SCREENING, ISOLATION AND IDENTIFICATION OF MICROORGANISMS FROM PRE-TREATED SEWAGE WATER

Thesis submitted in partial fulfillment of the requirements for the degree

of

MASTERS OF PHILOSOPHY

in

MICROBIOLOGY

Submitted by

Pooja Pradhan

Roll. No. 12PDMB06

Reg No. 12SU6400

Department of Microbiology

Sikkim University

Tadong Gangtok-737102

2014

सिक्किम विश्वविद्यालय
(भारतीय संसद के अधिनियमद्वारा स्थापित केन्द्रीय विश्वविद्यालय)
गुणवत्तापूर्ण प्रबंधन प्रणाली ISO 9001:2008 हेतु प्रमाणित संस्थान
SIKKIM UNIVERSITY
[A Central University established by an Act of Parliament of India in 2007]
An ISO Quality Management System 9001:2008 Certified Institution

DECLARATION

I declare that the thesis entitled **"Screening, Isolation and Identification of Microorganisms from Pre-Treated Sewage Water"** submitted by me for the award of **Master of Philosophy (M.Phil) Degree in Microbiology** of Sikkim University is my original work. The content of this thesis is based on the experiments which I have performed myself. This thesis has not been submitted for any other degree to any other University.

P. Pradhan

Date: 22nd July 2014

Pooja Pradhan

Roll No: 12PDMB06

Regn.No: 12SU6400

We recommend that the thesis be placed before the Examiners for evaluation.

HK

Signature of Head of the Department

Dr H K Tiwari

Bimala Singh

Signature of the Supervisor

Dr Bimala Singh

ASSISTANT PROFESSOR
Department of Microbiology
School of Life Sciences
SIKKIM UNIVERSITY
6th Mile Samdur P.O. Tadong Sikkim

6 माईल, सामदुर, तादोंग, गगंटोक - 737102 सिक्किम, भारत
दूराभाष : 00-91-3592 - 251067, 251403, फैक्स :- 251067/251757

6th Mile, Samdur, PO Tadong 737102, Gangtok, Sikkim, India
Phones : 00-91-3592-251067, 251403, Fax - 251067/251757
website : www.sikkimuniversity.in/www.sikkimuniversity.ac.in
Email : sikkimuniversity@gmail.com

सिक्किम विश्वविद्यालय
(भारतीय संसद के अधिनियमद्वारा स्थापित केन्द्रीय विश्वविद्यालय)
गुणवत्तापूर्ण प्रबंधन प्रणाली ISO 9001:2008 हेतु प्रमाणित संस्थान
SIKKIM UNIVERSITY
[A Central University established by an Act of Parliament of India in 2007]
An ISO Quality Management System 9001:2008 Certified Institution

CERTIFICATE

This is to certify that the thesis entitled **"Screening, Isolation and Identification of Microorganisms from Pre-Treated Sewage Water"** submitted to the **Sikkim University** for the award of **Master of Philosophy (M.Phil) Degree in Microbiology,** embodies the results of *bona fide* research work carried out by Ms. Pooja Pradhan under my guidance and supervision. No part of the thesis has been submitted for any other degree, diploma, associate-ship and fellowship.

All the assistance and help received during the course of the investigation have been acknowledged by her.

Bimala Singh

Place: Gangtok, Sikkim

Date: 22nd July 2014

Dr. Bimala Singh

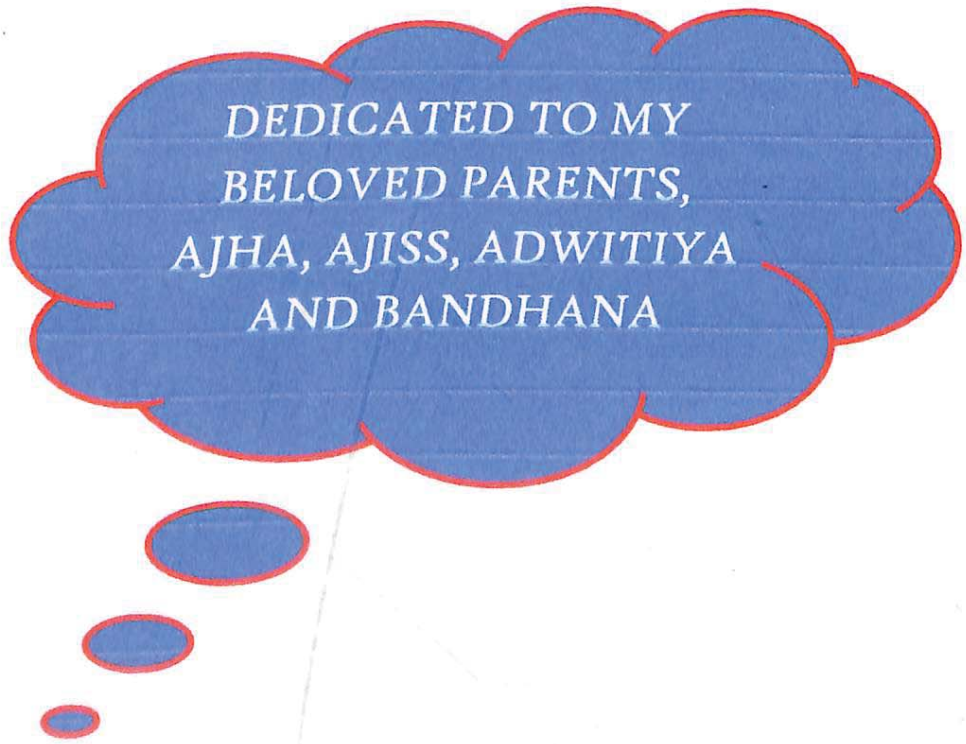
(Supervisor)

Department of Microbiology

ASSISTANT PROFESSOR
Department of Microbiology
School of Life Sciences
SIKKIM UNIVERSITY
6th Mile Samdur P.O. Tadong-Sikkim

6 माईल, सामदुर, तादोंग, गगंटोक - 737102 सिक्किम, भारत
दूराभाष : 00-91-3592 - 251067, 251403, फैक्स :- 251067/251757

6th Mile, Samdur, PO Tadong 737102, Gangtok, Sikkim, India
Phones : 00-91-3592-251067, 251403, Fax - 251067/251757
website : www.sikkimuniversity.in/www.sikkimuniversity.ac.in
Email : sikkimuniversity@gmail.com


DEDICATED TO MY
BELOVED PARENTS,
AJHA, AJISS, ADWITIYA
AND BANDHANA

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my research supervisor Dr. Bimala Singh, for her patient guidance and enthusiastic encouragement. I would also like to thank Honorable Vice – Chancellor of Sikkim University Prof T.B Subba, for providing us with requisite infrastructure. My grateful thanks are also extended to Prof. Jyoti Prakash Tamang (Dean, Sikkim University and Professor, Department of Microbiology, Sikkim University, Dr. Hare Krishna Tiwari (Associate Professor), Dr. Buddhiman Tamang (Assistant Professor) and Dr. Nagendra Thakur (Assistant Professor), Department of Microbiology, Sikkim University for their guidance and advice. Special thanks to Dr. Laishram Shanti Singh (Scientist B, RCIBSD) for his support and valuable suggestions.

I would also like to thank the staff of the following organizations for their co-operation and for enabling me to visit their offices to observe their daily operations:
P.H.E Department, Govt of Sikkim,India
Adampool Sewage Treatment Plant, Adampool, Gangtok. East Sikkim.
Regional Center of Institute of Bioresource and Sustainable Development (RCIBSD), Sikkim.

I would also like to extend my thanks to the technician of Microbiology and Chemistry Department for their help in offering me the resources during my research, my friends and my seniors for their advice and guidance.

Finally, I wish to thank my parents for their support and encouragement throughout my study.

Pooja Pradhan

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Sewage or the wastewater is defined as a mixture of liquid and waste discarded from residents, institutions, hospitals, commercial and industrial establishments together with ground water, rain water and surface water (Cheremisinff, 2002). The waste water discharged from domestic premises such as community and commercial establishments is generally termed as "sewage /community wastewater" (Niraj *et al*, 2011). Composition of waste water consist of organic material like human waste, paper, plastics, vegetable matter, toxic chemicals, 99% water and 0.1% solids (Niraj *et al*, 2011). In addition to domestic waste, sewage also consists of industrial and hospital wastes (Niraj *et al*, 2011). The high load of organic compounds in the sewage makes it a suitable and highly optimal environment for the survival and growth of various microorganisms which also includes pathogenic microbes. Sewage also consists of influx from hospitals; it harbors various highly pathogenic organisms. However, since the pathogenic microorganisms require certain optimal conditions and enrichment for its growth, the population of pathogens compared to those of common microorganisms is low (Gerba *et al*, 2009). Thus the treatment of sewage to reduce both organic and microbial load and removal of pathogens becomes essential before its discharge into the water bodies. Various technologies have been designed since 1900 for the treatment of wastewater before its disposal to the water bodies and its reuse especially in developing countries (Niraj *et al*, 2011).

Various adverse effects of releasing untreated sewage into the environments are as follows:

- Production of large quantity of malodorous gases due to the decomposition of organic matter in the sewage, which leads to air pollution (Niraj *et al*, 2011).
- Due to high organic matter load in the sewage there will be rapid consumption of dissolved oxygen to meet the BOD requirement, which will be unfavorable to the aquatic life along with other undesirable effects (Spellman, 1999).

- Wastewater may also contain nutrients, which can stimulate the growth of aquatic plants and algal blooms; thus, leading to eutrophication of the lakes and streams (Niraj *et al*, 2011) and
- Untreated sewage contains various pathogens from human intestine and toxic compounds from the industries which when released in the land or the water bodies may contaminate it (Niraj *et al*, 2011; Spellman, 1999).

Various wastewater treatment plant (WWTP) have been designed which works on various processes to treat the sewage water. (Mara & Horan, 2003). The WWTP works to reduce the organic matter load and removal of microorganisms from the sewage. Removal of pathogens from the sewage depends strongly on the treatment process used (aerobic/anaerobic) and the duration of the process (Hoogenboezem, 2007). Most of the treatment plant is designed to remove the organic matter and removal of pathogen during this process is just a matter of chance (Mara and Horan, 2003). Common microorganisms present in sewage include bacteria, protozoa, helminthes and viruses. These pathogenic microorganisms survive in the environment for a long period and usually pathogenic bacteria will be present in much lower levels as compared to coliform group of bacteria (FAO, 2004). Some of the pathogenic bacteria present in raw sewage are *Campylobacter, Enterohaemoragic E.coli, Helicobacter, Salmonella typhi, Salmonella enterica, Shigella, Vibrio cholerae* (Mara and Horan, 2003), *Streptococcus pneumoniae* (Agarwal *et al*, 2010). *Entameoba histolytica* ,*Giardia lambli* and *Balantidium coli* are the protozoan commonly present in raw sewages (FAO, 2004) and among helminthes *Ascaris lumbricoides, Schistosoma mansoni, Taenia saginata,* and *Trichuris trichiura* are the most commonly found (Feachem *et al*, 1983) Some of these microorganisms present in the raw sewage become aerosolized during the aeration used in treatment process (Teltsch *et al*, 1980). Thus many of the pathogenic microbes present in the raw sewage gets transmitted through air causing health hazards to the workers in the treatment plant (Teltsch *et al*, 1980).

Another matter of concern is the increasing incidence of resistant microorganisms to a wide spectrum of antibiotics. Studies have shown the presence of antibiotics in trace amounts in sewage treatment plants influent as well as in effluent. The intensive use of antibiotics in both animal husbandry and pharmaceuticals and the incomplete metabolism of these antibiotics results in the release of large amounts of these chemicals in the sewage water (Pathak *et al*, 2011). The constant exposure of the microorganisms to these antibiotics has resulted in the development and selection of resistant species of bacteria to a wide spectrum of antibiotics (Christian *et al*, 2003; Close, 2007).

Due to the rapid growth in the population and increase in industrialization in the recent years, the rate of waste disposal in the environment has exceeded the rate of its purification. This has led to the rapid pollution of the environment specially the water bodies, which are the basic and vital resource of our planet (Kumar *et al*, 2012). In country like India 70% of the available water is polluted, with the major pollutant being identified as sewage (Kumar *et al*, 2012; Agarwal *et al*, 2010).

The present study focuses on the topic screening, isolation and identification of microorganisms from pretreated sewage water of the Adampool sewage treatment plant (STP), Gangtok. The study becomes important as Adampool STP is the only functional Waste Water Treatment Plant (WWTP) in the town of Gangtok. This WWTP receives waste influents from the entire town, which includes domestic, hospital and municipal wastes. The treated effluent is released in the river Rongni Chu. The present treatment capacity of the plant at Adampool is 10 million litres per day (MLD) although the liquid waste generation is 20 MLD (Annual Report of Water security and Public Health Engineering Department, Govt of Sikkim, India, 2012). Therefore, it becomes necessary for the microbial analysis of the sewage influent to estimate the hazard due to fecal pollution as well as to evaluate the presence and the concentration of pathogenic organisms.

CHAPTER 2

RATIONALE AND SCOPE OF STUDY

2. RATIONALE AND SCOPE OF STUDY

Water quality has always been a matter of concern, as it is one of the most important resources required for man. However, the quality of water is rapidly deteriorating due to the constant input of waste materials resulting from domestic and industrial use into these water bodies. One of such waste materials includes raw sewage, which is one of the major source of water pollution and spread of certain human diseases. Thus, the proper treatment of sewage water before its release into the water bodies becomes highly essential to maintain the water quality.

In Sikkim, Gangtok is the only town to have a full-fledged sewerage system and it caters to approx 25,000 people. This sewerage system has been an immense help for the community. It is also helping to keep river *Rongni Chu* from being excessively polluted. Sewage is treated at Adampool Treatment Plant to render it harmless and finally disposes off into the river body .This Treatment plant receives sewage from the entire town of Gangtok. The sewage coming to this plant contains influent from domestic waste and hospital waste. After the treatment, the effluent is released into the river (*Rongni Chu*), (Annual Report of Water security and Public Health Engineering Department, Govt of Sikkim, India). Since this treatment plant operates to treat the wastewater coming from the entire town it is necessary to evaluate its working efficiency. Thus, the study and analysis of microbial population and the physical parameters of the sewage water in both the pre-treated as well as post-treated sewage water becomes highly essential both to study the type of microbial population harbored by the influent and also to evaluate the working efficiency of the sewage plant.

The present study therefore focuses on analysis of microbial flora and physical parameters of raw sewage sample from Adampool sewage treatment plant and the isolation of some bacterial population belonging to *Enterobactericeae* along with the identification of these isolated bacterial population based on morphological and biochemical characterization.

CHAPTER 3

AIMS AND OBJECTIVES

3. AIM AND OBJECTIVES OF THE STUDY

- To study some physico-chemical parameters of the Pre-Treated sewage water.
- To isolate and characterize some bacterial population present in Pre-Treated sewage water.
- To identify the isolated Microorganisms based on morphological and biochemical characteristics (presumptive identification).

CHAPTER 4

REVIEWOF LITERATURE

4. REVIEW OF LITERATURE

Water is the most important factor for the sustenance of mankind. Water is used by both terrestrial as well as aquatic environments for various activities including the balancing of ecological system of global environment. Water is an important natural source and is abundant in nature covering over 2/3rd of earth surface. However only 1% of the fresh water present is available for human consumption and other activities. The major uses of water are for irrigation (30%), thermal power plants (50%), while other uses are domestic (7%) and industrial consumption (~12%) (De, 2002).As the human population grows there is an increased pressure on quality and quantity of drinking and fresh water resources and on their availability (Pindi *et al*, 2013). Adequate supply of drinking water is a prerequisite of a healthy life (Fawell *et al*, 2003). Today safe drinking water remains unavailable to several billion people around the globe and is more prevalent in developing countries (Pindi *et al*, 2013). Poor water quality is one of the reasons of child mortality in developing countries, which is mainly through infectious diarrhea (Pindi *et al*, 2013). According to WHO estimates, around 110 million people in the world drink unsafe water and about 80% of diarrheal disease across the globe is due to contaminated water, poor sanitation and hygiene conditions (WHO, 2013). Drinking water is derived from two sources:

1. Ground water/ sub surface sources: wells, tube wells, springs, infiltration, wells etc (Fawell *et al*, 2003).
2. Surface water such as rivers, ponds, lakes, streams, oceans and reservoirs (Fawell *et al*, 2003).

Ground water is the chief source of drinking water in India and constitutes 0.61% of total available water on earth (Singh, 2011). Drinking water is polluted in number of ways. Pollution which generally means to make water foul or unclean occurs when large amount of materials and wastes are added to water bodies which makes it unfit for use. Natural processes are some of the ways by which water gets polluted such as inorganic

contaminants are mostly added as the water flows through various geological strata but human activities play a major role in water pollution (Fawell *et al*, 2003).

Anthropogenic activities play a major role in polluting the water bodies. Such activities detrimentally affect the quality and quantity of remaining fresh water resources on earth which is an issue of major concern at present. However, ground water is less contaminated than surface water and all the sources of contamination can be broadly classified into two types (Fawell *et al*, 2003):

1. Point source: sources of contamination are industrial and sewage discharge.
2. Diffuse source: run off from agricultural land and roads, traffic, corrosion from roofing and deposition

Among the various point sources, improperly treated sewage discharge is one of the major pollutants of water bodies. Sewage is the waste water resulting from the discharge of human excreta, households, hospitals, slaughter houses and industries into sewers (Hoogenboezem, 2007; Guardabassi *et al*, 2002). Sewage contains various mixtures of organic and inorganic matters along with various microorganisms some of which are pathogenic .The hospital and slaughter house discharge contains important microbial contaminants and the industrial discharge contains contaminants of chemical nature (Hoogenboezem, 2007).

The sewage constitutes 99.9% water, suspended solid materials, organic matter, inorganic matter,various nutrients, toxic chemicals, elements, antibiotics from industries and hospital influents (Xiaochang *et al*, 2007) and high density of living organisms which includes commensals, pathogens and environmental bacteria (Eze *et al*, 2009). The organic matter comprises of carbohydrates, proteins, and fats however not much is known about these primary organic constituents (Heukelekian *et al*, 1959). The various minerals present in the sewage are calcium, chlorine, magnesium, manganese, sodium, zinc, lead, sulphur, aluminum, silicon, potassium, iron, copper and phosphorous (Heukelekian *et al*, 1959). The carbohydrates present in sewage are cellulose, hemicelluloses, lignin and pectin (Heukelekian *et al*, 1959).

Various toxic chemicals are also released in the sewage from industries and hospitals. Examples of such toxic chemical are endocrine disrupting chemical (EDC), 17α-ethynylestradiol (EE2) which is major component of oral contraceptive pills, carbamazepine and diclofenac used as anti-epileptic and anti-inflammatory drugs, respectively (Zhang *et al*, 2008). Such chemicals are very harmful and interfere with proper functioning of endocrine system of human as well as animals (Zhang *et al*, 2008).These organic chemicals are released in the sewage and environment via various anthropogenic activities (Zhang *et al*, 2008).

