CHAPTER I

1. INTRODUCTION

1.1. River and Its Importance

A river is a natural flowing or lotic system of fresh water that provides an avenue for drainage of water from higher elevation to standing body of water at lower elevation (Lerner et al., 2001). Rivers have always been the most important freshwater resources, along the banks of which our ancient civilizations have flourished and still most of the developmental activities are dependent upon them. River water finds multiple uses in every sector of development like agriculture, industry, transportation, aquaculture, public water supply and so on. In early days, water was primarily used for domestic needs like drinking, washing, bathing and cooking. But due to industrial and urban development, requirement of water for these activities has increased along with domestic purpose. Clean, fresh drinking water is essential to living beings. Some observers have estimated that by 2025, more than half of the world population will be facing water-based vulnerability, a situation which has been called a 'water crisis' by the United Nations (Kulshreshtha, 1998). A recent report suggests that by 2030, in some developing regions of the world, water demand will exceed supply by 50% (Anon, 2011).

1.2. Water Pollution

Pure uncontaminated water does not occur in nature. Water contains mainly two kinds of impurities that are natural and human mediated. The natural impurities are not essentially dangerous. These comprise dissolved gases (nitrogen, CO_2, H_2S and so on which may be picked up during rainfall), dissolved minerals (salts of Ca, Mg, Na) which are natural constituents of water following its contact with soil, suspended impurities (clay, silt, sand and mud) and microscopic organisms. These impurities are derived from the atmosphere, catchment area and the soil. The more serious aspect of water pollution is that caused by human activity (Joseph and Selvanayagam, 2011). There are many types of human mediated water pollution, including runoff of pesticides, herbicides and faeces of agricultural land, runoff of gasoline and other chemical from concrete in urban setting and house hold chemicals (medicines or household cleansers) discarded by flushing down the toilet. Runoff occurs when pollutants are washed into a water body by the movement of water. As the water travels, it picks up sediment and pollutant, which are eventually deposited into water bodies. The river may carry with it pollutant that it has picked up along its entire length (McKinney et al., 2007 and Gambhir et al., 2012). Industrialization also increases runoff because manufacturing industries like chemical, oil refining, steel contribute many of the most highly toxic pollutants, including a variety of organic chemicals and heavy metals (Gambhir et al., 2012).

1.3. Role of Microorganism in Water Pollution

Normally, water bodies possess many microorganisms like bacteria, algae, protozoa and others. Populations of these microorganisms are able to increase rapidly due to deteriorating water quality of river or stream. Among these microorganisms, bacteria frequently occurred and utilized the greater amounts of organic nutrients in water. As a result, the faster is the biological contamination of water. Bacteria directly take an active part in the formation of all aquatic pollution. Along with their inactivate property such natural microbial population, in turn, function as vectors causing rapid and wide spread dissemination of the water borne diseases leading to high rates of morbidity and mortality. Since their first isolation from faeces in the late 19^{th} century, the coliform group of bacteria has been used as an indicator of the bacteriological safety of water (APHA, 1989). High level of fecal indicator bacteria in rivers and streams can indicate the possible presence of pathogenic microorganisms. In many cases, the extent of pollution causes increase in number of faecal bacteria and may contain pathogenic microorganisms like *Enterobacteriaceae* family, *Aeromonas* spp. and *Salmonella* spp. in high numbers beyond the assimilation capacity of the receiving water bodies (Mato, 2002). These enteric bacterial pathogens are variously incriminated in cases of diarrhoea, which accounts for a substantial degree of morbidity and mortality in different age groups worldwide (Black, 1993; Nath et al., 1993; Prado and O'ryan, 1994, Obi et al., 1997 and El-Sheikh and El-Assouli, 2001). *Pseudomonas* spp. exist throughout the aquatic environment and is associated with both healthy and diseased fish. It is generally believed that Pseudomonads can be opportunistic pathogens or produce damaging secondary infections (Woo and Bruno, 1999). In addition to this, other water borne diseases namely cholera, typhoid fever, hepatitis and cryptosporidiosis spread through water contaminated with fecal matter. However, in many fresh water systems, fecal bacteria are of little numerical significance despite the fact that they are discharged almost into the inland water.

1.4. Antibiotic Act as a Pollutant

Antibiotic pollution has been a growing concern for its human and environmental health impacts. Antibiotics once seemed like a miracle weapon against infections but as the weapon is beginning to lose its force, physicians are struggling against a shrinking number of still-effective drugs. Antibiotic pollution is increasingly being recognized as a promoter of antibiotic resistance that may add to over-prescription and misuse of antibiotics (JETACAR, 1999). Human and veterinary antibiotics enter the environment through wastewater, agricultural use of manure, and leakage from waste storage facilities (Costanzo et al., 2005). In addition to pollutants, the contamination of antimicrobial agents in river water has become a major threat to public health. The investigation on antimicrobial resistance of river microflora is the most essential part in water pollution studies because antibiotics have a direct biological action on microbes (Waksman, 1947). Spread of antibiotic resistant bacteria in river water may be due to direct or indirect human and animal interference in river system. In this perspective, cells have been made for antibiotic resistance is to be considered when establishing bacteriological water quality criteria. All the known antibiotic resistance mechanism, acquired by opportunistic and pathogenic bacteria, evolve by means of mutations occurring in pre-existing genes of the bacterial chromosome positively selected by environmental forces (Gullberg et al., 2011 and Zhang et al., 2011). According to Davies (1994); Tenover and McGowan (1996); Whitte (1998) and Schmitt et al. (2006) selection of resistant organisms in nature may result from natural production of antibiotics by runoff of animal feed or crops, waste product from treated animals or humans. Depending on their concentrations, contaminating antimicrobials exert a selective pressure and may thus favour the emergence and spread of resistances among aquatic bacteria. Therefore, greater use of antibiotics will exert selective pressure and resistant pathogens will be encountered more frequently (MacMillan, 2001). The impact of these substances on the resident microflora is difficult to assess because of the complexity of the aquatic environment, whilst the resistance patterns of bacterial fish pathogens often reflect an intensive use of antimicrobial substances (Smith et al., 2007). Many antibiotics have been used in the last several decades in medical, veterinary, agriculture and aquaculture practices (Chelossi et al., 2003; Costanzo et al., 2005 and Alpay-Karaoglu et al., 2007) and there has also been a growing interest in the presence of different pharmaceutical substances, namely antibiotics in the aquatic environment (Gòmez et al., 1996 and Hirsch et al., 1999).

1.5. Importance of Water Quality Monitoring

Water of good quality is required for living organisms. The quality of water is one of the most critical factors affecting the quality of life on Earth. Hence, monitoring water quality is an urgent and important task. The quality of water is described by its physical, chemical and biological characteristics. One of the most important issues that must be addressed in any global programme of water quality monitoring is the need for more biological monitoring (Cairn et al., 1973). It has been shown that chemical monitoring alone fails to identify many instances in which freshwater ecosystems are stressed or threatened by nonchemical factors. In the environment water pollution can alter or destroy a water source directly by the addition of the pollutant that can be used as food source by microorganisms, whose explosive increase in growth and total number depletes the water of oxygen.

Biological monitoring is needed to give greater information about overall ecosystem health and integrity. Because one of the most important factors of water pollution is the microbial contamination, enteric pathogens are typically responsible for waterborne sickness (Karaboze et al., 2003). However, biological monitoring plays an important role in pollution monitoring programme and provide information not available through conventional physical and chemical monitoring. Information about freshwater biotic integrity is highly dependent on local circumstances, such as the numbers and types of organisms and the ways in which freshwater biological communities are structured (Cairn et al., 1973). Limnochemistry study gives the proper direction in decision making processes for problems like pollution control, fish and other aquatic lives. This represents the organic material available in particulate form on which the animal population of aquatic ecosystem depends directly or indirectly. The studies on limnochemistry parameters are used to detect the effects of pollution on the water quality.

1.6. River Pollution in India

The river system of India comprises 14 major rivers (catchments >2000 km^2), 44 medium rivers (catchments 2,000 to 20,000 km^2) and innumerable small rivers and streams (Sambasivan, 2008). These rivers and rivulets play a major supporter of all possible ways of life of India. River pollution in India is now one of the important environmental problems and threat raised to public health in both rural and urban population. India faces the challenge of providing clean and safe drinking water to all citizens due to growing urbanization and industrialization. In the name of economic growth most rivers and streams are turning into sewers. As more and more rivers are getting polluted the municipalities are finding it difficult to treat river water to safe levels and supply it to citizens. Rapid industrialization in the country poses problems of environmental pollution and consequently, adverse health effects. This growth, however, cannot be stunted or dwarfed. With regard to water pollution in India, the situation could be improved by a proper dispersal of industries, distribution of pollution load in the river and suitable physicochemical/biological treatment of the effluents.

1.7. Rivers of Terai region of Northern Part of West Bengal

The northern parts of West Bengal are blessed with vast water resources and therefore are a great potential. The rivers of Himalayan foothills presumably do not run the risk of being contaminated with toxic industrial elements except, surface runoff from domestic sewage, tea gardens and agricultural fields. Monitoring of river water quality of North Bengal has thus become a commitment, which has generally being overlooked for various reasons, amongst which are primarily the resource and man power constraint, institutional inertia and public apathy due to lack of awareness. The entire stretch of many rivers and streams flows through the Terai region of Darjeeling district. Main rivers of this region belong to Ganga river system (for example, Mahananda and Balason) and other small rivers or streams like Panchanoi, Lotchka, Buri Balason, Monjha, Chengo Dumri and Buri Mechi are the parts of the two main rivers Mahanada and Balason. These rivers have two types of drainage system one has its origin in the hills and the other in plains through seepage and whose prominence is generally determined by the water table fluctuations (Haque, 2001). One of the most well known small rivers of this region is Lotchka. River Lotchka (located between the latitudes 26°43’46.98”N to 26°39’31.62”N and longitudes 88°19’53.33”E to 88º23’36.89”E) originated from the plains through seepage and mixes with the river Mahanada. The river flows through the Terai region of Darjeeling district covering an entire length of 18.2 kms. Inhabitants are situated on either sides of the entire stretch of the river and there are run-offs such as agricultural contaminants, domestic and human sewage especially from the traditional toilets and seepage from septic tanks into the River Lotchka.

1.8. Bacterial Contamination on Fish

Fish faunal diversity is thus, one of the potential and essential characteristics of life for aquatic eco-system. Relation between fish faunal diversity and water quality play as an indicator of health of river and its drainage basin. Since the microorganisms associated with fish is the reflection of their surrounding environment (Chill, 1999). Reduction in the quality of environment will lead to a marked increase in the frequency and severity of disease because interrelationship between the host organisms, their pathogens and their environmental factors play a crucial role. Lotic aquatic ecosystem revealed, bacterial population flourishes with release of organic wastes. As bacterial load increases dissolved oxygen level declines, causing trouble for fish and other aquatic organisms by either inciting fish to migrate to new environment or result in fish kills (Chiras, 2010).

Aquatic micro flora is associated with physiology of fish. There is an indication that the type of bacteria carried in the gastrointestinal tract of fish is related to a certain degree on the level of contamination of water by enteric bacteria. The majority of these bacteria are carried in the gastrointestinal tract and they are of primary significance as a source of occupational disease in fish handlers or consumers (Roy and Barat, 2011). Many pathogenic bacteria coexist with their host without causing disease under natural condition. Fish spend all their life stages in an aqueous environment that allows intimate contact with a variety of microflora, including pathogenic and opportunistic bacteria, which may colonize their various external and internal body surfaces. The epidermal mucus layer constitutes the primary biological interface between fish and the aqueous environment (Hansen and Olafsen, 1999). The mucous coat may be an adhesion site for bacteria (Conway, 1989 and Speare and Mirsalimi, 1992), but it has also been suggested that it may function to prevent firm attachment of bacteria to the skin (Crouse-Eisnor et al., 1985).

1.9. Antibiotic resistant bacteria in fish

The microorganisms in fishes are influenced by their surrounding environment. It is indicated, that if river water is contaminated with antibiotic resistant organisms there is a possibility for fish to harbour antibiotic resistant organisms from their surrounding environment. From pollution point of view, worldwide raising problem is the increase of antibiotic resistant bacterial population in river systems. In the past few years many researchers are working in this field but, work is scanty in the area of antibiotic resistant bacterial population in river water and resident fishes as well.

1.10. The loach *Lepidocephalichthys guntea* as tool of study

Lepidocephalichthys guntea (Hamilton Buchanan), is a fresh water indigenous loach. The fish has both ornamental (Panigrahi, 2009) and edible value (Mondal et al., 2007). It is widely distributed in the region of Northern India, Pakistan, Nepal, Myanmar, Thailand and Bangladesh (Talwar and Jhingran, 1991) and also very commonly found in the river Lotchka and other Terai rivers of Darjeeling district, West Bengal, India. Jha et al. (2010) stated that the loach is readily available in the local markets of Siliguri city of West Bengal, India.

1.11. Aims and Objectives

With the background, that the water quality of river water may be deteriorate due to anthropogenic pressures and harboured resident fish can be contaminated with antibiotic resistant entero-pathogenic bacteria. The nucleus of the present study entitled "**Investigation of pathogenic bacteria of a resident fish, *Lepidocephalichthys guntea* (Hamilton Buchanan), in relation to limnochemistry of a Terai River Lotchka in the Darjeeling foothills of West Bengal, India"** was therefore, executed to determine the abundance of opportunistic pathogenic bacteria with their antibiotic resistance ability in resident fish, *Lepidocephalichthys guntea* and also in river Lotchka water in relation to limnochemistry or water quality characteristics.

The work executed for a period of three years (2009 March-2012 February) based on the following objectives.

1. To study different physico-chemical parameters of Terai river Lotchka.

2. To determine the total counts of Heterotrophic bacteria, *Pseudomonas* spp., *Aeromonas* spp. and Total coliform counts in river water sample and skin, gills and gut of fish, *Lepidocephalichthys guntea.*

3. To isolate and identify opportunistic pathogenic bacteria from water samples and skin, gills and gut of fish, *Lepidocephalichthys guntea.*

4. Molecular identification of bacterial isolates.

5. To test antibiotic sensitivity of bacterial isolates.

CHAPTER II

2. REVIEW OF LITERATURE

2.1. FRESH WATER ECOLOGY

Communities of plants and animals living in water are known as aquatic ecosystem. Aquatic or watery environment are divided in to fresh water and marine water (Lerner et al., 2001). Flowing fresh water environment is called lotic ecosystem for obvious reason of unidirectional water movements along a slope in response to gravity (Wetzel, 2001). A lotic ecosystem is the ecosystem of a river, stream or spring. Lotic environments are fundamental components of regional and global biogeochemical cycles, acting as both transport pathways and sites of elemental transformations and storage and they act as sources of drinking water, fisheries resources, irrigation supplies, and waste removal systems. They are characterized by interactions among physical, chemical and biological processes, which reach a higher degree of complexity downstream (Wehr and Descy, 1998). The hydrology, chemical and biological characteristics of a stream or river reflect the climate, geology and vegetational cover of the drainage basin (Hynes, 1970 and Whitton, 1975).

High population densities and multiplicity of industrial and agricultural activities expose most hydrographic basins to heavy and rising environmental impacts especially to pollution by domestic and industrial waste residues (Salomoni et al., 2006). This increasing anthropogenic influence on lotic environments that parallels civilisation has captured public interest because of the consequent deterioration of water quality, health problems, pest plants and animals, and other problems (Salvia et al., 1999 and Bere, 2007).

2.2. STATUS AND NEED OF WATER QUALITY

Worldwide deterioration of surface water quality has been attributed to both natural processes and anthropogenic activities, including hydrological features, climate change, precipitation, agricultural land use, and sewage discharge (Ravichandran, 2003; Gantidis et al., 2007; Kundewicz et al., 2007 and Arain et al., 2008). Information on water quality and pollution sources is important for the implementation of sustainable water-use management strategies (Crosa et al., 2006; Sarkar et al., 2007 and Zhou et al., 2007). In addition, due to seasonality and regionality of river water, assessing spatio-temporal variations of river water quality at a watershed level has become an important aspect for the physical and chemical characterization of aquatic environments (Ouyang et al., 2006 and Sundaray et al., 2006). Rivers and streams are an important component of the natural environment and need to be protected from all sources of pollution because man's own survival depends on their sustainable use. Rivers, however, are increasingly under threat from different pollutants, which include conventional pollutants (organic matter and inorganic nutrients) and hazardous substances (organic contaminants and heavy metals) (Boorman, 2003 and DWAF,1995).

However one of the biggest challenges in future global water monitoring programmes is the integration of chemical and biological measures of water quality. Although the former can be carried out at national and even continental scales, the latter must be approached at the local and regional level. Yet, both have important messages to tell us about water quality and ecosystem health.

