	2	3
Mucor	1	2
Rhizopus	1	2
Fusarium	1	2
Acremonium	1	2
Cryptococcus	1	2
Saprolegnia	1	2

Table 11. Distribution record of species as per the families

Family	Number of species
Trichocomaceae	19
Saprolegniaceae	15
Saccharomycetaceae	4
Blastocladiaceae	4
Pythiaceae	4
Mucoraceae	2
Nectriaceae	1
Hypocreaceae	1
Tremellaceae	1

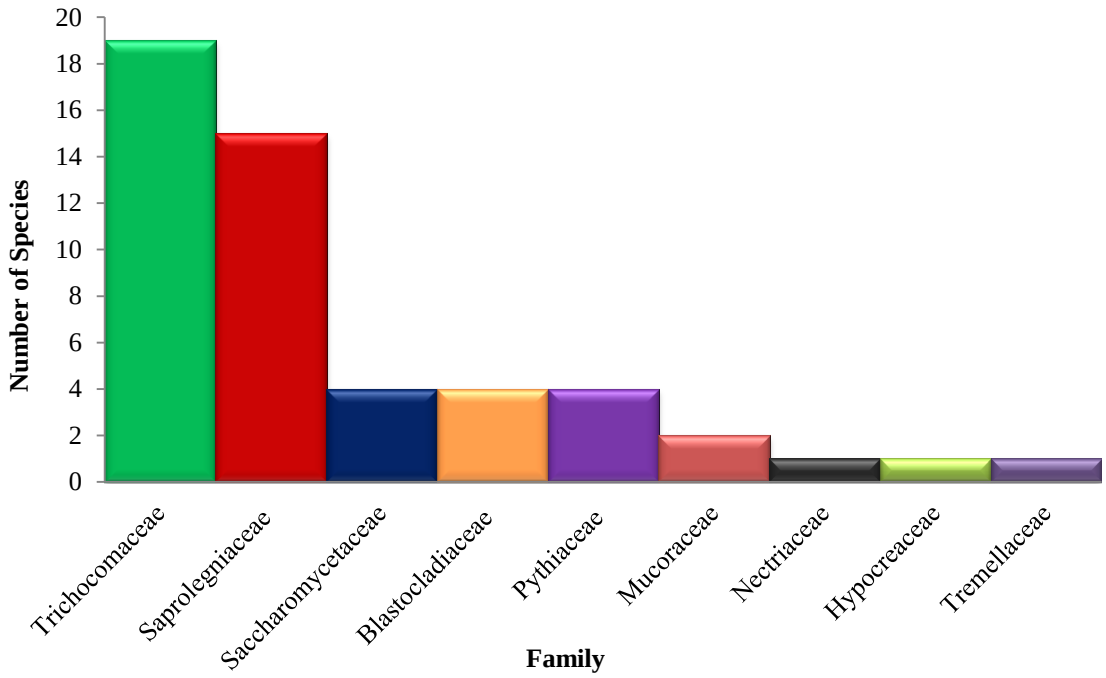


Fig. 2. Family wise distribution record of species

4.3. Distribution of colonies

The fungal community was assessed by plating serially diluted water samples in normal saline solution (0.9% NaCl) on Rose Bengal Agar and incubated at 25-37°C for seven days. The developed colonies in the plates were enumerated by Qubeck colony counter and the fungal load assessed in terms of colony forming units (cfu/ml)

revealed that the concentration of fungal colonies was comparatively higher in all seasons of second year of study as compared to the seasons of first year of study. The overall highest of 9.80×10^4 cfu/ml was observed at site 14 in summer season and lowest 0 cfu/ml was observed in winter seasons at site 12. The filamentous fungal load (Table 12) ranged between 0.20×10^4 cfu/ml at site 4 to 8.70×10^4 cfu/ml at site 1 in spring 2010, 0.80×10^4 cfu/ml at site 4 to 5.80×10^4 cfu/ml at site 16 in spring 2011, 0.50×10^4 cfu/ml at site 5 to 9.80×10^4 cfu/ml at site 14 in summer 2010, 0.80×10^4 cfu/ml at site 5 to 9.10×10^4 cfu/ml at site 16 in summer 2011, 0.80×10^4 cfu/ml at site 4 to 6.20×10^4 cfu/ml at site 14 in autumn 2010, 0.40×10^4 cfu/ml at site 11 to 4.90×10^4 cfu/ml at site 14 in autumn 2011, 0 cfu/ml at site 12 to 2.00×10^4 cfu/ml at site 10 in winter 2010 and 0.10×10^4 cfu/ml at site 12 to 3.30×10^4 cfu/ml at site 14 in winter 2011.

Table 12. Colony forming units (cfu/ml) of filamentous fungi at different sites

Sites	Spring		Summer		Autumn		Winter	
	2010	**2011**	**2010**	**2011**	**2010**	**2011**	**2010**	**2011**
S 1	8.70×10^4	5.00×10^4	1.60×10^4	2.20×10^4	2.90×10^4	1.20×10^4	1.40×10^4	0.30×10^4
S 2	2.60×10^4	4.40×10^4	1.70×10^4	2.50×10^4	3.30×10^4	2.20×10^4	1.30×10^4	2.40×10^4
S 3	1.10×10^4	2.30×10^4	1.30×10^4	1.40×10^4	1.80×10^4	1.40×10^4	0.40×10^4	0.50×10^4
S 4	0.20×10^4	0.80×10^4	0.70×10^4	1.20×10^4	0.80×10^4	1.10×10^4	0.70×10^4	0.30×10^4
S 5	0.60×10^4	2.00×10^4	0.50×10^4	0.80×10^4	1.30×10^4	2.00×10^4	0.50×10^4	0.20×10^4
S 6	0.60×10^4	1.30×10^4	1.00×10^4	1.00×10^4	2.10×10^4	0.90×10^4	0.20×10^4	0.20×10^4
S 7	3.60×10^4	3.00×10^4	2.80×10^4	4.50×10^4	0.90×10^4	2.50×10^4	0.90×10^4	0.70×10^4
S 8	6.80×10^4	3.00×10^4	3.60×10^4	4.60×10^4	3.00×10^4	1.50×10^4	1.70×10^4	1.30×10^4
S 9	1.20×10^4	3.30×10^4	3.70×10^4	2.60×10^4	1.60×10^4	1.10×10^4	1.00×10^4	1.50×10^4
S 10	2.00×10^4	2.70×10^4	3.50×10^4	4.00×10^4	1.60×10^4	1.50×10^4	2.00×10^4	1.10×10^4
S 11	0.90×10^4	2.70×10^4	5.30×10^4	5.00×10^4	1.90×10^4	0.40×10^4	0.90×10^4	0.50×10^4
S 12	0.30×10^4	1.20×10^4	2.30×10^4	3.00×10^4	1.40×10^4	0.70×10^4	0	0.10×10^4
S 13	3.20×10^4	5.40×10^4	7.10×10^4	8.90×10^4	3.90×10^4	2.70×10^4	1.70×10^4	1.50×10^4
S 14	6.30×10^4	7.30×10^4	9.80×10^4	8.40×10^4	6.20×10^4	4.90×10^4	1.40×10^4	3.30×10^4
S 15	1.00×10^4	3.30×10^4	5.30×10^4	3.60×10^4	3.20×10^4	1.80×10^4	1.80×10^4	0.60×10^4
S 16	4.70×10^4	5.80×10^4	7.80×10^4	9.10×10^4	1.10×10^4	3.30×10^4	1.00×10^4	0.30×10^4

The site wise distribution of fungal colonies (filamentous colonies, zoosporic colonies and total number of colonies) shown in Fig. 3 depicts the heavy fungal load at Site 13 and 14 followed by Site 16, Site 8 and all other sites.

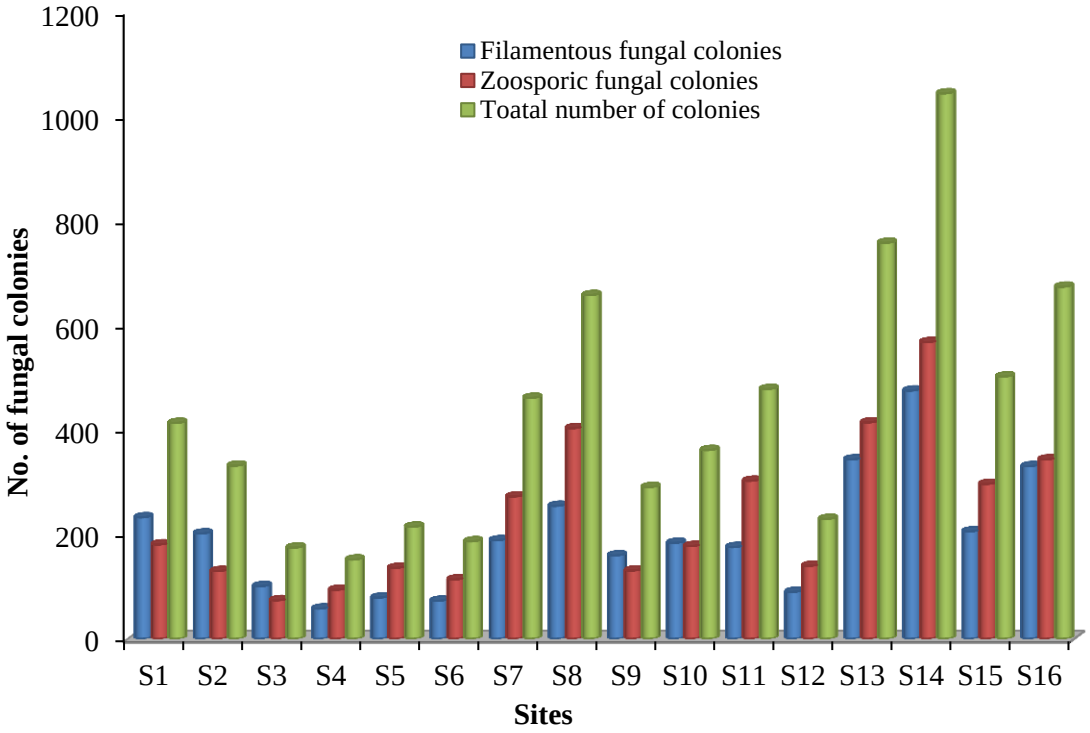


Fig. 3. Distribution of filamentous and zoosporic fungi at different sites

The family and species wise total count (TC), % total count (%TC), number of cases of isolation (NCI) and occurrence remarks (OR) shown in Tables 13 and 14 depicts that among the filamentous group different species of family trichocomaceae isolated in a low to high frequency contributed a total count of 2517 colonies constituting 79.72% of this group. The different species of genus *Aspergillus* contributed a total of 1073 colonies constituting 33.99% of the whole group and 42.63% of the family trichocomaceae. It included *Aspergillus flavus* with a total count of 178 colonies constituting 5.64%, *A. fumigatus* and *A. japonicus* with a total count of 98 colonies constituting 3.10% each, *A. niger* with a total count of 218 colonies constituting 6.90%, *A. oryzae* with a total count of 72 colonies constituting 2.28%, *A. terreus* with a total count of 110 colonies constituting 3.48%, *A. versicolor* with a

total count of 142 colonies constituting 4.5%, *A. wentii* with a total count of 157 colonies constituting 4.98%. Different species of genus *Penicillium* contributed a total of 1444 colonies constituting 45.74% of the whole group and 57.37% of the family trichocomaceae. It included *Penicillium caseicolum* with a total count of 123 colonies constituting 3.90%, *P. chrysogenum* with a total count of 334 colonies constituting 10.58%, *P. commune* with a total count of 63 colonies constituting 1.99%, *P. dimorphosporum* with a total count of 128 colonies constituting 4.05%, *P. funiculosum* with a total count of 236 colonies constituting 7.47%, *P. lilacinum* with a total count of 78 colonies constituting 2.47%, *P. olivicolor* with a total count of 15 colonies constituting 0.47%, *Penicillium* spp. I with a total count of 100 colonies constituting 3.17%, *Penicillium* spp. II with a total count of 84 colonies constituting 2.66%, *Penicillium* spp. III with a total count of 136 colonies constituting 4.30%, *Penicillium* spp. IV with a total count of 147 colonies constituting 4.65%. Family Mucoraceae isolated in a high frequency contributed a total count of 186 colonies constituting 5.9% of this group. It included two species of two different genera one which contributed 94 colonies constituting 2.98% of the whole group and 50.54% of the family i.e. *Mucor* spp. and another *R. stolonifer* which contributed 92 colonies constituting 2.91% of the whole group and 49.46% of the family. Families Nectriaceae, Hypocreaceae and Tremellaceae contributed with one species each viz. *F. oxysporum*, *A. strictum* and *Cryptococcus neoformans* respectively. *F. oxysporum* contributed a total of 80 colonies constituting 2.53% of the whole group and 100% of the family Nectriaceae isolated with a high frequency. *A. strictum* contributed a total of 36 colonies constituting 1.14% of the whole group and 100% of the family Hypocreaceae isolated with a rare frequency. *Cryptococcus neoformans* contributed a total of 56 colonies constituting 1.77% of the whole group and 100% of the family Tremellaceae isolated with a medium frequency. The family saccharomycetaceae isolated with a low to high frequency contributed four species with a total count of 282 colonies constituting 8.94% of the group. It included *Candida albicans* with a

total count of 84 colonies constituting 2.66% of the whole group and 29.78% of the family, *C. krusei* with a total count of 83 colonies constituting 2.63%of the whole group and 29.43% of the family, *C. parapsilosis* with a total count of 9 colonies constituting 0.29% of the whole group and 3.19% of the family, *C. glabrata* with a total count of 106 colonies constituting 3.36% of the whole group and 37.59% of the family. Among the different families of zoosporic fungi family Saprolegniaceae was the largest share-holder with a maximum number of 2474 colonies of fifteen (15) species constituting 65.39% of the group followed by family Blastocladiaceae with 659 colonies of four (4) species constituting 17.42% and family Pythiaceae with 650 colonies of four (4) species 17.19%. In the family Blastocladiaceae isolated with a high frequency consisting of only one genus Allomyces; *A. moniliformis* contributed a total of 220 colonies constituting 5.81% of the zoosporic group and 33.39% of the family, *A. anomalus* contributed 154 colonies constituting 4.07% of the zoosporic group and 23.37% of the family, *A. arbuscula* contributed 181 colonies constituting 4.79% of the group and 27.46% of the family and *Allomyces* spp. contributed 104 colonies constituting 2.75% of the group and 15.78% of the family. Family Saprolegniaceae included four genera with *Achyla* contributing 791 colonies belonging to five species constituting 31.97% family contribution, *Brevilegnia* 679 colonies (27.45%) of four different species, *Dictyuchus* 401 colonies (16.2%) of two species, *Aphanomyces* 323 colonies (13.06%) and *Saprolegnia* 280 colonies (11.32%) of only one species. However in family Pythiaceae the single genus *Pythium* contributed all the 650 colonies of four different species with *P. proliferum* contributing a total of 204 colonies constituting 5.4% of the group and 31.39% of the family, *P. elongatum* contributing a total of 124 colonies constituting 3.28% of the group and 19.07% of the family, *P. debaryanum* contributing a total of 206 colonies constituting 5.4% of the group and 31.7% of the family and *Pythium* spp. contributing a total of 116 colonies constituting 3.07% of the group and 17.84% of the family.

Table 13. Total count (TC), % total count (%TC), number of cases of isolation (NCI) and occurrence remarks (OR) of filamentous fungi

Family and Species	TC	% TC	NCI	OR
Trichocomaceae	**2517**	**79.72**	-	H
Aspergillus	**1073**	-	-	-
Aspergillus flavus	178	5.64	15	H
Aspergillus fumigatus	98	3.1	14	H
Aspergillus japonicus	98	3.1	15	H
Aspergillus niger	218	6.9	16	H
Aspergillus oryzae	72	2.28	11	H
Aspergillus terreus	110	3.48	10	H
Aspergillus versicolor	142	4.5	15	H
Aspergillus wentii	157	4.98	14	H
Penicillium	**1444**	-	-	-
Penicillium caseicolum	123	3.9	14	H
Penicillium chrysogenum	334	10.58	16	H
Penicillium commune	63	1.99	7	M
Penicillium dimorphosporum	128	4.05	14	H
Penicillium funiculosum	236	7.47	16	H
Penicillium lilacinum	78	2.47	7	M
Penicillium olivicolor	15	0.47	4	L
Penicillium spp. I	100	3.17	14	H
Penicillium spp. II	84	2.66	12	H
Penicillium spp. III	136	4.3	14	H
Penicillium spp. IV	147	4.65	11	H
Mucoraceae	**186**	**5.9**	-	-
Mucor spp.	94	2.98	15	H
Rhizopus stolonifer	92	2.91	9	H
Nectriaceae	**80**	**2.53**	-	-
Fusarium oxysporum	80	2.53	11	H
Hypocreaceae	**36**	**1.14**	-	R
Acremonium strictum	36	1.14	11	-
Saccharomycetaceae	**282**	**8.94**	-	-
Candida albicans	84	2.66	11	H
Candida krusei	83	2.63	14	H
Candida parapsilosis	9	0.29	3	L
Candida glabrata	106	3.36	10	H
Tremellaceae	**56**	**1.77**	-	-
Cryptococcus neoformans	56	1.77	6	M
Total	**3157**	-	-	-

Table 14. Total count (TC), % total count (%TC), number of cases of isolation (NCI) and occurrence remarks (OR) of zoosporic fungi

Family and Species	TC	% TC	NCI	OR
Blastocladiaceae	**659**	**17.42**	-	H
Allomyces moniliformis	220	5.81	15	H
Allomyces anomalus	154	4.07	12	H
Allomyces arbuscula	181	4.79	14	H
Allomyces spp.	104	2.75	12	H
Saprolegniaceae	**2474**	**65.39**	-	H
Achyla	**791**	**31.97**	-	-
Achyla klebsiana	131	3.46	15	H
Achyla apiculata	85	2.25	12	H
Achyla flagellate	105	2.77	11	H
Achyla cornuta	261	6.9	16	H
Achyla conspicua	209	5.52	16	H
Brevilegnia	**679**	**27.45**	-	-
Brevilegnia indica	136	3.6	16	H
Brevilegnia linearis	143	3.78	15	H
Brevilegnia diclina	221	5.84	15	H
Brevilegnia subclavata	179	4.73	15	H
Dictyuchus	**401**	**16.2**	-	-
Dictyuchus monosporus	133	3.52	16	H
Dictyuchus spp.	268	7.08	16	H
Aphanomyces	**323**	**13.06**	-	-
Aphanomyces laevis	76	2.01	11	H
Aphanomyces cladogamous	107	2.83	12	H
Aphanomyces spp.	140	3.7	16	H
Saprolegnia	**280**	**11.32**	-	-
Saprolegnia parasitica	280	7.4	16	H
Pythiaceae	**650**	**17.19**	-	H
Pythium proliferum	204	5.4	13	H
Pythium elongatum	124	3.28	12	H
Pythium debaryanum	206	5.44	16	H
Pythium spp.	116	3.07	14	H
Total	**3783**	-	-	-

4.4. Species diversity patterns

The site wise colony count of the fungal species presented in Tables 15 and 16 shows that a total of 3157 colonies of filamentous fungi with a contribution of twenty eight species and 3783 colonies of zoosporic fungi with a contribution of twenty three

species were isolated from the lake water samples. Amongst the filamentous fungi the maximum number of colonies 476 (15.08%) was obtained at site 14 followed by 344 colonies (10.90%) at site 13, 331 colonies (10.48%) at site 16, 255 colonies (8.08%) at site 8, 233 colonies (7.38%) at site 1, 206 colonies (6.52%) at site 15, 202 colonies (6.40%) at site 2, 189 colonies (5.99%) at site 7, 184 colonies (5.83%) at site 10, 176 colonies (6%) at site 11, 160 colonies (5.07%) at site 9, 101 colonies (3.20%) at site 3, 90 colonies (2.85%) at site 12, 79 colonies (2.50%) at site 5, 73 colonies (2.31%) at site 6 and 58 colonies (1.84%) at site 4. From the zoosporic group of fungi the maximum number of colonies 569 (15.04%) was obtained at site 14 followed by 415 colonies (10.97%) at site 13, 404 colonies (10.68%) at site 8, 344 colonies (9.10%) at site 16, 303 colonies (8%) at site 11, 297 colonies (7.85%) at site 15, 273 colonies (7.21%) at site 7, 181 colonies (4.78%) at site 1, 178 colonies (4.70%) at site 10, 140 colonies (3.70%) at site 12, 136 colonies (3.60%) at site 5, 131 colonies (3.46%) at site 9, 130 colonies (3.44%) at site 2, 114 colonies (3.01%) at site 6, 94 colonies (2.50%) at site 4 and 74 colonies (1.96%) at site 3.