Antibiotics used to treat humans also find its way into the sewage treatment plants. Antibiotics are a group of pharmaceuticals used to treat several kinds of diseases and have an important role in human and veterinary medicine (Kummerer, 2010). These substances are used in large quantities approximately 100000- 200000 ton per year all over the world (Lindberg *et al*, 2004). Many of these antibiotics are excreted unchanged/unmetabolized through the urine or faeces after administration (Deng, 2013). These antibiotics also enter the sewage and environment as as a result of improper disposal of unused medicine (Giger *et al*, 2003). Recent studies have shown the presence of trace level concentration of antibiotics in waste water treatment plant effluents (Christian *et al*, 2003; Close, 2007). The concentration of antibiotics in the sewage range between sub or low ng L^{-1} to mg L^{-1}. Antibiotics such as Sulfamethoxazole widely used in human and veterinary medicine and Fluroquinolones have been reported to be found in the sewage (Vieno *et al*, 2007; Golet *et al*, 2003; Hong *et al*, 2008). These antibiotics found in the sewage are either eradicated by sewage treatment process or is released with the effluent in the environment (Hirsch *et al*, 1999).

Another component of sewage is heavy metals which is widespread as a consequence of industrial influent. Heavy metals such as mercury, lead, cadmium, silver, chromium, nickel, molybdenum etc are found to be present in the sewage, (Jakubus *et al*, 2001). Most of these heavy metals are not removed during the treatment process and therefore, persistent in the sludge. These sludges due to their high organic matter content are used as a fertilizer in agricultural fields which results in the accumulation of these heavy metals in the environment (Taylor *et al*, 1995).

Sewage also harbors various microorganisms some of which are highly pathogenic. The rich organic and inorganic contents of the sewage serve as a suitable nutrient rich medium for the growth and survival of many microorganisms. However, the pathogenic microorganisms require certain optimal conditions and enrichment for its growth, thus the population of pathogens compared to those of common microorganisms is usually low in sewage (Gerba *et al*, 2009). The species and the quantity of pathogenic organisms in the sewage depend on the health status of the local community and vary substantially at different times (EPA, 2003). Common microorganisms reported from sewage are viruses, bacteria, protozoa, helminthes and fungi. Viruses that are reported from sewage includes plant viruses belonging to the members of the *Alphaflexiviridae*, *Betaflexiviridae*, *Bromoviridae*, *Closteroviridae*, *sobemovirus*, *Tombusviridae*, and *Tymoviridae* , insect viruses belonging to members of the *Dicistroviridae*, *Iridoviridae*, *Nodaviridae*, and *Parvoviridae* families, human viruses such as *Astroviruses, Norwalk virus, Aichi virus, Parechoviruses, Echoviruses, Reoviruses and Polyomavirus* (Cantalupo *et al*, 2011). Bacteria that are commonly found in sewage include *Bacillus sp, Pseudomonas sp, E.coli, Klebsiella sp, Campylobacter jejuni, Vibrio cholerae, Salmonella sp, Shigella sp,* and *Yersinia sp* (EPA, 2003).. Protozoans found in sewage include *Cryptosporidium, Entamoeba histolytica, Giardia limblia, Toxoplasma gondii* and *Balantidium coli* (EPA, 2003). Helminthes commonly reported from sewage are *Ascaris lumbricoides, Ascaris suum, Toxocara canis, Taenia saginafa, Taenia solium* and *Trichuris trichiura* (EPA, 2003).

Along with the diverse population of microorganisms found in the sewage, there are also reports of occurrence and emergence of drug resistant organisms. The intensive use of antibiotics in medicine and animal husbandry has resulted in inducing a selective pressure on various bacteria resulting in the development of multi drug resistance in them. The emergence of resistant strains is always associated with the introduction of new antimicrobial agent and its indiscriminate use (Pathak *et al*, 2011). Long term exposure of microorganisms to low concentrations of antibiotics found in the wastewater has the potential for the development of antibiotic resistance in these organisms. There are several reports, which document the presence of such multidrug resistant bacteria in

sewage. Vancomycin resistant *Enterococci* (VRE) have been reported in the sewage sample (Schwartz *et al*, 2003). It has been found that about 1 % of the total lactose fermenting enteric bacteria found in both raw as well as treated sewage are resistant to one or more antibiotic used in animal feed and therapeutics (Sturtevant *et al*, 1971). One of the clinically important Gram negative bacteria which have acquired resistance to multiple antibiotics belongs to the family Enterobactericeae (Pathak *et al*, 2011). Tetracycline resistant Enterobactericeae have been isolated from the sewage treatment plant of Hong Kong, Shanghai in China and the bay area in California of the United States (Tongzhang *et al*, 2009). On the examination of drug resistance among the fecal coliforms isolated from raw sewage sample, it was found that approximately 3% of the coliform bacteria in both total and fecal coliform group were found to show resistance to two or more antibiotics (Sturtevant *et al*, 1971). In one of the treatment plant effluents in Finland, it was found that more than 20% of fecal coliforms were resistant to ampicillin, sulfanomide, tetracycline and streptomycin (Niemi *et al*, 1983).

Along with the antibiotic resistance, metal tolerance characteristics were also found to be exhibited by bacteria isolated from raw sewage. Various bacteria such as *Pseudomonas sp, Enterobacter sp, Acinetobacter sp* ,isolated from raw sewage have been found to exhibit tolerance to many metals such as lead, zinc, mercury, aluminum etc. Considerable evidences have shown that antibiotic resistance is directly correlated to metal tolerance in bacteria. According to McArthur and Tuckfield (2000), metal tolerance and antibiotic resistance are closely linked and the expression of the antibiotic resistance may be dependent on exposure to metals (McArthur *et al*, 2000). This is supported by the finding that unspecified mechanisms conferring resistance to both metals and antibiotics exists in some bacterial species for example active pump-efflux system encoded by the *mar* A gene in *E.coli*. In addition, bacteria belonging to same genus isolated from heavy metal polluted marine sediment are significantly more resistant to antibiotics than their counterparts isolated from unpolluted sites (Rasmussen *et al*, 1998). The genes coding for the metal tolerance and antibiotic resistance are found in the extra chromosomal DNA also known as plasmids in bacteria. Resistant bacteria have the ability to transfer these plasmids to susceptible bacteria using various mechanisms such as conjugation, which

renders the susceptible bacteria resistant. Thus, sewage containing these resistant bacteria, if released in the environment becomes the source for the spread of resistant plasmids from these bacteria to other susceptible bacteria, which will result in the hazardous spread of multi drug and metal tolerance organism in the environment. Several studies have reported the occurrence of antibiotic resistant organisms in the environmental samples. For example the prevalence of antibiotic resistant bacteria in the major rivers of US (Ash *et al*, 2002). A recent study have shown the occurrence of multi-antibiotic resistant fecal coliforms and *Enterococci* in influent and effluent wastewater from treatment plants and the release of these antibiotic organisms into the streams through the wastewater effluent (Gallert *et al*, 2005).

There are various adverse effects of releasing the untreated sewage, treated effluent and sewage sludge in the environment. The sewage sludge is often used for agricultural purposes due to its abundance of organic matter and nutrients (Czekala, 1999). Currently the United States and a growing number of western European countries apply almost half of their sewage sludge onto the agricultural fields (Lewis *et all*, 2002). The application of sewage sludge has an advantageous effect on the physical and chemical properties of the soil. It increases the bioavailability of phosphorous, increase in cation exchange capacity and also supplies the soil with exchangeable ions of Ca^{2+} and Mg^{2+} (Cavallaro *et al*, 1993). However, one of the limitations and hazard of using sewage in agriculture is the presence of various chemical compounds including unstable amounts of metals and the formation of combinations with various degrees of their release to the environment (Czekala, 1999).

The release of sewage in the water bodies is one of the major causes of environmental pollution, mainly aquatic environment; it is also a source of spread of various diseases and affects the sustainability of living resources and public health (Ouseph *et al*, 2009). Wastewater discharge is considered as one of the most significant threats of the coastal environments worldwide as these areas of the world are inhabited by over 60% of the human population (GPA, 2001). There are various effects of sewage discharge on the water quality and on aquatic ecosystem. The deleterious effect of sewage effluent on the receiving water quality depends on the volume of the effluent discharged, its chemical

composition, and the concentration of the effluent, amount of suspended solids, organic matter, and hazardous pollutants like heavy metals, antibiotics and also on the characteristics of the receiving waters. The receiving water bodies with high flushing capacity dilute and eliminate most of the contaminants. However, there are some toxic compounds and pathogens, which are highly persistent and survive, contaminating the receiving water (NAP, 1984; Canter, 1996; Nemerow and Dasgupta, 1991). There are reports of occurrence of enteric bacteria such as *E.coli, Salmonella, Vibrio* and *Shigella* along the southern coast of Kerela. Pradeep and Lakshmanaperumal, (2009) have reported that the main source of fecal coliforms and other enteric pathogen in the Cochin transects is mainly through the river water which carries land runoff, sewage discharges and storm water (Pradeep *et al*, 1986; Ouseph *et al*, 2009).

The disposal of sewage in the water bodies also results in the accumulation of various chemical compounds in the aquatic environment. Organochlorine compounds such as the polychlorinated biphenyls (PCBs), dichloro diphenyltrichloroethane and metabolites (DDTs), chlordanes, dibenzo-p-dioxins (Ds) and dibenzofurans (Fs), polycyclic aromatic hydrocarbons (PAHs) and sulphur hetero cycles were found to accumulate in the marine harbor sediments, which received an inflow of raw sewage (Hellou *et al*, 1999).

Antibiotics such as Fluoroquinolones and Sulfamethoxole which are extensively used as antimicrobial in animals and humans have been found in both raw as well as treated sewage water (Peng *et al*, 2006). The release of these antibiotics into the environment via effluent discharge can cause adverse effects on aquatic microorganisms (Lindberg *et al*, 2007) and also remains persistent in the environment (Hong *et al*, 2008).Wastewater may also contain nutrients, which can stimulate the growth of aquatic plants and algal blooms; thus, leading to eutrophication of the lakes and streams. Reports have found that the discharge of raw sewage in the water bodies results in the blooming of algal species such as *Noctiluca scintillans* in much frequent rate. (Umani *et al*, 2004)

The sewage water is used as a potential source of irrigation by the farmers in India for raising vegetables and fodder crops. Soil which is contaminated by sewage water adversely affects both soil health and crop productivity as many of this untreated and

contaminated sewage harbors many heavy metals such as cadmium, lead, nickel and chromium and pathogens. The continued use of effluent irrigation has resulted in soil sickness (Narwal *et al*, 1988) along with the accumulation of harmful chemicals present in the effluent in the soil (Adhikari *et al*, 1993; Antil & Narwal, 2005, 2008; Antil *et al*, 2004, 2007; Gupta *et al*, 1986, 1994, 1998; Kharche *et al*., 2011; Narwal *et al*., 1993) which may pose serious impact on human and animal health. There are reports of health hazards due to the absorption of nickel by the crops grown in the fields irrigated with sewage containing industrial effluent (Antil, 2012).

The pathogen present in the sewage also gets aerosolized during the treatment by trickling filters, activated sludges and during spray irrigation of land (Hickey *et al*, 1975; Sorber *et al*, 1975). This air borne transmission of pathogens is of growing concern and there are presumptive evidences of increased incidence of enteric diseases due to such aerosolized wastewater pathogens among the nearby residence (Katzenelson, 1976). In one of the studies done on the wastewater irrigated fields, it was found that the frequency of airborne emissions of *Salmonella* and *Enteroviruses* were 18 % and 44% respectively (Teltsch, 1980).

Sewage also results in the air pollution by the production of malodorous gases produced during the decomposition of organic matter present in the sewage. Harmful gases such as hydrogen disulfide, methane, ammonia and carbon monoxide are produced which pollutes the air and causes severe respiratory disorders in persons who are exposed to such gases (Tiwari, 2003). Exposure to such gases produces several health problems such as cardiovascular degeneration, musculoskeletal disorders like osteoarthritic changes and intervertebral disc herniation, infections like hepatitis, leptospirosis and Helicobacter, skin problems, respiratory system problems and altered pulmonary function parameters (ILO, 1970; Tiwari, 2003). Watt *et al* found that 53.8% of sewer workers who are exposed to sewer gases developed sub-acute symptoms including sore throat, cough, chest tightness, breathlessness, thirst, sweating, irritability and loss of libido (Watt, 1997).

The heavy microbial load along with the presence of various harmful chemicals in the sewage makes the treatment of the sewage essential before its release in the environment. Various treatment plant have been designed which works on various technologies for the treatment of sewage water before its disposal to the water bodies and its reuse especially in developing countries (Niraj *et al*, 2011). The objectives of Sewage treatment plant are to produce an environmentally safe effluent suitable for disposal and a solid waste (sludge) for reuse as farm fertilizer which can be achieved by reduction of Biological Oxygen Demand (BOD), and removal of suspended solids, and pathogenic microorganisms (Khopkar, 2004). The contaminants of waste water are removed by physical, chemical and biological processes in the treatment plants (Brock, 2006). The heavy metals are removed from the raw sewage mainly during the physical and chemical treatment processes which involves sedimentation with suspended solids and activated sludge flocs, co-precipitation by organic compounds and chemical precipitation (Kulbat *et al*, 2003). Heavy metals are also removed by microbiological processes where they are actively bound by living organisms by means of intracellular accumulation, extracellular precipitation and chemical transformation such as oxidation, reduction, methylation and demethylation by these organisms and binding of heavy metals to active groups of chemical compounds of cell walls and membranes (Macaskie *et al*, 1989; Tobin *et al*, 1990). The removal of heavy metals by various processes depends on applied technology of wastewater treatment, type and concentration and oxidation state of metal, composition and pH of wastewater, type of microorganisms (Olanczuk-neyman *et al*, 1975) and mechanism of metal removal (Brierley, 1990).

The removal of antibiotics and other pharmaceutical compounds from the sewage depends on the nature of the drug, temperature of the wastewater, the hydraulic and solid retention time, age of the activated sludge, environmental conditions and characteristics of the raw influent (Vieno *et al*, 2007; Zuccato *et al*, 2010). Studies have also shown that dilution of raw sewage by heavy rain also reduces the efficiency of treatment plant in removal of pharmaceuticals (Ternes, 1998). Hydrophilic antibiotics such as fluoroquinolones are mostly removed from the sewage during the treatment via sorption to the sludge (Golet *et al*, 2003). However studies have shown that the antibiotics are not

efficiently removed from the influent during the treatment process and antibiotics such as fluoroquinolones and sulfamethoxazole are detected in the effluent even after the treatment (Hendricks *et al*, 2012).

Sewage treatment plants are designed to reduce and oxidize the putrescible organic matter in the sewage so that the resulting effluent does not reduce the dissolve oxygen content of the receiving water bodies. Studies have shown that the treated effluent satisfying the recommended standards may contain a large number of disease causing organisms (Kabler, 1959). It has been found that the chlorination which is normally used to disinfect the treated sewage effluent is inefficient to kill all the pathogens in the effluent (Periasamy *et al*, 2013). New approaches have been discovered which have been found to be effective in reducing the pathogen load in the effluent. One of the studies shows that the pathogen specific phage isolated from the sewage has a potential to eliminate a number of dreadful pathogens from the sewage. This study has paved a way for the use of pathogen specific phage as a biocontrol agent which could be a viable and effective method for controlling and reducing the pathogen load of the sewage effluent (Periasamy *et al*, 2013).

5. MATERIALS AND METHODS

5.1 Materials

All the chemicals used in this study were obtained from HiMedia and Merck, Mumbai.

5.1.1 Instruments used

Autoclave (Instrumentation India)

Laminar air flow (REMI/ROFV170)

Incubator (REMI/CSI-24BL)

Micro pipette (PIPETMAN)

Weighing machine (PL-202-S/03)

Hot air oven (NAINSTRUMENT NAHA0/031/09)

Micro oven (SAMGUNG)

Refrigerator (SAMSUNG RTZ4/2009)

pH meter (8102NUWP(THERMOSCIENTIFIC))

5.1.2 Culture media

Nutrient Agar

Beef extract	3g
Peptone	5g
Sodium chloride	5g
Agar	15g
Distilled water	1000 mL
pH	7.4± 0.2 at 25^{O}C

Nutrient Broth

Peptone	5g
Sodium chloride	3g
Beef extract	3g

Distilled water	1000mL
pH	7.4± 0.2 at 25°C

MacConkey Agar

Peptone	20 g
Trehalose	10 g
Bile salts	1.5 g
Sodium chloride	5 g
Neutral red	0.05 g
Crystal violet	0.001 g
Agar	15 g
Distilled water	1000 mL
pH	7.4

Tri-Citrate Bile Sugar media (TCBS) (M189 HiMedia)

MacConkey Sorbitol Agar (Sorbitol Agar) (M298 HiMedia)

Salmonella-Shigella Agar (SS Agar), Modified (M1032 HiMedia)

Eosine Methylene Blue Agar (EMB)

Peptone	10g
Lactose	5g
Dipottasium phosphate	2g
Agar	13.5g
Eosine Y	0.4g
Methylene blue	0.065g
pH	7.2

5.1.3 MEDIA FOR THE BIOCHEMICAL TESTS

Sim Medium (M181 HiMedia)

Simmons Citrate Agar

Ammonium dihydrogen phosphate	1g
Dipottasium phosphate	1g
Sodium chloride	5g
Sodium citrate	2g
Magnesium sulphate	0.2g
Bromothymol blue	0.08g
Agar	15g
Distilled water	1000 mL
pH	7

MR-VP Medium (Buffered Glucose Broth), (M070, HiMedia)

Triple Sugar Iron Agar

Beef extract	3g
Yeast extract	3g
Peptone	15g
Proteose peptone	5g
Lactose	10g
Saccharose	10g
Dextrose	1g
Ferrous sulphate	0.2g
Sodium chloride	5g
Sodium thiosulphate	0.3g
Phenol red	0.024g
Agar	12g
pH	7.4

Urea Broth Base (M111, HiMedia)

Urea GR (MJ7M572681, Merck)

Arabinose Fermentation Broth

Tripticase	10g
Arabinose	5g
Sodium chloride	5g
Phenol red	0.018g
Distilled water	1000 mL

Rhamnose Fermentation Broth

Tripticase	10g
Rhamnose	5g
Sodium chloride	5g
Phenol red	0.018g
Distilled water	1000 mL

Raffinose Fermentation broth

Tripticase	10g
Raffinose	5g
Sodium chloride	5g
Phenol red	0.018g
Distilled water	1000 mL

Arabinose Fermentation Broth

Tripticase	10g
Arabinose	5g
Sodium chloride	5g
Phenol red	0.018g
Distilled water	1000 mL