2.3. CATEGORY OF LIMNOCHEMISTRY

The Limnochemistry or water quality parameters of water are roughly divided into three categories that are (1) Physical (2) Chemical (3) Biological.

2.3.1. Physical

2.3.1.1.Temperature

The temperature is basically important for its effects on the chemistry (namely pH, dissolved oxygen and total alkalinity so on) and biological reactions in the organisms in water. Temperature is also very important to in the determination of various other parameters such as (Trivedy and Goel, 1984). Water temperature showed seasonal variation, minimum water temperature observed in winter months and maximum in pre monsoon and in monsoon (Pathani and Upadhyay, 2003). Lotic systems in regions of seasonal climates exhibit daily and annual seasonal temperature periodicity patterns (Ward, 1985). Hourly temperatures are generally lowest during the night time or early morning and highest in the mid to late afternoon, although this may shift with season and size of river (Hopkins, 1971). The effect of a change in water temperature may be direct including thermal discharges; or indirect including land-use changes, agricultural irrigation return-flows, flow modifications (river regulation), inter-basin water transfer, modification to riparian vegetation, and global warming (Dallas, 2008). All organisms have a range of temperatures at which optimal growth (adult size), reproduction and general fitness occur. This is often termed the 'optimum thermal regime'. Temperature outside of the 'optimum thermal regime' may affect the metabolism, growth, behaviour, food and feeding habits, reproduction and life histories, geographical distribution and community structure, movements and migrations, and tolerance to parasites, diseases and pollution; of aquatic organisms (Vannote and Sweeney, 1980).

2.3.1.2. pH

The pH value characterizes the acidity of river water. By definition, pH of a solution is the logarithm of the inverse of the concentration of hydronium ions (H_3O^+), produced as a result of dissociation of water (Faurie et al., 2001)**.** At extremely high or low pH levels, for example ≥9.6 or ≤4.5), the water becomes unsuitable for most organisms. Young fish and insects are also very sensitive to changes in pH. Most aquatic organisms adapt to a specific pH level and may die if the pH of the water changes even slightly (Mandal et al., 2012). According to Indian drinking water standards (2009) the permissible limit of pH concentration of river water should be pH 6.5 to 8.5. The pH does not have any adverse health effect but it alters the taste of water. Higher pH reduces the germicidal potentiality of chloride and induces the formation of toxic trihalomethanes (Maiti, 2004).

2.3.2. Chemical

2.3.2.1. Dissolved Oxygen

Dissolved oxygen is one of the most important parameters in water quality assessment and reflects the physiological and biological process prevailing in the waters. It enters the water mostly via diffusion at the water-air interface (Giller and Malqvist, 1998). Its presence is essential to maintain the higher forms of biological life in the water and affects of a waste discharge in a water body are largely determined by the oxygen balance of the system. Higher temperatures reduce the solubility of dissolved oxygen in water, decreasing its concentration and thus its availability to aquatic organisms. Chemical reaction rates and the toxicity of many substances (for example cyanide, zinc, phenol and xylene) and the vulnerability of organisms to these toxins, is intensified as temperature increases (Duffus, 1980). If the organic loading is high oxygen depletion is further accelerated by greater microbial activity at the higher temperature (Dalas, 2008). With dissolved oxygen ranging between 1-5 mg l^{-1} the fish can survive, but reproduction is poor and growth is slow if exposure is continued (Swingle, 1967). Dissolved oxygen concentration varies according to many factors; the main factors are photosynthesis and respiration by organisms. Dissolved oxygen can be removed from the water by discharges of the oxygen demanding wastes, other inorganic reductants like hydrogen sulphide, ammonia, ferrous, nitrate and other oxidable substances tends to decrease dissolved oxygen in water (Kumar, 2012).

2.3.2.2. Free Carbon dioxide

Stream and river dissolved carbon dioxide (CO_2) originates largely from terrestrial ecosystem respiration entering the hydrosphere as dissolved soil CO_2, the oxidation of allochthonous and emergent autochthonous organic carbon, the acidification of buffered waters, the precipitation of carbonate minerals, and the direct pumping of root respiration CO_2 from riparian vegetation. The relative importance of these sources coupled with their response to anthropogenic disturbance impacts how we include stream and river CO_2 evasion in global and regional budgets (Butman and Raymond, 2011). Carbon dioxide is an odourless, colourless gas produced during the respiration cycle of animals, plants and bacteria. CO_2 can build up to significantly high levels in systems with large numbers of fish and relatively slow water turnover (APHA, 2005). The pH is directly dependent on the amount of CO_2 present and inversely proportional to the activity of photosynthesis (Pandit et al., 2001). Excess of CO_2 in river water results from: (1) input from surrounding areas, including soils (Jones and Mulholland, 1998 and Barth et al., 1999), wetlands (Hamilton et al., 1995), intertidal marshes Cai et al. (1999) and urban areas Jarvie et al. (1997); (2) production by heterotrophic organisms and assimilation by phytoplankton and periphyton (Devol et al., 1987; Neal et al., 1998 and Barth et al., 1999).

2.3.2.3. Total Alkalinity

Total alkalinity is the sum of hydroxides, carbonates and bicarbonates. Alkalinity is a measure of the amount of strong acid needed to lower the pH of a sample to 8.3, which gives free alkalinity (phenolphthalein alkalinity) and to a pH 4.5 gives bicarbonate alkalinity (Shinde et al., 2011). Alkalinity of the water is its capacity to neutralize a strong acid and is characterized by the presence of all hydroxyl ions capable of combining with the hydrogen ion. Alkalinity is due to free hydroxyl ions and hydrolysis of salts formed by weak acid and strong bases. Alkalinity is primarily a function of carbonate, bicarbonate and hydro-oxide content; it is taken as an indication of concentration of these constituents (APHA, 2005). Alkalinity indicates a solution’s power to react with acid and "buffer" its pH. Alkalinity is important for fish and aquatic life because it protects or buffers against pH changes (Patra et al., 2011). According to Wetzel (2001), alkalinity is numerically the equivalent concentration of titrable base and is determined by titration with a standard solution of a strong acid to equivalency points dictated by pH values at which the alkaline contribution of hydroxide, carbonate and bicarbonate are neutralized.

2.3.2.4. Chloride

Chloride occurs naturally in all types of waters. However in natural fresh waters, however its concentration remains quite low and is generally less than that of sulphate and bicarbonate. The most important source of chloride is the discharge of domestic sewage and excreta of animals (Trivedy and Goel, 1984). Chlorides as chloride anions (Cl-) are major anions in wastewater. The chloride concentration is higher in organic wastes and its higher level in natural water is definite indication of pollution from domestic sewage. The ecological significance of chloride lies in its potential to regulate salinity of water and exert consequent osmotic stress on biotic communities. The increase in chloride concentration in lakes, rivers and dams is due to the discharge of municipal and industrial wastes as reported by Kant and Rain (1990).

2.3.2.5. Total Hardness

Total Hardness is due to concentration of alkaline earth metals. Ca^{2+} and Mg^{2+} ions are the principal cations imparting hardness, it prevents lather formation. Ca^{2+} and Mg^{2+} are the most abundant elements in natural surface and ground water and exist mainly as carbonates, bicarbonates and carbon dioxide constituted major source of inorganic carbon to producers in an aquatic ecosystem. They also act as buffers regulating the pH of the medium (Shinde et al., 2011). Kannan (1991) had classified water on the basis of hardness values in the following manner, 0- 60 mg l^{-1} soft, 61-120 mg l^{-1} moderately hard, 121-180 mg l^{-1} hard and above 180 mg l^{-1} very hard.

2.3.2.6. Nutrients

Nutrients are mainly constituted by nitrogen and phosphorus. They are essential to the composition of living matters. Nitrogen is present in river water, in five different chemical forms (NO_3^-, NO_2^-, NH_4^+, NH_3 and organic nitrogen for example urea) apart from its molecular form (N_2). The quantities of dissolved inorganic (NO_3^-, NO_2^-, and NH_4^+) and organic nitrogen compounds are highly diverse and variable because of marked differences in the inputs from surface and ground water sources, particularly as affected by human activities (Spalding and Exner, 1993), and because of many competing reactions occurring in the nitrogen cycle (Wetzel, 2001).

Ammonium-N (NH_4-N)

Ammonia is naturally present in surface water and groundwater and can be produced by the deamination of organic nitrogen containing compounds and by the hydrolysis of urea. The problem of taste and odour may, however, arise when the ammonium-N (NH_4-N) level is greater than 2 mgl^{-1}. Greater than 10 mgl^{-1}, appreciable amounts of NO_3-N may be produced from NH_4-N under suitable anaerobic conditions (WHO, 1993 and Kempster et al., 1997). If only ammonia is present, pollution of sewage must be recent. It is also produced by fishes and zooplanktons, which lead to very rapid development of phytoplanktons in water in which this chemical form of nitrogen cannot be detected by chemical analysis (Faurie et al., 2001).

Nitrite-N (NO_2-N)

Nitrites (NO_2-N) are the chemical form that is not abundant in water and are generally formed in water due to bacterial action on ammonia and organic nitrogen. They are rare because they constitute only a passing form between the nitrate and ammonia, during the processes of nitrification and denitrification. Since, nitrites are readily oxidized to nitrates, they are seldom present in significant concentration in surface waters. In natural waters, any concentration higher than 10µg l^{-1} indicates a dysfunction of microbial mechanisms involved in the nitrogen cycle (Faurie et al., 2001) and water is not for drinking purpose if concentration greater than 0.05 mg l^{-1} (WHO, 1992).

Nitrate-N (NO_3-N)

Nitrate-N in surface water is an important factor for water quality assessment (Johnes and Burt, 1993). Nitrate is the most highly oxidized form of nitrogen compounds commonly present in natural waters, because it is a product of aerobic decomposition of organic nitrogenous matter. Significant sources of nitrates are fertilizers, decayed vegetable and animal matter, domestic and industrial effluents and atmospheric washouts. Unpolluted natural water contain usually only minute amount of nitrate (Shinde et al., 2011). Usually Nitrate-N levels in excess of 0.2 mg l^{-1} show possible eutrophic conditions (WHO, 1992).

Phosphorus-P

Phosphorus is found in water in two chemical forms; the orthophosphate and organic matter compounds (ATP, NADP and so on). This element is essential to life and is thus the limiting factor in the development of living things. The environmental significance of phosphorus (in addition to phosphates, phosphorus of organic origin by means of appropriate enzyme, phosphatases) arises out of its role as a major nutrient for both plants and microorganisms (Vanloon and Duffy, 2000 and Faurie et al., 2001). Phosphorus is a nutrient for plant growth and a fundamental element in the metabolic reaction of plants and animals. It controls algal growth and primary productivity. In most natural waters, phosphorus ranges from 0.005 to 0.020 mg l^{-1}. Algae require only small amounts of phosphorus. Excess amounts of phosphorus can cause eutrophication leading to excessive algal growth called algal blooms (WHO, 1992 and Shinde et al., 2011). Phosphate is an essential nutrient for the growth and development of flora in any ecosystem. The major sources of PO_4-P in natural waters are domestic sewage, detergents, agricultural effluents with fertilizers and industrial waste (Imnatoshi and Ahmed, 2012). Nutrients that contribute to eutrophication are most commonly phosphorus that can be considered as a pollutant if it is present at a higher concentration than the normal limit (Saad, 1973).

2.3.3. BIOLOGICAL

Microbes are present in large numbers in lotic waters. Free-living forms are associated with decomposing organic material, biofilm on the surface of rocks and vegetation, in between particles that compose the substrate, and suspended in the water column. Other forms are also associated with the guts of lotic organisms as parasites or in commensally relationship (Giller and Malmqvist, 1998). Bacteria play a large role in energy recycline (Allan, 1995). Biological parameters are perhaps of greatest importance from human point of view. In water receiving sewage, domestic waste and industrial wastes, a plethora of pathogenic organisms may be present. Besides pathogen unwanted, biological growth of algae, fungi and angiospermic plant is also of utmost importance.

Water is essential to all living organisms including pathogenic microbes. Man, in his effort to get rid of his wastes have introduced into natural water bodies, noxious substances including organic wastes that promote the growth of pathogenic bacteria, fungal, viral and protozoan microbes (Adams and Kolo, 2006). In recent times, environmentalists have become increasingly concerned about the pollution of surface waters. The World Health Organization (WHO) estimated that about 80% of ill-health especially in developing countries are water related (Cheesbrough, 2000). The access of faecal matter to water through direct contamination of surface run-off or sewage may add a variety of pathogens. Atiribom et al. (2007) reported that high concentrations of bacteria and nitrates discharged into water can occur from animal husbandry operations like grazing and that this can result in health hazards to man due to the presence of pathogens. Wastes from agricultural operations, which are usually discharged into surface waters have been reported to have serious environmental and human health concerns (Adams and Kolo, 2006).

2.4. WATER QUALITY MONITORING IN DIFFERENT RIVERS

River water pollution creates a serious problem throughout the globe. Worldwide studies carried out on the rivers of Cameroon (Djuikom et al., 2006), Canada (Cameron, 1996), China (Wang et al., 2008 and Zheng et al., 2008,) Germany (Kistemann et al., 2002), Ghana (Yidana et al., 2008), Korea (Chang, 2008), Nepal (Kannel et al., 2007), Nigeria (Kakulu and Osibanjo, 1992; Ayotamuno, 1994; Ajibade, 2004), Adamiec and Helios-Rybicka, 2002, Turkey (Parlak et al., 2006) and USA (Pennington et a., 2001) suggest that virtually one or more indicators of pollutions were higher than the permissible limits (WHO, 2006). In Indian context, the physico-chemical parameters of the rivers Gola (Chandra et al., 2006), Gomti (Singh et al., 2005a, b, 2007a, b), Mahanadi (Sundaray et al., 2006), and Narmada (Sharma et al., 2008) at selected sites exhibited considerable amount of contamination in terms of metals, organic pollutants, and coliform counts. The bacteriological parameters of many of these rivers are low compared to the coliform counts noted in river Damodar in the present study. The bacteriological levels of the Ganges nearer to its origin show less pollution (Sood et al., 2008) compared to river Damodar. It appears that the study site as a point source is highly contaminated with unwanted pollutants and coliform bacteria as in river Gomti (Singh et al., 2005a, b, 2007a, b). Studies on the effect of various industrial effluents on Damodar River Ecosystem, West Bengal was Undertaken by Ghatak and Konar (1992). The Physico-Chemical and Biological characteristics of Damodar river water (West Bengal, India) was found gradually changed due to toxic effects of various industrial effluents. The concentrations of Dissolved Oxygen, Total Alkalinity, Phosphate and Total Hardness of river water were significantly decreased but CO_2, was found significantly increased ($P<0.05$) at various sampling stations. This also resulted in reduction of planktonic population (both zooplankton and phytoplankton) and also bottom organisms significantly. Bhasker et al. (2003) have studied the physico-chemical and bacteriological parameters on certain locations of the river Torsa and reported that the water was highly alkaline with high concentration of free ammonia. Gupta and Pankaj (2006) reported organic pollution of river Gomati due to anthropogenic activities. The sewage from many parts of Tripur and discharged from the surrounding areas, gets into Noyyal river, which are responsible for the decreased in water quality. Okendro and Mahanta (2007) described chloride, bicarbonate alkalinity and pH as indicators of three significant component namely, animal waste, sewage and industrial discharges into the Narmada river. Verma and Khan (2007) reported that rapid urbanization and increased anthropogenic activities have deteriorated the water quality parameter of Arpa river water of Bilaspur in Chattisgarh. Majority of the water characteristics of river Gomati at Sultanpur (Uttar Pradesh) were found to exceed the permissible limits due to sewage discharge and posed problems for the survival of the aquatic life and human beings. Roy and Barat (2011) clearly indicated that opportunistic pathogenic bacterial population increased down the stretch of the river with deteriorating water quality and have been successfully related with the human activity and anthropogenic pressures contributing to pollution of River Lotchka of Darjeeling District West Bengal, India. Trivedi et al. (2009) concluded, that Ganga water of Kanpur though fit for drinking purpose yet it needs treatment to minimize the contamination especially turbidity and Fe^{2+} contents. Higher nutrients were recorded due to eutropic condition by Paria and Konar (2003) in some rivers of West Bengal. Other than these some important studies were also conducted on water quality parameters of river water (Dhanze et al., 1998; Pathani and Upadhyay, 2003 and Agarwal and Thapliyal, 2005).

2.5. ANTIBIOTICS

An antibiotic, in a broader sense, is an antimicrobial agent that inhibits or abolishes the growth of microorganisms, such as bacteria, fungi or protozoa and also be effective against viruses. The classical definition of an antibiotic is, “a compound produced by microorganisms which inhibits the growth of another microorganism”. The term antibiotic originally referred to any agent with biological activity against living organisms; however, ‘‘antibiotic” now refers to substances with antibacterial, anti-fungal, or anti-parasitical activity (Kümmerer and Henninger, 2003).