The percentage occurrence of the different fungal species isolated from the lake water samples from different sites shown in Tables 17 and 18 explains that from among the filamentous group of fungi the percentage occurrence of *Penicillium caseicolum* ranged between a maximum of 17.89% at site 16 to a minimum of 0.81% both at site 5 and 6, *P. chrysogenum* ranged between 14.97% at site 14 to 2.09% at site 6, *P. commune* between 31.74% at site 14 to 1.6% at site 1, *P. dimorphosporum* between 16.4% at site 14 to 1.56% at site 6, *P. funiculosum* between 15.68% at site 14 to 1.7% at site 5, *P. lilacinum* between 21.79% at site 16 to 8.98% both at site 2 and 12, *P. olivicolor* between 46.67% at site 1 to 13.33% at site 3, *Penicillium* spp. I between 20% at site 16 to 1% at site 6, *Penicillium* spp. II between 32.14% at site 14 to 1.2% both at site 1 and 5, *Penicillium* spp. III between 18.38% at site 14 to 1.47% at site 5 and 12, *Penicillium* spp. IV between 25.17% at site 14 to 2.04% at site 2, *Aspergillus flavus* between 23.6% at site 14 to 1.7% at site 12, *A. fumigatus* between 18.37% at site 16 to 1.02% at site 4, *A. japonicas* between 13.27% at site 14 to 1.02% both at site 3 and 6, *A. niger* between 17.89% at site 14 to 1.38% at site 3, *A. terreus* between 28.18% at site 8 to 0.91% at site 4, *A.versicolor* between 17.6% at site 14 to 0.7% at site 6, *A. wentii* between 17.83% at site 14 to 1.27% at site 4, *A. oryzae*. between 20.83% at site 16 to 1.39% at site 4, 5 and 8, *R. stolonifer* between 14.13% at site 16 to 1.09% at site 4,

A. strictum between 30.55% at site 16 to 2.78% at sites 2,4,7 and 8, *F. oxysporum* between 23.75% at site 1 to 2.5% at site 5, *Mucor* spp. between 32.97% at site 14 to 1.07% both at site 3 and 4, *Candida albicans* between 20.24% at site 16 to 1.19% both at site 5 and 8, *C. krusei* between 15.66% at site 1 to 1.2% at site 5,*C. parapsilosis* between 44.45% both at site 1 and 4 to 11.11% at site 3, *C. glabrata* between 19.81% at site 8 to 3.77% at site 4 and *Cryptococcus neoformans* ranged between 23.21% both at site 1 and 10 to 1.79% both at site 5 and 8. From among the zoosporic group of fungal species the percentage occurrence of *Allomyces moniliformis* ranged between a maximum of 16.82% at site 16 to a minimum of 0.46% at site 4, *A. anomalus* ranged between 17.53% at site 14 to 1.95% at site 4, *A. arbuscula* between 19.33% at site 14 to 1.1% both at site 2 and 3, *Allomyces* spp. between 14.42% at site 10 to 4.8% both at site 5 and 7, *Achyla klebsiana* between 12.98% at site 10 to 2.3% at site 3, 6 and 12, *A. apiculata* between 14.11% at site 16 to 1.18% at site 1, 4 and 6, *A. flagellate* between 20.95% at site 14 to 1.9% at site 5, *A. cornuta* between 13.02% at site 14 to 1.91% at site 5, *A. conspicua* between 16.75% at site 8 to 0.96% at site 3, *Brevilegnia indica* between 14.7% at site 14 to 0.73% at site 4, *B. linearis* between 15.38% at site 14 to 2.1% at site 2, *B. diclina* between 19.45% at site 14 to 2.26% at site 15, *B. subclavata* between 14.52% both at site 8 and 14 to 1.11% at site 9, *Dictyuchus monosporus* between 17.29% at site 14 to 2.26% at site 1,4,9 and 12, *Dictyuchus* spp. between 13.43% at site 14 to 0.37% at site 4, *Aphanomyces laevis* between 21.05% at site 8 to 2.63% at site 4, *A. cladogamous* between 15.89% at site 8 to 1.87% at site 9, *Aphanomyces* spp. between 20.71% at site 14 to 1.43% at site 6, *Pythium proliferum* between 23.03% at site 14 to 2.45% at site 6, *P. elongatum* between 20.16% at site 14 to 0.8% at site 1, *P. debaryanum* between 21.36% at site 14 to 0.97% both at site 4 and 6, *Pythium* spp. between 16.38% at site 13 to 1.72% at site 9 and *Saprolegnia parasitica* between 16.43% at site 14 to 1.79% at site 3.

4.5. Dominance Pattern

The most dominantly occurring ten species at each site from both fungal groups shown in the Tables 19 and 20 depicts that *P. olivicolor* was dominating at two sites, *C. neoformans* at three sites, *C. glabrata* at two sites, *A. terreus* at two sites, *C. albicans* at two sites, *Mucor* spp. at two sites, *C. parapsilosis*, *P. lilacinum* and *Acremonium* sp at one site each from among the filamentous group while as from zoosporic group *A. klebsiana* dominated two sites, *A. laevis* dominated five sites, *A. apiculata* dominated two sites while as *A. cornuta*, *A. conspicua*, *Allomyces* spp., *P. elongatum*, *P. proliferum*, *A. cladogamous* and *A. moniliformis* dominated one site each.

Table 19. Percentage occurrence of dominant filamentous fungal species

Site 1		**Site 2**		**Site 3**		**Site 4**	
P. olivicolor	46.67	*C. neoformans*	25	*P. olivicolor*	13.33	*C. parapsilosis*	44.45
C. parapsilosis	44.45	*P. olivicolor*	20	*C. glabrata*	11.32	*A. japonicus*	7.14
F. oxysporum	23.75	*C. glabrata*	14.15	*C. parapsilosis*	11.11	*Penicillium* spp. IV	5.44
C. neoformans	23.21	*F. oxysporum*	11.25	*C. krusei*	8.43	*C. glabrata*	3.77
C. krusei	15.66	*A. fumigatus*	11.22	*F. oxysporum*	7.5	*F. oxysporum*	3.75
A. terreus	12.73	*P. commune*	11.11	*P. funiculosum*	5.51	*C. krusei*	3.61
P. dimorphosporum	10.93	*C. krusei*	10.84	*Penicillium* spp. III	5.15	*P. dimorphosporum*	3.12
P. caseicolum	9.76	*A. terreus*	10	*A. fumigatus*	5.1	*A. strictum*	2.78
P. funiculosum	8.9	*A. oryzae*	9.72	*P. chrysogenum*	5.09	*P. chrysogenum*	2.7
Mucor spp.	8.51	*P. lilacinum*	8.98	*A. wentii*	3.82	*P. funiculosum*	2.12
Site 5		**Site 6**		**Site 7**		**Site 8**	
C. glabrata	8.5	*C. glabrata*	5.66	*A. terreus*	23.64	*A. terreus*	28.18
A. flavus	6.18	*Penicillium* spp. IV	5.44	*C. glabrata*	11.32	*C. glabrata*	19.81
A. terreus	4.54	*F. oxysporum*	5	*R. stolonifer*	9.78	*Penicillium* spp. IV	13.6
Penicillium spp. IV	4.08	*A. wentii*	4.46	*Penicillium* spp. IV	9.52	*R. stolonifer*	11.96
Penicillium spp. I	4	*Penicillium* spp. III	4.41	*F. oxysporum*	8.75	*F. oxysporum*	11.25
P. chrysogenum	3.59	*P. funiculosum*	3.76	*Penicillium* spp. III	8.09	*A. wentii*	10.19
A. fumigatus	3.06	*C. krusei*	3.61	*A. wentii*	7.64	*Penicillium* spp. III	9.56
A. wentii	2.55	*A. fumigatus*	3.06	*P. chrysogenum*	6.89	*A. fumigatus*	9.2
F. oxysporum	2.5	*A. flavus*	2.81	*P. funiculosum*	6.35	*P. chrysogenum*	8.38
A. niger	2.3	*A. terreus*	2.73	*P. dimorphosporum*	6.25	*A. japonicus*	8.16
Site 9		**Site 10**		**Site 11**		**Site 12**	
C. neoformans	25	*C. neoformans*	23.21	*C. albicans*	14.29	*C. albicans*	9.52
A. oryzae	18.05	*P. olivicolor*	20	*P. lilacinum*	14.1	*Penicillium* spp. I	9
Penicillium spp. II	10.71	*A. oryzae*	16.67	*Penicillium* spp. I	13	*P. lilacinum*	8.98
C. glabrata	10.38	*F. oxysporum*	15	*A. fumigatus*	12.24	*A. fumigatus*	7.14
Penicillium spp. IV	8.16	*P. dimorphosporum*	10.16	*A. japonicus*	11.22	*A. japonicus*	7.14
A. terreus	6.36	*C. krusei*	9.64	*R. stolonifer*	9.78	*R. stolonifer*	6.52
C. krusei	6.02	*C. glabrata*	8.5	*P. commune*	9.52	*A. versicolor*	5.63
A. wentii	5.73	*A. terreus*	8.18	*A. versicolor*	9.15	*A. niger*	5.04
A. strictum	5.55	*R. stolonifer*	7.61	*P. chrysogenum*	8.08	*P. caseicolum*	4.06
P. dimorphosporum	5.47	*Penicillium* spp. I	7	*A. niger*	7.8	*P. funiculosum*	2.97
Site 13		**Site 14**		**Site 15**		**Site 16**	
Mucor spp.	28.72	*Mucor* spp.	32.97	*P. lilacinum*	17.95	*A. strictum*	30.55
A. flavus	19.66	*Penicillium* spp. II	32.14	*C. albicans*	17.86	*P. lilacinum*	21.79
A. strictum	19.44	*P. commune*	31.74	*P. caseicolum*	13.82	*A. oryzae*	20.83
P. commune	19.04	*Penicillium* spp. IV	25.17	*A. versicolor*	11.27	*C. albicans*	20.24
Penicillium spp. IV	17	*A. strictum*	25	*P. commune*	11.11	*Penicillium* spp. I	20
Penicillium spp. II	14.29	*A. flavus*	23.6	*Penicillium* spp. I	11	*A. fumigatus*	18.37
A. niger	14.22	*Penicillium* spp. III	18.38	*A. japonicus*	10.2	*P. caseicolum*	17.89
P. lilacinum	14.1	*A. niger*	17.89	*A. fumigatus*	9.2	*P. commune*	15.87
A. wentii	12.1	*A. wentii*	17.83	*Penicillium* spp. II	8.33	*A. wentii*	14.65
C. albicans	11.9	*A. versicolor*	17.6	*A. strictum*	8.33	*A. niger*	14.22

Table 20. Percentage occurrence of dominant zoosporic fungal species

Site 1		Site 2		Site 3		Site 4	
A. klebsiana	10.68	*A. klebsiana*	8.4	*A. cornuta*	5.37	*A. laevis*	7.9
A. laevis	9.21	*B. diclina*	7.24	*B. diclina*	4.08	*A. cladogamous*	6.54
Allomyces spp.	7.7	*A. cornuta*	6.51	*B. subclavata*	3.91	*Allomyces* spp.	5.77
A. cornuta	7.28	*P. proliferum*	6.37	*D. monosporus*	3.76	*A. klebsiana*	5.34
P. proliferum	6.37	*S. parasitica*	5.36	*P. proliferum*	3.43	*A. conspicua*	5.26
P. debaryanum	6.31	*P. debaryanum*	4.37	*Dictyuchus* spp.	3.36	*Pythium* sp	5.18
B. linearis	6.3	*Dictyuchus* spp.	4.1	*A. klebsiana*	2.3	*B. linearis*	4.89
A. conspicua	6.22	*B. subclavata*	3.91	*A. moniliformis*	2.27	*S. parasitica*	4.64
B. subclavata	6.15	*D. monosporus*	3.76	*B. indica*	2.21	*P. elongatum*	3.22
B. indica	5.88	*B. indica*	3.7	*S. parasitica*	1.79	*A. flagellate*	2.86
Site 5		**Site 6**		**Site 7**		**Site 8**	
A. laevis	10.52	*A. conspicua*	5.26	*A. laevis*	13.16	*A. laevis*	21.05
Pythium spp.	7.76	*D. monosporus*	5.26	*A. apiculata*	11.77	*A. conspicua*	16.75
A. cladogamous	7.47	*A. laevis*	5.26	*A. conspicua*	10.04	*A. cladogamous*	15.89
P. elongatum	7.26	*A. cladogamous*	4.68	*Dictyuchus* spp.	9.33	*A. apiculata*	15.3
A. klebsiana	6.87	*A. arbuscula*	4.42	*A. anomalus*	9.09	*B. subclavata*	14.52
A. conspicua	6.7	*B. diclina*	4.08	*A. flagellate*	8.57	*A. anomalus*	13.64
A. arbuscula	6.63	*A. flagellate*	3.81	*A. cladogamous*	8.41	*A. arbuscula*	12.7
Allomyces spp.	4.8	*Dictyuchus* spp.	3.73	*B. linearis*	7.7	*Dictyuchus* spp.	10.82
A. anomalus	4.54	*A. cornuta*	3.45	*A. cornuta*	7.28	*B. linearis*	10.49
B. linearis	4.19	*Pythium* sp	3.44	*A. moniliformis*	7.27	*P. elongatum*	10.49
Site 9		**Site 10**		**Site 11**		**Site 12**	
A. apiculata	10.59	*Allomyces* spp.	14.42	*A. apiculata*	12.94	*A. laevis*	9.21
Allomyces spp.	7.7	*A. klebsiana*	12.98	*Allomyces* spp.	12.5	*A. apiculata*	8.23
A. klebsiana	7.63	*A. cornuta*	8.04	*A. cladogamous*	12.15	*A. cladogamous*	7.48
P. debaryanum	6.8	*P. debaryanum*	7.3	*A. laevis*	11.85	*A. moniliformis*	5.91
A. cornuta	5.75	*B. subclavata*	7.26	*A. moniliformis*	11.82	*A. conspicua*	5.26
P. proliferum	5.4	*A. apiculata*	7.05	*Aphanomyces* spp.	10.71	*Pythium* spp.	5.18
B. diclina	4.98	*B. indica*	6.62	*P. debaryanum*	9.22	*B. linearis*	4.89
Dictyuchus spp.	4.85	*Dictyuchus* spp.	6.34	*B. linearis*	9.09	*P. elongatum*	4.84
B. indica	4.41	*B. linearis*	5.6	*A. flagellate*	8.57	*A. flagellate*	4.76
A. laevis	3.94	*P. proliferum*	5.4	*A. anomalus*	8.44	*A. arbuscula*	4.42
Site 13		**Site 14**		**Site 15**		**Site 16**	
P. elongatum	20.16	*P. proliferum*	23.03	*A. cladogamous*	13.08	*A. moniliformis*	16.82
Aphanomyces spp.	19.3	*P. debaryanum*	21.36	*A.apiculata*	12.94	*Aphanomyces* spp.	15
Pythium spp.	16.38	*A. flagellate*	20.95	*A. moniliformis*	12.72	*A. apiculata*	14.11
P.proliferum	16.17	*Aphanomyces* spp.	20.71	*A. flagellate*	12.38	*P. elongatum*	13.71
A. arbuscula	15.47	*P. elongatum*	20.16	*A. anomalus*	12.33	*A. flagellate*	13.33
B. diclina	14.48	*B. diclina*	19.45	*Aphanomyces* spp.	12.14	*A. cladogamous*	13.08
A. anomalus	14.29	*A. arbuscula*	19.33	*S. parasitica*	10.71	*Allomyces* spp.	12.5
B. subclavatah	13.41	*Pythium* spp.	18.1	*Allomyces* spp.	10.58	*B. indica*	12.5
P. debaryanum	13.1	*A. anomalus*	17.53	*P. elongatum*	10.49	*D. monosporus*	10.52
B. linearis	12.58	*D. monosporus*	17.29	*D. monosporus*	9.77	*Dictyuchus* spp.	9.7

4.6. Seasonal variation of fungal population

Showing considerable seasonal variations at all the selected sites (Table 21) highest number of filamentous fungal colonies 1208 (38.26%) was registered in summer season followed by 972 colonies (30.79%) in spring, 660 colonies (20.91%) in autumn and the least number of 317 colonies (10.04%) in winter season. One way analysis of variance (ANOVA) showed that the observed distribution of the filamentous fungal colonies in different seasons are statistically significant ($F=7.06$, $F_{cric}=2.75$, $p<0.01$) The occurrence of zoosporic fungal species (Table 22) was also

found statistically significant by the one way analysis of variance (ANOVA) carried out between different seasons ($F=3.25$, $F_{cric}=2.75$, $p<0.05$). It showed considerable seasonal variation with a maximum number of fungal colonies 1380 (36.48%) in summer followed by 1251 colonies (33.07%) in spring, 709 colonies (18.74%) in autumn and least number of colonies 443 (11.71%) in winter. Visible from the Tables (21 & 22) the seasonal variation of occurrence of fungal colonies was also observed between different study sites of the lake.

Table 21. Variation of total colony count and percentage occurrence of filamentous fungal colonies at different sites

Sites	Spring	Summer	Autumn	Winter	Total	% Occurrence
Site1	137	38	41	17	233	7.38%
Site2	70	42	53	37	202	6.40%
Site3	33	27	32	9	101	3.20%
Site4	10	19	19	10	58	1.84%
Site5	26	13	33	7	79	2.50%
Site6	19	20	30	4	73	2.31%
Site7	66	73	34	16	189	5.99%
Site8	98	82	45	30	255	8.08%
Site9	45	63	27	25	160	5.07%
Site10	47	75	31	31	184	5.83%
Site11	36	103	23	14	176	6%
Site12	15	53	21	1	90	2.85%
Site13	86	160	66	32	344	10.90%
Site14	136	182	111	47	476	15.08%
Site15	43	89	50	24	206	6.52%
Site16	105	169	44	13	331	10.48%

Total	**972**	**1208**	**660**	**317**	**3157**	**100%**
% Occurrence	30.79%	38.26%	20.91%	10.04%	100%	-

The seasonal comparison of the total fungal load in addition to the filamentous fungal load and zoosporic fungal load shown in Fig. 4 & 5 depicts that the heavy load of fungi was observed in summer contributing a fungal load of 2588 colonies followed by spring (2223 colonies), autumn (1369 colonies) and winter (760 colonies).

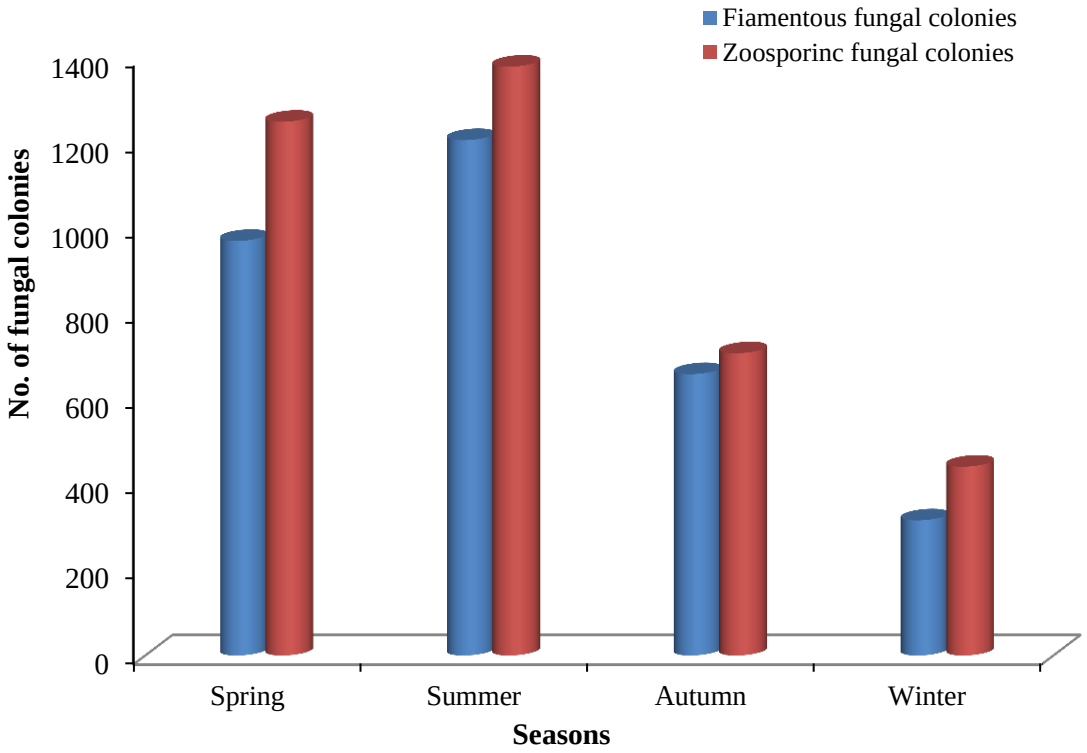


Fig. 4. Seasonal fungal load of filamentous and zoosporic fungi

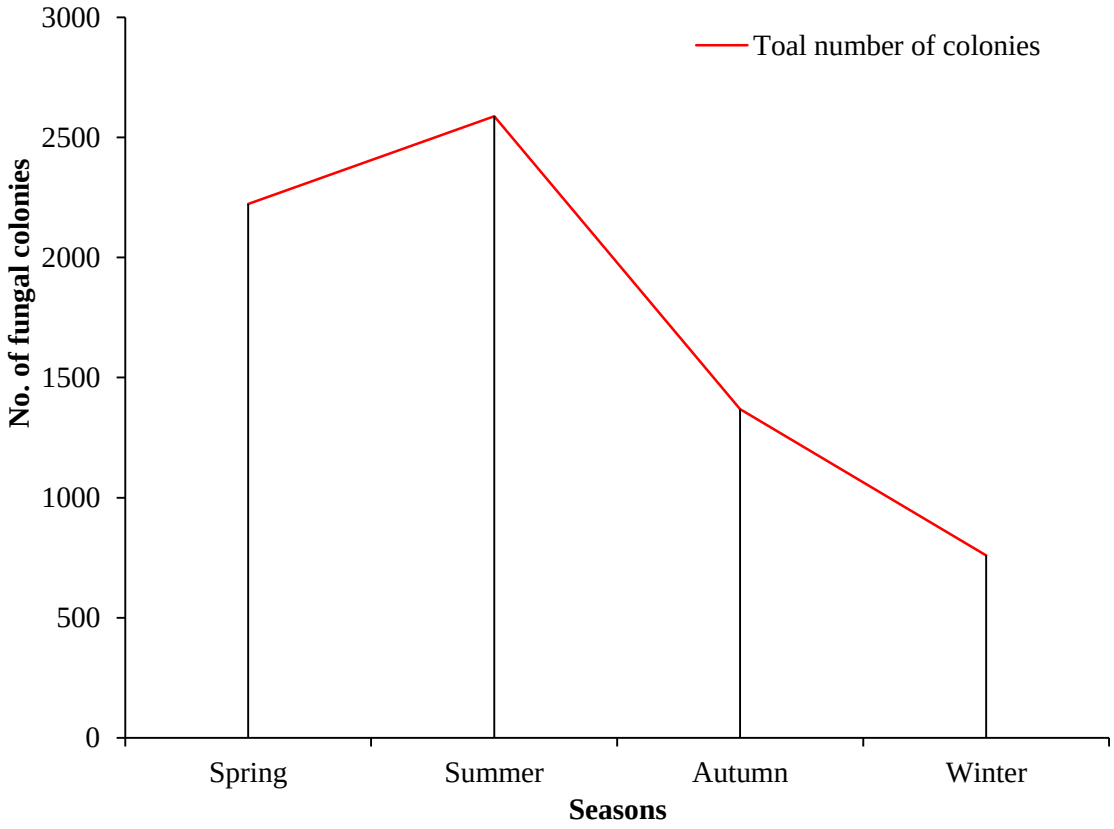


Fig. 5. Seasonal fluctuation of fungal load

Table 22. Variation of total colony count and percentage occurrence of zoosporic fungal colonies at different sites

Sites	Spring	Summer	Autumn	Winter	Total	% Occurrence
Site1	93	16	38	34	181	4.78%
Site2	20	13	57	40	130	3.44%
Site3	11	15	21	27	74	1.96%
Site4	36	12	32	14	94	2.50%
Site5	39	20	51	26	136	3.60%
Site6	52	4	33	25	114	3.01%
Site7	210	37	11	15	273	7.21%
Site8	281	84	18	21	404	10.68%
Site9	36	29	50	16	131	3.46%
Site10	26	55	81	16	178	4.70%
Site11	8	233	41	21	303	8.00%
Site12	54	44	35	7	140	3.70%
Site13	76	156	100	83	415	10.97%
Site14	148	266	91	64	569	15.04%
Site15	70	183	17	27	297	7.85%
Site16	91	213	33	7	344	9.10%
Total	**1251**	**1380**	**709**	**443**	**3783**	**100%**
% Occurrence	33.07%	36.48%	18.74	11.71%	100%	-

Although the species composition varied between different sites and season of the study, the overall highest species variety was observed in summer season during both the years (Table 23) followed by the other seasons. During spring 2010 the highest number of 40 species was recorded at site 8 while as a maximum of 36 species was recorded at site 1 in spring 2011. In summer 2010 and 2011 a highest of 41 species and 40 species respectively was recorded at site 16. A maximum of 30 species was observed at site 14 in Autumn 2010 compared to Autumn 2011 in which a maximum of only 23 species was recorded at site 1 however in winter 2010 and 2011 the highest number of 19 species at site 9 and 22 species at site 14 was recorded in the same season of two consecutive respectively.