Xylose Fermentation Broth

Tripticase	10g
Xylose	5g
Sodium chloride	5g
Phenol red	0.018g
Distilled water	1000 mL

Inositol Fermentation Broth

Tripticase	10g
Inositol	5g
Sodium chloride	5g
Phenol red	0.018g
Distilled water	1000 mL

Dulcitol Fermentation Broth

Tripticase	10g
Dulcitol	5g
Sodium chloride	5g
Phenol red	0.018g
Distilled water	1000 mL

5.1.4 REAGENTS

Gram's Crystal Violet (S012, HiMedia)

Gram's iodine

Iodine	1g
Potassium iodide	2g
Distilled water	300 mL

Safranin (S027, HiMedia)

Kovac's reagent

p Dimethylaminobenzaldehyde	5g
Amyl alcohol	75 mL
Hydrochloric acid	25 mL

Barrit's reagent

Solution A

α- Napthonal	5g
Ethanol	95 mL

Solution B

Potassium hydroxide	40 g
Creatinine	0.3 g
Distilled water	1000 mL

Methyl Red Solution

Methyl red	0.1g
Ethyl alcohol	300 mL
Distilled water	200 mL

***P*- Amino-N,N-dimethylanilineine Oxalate (RM641, HiMedia)**

p- aminodimethylalanine oxalate	0.5g
Distilled water	50 mL

5 % Aqueous Malachite Green

Malachite green	100 g
Distilled water	2 L

0.25 % Methylene blue

Methylene blue	0.25 g
Glacial acetic acid	1 mL
Distilled water	99 mL

Carbol fuschin

Basic fuschin	1g
Phenol crystal	5g
Absolute ethanol	10 mL
Distilled water	100 mL

5.1.5 Chemicals

Concentrated Hydrochloric acid	HG8H580592 (MERCK)
Absolute ethyl alcohol	MB106 (HI MEDIA)
Glacial acetic acid	SRL
Hydrogen peroxide	HI6H560662 (MERCK)
Agar –Agar	RM666-500G (HI MEDIA)
Beef Extract	RM002-500G (HI MEDIA)
Peptone	RM001-500G (HI MEDIA)
NaCl	QE2Q620906 (MERCK)
Phenol	AF7AF57246 (MERCK)
Phenol red	MH5M552152 (MERCK)
Crystal violet	RM961-25G (HI MEDIA)
Grams iodine	10225 (STANBIO)
Safranine	RM1315-100G (HI MEDIA)
Bile salts	RM008-500G (HI MEDIA)
Neutral red	1008-100g (HI MEDIA)
Sodium citrate	RM3935-250G (HI MEDIA)
Sodium thiosulphate	M314-500G (HI MEDIA)
Ferric citrate	M563-500G (HI MEDIA)

5.2 METHODOLOGY

5.2.1 Sampling location

Samples were collected from sewage treatment plant (STP) Adampool, located 10 kms from Gangtok, East Sikkim, 27.3065°N, 88.5828°E.

5.2.2 Collection of samples

Raw sewage sample was collected from Adampool Sewage Treatment Plant, Adampool, Gangtok, East Sikkim. Sewage samples were collected in sterile BOD bottles of 250 mL capacity and immediately transported to the laboratory and stored at 4-8^{0}C until microbial analysis (Atieno *et al*, 2013). The BOD bottles were rinsed three times with the sample water before collection (Young *et al*, 2010; Kumar *et al*, 2012). The samples were analyzed on the same day within 2 hours of collection. All the samples were collected in duplicate during each sampling. Total of 4 sampling were done during the month of November (2013), February (2014), May (2014) and June (2014). The samplings were done in the morning time from 9am-12am.

5.2.3 Period of study

The samples were studied between the months of November 2013 to June 2014.

5.2.4 Isolation of microorganisms

1 mL of the raw sewage sample collected in duplicate (sample A and sample B) was transferred in tube containing 9 mL sterile distilled water. Serial dilutions (10^{-1} – 10^{-9}) was prepared for both samples taking the initial dilution as 10^{-1}. From the dilution range of 10^{-4}, 10^{-5} and 10^{-6}, 0.1 mL of the sample was inoculated in nutrient agar (NA) , EMB, MacConkey agar, Salmonella-Shigella agar, Tri-citrate bile sugar media and Sorbitol MacConkey agar using spread plate technique (Kumar *et al*, 2012; Eze, 2009; Sherman *et al*, 2009). The inoculated plates were incubated at 37^{0}C for 24 hrs (Eze, 2009). After incubation individual colonies were isolated in pure culture using streak plate technique Eze, 2009; Sherman *et al*, 2009).

5.2.5 Preservation of pure culture

Pure cultures isolated by streak plating technique from nutrient agar and various selective media were preserved in nutrient broth using 15% (v/v) glycerol at -80^{0}C. Slants of pure isolates were prepared in nutrient agar and preserved at -20^{0}C for working culture.

5.2.6 Determination of microbial load

a. Plate count method

R2A agar was used for the determination of microbial load in terms of CFU/mL (Reasoner, 1985). The raw sewage sample was serially diluted up to a dilution of 10^{-9}. Dilutions of 10^{-5}, 10^{-6} and 10^{-7} were selected and 0.1 mL of the above dilution was plated in R2A agar in triplicates using spread plate technique. The plates were incubated at 37°C for 24 hours (Reasoner, 1985). After 24 hours the plates were observed for growth and the number of bacteria was counted in terms of CFU/mL for each dilution and average number of bacteria in the sample was calculated.

b. Most probable number (MPN)

MPN method according to the standards of EPA, 2003 was used to estimate the concentration of viable microorganisms in the sewage sample. In this method five tubes of each of the three dilutions 10 mL, 1 mL and 0.1 mL of raw sewage sample was taken and inoculated in lactose broth with Durham's tube to check for lactose fermentation and gas production. Only the presumptive test was performed. Growth of the bacteria was observed as turbidity in lactose broth and gas production as formation of gas bubble inside the inverted Durham's tube. Tubes with bacterial growth (turbid) and gas production were taken as positive tube and the results were tabulated. The MPN index and 95 % confidence limit per 100 mL is calculated by referring the results with the standard chart of MPN index (EPA, 2003; American Public Health Association, 1916)..

5.2.5 Preservation of pure culture

Pure cultures isolated by streak plating technique from nutrient agar and various selective media were preserved in nutrient broth using 15% (v/v) glycerol at -80^{0}C. Slants of pure isolates were prepared in nutrient agar and preserved at -20^{0}C for working culture.

5.2.6 Determination of microbial load

a. Plate count method

R2A agar was used for the determination of microbial load in terms of CFU/mL (Reasoner, 1985). The raw sewage sample was serially diluted up to a dilution of 10^{-9}. Dilutions of 10^{-5}, 10^{-6} and 10^{-7} were selected and 0.1 mL of the above dilution was plated in R2A agar in triplicates using spread plate technique. The plates were incubated at 37°C for 24 hours (Reasoner, 1985). After 24 hours the plates were observed for growth and the number of bacteria was counted in terms of CFU/mL for each dilution and average number of bacteria in the sample was calculated.

b. Most probable number (MPN)

MPN method according to the standards of EPA, 2003 was used to estimate the concentration of viable microorganisms in the sewage sample. In this method five tubes of each of the three dilutions 10 mL, 1 mL and 0.1 mL of raw sewage sample was taken and inoculated in lactose broth with Durham's tube to check for lactose fermentation and gas production. Only the presumptive test was performed. Growth of the bacteria was observed as turbidity in lactose broth and gas production as formation of gas bubble inside the inverted Durham's tube. Tubes with bacterial growth (turbid) and gas production were taken as positive tube and the results were tabulated. The MPN index and 95 % confidence limit per 100 mL is calculated by referring the results with the standard chart of MPN index (EPA, 2003; American Public Health Association, 1916)..

5.2.5 Preservation of pure culture

Pure cultures isolated by streak plating technique from nutrient agar and various selective media were preserved in nutrient broth using 15% (v/v) glycerol at -80^{0}C. Slants of pure isolates were prepared in nutrient agar and preserved at -20^{0}C for working culture.

5.2.6 Determination of microbial load

a. Plate count method

R2A agar was used for the determination of microbial load in terms of CFU/mL (Reasoner, 1985). The raw sewage sample was serially diluted up to a dilution of 10^{-9}. Dilutions of 10^{-5}, 10^{-6} and 10^{-7} were selected and 0.1 mL of the above dilution was plated in R2A agar in triplicates using spread plate technique. The plates were incubated at 37°C for 24 hours (Reasoner, 1985). After 24 hours the plates were observed for growth and the number of bacteria was counted in terms of CFU/mL for each dilution and average number of bacteria in the sample was calculated.

b. Most probable number (MPN)

MPN method according to the standards of EPA, 2003 was used to estimate the concentration of viable microorganisms in the sewage sample. In this method five tubes of each of the three dilutions 10 mL, 1 mL and 0.1 mL of raw sewage sample was taken and inoculated in lactose broth with Durham's tube to check for lactose fermentation and gas production. Only the presumptive test was performed. Growth of the bacteria was observed as turbidity in lactose broth and gas production as formation of gas bubble inside the inverted Durham's tube. Tubes with bacterial growth (turbid) and gas production were taken as positive tube and the results were tabulated. The MPN index and 95 % confidence limit per 100 mL is calculated by referring the results with the standard chart of MPN index (EPA, 2003; American Public Health Association, 1916)..

5.3 Phenotypic Characterization of bacterial isolates

5.3.1. Gram staining

A smear of 24 hr old bacterial culture of all isolates was prepared on a grease free glass slide. The smear was first air dried and then heat fixed (Bartholomew, 1962). The heat fixed smear was then flooded with crystal violet stain for 1 minute and then washed with distilled water for 5 seconds. The washed slides were then flooded with Gram's iodine solution for 1 minute and then again washed for 5 seconds with distilled water. The slides were then washed with 95% ethanol in drop wise fashion. After washing with distilled water again the smear was flooded with safranin for 1 minute (Bartholomew, 1962). The stained slides were finally washed with distilled water, air dried and observed under microscope. All the observation was done under oil immersion objective.

5.3.2. Endospore staining

A loop full culture of the isolates was taken on a grease free slide and a smear was prepared. The smear was air dried and heat fixed. The heat fixed slides were then placed over a steaming water bath with a piece of paper towel on the slides to prevent the stain from drying. The slides were then flooded with 5% aqueous malachite green (Mormak *et al*, 1985). Malachite green was added periodically to prevent the drying of the stain. The slides were then heated till the dye started to steam. The slides were then washed with water and counterstained with crystal violet for two minutes (Mormak *et al*, 1985). Subsequently the slides were washed with water, dried and observed under 100X microscope.

5.3.3. Acid fast staining

Smear of the isolates were made in a grease free glass slides. The slides were air dried and then heat fixed. The smear was then flooded with carbol fuschin and then steamed on a water bath for 5 minutes (Brown, 2005). The stain was added to the slides at regular intervals to avoid the stain from drying. After cooling the slides were then decolorized with acid alcohol (3% hydrochloric acid in 95% ethyl alcohol) (Ellis *et al*,

1993) until no more color ran off from the slide. The slides were then washed with water and counterstained with methylene blue (0.25% methylene blue in 1% acetic acid) for 30 seconds (Ellis *et al*, 1993). Subsequently the slides were washed with water, dried and observed under oil immersion (Brown, 2005).

5.3.4. Motility test

The motility tests of the isolates were done by two methods namely stab inoculation in SIM agar and by Hanging Drop Technique. In SIM agar, the motility of the bacteria can be determined by observing the spreading growth of organism from the line of inoculation. If the organism is motile it swims away from the line of inoculation into the uninoculated surrounding medium (Brown, 2005). In Hanging Drop Technique, a drop of culture was placed on a cover slip, which was then placed over a special glass slide that has a concave depression at the center. The glass slide was held in place by applying petroleum jelly on the ends of cover slip. The swimming motility of the organism was then observed under phase contrast microscope (Brown, 2005).

5.4 Physiological characteristics of bacterial isolates

5.4.1. Production of the enzyme catalase

The production of enzyme catalase by the isolates was detected as gas bubbles on addition of 3% hydrogen peroxide solution to the cultures (Taylor *et al*, 1972). A smear of each isolate was prepared on glass slides on top of which a drop of 3% hydrogen peroxide solution was added (Taylor *et al*, 1972). Detection of gas bubble was interpreted as a positive reaction indicating that the isolate produces the enzyme catalase.

5.4.2. Production of enzyme oxidase

The oxidase test is used to analyze the ability of bacteria to produce the enzyme cytochrome oxidase. This enzyme catalyses the transfer of electrons from cytrochrome c to molecular oxygen and is usually found in bacteria which uses oxygen as the terminal electron acceptor during respiration (Brown, 2005). The oxidase test is most useful in characterization of Gram negative bacteria (Blackman *et al*, 1978). In this test artificial

electron acceptor *p*- aminodimethylalanine oxalate is used which turns purple in color from yellow when it is reduced by transfer of electrons from cytochrome c. Production of oxidase enzyme in bacterial isolates was detected by production of purple color on exposure to the chemical *p*- aminodimethylalanine oxalate . The isolated colonies were picked on a sterile cotton swabs and the inoculums were allowed to dry. The cotton swabs were then tamped lightly for 10 times on the filter paper wet with *p*-aminodimethylalanine oxalate solution (Tarrand *et al*, 1982). Detection of purple colour on the tip of cotton swab was interpreted as a positive reaction (Brown, 2005).

5.4.3. Production of enzyme urease

This test is used for the identification of several genera and species of Enterobactericeae including the genera *Proteus*, *Providencia, Klebsiella* and *Morganella.* Urease broth was used for the test, which contains the substrate urea. The urease enzyme breaks down urea releasing ammonia which converts the pH of the medium alkaline .Increase in pH causes the phenol red indicator present in the media to turn from yellow to bright pink indicating a positive reaction (Brown, 2005).

5.5BIOCHEMICAL TESTS

5.5.1. IMViC TEST

- **INDOLE PRODUCTION TEST**

SIM media was used to evaluate the production of indole by the isolates. SIM media contains the amino acid tryptophan which is converted to indole by the enzyme tryptophanase. The indole is detected by the addition of Kovac's reagent, which produces cherry red colour if indole is present. The appearance of cherry red color gives a positive reaction (Sherman and Cappuccino, 2009). Tubes containing SIM media was inoculated with the isolates using stab inoculation. The inoculated tubes were then incubated for 24 hours at 37 ^{0}C. After 24 hours the tubes were checked for indole production using Kovac's reagent.

- **METHYL RED TEST**

This test is used to differentiate a number of enteric bacteria. MRVP broth was used which contains glucose. This test evaluates the organism's ability to ferment glucose. On fermentation of glucose various acidic end products are produced such as acetic acid, succinic acid etc which lowers the pH of the medium. The methyl red indicator added to the broth turns red as the pH of the medium lowers, indicating a positive reaction. The broth however turns yellow on addition of methyl red if glucose fermentation has not taken place (Sherman and Cappuccino, 2009; Brown, 2005). A loop full of culture was inoculated in tubes containing MRVP broth for all the isolates and incubated at 37^0C for 24 hours. After the incubation, the glucose fermentation was checked by addition of methyl red indicator in all the tubes.

- **VOGES PROSKAUER TEST**

Some of the organisms produce non acidic products such as 2, 3-butanediol ethanol and acetoin on fermentation of glucose, which gives a negative test for methyl red. Voges Proskauer test evaluates the organism's ability to produce these non acidic products on fermentation of glucose. Barrit's reagent A and B is used in this test which gives a Red coloration when these non acidic products are oxidized to diacetyl compounds, indicating a positive reaction (Sherman and Cappuccino, 2009; Brown, 2005). Loop full of culture was inoculated in MRVP broth and incubated for 24 hours at 37 ^{0}C for all the isolates. After the incubation 15 drops of Barrit's reagent A was added followed by the addition of 15 drops of Barrit's reagent B. The tubes were shaken after every few minutes and results were observed after 15 minutes.

organism. When the reaction tube is overlaid with acid, over acid in both the slant (yellow) and the butt (yellow) then the bacteria are said to be glucose, lactose and / or sucrose fermenters. When there is no acid produced in both the slant and the tube the bacteria are said to be glucose, lactose and sucrose non fermenter.

5.5.3. PRODUCTION OF HYDROGEN SULPHIDE

The production of hydrogen sulphide by the isolates was observed by making a stab inoculation of the isolates in tubes containing SIM agar. SIM agar contains iron salts that react with hydrogen sulphide to form dark precipitates of iron sulphide. The inoculated tubes were incubated for 24hrs at 37^0C. Appearance of black colour in SIM agar stabs indicated a positive reaction (Brown, 2005)

5.5.4. SUGAR FERMENTATION TEST

The isolates were screened for their ability to ferment 8 different carbohydrates. Tubes containing 5mL carbohydrate broth (Tripticase 10g/l, 5g/l of different carbohydrate, Sodium chloride 5g/l, and Phenol red 0.018g/l) were inoculated with the isolates and incubated at 37 ^{0}C for 24 hours. For the observation of gas production Durham's tubes were used. Color change from red to yellow indicates that that the carbohydrate has been utilized along with the production of acidic products which causes the lowering of the pH. This causes the phenol red indicator to turn yellow in color. The appearance of yellow color indicates a positive reaction (Sherman and Cappuccino, 2009). The gas production is also observed as a gas bubble or a void inside the inverted Durham's tube (Brown, 2005).

CHAPTER 6

RESULTS

6. RESULTS

6.1 Results of Physico – chemical analysis of Raw Sewage Sample:

Various physical and chemical parameters of Raw Sewage sample were observed and readings were taken for all the samples collected during the four months. The TDS, conductivity and temperature was measured using la motte tracer pocke tester (1766). pH was measured using automatic pH meter (Orion Z Star Thermoscientific). The pH, conductivity, temperature, odour and TDS were measured for all the samples collected during the 4 months and the results were tabulated and shown in Table 6.1.1. A linear regression of conductivity on TDS is also plotted in Fig 6.1.1.

TABLE 6.1.1: Physico-Chemical analysis of raw sewage sample

SAMPLES	PARAMETERS						
	TDS (mg/L)	Conductivity (µS/ cm)	Temperature (^{O}F)	pH	COLOUR	ODOUR	Month of sampling
Sample 1	310	525	62.4	7.24	Light brown	Foul smell	November 2013
Sample 2	360	530	58.6	7.21	Light brown	Foul smell	February 2014
Sample 3	390	570	67.8	7.24	Light brown	Foul smell	May 2014
Sample 4	320	470	74.6	7.31	Light brown	Foul smell	June 2014

TDS: Total dissolve solid ; ^{O} F : Fahrenheit
Sample 1 : collected during the month of November
Sample 2 : collected during the month of February
Sample 3 : collected during the month of May
Sample 4 : collected during the month of June.