2.5.1. Classes of Antibiotics

The first antibiotics were of natural origin, for example, penicillins produced by fungi of the genus *Penicillium* or streptomycin from bacteria of the genus *Streptomyces*. Currently, antibiotics are obtained by chemical synthesis, such as the sulpha drugs (for example, sulfamethoxazole), or by chemical modification of compounds of natural origin. An overview of the most important classes and groups is given in Table 2.5-1. Antibiotics can be grouped by either their chemical structure or mechanism of action. They are a diverse group of chemicals that can be divided into different sub-groups such as ß-lactams, quinolones, tetracyclines, macrolides, sulphonamides and others. They are often complex molecules which may possess different functionalities within the same molecule.

Table 2.5-1. Important classes and groups of antibiotic compounds (Kümmerer, 2009)

Class	Group	Sub-group	Example
β-lactams	Penicillins	Benzyl-penicillins	Phenoxypenicillin
		Isoxazolylpenicillins	Oxacillin
		Aminopenicillins	Amoxicillin
		Carboxypenicillins	Carbenicillin
		Acylaminopenicillins	Piperacillin
	Cephalosporins	Cefazolin group	Cefazolin
		Cefuroxim group	Cefuroxim
		Cefotaxim group	Cefotaxim
		Cefalexin group	Cefprozil
	Carbpenems		Meropenem
Tetracyclines			Doxycycline
Aminoglycosides			Gentamicin 1c
Macrolides			Erythro-mycin A
Glycopeptides			Vancomycin
Sulfonamides			Sulfamethoxazole
Quinolones			Ciprofloxacin

2.5.2. Sources and application of antibiotics

Natural background

Antibiotics are likely to be released into the aquatic environment via wastewater effluent and agricultural runoffs as a result of incomplete metabolism, ineffective treatment removal or improper disposal because large quantities of antibiotics are used annually in human therapy and in agriculture. Occurrences of antibiotics in the aquatic environment confirm the persistence of certain antibiotics. Although information is limited, studies on the transformation and sorption of antibiotics indicate that these processes significantly affect the fate of most classes of antibiotics. By combining information on environmental fate with the predicted concentrations, we identify that antibiotic of sulfonamides and fluoroquinolones as the most likely water contaminants, followed by macrolides. Among sulfonamide and fluoroquinolone antibiotics, sulfamethoxazole and ciprofloxacin are most likely to be present in municipal wastewater effluent and sulfamethazine is most likely to be present in agricultural runoff. Azithromycin and tylosin are the most likely macrolides present in municipal wastewater effluent and in agricultural runoff, respectively **(**Kümmerer, 2009**)**.

Human Therapy

Consumption for humans in total, per capita and the individual share of each compound varies from country to country. Antibiotic prescription rates and intake without prescription vary markedly between countries (Mölstad et al., 2002). It was found that ß-lactam antibiotics, including the sub-groups of penicillins, cephalosporins and others, make up the largest share of human use antibiotics in most countries. They account for approximately 50–70% of total antibiotic use. In most countries, sulphonamides, macrolides, and fluoroquinolones follow in decreasing order of use **(**Kümmerer, 2009**)**. Cars et al., (2001) obtained data for non-hospital antibiotic sales for 1997 from the 15 member states and analysed these according to the Anatomic Therapeutic Chemical (ATC) classification system, and expressed them as defined daily doses per 1000 people per day.

Applications in Animals

Antibiotics consumption by animals for the purposes of prevention or therapy is largely determined by modern animal breeding and fattening methods and conditions (Table 2.5-2). Antibiotics are also used to promote the growth of animals (Gaskins et al., 2002) in some countries where they are used at low doses in animal feeds and are considered to improve the quality of the product, with a lower percentage of fat and higher protein content in the meat (Cromwell, 2002). The use of even small amounts of antibiotics is associated with the selection of resistance in pathogenic bacteria. It has been argued that the use of antibiotic growth promoters imposes a selective pressure for bacteria that are resistant to antibiotics that may be used in clinical or veterinary practice, thus compromising the continued use of antimicrobial chemotherapy. Alternatives to improve the quality of the product, with a lower percentage of fat and higher protein content in the meat without using antibiotics are available **(**Kümmerer, 2009**)**.

Applications in Aquaculture

In aquaculture, antibiotics have been used mainly for therapeutic purposes and as prophylactic agents. Antibiotics authorized for use in aquaculture are oxytetracycline, florfenicol, premix, sarafloxacin, erythromycin sulphonamides potentiated with trimethoprim or ormethoprim (Serrano, 2005).

Table-2.5-2. Important antibiotics in human and animal medicine (Kemper, 2008**)**

Class	Compounds	Primary usage
Amino glycosides	Apramycin	Pigs only
	Gentamycin	All animals, humans
	Kanamycin	Dogs, pigs, cattle, horses
	Neomycin	All animals
	Sisomycin	Humans only
	Spectinomycin	Pigs, cattle, poultry, sheep
	Streptomycin	Obsolete
β-Lactams: Penicillins	Amoxicillin	All animals
	Ampicillin	All animals
	Azlocillin	Human
	Benzylpenicillin	All animals
	Cloxacilin	Cattle
	Dicloxacilin	Cattle
	Flucloxacillin	Human
	Meticillin	Human
	Mezocillin	Human
	Nafcillin	Human
	Oxacillin	Cattle
	Piperacillin	Human
	Phenoxymetylcillin	Human
	Penicillin-G	Human
Cephalosporines	Cefalexin	Dogs

	Cefalotin	Human
	Cefazolin	Human
	Ceftiofur	Cattle, pig
	Cefotaxim	Human
	Cefotiam	Human
	Cefquinom	Cattle, pig
Fenicoles	Chloramphenicole	Cats, dogs
Fluorochinolones	Ciprofloxacin	Humans
	Enrofloxacin	All animals
	Marbofloxacin	All animals
	Flumequin	Humans
	Ofloxacin	Humans
Lincosamides	Clindamycin	Dogs, humans
	Lincomycin	Pigs, cats, dogs, cattle
Macrolides	Azithromycine	Humans
	Clarithromycin	Humans
	Erythromycin	Humans, cattle, chiken
	Roxithromycin	Human
	Spiramycin	All animals
	Tylosin	Animals only
	Vancomycin	Humans
Sulponamides	Sulpanilamide	Humans
	Sulphadimethoxine	Cattle, pigs, chicken
	Sulphadimidine	Cattle, Sheep, Chicken
	Sulphamethoxazole	Humans
	Sulphapyridine	Pigs
	Sulphatiazole	Humans
Trimethoprim		In combination with sulphonamides
Tetracyclines	Chlortetracycline	Cattle, pig
	Doxycyline	Humans, cats, dogs
	Oxytetracycline	Humans, cattle, Sheep,

	Tetracycline	Human, Horse, Sheep, Pigs

2.5.3. Antibiotic-Resistant Bacteria in Aquatic Environment

2.5.3.1 Origin of Antibiotic Resistance

When concerned about the origin of antibiotic resistance in the environment, the antibiotic resistance of bacteria can be divided into two groups of origin, (a) *anthropogenic* and (b) *autochthonous*:

(a) *Anthropogenic*: The resistance present is the result of human activities. Enteric bacteria in humans and husbandry animals treated with antibiotics may develop and proliferate resistance to these substances (Schmitt et al., 2006). Treatment with (man-made) antibiotics creates conditions where selection advantages will thrive. Human and animal faeces are the direct sources of this resistance. Presence of resistance against man-made antibiotics as such is no conclusive proof on the origin. Chromosomal-lactamases, antibiotic multi-resistance genes (AMRG), and some aminoglycoside inactivating enzymes are assumed to have an anthropogenic origin.

(b) *Autochthonous:* Antibiosis to other microorganisms to compete for particular niches is a common phenomenon in microbial communities present in terrestrial and aquatic environments. Various therapeutic antibiotics are derived from, for example, soil actinomycetes (Brock et al., 2002). It is thought that most acquired resistance mechanisms originate in producers of antibiotics, such as *Streptomyces* sp. or *Penicillium notatum*. Autochthonous bacteria can also be resistant to more than one antibiotic without direct contact with therapeutic antibiotics (Seveno et al., 2002). Various plasmids belong to a class of plasmids found in bacteria from human intestines which may show resistance to a wide variety of antibiotics (Smalla et al., 2002). It is necessary to discern *in situ* between a 'natural' background of Antibitic Resistant Genes (ARGs) and an 'added' anthropogenic level. However, D`Costa et al. (2006) proved that fluoroquinolone resistance in soil was autochthonous, whereas it was previously assumed to have an anthropogenic origin only. Jones et al. (1986) found that the incidence of antibiotic resistance in aquatic bacteria was lower than that in *Pseudomonas* spp. and *E. coli*, but higher than in coliforms and faecal Streptococci.

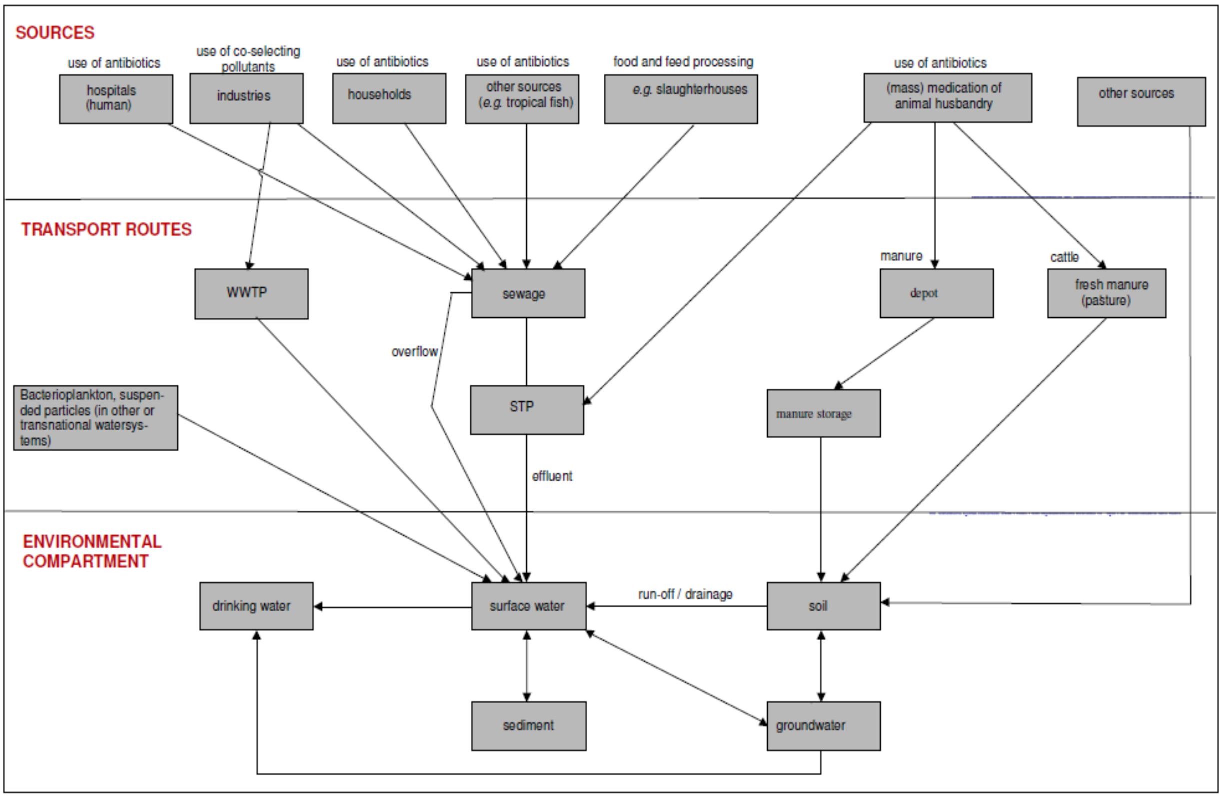


Figure1. Transport routes for antibiotic resistance (Mensink and Montforts, 2012).

It was coined that nutrient poor conditions associate with increased resistance. Pirnay et al. (2005) found no real difference between *Pseudomonas aeruginosa* strains from clinical and environmental strains. The pattern of biodiversity within clinical samples and aquatic samples from a Belgium river, resembled that of all global samples (clinical and environmental). Likewise, the origin of antibiotic resistance has been investigated in a Portuguese mesotrophic estuary affected by harbour facilities, industrial plants, domestic sewage inputs and aquaculture. This estuary was also used for recreational purposes. (Henriques et al., 2006) focused on the genes encoding for β-lactamases, the enzymes that de-activate β-lactam antibiotics. It was shown that various bacterial DNA sequences extracted from the water samples were (almost) identical to β-lactamase sequences of enteric isolates. The patterns of molecular diversity of the bacterial DNA sequences, however, indicated that the origin of the β-lactam resistance in the estuary was mixed and that there were sequences of genes encoding for β-lactamases that were more ancient than the resistance genes clusters of enteric isolates, and therefore not of anthropogenic origin. This investigation showed that in situ antibiotic resistance in aquatic environments can be autochthonous, in spite of the anthropogenic pollution with antibiotic resistance (Henriques et al., 2006). It is expected that recent improvements in DNA-based analytical techniques and the combinations of these techniques will be helpful to discriminate the anthropogenic from the autochthonous contributions to resistance (Schwartz et al., 2003).

In marine water in China, resistance against chloramphenicol was measured both in indigenous estuarine or marine bacteria, and in potential human or marine animal pathogens, although chloramphenicol had been banned in China in 1999. The *cat*I gene in the marine bacteria had probably the same clinical origin as the gene in the *cat*-positive *Enterobacteriaceae* (Dang et al., 2007).

Other substances than clinical antibiotics might be involved in increasing antibiotic resistance in the environment. Therefore, within the context of this review, other substances with an antibiotic mode of action or antibiotics used for other purposes than clinical antibiotics are defined as antibiotics as well. Such substances may be biocides used for industrial or sanitary purposes as antimicrobial slimicides or disinfectants, but also agricultural pesticides. Examples of the former are quaternary ammonium compounds, used as disinfectants in the food and feed industries and for veterinary hygiene. Examples of the latter are streptomycin and kasugamycin used as fungicide in various crops and arboriculture. In this way other substances than clinical antibiotics may select for resistance. Also organic solvents and detergents have been discussed as possible stressors to select for mutant bacteria with higher expressions of multiple antibiotic resistance (Alonso et al., 2001).

Cross-resistance of antibiotics or heavy metals is indeed possible under laboratory conditions, as the genes for resistance to these compounds may be located in close proximity and may therefore be transferred in tandem (Russell, 2002 and Schwartz, 2006). Also, the mechanism for resistance could be effective against multiple substrates. For instance, the bacterial cell pump from the human pathogen, *Listeria monocytogenes* that excretes antibiotics has been shown to excrete heavy metals as well (Alonso et al., 2001). There is evidence for *in situ* cross-resistance to metals in the aquatic environment (Russell, 2002). Recent experiments on the co-selection for microbial resistance to metals and antibiotics in freshwater environments showed that exposure of bacterioplankton to cadmium or nickel resulted in co-selection for antibiotic resistant strains (Stepanauskas et al., 2006). Berg et al., (2005) have shown that antibiotic resistance evolved in agricultural soils treated with copper, thus indicating co-selection for terrestrial environments. Pirnay et al. (2005) found one Multi Drug Resistant (MDR) clinical strain of *P. aeruginosa* in a Belgium river, among many other strains. The resistance level of this environmental strain was not as high as that of clinical strains in hospitals. However, this strain is hypothesized to have been selected for in the environment by noxious substances. Since all *P. aeruginosa* strains should be considered as potential pathogens, these observation shows that MDR strains can be disseminated through rivers and that the emergence of antibiotic resistance in the environment should be taken seriously (Pirnay et al., 2005).

` Various anthropogenic sources contribute to the spread of antibiotic resistance into the environment. The treatment of infected patients and husbandry animals is the most relevant in this respect. Waste streams that contain faeces and their residues may therefore contain antibiotic resistance genes. As manure used for fertilization of grassland or arable land may contain antibiotic resistance genes as well, manure may contribute to the dissemination of resistance as well. The most relevant sources of antibiotic resistance in European Union countries have been listed in Table 2.5-3.