Table 23. Season and site wise variation of species abundance

Sites	Spring		Summer		Autumn		Winter	
	2010	2011	2010	2011	2010	2011	2010	2011
Site1	37	36	18	19	21	23	15	16
Site2	22	34	14	23	22	21	16	13
Site3	5	20	15	16	15	15	10	13
Site4	11	20	6	18	14	16	11	8
Site5	16	23	11	11	15	22	13	9
Site6	13	21	6	9	15	13	9	9
Site7	33	34	25	21	9	16	11	12
Site8	40	35	25	24	15	16	10	16
Site9	9	23	17	18	13	16	19	12
Site10	10	25	24	26	21	22	16	12
Site11	11	13	34	34	6	13	13	10
Site12	10	17	26	20	13	16	4	2
Site13	25	27	26	30	22	20	14	19
Site14	28	21	39	33	30	16	9	22
Site15	22	19	31	34	17	16	9	14
Site16	30	25	41	40	11	22	10	4
All figures indicate number of species isolated in different season and sites								

The occurrence values, calculated on the basis of presence and absence of a particular species at a particular site out of eight collections of two consecutive study years presented in Table 24 shows that the occurrence values of different species varied between a maximum of eight (8) indicating its occurrence in all the eight collections to a minimum of zero (0) indicating its absence in all the collections. The occurrence value of *A. strictum*, *Candida parapsilosis*, *Penicillium olivicolor*, *Achyla apiculata*, *Achyla flagellate* and *Brevilegnia linearis* ranged between 0 and 4, *Cryptococcus neoformans*, *Penicillium commune*, *Penicillium* spp. III, *Allomyces anomalus*, *Allomyces* spp., *Pythium* spp. *Pythium proliferum*, *Pythium elongatum* between 0 and 5, *Aspergillus fumigatus*, *Aspergillus japonicas*, *Aspergillus versicolor*, *Candida albicans*, *F. oxysporum*, *Mucor* spp., *Penicillium dimorphosporum*, *Penicillium lilacinum*, *Penicillium* spp. I., *Penicillium* spp. II., *Allomyces moniliformis*, *Achyla klebsiana*, *Brevilegnia diclina*, *Brevilegnia subclavata*, *Aphanomyces laevis* and *Aphanomyces cladogamous* between 0 and 6, *A. oryzae*, *Aspergillus terreus*, *Candida glabrata*, *Candida krusei*, *Penicillium caseicolum*, *Penicillium* spp. IV, *R. stolonifer* and *Aphanomyces* spp. between 0 and 7, *Aspergillus flavus*, *Aspergillus wentii* and *Allomyces arbuscula* between 0 and 8, *Penicillium funiculosum* and *Achyla cornuta* between 2 and 7, *Dictyuchus monosporus* between 2 and 5, *Brevilegnia indica* between 1 and 4, *Achyla conspicua* and *Dictyuchus* spp. between 1 and 7, *Aspergillus niger* between 1 and 8, *Penicillium chrysogenum* between 3 and 8, *Pythium debaryanum* between 1 and 6, *Saprolegnia parasitica* between 2 and 7.

4.7. Spatial variation of species

Spatial variation of the fungal species is visible from the data (Fig. 6) as the highest number of 46 species were isolated at site 8 followed by 45 species at site 1, 43 species each at site 7, 10, 15 and 16; 42 species at site 5; 41 species at site 14; 40 species each at site 9 and 11; 39 species at site 2, 6 and 13; 37 species at site 4; 36 species at site 12 and the least number of 32 species at site 3.

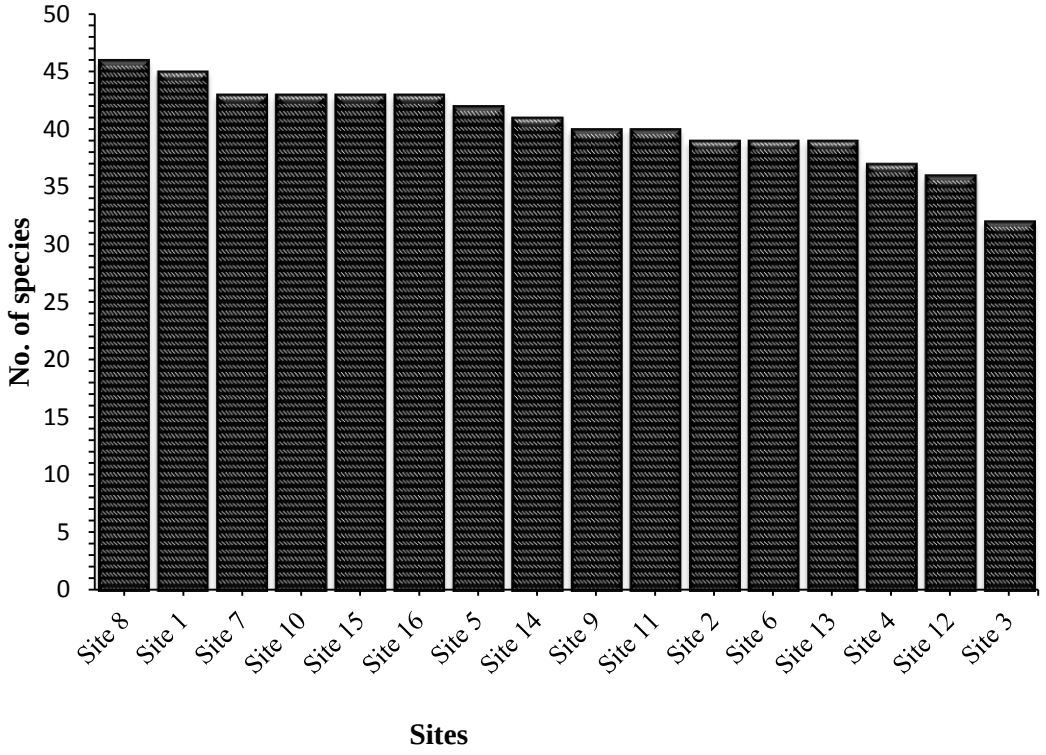


Fig. 6. Spatial variation of species

The site wise relative abundance and species contribution of different genera shown in Table 25 indicates that at site 1 maximum number of nine (9) species with a relative abundance of 20% was contributed by genus *Penicillium* followed by eight (8) species (17.78%) by genus *Aspergillus*, four (4) species (8.89%) each by genera *Allomyces*, *Achyla*, *Brevilegnia* and *Pythium*, three (3) species (6.66%) by genus *Candida*, two (2) species (4.45%) each by *Dictyuchus* and *Aphanomyces*, one (1) species (2.22%) each by *Mucor*, *Fusarium*, *Rhizopus*, *Cryptococcus* and *Saprolegnia*. However, genus *Acremonium* contributed no species at this site during the study. At site 2 out of a total of 39 species, maximum number of ten (10) species with 25.64% relative abundance was contributed by genus *Penicillium* followed by seven (7) species (17.64%) by genus *Aspergillus*, four (4) species (10.26%) by genus

Brevilegnia, three (3) species (7.7%) each by genera *Candida* and *Achyla*, two (2) species (5.13%) each by *Allomyces*, *Dictyuchus* and *Pythium*, one (1) species (2.56%) each by *Rhizopus*, *Fusarium*, *Acremonium*, *Cryptococcus*, *Aphanomyces*, *Saprolegnia* and with no contribution from genus *Mucor*. Out of 32 species recorded at site 3, maximum contribution of seven (7) species with a relative abundance of 21.88% was of genus *Aspergillus* followed by genus *Penicillium* six (6) species (18.75%), *Candida*, *Achyla* and *Brevilegnia* three (3) species (9.38%) each, *Allomyces*, *Dictyuchus* and *Pythium* two (2) species (6.25%) each and *Mucor*, *Rhizopus*, *Fusarium* and *Saprolegnia* one (1) species (3.12%) each. Here no contribution was given by the genera *Acremonium*, *Cryptococcus* and Aphanomyces. At site 4 out of 37 species recorded maximum contribution of six (6) speccies was from genus *Aspergillus* with 16.22% relative abundance followed by *Penicillium* and *Achyla* five (5) species (13.52%) each, *Allomyces* four (4) species (10.81%), *Candida* and *Pythium* three (3) species (8.11%) each, *Brevilegnia*, *Dictyuchus* and *Aphanomyces* two (2) species (5.4%) each; *Mucor*, *Fusarium*, *Rhizopus*, *Acremonium* and *Saprolegnia* with one (1) species (2.7%) each and *Cryptococcus* with no species contribution. At site 5 out of 42 species recorded maximum contribution was from genus *Aspergillus* with eight (8) species (19.04%) followed by *Penicillium* seven (7) species (16.66%), *Achyla* five (5) species (11.9%), *Brevilegnia* four (4) species (9.52%), *Candida*, *Allomyces*, *Aphanomyces* and *Pythium* three (3) species (7.14%) each, *Dictyuchus* with two (2) species (4.76%), *Rhizopus*, *Fusarium*, *Cryptococcus* and *Saprolegnia* with one (1) species (2.39%) each, *Mucor* and *Acremonium* with no contribution. At site 6 out of 39 species recorded maximum contribution was from genus *Penicillium* and *Aspergillus* with seven (7) species (17.94%) followed by *Achyla* five (5) species (12.82%), *Brevilegnia* four (4) species (10.25%), *Allomyces*, *Aphanomyces* and *Pythium* three (3) species (7.7%) each, *Candida* and *Dictyuchus* with two (2) species (5.13%) each, *Rhizopus*, *Fusarium*, and *Saprolegnia* with one (1) species (2.57%) each, *Mucor*, *Acremonium* and *Cryptococcus* with no contribution. At site 7 out of 43 species recorded maximum contribution was from genus *Penicillium* with eight (8) species (18.6%) followed and *Aspergillus* seven (7) species (16.28%), *Achyla* five (5) species (11.63%), *Allomyces*, *Brevilegnia* and *Pythium* four (4) species (9.3%) each, *Aphanomyces* three (3) species (6.98%),

Candida and *Dictyuchus* with two (2) species (4.66%) each, *Rhizopus*, *Fusarium*, *Acremonium* and *Saprolegnia* with one (1) species (2.32%) each, *Mucor* and *Cryptococcus* with no contribution. At site 8 out of 46 species recorded maximum contribution was from genus *Penicillium* and *Aspergillus* with eight (8) species (17.4%) each followed by *Achyla* five (5) species (10.87%), *Allomyces*, *Brevilegnia* and *Pythium* four (4) species (8.7%) each, *Candida* and *Aphanomyces* three (3) species 6.52%, *Dictyuchus* with two (2) species (4.34%), *Rhizopus*, *Fusarium*, *Acremonium*, *Cryptococcus* and *Saprolegnia* with one (1) species (2.17%) each and *Mucor* with no contribution. At site 9 out of 41 species recorded maximum contribution was from genus *Penicillium* and *Aspergillus* with eight (8) species (19.51%) each followed by *Achyla* and *Brevilegnia* four (4) species (9.75%) each, *Candida* and *Pythium* three (3) species (7.32%) each, *Allomyces*, *Dictyuchus* and *Aphanomyces* two (2) species (4.88%) each, *Mucor*, *Fusarium*, *Acremonium*, *Cryptococcus* and *Saprolegnia* with one (1) species (2.44%) each, *Rhizopus* with no contribution. At site 10 out of 43 species recorded maximum contribution was from genus *Penicillium* with nine (9) species (20.93%) followed by *Aspergillus* eight (8) species (18.6%), *Achyla*, *Brevilegnia* and *Pythium* four (4) species (9.3%) each, *Candida* three (3) species (6.98%), *Allomyces*, *Dictyuchus* and *Aphanomyces* two (2) species (4.66%) each, *Mucor*, *Rhizopus*, *Fusarium*, *Cryptococcus* and *Saprolegnia* with one (1) species (2.32%) each, *Acremonium* with no contribution. At site 11 out of 40 species recorded maximum contribution was from genus *Penicillium* with eight (8) species (20%) followed by *Aspergillus* and *Achyla* five (5) species (12.5%) each, *Allomyces*, *Brevilegnia* and *Pythium* four (4) species (10%) each, *Aphanomyces* three (3) species (2.5%), *Dictyuchus* two (2) species (5%), *Mucor*, *Rhizopus*, *Fusarium*, *Candida*, and *Saprolegnia* with one (1) species (2.5%) each, *Acremonium* and *Cryptococcus* with no contribution. At site 12 out of 36 species recorded maximum contribution was from genus *Penicillium* with six (6) species (16.67%) followed by *Aspergillus* and *Achyla* five (5) species (13.89%) each, *Allomyces* and *Brevilegnia* four (4) species (11.11%) each, *Aphanomyces* and *Pythium* three (3) species (8.33%) each, *Dictyuchus* two (2) species (5.55%), *Mucor*, *Rhizopus*, *Candida* and *Saprolegnia* with one (1) species (2.78%) each, *Fusarium*, *Acremonium* and *Cryptococcus* with no contribution. At site 13 out of 39 species recorded maximum contribution was from genus *Penicillium* with ten (10) species (25.64%) followed by

Aspergillus six (6) species (15.4%), *Brevilegnia* and *Pythium* four (4) species (10.26%) each, *Allomyces* and *Achyla* three (3) species (7.69%) each, *Candida* and *Dictyuchus* two (2) species (5.13%) each, *Mucor*, *Rhizopus*, *Acremonium*, *Aphanomyces* and *Saprolegnia* with one (1) species (2.56%) each, *Fusarium* and *Cryptococcus* with no contribution. At site 14 out of 41 species recorded maximum contribution was from genus *Penicillium* with ten (10) species (24.4%) followed by *Aspergillus* six (6) species (14.64%), *Allomyces*, *Achyla*, *Brevilegnia* and *Pythium* four (4) species (9.75%) each, *Candida* and *Dictyuchus* two (2) species (4.88%) each, *Mucor*, *Rhizopus*, *Acremonium*, *Aphanomyces* and *Saprolegnia* with one (1) species (2.44%) each, *Fusarium* and *Cryptococcus* with no contribution. At site 15 out of 43 species recorded maximum contribution was from genus *Penicillium* with nine (9) species (20.93%) followed by *Aspergillus* seven (7) species (16.27%), *Achyla* with five (5) species (11.63%), *Allomyces*, *Brevilegnia* and *Pythium* four (4) species (9.3%) each, *Candida*, *Dictyuchus* and *Aphanomyces* two (2) species (4.65%) each, *Mucor*, *Rhizopus*, *Acremonium*, and *Saprolegnia* with one (1) species (2.33%) each, *Fusarium* and *Cryptococcus* with no contribution. At site 16 the different genera showed the same trend in species distribution and relative abundance as in site 15.

Distribution of various fungal species isolated from different sampling stations of the lake shown in Table 26 reveals that some of the fungal species showed a broad spectrum distribution with their presence in all the sampling stations in all the seasons while as some of the species showed a restricted distribution with their presence in only few sampling stations and seasons. This distribution pattern characterized the sampling stations along with the influence of seasonal variation on the species diversity. From genus *Penicillium*, the species *P. caseicolum* was found most widely distributed with its presence in all the sampling stations in spring and summer seasons, besides its presence in most of the sampling stations in the autumn and winter seasons. As visible from the data the rest of the species of this genus except *P. olivicolor* were also found well distributed in this aquatic ecosystem displaying a great seasonal variation also. This was the only species of this genus which was found to be absent in winter. Furthermore it was isolated only from four sampling stations viz. Hazratbal open (S1), Hazratbal Littoral (S2), Nigeen Open (S3) and Dal Lock Gate II (S10) in rest three seasons. Like the different species of genus *Penicillium*, various species of genus *Aspergillus* also showed considerable spatial and temporal variation in their occurrence.

From among this genus *A. versicolor* was the only species which was isolated from all the sampling stations in spring along with the rest three seasons of the year. *A. terreus* was found to be comparatively less spatially distributed in all the seasons. *Mucor* spp. was found to be present only in two sampling stations in winter in contrast to its station wise distribution in rest of the seasons and almost similar distribution pattern was shown by *R. stolonifer* and *F. oxysporum* The *Acremonum* spp. was absent in all the sampling stations in spring and was found present only in three stations in autumn and winter. Its wide distribution was restricted to the summer season only. The species of genus *Candida* were found to be well distributed across the lake except *C. parapsilosis* which was isolated only from three sampling stations viz. Hazratbal open (S1), Nigeen Open (S3) and Nigeen littoral (S4) in all the seasons. Like this species of *Candida*, *Cryptococcus neoformans* also showed a restricted distribution in terms of its isolation during the study. In case of the zoosporic group of fungi all the species belonging to this group showed a broad spectrum spatial and seasons distribution as they were found present in almost all sampling stations in all the seasons of the study. However, the different species of genus *Aphanomyces* like *A. laevis*, *A. cladogamous* and *Aphanomyces* spp. showed a little restricted distribution pattern.

4.8. Comparison of different microhabitats

The Bray Curtis Cluster Analysis diagram of the study site (Fig. 7) developed on the basis of presence and absence of a species at the respective sites shows four groups of the sites with group 1 including three sites, group 2 including six sites, group three including four sites and group four including three sites. Group one including site 2, 3 and 4 is quite different from the rest three groups in the respect that they are more dissimilar to the other sites in terms of the species composition. However some of the sites like site 15 & 16, site 13 & 14, site 11 & 12, site 7 & 8, site 9 & 10 show similarity ranging from 92% to 100%, with the least similarity of 81.69% between site 1 & 3 and maximum similarity of 100% between site 15 & 16. The sites which were very closely located to each other showed higher similarity compared to those which were distantly located.

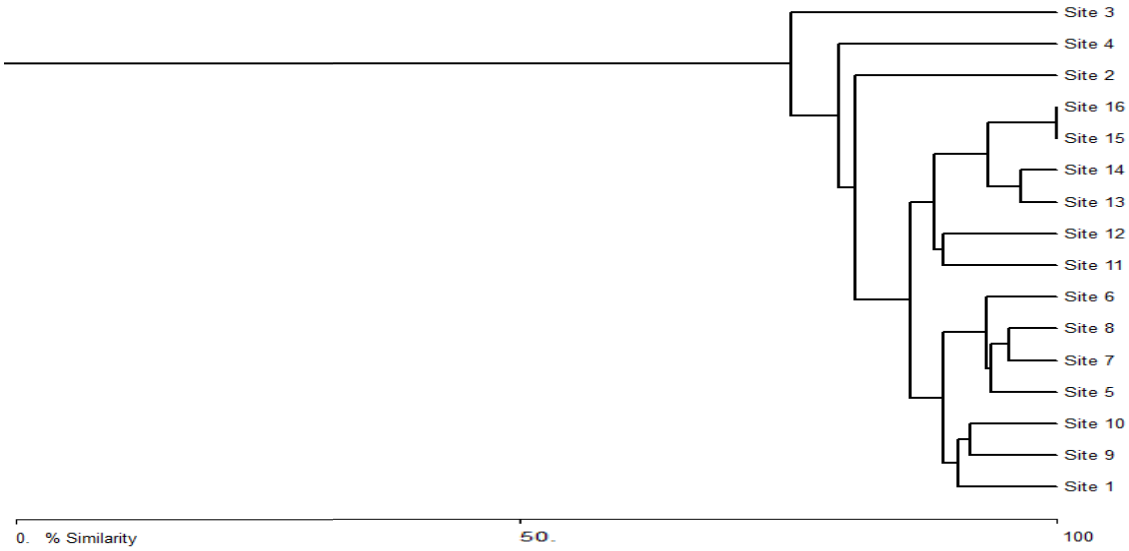


Fig. 7. Bray-Curtis Cluster Similarity diagram of different microhabitats

The similarity indices given in Table 27 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. However the similarity of sites lying within the predefined categories was more than between the sites of the categories i.e. two sites selected in an outlet were more similar to each other than when compared to a site from inlets or the lake basins; with highest similarity between site 15 and site 16 and lowest between site 1 and site 3.

Table 27. Similarity indices of different study sites

Study site groups		Similarity index	
I	**II**	**(Jaccard)**	**Bray Curtis**
15	16	100	100
13	14	95.12	97.5
7	8	93.47	96.63
5	7	91.3	95.45
13	15	90.91	95.23
5	6	90.69	95.12
9	10	88.63	93.97
1	9	87.23	93.18
1	5	85.41	92.13
11	12	85.36	92.1
11	13	84.44	91.56
1	11	81.63	89.88
1	2	75.55	86.07
1	4	73.91	85
1	3	69.04	81.69

The occurrence of fungal community in the lake consisting of diverse life forms from filamentous fungi to zoosporic fungi and some yeasts corresponded to the different microhabitats across the lake with some sites characterized as open water sites some characterized as littoral sites and some others as inlet sites or outlet sites. The overall analysis of the data showed that the occurrence of these fungal species displayed a remarkable spatial variation across the lake with *P. chrysogenum, S. parasitica*, *Dictyuchus* spp., *A. cornuta* and *P. funiculosum* as the five most dominant and prominent species. Species like *P. chrysogenum, S. parasitica, A. cornuta, A. conspicua*, *P. funiculosum*, *Dictyuchus* spp., *A. terreus* and *B. diclina* are a few dominant species occurring at the open water sites however *P. chrysogenum, A. conspicua*, *P. funiculosum, Dictyuchus* spp., *A. cornuta, A. terreus*, *S. parasitica* and *C. glabrata* are the most dominant representative of the littoral sites. In case of the outlet sites the most prominent species included *P. chrysogenum, P. proliferum, P. debaryanum, S. parasitica*, *B. diclina*, *A. flavus, A. cornuta* and *Dictyuchus* spp. while as for the inlet sites they included *A. moniliformis, S. parasitica, P. chrysogenum, Dictyuchus* spp., *A. niger*, *A. cornuta*, *A. versicolor* and *Aphanomyces* spp. Although the record for the five most dominant representatives of fungi in different microhabitats of the lake (Table 28) showed that different species were dominating different sites, *P. chrysogenum* dominated six sites, *A. conspicua* dominaed three sites, *S. parasitica* dominated two sites, *A. terreus* dominated only one site, *A. cornuta* dominated two sites and *A. moniliformis* dominated two sites.