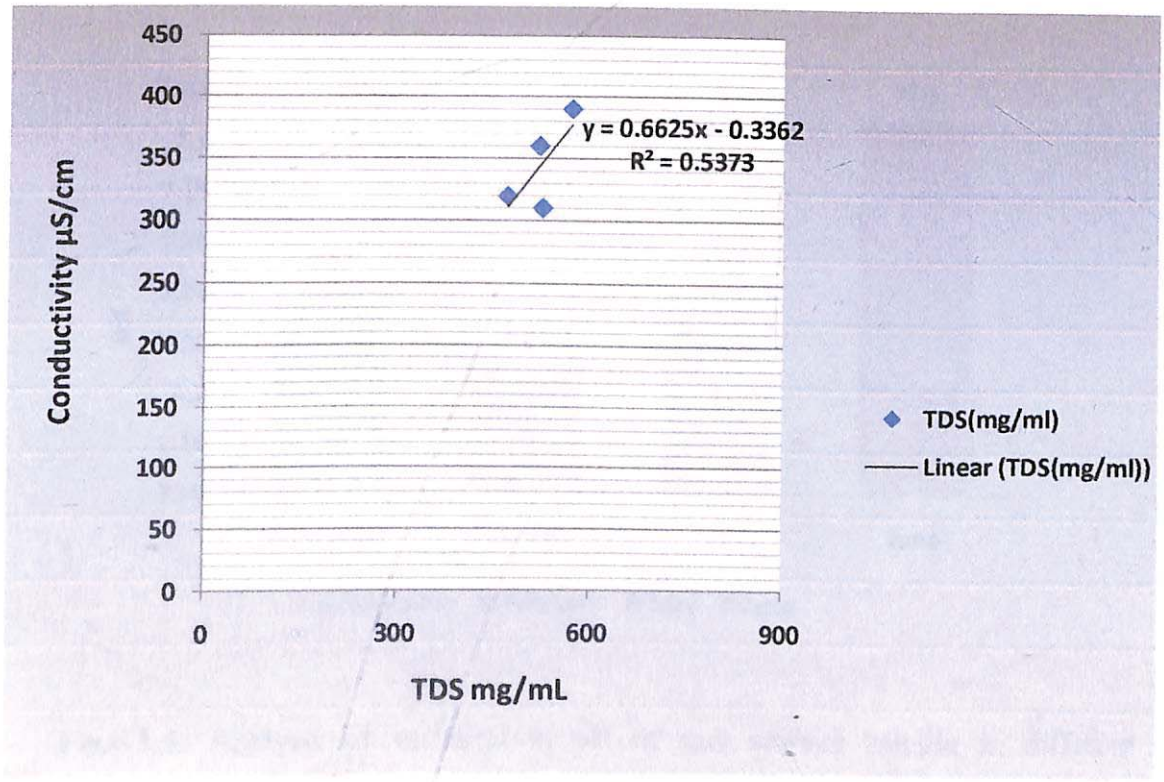


Fig. 6.1.1: Linear regression of conductivity on TDS

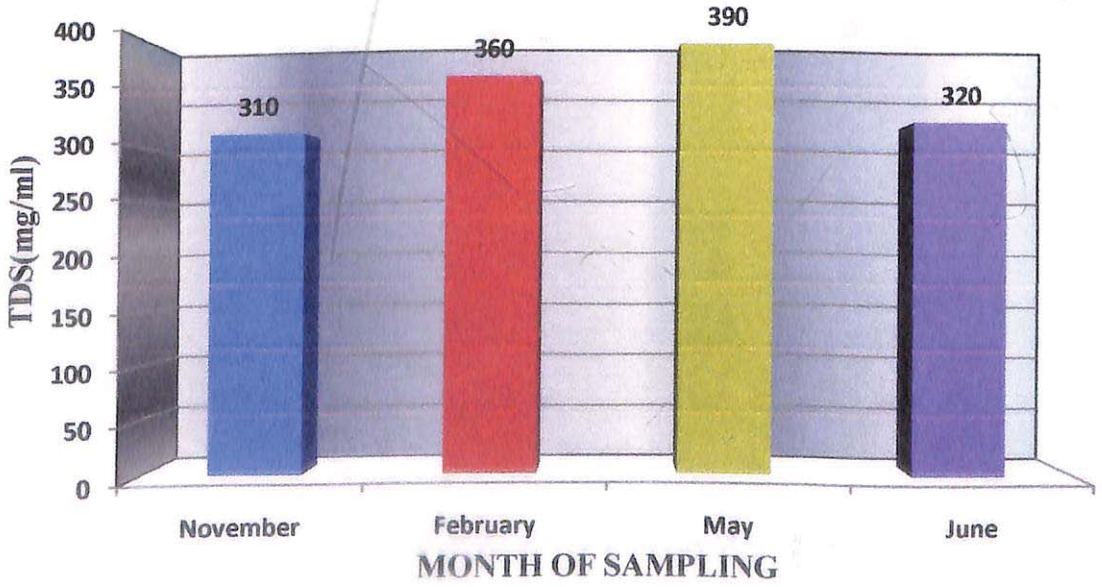


Fig.6.1.2: Analysis of variation in TDS of raw sewage sample in different months

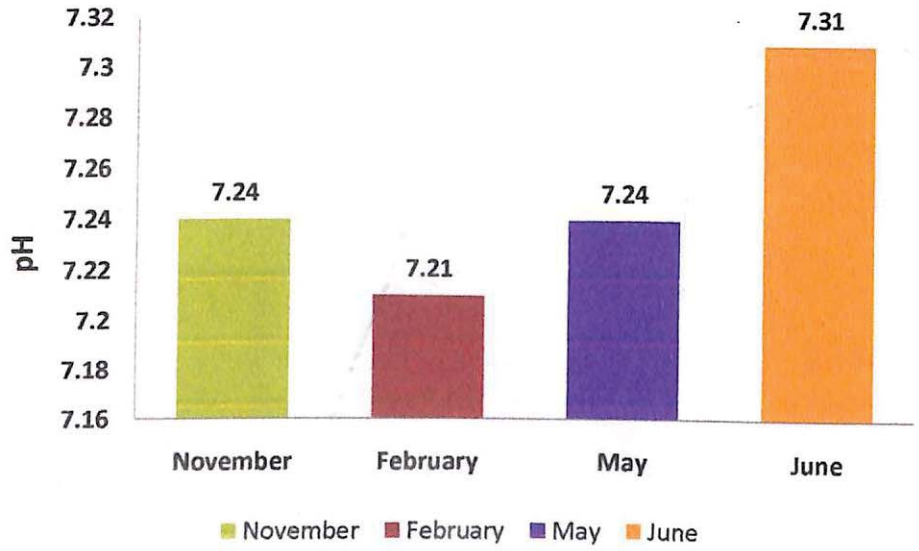


Fig.6.1.3: Analysis of variation in pH of raw sewage sample in different months

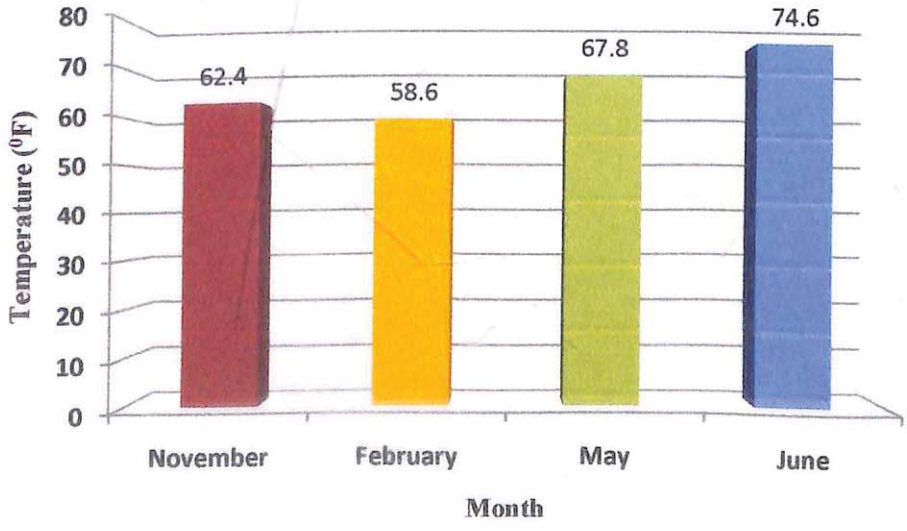


Fig.6.1.4. Analysis of variation in temperature of raw sewage sample in different months.

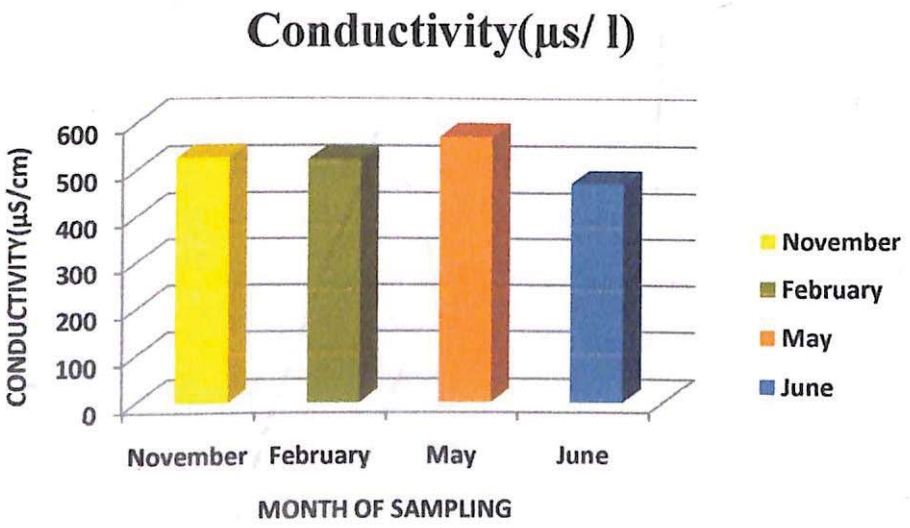


Fig.6.1.5: Analysis of variation in conductivity of raw sewage sample in different months

6.2 Results of phenotypic characterization of bacteria.

6.2.1 Morphological and cultural characteristics of the isolates of Pre-Treated sewage sample

The cultural characteristics of all the isolates were analyzed in Nutrient Agar and MacConkey Agar. Gram staining was done to observe the morphological characteristics of the isolates. The results are tabulated in Table 6.2.1. Out of 72 bacterial isolates from the raw sewage sample, the percentage of Gram positive rods, Gram negative rods, Gram positive cocci and comma shaped bacteria isolated are 20.83%, 62.5%, 16.66% and 8.3% respectively.

TABLE 6.2.1: Morphological and cultural characteristics of the isolates.

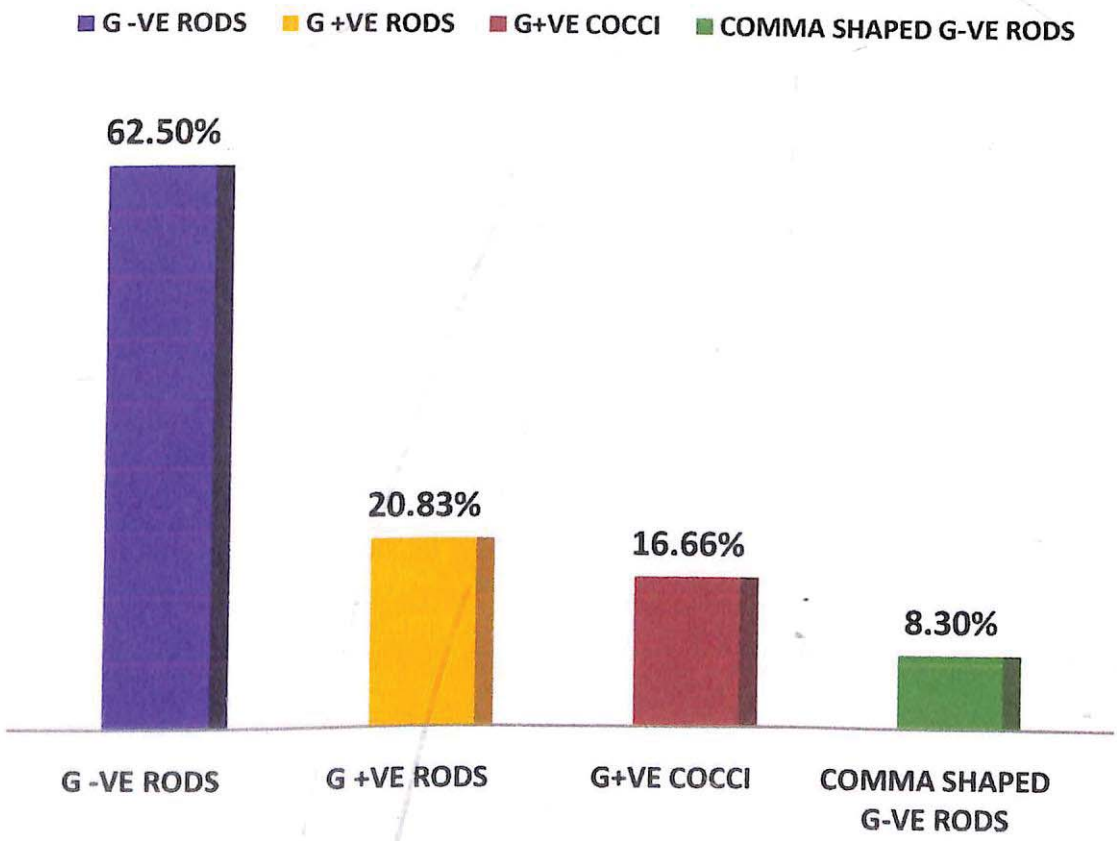
ISOLATES	MORPHOLOGY AND GROWTH CHARACTERISTICS IN NUTRIENT AGAR	MORPHOLOGY AND GROWTH CHARACTERISTICS IN MACCONKEY AGAR	GRAM STAINING
PT:IF:1	Small creamy white coloured colony, round in shape and opaque.	Yellow coloured colonies	Gram positive Rods
PT:IF:2	Transparent white colonies oval in shape with irregular edges.	Pink coloured colonies	Gram positive cocci
PT:IF:3	Cream coloured colonies, small and round in shape with shiny surface, with spreading growth on plates.	Colourless colonies	Gram negative Rods
PT:IF:4	Small white colonies, round to oval shaped, slightly gummy consistency.	Colourless colonies	Gram negative Rods
PT:IF:5	Transparent yellow coloured colonies, small in size with shiny surface.	Pink coloured colonies	Gram positive Rods
PT:IF:6	Cream coloured colonies, small and round in shape with shiny surface, with spreading growth on plates	Colourless colonies	Gram negative Rods
PT:IF:7	Very small shiny colonies producing brown pigmentation.	Yellow coloured colonies	Gram negative Rods

PT:IF:8	Translucent white colonies, small in size.	Pink coloured colonies	Gram positive cocci
PT:IF:9	Creamy white coloured colonies, mucoid, round shaped and intermediate in size.	Pink coloured colonies	Gram negative Rods
PT:IF:10	Opaque and round shaped colonies with intermediate size.	Pink coloured colonies	Gram negative Rods
PT:IF:11	Brownish coloured colonies with shiny surface, very small in size.	Colourless colonies	Gram negative Rods
PT:IF:12	Cream coloured colonies, thick in consistency, with irregular edges	No growth was observed	Gram positive Rods
PT:IF:13	White coloured very small colonies with shiny surface.	Yellow colored colonies	Gram negative Rods
PT:IF:14	Transparent white colonies, intermediate in size.	Yellow colored colonies	Gram positive cocci
PT:IF:15	Dense white coloured colonies, large in size.	No growth was observed	Gram positive Rods
PT:IF:16	Small glistening transparent colonies with regular edge and round shaped.	No growth was observed	Gram positive Rods
PT:IF:17	Very small glistening white colonies with regular edge, not mucoid and showed spreading in the agar plates.	Pink coloured colonies	Gram negative Rods
PT:IF:18	Lemon yellow coloured colonies, small in size with glistening surface.	No growth was observed	Gram negative Rods
PT:IF:19	Yellowish White coloured colonies, large in size, shows spreading from the line of inoculation.	Colorless colonies	Gram positive cocci
PT:IF:20	Large confluent growth, white coloured colonies with filamentous edges.	No growth was observed	Gram positive Rods
PT:IF:21	Confluent growth, large white colonies with filamentous edges.	No growth was observed	Gram positive Rods
PT:IF:22	Dense creamy colonies, white in colour ,round edges with large size.	No growth was observed	Gram positive Rods
PT:IF:23	Yellow coloured colonies with thick dense consistency, round shaped colonies with regular edge.	No growth was observed	Gram positive cocci
PT:IF:24	Fuzzy white coloured colonies with filamentous edges and thick in consistency.	No growth was observed	Gram positive Rods

PT:IF:25	Fuzzy white coloured colonies with filamentous edges and thick in consistency.	No growth was observed	Gram positive Rods
PT:IF:26	Small transparent colonies, round in shape with regular edges.	No growth was observed	Gram negative Rods
PT:IF:27	Confluent growth, large white coloured colonies with filamentous edges.	No growth was observed	Gram negative Rods
PT:IF:28	Confluent growth, large white coloured colonies with filamentous edges.	No growth was observed	Gram positive Rods
PT:IF:29	Confluent growth, large white coloured colonies with filamentous edges.	Pink colored colonies	Gram positive cocci
PT:IF:30	Yellow coloured colonies with thick dense consistency, round shaped colonies with regular edge.	No growth was observed	Gram positive cocci
PT:IF:31	Round shaped small colonies, transparent and shiny with gummy consistency.	Pink colored colonies	Gram negative Rods
PT:IF:32	Round shaped small colonies, transparent and shiny with gummy consistency.	No growth was observed	Gram positive Rods
PT:IF:33	Small white coloured colonies with regular edge, not shiny in appearance.	Pink colored colonies	Gram negative Rods
PT:IF:34	Gummy yellowish white coloured colony with thick consistency.	No growth was observed	Gram negative Rods
PT:IF:35	Gummy yellowish white coloured colony with thick consistency.	No growth was observed	Gram negative Rods
PT:IF:36	Small white colonies with regular edge and shiny surface, round in shape.	No growth was observed	Gram negative comma shaped
PT:IF:37	Gummy yellowish white coloured colony with thick consistency.	No growth was observed	Gram negative Rods
PT:IF:38	Small white coloured colonies with regular edge and shiny surface, round in shape.	No growth was observed	Gram negative Rods
PT:IF:39	Small white coloured colonies with regular edge and shiny surface, round in shape.	No growth was observed	Gram negative comma shaped
PT:IF:40	Yellowish white coloured slightly mucoid colonies with spreading growth on the agar surface.	Colorless colonies	Gram negative Rods
PT:IF:41	Yellowish white coloured slightly mucoid colonies with intermediate sized colonies.	Colorless colonies	Gram negative Rods