Table 2.5-3. Sources of antibiotic resistance (determinations based on genetically based tests such as PCR) (Mensink and Montforts, 2007**)**

ANTHROPOGENIC SOURCES	REMARKS
a) Hospital waste water and waste water from other clinical settings	Waste water from hospitals in Gent (Belgium) and Rotterdam (The Netherlands) was shown to contain various ARG types in bacterial hosts (Hamelin et al., 2006). German hospital and municipal waste water and other studies (Schwartz et al., 2006) also indicated the occurrence of antibiotic resistance in wastewater (Volkmann et al., 2004). The abundance of vancomycin resistant enterococci and streptococci was circa 25% in waste water of a German hospital (45% was resistant to imipenem and circa 45% to ampicillin) (Obst et al., 2006). Biofilms in German hospital waste water (n = 5): the average percentage of antibiotic resistant bacteria is 6.8-71% (Schwartz, 2003). Sewage systems in a German hospital area showed particular hotspots with high levels of vancomycin resistant enterococci and multiresistant pathogens [personal communication of T. Schwartz, ITC-WGT]. Water in a city lake by a hospital contained as much resistant bacteria (75%) as dairy farm canal water (Esiobu et al., 2002).
b) Sewage from households	Sewage contains antibiotic resistance (Volkmann et al., 2004); (Cooke, 1983) a.o. due to antibiotics for public health care but also used for private tropical fish cultures. Water of an artifical residential lake (receiving no discharge) contained only 1.5%

	resistant bacteria; whereas recreation park canal water contained 54.2% resistant bacteria (Esiobu et al., 2002).
c) Manure of industrial farms with husbandry animals, incl. farms for veal calves	All faecal samples of pigs (n = 12) from German commercial animal farms showed Enterococcus faecium resistant to quinupristin/dalfopristine (Klare, 2003 and Schwartz, 2003). There is a relatively low number of Dutch veal calve farms and they generally discharge their waste water on prepurification plants, before entering the sewage.
d) Waste water of facilities in the feed and food industries (e.g., slaughterhouses)	Slaughterhouses are likely sources of antibiotic resistance as organic waste fluids of treated animals will be discharged via the sewer. Indeed, waste water from Portuguese poultry slaughterhouses contained a wide variety of antibiotic resistance (Da Costa et al., 2006). No significant differences were found in the resistance to various antibiotics of inflow versus effluent of eight Portuguese waste water treatment plants of poultry slaughterhouses. All samples contained high percentages of resistant isolates (e.g., up to 86% for tetracycline, and up to 46% for erythromycin) (Klare et al., 2003).
e) (Industrial) facilities discharging antibiotics	No data available, therefore a potential source. There is debate on the role of biocides and heavy metals as pollutants that may co select for antibiotic resistance (cross-resistance) (Bloomsfield et al., 2002 and Russell, 2002).

2.5.3.2. Transfer mechanisms of antibiotic resistance

Many studies indicate that antibiotic resistance is transferred via the genes (Kümmerer, 2004 and Seveno et al., 2002).

Transfer mechanism 1 refers to the horizontal gene flow (HGF) that is when a recipient bacterium acquires genetic material from a donor that is not its ‘parent’. This horizontal gene flow (HGF) is achieved via conjugation, transformation or transduction of mobile DNA fragments (plasmids, transposons and integrons). Davison (1999) provides more details. HGF is very common within bacterial communities and most of the resistance present in a community is indeed assumed to be obtained by HGF (Alonso et al., 2001). Antibiotic resistance can be transferred by HGF from enteric bacteria to autochthonous bacteria. Since the latter may be much more resilient to the in situ environment than the bacteria originally carrying the resistance, the resistance can remain present while the donors do not persist.

Transfer mechanism 2 is the parental or vertical gene flow (VGF), that is from bacterial ‘parents’ to ‘offspring’ by cloning, the regular process of cell and plasmid division. VGF will predominantly occur in case the microbiological conditions for bacteria in a particular niche are optimal, favouring proliferation. Hotspots are physical locations where relatively high resistance transfer rates may occur. Such hotspots may be the guts of soil arthropods (Hoffmann et al., 1998), manure (Smalla et al., 2000), but also biofilms (Schwartz et al., 2003) and upper aerobic sediment layers with high microbial densities (Montforts et al., 2007). Biofilms may be present in various aquatic environments varying from industrial process water and surface water to drinking water systems. High microbial densities indicate favourable conditions for the growth and proliferation of bacteria. *In situ* transfer rates of ARGs in the aquatic environment are needed for a more mathematical approach to quantify the dissemination and fate of antibiotic resistance. Under laboratory conditions, the transfer frequencies could be determined in mating experiments in which donors, that is, from sewage, are inoculated into media with reference recipient bacteria.

2.5.4. Antibiotic Resistant Bacteria act as a Pollutant

The intensive and wide use of antibiotics for controlling pathogens in humans, animals and plants have contributed to the increase of antibiotic resistant bacteria populations in the environment (Hirsch et al., 1999; Mazel and Davis, 1999 and Gordon et al., 2007). Furthermore, other wastes such as waste effluents from hospitals, pharmaceutical plants (Goni-Urriza et al., 2000), agricultural sewage (Halling-Sorensen et al., 1998) and wastewater treatment plants (Tennstedt et al., 2003) are discharged into the environment, which is also a main factor leading to the increasing cases of antibiotic and heavy metal resistance among bacterial population in the environment.

An important part of the dispersal and evolution of antibiotic resistant bacterial organisms depends on water environments. In water, bacteria from different origins (human, animal, environmental) are able to mix, and resistance evolves as a consequence of promiscuous exchange and shuffling of genes, genetic platforms, and genetic vectors. At the same time, antibiotics, disinfectants, and heavy metals are released in water, and might exert selective activities, as well as ecological damage in water communities, resulting in antibiotic resistance. The loss of antibiotic susceptibility among the aquatic bacteria has been observed to be affected to a considerable extent by the physico-chemical qualities of water (Pathak et al., 1993).

Most studies on bacterial antibiotic resistances in sewage (Al-Jebouri 1995, Fernandez et al., 1990), freshwater (Al-Jebouri, 1995; Jones et al., 1986; Ogan et al., 1993 and Pathak, et al., 1993) and seawater (Baya et al., 1986, Sabry et al., 1997) did not take into account these elements, leading to unexpected conclusions. For example, Jones et al. (1986)

found a higher incidence of resistance in the bacteria isolated from remote upland tarns than in those isolated from a polluted lake or sewage, with the highest values being observed for pseudomonads, which are naturally multi resistant organisms. Similarly, McKeon et al. (1995) have reported that 100% of *A. hydrophila* strains isolated from rural groundwater supplies were resistant to at least two antibiotics, but among the tested antibiotics were ampicillin and cephalothin, to which *A. hydrophila* is naturally resistant (Walsh et al., 1995). Mukherjee and Chakraborty, (2006) found several Multiple-Antibiotic-Resistant (MAR) Gram-negative copiotrophic bacteria in the River Torsa in West Bengal, India.

A correlation between resistant bacteria in rivers and urban water input has been found to have antibiotic resistance genes (ARGs) (Kümmerer, 2004; Edge and Hill, 2005; Salmore et al., 2006 and Watkinson et al., 2007). Vanneste and co-workers (2008) isolated plant pathogenic bacteria that were resistant to copper and/or streptomycin from waterways even in areas where no agriculture or horticulture is present and waterways are not used for crop irrigation. These results suggest that natural waterways could be a source of genes that confer streptomycin resistance. Antimicrobial resistance has also been found in marine bacteria (Neela et al., 2007) and bacteria living in estuaries or coastal waters polluted with sewage water (Kümmerer, 2004 and Kimiran-Erdem et al., 2007). Even in remote places such as the Arctic Sea, *E. coli* isolates originating from Arctic birds carry antimicrobial drug resistance. These results show that resistance genes can even be found in a region where no selection pressure exists (Sjölund et al., 2008).

Past studies have concluded that the concentration of mesophilic *Aeromonas* spp. is strongly correlated with common water quality parameters (Araujo et al., 1989; Araujo et al., 1990 and Rippey et al., 1989). As previously noted, (Hassani et al., 1992 and Imziln et al., 1996), all isolates of mesophilic aeromonads from rivers were found to be resistant to ampicillin and amoxicillin, and just a few (<5%) strains were found to be susceptible to novobiocin. Among the 841 isolates studied and according to the calculated ARI, *Aeromonas sobria* strains were most susceptible to antibiotics used in this study ($p < 0.05$). The most striking difference among the three phenospecies was in their resistance to cefalotin followed by cefamandole, erythromycin and sulfamethoxazole. The difference in the distribution of susceptibility to cefalotin among aeromonads species was described previously (Hassani et al.,1992; Urbaskova, 1993 and Imziln et al., 1996).

Antibiotics not only select for resistant strains of bacteria in clinical settings, but they are also poorly absorbed in both humans and animals and waste in the form of agricultural runoff into streams and waste water treatment facility effluent often containing antibiotics that continue to select for resistant strains in the river water. Multi antibiotic resistant bacterial population may act as an indicator of agricultural runoff and waste water treatment facility effluent contribute to the river pollution. The detection of antibiotic resistant bacterial population and regular monitoring of limnological parameters should be used to assess the pollution status of river system.

2.6. BACTERIAL CONTAMINATION OF FISH

Earlier researcher showed that fishes consist of diverse bacterial population in their skin, gills, gut and other parts of body. Surrounding environment play a crucial role in bacterial association in the skin and gills surfaces of fishes (Sugita et al., 1985; Sakata, 1988; Toranzo et al., 1985, 1993 and Cahill, 1990). Therefore, bacteria mainly vary in marine, estuarine and freshwater species.

2.6.1. Bacteria in Skin

Establishment of bacterial population in skin of fish mainly depends on their environment. Previous researcher Horsley (1973) examined bacteria from the skin of Atlantic salmon in various environmental conditions that is marine, estuarine and fresh waters. The results of this study showed that isolated bacterial genus varied at different sampling sites and the major components of the skin flora were similar to those present in water. Gillespie and MacRae (1975) and Gilmour et al. (1976) also found similar conclusion with the above studies that composition of bacterial genus on fish skin is directly proportional with the different geographical location. These studies are strongly indicating that the external floras of fish are a reflection of their environment. Austin (1982) and Austin (1983) identified up to the species level of the bacteria. It was found that the greatest diversity of bacterial taxa was isolated from the skin of healthy turbot. Examples of bacterial genera isolated from fish skin are given in Table 2.6-4.

After complete taxonomic study many researchers showed that some different bacterial species were present in fish skin, but these were not found in water. Whether bacteria isolated from fish skin are a true resident microflora, or are merely loosely associated with the surface, is debatable (Chill, 1990). However, bacteria present on the skin in addition of pathogenic property have many other characteristics. Bacteria of skin surface of fast-swimming fish may change the surface properties of fish skin to reduce drag and thus assist in locomotion (Sar and Rosenberg, 1987). Bacteria resident on the skin of such fish might have (a) the ability to attach strongly to the skin (b) high surface hydrophobicity (c) the ability to release drag-reducing polymers to dampen local turbulent flow and (d) mechanisms for overcoming antimicrobial agents in fish skin or mucus. An initial study of bacteria adherent to the skin of fast-swimming marine fish showed that two of 13 isolates had very high colonial hydrophobicity (measured as the ability of the edge of a lawn of bacteria to repel water droplets). There was evidence that some isolates could produce a hydrophobic slime which might reduce drag. The isolates were not identified, and further investigations into the surface properties of these bacteria and their densities on fish skin would be needed to support claims for their ability to help fish swim faster.

Table 2.6.-4. Different studies on bacterial flora in fishes.

Fish species	Bacteria isolated	References
Various	*Pseudomonas* spp., *Vibrio* spp, *Achromobacter* spp., *Flavo- bacterium* spp.,*Corynebacterium* spp., *Micrococcus* spp., *enterobacteria* spp., *Bacillus* spp.	Colwell (1962)
Atlantic salmon (*Salmo salar)*	*Moraxella* spp., *Acinetobacter* spp., *Pseudomonas*spp., *Flavobacterium/Cytophaga* spp., *Enterobacteriaceae* spp., *Micrococcaceae* spp., *Bacillus* spp., *Aeromonas* spp.	Horsley (1973)
Sea mullet (*Mugil cephalus*), whiting (*Sillago ciliata*), bream (*Mylio aus- tralis*), and flathead (*Platycephalus fuscus*)	*Micrococcus* 49%, *Pseudomonas* 18%, *Coryneforms* 12%, *Moraxella* 8%, *Flavobacterium/Cytophaga* 8%, plus *Staphylococcus,Bacillus*, *Acinetobacter*, *Aeromonas* and *Vibrio*	Gillespie and MacRae (1975)
Plaice (*Pleuronectes* and *Platessa L.*)	*Pseudomonas* Groups I, II, IV/*Achromobacter*/*Alcaligenes*/ *Agrobacterium*, *Vibrio*/anaero-genic *Aeromonas,Enterobacteriaceae,*	Gilmour et al. (1976)

	Moraxella	
Turbot (*Scophthalmus maximus L.*)	*Acinetobacterc alcoaceticus, Alcaligenes* spp., *Bacillus* sp p., *Caulobacter* spp., *Coryneforms, Cy-tophaga* *Flavobacterium- Flexibacter* spp., *Enterobacteriaceae, Hjyphomicrobium Hyphomonas* spp., *Lucibacterium harveyi, Photobacterium* spp., *Prosthecomicrobium* spp., *Pseudomonas* spp., *Vibrio* spp., unidentified spp.	Austin (1983)

2.6.2. Bacteria in Gills

Bacterial compositions of gills always have some relation with surrounding environment. Horsley (1973) had studied on the bacterial genera of gills of migrating Atlantic salmon and showed that the relative numbers of the different genera changed with changes in the environment of the fish, from marine to freshwater. In marine water, *Moraxella* spp. harboured 32% of the total bacterial flora in gills but only 8% present in upland sites of river water. *Aeromonas* spp. was present in highest numbers on gills of fish taken from upstream waters. *Vibrio* spp. was isolated only from gills of marine fishes. Trust (1975) studied on bacteriological status on gills in free-living and hatchery-reared salmonids from British Columbia and also demonstrated a difference between the gill microfloras of freshwater and marine fish. These studies also supported the hypothesis that the external flora of fish is a reflection of their environment. Table 2.6-5 represent that reported different bacterial flora in gills of fishes by several workers.

Table 2.6-5. Different studies on bacterial flora in gill of fishes.

Fish species	Isolated Bacteria	References
Rainbow trout (*Salmo gairdneri*)	*Pseudomonas* spp., *Xanthomonas* spp., *Aeromonas* spp., *Vibrio* spp., *Flavobacterium* spp., *Enterobacteria* spp., *Corynebacteria* spp., gram-positive cocci	Nieto et al. (1984)
Turbot (*Scophthalmus maximus)*	*Janthinobacterium lividum, Vibrio* spp., *Hyphomicrobium* spp., *Pseudomonas*	Mudarris and Austin (1988)

	fluorescens, Asticacaulis spp., *Prosthecomicrobium* spp.	
Fourteen different fishes	*Pseudomonas* spp., *Aeromonas* spp., *Vibrio* spp., *Flavobacterium* spp., *Enterobacteria* sp.	Sousa & Sauza (2001)
Tilapia (*Oreochromis niloticus*)	*Aeromonas hydrophila, A. caviae, Pseudomonas putida, Pseudoonas fluorescens* and *Vibrio spp.,*	Ahmed and Shoreit (2001)
Fresh water fishes	*Aeromonas hydrophila, A. caviae, Pseudomonas putida, Pseudomonas fluorescens* and *Vibrio anguillarum*	Laila El-Seedy et al. (2004)
Several fresh water fishes	*Pseudomonas* spp.	Hnadi (2008)

2.6.3. Bacteria in Intestine

There is evidence that diverse and higher microbial populations occur within the intestinal tract of fishes than their surrounding water. This is also indicates that the intestines provide favorable ecological niches for these organisms. Trust and Sparrow (1974) found that numbers of bacteria in freshwater salmonids increased between the stomach and the posterior portion of the intestine. They suggested that these numbers must represent active multiplication in the tract as they could not be accounted for by ingestion. The predominant species were *Enterobacter*, *Aeromonas* and *Acinetobacter* in these freshwater fishes. Many workers proved that the bacteria present in the aquatic environment influence the composition of the gut flora. MacFarlane et al. (1986) found that bacterial numbers were consistently higher in the intestines of the latter fish of higher organic content of estuary water (Hudson River) as compared to marine water (Long Island Sound). It was also found that the numbers of the various genera isolated varied from month to month in both environments. Similar study had been done on the intestinal microflora of salmonids in different environment like river water, lake and sea water to compare the microfloral composition in intestine of the fish in different environment (Yoshimizu et al. 1976 a and b). The intestinal microfloras were simpler than those of the surrounding waters, consisting of *Aeromonas* and *Enterobacteriaceae* in fresh water-reared fish and *Vibrio* in fish from sea water.