The sites were classified into four different categories with four (4) open water sites designated as category I, four (4) littoral zone sites as category II, four (4) outlet sites as category III, four (4) open inlet sites as category IV and the most common species with their occurrence in all the sites within the group from among both the fungal groups were recorded as depicted in Table 29 showing that among the filamentous group twelve (12) species viz. *Aspergillus fumigatus, A. niger, A. terreus, A. wentii*, *Penicillium chrysogenum, P. dimorphosporum, P. funiculosum, Penicillium* spp. IV, *R. stolonifer*, *F. oxysporum*, *Candida krusei*, *C. glabrata* were most common in category I, ten (10) species viz. *Aspergillus terreus*, *A. versicolor*, *A. wentii*, *Penicillium chrysogenum*, *P. funiculosum*, *Penicillium* spp. III, *R. stolonifer*, *F. oxysporum, Candida krusei*, *C. glabrata* were most common in category II, seventeen (17) species viz. *Aspergillus flavus, A. japonicas, A. niger, A. oryzae, A. versicolor, A. wentii,*

Penicillium caseicolum, *P. chrysogenum*, *P. dimorphosporum*, *P. funiculosum*, *Penicillium* spp. I, *Penicillium* spp. II, *Penicillium* spp. III, *Penicillium* spp. IV, *Mucor* spp., *Candida albicans*, *C. krusei* were most common in category III and thirteen (13) species viz. *Aspergillus flavus A. fumigatus*, *A. japonicas*, *A. niger*, *A. versicolor*, *Penicillium caseicolum P. chrysogenum*, *P. funiculosum*, *P. lilacinum*, *Penicillium* spp. I, *R. stolonifer*, *Mucor* spp., and *Candida albicans* were most common in category IV. From among the zoosporic group of fungi twelve (12) species viz. *Allomyces moniliformis*, *A. arbuscula*, *Achyla klebsiana*, *A. cornuta*, *A. conspicua*, *Brevilegnia indica*, *B. linearis*, *Dictyuchus monosporus*, *Dictyuchus* spp., *Aphanomyces laevis*, *Pythium debaryanum* and *Saprolegnia parasitica* were most common in category I, eleven (11) species viz. *Allomyces arbuscula*, *Achyla klebsiana*, *A. cornuta*, *A. conspicua*, *Brevilegnia indica*, *B. diclina*, *B. subclavata*, *Dictyuchus monosporus*, *Dictyuchus* spp., *Pythium debaryanum* and *Saprolegnia parasitica* were most common in category II, thirteen (13) species viz. *Allomyces moniliformis*, *Achyla cornuta*, *A. conspicua*, *Brevilegnia indica*, *B. linearis*, *B. diclina*, *B. subclavata*, *Dictyuchus monosporus*, *Dictyuchus* spp., *Pythium proliferum*, *P. debaryanum*, *Pythium* spp. and *Saprolegnia parasitica* were most common in category III, twenty one (21) species viz. *Allomyces moniliformis*, *A. anomalus*, *A. arbuscula*, *Allomyces* spp., *Achyla klebsiana*, *A. apiculata*, *A. flagellate*, *A. cornuta*, *A. conspicua*, *Brevilegnia indica*, *B. linearis*, *B. diclina*, *B. subclavata*, *Dictyuchus monosporus*, *Dictyuchus* spp., *Aphanomyces cladogamous*, *Aphanomyces* spp., *Pythium elongatum*, *P. debaryanum*, *Pythium* spp. *and Saprolegnia parasitica* were most common in category IV.

As a series of study sites from the littoral areas, open water areas, outlets and inlets were selected for the present study, a comparison of the lake basin in terms of the fungal colonies of both the groups (filamentous and zoosporic) and the total fungal load shown in Fig. 8 indicated the heavy fungal load in Nishat basin followed by Hazratbal, Gagribal and Nigeen basins. The comparative analysis of outlet sites in general and the extreme ends of the outlets shown in Fig. 9 & 10 depicts the heavy fungal load in Pokhribal outlet compared to the Dal Lock gate outlet. The comparison drawn within the outlet sites showed that the outer extreme of the outlet (lying much away from the main lake basin) carry much higher load of fungi as compared to the

inner extreme (connected with the main lake basin). Similarly a comparison drawn in case of the inlet sites shown in Fig. 11 depicts that the heavy load of fungi in Tailbal nallah compared to the Boathall nallah. However, the comparison within the inlet sites (Fig. 12) in contrary to the outlet sites showed heavy load of fungi in the inner extremes of the inlets compared to their outer extremes.

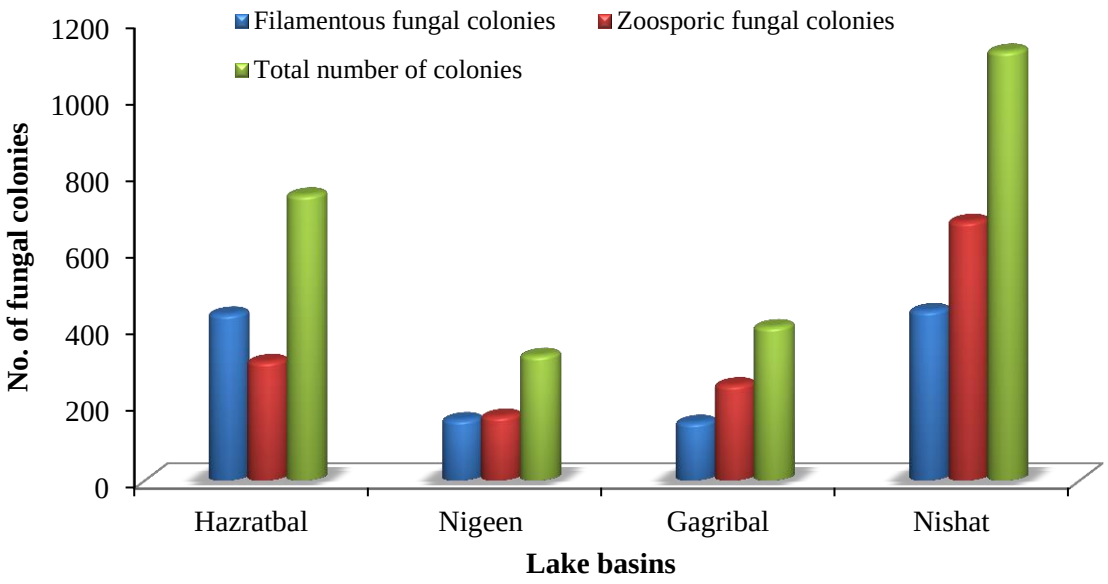


Fig. 8. Basin wise variation in fungal load

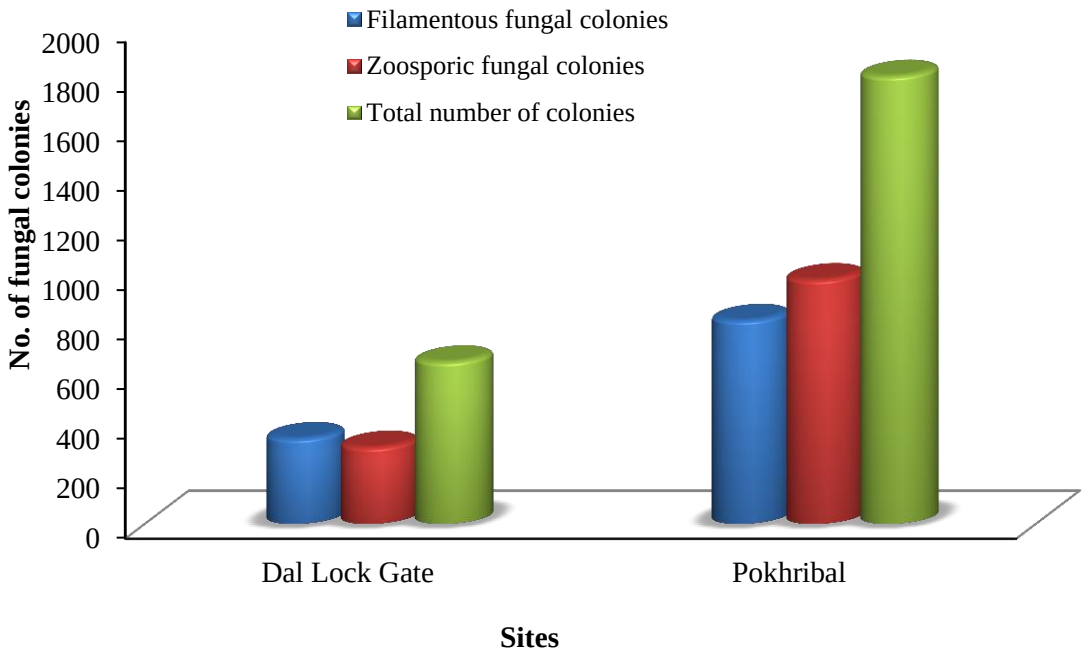


Fig. 9. Comparative fungal load of outlet sites

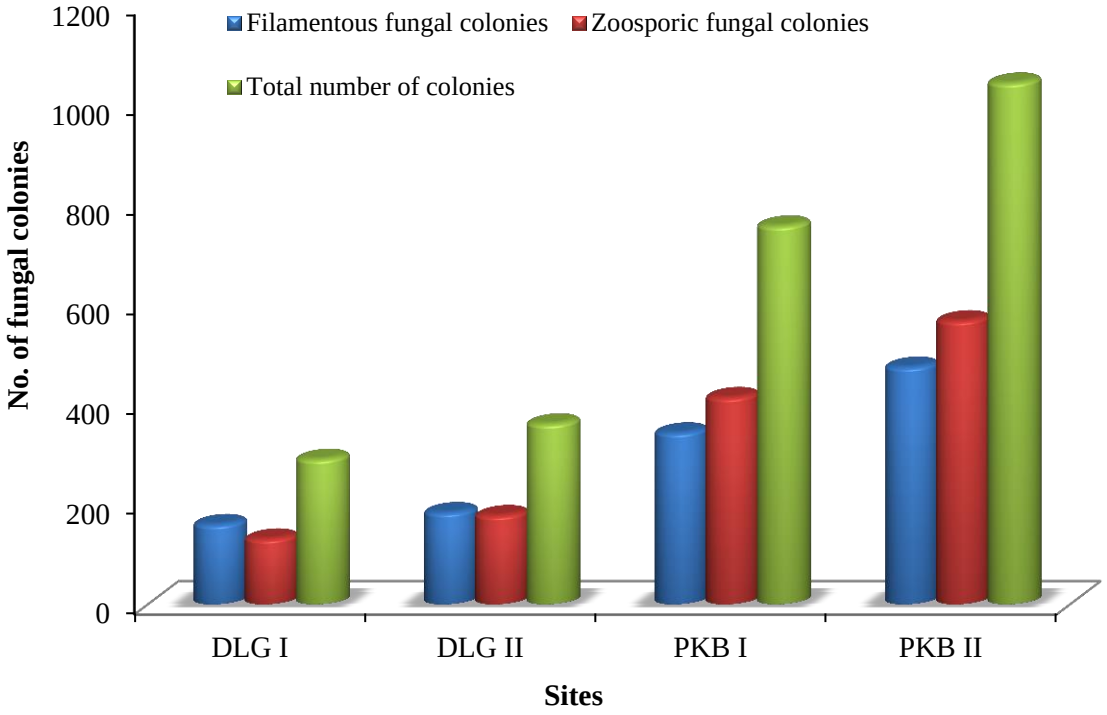


Fig. 10. Comparative fungal load within outlet sites

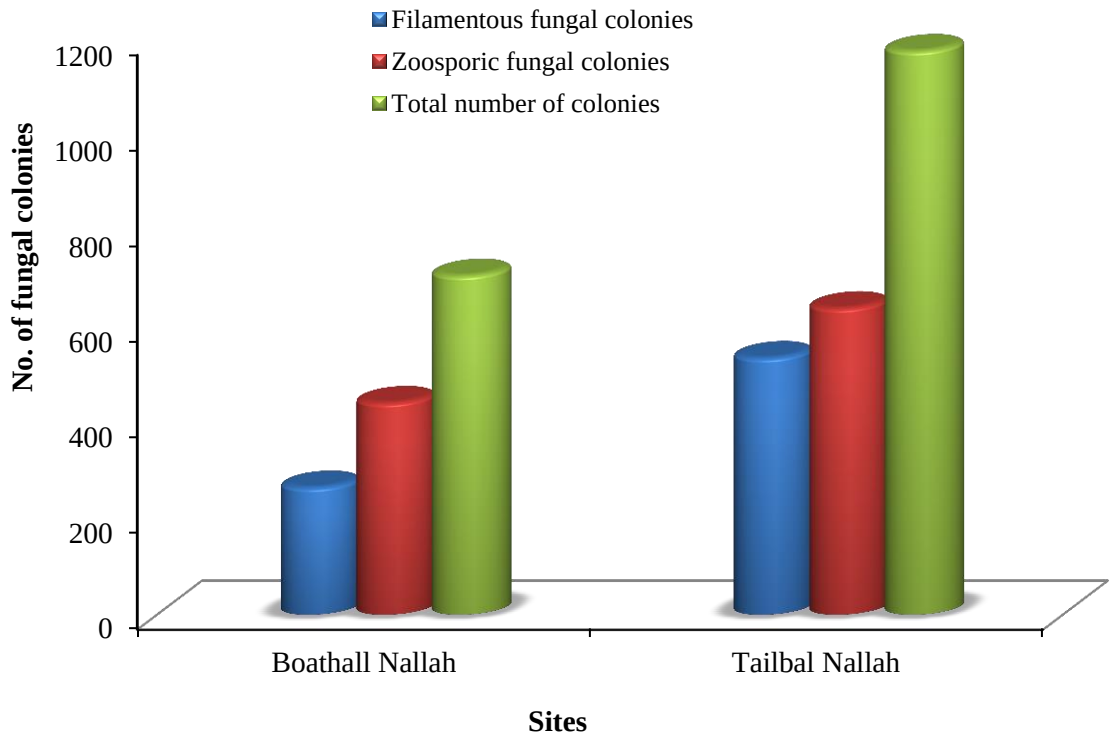


Fig. 11. Comparative fungal load of intlet sites

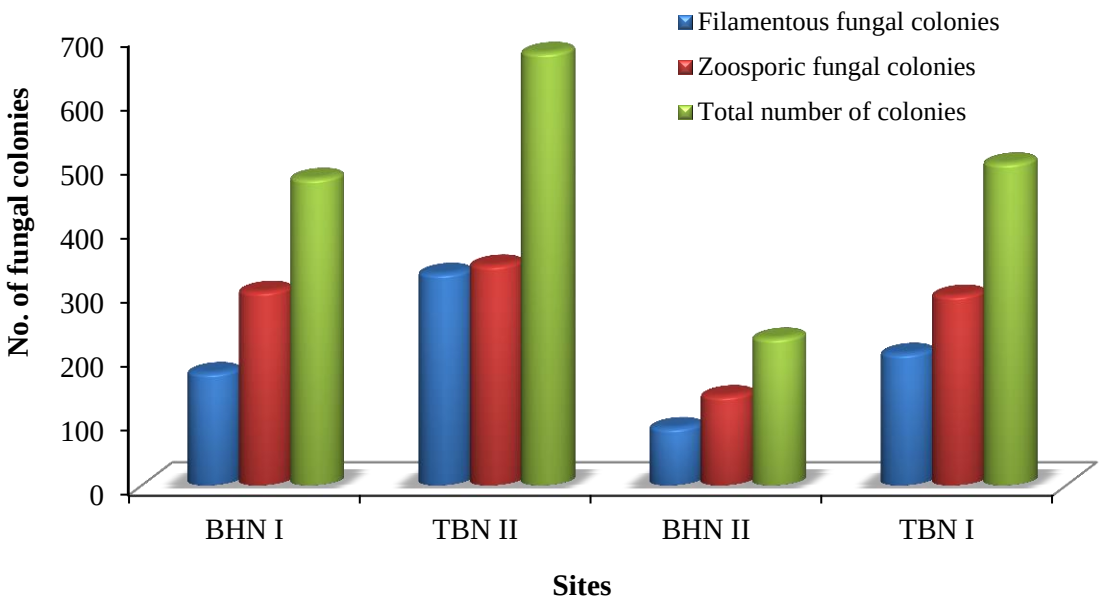


Fig. 12. Comparative fungal load within intlet sites

A comparison of four littoral sites of the four basins shown in Fig. 13 depicts that the site located near hotel Centaur was having a higher load of fungi followed by the Hazratbal littoral, littoral sites in Nigeen Lake and Gagribal. Similar results were displayed by the comparison of the four open water sites of the lake (Fig. 14). However in the entire, load was higher towards the littoral areas compared to the sites located in the middle of the lake basins (Fig. 15).