PT:IF:44	Small white coloured colonies, round to oval shaped, slightly gummy to mucoid consistency.	Pink colored colonies	Gram negative Rods
PT:IF:45	Transparent white coloured colonies, small in size with regular edges and shiny surface.	Colorless colonies	Gram negative Rods
PT:IF:46	Very small glistening white colonies with regular edge, not mucoid ,and showed spreading in the agar plates.	Pink colored colonies	Gram negative Rods
PT:IF:47	Very small glistening white colonies with regular edge, not mucoid ,and showed spreading in the agar plates.	Pink colored colonies	Gram negative Rods
PT:IF:48	Small white coloured colonies, round to oval shaped, slightly gummy to mucoid consistency.	Pink colored colonies	Gram negative Rods
PT:IF:49	Small white coloured colonies with regular edge and shiny surface, round in shape.	Pink colored colonies(slow grower)	Gram negative Rods
PT:IF:50	Small white coloured colonies with regular edge and shiny surface, round in shape.	No growth was observed	Gram negative Rods
PT:IF:01	White mucoid colonies intermediate in size with regular edges.	Pink colored colonies	Gram positive cocci
PT:IF:02	White mucoid colonies intermediate in size with regular edges.	Pink colored colonies	Gram positive cocci
PT:IF:03	Gummy yellowish white coloured colonies with thick consistency.	Pink colored colonies	Gram negative Rods
PT:IF:04	Gummy yellowish white coloured colonies with thick consistency.	Pink colored colonies	Gram negative Rods
PT:IF:05	White coloured colonies, round edge, not mucoid, thick consistency.	Yellow colored colonies	Gram negative Rods
PT:IF:06	Gummy yellowish white coloured colonies showing slight spreading on agar surface.	Pink colored colonies	Gram positive cocci
PT:IF:07	Yellowish white slightly mucoid colonies with intermediate sized colonies.	Colorless colonies	Gram negative Rods
PT:IF:08	Small white coloured colonies, round to oval shaped, slightly gummy to mucoid consistency.	Pink colored colonies	Gram negative Rods
PT:IF:09	Greenish coloured colonies, small round in shape.	Pink colored colonies	Gram negative Rods
PT:IF:010	Small white coloured colonies, round to oval shaped, slightly gummy to mucoid consistency.	Pink colored colonies	Gram negative Rods

PT:IF:011	Gummy yellowish white coloured colonies showing slight spreading on agar surface.	No growth was observed	Gram positive Rods in chain
PT:IF:012	Cream coloured colonies, thick in consistency, with irregular edges.	Pink colored colonies	Gram negative Rods
PT:IF:013	Yellow coloured colonies with shiny surface round and intermediate in size.	Pink colored colonies	Gram positive Rods
PT:IF:015	Green coloured colonies, small round in shape.	Pink colored colonies	Gram negative Rods
PT:IF:016	Green coloured colonies, small round in shape.	Pink colored colonies	Gram negative Rods
PT:IF:018	Yellow coloured colonies with thick dense consistency, round shaped with regular edge.	No growth was observed	Gram positive cocci in cluster
PT:IF:019	White coloured colony showing confluent growth with sticky consistency.	No growth was observed	Gram positive Rods
PT:IF:020	Small white coloured colonies, round to oval shaped, slightly gummy to mucoid consistency.	Pink colored colonies	Gram negative Rods
PT:IF:021	Yellowish white slightly mucoid colonies showing spreading on the agar surface.	Colorless colonies	Gram negative Rods
PT:IF:022	Thick gluey yellowish white colonies with irregular edge.	Colorless colonies	Gram negative Rods
PT:IF:023	Thick gluey yellowish white colonies with irregular edge.	Colorless colonies	Gram negative Rods
PT:IF:025	Yellowish white slightly mucoid colonies showing spreading on the agar surface.	Colorless colonies	Gram negative Rods
PT:IF:027	Yellowish white slightly mucoid colonies showing spreading on the agar surface.	Colorless colonies	Gram negative Rods
PT:IF:028	White coloured confluent colonies with sticky consistency.	No growth was observed	Gram positive cocci in chain



G –VE : Gram Negative; G +VE: Gram Positive

Fig. 6.2.1: Percentage of different bacteria present in raw sewage sample based on morphological and Gram stain characteristics.

6.3 Analysis of microbial load of raw sewage sample

a. Using agar plate count method

The bacterial count for the Pre-treated sewage samples were done using R2A agar with dilutions 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}. All the experiments were performed in triplicates. The bacterial count for the dilution 10^{-5} was too many to count (>300) so this data was not analyzed .The number of colonies was counted using the colony counter pen (Cole Parmer) .The cfu/mL for all the triplicate plates for each dilution was then calculated. The bacterial load of the samples collected during the four months for each dilution is given in Table 6.3.1. The bacterial count for the month of November was 650±1, 540.7±3.5 and 230±2.65 cfu/L mL for the dilutions 10^{-6}, 10^{-7} and 10^{-8} respectively. For the month of February it was 690.33±0.57, 440±2.64 and 180.66±1.15 cfu/mL for the dilutions 10^{-6}, 10^{-7} and 10^{-8} respectively. For the month of May it was 760.33±1.52, 610±3.6 and 360.33±1.52 cfu/ mL for the dilutions 10^{-6}, 10^{-7} and 10^{-8} respectively. For the month of June it was 570±3.6 and 320.66±2.08 cfu/mL for the dilutions 10^{-7} and 10^{-8} respectively. For the month of June the bacterial count for the dilution 10^{-6} was not considered as the count was more than 300.

TABLE 6.3.1: Analysis of microbial load in raw sewage sample

DETERMINATION OF MICROBIAL LOAD BY AGAR PLATE TECHNIQUE				
NUMBER OF COLONIES COUNTED IN CFU/mL				
MONTH OF SAMPLING	**DILUTION 10^{-5}**	**DILUTION 10^{-6}**	**DILUTION 10^{-7}**	**DILUTION 10^{-8}**
NOVEMBER	TMTC	650±1	540.7±3.5	230±2.65
FEBRUARY	TMTC	690.33±0.57	440±2.64	180.66±1.15
MAY	TMTC	760.33±1.52	610±3.6	360.33±1.52
JUNE	TMTC	>300	570±3.6	320.66±2.08

+/-: Standard Deviation; TMTC: Too many to count (greater than 300 colonies). N=3

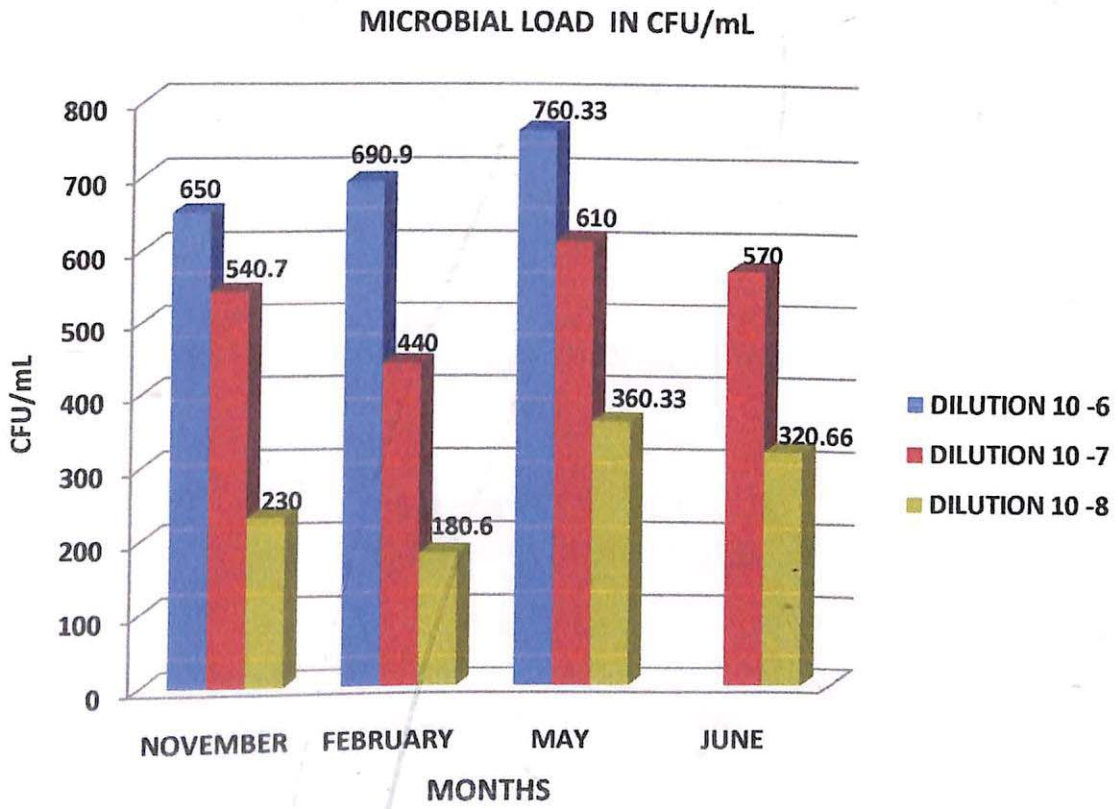


Fig.6.3.1: Analysis of microbial load of Pre-Treated sewage sample.

b. Analysis of microbial load of raw sewage sample using Multiple Tube Most Probable number technique.

The microbial load of the raw sewage sample was also calculated using the Multiple Tube Most Probable Number Test (MPN) technique. The MPN index per 100 mL was found to be >1600 as compared with the standard chart given in Table 6.3.3. The results of MPN are tabulated in table 6.3.2.

TABLE 6.3.2: MPN index and 95 per cent confidence limits for various combinations of positive results for a set of 5 10 mL, 5 1mL and five 0.1 mL portions of sample.

MULTIPLE TUBE MOST PROBABLE NUMBER TEST								
DILUTIONS	TUBES					MPN INDEX	95% CONFIDENCE LIMITS	
	TUBE 1	TUBE 2	TUBE 3	TUBE 4	TUBE 5		UPPER	LOWER
0.1 mL (1 X)	+	+	+	+	+	>1600	------	-----
1 mL (1 X)	+	+	+	+	+	>1600	------	------
10 mL (2 X)	+	+	+	+	+	>1600	-------	------

+ : Growth observed

TABLE 6.3.3: Standard chart for the MPN index and 95 % confidence limits for various combinations of positive results when five tubes are used per dilution (10 mL, 1mL and 0.1 mL portions of sample).

COMBINATION OF POSITIVES	MPN INDEX PER 100 mL	95 % CONFIDENCE LIMITS		COMBINATION OF POSITIVES	MPN INDEX PER 100 mL	95 % CONFIDENCE LIMITS	
		UPPER	LOWER			UPPER	LOWER
0-0-0	<2	-	-	4-2-1	26	12	65
0-0-1	2	1.0	10	4-3-0	27	12	67
0-1-0	2	1.0	10	4-3-1	33	15	77
0-2-0	4	1.0	13	4-4-0	34	16	80
1-0-0	2	1.0	11	5-0-0	23	9	86
1-0-1	4	1.0	15	5-0-1	30	10	110
1-1-0	4	1.0	15	5-0-2	40	20	140
1-1-1	6	2.0	18	5-1-0	30	10	120
1-2-0	6	2.0	18	5-1-1	50	20	150
2-0-0	4	1.0	17	5-1-2	60	30	180
2-0-1	7	2.0	20	5-2-0	50	20	170
2-1-0	7	2.0	21	5-2-1	70	30	210
2-1-1	9	3.0	24	5-2-2	90	40	250
2-2-2	9	3.0	25	5-3-0	80	30	250
2-3-0	12	5.0	29	5-3-1	110	40	300
3-0-0	8	3.0	24	5-3-2	140	60	360
3-0-1	11	4.0	29	5-3-3	170	80	410
3-1-0	11	4.0	29	5-4-0	130	50	390
3-1-1	14	6.0	35	5-4-1	170	70	480
3-2-0	14	6.0	35	5-4-2	220	100	580
3-2-1	17	7.0	40	5-4-3	280	120	690
4-0-0	13	5.0	38	5-4-4	350	160	820
4-0-1	17	7.0	45	5-5-0	240	100	940
4-1-0	17	7.0	46	5-5-1	300	100	1300
4-1-1	21	9.0	55	5-5-2	500	200	2000
4-1-2	26	12.0	63	5-5-3	900	300	2900
4-2-0	22	9.0	56	5-5-4	1600	600	5300
				5-5-5	>1600	-----	-------

Source: APHA 1992

6.4 Biochemical tests for the presumptive identification of isolates.

The isolates were grouped in two categories according to their staining properties as Gram positive and Gram negative. No Gram negative cocci were found among the isolates. Various biochemical tests were done for the identification of isolates .The test included IMViC, urease, catalase, motility, oxidase, TSI and various carbohydrate fermentation tests. Eight sugars were used for the evaluation of the ability of isolates to ferment various carbohydrates. Presumptive identification of Gram negative isolates which were rods were done on the basis of these biochemical tests. The results are tabulated in Table 6.4.1 and 6.4.2. Graphical representation of the percentage occurrence of these isolates in raw sewage sample is shown in Figure 6.10.1. The Gram positive isolates however were grouped into three groups based on their acid fast and endospore staining properties and catalase test. All the non spore forming, catalase positive, Gram positive cocci were grouped under Group I which consist of *Micrococcus sp, Planococcus sp* and *Staplylococcus sp.* Endospore producing Gram negative rods were grouped under Group II which consist of *Bacillus sp, Clostridium sp and Sporolactobacillus sp.* The non spore forming, catalase positive Gram positive cocci were gouped under Group III consist of *Lactobacillus sp, Listeria sp and Kurthia sp.* The results are tabulated in Table 6.4.3.

TABLE 6.4.1: Presumptive identification of isolates based on biochemical tests

	BIOCHEMICAL TEST									
ISOLATES	INDOLE	METHYL RED	VOGES PRAUSKEUR	CITRATE	TSI	UREASE	CATALASE	OXIDASE	MOTILITY	TENTATIVE IDENTIFICATION
PT:IF:1	-	+	-	+	A/AB/G	-	+	-	+	*Salmonella sp*
PT:IF:2	-	+	-	+	A/A/G	-	-	-	+	*Escherichia sp*
PT:IF: 3	+	+	-	+	AB/AB/G	-	+	-	+	*Proteus sp*

PT:IF:4	+	+	-	+	A/B/G	+	+	-	+	*Klebsiella sp*
PT:IF:5	+	+	-	-	K	-	+	-	-	*Shigella sp*
PT:IF:6	-	+	+	+	A/A/G	+	+	-	+	*Proteus sp*
PT:IF: 7	-	+	-	+	K	-	+	-	+	*Providencia sp*
PT:IF:8	+	+	-	+	A/A	-	+	-	+	*Gram positive cocci*
PT:IF:9	-	+	-	+	A/A/G	-	-	-	+	*Citrobacter sp*
PT:IF:10	+	+	-	-	A/A/G	-	+	-	+	*Escherichia sp*
PT:IF:11	-	+	-	+	K	-	-	-	-	*Gram negative rods*
PT:IF:12	-	+	-	-	K	-	+	-	+	*Bacillus sp*
PT:IF:13	-	-	+	+	A/A/G	-	+	-	-	*Enterobacter sp*
PT:IF:14	-	-	+	-	K	-	-	-	+	*Gram positive cocci*
PT:IF:15	-	-	+	-	K	-	+	-	-	*Bacillus sp*
PT:IF:16	-	+	-	-	K	-	+	-	+	*Gram positive rods*
PT:IF:17	+	+	-	-	A/A/G	-	+	-	+	*Escherichia sp*
PT:IF:18	-	-	+	-	K	-	-	-	+	*Gram negative rods*
PT:IF:19	+	+	-	-	K	-	+	-	+	*Gram positive cocci*
PT:IF:20	-	-	+	-	K	-	+	-	+	*Bacillus sp*
PT:IF:21	-	+	+	-	K	-	+	-	+	*Bacillus sp*
PT:IF:22	-	+	-	-	K	-	+	-	+	*Gram positive rods*
PT:IF:23	-	-	+	-	K	-	+	-		*Gram positive cocci*
PT:IF:24	-	+	-	-	K	-	+	-	+	*Bacillus sp*
PT:IF:25	-	+	-	-	K	-	+	-	+	*Bacillus sp*
PT:IF:26	-	+	-	-	K/A	-	+	-	+	*Bacillus sp*
PT:IF:27	+	+	-	-	A/A	-	+	-	+	*Bacillus sp*
PT:IF:28	-	-	+	-	K/A	-	+	-	+	*Bacillus sp*
PT:IF:29	-	+	-	-	K/A	-	-	-	+	*Gram positive rods*
PT:IF:30	-	+	-	-	K	-	+	-	-	*Gram positive cocci*

PT:IF:31	+	+	-	-	A/A/G	-	+	-	+	*Escherichia sp*
PT:IF:32	-	+	-	-	K	-	+	-	+	*Gram positive rods*
PT:IF:33	+	+	+	-	A/A/G	-	+	-	+	*Escherichia sp*
PT:IF:34	-	+	-	-	K/A/G	+	+	-	+	*Klebsiella sp*
PT:IF:35	-	+	-	-	K	+	+	-	+	*Klebsiella sp*
PT:IF:36	-	+	-	-	A/A	-	-	-	+	*Vibrio sp*
PT:IF:37	+	+	-	+	A/AB	-	-	-	+	*Vibrio sp*
PT:IF:38	-	+	-	-	K/A	-	+	-	+	*Vibrio sp*
PT:IF:39	-	+	-	-	A/A	-	-	+	+	*Vibrio sp*
PT:IF:40	+	+	-	+	K/B/G	-	+	+	+	*Salmonella sp*
PT:IF:41	-	+	-	-	K/A	-	+	-	-	*Shigella sp*
PT:IF:44	+	+	-	-	A/A/G	-	+	-	+	*Escherichia sp*
PT:IF:45	-	+	-	-	K/A/G	-	+	-	-	*Shigella sp*
PT:IF:46	+	-	+	+	K/A/G	-	+	-	+	*Klebsiella sp*
PT:IF:47	+	-	+	+	A/A/G	-	+	-	+	*Klebsiella sp*
PT:IF:48	+	+	-	-	A/A/G	-	+	-	+	*Escherichia sp*
PT:IF:49	-	+	-	-	A/A	-	-	-	+	*Vibrio sp*
PT:IF:50	-	+	-	-	A/A	-	-	+	+	*Vibrio sp*
PT:IF:01	-	+	+	+	A/A/G	-	-	+	-	*Gram positive cocci*
PT:IF:02	-	+	+	+	A/A	-	-	-	-	*Gram positive cocci*
PT:IF:03	+	-	+	+	A/A/G	-	+	-	+	*Klebsiella sp*
PT:IF:04	+	-	+	+	A/A/G	-	+	-	+	*Klebsiella sp*
PT:IF:05	-	-	+	+	A/A/G	-	+	-	-	*Enterobacter sp*
PT:IF:06	-	+	-	+	A/A/G	-	-	-	-	*Gram positive cocci*
PT:IF:07	+	+	-	-	K/A	-	+	-	-	*Shigella sp*
PT:IF:08	+	+	-	-	A/A/G	-	+	-	+	*Escherichia sp*
PT:IF:09	-	-	-	+	K	+	+	+	+	*Pseudomonas sp*

PT:IF:010	+	+	-	-	A/A/G	-	+	-	+	*Escherichia sp*
PT:IF:011	+	+	-	-	A/A/G	-	-	-	-	*Gram positive cocci*
PT:IF:012	+	+	-	-	K	-	-	-	-	*Gram positive rods*
PT:IF:013	-	-	+	-	A/A	-	-	-	-	*Gram positive cocci*
PT:IF: 015	-	-	-	+	K	+	+	+	+	*Pseudomonas sp*
PT:IF:016	-	-	-	+	K	+	+	+	+	*Pseudomonas sp*
PT:IF:018	-	-	+	-	K/A	-	-	-	-	*Gram positive cocci in cluster*
PT:IF:019	-	-	+	-	K	-	-	-	-	*Gram positive rods*
PT:IF:020	+	+	-	-	A/A/G	-	+	-	+	*Escherichia sp*
PT:IF:021	-	+	-	+	AB/AB/G	-	+	-	+	*Salmonella sp*
PT:IF:022	+	+	-	+	K	-	+	-	+	*Providencia sp*
PT:IF:023	+	+	-	+	K	-	+	-	+	*Providencia sp*
PT:IF:025	-	+	-	+	A/B/G	-	+	-	+	*Salmonella sp*
PT:IF:027	-	+	-	+	A/B/G	-	+	-	+	*Salmonella sp*
PT:IF:028	+	+	-	+	A/A/G	-	-	-	-	*Gram positive cocci*

Source: Bergey's Manual

K: Alkaline (slants were orange in colour)

K/A: alkaline slant/acidic butt

A/B/G: Acidic slant/ black butt/gas production

A/A/G: Acidic slant/acidic butt/gas production

AB/AB/G: Acidic slant with black colouration/ Acidic butt with black colouration/ gas production ;

K/A/G: alkaline slant/acidic butt/gas production

.Acidic: colour of the media changed to yellow.