Fish taken from rivers and lakes also had *Aeromonas* and *Enterobacteriaceae* as the predominant intestinal microflora, and again this microflora was simpler than that of the water. In this case there was seasonal variation, with counts being higher from spring to summer and lower in winter. In anadromous salmon, the intestinal slime of nonfeeding fish was sampled and cultured, and its microflora was found to consist of *Vibrio, Pseudomonas*, and *Aeromonas*. In mature Masu Salmon cultured in fresh water, *Aeromonas* was the predominant genus isolated from intestinal slime (Yoshimizu 1976c). A similar study was undertaken (Sugita 1983) in which aerobic and anaerobic bacteria were isolated from the guts of seven types of fish from different sites along a river in Japan and were compared with the bacterial flora of water, sediment and aquatic insects. Al-Harbi and Uddin (2004) also identified huge bacterial diversity in cultured hybrid Tilapia with the comparison of surrounding environment.

Researchers concentrated recently on study of bacterial diversification based on different molecular techniques, because bacterial pathogens that infect fish include a phylogenetically diverse collection of organisms. Consequently, detection of these pathogens requires a wide diversity of sometimes time consuming assays (Fryer and Hedrick, 2003). Thus, the heterogeneity of assays combined with often fastidious growth conditions makes these organisms excellent candidates for detection by molecular methods, such as Polymerase Chain Reaction (PCR). PCR-based assays are designed to amplify specific fragments of DNA that are subsequently identified by size using gel electrophoresis (Brasher et al., 1998; Del Cerro et al., 2002 and Gonza´lez et al. 2003). By combining primer sets for multiple species-specific markers, a single PCR can be used to simultaneously detect several microbes.

Lee et al., (2002) identified total of seven different species of *Aeromonas* spp., in the intestine of trout. Vesta et al., (2008) found a greater diversity of the *Enterobacteriaceae* in the intestinal tract of the juvenile sea trout than that in the salmon. *Buttiauxella*, *Enterobacter*, *Moellerella wisconsensis*, *Pantoea aglomerans*, *Pantoea* sp., *Rahnella* and *T. arctica* have been detected in the intestinal tract of sea trout. The *Pragia fontium*, *Pragia* sp. and *Serratia* from the *Enterobacteriaceae* family have been detected in the salmon intestinal tract.

2.7. ANTIBIOTIC RESISTANT BACTERIA IN FISH

It had been pointed earlier that antibiotic resistant bacteria play as pollutant in river water and develop due to anthropogenic pressure. It is expected that resident fish harbour antibiotic resistant bacteria from antibiotic resistant bacterial polluted river. Pathak and Gopal (2005) studied the antibiotic resistant bacterial profile in various fishes as well as river water also. Further, Pathak and Gopal (2009) also found multi antibiotic resistant *Aeromonas* sp in fresh water *Channa punctatus* and indicated that pollution due to toxic metal or antibiotics in ecosystem enhances density and pathogenic characteristic of bacteria in fish organs, which may pose risk to fish fauna and public health. Several molecules belonging to the same chemical family are usually affected by a single mechanism of resistance (cross-resistance). Both ampicillin and cephalothin, for example, are inactivated by chromosomal β- lactamases produced by many *Aeromonas* species (Walsh et al., 1995). An increase in resistance levels of the genus *Aeromonas*, particularly to β-lactam antibiotics has been observed previously (Rowe-Magnus et al., 2002). Antibiotic resistance motile Aeromonad were also found in fresh water fishes (Hatha et al., 2005) and in Danish rainbow trout fish (Schmidt et al., 2000). Many other researchers also reported on multi-antibiotic resistant bacteria found in healthy and infected fishes; Shome and Shome (1999); Wei et al. (2011); Noorlis et al. (2011) and Roy et al. (2013) and (2014).

2.8. CONCLUSION

Worldwide river pollution is now major warning to total ecosystem. Anthropogenic pressure is a crucial factor for river water pollution. From pollution point of view, one of the most recent threatening pollutants in river water are antibiotics and multi antibiotic resistant bacterial population. Anthropogenic pressure is playing a major role for the development of this pollutant in aquatic environment and also deteriorating of water quality. Due to water quality deterioration, many pathogenic organisms are also increasing in water bodies. It is obvious, if water body is contaminated with antibiotic resistant pathogenic organisms, they will affect on the aquatic animals directly.

The detection of antibiotic resistant bacteria in river water and resident fishes along with regular monitoring of limnological parameters of river should therefore be done to assess the pollution status of Riverine system.

CHAPTER III

3. MATERIALS AND METHODS

3.1. EXPERIMENTAL DESIGN

With a view to determining the water quality parameters of river Lotchka and the abundance of opportunistic pathogenic bacteria with their antibiotic resistance pattern being harboured both in the river water and resident fish, *Lepidocephalichthys guntea*, a study was conducted by selecting four different sampling sites (S1, S2, S3, and S4) over the entire stretch of 18.2 km river Lotchka of Darjeeling district, West Bengal, India, located between the latitudes 26°42’39.71”N to 26°41’22.47”N and longitudes 88°20’54.78”E to 88º22’00.20”E (Fig. 3.1-1). S1 was situated on the west side of the University tea garden and received agricultural contaminants; S2 and S3 were contaminated by run-offs such as, domestic and human sewage because of the inhabitants and pig farms located on either side of these two sites whereas, S4 was located near the cremation site and agricultural farms.

3.2. DURATION AND PLACE OF STUDY

The study was performed for a period of three years from April 2009 to March 2012. Water samples, both for water quality and bacterial analysis and live healthy loach, *Lepidocephalicthys guntea,* were collected from the river Lotchka at monthly interval and analyzed in the Aquaculture and Limnology Research Unit, Department of Zoology, University of North Bengal. The collected water samples were subjected to water quality and bacteriological analysis, where as the fish was used for the estimation of both the quantitative counts and isolation of bacteria.

3.3. WATER QUALITY ANALYSIS

Water quality analyses were performed following Trivedy and Goel (1984) and APHA (2005).

3.3.1. Water Sample Collection Procedure

Water samples were collected in sterile Borosil-make BOD bottles and sample bottles (Tarson make) from four sites (S1, S2, S3 & S4) between at 8 A.M to 10 A.M. for water quality analysis at monthly interval.

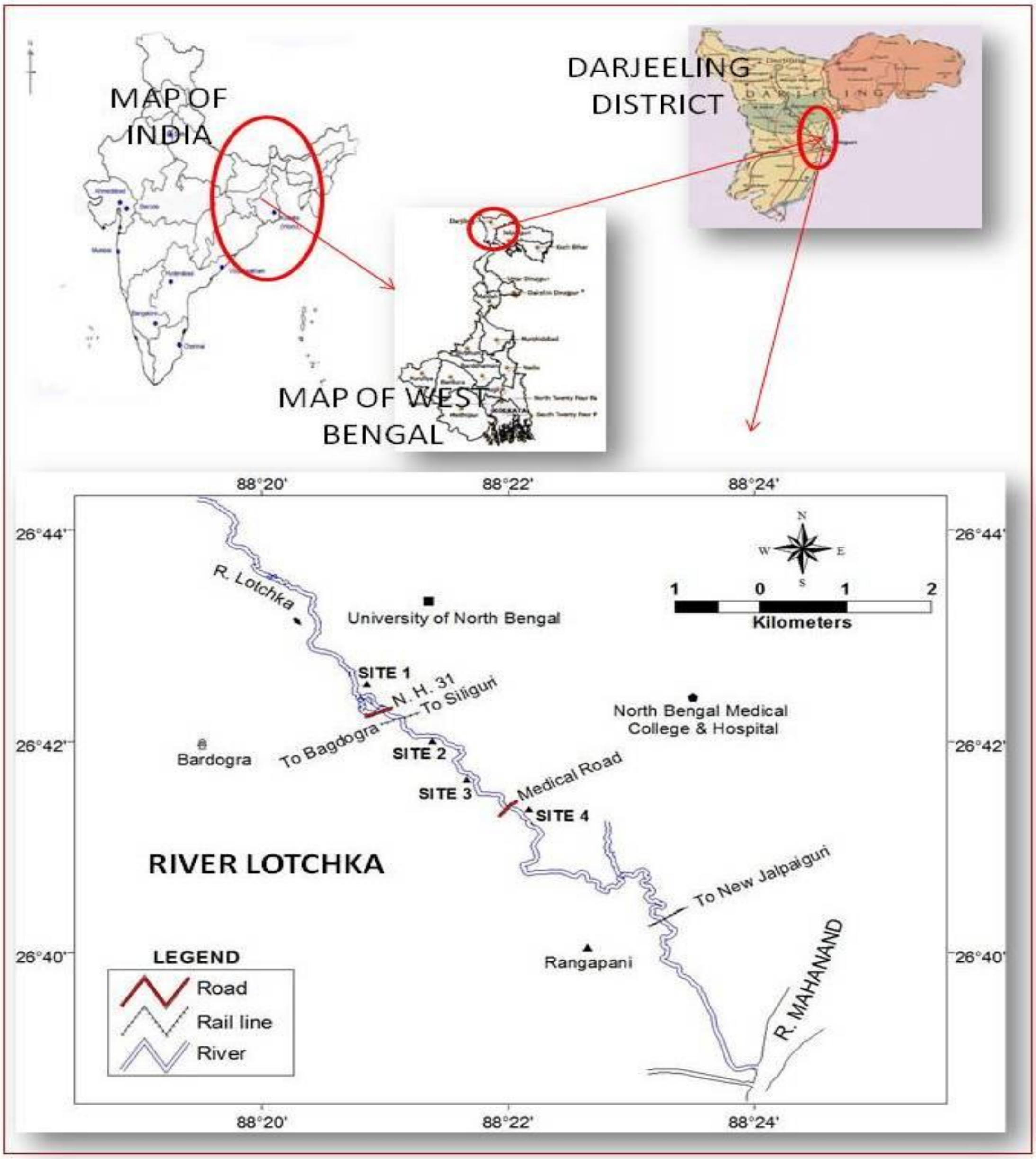


Fig 3.1-1: Study area and location of the river Lotchka in Darjeeling District, West Bengal, India.

SITE 1

SITE2

SITE 3

SITE 4

Fig.3.1-2: Images of four different sites of river Lotchka

3.3.2. Examination Procedure of Water Quality Parameters

3.3.2.1. *Temperature*

Temperature in degree Centigrade (^0C) was recorded by using a standard mercury thermometer by dipping the thermometer bulb in water. The readings were recorded only after getting constant mercury level in the thermometer column.

3.3.2.2. *pH (Hydrogen ion concentration)*

pH was measured using a portable pH meter (Hanna make). Before using the meter, it was standardized at pH buffer 4 and 7.

3.3.2.3. *Dissolved Oxygen (DO)*

For the estimation of Dissolved Oxygen of water modified Winkler's Method (APHA, 2005) was performed. The method involved taking 300 ml water sample in a glass stoppered BOD bottle avoiding air bubbles. 2 ml of Manganous Sulphate and Potassium Iodide each were added to the sample water for fixation of the DO. After obtaining a precipitate 2ml of concentrated H_2SO_4 was added to dissolve the precipitate. About 100 ml of sample was then taken in a clean conical flask and titrated against 0.025N Sodium Thiosulphate ($Na_2S_2O_3$) solution using starch as an indicator and get a colourless end point. The calculations were made using the following formula.

Concentration of

$$\text{Dissolved Oxygen in } (mgl^{-1}) = \frac{\text{Volume of sample consumed (ml) X 8 X 1000}}{V_2\,(V_1 - V)/V_1}$$

Where, V_1= volume of sample bottle after placing the stopper.

V_2= volume of the part of the contents titrated.

V= Volume of Manganous Sulphate and Potassium Iodide.

3.3.2.4. ***Free Carbon dioxide***

Free Carbon dioxide was measured by titrimetric method (APHA, 2005) using phenolphthalein as indicator. Water sample (100ml) was taken in a conical flask and a few drops of phenolphthalein indicator added. If no pink colour appeared it indicated the presence of free carbon dioxide. This colourless solution was then titrated against N/44 NaOH until appeared faint pink colour appeared. The volume of NaOH consumed was noted and final calculation made as follows:

$$\text{Concentration of Free Carbon dioxide } (mgl^{-1}) = \frac{\text{Volume of sample consumed (ml) X 1000}}{\text{Volume of water sample}}$$

3.3.2.5. ***Total Alkalinity***

The total alkalinity which consisted of both Bicarbonate (Methyl orange) and Carbonate (Phenolaphthalein) was measured titrimetrically using the method of Trivedy and Goel (1984). 100ml of water sample was taken in a 250 ml conical flask and a few drops of phenolphthalein indicator added to it. If the sample turned pink, then it was titrated against N/50 H_2SO_4 solution until the pink colour disappeared. This gave the Carbonate or Phenolaphthalein alkalinity. To the same titrated solution 2 to 3 drops of Methyl Orange indicator was added to obtain a yellow colour on shaking. This solution was then further titrated with N/50 H_2SO_4 to obtain an orange colour. The end point reading was recorded. This was the Bicarbonate or Methyl orange alkalinity. The total alkalinity was thus the sum of carbonate and bicarbonate concentrations. The volumes were recorded and utilized to calculate the total alkalinity:

$$\text{Concentration of Total alkalinity } (mgl^{-1}) = \frac{\text{Volume of sample consumed (ml)by N/50 } H_2SO_4 \text{ X1000}}{\text{Volume of water sample taken}}$$

3.3.2.6. ***Total Hardness***

Following the EDTA titrimetric method Total Hardness of sample was estimated (Trivedy and Goel, 1984). For the titration, 100 ml of water sample taken in a conical flask and 1 ml of ammonia buffer solution added. Then a few drops of Ericrome Black T indicator solution was added. The solution turned to wine red colour. This solution was then titrated against 0.01M EDTA solution until the colour changed to blue.

$$\text{Concentration of Total Hardness } (mgl^{-1}) = \frac{\text{Volume of sample consumed (ml) X 1000}}{\text{Volume of water sample}}$$

3.3.2.7. *Chloride*

The estimation of Chloride in water sample was determined by Argentometric method (Trivedy and Goel, 1984). 100ml of sample water was taken in a conical flask and 2 ml of Potassium Chromate solution (K_2CrO_4) solution added. This was then titrated against 0.02N $AgNO_3$. A permanent brick red colour determined the end point of titration. The consumption volume of titrate was noted and the final calculation was done as follows.

$$\text{Concentration of Chloride } (mgl^{-1}) = \frac{\text{Volume of } AgNO_3 \text{ consumed X1000X35.5}}{\text{Volume of water sample}}$$

3.3.2.8. *Ammonium-N*

Ammonium-N of water sample was determined by Phenol-Hypochlorite Method (APHA, 2005). 50 ml of water sample was taken in a 250 ml conical flask and 2 ml phenol solution was added. After shaking, 2 ml sodium nitroprusside was mixed. Finally, 5 ml of an oxidizing solution (Sodium hypochloride and Alkaline solution) were added and allowed to stand for one hour. Absorbance or Optical Density at 640 nm was noted in a spectrophotometer (Ray-Leigh) and the concentration obtained from a standard curve.

3.3.2.9. *Nitrite-N*

Following the method described in APHA, (2005) Nitrite-N of water sample was estimated using α-Napthalamine and Sulphanilic Acid Method. 100ml filtered sample was taken in a 250 ml conical flask. To this 1 ml α-Napltholamine solution and 1 ml Sulphanilic acid were added. Sample was kept at room temperature for 10 minutes. A pink colour developed. The absorbance was noted in a spectrophotometer (Ray-Leigh-2601, China) at 540 nm and the concentration obtained from a standard curve.

3.3.2.10. *Nitrate-N*

The Brucine Method was followed is determining Nitrate-N of water sample (Trivedy and Goel, 1983). Water sample of 2 ml was taken and mixed with 3 ml distilled water. To this 1 ml Brucine-Sulphanilic acid was added and mixed carefully with 10 ml concentrated H_2SO_4 solution and kept in the dark for 10 minutes. After 10 minutes the sample was taken out from the dark and 10 ml distilled water was added to the sample. Again, it was kept in the dark for another 30 minutes. After 30 minutes the absorbance at 410 nm in a spectrophotometer (Ray-Leigh-2601, China) was observed and the concentration obtained through a standard curve.

3.3.2.11. *Phosphate-P*

Phosphate-P of water sample was determined by Stannous Chloride Method (APHA 2005). 100ml of filtered water sample was taken in a 250 conical flask and 4 ml Ammonium Molybdate reagent was added. Then, 0.5 ml of stannous chloride in glycerine was mixed. After 10 to 12 minutes, absorbance at 690 nm with blue colored was observed and the concentration obtained from the standard curve.