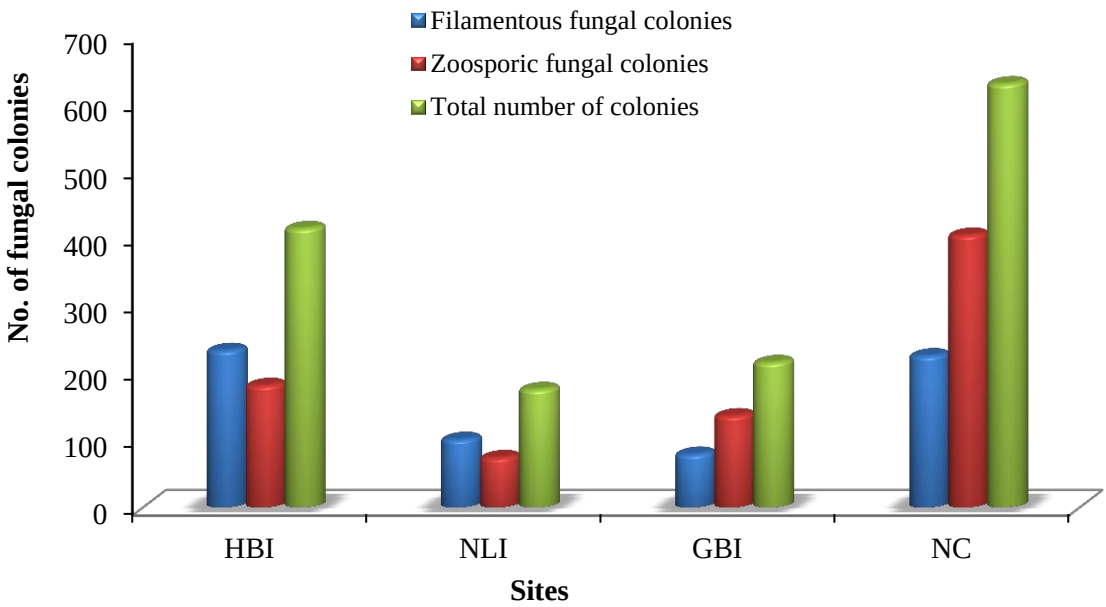


Fig. 13. Comparative fungal load within littoral sites

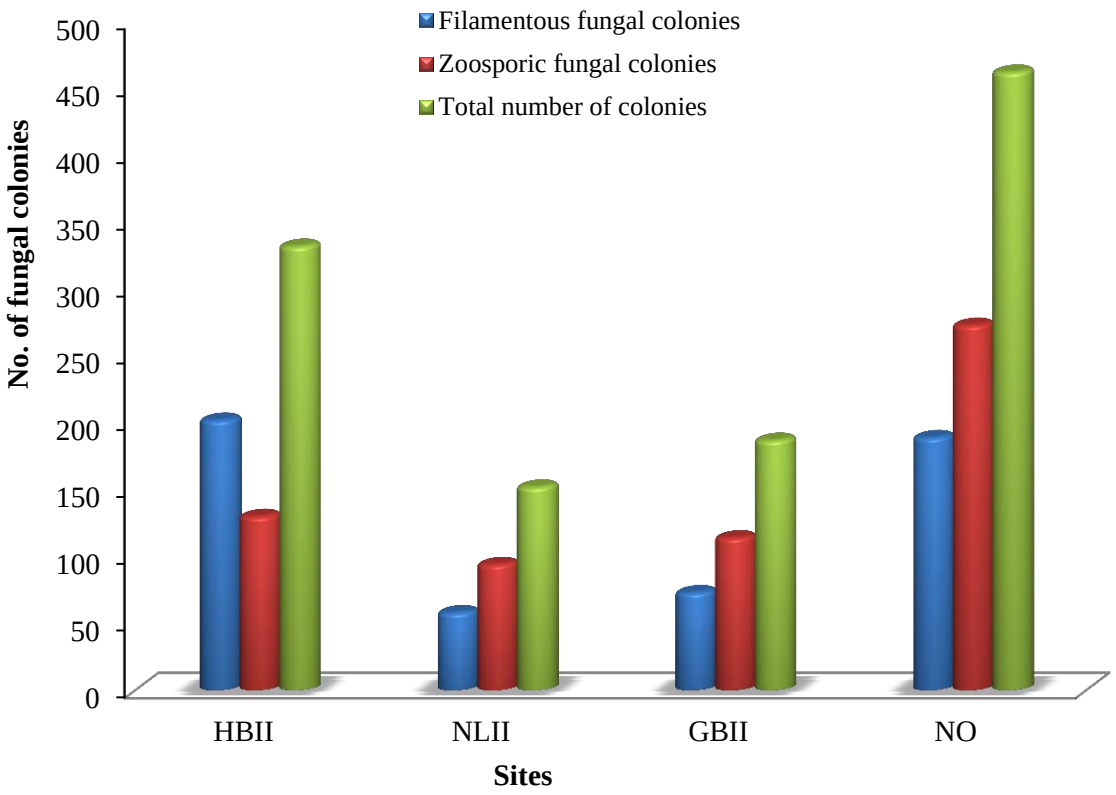


Fig. 14. Comparative fungal load within open water sites

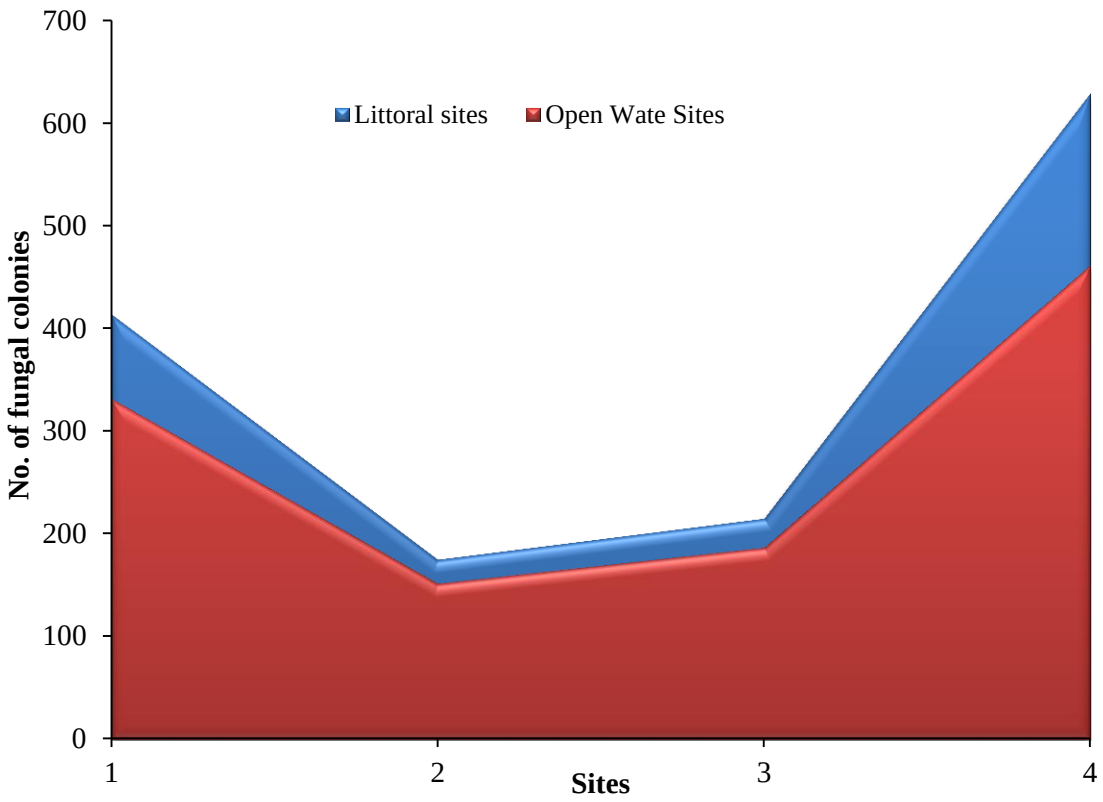


Fig. 15. Comparative fungal load between littoral and open water sites

4.9. Explanatory data analysis

The explanatory data analysis by Box and Whisker Plot model shown in Fig. 16-19 on the basis of all experimental data for both the filamentous and zoosporic group of fungi from all the sixteen microhabitats studied as an indicator of centrality, spread and similarity lined up side by side shows the main characteristics of their distribution.

4.9.1. Filamentous fungi

From the Box Plot diagram of filamentous fungi (Fig. 16) it can be seen that site 14 and 16 appear to have similar centers which exceed the rest of the sites while as sites 3, 4, 5, 6 and 12 appear to have similar centers lying towards the base of the graph. Site 14 appears to have the largest variability along with site 13 and 16 than the other thirteen sites. However, the sites 4, 5, 6 and 12 are reasonably symmetric compared to rest of the sites.

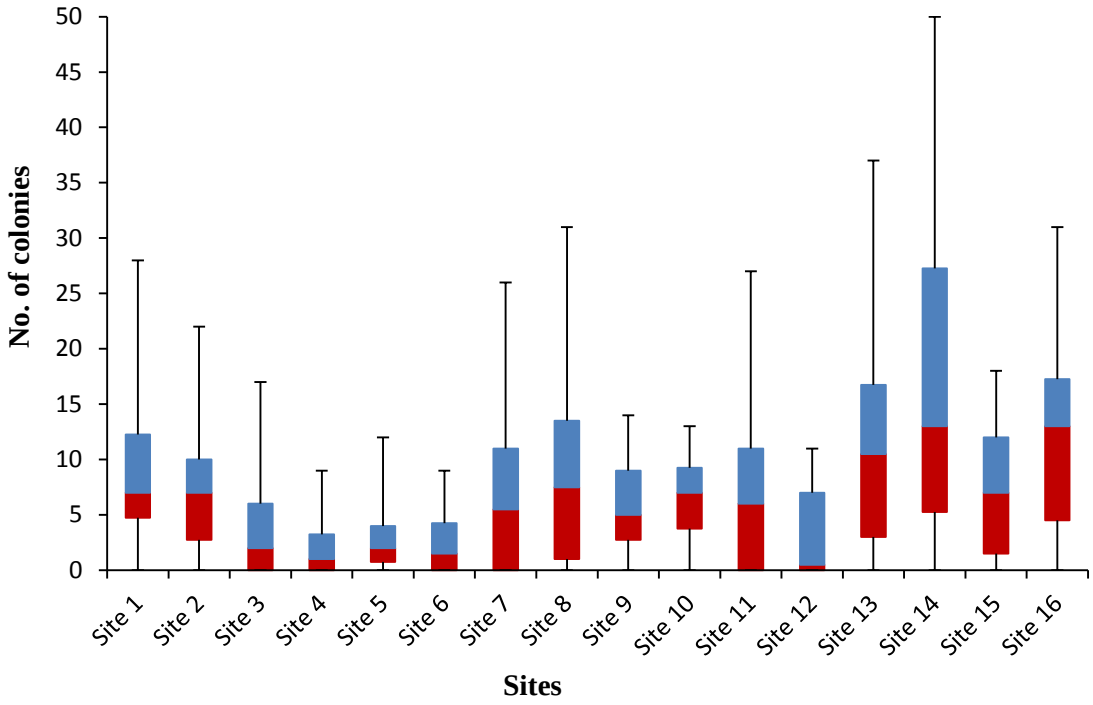


Fig. 16. Site wise box plot model of filamentous fungi

From Fig. 17 it can be seen that most of the filamentous species of fungi show a good variation with species 10 showing the largest variation followed by species 4, species 19 and species 13 however, species 15 and 26 showed the least variability. Species 3, 7, 8, 9, 18, 19, 20 and 27 appear to have same centrality which exceed or precede the rest of the species. However, the species 3, 20 and 25 are reasonably symmetric compared to rest of the species.

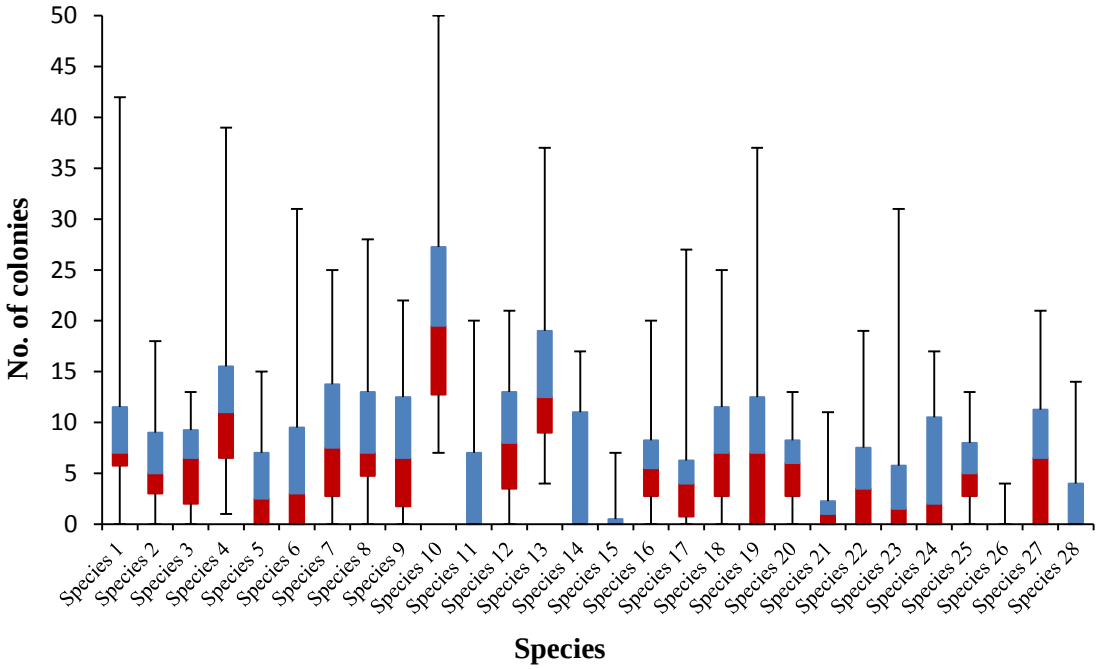


Fig. 17. Species wise box plot model for filamentous fungi (Species1 = *A. flavus*, 2 = *A. fumigatus*, 3 = *A. japonicas*, 4 = *A. niger*, 5 = *A. oryzae*, 6 = *A. terreus*, 7 = *A. versicolor*, 8 = *A. wentii*, 9 = *P. caseicolum*, 10 = *P. chrysogenum*, 11 = *P. commune*, 12 = *P. dimorphosporum*, 13 = *P. funiculosum*, 14 = *P. lilacinum*, 15 = *P. olivicolor*, 16 = *Penicillium* spp. I, 17 = *Penicillium* spp. II, 18 = *Penicillium* spp. III, 19 = *Penicillium* spp. IV 20 = *R. stolonifer*, 21 = *Acremonium strictum*, 22 = *F. oxysporum*, 23 = *Mucor* spp., 24 = *C. albicans*, 25 = *C. krusei*, 26 = *C. parapsilosis*, 27 = *C. glabrata*, and 28 =*C. neoformans*)

4.9.2. Zoosporic fungi

From the Box Plot diagram of zoosporic fungi (Fig. 18) it can be seen that site 14 appears to have the center which exceed the rest of the sites. Here also site 13 and 14 appear to have largest variability along with site 8 than the other thirteen sites and the sites 4, 5, 6 and 12 are reasonably symmetric compared to rest of the sites.

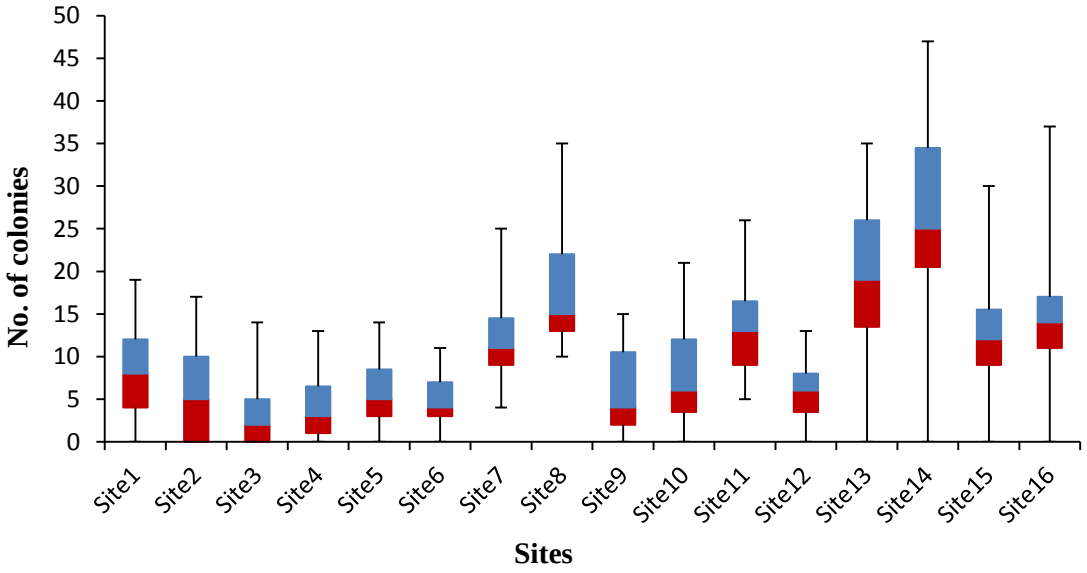


Fig. 18. Site wise Box Plot Model for zoosporic fungi

From Fig. 19 it can be concluded that most of the zoosporic species of fungi showed a good variation with species 23 showing the largest variation followed by species 1, 12, 15, 19 and species 21 however, species 14 showed the least variability. Species 6, 7 and 20 appear to have same centrality which exceed or precede the rest of the species. However, none of the two species are reasonably symmetric in terms of the distribution of their numbers.

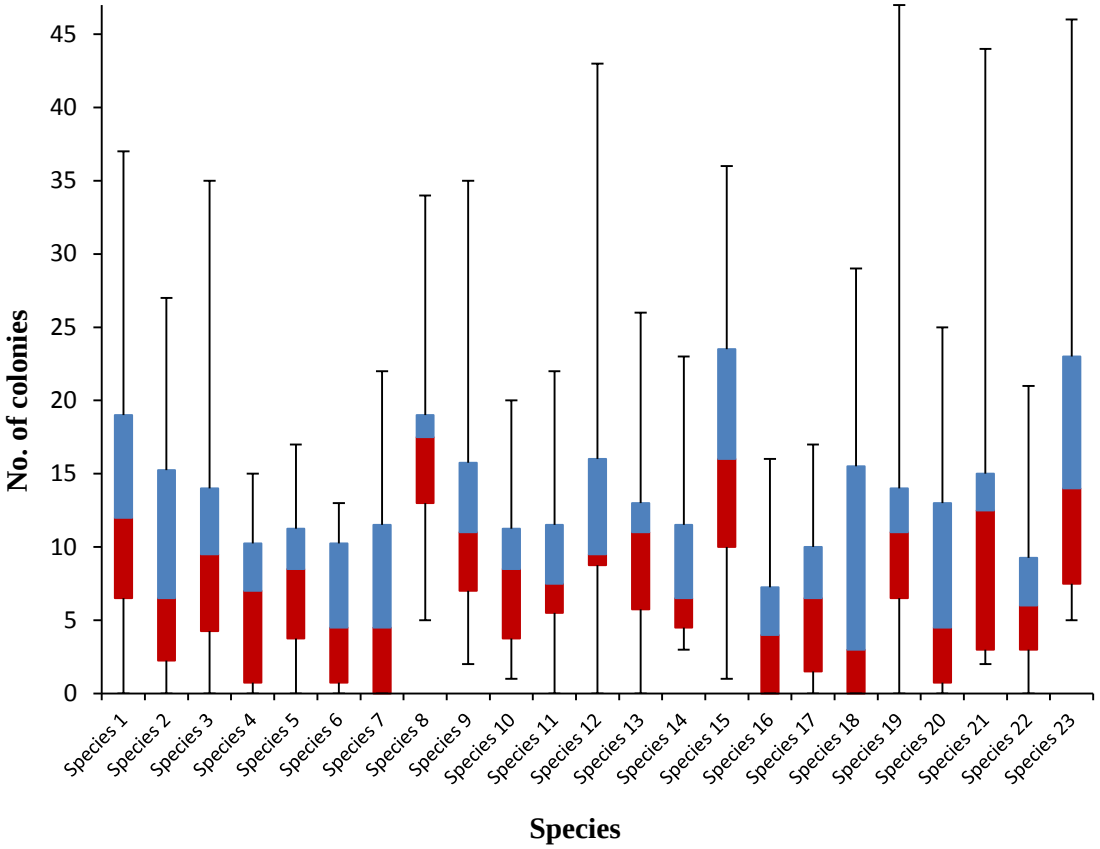


Fig. 19. Species wise Box Plot Model for zoosporic fungi (Species1 = *A. moniliformis*, 2 = *A. anomalus*, 3 = *A. arbuscula*, 4 = *Allomyces* spp., 5 = *A. klebsiana*, 6 = *A. apiculata*, 7 = *A. flagellate*, 8 = *A. cornuta*, 9 = *A. conspicua*, 10 = *B. indica*, 11 = *B. linearis*, 12 = *B. diclina*, 13 = *B. subclavata*, 14 = *D. monosporus*, 15 = *Dictyuchus* spp., 16 = *A. laevis*, 17 = *A. cladogamous*, 18 = *Aphanomyces* spp., 19 = *P. proliferum*, 20 = *P. elongatum*, 21 = *P. debaryanum*, 22 = *Pythium* spp., 23 = *S. parasitica)*

4.10. Statistical appraisal of the data

The Analysis of variance (ANOVA) carried out pair wise between the different sites for both the filamentous and zoosporic fungal species isolated from different microhabitats (Table 30) showed that 64% results were statistically significant with 54% as highly significant ($p<0.01$) and 10% as significant ($p<0.05$).

4.11. Diversity and evenness patterns

From the values of different indices computed for sixteen sites for the occurrence of different fungal species presented in Table 31 it can be seen that, Shannon-Wiener (H) index is highest (3.69) at site 16 followed closely by the species rich Site 8 (3.65), Site 15 (3.64), Site 1 and 10 (3.62) each and lowest at Site 3 (3.21). The one way Analysis of variance (ANOVA) carried out showed that the Dominance ($F=50.60$, $F_{crit}=4.17$, $p<0.01$), Diversity ($F=17.34$, $F_{crit}=4.17$, $p<0.01$) and Evenness ($F=41.25$, $F_{crit}=4.17$, $p<0.01$) varied significantly between different microhabitats of the lake. The evenness pattern calculated for the said sites was also highest (0.93) at Site 16 and lowest (0.76) at site 4.

Table 31. Diversity and evenness patterns

Sites	Dominance (D)	Shannon (H)	Evenness_e^H/S
Site 1	0.032	3.62	0.83
Site 2	0.034	3.48	0.83
Site 3	0.047	3.21	0.78
Site 4	0.041	3.34	0.76
Site 5	0.034	3.50	0.79
Site 6	0.033	3.49	0.84
Site 7	0.030	3.61	0.86
Site 8	0.028	3.65	0.83
Site 9	0.032	3.54	0.84
Site 10	0.029	3.62	0.87
Site 11	0.030	3.58	0.90
Site 12	0.033	3.47	0.89
Site 13	0.031	3.54	0.89
Site 14	0.029	3.59	0.89
Site 15	0.028	3.64	0.88
Site 16	0.026	3.69	0.93

4.12. Relation of water temperature and pH with fungal 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 fungal load shown in Figs. 20-35 of the sixteen sites respectively indicated that in the entire sites

and seasons there was a negative correlation of pH with the fungal load indicating that with increasing pH the fungal 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^{O}C in spring, 16-25.8^{O}C in summer, 12.5-19.4^{O}C in autumn and 3.2-6.4^{O}C in winter. Here the correlation analysis between water temperature and fungal load shown in Figs. 36-51 of the sixteen sites respectively showed that there was a strong positive correlation of fungal load with temperature all through the sites and seasons indicating that with increasing water temperature the fungal load got increased and the results were significant as shown in Table 32.