B: colour of the media changed to black due to production of Hydrogen sulphide by the organism.

TSI: Triple Sugar Iron Agar.

TABLE 6.4.2: Carbohydrate test for the presumptive identification of isolates

ISOLATES	SUGAR FERMENTATION TEST							
	INOSITOL	DULCITOL	RAFFINOSE	XYLOSE	SUCROSE	MANNOSE	D-MANNITOL	D-ARABINOSE
PT:IF:1	A/G	K	A/G	A	K	A/G	A/G	A/G
PT:IF:2	K	K	A	A	K	A/G	A/G	A/G
PT:IF:3	K	K	K	A	A	K	K	K
PT:IF:4	K	K	A	A	K	A/G	A/G	A
PT:IF:5	K	K	A	A	K	K	K	K
PT:IF:6	K	K	K	A	A/G	K	K	K/G
PT:IF:7	K	K	K	K	A	A/G	K	K
PT:IF:8	K	K	K	A	A	A/G	A/G	A/G
PT:IF:9	K	K	A	A	A/G	A/G	A/G	K
PT:IF:10	K	A/G	K	A	A/G	A	A/G	A/G
PT:IF:11	K	K	K	K	K	K	K	K
PT:IF:12	K	K	K	K	K	K	K	K
PT:IF:13	K	K	A	K	A	A	A	A
PT:IF:14	K	K	K	K	K	K	K	K
PT:IF:15	K	K	K	K	K	K	K	K
PT:IF:16	K	K	K	K	K	K	K	K

PT:IF:17	K	K	K	A	K	A/G	A/G	A/G
PT:IF:18	K	K	K	K	K	K	K	K
PT:IF:19	K	A/G	A	A	A	A/G	A/G	A/G
PT:IF:20	K	K	K	K	A	A	K	K
PT:IF:21	K	K	K	A	K	K	K	K
PT:IF:22	K	K	K	K	A	K	K	K
PT:IF:23	K	K	K	K	K	K	K	K
PT:IF:24	K	K	K	K	K	K	K	K
PT:IF:25	K	K	K	K	A	A	K	K
PT:IF:26	K	K	K	K	A	A	K	K
PT:IF:27	K	K	A	A/G	A	K	K	A/G
PT:IF:28	K	K	K	K	K	K	K	K
PT:IF:29	K	K	K	A	K	K	K	K
PT:IF:30	K	K	K	K	K	K	K	K
PT:IF:31	K	A/G	A	A	A/G	A/G	A/G	A/G
PT:IF:32	K	K	K	A	K	K	K	K
PT:IF:33	K	A/G	A	A	A	A/G	A/G	A/G
PT:IF:34	A/G	A/G	A	A	A/G	A/G	A/G	K
PT:IF:35	A/G	A/G	A	A	A/G	A/G	A/G	K
PT:IF:36	K	K	K	K	A	K	K	K
PT:IF:37	K	K	K	K	A	K	K	K

PT:IF:38	K	K	K	K	A	K	K	K
PT:IF:39	K	K	K	K	A	K	K	K
PT:IF:40	K	K	A	A/G	K	K	A/G	A
PT:IF:41	K	K	A	K	K	K	K	A
PT:IF:44	K	A/G	K	K	K	A/G	A/G	A
PT:IF:45	K	K	K	K	K	A/G	A/G	K
PT:IF:46	A/G	A/G	A	A	A/G	A/G	A/G	K
PT:IF:47	A/G	A/G	A	A	A/G	A/G	A/G	K
PT:IF:48	K	A/G	K	A	K	A/G	A/G	A
PT:IF:49	K	K	K	K	A	K	K	K
PT:IF:50	K	K	K	K	K	K	K	K
PT:IF:01	K	K	K	K	K	K	K	K
PT:IF:02	K	K	K	K	K	K	K	K
PT:IF:03	A/G	A/G	A	A	A/G	A/G	A/G	K
PT:IF:04	A/G	A/G	A	A	A/G	A/G	A/G	K
PT:IF:05	K	K	A	K	A	A	A	A
PT:IF:06	K	K	K	K	K	K	K	K
PT:IF:07	K	K	A	K	K	K	K	A
PT:IF:08	A/G	A/G	A	A	A/G	A/G	A/G	K
PT:IF:09	K	K	K	K	K	K	K	K
PT:IF:010	A/G	A/G	A	A	A/G	A/G	A/G	K

PT:IF:011	K	K	K	K	K	K	K	K
PT:IF:012	K	K	K	K	A	A	K	K
PT:IF:013	K	K	K	K	K	K	K	K
PT:IF:015	K	K	K	K	K	K	K	K
PT:IF:016	K	K	K	K	K	K	K	K
PT:IF:018	K	K	K	K	K	K	K	K
PT:IF:019	K	K	K	K	A	A	K	K
PT:IF:020	A/G	A/G	A	A	A/G	A/G	A/G	K
PT:IF:021	K	K	A	A/G	K	K	A/G	A
PT:IF:022	K	K	K	K	K	A	K	K
PT:IF:023	K	K	K	K	K	A	K	K
PT:IF:025	K	K	A	A/G	K	K	A/G	A
PT:IF:027	K	K	A	A/G	K	K	A/G	A
PT:IF:028	K	K	K	K	K	K	K	K

K: Alkaline, no change in colour of the broth, indicates negative reaction.

A/G: Acidic with gas production, yellow coloration of the broth, indicates positive reaction.

A: Acidic with no gas production, yellow coloration of the broth.

TABLE: 6.4.3: Grouping of Gram positive isolates based on endospore, acid fast staining and catalase test

ISOLATES	ENDOSPORE STAINING	ACID FAST STAINING	CATALASE	GRAM STAINING	GROUP
PT:IF:02	-	-	+	Gram positive cocci	I
PT:IF:06	-	-	+	Gram positive cocci	I
PT:IF:8	-	-	+	Gram positive cocci	I
PT:IF:011	-	-	+	Gram positive cocci	I
PT:IF:12	+	-	+	Gram positive rods	II
PT:IF:14	-	-	+	Gram positive cocci	I
PT:IF:15	+	-	+	Gram positive rods	II
PT:IF:16	+	-	+	Gram positive rods	II
PT:IF:018	-	-	+	Gram positive cocci	I
PT:IF:19	-	-	+	Gram positive cocci	I
PT:IF:019	-	-	+	Gram positive rods	III
PT:IF:20	+	-	+	Gram positive rods	II
PT:IF:21	+	-	+	Gram positive rods	II
PT:IF:22	+	-	+	Gram positive rods	II
PT:IF:23	-	-	+	Gram positive cocci	I
PT:IF:24	+	-	+	Gram positive rods	II
PT:IF:25	+	-	+	Gram positive rods	II
PT:IF:26	+	-	+	Gram positive rods	II
PT:IF:27	+	-	+	Gram positive rods	II
PT:IF:028	-	-	+	Gram positive cocci	I
PT:IF:28	+	-	+	Gram positive rods	II
PT:IF:29	-	-	+	Gram positive rods	III
PT:IF:30	-	-	+	Gram positive cocci	I
PT:IF:32	-	-	+	Gram positive rods	III

In accordance to Bergeys manual;

GROUP I: *Micrococcus sp, Planococcus sp* and *Staphylococcus sp.*

GROUP II: *Bacillus sp, Clostridium sp* and *Sporolactobacillus sp.*

GROUP III: *Lactobacillus sp, Listeria sp* and *Kurthia sp.*

6.5 Biochemical test for the presumptive identification of *Escherichia sp.*

The various biochemical tests for the presumptive identification of *Escherichia sp* were performed and tabulated as shown below in Table 6.5.1.

TABLE 6.5.1: Biochemical test for the presumptive identification of *Escherichia sp*

Isolates	INDOLE	METHYL RED	VOGES PROSKAUER	CITRATE UTILIZATION	LACTOSE UTILIZATION	MOTILITY	TSI TEST	GROWTH IN EMB AGAR	TENTATIVE IDENTIFICATION
PT:IF:10	+	+	-	-	+	+	A/A/G	Green metallic sheen	*Escherichia sp*
PT:IF:17	+	+	-	-	+	+	A/A/G	Green metallic sheen	*Escherichia sp*
PT:IF:31	+	+	-	-	+	+	A/A/G	Green metallic sheen	*Escherichia sp*
PT:IF:44	+	+	-	-	+	+	A/A/G	Green metallic sheen	*Escherichia sp*
PT:IF:48	+	+	-	-	+	+	A/A/G	Green metallic sheen	*Escherichia sp*
PT:IF:08	+	+	-	-	+	+	A/A/G	Green metallic sheen	*Escherichia sp*
PT:IF:010	+	+	-	-	+	+	A/A/G	Green metallic sheen	*Escherichia sp*
PT:IF:020	+	+	-	-	+	+	A/A/G	Green metallic sheen	*Escherichia sp*
PT:IF:2	-	+	-	+	+	+	A/A/G	Green metallic sheen	*Escherichia sp*
PT: IF: 33	+	+	+	-	+	+	A/A/G	Green metallic sheen	*Escherichia sp*

A/A/G: Acidic slant/acidic butt/gas production.

All the isolates were Gram negative and rod in shape.

6.6 Biochemical test for the presumptive identification of *Salmonella sp.*

Various biochemical tests were performed for the identification of *Salmonella sp* and the results are tabulated in Table 6.6.1.

TABLE 6.6.1: Biochemical test for the presumptive identification of *Salmonella Sp*

ISOLATES	INDOLE	METHYL RED	VOGES PROSKAUER	CITRATE UTILIZATION	TSI TEST	MOTILITY	SUGAR FERMENTATION					TENTATIVE IDENTIFICATION
							D-ARABINOSE	D-MANNITOL	SUCROSE	LACTOSE	D-XYLOSE	
PT:IF:1	-	+	–	+	K/A/B	+	+	+	–	–	+	*Salmonella sp*
PT:IF:40	-	+	–	+	K/A/B	+	+	+	–	–	+	*Salmonella sp*
PT:IF:021	-	+	–	+	K/A/B	+	+	+	–	–	+	*Salmonella sp*
PT:IF:025	-	+	-	+	K/A/B	+	+	+	-	-	+	*Salmonella sp*
PT:IF:027	-	+	-	+	K/A/B	+	+	+	-	-	+	*Salmonella sp*

K/A/G: alkaline slant/ acidic butt/gas production.

Culture characteristics: The colonies were black in Salmonella-Shigella agar.

All the isolates were Gram negative and rod in shape.

6.7 Biochemical tests for the presumptive identification of *Shigella sp.*

Various biochemical tests were performed for the identification of *Shigella sp* and the results are tabulated in Table 6.7.1

TABLE 6.7.1: Biochemical test for the presumptive identification of *Shigella sp.*

ISOLATES	INDOLE	METHYL RED	VOGES PROSKAUER	CITRATE UTILIZATION	UREASE	TSI TEST	MOTILITY	SUGAR FERMENTATION							TENTATIVE IDENTIFICATION
								MANNITOL	D-ARABINOSE	INOSITOL	D-XYLOSE	DULCITOL	LACTOSE	SUCROSE	
PT:IF:5	+	+	-	-	-	K/A	-	+	+	-	-	-	-	-	*Shigella sp*
PT:IF:41	-	+	-	-	-	K/A	-	+	+	-	-	-	-	-	*Shigella sp*
PT:IF:45	-	+	-	-	-	K/A	-	+	+	-	-	-	-	-	*Shigella sp*
PT:IF:07	+	+	-	-	-	K/A	-	+	+	-	-	-	-	-	*Shigella sp*

K/A: Alkaline slant/acidic butt

Cultural characteristics: all the colonies were colorless in Salmonella- Shigella agar.

6.7 Biochemical tests for the presumptive identification of *Vibrio sp.*

Various biochemical tests were performed for the identification of *Vibrio sp* and the results are tabulated in Table 6.8.1.

TABLE 6.8.1: Biochemical test for the presumptive identification of *Vibrio sp.*

ISOLATES	VOGES PROSKAUER	CITRATE UTILIZATION	OXIDASE	TSI TEST	SUGAR FERMENTATION			GROWTH IN TCBS	TENTATIVE IDENTIFICATION
					MANNITOL	SUCROSE	L-ARABINOSE		
PT:IF:36	-	+	+	A/A	-	+	-	Light yellow coloured small colonies	*Vibrio sp*
PT:IF:37	-	+	+	A/AB	-	+	-	Blue green colonies	*Vibrio sp*
PT:IF:38	-	+	+	K/A	-	+	-	Yellow coloured large colonies	*Vibrio sp*
PT:IF:39	-	+	+	A/A	-	+	-	Light yellow coloured small colonies	*Vibrio sp*
PT:IF:49	-	+	+	A/A	-	+	-	Light yellow coloured small colonies	*Vibrio sp*
PT:IF:50	-	+	+	A/A	-	-	-	Yellow coloured large colonies	*Vibrio sp*

K/A/G: alkaline slant/ acidic butt/gas production, K: alkaline, A/A: acidic slant/ acidic butt.

All the isolates were Gram negative and comma shaped.

6.9 Biochemical tests for the presumptive identification of *Klebsiella sp.*

Various biochemical tests were performed for the identification of *Klebsiella sp* and the results are tabulated in Table 6.9.1.

TABLE 6.9.1: Biochemical test for the identification of *Klebsiella sp*

ISOLATES	METHYL RED	VOGES PROSKAUER	UREASE	INDOLE	CITRATE	TSI	MOTILITY	SUGAR FERMENTATION						TENTATIVE IDENTIFICATION
								D-XYLOSE	RAFFINOSE	SUCROSE	DULCITOL	D-ARABINOSE	INOSITOL	
PT:IF:34	+	-	+	-	-	K/A/G	+	+	+	+	+	-	+	*Klebsiella sp*
PT:IF:35	+	-	+	-	-	K/A/G	+	+	+	+	+	-	+	*Klebsiella sp*
PT:IF:4	+	-	+	-	-	K/A/G	+	+	+	+	+	-	+	*Klebsiella sp*
PT:IF:46	+	-	+	-	-	K/A/G	+	+	+	+	+	-	+	*Klebsiella sp*
PT:IF:47	+	-	+	-	-	K/A/G	+	+	+	+	+	-	+	*Klebsiella sp*
PT:IF:03	+	-	+	-	-	K/A/G	+	+	+	+	+	-	+	*Klebsiella sp*
PT:IF:04	+	-	+	-	-	K/A/G	+	+	+	+	+	-	+	*Klebsiella sp*

K/A/G: alkaline slant/ acidic butt/gas production

Cultural characteristics: pink mucoid colonies in EMB

All the isolates were Gram negative and rod shaped.

6.10 Biochemical tests for the presumptive identification of *Proteus sp.*

Various biochemical tests were performed for the identification of *Proteus sp* and the results are tabulated in Table 6.10.1

TABLE 6.10.1: Biochemical test for the identification of *Proteus sp*

ISOLATES	METHYL RED	VOGES PROSKAUER	INDOLE	CITRATE	TSI	SUGAR FERMENTATION					UREASE	CATALASE	MOTILITY	TENTATIVE IDENTIFICATION
						MANNITOL	RAFFINOSE	INOSITOL	L-ARABINOSE	SUCROSE				
PT:IF:3	+	-	+	+	AB/AB/G	+	-	-	+	+	+	+	+	*Proteus sp*
PT:IF:6	+	+	-	+	A/A/G	+	-	-	+	+	+	+	+	*Proteus sp*

AB/AB/G: Acidic slant and acidic butt with H2S production/gas production.

A/A/G: Acidic slant/ acidic butt/gas production.

Cultural characteristics: spreading growth on agar indicating swarming movement of the organism.

All the isolates were Gram negative and rod shaped.