3.4. BACTERIOLOGICAL STUDY OF RIVER WATER AND FISH SAMPLES

3.4.1. SAMPLE COLLECTION

Water and fish samples were collected at monthly intervals from the four sites of the river Lotchka. The water samples for bacteriological study were collected in sterilized glass sampling bottles and were analyzed within two hours of bringing to the laboratory. For fish sampling, 10-12 freshly caught fishes were brought to the laboratory in sterile plastic bags containing the river water and processed within 2 hours of their collection.

3.4.2. BACTERIAL ENUMERATION OF WATER AND FISH SAMPLES

3.4.2.1. *Water Sample*

After sample collection, serial dilutions of water samples were prepared in sterile physiological saline and plated onto Nutrient Agar and different selective agar medium namely, *Aeromonas* Starch DNA Agar Base, *Pseudomonas* Agar Base, Bismuth Sulphite Agar and MacConkey Agar all manufactured by HiMedia Laboratories Ltd., Mumbai, India for colony counts and recovery of the selective organisms using Spread Plate technique (Okpokwasili and Alapiki, 1990). All the plates were incubated at 37 ±1 °C for 24–48 hours. After incubation, the selective media plates were examined and the growth of colonies on the plates was recorded for counts of Heterotrophic bacteria namely, *Aeromonas* spp., *Pseudomonas* spp., and Total Coliform.

3.4.2.2. *Fish Sample*

Through dissection, skin, gills and gut samples of each fish were removed aseptically and collected separately in sterile containers. Skin, gills and gut had been particularly selected because of their association with their surrounding environment. The dissected organs were homogenized in a sterile glass tissue homogenizer and serial dilutions of homogenized organs were prepared same as water samples in sterile physiological saline. The diluents were then plated onto Nutrient Agar and different selective agar medium namely, *Aeromonas* Starch DNA Agar Base, *Pseudomonas* Agar Base, Bismuth Sulphite Agar and MacConkey Agar all manufactured by HiMedia Laboratories Ltd., Mumbai, India, for colony count and recovery of the selective organisms using Spread Plate technique (Okpokwasili and Alapiki 1990) and then incubated at 37 ±1 °C for 24–48 hours. After incubation, the selective media plates were examined and the growths of colonies on the plates were recorded for count of Heterotrophic bacteria, *Aeromonas* spp., *Pseudomonas* spp. and Total Coliform.

3.4.3. ISOLATION OF PURE CULTURE AND THEIR PRESERVATION

After incubation, the selective media plates were examined and the growths of colonies on the plates were recorded for counts of *Aeromonas* spp., *Pseudomonas* spp., and total coliform. Growing colonies of selective media were followed by Streaking method for the isolation of pure cultures. Purification of culture was done by sub-culturing a part of a typical well separated colony on the corresponding medium. The process was repeated several times. The pure cultures were then stored at 4° C and used for studying the cultural and biochemical characteristics as well as the sensitivity of isolates to different antibiotics.

3.4.4. INITIAL IDENTIFICATION OF ISOLATES

3.4.4.1. Microscopic Examination

Smears were made from each type of colony on primary culture and from purified colonies. The smears were then fixed by heating and stained by Gram stain method as described by Barrow and Feltham (1993) and Holt et al. (1994). The stained smears were examined microscopically under oil immersion lens for cell morphology, cell arrangement and staining reaction.

3.4.4.2. Biochemical Procedures

All biochemical tests were performed as described by Barrow and Feltham (1993). The tests are explained as below.

3.4.4.2.1. *Motility Test*

The test culture was inoculated by stabbing with a straight inoculating wire into the Craigie tube in motility medium and then incubated at 37°C for 24 h. The organisms were considered motile if there was turbidity in the medium of the Cragie tube, while the growths of non-motile organisms were confined inside the Craigie tube.

3.4.4.2.2. *Catalase Test*

Catalase test was done to distinguish between the catalyzing enzyme producing bacteria and non-catalyzing enzyme producing bacteria. Trypticase Soy Agar Medium was used for this test. Preparation of Trypticase Soy Agar Medium was done as follows:

Trypticase Soy Agar Medium

Composition

Ingredients	**gm l^{-1}**
Trypticase	15g
Phytone	5g
Sodium Chloride	5g
Agar	15g
Distilled Water	1000ml
pH	7.3

Fresh cultures were inoculated in Trypticase Soy Agar Medium and incubated at 37° C for 24 to 48 hrs. 3 to 4 drops of H_2O_2 were smeared over the growth medium. Positive reaction was indicated by evolution of gas (air bubbles).

3.4.4.2.3. *Citrate Utilization Test*

Citrate test was used to differentiate among enteric bacteria on the basis of their ability to utilize citrate by the citrase enzyme. Simmon's Citrate medium was used for this test. Preparation of Simmon's Citrate Medium was done as follows:

Simmon's Citrate Medium

Composition

Ingredient	**gm l^{-1}**
Ammonium dihydrogen phosphate	1gm
Dipotassium phosphate	1gm
Sodium Chloride	5gm
Sodium citrate	2gm
Magnesium Sulphate	0.2gm
Agar	15gm
Bromophenol Blue	0.8gm
Distilled Water	1000ml
pH	6.9

The test culture was inoculated onto the Simmon's Citrate Medium and incubated at 37° C. The examination was done daily for 7 days. A blue color indicated a positive reaction.

3.4.4.2.4. *Hydrogen Sulphide Production Test*

The Hydrogen Sulphide Production test was performed to identify the H_2S producing bacteria. SIM (Sulphide-Indole-Motility) Agar medium was used as medium for this test. Preparation of SIM Agar Medium constituent is as follows:

SIM Agar Medium

Composition

Ingredient	**gm l^{-1}**
Peptone	30g
Beef extract	3g
Ferrous Ammonium Sulphate	0.2g
Sodium thiosulphate	0.025g
Agar	3g
Distilled Water	1000ml
pH	7.3

Fresh culture was inoculated on to SIM Agar medium and incubated at 35-36˚C for 48 hrs. Blackening of the culture medium was an indication of H_2S production.

3.4.4.2.5. *Aesculin Hydrolysis*

This test was performed to see if the microbes can hydrolyze the compound Aesculin as a carbon source. Aesculin Broth was used as liquid medium for Aesculin Hydrolysis test. Preparation of Aesculin Broth medium consists of the following composition.

Aesculin Broth

Composition

Ingredients	**gm l^{-1}**
Aesculin	1gm
Ferric citrate	0.5gm
Peptone water	1000ml

The fresh culture was incubated onto Aesculin Broth and examined daily up to 5 days for blackening. This indicates hydrolysis of the Aesculin.

3.4.4.2.6. *Arginine Hydrolysis*

The Arginine Hydrolysis was used to see if the microbe can utilize the amino acid arginine as a source of carbon and energy for growth. Use of arginine is accomplished by the enzyme arginine dihydrolase. Arginine broth medium was used for this test. Preparation of Arginine broth medium was prepared as follows:

Arginine broth

Composition

Ingredients	**gm l^{-1}**
Peptone	5gm
Yeast extracts	5gm
K_2HPO_4	2gm
Glucose	0.5gm
Arginine monohydrochloride	3gm
Distilled Water	1000ml
pH	7.0

5ml Arginine broth was incubated and kept for 24hrs at 37^0C. Then 0.25ml Nessler's Reagent was added. Arginine Hydrolysis was indicated by the development of a brown colour.

3.4.4.2.7. *Urease Test*

Urease test was performed to distinguish whether urease was produced or not in the bacteria. Urea agar was used as medium for this test. The constituent of Urea Agar medium is as follows:

Urea agar medium

Composition

Ingredients	**gm l^{-1}**
Peptone	1g
Sodium Chloride	5g
Dipotasium hydrogen phosphate	2g
Agar	20g
Distilled Water	1000ml
pH	6.8
Glucose	1g
Phenol Red (0.2%)	6ml
Urea	20%

The test culture was inoculated onto the slant of Urea Agar Medium and incubated at 37° C for 24-48 hours. This was examined daily for five days. The positive reaction was indicated by a pink color. Negative and weak tests were left for a week before final observation.

3.4.4.2.8. ***Indole Production Test***

This test was performed to determine the indole production from microbial catabolism of tryptophan. Tryptone broth was used as liquid medium. Preparation of tryptone broth medium was done as follows:

<u>Tryptone broth</u>

Composition

Ingredients	**gm l^{-1}**
Peptone	10g
Distilled water	1L

Culture was incubated in Tryptone broth at 37°C for 48 h. One ml of Kovac's reagent was added to the tube. The appearance of a pink color in the reagent layer within a minute indicated positive reaction.

3.4.4.2.9. ***Methyl Red and Voges- Proskauer Test (MR & VP Test)***

The Methyl Red (MR) and Voges- Proskauer (VP) test were used to differentiate two major types of enteric bacteria that produce large amounts of acid and those that produce the neutral product action as end product. Glucose phosphate medium was used for MR/VP test. The composition of Glucose Phosphate medium is as follows:

<u>Glucose Phosphate medium</u>

Composition

Ingredient	**gm l^{-1}**
Peptone	7g
Dextrose	5g
Potassium phosphate	5g
Distilled Water	1000ml
pH	6.9

The test culture was inoculated into Glucose Phosphate medium and then incubated at 37°C for 48 h. Two drops of Methyl Red indicator were added and shaken well. A red color indicated positive reaction. Yellow or orange colour indicated a negative reaction.

In another test, one ml of cultured medium was transferred aseptically into sterile test tubes and then 0.6 m1 of 5% alpha-naphthol solution was added, followed by 0.2 m1 of 40%

KOH aqueous solution. The test tubes were shaken well and kept at a slant position for 1 h. A positive reaction was indicated by a strong red color.

3.4.4.2.10. *Sugar Fermentation Test*

Sugar Fermentation test was performed to determine the fermentative degradation of various carbohydrates by bacteria. Carbohydrate medium was used for this test. Preparation of Carbohydrate medium constituent is as follows:

Carbohydrate medium

Composition

Ingredients	**gm l^{-1}**
Peptone	10g
Sugar	5g
Sodium Chloride	15g
Phenol Red	0.018g
Distilled Water	1000ml
pH	7.3

Carbohydrate medium was inoculated into the test culture and then incubated at 37°C. Examination was done daily for 7 days. The acid production was indicated by a change in colour and gas production by the presence of an empty space in Durham’s tube.

3.4.4.2.11. *Reagents used for Biochemical Characterization*

α-Naphthol solution

α- Naphthol	5gm
Ethanol	100ml

The above solution was stored in the dark at room temperature after preparation (Fulton et al., 1960).

Potassium Hydroxide

It was used for Voges Proskauer test and was prepared as 40% aqueous KOH solution.

Methyl red

Methyl red	0.04 gm
Ethanol	40.0 ml
Distilled Water	100.0 ml

Nessler's Reagent

Potassium iodide	5.0 gm
Mercuric iodide	11.5 gm
Distilled water	70.0 ml
6N NaOH	40.0 ml

Kovac's reagent

para-Dimethylaminobenzaldehyde	5.0 gm
Amyl alcohol	75.0 ml
Concentrated HCl	25.0 ml

The para-Dimethylaminobenzaldehyde was dissolved in Amyl alcohol by gentle warming in a water bath having a temperature of 50-55^{0}C. The reagent was cooled and stored at 4^{0}C (Kovac's, 1928).

3.4.4.3. Selective Medium used for bacterial culture and enumeration

Nutrient Agar

This medium was used for the culture of heterotrophic bacterial count. The composition of Nutrient Agar medium as given by Chen and Kueh, (1976) is as follows.

Composition

Ingredients	**gm l^{-1}**
Peptic digest of animal tissues	5
Sodium Chloride	5
Beef Extract	1.5
Yeast extract	1.5
Agar	15
pH	7.4

28 gms of the above Nutrient Agar powder was dissolved in 1000ml distilled water and sterilized by autoclaving at 15 lbs pressure (121^{0}C) for 15 minutes.

Nutrient broth

This medium was used for preparation of fresh culture of Heterotrophic bacterial isolates. Composition of Nutrient Broth medium as suggested by Chen and Kueh (1976) is follows as:

Composition

Ingredients	**gm l^{-1}**
Peptic digest of animal tissues	5
Sodium Chloride	5
Beef Extract	1.5
Yeast extract	1.5
pH	7.4

The medium was prepared by adding 13 g of nutrient broth powder to one litre of distilled water and mixed well. The pH was adjusted to 7.4. The mixture was distributed in 5 m1 volumes of clean vials, and then sterilized by autoclaving at 121 °C for 15 minutes (Chen and Kueh, 1976).

Aeromonas Starch DNA Agar Base (Hi Media Laboratory, India)

Aeromonas Starch DNA Agar Base was used for selective isolation of *Aeromonas* spp.
Its composition is as follows:

Composition

Ingredient	**gm l^{-1}**
Peptic digest of Animal tissue	15.0
Peptic digest of soyabean meal	5.0
Sodium chloride	5.0
Corn starch	10.0
Deoxyribonucleic acid (DNA)	2.0
Agar	15.0
pH	7.5

52 gms of the above ingredients were suspended in 1000ml distilled water and sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 minutes. After cooling, one vial of Ampicillin Supplement (FD082) was added.

Bismuth Sulphite Agar (HiMedia Laboratory, India)

For the selective isolation of *Salmonella* spp. Bismuth Sulphite Agar was used. The Composition of the Bismuth Sulphite Agar medium is given as follows.

Composition

Ingredients	**gm l^{-1}**
Pancreatic digest of casein	5.0
Peptic digest of Animal tissue	5.0
Beef extract	5.0
Dextrose	5.0
Sodium phosphate	4.0
Ferrous sulphate	0.3
Bismuth sulphite indicator	8.0
Brilliant green	0.025
Agar	20.0
pH	7.6

52.32 gms of the above ingredients were dissolved in 1000ml distilled water and then heated to boiling for complete dissolution.

Pseudomonas Agar Base (HiMedia Laboratory, India)

This medium was used for selective isolation of *Pseudomonas* spp. The composition of Pseudomonas Agar Base medium is given as follows:

Composition

Ingredients	**gm l^{-1}**
Pancreatic digest of Gelatin	16.0
Casien enzymic hydrolysate	10.0
Potassium Sulphate	10.0
Magnessium chloride, anhydrous	1.4
Agar	11.0
pH	7.1

The medium containing 48.4 gms of the above was dissolved in 1000 ml distilled water and then sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 minutes.

MacConkey Hi-Veg Agar w/CV and NaCl (HiMedia Laboratory, India)

This medium was used for Total Coliform count and isolation of *Enterobacteriaceae*. Composition of MacConkey Hi-Veg Agar medium is outlined below:

Composition Ingredients	**gm l^{-1}**
Peptone	1.5
hydrolysate	1.5
Peptone 2	17.0
Lactose	10.0
Synthetic Detergents	1.5
Sodium Chloride	5.0
Crystal violet	0.001
Neutral red	0.03
Agar	15.0
pH	7.1

51.5 gms of the ingredients were suspended in 1000ml distilled water and sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 minutes.

<u>Mueller Hinton Agar (HiMedia Laboratory, India)</u>

This medium was used for antibiotic resistant test and its composition is as follows.

Composition Ingredients	**gm l^{-1}**
HiVeg acid hydrolysate	17.5gm
Hivag infusion	2.0gm
Starch, soluble	1.5gm
Agar	17.0gm
pH	7.3

38 gm of the above composition was dissolved in 1000 ml distilled water and then autoclaved at 15 lbs pressure (121^0C) for 15 minutes.

3.4.5. MOLECULAR IDENTIFICATION OF BACTERIAL ISOLATES

3. 4.5.1. *DNA Extraction*

Genomic DNA was extracted from each bacterial isolates by Lysozymes, Freeze-thawing and Phenol-Chloroform method (Lee et al., 1996). The bacterial cell pellet, resuspended in 300 ml of lysozyme solution (0.15 M NaCl; 0.1 M EDTA [pH 8.0] and 15 mg of lysozyme), was incubated at 37˚C for 1 h with mixing by inversion every 15 minutes. After addition of 300 ml of SDS buffer (0.1 M NaCl, 0.5 M Tris-HCl [pH 8.0], 10 %

Sodium Dodecyl Sulfate [SDS]), the sample was incubated in an ice bath for 10 minutes and then allowed to stand at 55˚C for 10 minutes. The freezing-thawing process was repeated three times. Bacterial genomic DNA was extracted and purified from cell lysates by two sequential phenol-chloroform extractions and an isopropanol precipitation. The DNA pellet was washed with 70% ethanol and resuspended in sterile Tris-EDTA (TE) buffer. DNA was stored at -20^0C for further use.