Table 32. Correlation of fungal load at different sites with pH and temperature of water

Sites	pH		Temperature	
	r	p value	r	p value
Site 1	-0.019	0.96	0.621^{NS}	0.1
Site 2	-0.365	0.38	0.709*	0.04
Site 3	-0.017	0.96	0.542 ^{NS}	0.16
Site 4	-0.330	0.42	0.708*	0.04
Site 5	-0.164	0.69	0.383 ^{NS}	0.35
Site 6	-0.425	0.29	0.773*	0.25
Site 7	-0.387	0.34	0.756*	0.03
Site 8	-0.157	0.71	0.717*	0.04
Site 9	-0.356	0.38	0.762*	0.02
Site 10	-0.149	0.72	0.769*	0.02
Site 11	-0.066	0.87	0.712*	0.04
Site 12	-0.623	0.09	0.842**	0.009
Site 13	-0.479	0.23	0.708*	0.04
Site 14	-0.222	0.59	0.733*	0.02
Site 15	-0.346	0.4	0.707*	0.05
Site 16	-0.372	0.36	0.877**	0.004

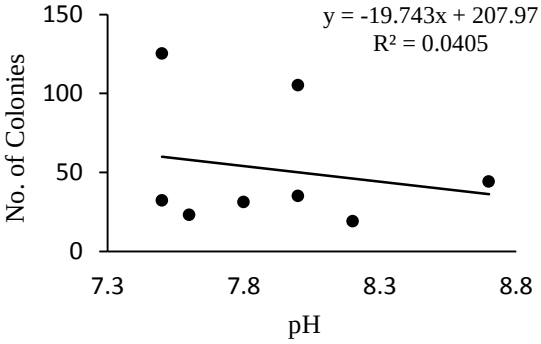


Fig. 20. Site 1

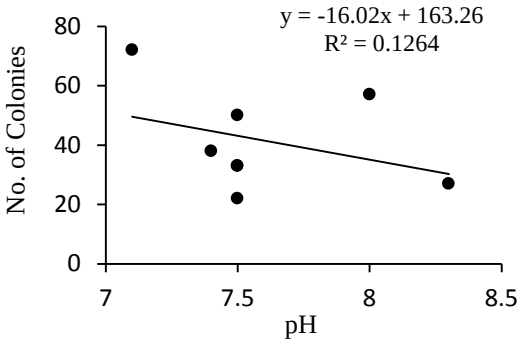


Fig. 21. Site 2

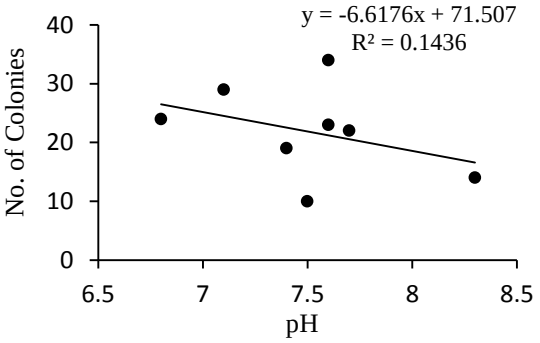


Fig. 22. Site 3

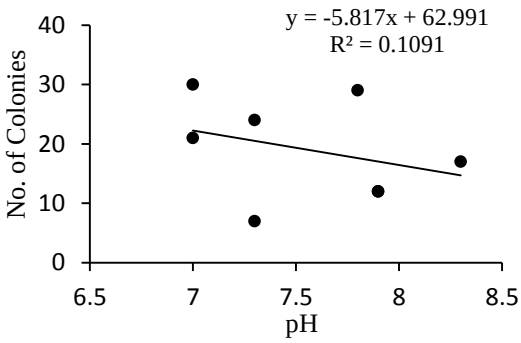


Fig. 23. Site 4

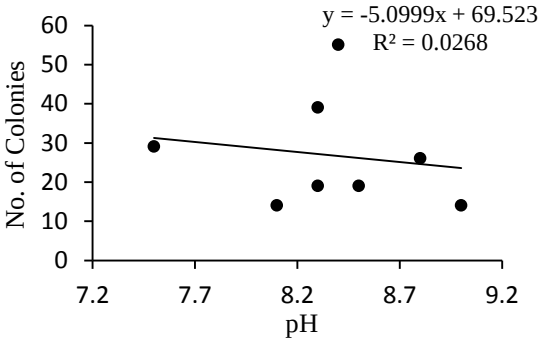


Fig. 24. Site 5

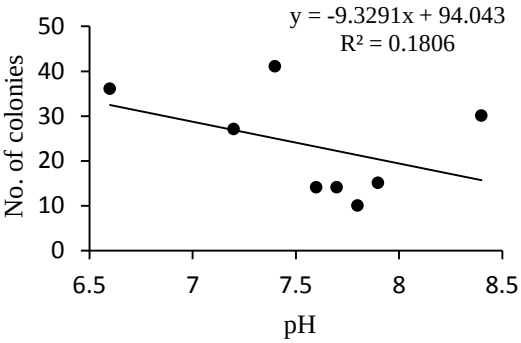


Fig. 25. Site 6

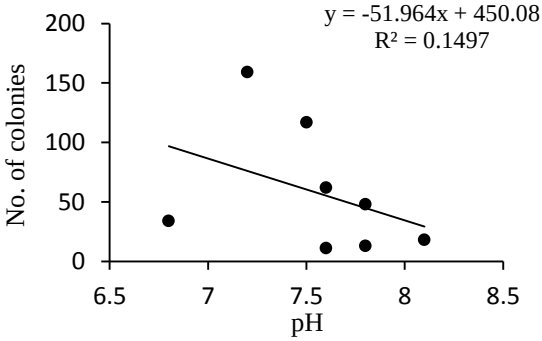


Fig. 26. Site 7

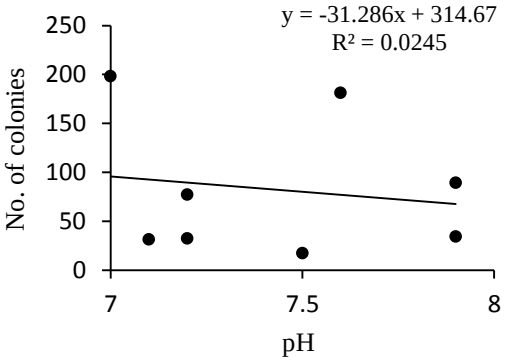


Fig. 27. Site 8

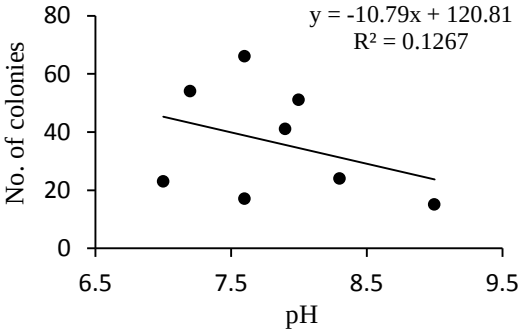


Fig. 28. Site 9

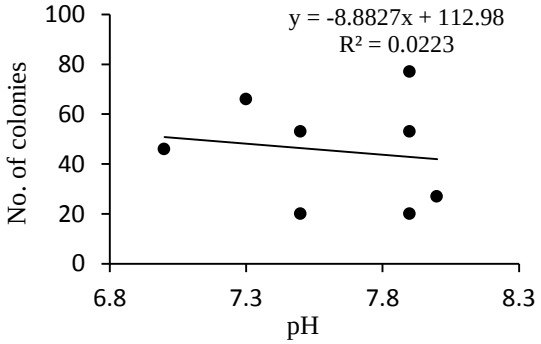


Fig. 29. Site 10

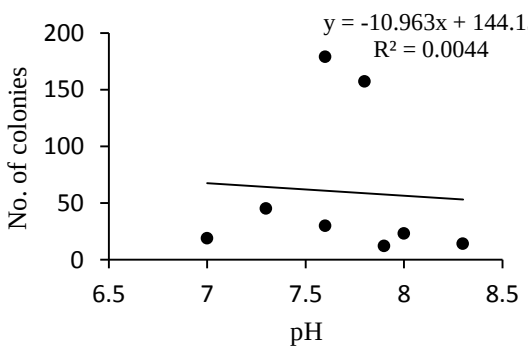


Fig. 30. Site 11

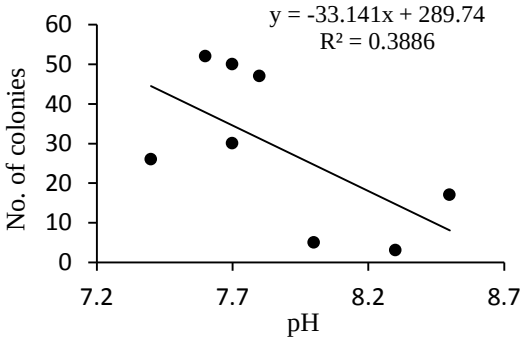


Fig. 31. Site 12

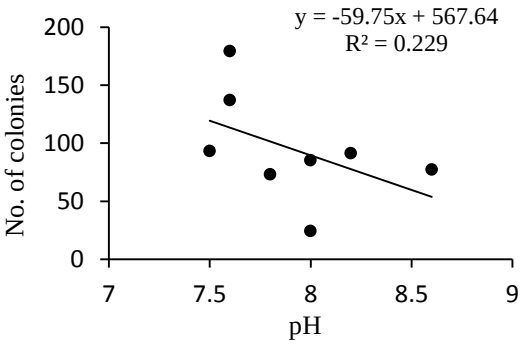


Fig. 32. Site 13

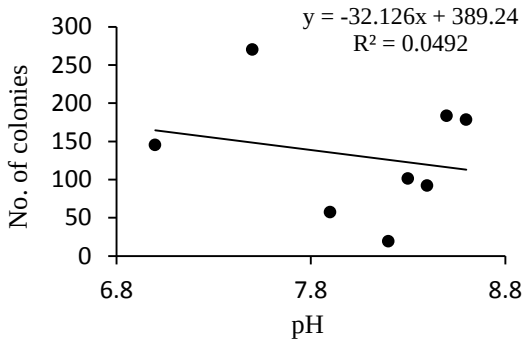


Fig. 33. Site 14

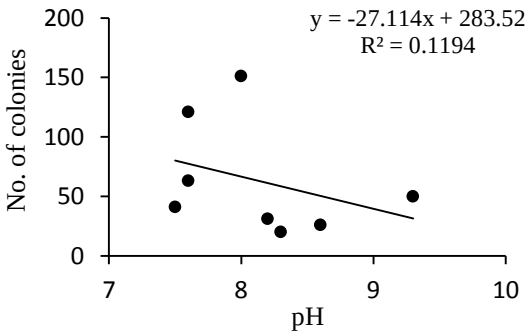


Fig. 34. Site 15

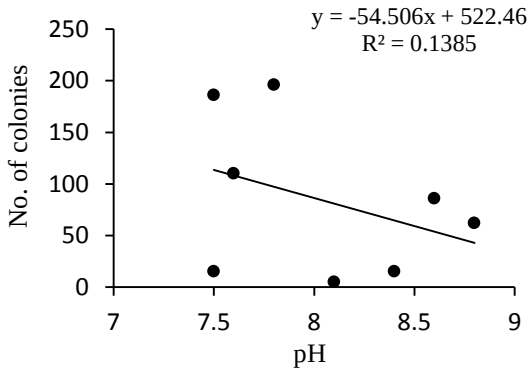


Fig. 35. Site 16

Fig. 20 - 35. Correlation of pH with fungal load

(The solid circles in the graphs indicate the averaged seasonal values)

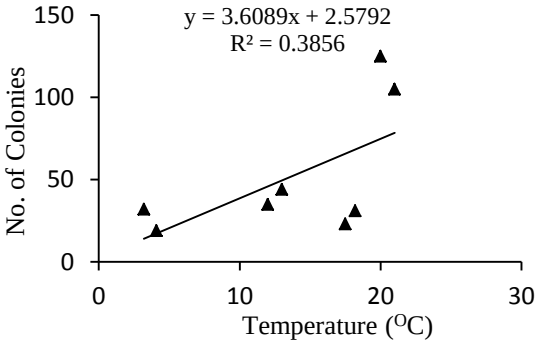


Fig. 36. Site 1

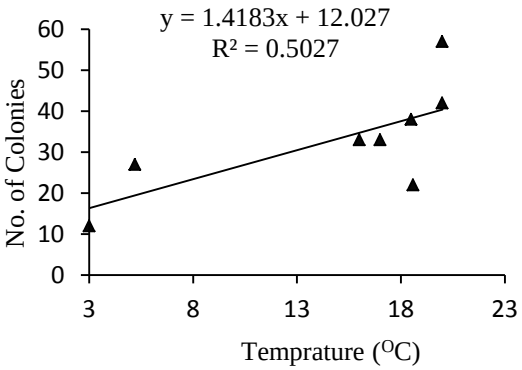
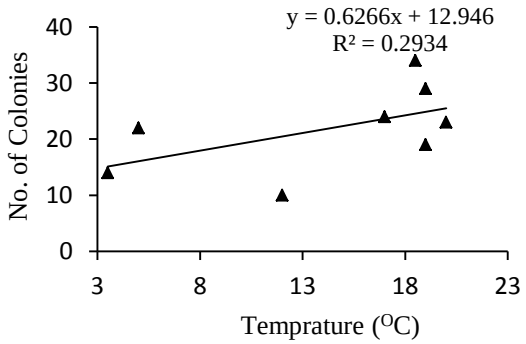


Fig. 37. Site 2



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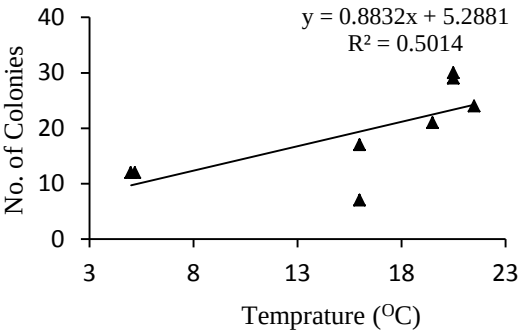

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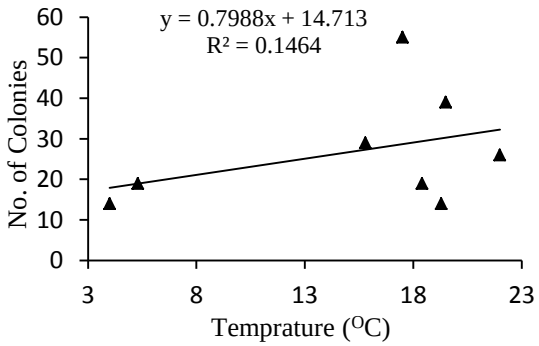

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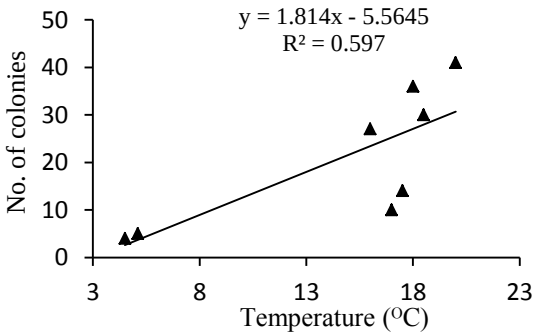

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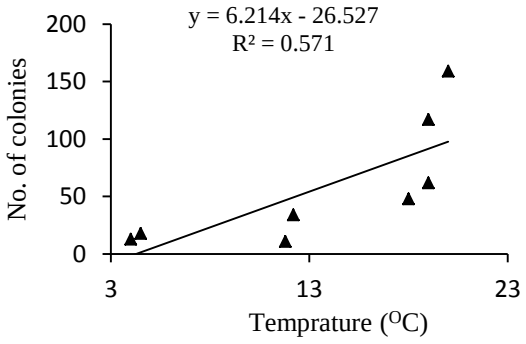

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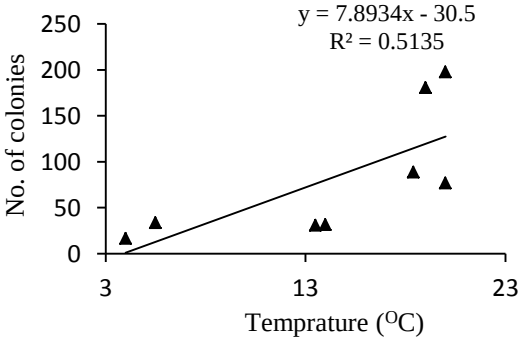


Fig. 43. Site 8

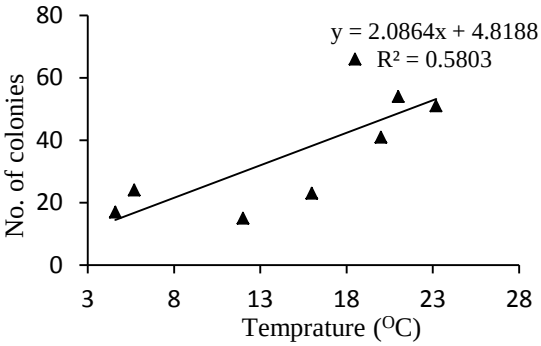
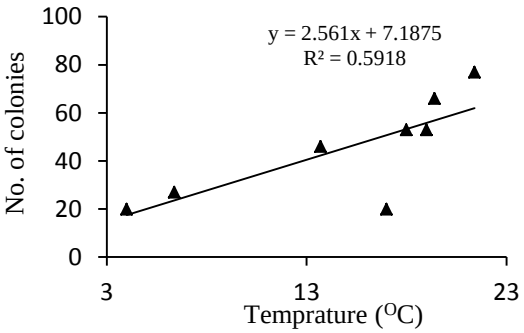


Fig. 44. Site 9



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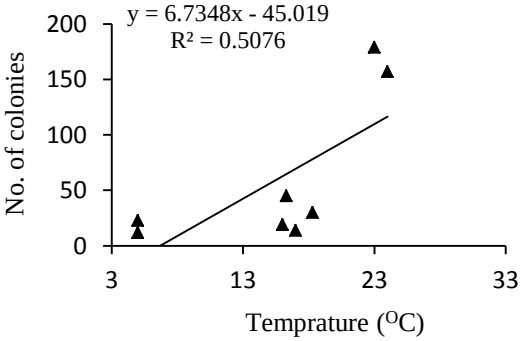

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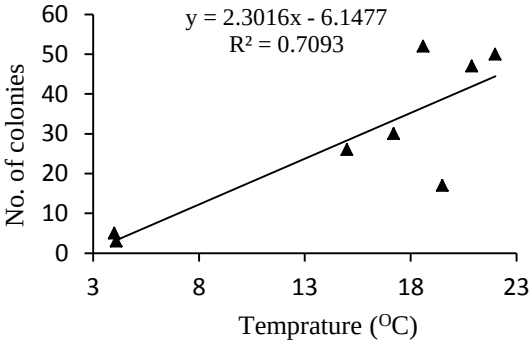
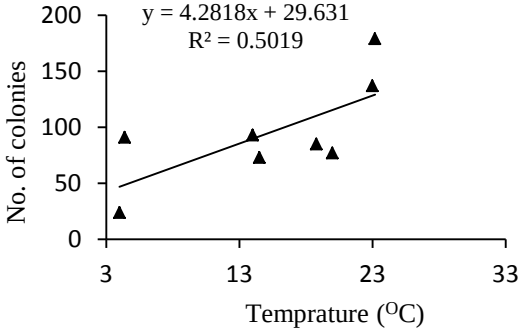


Fig. 47. Site 12



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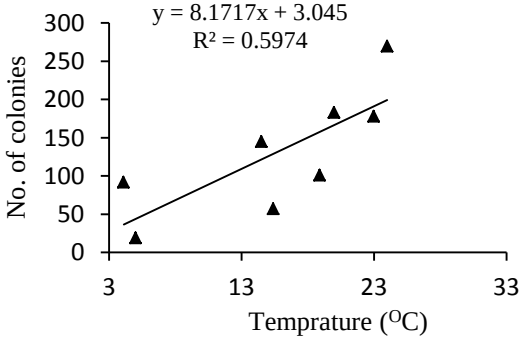

Fig. 49. Site 14

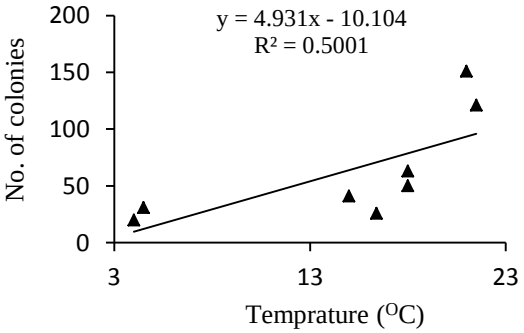

Fig. 50. Site 15

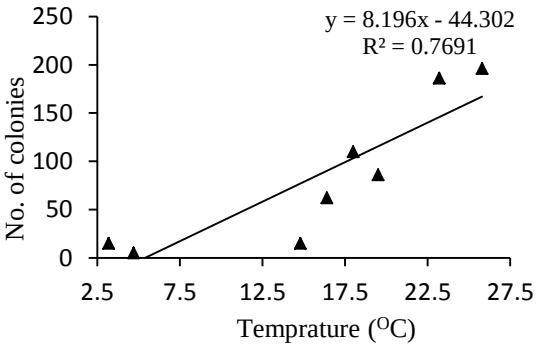

Fig. 51. Site 16

Fig. 36 - 51. Correlation of water temperature (^{O}C) with fungal load

(The solid circles in the graphs indicate the averaged seasonal values)

4.13. Pathogenic species

Out of 51 fungal species recovered during the present endeavor sixteen species were found pathogenic on the basis of earlier research carried out on these species around the world (Table 33). As such these pathogenic fungal species can infect a wide range of hosts but without taking into consideration the other host a questionnaire survey was carried out on the a human population of Dal inhabitants to get an insight into the prevalence of fungal infections in them.

4.14. Disease incidence in Dal inhabitants

It needs to be realized that a large number of people (more than 50,000) are living in the vicinity of Dal Lake in the Lake's 111 hamlets, basically on the peripheries of the lake. There are also 758 own House Boats (For commercialization as tourist attraction), however a population of some 1920 people (320 families) live in their own Dunga Boats who are actively engaged in one or the other activity related to the lake water including the rowing of shikaras, extraction of 'Nadru' from the lake, their women washing the clothes and other daily use items often in the lake water with just 2.11% of them fully aware about the problems of Dal Lake. Present study was conducted on these actively engaged inhabitants of Dal Lake, Kashmir to work out the prevalence of fungal disease and to identify the risk factors associated with disease prevalence. About 20% of this population including both male and female from all ages were interviewed and examined for any sort of superficial fungal infection on their body. 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 and examined was done. The people included for the survey were grouped in three age groups with an age of upto 20years as first age group, 20-40years as the second age group and above 40years as the third age group. For the sake of clear understanding, the observational part is presented under the following headings:

4.14.1. Gender distribution

About 20% of the population (n=1920) comprising some 384 individuals including 211 (54.95%) males and 173 (45.05%) females from different parts of the lake were studied for the incidences of fungal diseases (Table 34).

4.14.2. Age Distribution

With the view of maintaining age stratification, study population was divided into three age groups of upto 20 years, 20-40 years and above 40 years. In the first age group 172 individuals which included 87 (50.58%) males and 85 (49.42%) females were examined. In the second age group 139 individuals which included 71 (51.08%) males and 68 (48.92%) females were examined. While as in the third age group 73 individuals including 53 (72.60%) males and 20 (27.40%) females were examined (Table 34).

4.14.3. Prevalence of various fungal diseases

Among 384 individuals subjected to questionnaire survey and interview, data analysis revealed that 31 individuals were positive for different types of fungal diseases with 18 (4.68%) individuals positive for fungal skin infections followed by 9 (2.34%) individuals as Onchomycosis cases and 4 (1.04%) individuals positive for Candidiasis (Table 35). However, the *Candida* infection was only found to be present in the females. Statistically analyzing the data of prevalence of fungal diseases in the associated population by applying Chi-square test showed it was highly significant ($p<0.007$).

4.14.4. Gender wise prevalence of infection

Data analysis of the studied population (Table 36) reveals that from among the positive cases (31) the prevalence of fungal infections was higher in males than females as more number of positive cases 19 (61.30%) were seen in male population compared to a total of 12 (38.70%) positive cases in female population examined. Most of the positive cases were encountered in the first two age groups with 12 individuals each from first and second age group followed by the third age group with only 7 positive cases. In the first age group we encountered 7 (3.33%) positive cases from males and 5 (2.89%) from females, in second age group 9 (4.26%) positive cases were seen in the male population followed by female population with 3 (1.73%) positive cases of infection. In the third age group the number of positive cases from male side was only 3 (1.42%) compared to 4 (2.31%) positive cases from female side. Statistical analysis of the data by applying Chi-square test showed that the results were insignificant ($p<0.307$).

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

The prevalence of diseases also varied with the type of water being used for different purposes (Table 37). Higher prevalence of infection 6.77% (26 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 1.30% (5 positive cases among 120 individuals studied). Statistical analysis of the data by applying Chi-square test showed that the results again were insignificant ($p<0.09$).