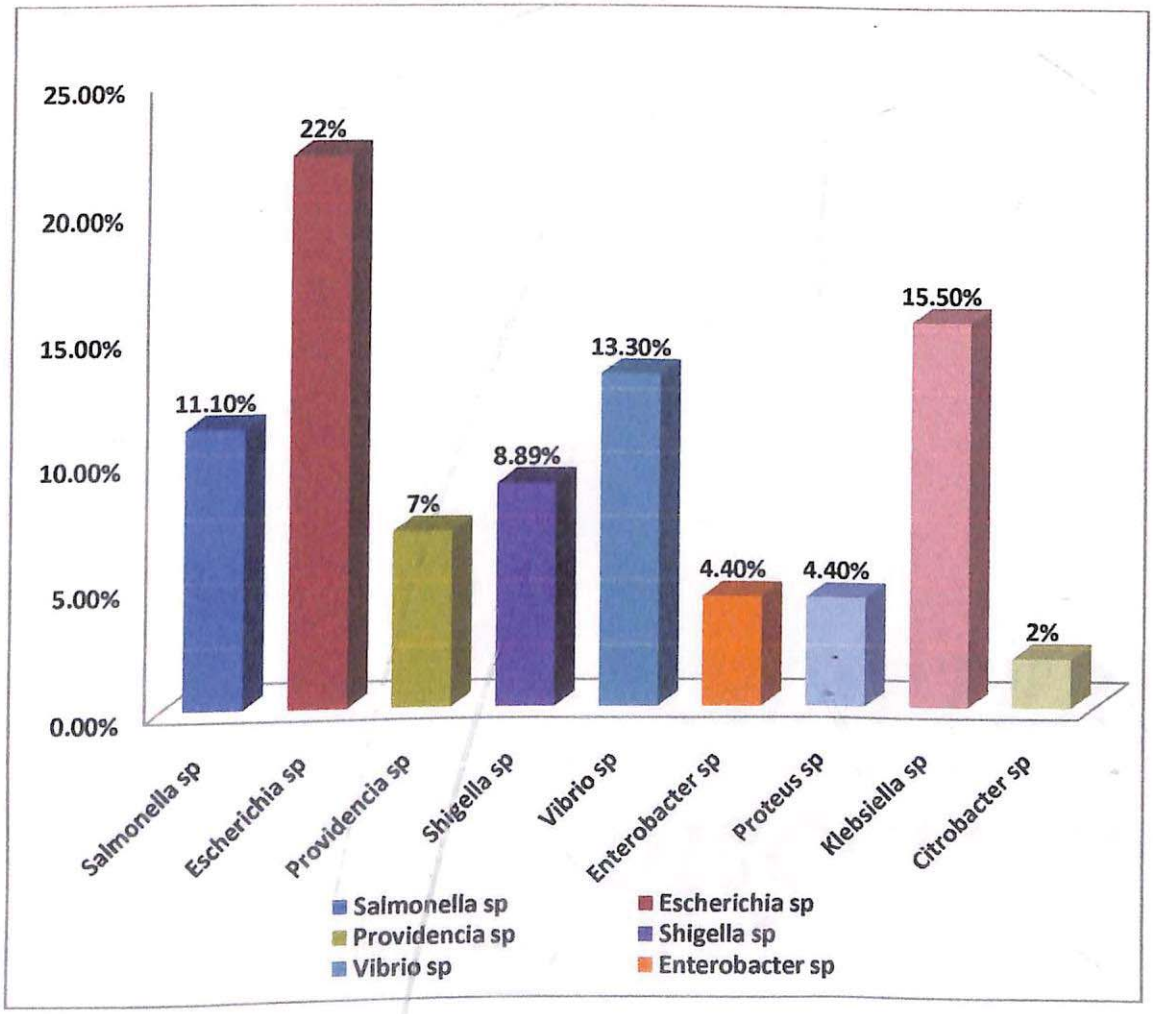


Fig.6.10.1. Percentage occurrence of some bacteria isolated from raw sewage sample.

CHAPTER 7

DISCUSSION

7. DISCUSSION

The main aim of this study was to determine the quality of the sewage influent in terms of certain physico-chemical parameters and on the types of bacterial population present. In the present study various physical and chemical parameters along with the type of bacterial flora present in raw sewage sample was studied for the four months- November, February, May and June, with November and February being the dry season and May and June being the wet ones. Various physico-chemical parameters such as pH, temperature, odor, smell, conductivity and TDS of the raw sewage sample of Adampool sewage treatment plant was examined and measured. The sewage sample had foul and offensive odor with light brown coloration. The temperature was measured in Fahrenheit and was found within the range of 58 °F – 75° F with minimum temperature of 58.6°F in the month of February and maximum of 74.6^{0}F in the month of June.

pH of the raw sewage is a very important parameter which determines the survival growth and existence of different biological forms in the sewage. The concentration range of pH is important as it affects the removal of various compounds from the sewage by the microorganisms. For carbonaceous removal a pH range of 6-9 is tolerable and optimum removal occurs at a neutral pH, optimal nitrification rates occurs at a pH range of 7.5 – 8. Sewage water with high hydrogen ion concentration is difficult to treat with biological process. The pH value in the present study ranges between 7.2 to 7.3 for all the four months. This pH range falls within the optimum value which lies between 6.8 – 7.5 levels that is conducive for the bacterial growth as suggested by Metcalf and Eddy (2004).

The total dissolved solids (TDS) were measured in mg/l for the four months and the TDS value was found within the range of 300-400. Among the four months the highest TDS value of 390 mg/L was observed during month of May and minimum during the month of November (525 mg/L). In a Similar study on the physico-chemical parameters of raw sewage by Chauhan *et all* (2012) the TDS value was found to be within the range of 300- 400 mg/l. Thus the TDS value obtained in my present study is

consistent with the earlier Chauhan *et all* (2012). The present obtained value of TDS falls under the category of medium concentration when compared with the standards given by Metcalf and Eddy (2004).

The conductivity of raw sewage sample was also analyzed and the values were found to range between 470-570 µS/cm with maximum conductivity observed for the month of May (570 µS/cm) and minimum value observed for the month of June (470 µS/cm). High conductivity shows that inorganic ions such as H^+, K^+, Mg^{2+}, Ca^{2+}, So_4^{2-}, HCO_3^- are present in reasonable concentration in sewage water. The TDS and conductivity are useful parameter for accessing the concentration of solid substances present in any waste water sample. The result shows that as the TDS increases the conductivity also increases and vice versa. The linear regression of conductivity and TDS shown in the figure 4.1.1 indicate that there was a positive and proportionate increase in both the parameters. The conductivity and TDS gave a correlation coefficient of r=0.5373 for the four months. Values of correlation coefficient can vary between +1 and -1 and a perfect correlation has a coefficient of +1. The regression analysis carried out to relate the two parameter with each other revealed that the conductivity and TDS gave a coefficient of 0.5373, which lies between +1 and -1. This shows that a positive correlation exist between the two parameters of raw sewage sample. This results show that the conductivity of the sewage water depends on the ionic solutes present in it (Adams, 1990).

Analysis of microbial load in the raw sewage sample of Adampool sewage treatment plant was also carried out. Determination of microbial load of raw sewage sample is an important parameter to study the percentage reduction of microorganisms by the treatment plant and thus to evaluate its working efficiency. In the present study the microbial load was calculated for all the samples collected during the four months for 10^{-6}, 10^{-7} and 10^{-8} dilutions. Values obtained are tabulated and were expressed as mean ± standard deviations from the mean. Maximum bacterial load was observed in the month of May as 610±3.6 cfu/mL for 10^{-7} dilution and 360.33±1.52 cfu/mL for 10^{-8} dilution. Minimum bacterial load was observed for the month of February as 440±2.64 cfu/mL for 10^{-7} dilution and 180.66±1.15 cfu/mL for 10^{-8} dilution.

Multiple tube fermentation (MPN) technique was also used to enumerate the bacterial load of raw sewage sample. This technique is based on the most probable number of bacteria present in a sample that produces gas in a number of fermentation tubes with various volumes of diluted samples. The MPN values are then obtained from the standard charts based on statistical studies of known concentration of bacteria (EPA, 2003; American Public Health Association, 1916). In the present study three dilutions, 10 mL, 1mL and 0.1 mL of raw sewage sample were chosen. The results of the MPN are tabulated in the Table 4.3.2. The MPN index per 100mL was calculated to be > 1600 MPN/100mL from the standard chart.

Microbial analysis of the present study shows that the maximum population of bacteria belonged to Gram negative rods followed by Gram positive rods then by Gram positive cocci and then by comma shaped organisms in the decreasing order of their percentage occurrence in raw sewage sample. Out of the total isolates 62.5 % were found to be Gram negative rods , 20.83 % belonged to Gram positive rods , 16.66 % were Gram positive cocci and 8.30% were comma shaped organisms. This data shows that the maximum number of organisms found in raw sewage samples is Gram negative and rod in shape. The abundance of Gram negative bacterial flora in raw sewage sample can be due to the presence of high amount of human waste in sewage. This data is analogous to the results of the study done by Edun and Efiuvwevwere in 2012 where they observed that the fecal coliform and Gram negative bacteria accounted for 61% of microbial flora in raw sewage sample which is consistent with the value obtained in the present study (Edun *et al*, 2012) .

The present study also focuses on the isolation and identification of bacterial population found in raw sewage sample of Adampool. The members of bacteria isolated during the study based on their presumptive identification were *Salmonella sp, Shigella sp, Vibrio sp, Escherichia sp, Enterobacter sp, Klebsiella sp, Providencia sp, Citrobacter sp* and *Proteus sp* with the percentage occurrence of 11.10%, 8.89%, 13.30%, 22%, 4.40%, 15.55% , 7%,, 2% and 4.40% respectively.

The final identification of the isolates was done based on the morphological characteristics and biochemical properties in accordance to the Bergey's Manual. This

study indicated that among the Enterobactericeae *Escherichia sp* are most abundantly found in raw sewage sample. The result obtained is consistent with the findings of Al-Zubeiry, (2005). Some species belonging to genus *Escherichia* are most common causative agents of diarrhea with *E.coli* being the most common one. Another predominant genus in the raw sewage sample was found to be *Klebsiella sp, Vibrio sp* and *Salmonella sp*. The main source of these organisms in raw sewage is human excreta. Human stools primarily act as an important reservoir of various serovars of these organisms. Many species belonging to *Klebsiella* are pathogenic to human mainly *K.pneumoniae* and *K.oxytoca. Salmonella sp* are a leading bacterial cause of acute gastroenteritis globally (Majowicz *et al*, 2010). It was reported by WHO in 1995 that 15 % of children younger than 5 years die due to diarrhea caused by *Salmonella sp. Vibrio sp* is well associated with gastrointestinal, extra intestinal and wound infections in India (Dumontet *et al*, 2000). Various species of *Vibrio* such as *V.choleraee, V.parahaemolyticus, V.furnissi* and *V.mimicus* are associated with gastroenteritis and found to be present in human faeces. In the present study low percentage of *Enterobacter sp* (4.40%) and *Proteus sp* (4.40%) and *Citrobacter sp* were isolated from raw sewage sample.

Gram positive rods and cocci isolates were grouped under three groups based on the information obtained from endospore staining, acid fast staining, and catalase test in accordance to Bergey's manual (A.E Brown, 2005). All the non spore forming, catalase positive, Gram positive cocci were grouped under Group I which consist of *Micrococcus sp, Planococcus sp* and *Staphylococcus sp.* Endospore producing Gram negative rods were grouped under Group II which consist of *Bacillus sp, Clostridium sp and Sporolactobacillus sp.* The non spore forming, catalase positive Gram positive cocci were gouped under Group III consist of *Lactobacillus sp, Listeria sp and Kurthia sp*

The study of the physico-chemical and microbial flora of raw sewage sample is an important area of study. The occurrence and prevalence of many multi drug resistant organisms and reports of emerging pathogens in sewage water increases the scope and necessity of the study in this field. The physical and chemical parameters of raw sewage play an important role in determining the type of microbial flora present in the sewage

water. These factors are important as the treatment methods in the sewage plant is dependent on the micro flora inhabited by it. Further the determination of chemical parameters of raw sewage is important as the presence of various chemicals, ions, inorganic solutes, metals etc has a great impact on the receiving water bodies when the treated sewage water is released into it. The most important need for the study of various parameters and microbial flora of raw sewage sample is to determine the percentage reduction of different unwanted substances, harmful chemicals and pathogens during the treatment process and thus to determine the working efficiency of sewage treatment plant.

CHAPTER 8

SUMMARY

8. SUMMARY

The present study is based on the determination of some physico-chemical parameters of raw sewage, isolation and characterization of bacterial population and isolation and identification of some members of Enterobactericeae from raw sewage sample. The raw sewage samples were collected from Adampool sewage treatment plant located in the East district of Gangtok. The study was done for four months November, February, May and June. The samples were collected during each of these months using standard procedures. The temperature, pH ,conductivity, TDS, odor and color of the raw sewage sample was examined for all the four months. The values obtained were found to range within 58 ^{o}F – 75^{o} F for temperature, 7.2 to 7.3 for pH, 300-400 mg/l for TDS and 470-570 µs/l for conductivity. Linear correlation of conductivity over TDS showed a positive correlation between the two parameters with correlation coefficient of r=0.5373 for the four months. The microbial load was also measured using plating technique on R2A media and Multiple Tube Fermentation (MPN) technique and maximum microbial load was measured in the month of May and minimum for the month of February . For the study of bacterial flora of the sample serial dilution was carried and isolation of bacteria was done using the spread plate technique on nutrient agar (NA), EMB, Mac conkeys agar, Salmonella-Shigella agar, Tri-citrate bile sugar media (TCBS) and Sorbitol Mac conkeys agar. Phenotypic characterization of these isolates were done based on Gram staining, endospore staining, Acid fast staining results, different biochemical tests and carbohydrate tests. Various biochemical test carried out were IMViC, urease test, catalase test, oxidase test, and triple sugar iron agar test .Eight carbohydrates (Inositol, Dulcitol, Sucrose, Raffinose, Mannose D-Mannitol, L-Arabinose and D-Xylose) were used to test the ability of the isolates to ferment them. Growth characteristics of the isolates on various specific media were also checked to aid the identification of these isolates.Gram negative bacteria was found to be dominant in the sewage sample and the percentage occurrence of various members were found to be 22% for *Escherichia sp,* 11.10 % for *Salmonella sp,*8.89 % for *Shigella sp,* 13.30 % for *Vibrio sp,* 15.50 % for *Klebsiella sp* ,7% for *Providencia sp,* 2% for *Citrobacter sp* and 4.40% for *Enterobacter sp,* and *Proteus sp.*

CHAPTER 9

CONCLUSION

9. CONCLUSION

From this study on the microbial flora and physico-chemical parameters of pre-treated sewage water the following conclusions were made:

- Raw sewage contains a very high load of microorganisms. This is due to the fact that Adampool STP receives municipal, domestic and hospital load from the entire town of Gangtok.
- Highest percentage occurrence of bacteria in the raw sewage sample was observed in case of Enterobactericeae indicating the presence of fecal matters.
- The data obtained in the present study shows that the raw sewage is highly polluted and proper treatment is essential before its release in the environment.

Further confirmed identification of the isolates has to be carried out in future work for which further experiments are required to be done. Experiments on molecular basis should be carried out to identify and confirm the identification of the isolates to genus and species level.

Further research can be carried out for the analysis of different other microorganisms present in the raw sewage water.

Research on the isolation of bacteriophage from the raw sewage of Adampool STP and its use in the removal of pathogens from the sewage can be carried out in future work.

REFERENCES

REFERENCES

Adams V.D. (1990). Water and Wastewater Examination Manual. *Chelsea, Michigan: Lewis Publishers Inc,* 1st Edition.

Adhikari S., Gupta S.K., and Banerjee S.K. (1993). Heavy metals content of city sewage and sludge. *Journal of Indian Society of Soil Science*, vol 41, pp.160-172.

Agarwal K.A. and Govind S. Rajwar. (2010). Physico-Chemical and Microbiological Study of Tehri Dam Reservoir, Garhwal Himalaya, India. *Journal of American Science,* vol 6, pp.65-71.

Al-Zubeiry, A.H.S. (2005). Microflora Inhabiting Raw Sewage, Secondary Effluent and Dewatered sludge in IBB, Yemen Republic. *The Assiut University Bulletin for Environmental Researches,* vol 8, pp. 1-15.

American Public Health Association and the American Water Works Association. (1916). Standard Methods for the Examination of Water and Sewage , 9th Edition, New York,

Annual Report of Water security and Public Health Engineering Department (2012), Govt of Sikkim, India (http://www.sikkim-wsphed.gov.in/areas_of_operation.htm).

Antil R.S. (2012). Impact of Sewage nad Industrial Effluents on Soil-Plant Health, Department of Soil Science, CCS Haryana Agricultural University, Hisar, India, pp.1-21.

Antil R.S. and Narwal R.P. (2008). Influence of sewer water and industrial effluents on soil and plant health. In: *Groundwater resources: Conservation and management*, Puranik V.D., Garg V.K., Kaushik A., Kaushik C.P., Sahu S.K., Hegde A.G., Ramachandarn T.V., Saradhi I.V. & Prathibha P., Department of Environmental Science and Engineering, GJU Science and Technology Hisar ,India, pp. 37-46.

Antil R.S. and Narwal R.P. Problems and prospectus of utilization of sewer water in Haryana, In: Management of Organic Wastes for Crop Production, Kapoor K.K., Sharma P.K., Dudeja S.S.and Kundu B.S. (2005). Department of Microbiology, CCS Haryana Agricultural University, Hisar, India, pp. 159-168.

APHA 1992 *Standard Methods for the Examination of Water and Wastewater*. 18th edition, American Public Health Association (APHA), American Water Works Association (AWWA) and Water Pollution Control Federation (WPCF), *Washington, D.C.*

Ash R.J., Mauck B., Morgan M. (2002). Antibiotic Resistance of Gram Negative Bacteria in Rivers, United States. Emerging Infectious Diseases, vol 8, pp. 713-716.

Atieno N.R., Paul Owuor O., Omwoyo O. (2013). Isolation of High Antibiotic Resistant fecal bacteria indicators, *Salmonella* and *Vibrio* species from Raw Abattoirs Sewage in Peri-Urban locations of Nairobi, Kenya, *Greener Journal Of Biological Sciences*, vol 3, pp.172-178.

Bartholomew J.W. (1962). Variable influencing results and the precise definition of steps in Gram staining as a means of standardizing the results obtained, *Stain Technology*, pp.139-155.

Blackman U. and Pickett M.J. (1978). Unusual aerobic bacilli in clinical bacteriology, *Scientific Development Press*, pp. 83.

Brierley C.L. (1990). Mineral immobilization using bacteria. *In:* Microbial Mineral Recovery. *Mc.Graw-Hill Publishing Company, New York*, pp. 303-323.

Brown A.E. (2005). Benson's Microbiological Applications, *Laboratory Manual in General Microbiology*, 9th Ed, pp. 249-263.

Cantalupo G.P, Calgua B., Zhao G., Hundesa A., Wier A. D., Katz J. P., Grabe M., Hendrix R.W., Girones R., Wang D. And Pipas M.J. (2011). Raw Sewage Harbors Diverse Viral Populations, *American Society For Microbiology*, vol 2, pp.1-12.

Canter L.W. (1996). Prediction and Assessment of Impacts on the surface-Water Environment, University of Oklahoma, *Mc. Graw-Hill publishing company,* 2nd Edition, pp. 189-220.

Cavallaro N., Padilla N., Villarrubia. J. (1993). Sewage Sludge Effects on Chemical Properties of Acid Soil, *Soil Science*, vol 156, 62-70.

Chauhan R.K. and Bhardwaj R.K. (2012). Physico-chemical analysis of sewage water of Yamuna Nagar district of Haryana before and after treatment using USAB. *Journal of Advance Scientific Reasearch,* vol 3, pp. 42-44.