3.4.5.2. *Primer Selection and PCR amplification*

Genus specific primers based on 16s rDNA, transcription factor (tuf 1 & 2) and ATPase D (A1, A2 & A3) gene were selected (Table 3.4-6) for Polymerase Chain Reaction (PCR) identification. PCR was performed with a thermal cycler (PeQ Lab, Germany) and amplification conditions were described as in Table 3.4-7. The PCR mixtures consisted of 2.5 µl of 10X buffer (with $MgCl_2$), 3 µl of dNTP mixtures (2.5 µM each), 1 µl of each primers and 0.5 µl of Taq DNA polymerase (3 U/µl) (Genei, Bangalore), in a final volume of 25 µl. Finally, 50 ng DNA template were used in each mixture.

Table 3.4-6. Genus specific 16s r DNA and tuf gene PCR primers

Primers	Sequence (5'-3')	Length	References
Ps-F **Ps-R**	GGTCTGAGAGGATGATCAGT TTAGCTCCACCTCGCGGC	990bp	Widmer et al. (1998)
Ar-F **Ar-R**	CTACTTTTGCCGGCGAGCGG TGATTCCCGAAGGCACTCCC	953bp	Lee et al. (2002)
Sal-F **Sal-R**	CGGGCCTCTTGCCATCAGGTG CACATCCGACTTGACAGACCG	396bp	Amit-Romach et al. (2004)
tuf **T1** **T2**	AAYATGATIACIGGIGCIGCICARATGGA CCIACIGTICKICCRCCYTCRCG	884bp	Paradis et al. (2005)

Table 3.4-7. Conditions of PCR amplification

	Ar-R and Ar-R	Ps-F and Ps-R	Sal-F and Sal-R	Tuf 1 and 2
Initial denature and time	94^0C (4 min)	95^oC (5 min)	94^0C(3 min)	95^oC (3 min)
Denature and time	94^0C (1 min)	94^0C (15s)	94^0C (30s)	95^oC (1 min)
Annealing Temperature and time	68^0C (30s)	68^0C (30s)	60^0C (1 min)	57^0C (1 min)
Extension Temperature and time	72^0C (45s)	72^0C (45s)	68^0C (2 min)	72^0C (1 min)
Final Extension Temperature and time	72^0C (10 min)	72^0C (10 min)	68^0C (7 min)	72^0C (1 min)
Cycles	35	35	35	35

3.4.5.3. *Sequencing and Phylogenetic Analysis*

A 953bp of 16s rDNA *Aeromonas* specified gene and 884 bp of tuf gene for selected strains were sequenced. The sequence was compared and aligned with sequence deposited in the NCBI-Gen Bank database using BLAST (Altschul et al., 1997) for identification of bacteria. The sequences were aligned in CLUSTAL X (Thompson et al., 1997). Pair wise evolutionary distances were computed using the MEGA 4 program and a Phylogenetic tree was constructed by the neighbour-joining method programme available online (Tamura et al., 2004).

3.5. ANTIBIOTIC SUSCEPTIBILITY TEST

Antibiotic susceptibility test was carried out to determine the susceptibility of bacteria to antibiotics. Testing for antibiotic sensitivity was done by the Kirby-Bauer method (Bauer et al., 1966). Total of ten antibiotics manufactured by HiMedia Laboratories Ltd, Mumbai, India, were used for Antibiotic Susceptibility Test namely Penicillin (10 µg/ml), Ampicilliin (10 µg/ml), Streptomycin (10 µg/ml), Gentamycin (10 µg/ml), Tetracycline (30 µg/ml), Kanamycin (30 µg/ml), Ciprofloxacin (5 µg/ml), Moxifloxacin (5 µg/ml), Erythromycin (15 µg/ml) and Cephalothin (30 µg/ml).

Fresh cultures of all isolates in Nutrient Broth were inoculated on a Mueller-Hinton Agar (HiMedia Laboratories Ltd, Mumbai, India). Antibiotic discs were placed on inoculated agar plates and incubated overnight at 37°C. Resistance was estimated by measuring the inhibition zone which appeared on the plates. The diameters of the zones were recorded using calibrated instruments.

3.6. STATISTICAL ANALYSIS

To verify the results of different aspects of the study various statistical methods were performed using SPSS 16 software. Mean and Standard Deviation were performed for water quality parameters and bacterial counts. Karl Pearson Correlation Coefficient was done to determine the significant linear relationship between different water quality parameters and bacterial counts. To detect the significant differences of Means in the four different sites of the river and water quality parameters ANOVA (one-way) was performed. Z-test (Standard Normal) for equalities proportion was performed to determine the significant differences of antibiotic resistant pattern of bacterial isolates.

CHAPTER IV

4. RESULTS

4.1. LIMNOCEMISTRY PARAMETERS ANALYSIS

4.1.1. Temperature

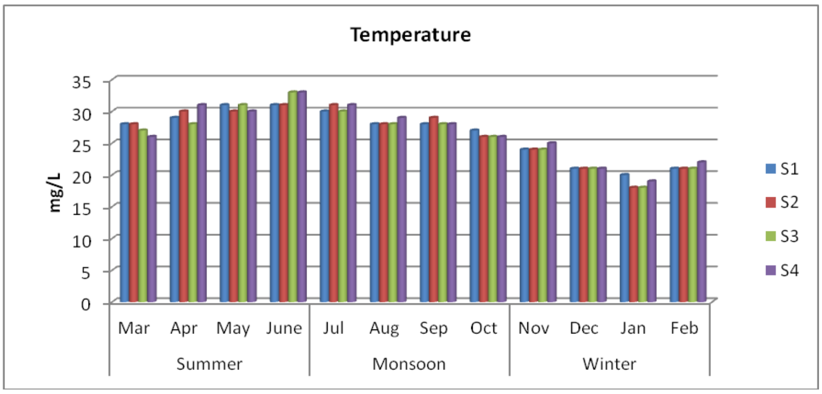
In the present study the temperature of the river water varied from 26.5±4.6 to 26.9±4.6 ^{o}C (Table 4.1-8a). As observed from Table 4.1-8b it is seen that water temperature of the four sites of the river ranges from a maximum summer temperature of 30.3^{0}C to a minimum of winter temperature of 21^{0}C. Throughout the three seasons (summer, monsoon and winter) however no significant variation of water temperature was found between the four different sites of the river water. The graphical representation showed that steady increase of temperature was observed from the month of June and then gradually decreased to lowest point in the month of January (Fig 4.1-3a).

4.1.2. pH

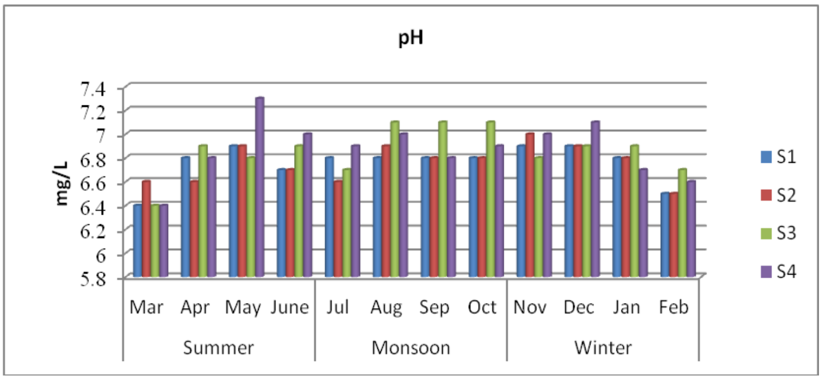
The pH recorded at the sampling sites of the river ranged from 6.7 ± 0.2 to 6.9 ± 0.3, thereby indicating acidic nature of water (Table 4.1-8a). Representation of Table 4.1-8b shows that minimum pH 6.7 was recorded in summer at Site 1 and maximum 7.0 in winter at Site 3. Analysis of variance was performed and no significant difference was found between the four different sites of river ($P \leq 0.05$). The graphical representation (Fig 4.1-3b) showed that the trend of pH was always uniform right throughout the summer, monsoon and winter seasons.

4.1.3. Dissolved Oxygen (DO)

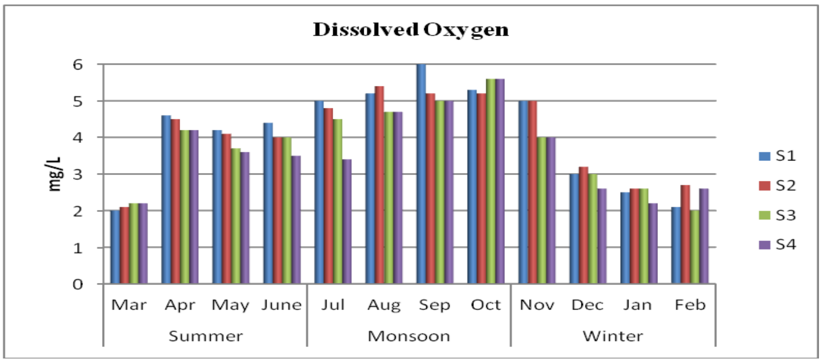
As the Table 4.1-8a represented that throughout the study period average dissolved oxygen (DO) concentration varied between 3.6±1.2 to 4.1±1.5 mg l^{-1}. No significant variation ($P \leq 0.05$) was found between the four different sites of the river. Maximum value of DO was observed in monsoon 5.3±1.3 mg l^{-1} at Site 1 and minimum in winter 3.0±1.0 mg l^{-1} at Site 3 as represented in Table 4.1-8b. Graphical representation (Fig 4.1-3c) shows that concentration of dissolved oxygen was increased from May and touched peak in the month of September then gradually descended to be lowest in January. Negative correlation was found between Dissolved oxygen with free Carbon dioxide ($r = -0.224$; $P \leq 0.01$).



(a)



(b)



(C)

Figure 4.1-3.Spatial and temporal variation of temperature (a) pH (b) and dissolved oxygen (c) of the river Lotchka during three years of the study period (March 2009-February 2012).

4.1.4. Free Carbon dioxide

Table 4.1-8a represented that mean value of free carbon dioxide ranged from15.0±5.8 to 16.7±6.1 mg l^{-1} and did not showed significant variation between four different sites of river. The free carbon dioxide value was minimum in summer (11.6± 4.8 mg l^{-1}) at Site 4 and maximum in winter (19.0±6.0 mg l^{-1}) at Site 1. Monthly variations (Figure 4.1-3d) of free carbon dioxide exhibited a trend of gradual ascendation from July to be found highest peak in December then gradually descended to be lowest in the month of June. Karl Pearson Correlation Coefficient test showing that free carbon dioxide was negatively correlated with pH ($r = -0.214$; $P \leq 0.01$).

4.1.5. Total Alkalinity

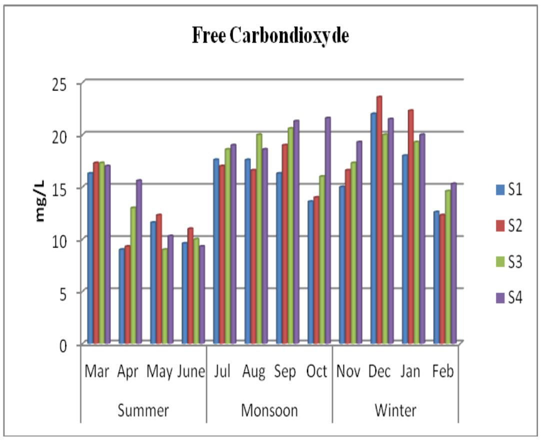
Average value of total alkalinity was varied from 19.8±7.6 to 21.4±7.4 mg l^{-1} throughout the study period (Table 4.1-8a). In the present investigation as above observed from Table 4.1-8b total alkalinity was maximum in monsoon (22±6.0 mg l^{-1}) at Site 4 and minimum in winter (18.5± 9.1 mg l^{-1}) at Site 1. Throughout the three seasons graphical representation showed that there was an increasing trend of total alkalinity from March and found to be highest peak in the month of June and gradually declined to be lowest in the month of December (Fig 4.1-3e). Karl Pearson Correlation Coefficient test proved that total alkalinity was positively correlated with total hardness ($r = -0.308$; $P \leq 0.01$).

4.1.6. Chloride

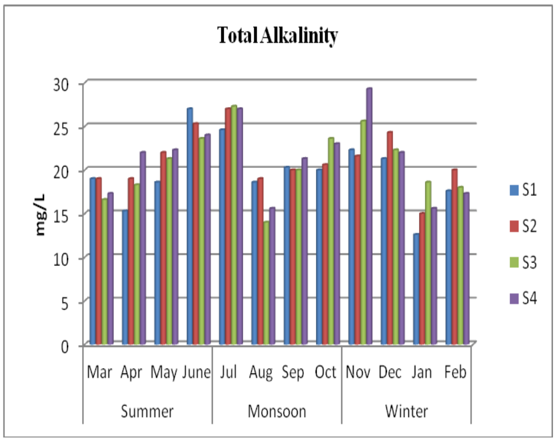
In this investigation chloride average concentration varied from 20.0±4.4 to 23.1±5.3 mg l^{-1} (Table 4.1-8a). Chloride values was highest (24.7±5.2 mg l^{-1}) in winter at site 4 and lowest (19.1± 5.0 mg l^{-1}) in the summer at Site 1 of the river (Table 4.1-8b). As per the graphical representation increasing trend was found in June. Maximum peak was recorded in the month of December then gradually descended to a minimum in the month of May (Fig 3f).

4.1.7. Total Hardness

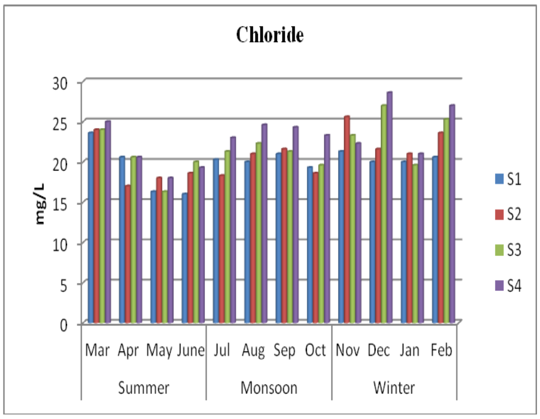
The maximum concentration of total hardness was observed in the summer (73±4.4 mg l^{-1}) at Site 4 and minimum in winter (56±7.8 mg l^{-1}) at Site 1 (Table 4.1-8b). The peak of maximum concentration was in the month of July and minimum in January (Fig 4.1-3g). Total hardness was varied between 62.6±8.6 and 68.5±9.7 mg l^{-1} throughout the study period.



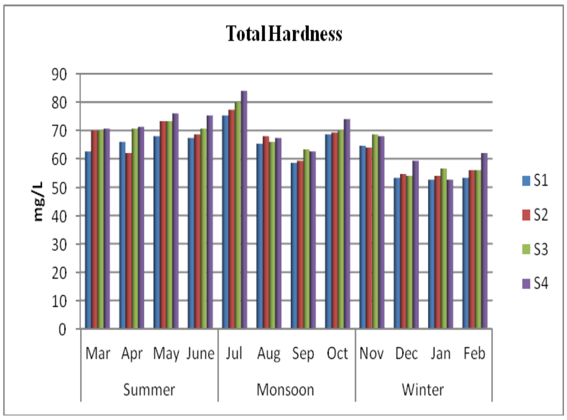
(d)



(e)



(f)



(g)

Figure 4.1-3. Spatial and temporal variation of free carbon dioxide (d), total alkalinity (e), chloride (f) and total hardness (g) of the river Lotchka during three years of the study period (March 2009-February 2012).

4.1.8. Ammonium-N (NH_4-N)

Ammonium-N usually occurs in water in the unionized form. Table 4.1-8a representing that average concentration of Ammonium-N throughout the study period ranged between 0.08±0.13 to 0.10±0.18 mg l^{-1}. No significant variation ($P \leq 0.05$) was found between the four different sites of river. A maximum value (0.15± 0.3 mg l^{-1}) was found in winter at Site 2 and minimum (0.06±0.04 mg l^{-1}) in summer at S1 as represented in Table 4.1-8b. Throughout the three years no significant variation of Ammonium-N concentration was observed among the four sites of the river. The maximum graphical peak was observed in the month of February and minimum in May (Fig 4.1-3h).

4.1.9. Nitrite-N (NO_2-N)

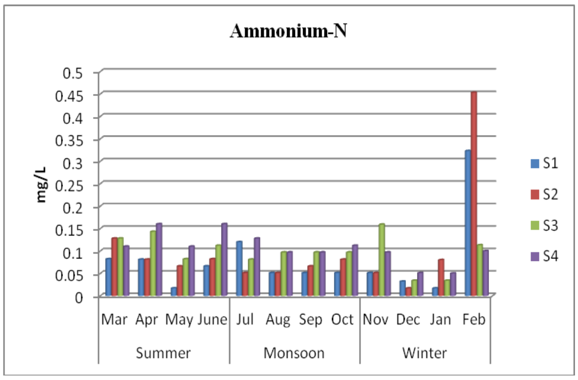
Throughout the three years Nitrite-N concentration maintained an average value between 0.07±0.07 to 0.12±0.09 mg l^{-1}. Nitrite-N concentration was highest in monsoon (0.20±0.11 mg l^{-1}) at Site 4 and minimum (0.00±0.0 mg l^{-1}) in winter at Site 2 (Table 4.1-8b). Monthly variations of NO_2-N showed a trend of slow rise from May and touched the peak in the month of September and then gradually declined to be lowest in February (Fig 4.1-3i).