Table 34. Gender and age distribution of population (n=384)

Age group	Male	Female	Total (%)
< 20 yrs	87	85	172
20-40 yrs	71	68	139
> 40 yrs	53	20	73
Total	211	173	384

Table 35. Prevalence of various fungal diseases in the study population

Disease	Positive	% age	$\chi 2$	*p-value*
Skin Infection	18	4.68	10.01	0.007
Onchomycosis	9	2.34		
Candidiasis	4	1.04		
Total	31	8.08		

Table 36. Gender and age wise infection in study population

Age group	No. examined	No. positive	Male (%)	Female (%)	$\chi 2$	*p-value*
< 20 yrs	172	12 (6.97)	7 (4.06)	5 (2.90)	1.997	0.307
20-40 yrs	139	12 (8.33)	9 (6.47)	3 (2.15)		
> 40 yrs	73	7 (9.58)	3 (4.10)	4 (5.47)		
Total	384	31 (8.07)	19 (4.94)	12 (3.12)		

Table 37. Prevalence of fungal diseases with respect to water used

Type of water	No. examined	No. positive	% Prevalence	$\chi 2$	*p-value*
Lake water	264	26	6.77	2.86	0.09
Tap Water	120	5	1.30		

Chapter 5

Discussion

Water from Dal Lake gives a unique opportunity to investigate the microbiology of a typical low altitude urban lake environment that remains completely influenced by different types of anthropogenic activities all through the year. Water samples collected from different microhabitats of the lake were extensively studied in terms of the fungal component and some physical parameters like pH and temperature. Although till now a lot of work has been carried out on different biodiversity components of the lake but the present findings are the attempts offered for the first time where fungi have been specifically targeted for culture and molecular identification. As such, this is the initial detailed study of known heterotrophic microbes 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 fungi and yeasts, 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 urban environmental setups offer a potentially good habitat for microorganisms due to the presence of nutrients and minerals (Sharp *et al*., 1999). Dal Lake 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. Isolation and identification of fungi from the water samples of the lake adds its first evidence to the range of microbial diversity in the lake, and further supports the likelihood of a complex ecosystem within the lake.

Microorganisms play important roles in the environment. Studies of microbial community composition can, therefore, provide potentially descriptive information about ecosystem function. Anthropogenic activities, such as urban development, agriculture, use of pesticides and pollution are believed to have significant effects on microbial communities (Kirk *et al.*, 2004). The characterization of microbial communities is therefore important to determine what kinds of microorganisms are associated with specific water samples and habitat properties as well as to gain an understanding of the ecological functions of these microbes. The role of such fungi in ecosystem-level processes like organic matter processing is largely unknown (Nikolcheva and Barlocher, 2004) however, several studies have documented the presence of some fungal taxa belonging to Basidiomycota, Chytridiomycota (Nikolcheva and Barlocher, 2004), Oomycota and Zygomycota (Barlocher and Kendrick, 1974; Nikolcheva and Barlocher, 2004) on decomposing plant litter. Fungi and fungus-like organisms show a high capacity for adaptation to the environmental conditions, and are therefore widely spread in nature. They can be found in water, soil and air, being a source of infection both for plants, animals and humans. The overall goal of this thesis was to provide an insight into the diversity of fungal communities in Dal Lake waters, as well as to identify their possible spatial and seasonal variation in the lake. However, it is possible that at the time of collection, all the fungi present in the lake ecosystem may not be capable of sporulation (Nikolcheva, 2003). For

example, high conidia production by a dominant hyphomycete species might mask spore production of other species, which thus escape detection if diversity assays are solely based on conidia formation. The fungal communities in this study were assessed using the traditional method of culturing and baiting as well as the molecular techniques. The use of these techniques is confirmed by the studies conducted by Das (2006) while working on the microbial community structure and interactions in leaf litter in a stream in North-eastern Ohio. The use of these techniques for the isolation of fungal communities from different water bodies and other environmental samples is further confirmed by many other studies (El-Nagdy, and Nasser, 2000; Czeczuga and Muszynska, 2004; Steiman *et al*., 2004; Marano and Steciow, 2006; D'Elia, 2008; Kiziewicz and Nalepa, 2008; Sathiyavathi and Parvathane, 2011; Saju, 2011 Bandh *et al*., 2011a and 2012a).

5.1. Identification

The primary identification of the isolated fungal colonies done on the basis of morphological characteristics including both the macro-morphological features and micro-morphological features on different types of culture media in our study was in accordance with the work of Klich (2002). The use of Riddle's classic slide culture method (Riddle, 1950) for microscopic study of the fungal isolates from Dal lake has also been used by many workers for the study of various microscopic features of fungal species (Bandh *et al*., 2011), the technique of James and Natalie (2001) adopted for identification of the unknown isolated fungi was in accordance with various mycological texts (Cheesbrough, 2000). A survey by the American society for microbiology (Anonymous, 2004) documented that 89% of laboratories still perform morphology based mycological examinations, 16% of them use serologic tests and fewer than 5% use molecular tests for identification of microbial pathogens. Only 3% of reporting laboratories use 'homebrew' molecular testing for microbial pathogens. The use of these techniques for the identification of the filamentous fungi usually belonging to genus *Penicillium* and *Aspergillus* is confirmed by some studies conducted by Bandh *et al*. (2011b and 2012b). It is further authenticated by the work that has been conducted on various *Aspergillus* species with a similar design for their identification (Klich, 2002; Curtis and

Baker, 2005; McClenny, 2005). Some recent similar studies (Leenders and Belkum, 1999; Anaissie *et al*., 2003) also confirm the usage of similar approaches for the identification of the molds. The color of the mold's surface differs from species to species and can be used to identify the type of mold. The rate of growth can also be used to identify *Aspergillus*, with most species growing quite quickly. After 1 week of growth at around 25°C, an *Aspergillus* colony will generally be 1 to 9 cm in diameter, with a little bit of variability from species to species which helped us in the proper identification of these molds.

The use of biochemical tests including some physiological, fermentation and assimilation tests along with the growth of the *Candida* species on the *candida* specific CHROM Agar for the observation of color of the colonies which facilitated their colonial or cellular morphology and characteristics helped us to know the details about these yeast specimens. Similar protocol was followed in various other studies for the identification of yeasts (Louvois *et al*., 1979; Rose and Harrison, 1987-1993; Mushtaq *et al*., 2004; Waema *et al*., 2009; Ghosh, 2011). The use of physiological profiling is also confirmed by the study of Junior *et al*. (2008). The results of the present study confirm that the chromogenic medium helps in the presumptive identification of *C. albicans*, *C. krusei* and *C. Parapsilosis* as reported in many other earlier studies (Pfaller *et al*., 1996; Odds and Bernaerts, 1994; Agarwal *et al*., 2011). However, the detailed and authentic identification depends on the biochemical reaction shown by different species.

Besides these methods polymerase chain reaction (PCR) based molecular approach was also used to identify some strains of fungi isolated from the lake water samples as DNA-based molecular methods are used to differentiate genera, species, subspecies, races, and strains of fungi as well as to identify individuals or clones within fungal populations in ecological studies. The development and application of molecular techniques, particularly those based on the analysis of specific genes amplified from extracted DNA as has been done in this study by targeting the highly conserved and highly variable internal transcribed spacer region, have transformed studies of fungal diversity in natural environments (Kowalchuk *et al*., 1997; Kowalchuk, 1998; Smit *et al*., 1999; Borneman and

Hartin, 2000; Vainio and Hantula, 2000; van Elsas *et al*., 2000; Lowell and Klein, 2001; Mohlenhoff *et al*., 2001; Pennanen *et al*., 2001; Schabereiter-Gurtner *et al*., 2001). Following the same trend here we targeted the nuclear rDNA region containing the two internal transcribed spacer (ITS) regions (ITS1 and ITS2) for amplification from the extracted DNA of the isolated fungal species by using the universal fungal primers (ITS1-TCCGTAGGTGAACCTGCGG, ITS2-GCTGCG TTCTTCATCGATGC, ITS3-GCATCGATGAAGAACGCAGC and ITS4-TCCT CCGCTTATTGATATGC) often used for estimating fungal diversity in environmental samples and considered important in modern microbiological assays. The use of this approach is authenticated by the work of Hibbet and Vilgalys, (1991), Nazar *et al*. (1991) and LoBuglio *et al*. (1993) reporting that molecular methods based on PCR could be used as a modern tool in the detection and identification of fungi besides the study of fungal taxonomy and population structure. The advent of the PCR (Mullis and Faloona, 1987) has expedited the molecular analysis of fungal genomes for both phylogenetic and population structure studies. Ribosomal genes and spacers regions within the fungal genome proved good targets for amplification via PCR because they are comprised of highly conserved tracts with heterogeneous regions in between as most molecular fungal species identification relies on the amplification and sequencing of the internal transcribed spacer (ITS) region of the fungal genome, which is highly variable among species or even populations of the same species (Hibbett, 1992; Horton and Bruns, 2001). The use of this approach is also confirmed by the work of Gardes *et al*. (1991a & b). In fungi, the ITS region is usually amplified by the universal primer pair ITS-1 and ITS-4 designed by White *et al*. (1990). Gardes and Bruns (1993) designed ITS1-F and ITS4-B primers specifically to amplify fungal internal transcribed spacer (ITS) regions which were also used in our study. The use of these primers to study the fungal diversity of different ecosystems is confirmed by many other studies (Luo and Mitchell, 2002; Wu *et al*., 2002; Anderson *et al*., 2003; Kumar and Shukla, 2005). Once the target region got amplified by PCR, it was sequenced and the sequence was compared to those of known species by BLAST search to reach their species level identification.

5.2. Diversity Pattern of Fungal Community

The fungal community in the lake showed a great deal of variation within and between the sites depending upon their characteristic features because the community composition of the fungal component of any water body depends upon various external and internal influences on the system and photosynthetically produced organic matter as well. Consequently, composition and abundance of fungi in the lake differed significantly between the different micro habitats. The variations in the fungal community characteristics within and between the sites of the lake can be attributed to the heterogeneity in the water characteristics of these microhabitats as aquatic habitats are heterogeneous in time and space and greatly differ in their physico-chemical features. Favoring the current observation, a recent review of the ecology of fungi in lake ecosystems by Wurzbacher *et al.* (2010) mentioned that the fungal communities differ significantly between and within the different water bodies as the aquatic habitats are characterized by a unique balance of allochthonous (external) and autochthonous (internal) organic matter supply, controlled largely by watershed characteristics, surface area and location. In aquatic systems, the fungal community structure greatly differs between substrates (Shearer and Webster, 1985; Findlay *et al*., 1990; Barlocher and Graca, 2002; Graca *et al*., 2002; Mille-Lindblom *et al*., 2006) and with the physico-chemical properties of the respective habitats, such as flow (Pattee and Chergui, 1995; Baldy *et al*., 2002), dissolved oxygen concentration (Field and Webster, 1983; Medeiros *et al*., 2009), nutrient concentrations (Gulis and Suberkropp, 2004; Rankovic, 2005), salinity (Hyde and Lee, 1995; Roache *et al*., 2006), temperature (Barlocher and Seena, 2008) and depth (Wurzbacher *et al*., 2010). Belonging of over 50% of the isolated fungi from the lake to phylum *Ascomycota* and only a meager percentage (0.5%) to phylum *Basidiomycota* may be attributed to the fact the water of Dal Lake is not that deficient in dissolved oxygen as proved by some limnological studies carried out recently on this water body. The results here are well supported by the work of Shearer *et al*. (2007) reporting that taxonomically, the aquatic fungi known as “aquatic hyphomycetes” found most commonly in clean and well oxygenated (Ingold, 1975; Barlocher,

1992) are mainly associated with the *Ascomycota*, and only a small percentage is affiliated with the *Basidiomycota*. The dominance of *Ascomycota* in the water samples of Dal lake is also confirmed by another study in which Gessner and Van Ryckegem, (2003) isolated over 600 species of fungi from the litter of *Phragmites australis* alone with 94% of them belonging to *Ascomycota* and only 6% belonged to *Basidiomycota*. The occurrence of zoosporic fungi in the lake waters is not anything surprising as they have been found to be universally present in all types of freshwater systems and occur as saprotrophs on a wide variety of substrata, playing a key role in those ecosystems as decomposers of organic materials (Mueller *et al*., 2004). Most studies dealing with zoosporic fungi have provided extensive inventories of taxa from specific sites or geographic regions, but often without characterizing the microhabitat, determining frequencies of occurrence or relative abundance of species (Coker, 1923; Sparrow, 1960; Scott, 1961; Karling, 1977). The isolation of different fungal species belonging to both the zoosporic group and filamentous group of fungi in the present study corroborates well with the two year study of Rajanaika *et al*. (2008) in which they isolated some 12 zoosporic fungal species and 14 filamentous fungal species from the Shanthi Sagar Lake.

A number of studies have examined the occurrence of fungal communities in Great Lakes waters (Paterson, 1967; Qureshi and Dutka, 1974; El Shaarawi *et al*., 1977; Sherry, 1986; Kwasniewska, 1988), but it is difficult to compare our results to those of the previous studies because of different techniques of collection, culturing mediums, and levels of identification. Qureshi and Dutka (1974) used various agar medias and found 25 genera in Lake Ontario waters off the Niagara River mouth, of which only three were found in Dal Lake Kashmir (*Aspergillus, Fusarium* and *Penicillium*).

Useful correlations could be made between temperature and the occurrence of Oomycetes in this study as this was probably because the study extended over two (2) years of essentially variable climatic conditions and because there was more variation in temperature within different seasons of a year. Seasonal variation observed in the fungal population was possibly due to the temperature

variations, as the maximum percentage of fungi was found during high temperature (summer) and minimum during low temperature (winter) season. Similar results were obtained by Khulbe and Durgapal, (1992) in their study on the population dynamics of geofungi in a polluted freshwater body at Nainital. A preliminary mycological study on the same lake by Bhat and Kamili (2004) had also reported the presence of filamentous fungal genera especially *Aspergillus* and *Penicillium* with a similar fluctuation in their numbers during different seasons. The seasonal variation found in this study is also in consonance with the results of Sharma and Parveen (2011). Another study on the diversity, distribution and periodicity of aquatic and extra aquatic fungi in Shanthi Sagar Lake by Rajanaika *et al*. (2008) confirms the seasonal variation of both aquatic and extra aquatic fungi reporting the maximum fungal distribution during the monsoon period followed by pre-monsoon periods attributed to the temperature, organic matter and pH of water thus confirming the seasonal variation of fungal population in our study. Khulbe and Bhargava (1977) while studying the distribution and seasonal periodicity of watermoulds in some lakes in Nainital hills (India), reported maximum numbers of watermoulds during summer as has been observed in present study.

The influence of temperature on the fungal load of Dal Lake, Kashmir is further supported by the work of Stoll (1936) mentioning that the occurrence and distribution of watermolds is directly related to the water temperature. Well in support of the results of present study Ho (1975) also found a positive correlation between the water temperature and the isolation frequency of watermolds. The presence of the terrestrial molds like *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. terreus*, *Acremonium* spp., *Penicillium chrysogenum*, *P. caseicolum*, *P. commune*, *P. lilacinum*, *Fusarium* spp. and *Rhizopus* spp., in the Dal waters may be attributed to the influence of catchment area like entry of litter, soil, dead plant parts, sewage and the influence of air as the occurrence of these terrestrial fungal species in aquatic systems are likely to originated from air (Sparrow, 1968) as well as from living or dead animal and plant parts, soil and litter being in contact with water (Park, 1972).

The percent relative abundance of the genus *Penicillium* (21.6%) and genus *Aspergillus* (15.7%) followed by all other genera obtained in the present study can be attributed to the fact that species of genus *Penicillium* and *Aspergillus* are biologically most successful of all fungi and are expected to occur in all sorts of habitats and was confirmed by a recent Nigerian study of Eze and Ogbaran (2010) who in their study on pond water found that species of genus *Penicillium* showed a maximum contribution of 55.8% followed by species of genus *Aspergillus* (44.1%). Their spores are the most widespread aeroallergens in the world. According to qualitative and quantitative reports, the former is the dominant species in tropical regions whilst the latter is dominant all over the world (Rosas *et al.,* 1992). *A. niger* and *A. flavus* found with a maximum percent total count among the species of genus *Aspergillus* in our study may be attributed to the influence of atmosphere as it is one of the most ubiquitous airborne saprophytic fungi (Asan *et al*., 2003). *Penicillium*, the most dominant genus as compared to *Aspergillus* observed in the study concurs with the results of Kinsey *et al*. (1999), who reported that certain fungi such as *Penicillium*, *Aspergillus*, *Cladosporium*, *Epicoccum*, and *Trichoderma* species appear more frequently than others in water. The results corroborate with theirs except that this study was not confined to the isolation and distribution of terrestrial fungi only but was a widespread study pertaining to the fungal diversity of Dal Lake. *P. chrysogenum*, *A. fumigatus* and *A. niger* many other species belonging to the two genera, isolated and identified in the current study with a high percentage occurrence and colony number have also been found to be widespread in Turkey and have been reported in many studies (Asan, 2000). The overwhelming presence of these terrestrial molds in the water samples of the lake supports the paradigm that their deposition is attributable to contamination of the water body due to the entry of sewage from the catchment areas, as they survive conventional treatment strategies and enter the distribution through the sewage coming out from the sewage treatment plants (Neimi *et al*., 1982). It can be attributed to the entry of sewage from the drains into the lake, as these genera have been reported frequently from the drain waters with maximum densities during higher pollution (Khulbe and Drugapal, 1994) and can therefore

be inferred that these species are good indicators of pollution. The genera and species of filamentous fungi isolated in the present study were previously isolated, but with varying numbers and frequencies, from different substrates such as rainfall water and mud in Saudi Arabia (El-Nagdy *et al*., 1992), soil (Abdel-Hafez, 1982a) and ferns (Abdel-Hafez, 1984). The occurrence of almost all filamentous fungal genera recovered in this study is nothing unusual as these molds have also been found present in the water bodies of India and many other countries (Saju 2011; Shafi *et al*., 2011, Bandh *et al*., 2011a & 2012a; Abdel-Hafez *et al.,* 1978; Abdel-Hafez, 1982b; Abdel-Kader *et al.*, 1983; Abdel-Hafez and Bagy, 1985; EI-Hissy *et al.,* 1990; Moharrum *et al.,* 1990; EI-Nagdy and AbdelHafez, 1990; Barlocher and Kendrick, 1974; Bettucci *et al.,* 1993; Bettucci and Roquebert, 1995). The occurrence of these fungal species in the different study sites of the Dal Lake with varying frequencies is also in consonance with the studies by El-Hissy and El-Nagdy (1983) and El-Hissy *et al*. (1996).

Penicillium was the most frequent and predominant genus detected in our study, followed by *Aspergillus*. According to the Kinsey *et al*. (1999) certain fungi such as *Aspergillus, Cladosporium, Epicoccum, Penicillium, Trichoderma, Arthrinium phaeospermum, A. flavus, C. cladosporioides, Fusarium culmorum, Mucor hiemalis* and *Trichoderma harzianum* species appear more frequently than others in water. Our results also concur with the results of Tothova (1999) except that we did not find *Epicoccum, Cladosporium* and *Trichoderma*. Species of genus *Aspergillus* and *Penicillium* are major contaminants of the environments and occur as ubiquitous saprophytes, with their spores able to survive and reproduce in water as well. The present results are in confirmation to a Brazilian study on filamentous fungi of water by Gomes *et al*. (2008) showing that *Aspergillus* and *Penicillium* were the most frequent genera isolated from water. The ecology of aquatic fungi affects their distribution both locally and globally, and the factors influencing the fungi are complex and vary depending on the aquatic environment. What governs the distribution of freshwater fungi is difficult to determine, although some species appear to be more common either in temperate or tropical regions (Shearer *et al*., 2007; Raja *et al*., 2009).

The zoosporic fungi isolated from Dal Lake in our study consisted of *Achyla*, *Aphanomyces*, *Brevilegnia*, *Dictyuchus*, *Saprolegnia* and *Pythium*. Occurrence of species belonging to these fungal genera in Dal Lake waters is off course a first report but is nothing new as the zoosporic fungi occurring as saprotrophs on a wide variety of substrates and playing a critically important role as decomposers of organic matter have been found universally present in all types of freshwater ecosystems (Muller *et al*., 2004). El-Hissy *et al*. (1982), El-Hissy and Khallil (1989) reported that zoosporic fungal communities in freshwater habitats are mainly composed of *Saprolegnia, Dictyuchus, Achlya* and *Pythium*. Various species of genus *Achyla*, *Aphanomyces*, *Brevilegnia*, *Dictyuchus*, *Saprolegnia* and *Pythium* isolated from diverse range of microhabitats of the study area under consideration in present study were previously isolated from different aquatic habitats including lakes, ponds, tanks and rivers (Manoharacharya, 1977; Chowdhry and Agarwal, 1980; Gupta and Mehrotra, 1989a & b and 1992) and could be considered as the normal mycoflora of the lake. The occurrence of different species of these fungal genera may be attributed to the favorable pH and temperature range available in the lake environment as these species have been isolated by many workers (Bhargava and Singh, 1965; Khulbe and Bhargava, 1977; Manoharacharya *et al*., 1983; Mishra *et al*., 1990) under similar conditions of temperature and pH and our results are similar to theirs. The correlation which we got between the pH and temperature of water samples and the fungal load present in them showed that the fungal load increase with increasing temperature while as it decreased with increasing pH and these findings are in accordance with the findings of many studies (Manohaachary, 1979; Manoharachary *et al*., 1983; Gupta and Mehrotra, 1989a & b and 1992; Khulbe 1980a & b) who also suggested that the pH and temperature range which was observed in the different water samples of present study supports the growth of both filamentous and zoosporic fungi.

Various studies (Coker, 1927; Sparrow, 1960; Scott, 1961 and Karling, 1977) dealing with the zoosporic fungi have provided extensive inventories of taxa from different specific sites or geographic regions, but often without

characterizing the microhabitats, determining frequencies of occurrence or relative abundance of species. The different microhabitats of a similar bigger habitat (Dal Lake) studied have approximately the same generic composition but vary in their percentage occurrence, frequency of occurrence and abundance distribution. The findings here show that zoosporic fungi, which exhibited high frequencies, also exhibited high values of abundance. These frequently found fungi could be considered as indicators of the generic composition of the water body. The occurrence of these zoosporic fungal species in water samples of Dal Lake is not new but it is a new report in the lake as no study has been carried out till now on the fungal community of the lake. These species have been isolated from a wide range of habitats including freshwater lakes, rivers, streams, ponds, water tanks and soils at different ranges of temperature, pH and organic matter by many workers from time to time. Some of the taxa found in this study, were previously isolated in other studies of zoosporic fungal communities in the neotropics (Schoenlein-Crusius *et al*., 1992). El-Hissy *et al*. (1982) and El-Hissy and Khallil (1989) reported that zoosporic fungal communities in freshwater habitats composing mainly of *Saprolegnia, Dictyuchus, Achlya* and *Pythium*. In consonance with their results we also noticed a varied kind of fungi *Pythium*, in the water samples collected from Dal Lake during our study.