Cheremisinff N.P. (2002). *Hand Book of Water and Wastewater Treatment Technologies, An Overview of Water and Water Treatments. Butterworth-Heinemann Publication*, pp. 1-60.

Christian T., Schneidera R.J., Farber H.A., Skutlarek D., Goldbache H.E. (2003). Determination of Antibiotic Residues in Manure, Soil and Surface Waters. *Acta Hydrochimica Et Hydrobiologica,* vol 31, pp. 36-44.

Close L. (2007). Occurrence of Ciprofloxacin, Sulfamethoxazole and Azithromycin in Municipal Wastewater Treatment Plants.MS Report. Department of Civil Engineering, Kansas State University,

Czekala J. (1999). Osady sciekowe zrodlem materii organiczneji skladnikow pokarmowych. *Fol. Univ. Agric. Stetin. 200 Agricultura*, vol 77, pp. 33-38 (in Polish, with English abstract).

Deng P., Zhong D., Yu K., Zhang Y., Wang T. and Chen X. (2013). Pharmacokinetics, Metabolism, and Excretion of the Antiviral Drug Arbidol in Humans. *Antimicrobial Agents and Chemotherapy*, vol 54, pp. 1743-1755.

Dumontet S., Krovacek K., Stevenson S.B, Pasquale V., Baloda S.B., Figliuolo G. (2000). Prevelance and Diversity of Aeromonas and Vibrio spp. in Coastal Waters of Southern Italy. *Comparative, Immunology, Microbiology and Infectious Diseases*, vol 23, pp. 53-72.

Edun O.M., Efiuvwevwere B.J.O. (2012). Bacterial Profiles and Physico-chemical Parameter of Water Samples from Different Sites of the New Calabar River, Nigeria. *Sustainable Agriculture Research*, vol 1, pp. 162-169.

Ellis R.C. and Zabrowarny L. A. (1993). Safer staining method for acid fast bacilli, *Journal of Clinical Pathology*, vol 46, pp. 559-560.

Environment Regulations and Technology, U.S. Environment Protection Agency. (2003). Control of Pathogens and Vector Attraction in Sewage Sludge, pp.8-19.

EPA. (2003). US Standard Methods 9221 B. Standard Total Coliform Fermentation Technique, reprinted from 20th Edition, pp. 1-14.

Eze E., Eze U., Eze C., and Ugwu K. (2009). Association Of Metal Tolerance With Multidrug Resistance Among Bacteria Isolated From Sewage. *Journal of Rural and Tropical Public Health,* vol 8 ,pp.25-29.

FAO . (2004). Quality control of wastewater for irrigated crop production. (Water reports - 10). D.W. Westcot, Health risks associated with wastewater use.

Fawell J. and Nieuwenhuijsen M..J. (2003) Contaminants In Drinking Water Environmental Pollution And Health, *British Medical Bulletin*, vol 68, pp.199-208.

Feachem R.G., Bradley D.J., Garelick H. and Mara D.D. (1983) Sanitation and Disease: *Health Aspects of Excreta and Wastewater Management*. John Wiley, Chichester.

Gallert C.,Fund K., Winter J. (2005). Antibiotic Resistance of Bacteria in Raw and Biologically Treated Sewage and in Groundwater below Leaking Sewers. *Applied Microbiology and Biotechnology*, vol 69, pp. 106-112.

Gerba, C.P., Pepper, I.L. and Maier, R.M. (2009). *Environmental Microbiology*, 2nd Edition, *Elsevier publication*, pp. 503-528.

Giger W., Alder A.C., Golet E.M., Kohler H.P.E., McArdell C.S., Molnar E., Siegrist H., Suter M.J.F. (2003). Occurrence and fate of antibiotics as trace contaminations in wastewaters, sewage sludges, and surface waters. *Environmental Analysis*, vol 57, pp. 485-491.

Golet E., Xifra I., Siegrist H., Alder A., Giger W. (2003). Environmental exposure assessment of fluoroquinolone antibacterial agents from sewage to soil, *Environmental Science Technology*, Vol 15, pp.3243–3249.

GPA. (2001). National Report for the Intergovernmental Review of the GPA 2001. Kenya.

Guardabassi L. and Dalsgaard A. (2002). Occurrence and fate of antibiotic resistant bacteria in sewage. The Royal Veterinary and Agriculture University, Department of Veterinary Microbiology. Danish Environmental Protection Agency. Environmental Project No. 722 2002, pp. 1-59.

Gupta A.P, Antil R.S, Singh A. and Poonia S.R. (1994). Impact of sewage water irrigation on soil health. In: Impact of Modern Agriculture on Environment, Behl R.K., Arora S.K. and Tauro P. CCS Haryana Agricultural University Hisar and Max Muller Bhawan, New Delhi, pp. 109-117.

Gupta A.P., Antil R.S, and Singh A. (1986). Proceedings of National Seminar on Environmental Pollution Control and Monitoring, CSIO Chandigarh, India, pp. 419-425.

Gupta A.P., Antil R.S, and Singh A. (1998). Sewer waters composition and their effect on soil properties. *Bioresource Technology*, vol 65, pp. 171-173.

Hellou J., Mackay D., Banoub, J Levels. (1999). Persistence and Bioavailability of Organic Contaminants present in Marine Harbor Sediments impacted by Raw Sewage. *Chemosphere [Chemosphere]*, vol. 38, pp. 457-473.

Hendricks R. and Pool J.E. (2012). The effectiveness of sewage treatment processes to remove faecal pathogens and antibiotic residues. *Journal of Environmental Science and Health, part A Toxic and Hazard Substances and Environmental Engineering*, vol 47(2), pp. 289–297.

Heukelekian H. and Balmat J.L. (1959). Chemical Composition Of The Particulate Fractions Of Domestoc Sewage. *Water Environment Federation*, vol 31, pp. 413-423.

Hickey J.L.S. and Reist P.C. (1975). Health significance of airborne microorganism from wastewater treatment processes, Part II, Health significance and alternatives for action, *Journal of Water Pollution*, vol 47, pp. 2758-2773.

Hirsch R., Ternes T., Haberer K., Kratz K. (1999). Occurrence of antibiotics in the aquatic environment. *Science of Total Environment.* vol 225, pp.109–118.

Hong C., Ying H., LeZheng W., Bing S. (2008), Occurrence of sulphonamide antibiotics in sewage treatment plants. *Chinese Science Bulletin*, vol 53(4), pp.514–520.

Hoogenboezem W. (2007). Influences of sewage treatment plant effluents on the occurrence of emerging waterborne pathogens in surface water, Association of River Waterworks – RIWA, Rhine Water Works The Netherlands, pp. 11- 24.

ILO. (1970). Encyclopedia of Occupational Health and Safety, International Labour Organization. Geneva, pp. 1251–3.

Jakubus M., Czekala J. (2001). Heavy Metal Speciation in Sewage Sludge. *Polish Journal of Environmental Studies*, vol 10, pp.245-250.

Kabler P. (1959). Removal of Microorganisms by Sewage Treatment Processes. *Sewage and Industrial Wastes*, vol 31, pp. 1373-1382.

Katzenelson E., Buium I. and Shuval H. I. (1976). Risk of communicable infection associated with wastewater irrigation in agricultural settlement. *Science*, pp. 944-946.

Kharche V.K., Desai V.N, and Pharande A.L. (2011). Effect of sewage irrigation on soil properties, essential nutrients and pollutant element status of soils and plants in a vegetable growing area around Ahmednagar city in Maharashtra. *Journal of Indian Society of Soil Science,* vol 59, pp. 177-184.

Khopkar S. M. (2004). Environmental Pollution Monitoring and Control. New Delhi: New Age International, ISBN 81-224-1507-5, pp. 299.

Kliger I.J. (1917). A Simple Medium for the Diffrentiation of Members of the Typhoid-Paratyphoid Group. *American Journal of Public Health,* vol 7, pp.1042-1044.

Kulbat E., Olańczuk-Neyman K., Quant B., Geneja M. and Haustein E. (2003). Heavy Metals Removal in the Mechanical-Biological Wastewater Treatment Plant "Wschód" in Gdańsk. *Polish Journal of Environmental Studies*, vol. 12, pp.635-641.

Kumar A., Rawat S., Srivastava M. and Bhusan V. (2012). Physico-chemical analysis and isolation of bacteria from water samples of Maharana Pratap Sagar, Kangra district of Himachal Pradesh. *Journal of Applied Sciences in Environmental Sanitation*, vol 7, pp.161-166.

Kumar K.R., Suman M. and singh A. (2012). Water quality assessment of Raw Sewage and final treated water with special reference to waste water treatment plant Bhopal, MP,India. *Research Journal of Recent Sciences*, vol 1, pp.185-190.

Kummerer K. (2010). Pharmaceuticals in the Environment; sources. *Annual Review of Environment and Resources,* vol 35, pp. 60-66.

Lampert C., Zessner M., Kroiss H. Sewage sludge composition – A multifunctional information. *Vienna University of Technology, Institute for Water Quality, Resources and Waste Management Karlsplatz 13, 1040 Vienna*, pp. 1003-1009.

Lewis L.D. and Gattie K.D. (2002). Pathogen Risk from Applying Sewage Sludge to Land. *American Chemical Society, Environmental Science and Technology*, pp.287-293.

Lindberg R., Björklund K., Rendahl P., Johansson M., Tyskling M. Andersson B. (2007). Environmental risk assessment of antibiotics in the Swedish environment with emphasis on sewage treatment plants. *Water Research*, vol 41, pp.613–619.

Lindberg R., Jarnheimer P., Olsen B., Johansson M., Tysklind M. (2004). Determination of antibiotic substances in hospital sewage water using solid phase extraction and liquid chromatography/mass spectrometry and group analogue internal standards. *Chemosphere*, vol 57, pp. 1479-1488.

Macaskie L.E. and Dean C.R. (1989). Microbial metabolism, desolubilization, and deposition of heavy metals: metal uptake by immobilized cells and application to the detoxification of liquid wastes. *Biological Waste Treatment, ed. A. Mizrahi. Liss, New York*, pp. 159-201.

Madigan T.M., Martinko M. J. (2006). Brock Biology Of Microorganisms. *Pearson Prentice Hall*, Eleventh Edition, pp. 916.

Mara Ducan & Nigel Horan Nigel. (2003). *Handbook of water and wastewater microbiology*, Bacterial pathogen removal from wastewater treatment plants. 1st Edition, *Elsevier Ltd.* pp. 477-490.

McArthur J.V. and Tuckfield R.C. (2000). Special Patterns in Antibiotic Resistance among Steam Bacteria: Effects of Industrial Pollution. *Journal of Applied and Environmental Microbiology*, vol 66, pp. 3722-6.

Metcalf and Eddy. (2004). Waste Water Engineering, Treatment and Reuse, 4th Edition, *McGraw Hill Publication, USA*. pp. 1345-1399.

Mormak D. A. and Casida Jr. L. E. (1985). Study of *Bacillus subtilis* Endospores in Soil by Use of a Modified Endospore Stain. *Applied and Environmental Microbiology*, vol 49, pp. 1356-1360.

Narwal R.P., Singh M. and Gupta A.P. (1988). Effect of different sources of irrigation on the physico-chemical properties of soil. *Indian Journal of Environment and Agriculture*, vol 3, pp.27-34.

Nemerow N.L and Dasgupta A. (1991). Industrial and Hazardous Waste Treatment. New York, N.Y, *Van Nostrand Reinhold*, pp.743.

Niemi M., Sibakov M., Niemela S. (1983). Antibiotic resistance among different species of fecal coliforms isolated from water samples. *Applied Environmental Microbiology*, vol 45, pp. 79–83.

Niraj S.T., Attar S.J. and Mosleh M.M. (2011). Sewage/Wastewater Treatment Technologies:A Review. *Scientific Reviews And Chemical Communications*, vol 1, pp. 18-24.

NPA. (2001). Iceland National Programme of action for the protection of the marine environment from land-based activities, Printed in Iceland. http://www.hollver.is/np.

Olanczuk-neyman K., Prejzner J. (1975). Influence of some heavy metals on biological processes of wastewater treatment. *Water Management*, vol 1, pp. 27-30, in Polish.

Ouseph P.P., Prasanthan V., Abhilash P.P.and Udayakumar P. (2009). Occurencce and Distribution Of Some Enteric Bacteria Along the South Coast of Kerela. *Indian Journal Of Marine Sciences,* vol 38, pp. 97-103.

Pathak K.A. (2011). Antibiogram of Some Selected Species of Gram Negative Bacteria Isolated From Hospital Environment. *Indian Journal Of Fundamental and Applied Life Sciences,* vol 1, pp. 145-150.

Peng X., Wang Z., .Kuang W., Tan J., Li K. (2006). A preliminary study on the occurrence and behaviour of sulphonamides, ofloxacin and chloramphenicol antimicrobials in wastewaters of two sewage treatment plants in Guangzhou, China. *Science of Total Environment*, vol 371, pp.314–322.

Periasamy D. and Sundaram A. (2013). A novel approach for pathogen reduction in wastewater treatment. *Journal of Environmental Health Science and Engineering*, vol 11:12, pp. 1-9.

Pindi P.K., Yadav P. R. and Shanker A. S. (2013). Identification of Opportunistic Pathogenic Bacteria in Drinking Water Samples of Different Rural Health Centers and Their Clinical Impacts on Humans. *BioMedical Research International* ,Volume2013, pp.1-10.

Pradeep and swamy P. L. (1986). Distribution of feacal indicator bacteria in Cochin backwater. *Indian Journal of Marine Siences*, vol 15, pp. 99-101.

Rasmussen L.D. and Sorensen S.J. (1998). The Effect of Long Term Exposure to Mercury on the Bacterial Community in Marine Sediment. *Current Microbiology*, vol 36, pp. 291-297.

Reasoner D.J. and Geldreich E.E. (1985). A New Medium for the Enumeration and Subculture of Bacteria from Potable water. *Applied and Environmental Microbiology*, vol 49, pp. 1-7.

Russell F.F. (1911). The Isolation of Typhoid Bacilli from Urine and Feces with the Description of a New Double Sugar Tube Medium. *Journal of Mediciene,* Vol 25, pp. 217-229.

Schwartz T., Kohnen W., Jansen B.and Obst U. (2003). Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water and drinking water bioflims. *FEMS Microbiology Ecology*, vol 43, pp. 325-335.

Sherman N. and Cappuccino G.J. (2009). Biochemical activities of microorganisms. *Microbiology a Laboratory Manual* , 7th Ed, pp. 143-203 .

Singh R. (2011). Study of Drinking Water in India, *Archives of Applied Science Research*, vol 1, pp. 444-449.

Sorber C.A. and Guter K.J. (1975). Health and Hygiene Aspects of Spray Irrigation. *American Journal of Public Health*, vol 55, pp. 47-52.

Spellman F. R. (1999-2000). Spellmann's Standard Handbook for Wastewater Operations, Vol. 1, 2 and 3, Lancaster PA: *Technomic Publishers*, pp. 60-80.

Sturtevant A.B., Cassell H.G and Feary W.T. (1971). Incidence of Infectious Drug Resistance Among Fecal Coliforms Isolated From Raw Sewage, *American Society For Microbiology,* vol 21, pp. 487-491.

Tarrand J.J. and Groschel D.H.M. (1982). Rapid, Modified Oxidase Test for Oxidase-Variable Bacterial Isolates. *Journal of Clinical Microbiology*, vol 16, pp. 772-774.

Taylor I.W. and Achanzar D. (1972). Catalase test as an aid to the identification of Enterobactericeae. *Applied and Environmental Microbiology*, vol 24 pp. 58-61.

Taylor R.W., Xiu H., Mehadi A.A., Shuford J.W. and Tadesse W. (1995). Fractionation of residual cadmium, copper, nickel, lead and zinc in previously sludge - amended soil. *Communication in Soil Science and Plant Analysis*, vol 26, pp. 2193-2204.

Teltsch B., Kedmi S., Bonnet L., Borenzstajn-rotem Y., and Katzenelson E. (1980). Isolation and Identification of Pathogenic Microorganisms at Wastewater-Irrigated Fields: Ratios in Air and Wastewater. *Journal of Applied and Environmental Microbiology,* vol 39, pp. 1183-1190.

Ternes T. (1998). Occurrence of drugs in German sewage treatment plants and rivers, *Water Research*, vol 32, pp.3245–3260.

Tiwari R.R. (2003), Occupational Health Hazards in Sewage and Sanitary Workers. *Indian Journal of Occupational and Environmental Medicine*, vol 12, pp. 112-115.

Tobin J.M., Cooper D.G. and Neufeld R.J. (1990). Investigation of the mechanism of metal uptake by denatured *Rhizopus arrhizus* biomass. *Enzyme and Microbial Technology,* Vol. 12, pp. 591-595.

Tongzhang, Mingzhang, Xuxiangzhang and Herberthanpingfang. (2009). Tetracycline Resistance Genes and Tetracycline Resistance Lactose Fermenting Enterobacteriaceae in Activated sludge of Sewage Treatment Plants. *Environmental Science Technology*, vol 43, pp. 3455-3460.

Umani S. F., Beran A., Parlato S., Virgilio D., Zollet T., Olazabal A. D., Lazzarini B. and Cabrini M. (2004). *Noctiluca scintillans* MACARTNEY in the Northern Adriatic Sea: long-term dynamics, relationships with temperature and eutrophication, and role in the food web. *Journal of Plankton Research.* Vol 26, pp 545-561.

Vieno N., Tuhkanen T., Kronberg L. (2007). Elimination of pharmaceuticals in sewage treatment plants in Finland. *Water research*, vol 41, pp. 1001-1012.

Watt M.M ., Watt S.J. and Seaton A. (1997). Episode of toxic gas exposure in sewer workers. *Journal of Occupational and Environmental Medicine*, vol 54, pp.277–280.

WHO, (2003). Emerging Issues in Water and Infectious Disease, World Health Organization (WHO), Geneva, Switzerland.

Xiaochang W., Pengkang J., Hongmei Z., and Lingba M. (2007). Classification of contaminants and treatability evaluation of domestic wastewater. *Frontiers of Environmental Science and Engineering*, China, vol 1, pp. 57-62.

Young S., and O'Mullan G. (2012). Quantification and identification of antibiotic resistant microbes in Hudson river and flushing bay, final reports of the Tibor T. Polgar Fellowship Program, 2010. Hudson River foundation. Section III, pp.1-29.

Zhang Z., Hibberd A. and Zhou J.L. (2008). Analysis of Emerging Contaminants in Sewage Effluent and River Water : Comparison Between Spot and Passive Sampling, *Analytica Chimica Acta*, pp. 37-44.

Zuccato E., Castiglioni S., Bagnati R., Melis M. and Fanelli R. (2010). Source, Occurrence and Fate of Antibiotics in the Italian Aquatic Environment. *Journal of Hazardous Material,* Vol 179, pp 1042–1048.