The overall highest percentage occurrence of the fungi in the four basins of Dal Lake was observed towards the areas dominated by residential houseboats, in the littoral zones of the lake basins, witnessing entry of sewage from the catchment areas. In the outlets the dominating occurrence of the fungal load was seen towards the extremes of the exit points carrying the full load of the sewage from the adjoining catchments, domestic effluents from the houseboats, rain-washed runoff from the agricultural areas and kitchen gardens. The sequence of fungal percentage occurrence at the sixteen microhabitats of the lake indicated that the highest total was observed at site Pokhribal outlet II followed by Pokhribal outlet I, Tailbal nallah II, Near Centaur, Tailbal nallah I, Boathall nallah I, Nishat open, Hazratbal I, Dal Lock Gate II, Hazratbal II, Dal Lock Gate I, Boathall nallah II, Gagribal I, Gagribal II, Nigeen Lake I, Nigeen Lake II. The heavy fungal load

in the littoral zones and the extreme exit points of the lake was because of the sewage entering the lake system from the drains, as these genera have also been reported frequently from the drain waters with maximum densities during higher pollution by Khulbe and Durgapal (1994). Fungi tend to be more abundant and diverse in areas subjected to organic enrichment (Quershi and Dutka, 1974). Littoral regions (or regions bordering the open water areas) of the four lake basins are rich enough in total fungal load. In these shoreline regions there remains a possibility that rainwater runoff from the catchment area harboring a diversity of habitats like urban settlements, orchards, recreational gardens, mountains and some agricultural fields releases microbes into the lake. However, the low cell counts and the absence of viable microbes in open water zones in the lake indicate that the microorganisms have to do something with the influence of catchment area. Additionally, the isolated fungi from Dal Lake are similar to fungi isolated from other similar aquatic environments including the freshwater lakes. Aquatic fungi utilize a variety of organic substrates for growth, and likely enter lakes through watershed inputs (Dick, 1971). Thus, more fungal species might be expected in near shore areas and near the bottom sediments where organic matter content would be highest. The greater occurrence of water molds at the shore areas of the lake agrees with various previous reports which appear to be generally true for aquatic fungi. Willoughby (1961 and 1962) reported that lake margins are "exceptionally favorable" for the growth of saprophytic chytrids and much higher numbers of Saprolegniales from the lake margin than from surface waters from the center of the lake. Willoughby and Collins (1966) suggest that "the Saprolegniaceous spora in open water receives local reinforcement as a result of fungal activity at the lake margin". The ubiquitous presence of one or more genera of Oomycetes indicates that these organisms do have a role in biological recycling in the lake. Their relative abundance on the shore line and in shallow water as opposed to deep water would indicate a greater abundance of energy materials in the littoral zone than in the deeper water. It could therefore be inferred that these species are good indicators of pollution due to the sewage from the adjoining residential and commercial catchment area. The species of genus *Penicillium* and

Aspergillus are usually found in polluted lake waters and act as cellulose decomposers as opined by Kellermann and McBeth (1912).

Species of genus *Achlya* recovered here had a moderate frequency and contributed the broadest spectrum of five species among the zoosporic group, of which *Achyla cornuta, Achyla conspicua* and *Achyla klebsiana* were the most common. Similar results were found by El-Nagdy *et al.* (1992) from the accumulated rainfall water and mud in Taif, Saudi Arabia. The recoverable *Achlya* species were encountered, but with different incidences, from various water habitats in Egypt (EI-Nagdy and Abdel-Hafez, 1990; Khallil *et al*., 1993; El-Hissy *et al*., 1996) and many other parts of the world (Alabi, 1973; Khulbe, 1980a; Misera, 1982; Klich and Tiffany, 1985; El-Hissy, 1994).

5.3. Disease 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 makes them to come in contacts with the lake water off and on during their day to day activities, examined as per the questionnaire and interview method in the study showed a relatively lesser prevalence of the fungal diseases except a few cases of fungal skin infections, candidiasis and Onchomycosis. The lesser prevalence of these fungal diseases 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 of the already mentioned diseases among the studied population could be correlated to the presence of fungal pathogens in the lake water which are the causative agents of such infections as we are familiar with the fact that majority of fungal infections of the skin are caused by dermatophytic fungi while as other fungal elements such as yeasts are also responsible for skin disease. Favoring the statement deHoog *et al.* (2000) reported some 40 species of fungi as causal agents of opportunistic infections in humans. Many species are thermo tolerant and their dry hydrophobic conidia are easily inhaled. A few of the *Aspergillus* species have been reported to produce potent

toxins of the aflatoxin family, which can cause cancer. Toxic death due to aflatoxins has been reported in humans, animals, and birds. Among these, *A. fumigatus* found in the lake water samples widely distributed in nature also is the most frequent etiological agent followed by *A. flavus* and *A. niger* causing infection or allergy in susceptible individuals. Some other species of the genus like *A. terreus*, *A. versicolor*, and *A. wentii* and several others which were found present in the lake water have also been less commonly or rarely recovered as opportunistic pathogens (Summerbell, 1998; de Hoog *et al*., 2000 and Richardson, 1998). The occurrence of these infections in a few individuals is not strange as the species of genus *Aspergillus* along with some other genera cause a variety of opportunistic infections, such as mycotic keratitis, otomycosis, nasal sinusitis, allergic bronchopulmonary aspergillosis (ABPA), aspergilloma and invasive aspergillosis (Richardson, 1998). The frequency of invasive *Aspergillus* infections has increased in recent years due to increasing number of patients receiving aggressive chemotherapy and immunosuppressive agents.

The yeast *Candida albicans* and the filamentous fungus *Aspergillus fumigatus* are by far the most important causes of life-threatening invasive mycoses. Apart from *A. fumigatus*, around 10% of the more than 200 species of the genus *Aspergillus* like *A. terreus*, *A. flavus*, and *A. niger* are regarded as human pathogens or as having other adverse effects (Brakhage, 2005). The prevalence of *C. albicans* in clinical *Candida* samples is 50–70%, followed by infections with *Candida glabrata*, which is found in 20–25% of clinical *Candida* samples. Other pathogenic *Candida* species include *C. tropicalis*, *C. dubliniensis*, *C. krusei*, and *C. parapsilosis* (Pfaller and Diekema, 2007). Another important human-pathogenic fungus of clinical relevance is the fungus *Cryptococcus neoformans*. The most common fungal infection among AIDS patients, cryptococcal meningitis, is caused by this basidiomycete. Furthermore, other fungal species, such as *Pneumocystis jiroveci*, *Zygomycetes*, *Fusarium* species, and *Scedosporium* species, have emerged as causal agents of invasive mycoses (Pfaller and Diekema, 2007). Thus the prevalence of some fungal infection cases in the studied population was not surprising.

Chapter 6

Summary, Conclusions and Recommendations

SUMMARY

A detailed knowledge of the diversity and functioning of microorganisms dewelling in the freshwater ecosystems (like Dal lake) is crucially important in the sustainable management of the freshwater resources of Kashmir. Hence the present work entitled *"Study of fungal diversity of Dal Lake, Kashmir with particular reference to pathogenic species"* was undertaken with the following objectives:

- Isolation, identification, characterization and classification of fungal diversity of Dal Lake, Kashmir.
- Documentation of the fungal community characteristics (abundance, diversity and density).
- Identification of disease causing species of fungi.

The study focused on the unexplord and undocumented fungal diversity of this important water body of Kashmir valley. The study was carried out for two consecutive years to achieve the above mentioned objectives.

- The study area (Dal Lake; Latitude 34^0 07′ N, longitude 74^0 52′ E, altitude 1583 m) selected for this work is a multibasined 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, Nageen Open, Nageen 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 was 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 limnitic zones.
- The water samples from the different sites of the lake, under consideration for exploring the fungal diversity 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 just below the surface 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 fungal 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 fungi from the water samples included spread plate technique, pour plate technique, streak plate technique, enrichment culture technique and baiting approach. The species isolated were identified on the basis of morphological observation and culture using differential culture media like Czepek dox agar (CZ) media, Czepek yeast agar (CYA) media, Malt extract agar (MEA) media, Potato dextrose agar (PDA) media, CHROM Agar *candida* and classical slide culture techniques besides the use of biochemical tests for the identification of yeast specimens and molecular approach.
- The biochemical tests conducted for the identification of the yeast specimens included three types of tests viz. Physiological tests including Germ Tube Test, Hydrolysis, Growth on Cycloheximide medium and Growth at $37^{O}C$; Fermentation tests including Glucose, Trehalose, Lactose, Galactose, Maltose, Sucrose fermentation and Assimilation tests including Glucose, Trehalose, Lactose, Galactose, Maltose, Sucrose, Raffinose, D-Manitol, Soluble Starch, Glycerol, Lactic acid, Potassium nitrate, Citric acid and Succinic acid assimilation with the species showing positive, negative and variable results for the respective tests for which they are positive, negative and variable.
- The molecular level identification of various species was done by using Polymerase chain reaction (PCR) targeting of internal transcribed spacer (ITS) region by universal fungal primers (ITS1-TCCGTAGGTGAACCTGCGG, ITS2-GCTGCGTTCTTCATCGATGC, ITS3-GCATCGATGAAGAACGCAGC and ITS4-TCCTCCGCTTATTGATATGC) often needed for estimating fungal diversity in environmental samples and considered important in modern microbiological assays.
- DNA from all the unknown cultures was successfully extracted and amplified using primer pair ITS1 and ITS4. The PCR products obtained were sequenced and

then identified by using the BLAST search program. All the sequences were found to be 90 to100% similar to the sequences of the ITSI, 5.8S rRNA gene, and ITSII regions of the respective fungi.

- Fungal population in the water samples collected from Dal Lake Kashmir on seasonal basis from sixteen different microhabitats of the Lake was assessed in terms of colony forming units (CFU/ml) and total viable counts on Rose Bengal Streptomycin Agar (RBSA) and Potato Dextrose Agar (PDA). The samples were serially diluted five folds using normal saline solution to get the desired number of colonies per plate so as to avoid any ambiguity in the results by the overlapping of different colonies.
- In all a total of fifty one species of fungi belonging to two groups viz. the filamentous groups contributing twenty eight (28) species and the zoosporic groups contributing twenty three (23) species were isolated from the water samples.
- As per the classification approach of National Center for Biotechnological Information (NCBI) he fifty one (51) species of fungi belonged to two major kingdoms (Fungi and Chromista) and five major phyla viz. Ascomycota contributing four classes (Eurotiomycetes, Sordariomycetes, Ascomycetes, Saccharomycetes) and twenty five (25) species, Oomycota contributing one class (Oomycetes) and nineteen (19) species, Blastocladiomycota contributing one class (Blastocladiomycetes) and four (4) species, Zygomycota contributing one class (Zygomycetes) and two (2) species and Besidiomycota contributing one class (Tremellomycetes) and only one (1) species.
- The contribution of different genera in terms of species number and relative abundance (%) depicts that genus *Penicillium* contributed 11 species with a % relative abundance of 21.6% followed by genus *Aspergillus* 8 species (15.7%), *Achyla* 5 species (9.8%), *Candida*, *Allomyces*, *Brevilegnia* and *Pythium* 4 species (8%) each, *Aphanomyces* 3 species (5.9%), *Dictyuchus* 2 species (3%), *Mucor*, *Rhizopus*, *Fusarium*, *Acremonium*, *Cryptococcus* and *Saprolegnia* 1 species (2%) each.
- The colony count of the fungal species showed a total of 3157 filamentous fungal colonies and 3783 zoosporic fungal colonies from the lake water samples.

- The most dominantly occurring ten species at each site from both fungal groups show that among the filamentous group of fungi *P. olivicolor* dominated two sites, *C. neoformans* three sites, *C. glabrata* two sites, *A. terreus* two sites, *C. albicans* two sites, *Mucor* sp. two sites, *C. parapsilosis*, *P. lilacinum* and *Acremonium* sp one site each. In zoosporic group *A. klebsiana* dominated two sites, *A. laevis* dominated five sites, *A. apiculata* dominated two sites while as *A. cornuta A. conspicua, Allomyces* sp, *P. elongatum, P. proliferum, A. cladogamous* and *A. moniliformis* dominated one site each.
- The seasonal influence on the fungal population of the lake at all the selected sites showed considerably significant variations. The Analysis of variance (ANOVA) showed that the observed distribution of the filamentous fungal colonies ($F=7.06$, $F_{cric}=2.75$, $p<0.01$) and Zoosporic fungal colonies ($F=3.25$, $F_{cric}=2.75$, $p<0.05$) in different seasons was statistically significant. Visible from the results the seasonal variation of occurrence of fungal colonies was also observed between different study sites of the lake.
- The species composition in the lake water samples showed a great deal of seasonal and temporal variation with overall highest variety of species composition in summers seasons followed by other seasons.
- 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 that the sites which were much more closer to each other showed higher similarity while as those which were more distantly located showed less similarity. the sites with group 1 including site 2, 3 and 4 being quite different from the rest three groups in the respect that they are more dissimilar to the other sites in terms of the species composition.
- The explanatory data analysis by Box and Whisker Plot models on the basis of all experimental data for both the filamentous and zoosporic group of fungi from all the sixteen microhabitats studied as an indicator of centrality, spread and similarity shows the main characteristics of their distribution. The sites 13 and 14 showed a large variability in terms of both the fungal groups.
- The Analysis of variance (ANOVA) carried out pair wise between the different sites for both the filamentous and zoosporic fungal species isolated from different

microhabitats showed that 64% results were statistically significant with 54% as highly significant ($p<0.01$) and 10% as significant ($p<0.05$).

- From the values of different indices computed for sixteen sites showed that the Shannon-Wiener (H) index was highest (3.69) for site 16 followed closely by the species rich Site 8 (3.65), Site 15 (3.64), site 1 and 10 (3.62) each and lowest at Site 3 (3.21). The one way Analysis of variance (ANOVA) carried out showed that the Dominance ($F=50.60$, $F_{crit}=4.17$, $p<0.01$), Diversity ($F=17.34$, $F_{crit}=4.17$, $p<0.01$) and Evenness ($F=41.25$, $F_{crit}=4.17$, $p<0.01$) varied significantly between different microhabitats of the lake. The evenness pattern calculated for the said sites was also highest (0.93) for site 16 and lowest (0.76) for site 4.
- Among 384 individuals subjected to questionnair survey and interview, data analysis revealed that 31 individuals were positive for different types of fungal diseases with 18 (4.68%) individuals positive for fungal skin infections followed by 9 (2.34%) individuals as Onchomycosis cases and 4 (1.04%) individuals positive for Candidiasis. However, the Candida infection was only found present in the females.
- The prevalence of diseases also varied with the type of water being used for different purposes. Higher prevalence of infection 6.77% (26 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 1.30% (5 positive cases among 120 individuals studied).

CONCLUSIONS

From the foregoing account the following conclusions can be drawn:

- A single medium potato dextrose agar (PDA) is only useful to identify the fungal species upto genus level, but the use of some differential culture media like Czepek Dox Agar, Malt Extract Agar, Czepek Yeast Agar, Creatinine Agar and CHROM Agar *candida* to observe the morphological features of cultivated fungi can help us in identifying the strains even upto the species level. Furthermore a culture time of 5 days or more is generally required for identification of these fungal species.
- The biochemical tests including the physiological, fermentation and the assimilation tests along with the growth of the *Candida* species on CHROM Agar *candida* were quite suitable for their identification upto species level.
- The classical morphological approach and the biochemical testing approach both are good for the identification upto species level, but the molecular identification approaches based on polymerase chain reaction (PCR), amplification of genomic DNA followed by sequencing of resulting amplicons is the most promising technique for their identification. The identification and taxonomic analyses of fungal pathogens are increasingly becoming dependent on modern molecular techniques, based on PCR amplification of conserved regions of the genome and sequencing of the resulting PCR products.
- The universal fungal primers relating to the internal transcribed spacer region (ITS1-TCCGTAGGTGAACCTGCGG, ITS2-GCTGCGTTCTTCATCGATGC, ITS3-GCATCGATGAAGAACGCAGC and ITS4-TCCTCCGCTTATTGATATGC) are quite effective for estimating fungal diversity in environmental samples as they amplified the genomic DNA extracted from all the fungal strains used in this study quite effectively.
- The Dal Lake is not only rich interms of the diversity of other biodiversity components like phytoplanktons, zooplanktons, periphytons and macrophytes but

also interms of the fungal diversity which was assessed for the first time with various isolation approaches (like direct plating and baiting methods) and identification approaches (like morphological approach, biochemical approach and molecular approach).

- Dal Lake is a species rich habitat in terms of the fungal diversity also as a large collection of the fungal component in the lake belonging to two main groups viz. the filamentous group and the Zoosporic group with twenty eight (28) species and twenty three (23) species belonging to the two groups respectively were observed during the study.
- Dal Lake gives a unique opportunity to investigate the microbiology of the typical low altitude urban lake environment that remains completely influenced by different types of anthropogenic activities all through the years.
- The variation in the environmental setup (including the human interference, catchment area conditions, catchment area activities, commercial activities and other realted activities) of various microhabitats selected for the present study with respect to the fungal diversity in a vast habitat like Dal Lake is having a profound influence on the percentage occurrence, diversity, density, evenness, dominance and species variability of the fungal species.
- The species of filamentous group of fungi were mainly dominating the outlet sites, thus indicating the influence of sewage and other surface runoff being added in heavy loades to these sites.
- Seasonal variation has got a prominent effect on the fungal load of water bodies as the heavy loads of fungal colonies were registered in the warm temperature seasons compared to the cold temperature seasons.
- The occurrence of fungal species showed a remarkable seasonal and temporal variation across the lake with *P. chrysogenum, S. parasitica, Dictyuchus* spp., *A. cornuta* and *P. funiculosum* as the five most dominant and prominent species across the lake.
- Species like *P. chrysogenum, S. parasitica, A.cornuta, A. conspicua* and *P.*

funiculosum are a few dominant species occurring at the open water sites however *P. chrysogenum*, *A. conspicua*, *P. funiculosum*, *Dictyuchus* spp., and *A. cornuta* are the most dominant representative of the littoral sites.

- In case of the outlet sites the most prominent species included *P. chrysogenum*, *P. proliferum*, *P. debaryanum*, *S. parasitica* and *B. diclina* while as for the inlet sites they included *A. moniliformis, S. parasitica, P. chrysogenum*, *Dictyuchus* spp. and *A. niger*.
- A number of pathogenic fungal species like *A. flavus*, *A. fumigatus*, *C. albicans*, *C. krusei* and *C. neofromans* were also found present in the lake water, thus infecting a great deal of 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 diseases varies with the type of water being used for different purposes. Higher prevalence of infection 6.77% (26 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 1.30% (5 positive cases among 120 individuals studied).
- As majority of the people are not using the water of this lake for drinking, washing, bathing and other domestic purposes it is not emerging as a serious threat of human fungal infections, but where and when the human beings come in contact with it for a longer time various diseases like skin infections, candidiasis and onchomycosis appear in them.

RECOMMENDATIONS

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

- ✓ Regular studies on the fungal component of the freshwater lakes of Kashmir valley like all other components of biodiversity should be undertaken to know the fungal wealth (changing) of these water bodies. So, that the fungi which could essentially be used for lake restoration does not skip away from our attention.
- ✓ The faungal biodiversity present in existing aquatic habitats should be documented so that the effects of habitat alterations on species composition and functions could be detected.
- ✓ Mycological studies of aquatic habitats should be given due emphasis and preference due to the relevance of fungi and their activities in water as indicator organisms, as human, animal and plant pathogens, as a source of food and energy for macrofauna, as agents in self-purification process, in the remineralisation of organic materials and as a detector of influence of catchment area upon the lake.
- ✓ Studies on modern scientific lines on the use of these fungal species as the agents of bioremediation should be prioritized in order to restore the ever degrading lake habitats.
- ✓ Beside the use of classical approaches of culturing of microorganisms from different environmental samples, the community DNA approach, Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE) should be used to determine the genotypic diversity of fungi occurring in the different lake substrates as the fungi revealed by DGGE comes out to be completely different fom those revealed by the traditional culture-based methodology. This has important scientific implications in fungal ecology as well diversity and functions of fungi.
- ✓ Use of specifically designed primers is recommended to know the

phylogenetic relationship of the unknown microbial cultures.

- ✓ Work on the extraction, identification and characterization of active principles from the isolated fungal strains from the lakes should be carried out in depth as this particular aspect has a great commercial and industrial relevance.
- ✓ The drainage from the adjoining areas of the lake should be managed in such a way that it doesn't find its way directly into the lake. By doing so we can prevent the lake water from becoming a life threatening entity.
- ✓ Besides monitoring the lake water for various physico-chemical parameters it should be monitored regularly in order to check the presence of life threatening microbes.
- ✓ The Dal dwellers and others who are living in the catchment areas of the lake need to be made aware of the deadly fungal infections and other related diseases that can be caused by the polluted lake water.
- ✓ The association of fungi with other biodiversity components of the lakes needs to be studied in order to know the details about their relationships.
- ✓ Research in the area of applied microbiology should continue with an interdisciplinary approach where traditional microbiologists collaborate with molecular biologists, animal scientists, biologists and environmentalists.

Chapter 7

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Questionnaire for Surveillance of Fungal Diseases

S. No. **Date:**

Location: **Age:**

Name: **Sex:**

Parentage: **Merital status:**

Occupation:

Source of water supply:

Piped water Lake Others

Condition of drinking water: Boiled/Un-boiled

Have you noticed visible stains on the walls? *Yes/No*

Have you noticed visible stains on the ceiling tiles? *Yes/No*

Does your home/apartment have a musty odor? *Yes/No*

Have you noticed mold or mildew? *Yes/No*

Personal hygiene: Clean/Dirty

Clinical symptoms:

Skin irritation	□	Itching	□	Hair loss	□
Dry skin	□	Allergy	□	Dandruff	□
Deformed nails	□	Conjunctivitis	□	Others	□
Candidiasis	□	Red spots	□		□

Do you take any kind of medicine regularly? *Yes/No*

If YES, what kind?