4.1.10. Nitrate-N (NO_3-N)

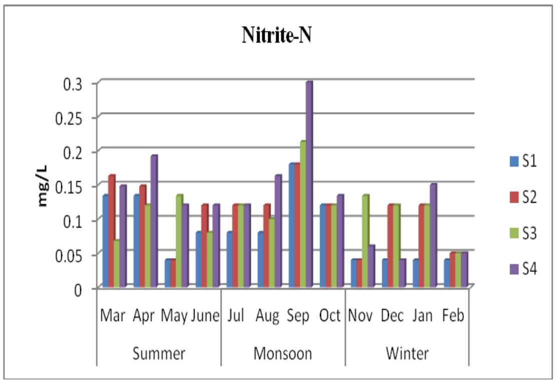
Throughout the study period mean value of the Nitrate -N was varied from 0.17±0.08 to 0.24±0.20 mg l^{-1}. ANOVA analysis showed no significant variation of Nitrate -N concentration between the four different sites of the river ($P \leq 0.05$). The Nitrate -N concentration was distinctly higher in the monsoon (0.3±0.03 mg l^{-1}) at site 4 and minimum in the winter (0.14±0.2 mg l^{-1}) at site 1. Graphical representation shows that the trend of Nitrate –N was always uniform right through the summer, monsoon and winter seasons. Maximum peak was to be found in the month of July and minimum in the month of December (Fig 4.1-3j).

4.1.11. Phosphate-P (PO_4-P)

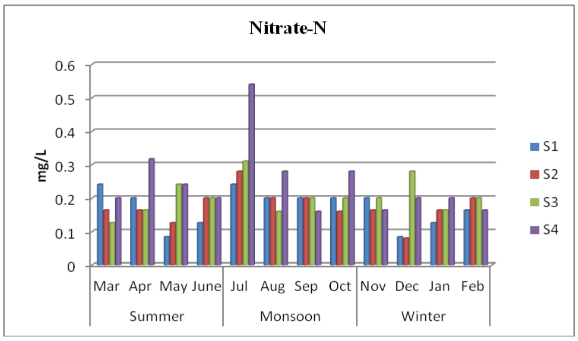
As observed from Table 4.1-8a the average concentration of Phosphate-P ranged between 0.23±0.11 and 0.33±0.24 mg l^{-1}. No variation of concentration of Phosphate- P was observed in the four different sites of the river. Phosphate-P concentration was maximum in summer (0.47±0.33 mg l^{-1}) at Site 3 and minimum (0.19±0.11 mg l^{-1}) at Site 2 in winter. Monthly variations of Phosphate-P concentration showed steady rise from February and touched a peak in the month of July and then gradually declined to be lowest in January (Fig 4.1-3k).



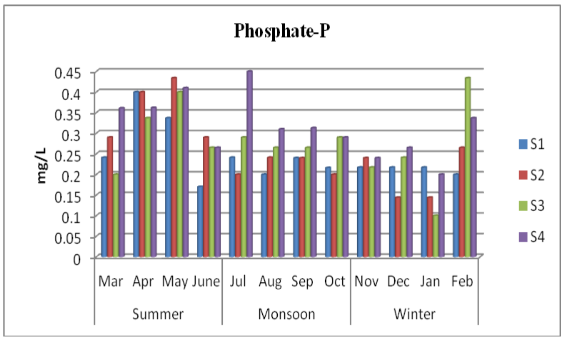
(h)



(i)



(j)



(k)

Figure 4.1-3. Spatial and temporal variation of Ammonium-N (h), Nitrite-N (i), Nitrate-N (j) and Phosphate-P (k) of the river Lotchka during three years of the study period (March 2009-February 2012).

Table 4.1-8a. Overall Mean Values (± Standard Deviation) of Water Quality parameters of four different sites of river Lotchka during the three years of study (March, 2009-February, 2012) of the study period.

Sites	Temp	pH	DO	FCO_2	TA	Cl	TH	NH_4^-N	NO_2^-N	NO_3^-N	PO_4^-P
1	26.8±4.3	6.7±0.2	4.1±1.5	15.0±5.8	19.8±7.6	20.0±4.4	62.6±8.6	0.08±0.13	0.07±0.07	0.17±0.09	0.23±0.11
2	26.7±4.5	6.7±0.2	4.0±1.3	16.0±6.6	21.0±7.5	20.7±4.4	64.4±8.7	0.10±0.18	0.09±0.06	0.17±0.08	0.25±0.14
3	26.5±4.6	6.8±0.2	3.8±1.2	16.3±6.7	20.7±7.6	21.7±5.2	66.3±8.6	0.09±0.05	0.11±0.06	0.20±0.10	0.33±0.24
4	26.9±4.6	6.9±0.3	3.6±1.2	16.7±6.1	21.4±7.4	23.1±5.3	68.5±9.7	0.10±0.04	0.12±0.09	0.24±0.20	0.31±0.15

Temp= Temperature, pH= Power of hydrogen ion concentration; DO= Dissolved oxygen; FCO_2= Free carbon dioxide, TA= Total alkalinity; Cl= Chloride; TH= Total hardness; NH_4-N=Ammonium-nitrogen; NO_2-N= Nitrite-nitrogen; NO_3-N= Nitrate- nitrogen; PO_4-P = Phosphate-phosphorous.

Table 4.1-8b. Mean Values (± Standard Deviation) of Seasonal Water Quality parameters of four different sites of river Lotchka during the three years (March, 2009-Feb, 2012) of the study period.

Seasons	Sites	Temp	pH	DO	FCO_2	TA	Cl	Hard	NH_4-N	NO_2-N	NO_3-N	PO_4-P
Summer	1	30±3.0	6.7±0.2	4.0 ±1.2	11.6±4.8	20±7.6	19.1±5.0	65±6.0	0.06±0.04	0.09±0.05	0.16±0.1	0.28±0.17
	2	30±2.5	6.7±0.2	4.0±1.1	12.5±6.4	21.3±7.0	19.4±3.6	67.6±5.1	0.08±0.04	0.09±0.05	0.16±0.07	0.34±0.2
	3	30.1±2.9	6.7±0.3	3.5±1.0	12.3±5.1	20±9.1	20.2±4.0	70.5±4.0	0.11±0.05	0.09±0.05	0.18±0.11	0.47±0.33
	4	30.3±3.3	6.9±0.4	3.3±1.0	13.0±4.1	21.4±6.6	20.7±3.5	73±4.4	0.13±0.03	0.13±0.01	0.24±0.12	0.34±0.12
Monsoon	1	28±2.0	6.8±0.1	5.3±1.3	16.5±6.1	21±6.4	20.1±5.2	67±8.1	0.08±0.1	0.14±0.12	0.21±0.09	0.22±0.1
	2	28±2.1	6.8±0.2	5.1±1.0	16.7±6.7	21.6±6.5	19.9±5.0	68.7±9.1	0.06±0.02	0.14±0.03	0.21±0.08	0.21±0.1
	3	28.3±2.1	7.0±0.3	5.0±1.0	19±8.1	21.2±6.4	21.1±6.3	69.8±8.7	0.09±0.01	0.16±0.05	0.22±0.01	0.27±01
	4	28.6±2.7	6.9±0.2	4.6±1.1	18.7±6.5	22±6.0	23.8±6.3	72±10.5	0.1±0.02	0.20±0.11	0.31±0.03	0.34±0.3
Winter	1	21±1.0	6.8±0.2	3.1±1.2	16.9±5.3	18.5±9.1	20.5±3.0	56±7.8	0.1±0.2	0.00±0.0	0.14±0.2	0.20±0.04
	2	21.4±2.6	6.8±0.2	3.1±1.0	18.7±5.4	20.2±9.3	23±4.0	57.1±6.7	0.15±0.3	0.06±0.06	0.15±0.3	0.19±0.11
	3	21.1±2.4	6.8±0.2	3.0±1.0	17.8± 4.8	21.0±9.7	23.8±5.0	58.8±7.5	0.08±0.07	0.09±0.05	0.21±0.07	0.24±0.2
	4	30.3±3.3	6.9±0.4	3.3±1.0	13.0±4.1	21.4±6.6	24.7±5.2	60±8.0	0.13±0.03	0.13±0.01	0.24±0.12	0.34±0.12

Temp= Temperature, pH= Power of hydrogen ion concentration; DO= Dissolved oxygen; FCO_2= Free carbon dioxide, TA= Total alkalinity; Cl= Chloride; TH= Total hardness; NH_4-N=Ammonium-nitrogen; NO_2-N= Nitrite-nitrogen; NO_3-N= Nitrate- nitrogen; PO_4-P = Phosphate-phosphorous.

4.2. BACTERIAL ENUMERATION IN FOUR DIFFERENT SITES OF RIVER LOTCHKA

4.2.1. Heterotrophic Bacteria

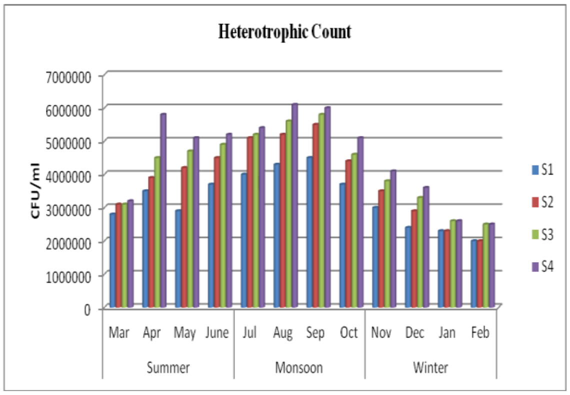
The heterotrophic bacterial count in the four sites of river average ranged from 3.3±1.1 x 10^6 and 4.6±1.4 x 10^6 cfu ml l^{-1} throughout the study period are represented in Table 4.2-9a. The maximum heterotrophic bacterial counts was found at Site 4 in the monsoon 5.6±1.1 x 10^6 cfu ml l^{-1} and minimum at Site 1 in winter (2.7 x 10^6 cfu ml l^{-1}). Monthly variations of heterotrophic bacterial counts exhibited a slow rise from March with a peak in the month of August and then steadily directly to its lowest level in January (Fig 4.2-4a). No significant difference of heterotrophic bacterial count was found among the four sites of the river. Heterotrophic bacterial population showed positive correlation with temperature ($r = 0.408$; $P \leq 0.01$), Dissolved oxygen ($r = 0.471$; $P \leq 0.01$), Nitrite-N ($r = 0.448$; $P \leq 0.01$), Nitrate-N ($r = 0.274$; $P \leq 0.01$) and Phosphate-P ($r = 0.262$; $P \leq 0.01$) (4.2-10).

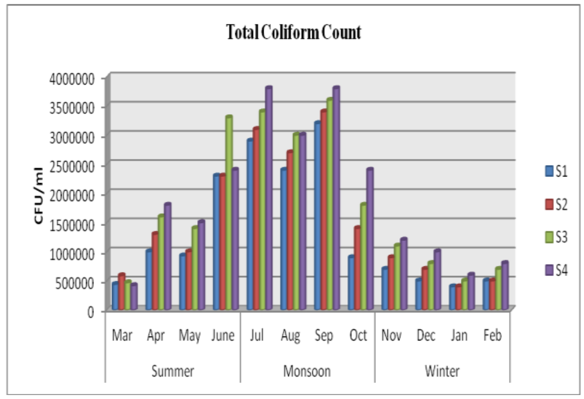
4.2.2. Total Coliform

The total coliform counts in water recorded ranged from 1.1±1.3 x 10^6 and 1.6±2.6 x 10^6 cfu ml^{-1} (Table- 4.2-9b). The maximum total coliform counts in water was recorded also in monsoon, (3.3 ±1.3 x 10^6 cfu ml^{-1}) at Site 4 and minimum in winter (0.4 ±0.5 x 10^6 cfu ml^{-1}) at Site 1 (Table 4.2-9b). Graphical representation explained that the slow rise of total coliform counts from May and touched peak in the month of September then gradually descended to be lowest peak in January (Fig 4.2-4b). Analysis of Karl Pearson Correlation Coefficient revealed Total Coliform Count to positively correlated with Temperature ($r = 0.193$; $P \leq 0.05$), Dissolved oxygen ($r = 0.376$; $P \leq 0.05$) and Nitrite-N ($r = 0.358$; $P \leq 0.01$) (Table-4.2-10).

4.2.3. *Aeromonas* spp. and *Pseudomonas* spp.

Counts of *Aeromonas* spp. and *Pseudomonas* spp. showed a maximum trend in monsoon (3.4 x 10^6 cfu ml^{-1} and 3.4 x 10^6 cfu ml^{-1} respectively) at Site 4 and minimum (0.7 x 10^6 cfu ml^{-1} and 0.1 x 10^6 cfu ml^{-1}) in winter season at Site 1 as represented in Table 4.2-9. Statistical study by ANOVA reveled that no significant variation of *Aeromonas* spp. and *Pseudomonas* spp. among the four different sites of the river.





(a) (b)

Figure 4.2-4. Spatial and temporal variations of average value of Heterotrophic Bacterial Count (l) and Total Coliform Count (b) of the four different sites of River during three years of the study period (March 2009-February 2012).

Table 4.2-9a. Mean ± Standard Deviation values of bacterial counts (cfu ml-^{1}) at four sites of river Lotchka throughout the three years of the study period (March, 2009-February, 2012)

Sites	HC(10^6)	TC(10^6)	*Aeromonas*spp.(10^6)	*Pseudomonas* spp.(10^6)
1	3.3±1.1	1.1±1.3	1.4±1.0	1.7±1.3
2	4.0±1.3	1.6±2.6	1.8±1.1	2.0±1.3
3	4.4±1.3	1.4±1.6	2.0±1.1	2.4±1.4
4	4.6±1.4	1.6±1.7	2.2±1.3	2.6±1.6

HC=Heterotrophic Count, TC=Total Coliform

Table 4.2-9b. Mean ± Standard Deviation values of seasonal bacterial counts (cfu ml-^{1}) at four sites of river Lotchka during the three years of the study period (March, 2009-February, 2012)

Seasons	Sites	HC(10^6)	TC(10^6)	*Aeromonas* spp.(10^6)	*Pseudomonas* spp.(10^6)
Summer	1	3.2±1.1	0.7±1.2	1.2±1.0	1.3±1.1
	2	4.0±1.3	0.7±1.2	1.6±0.8	1.5±1.0
	3	4.3±1.5	0.7±1.1	1.7±0.8	1.8±1.0
	4	4.5±1.4	0.8±1.3	2.0±1.0	1.8±1.1
Monsoon	1	4.1±0.7	2.3±1.3	2.3±1.0	3.1±1.1
	2	5.0±1.0	2.6±1.5	2.8±1.0	3.4±1.1
	3	5.3±1.1	3.0±1.6	3.1±0.8	4.0±1.0
	4	5.6±1.1	3.3±1.6	3.4±1.0	4.4±1.0
Winter	1	2.7±1.0	0.4±0.5	0.7±0.5	1.0±0.4
	2	3.0±1.0	1.6±0.4	1.6±4.0	1.2±0.5
	3	3.5±0.8	0.6±0.7	1.1±0.8	1.5±1.0
	4	3.8±1.0	0.7±0.8	1.2±1.0	1.5±1.0

HC=Heterotrophic Count, TC=Total Coliform

Table 4.2-10. Coefficient of Correlation values of selected water quality parameters and heterotrophic bacteria count of river Lochka.

	Temp	pH	DO	FCO_2	TA	TH	MH_4	NO_2	NO_3	PO_4	HC	TC
Temp	1											
pH	.017	1										
DO	.372**	.141	1									
FCO_2	-.333**	.214**	-.224**	1								
TA	.111	.026	.295**	-.078	1							
TH	.566**	.138	.349**	-.343**	.308**	1						
MH_4	-.007	-.253**	-.107	-.011	.150	.030	1					
NO_2	.325**	.079	.357**	.110	.019	.124	-.082	1				
NO_3	.093	-.038	.119	-.170*	.092	.250**	.080	.143	1			
PO_4	.207*	-.153	-.072	-.202*	-.020	.244**	.156	-.112	.170*	1		
HC	.408**	.080	.471**	-.324**	-.059	.368**	.014	.448**	.274**	.262**	1	
TC	.193*	-.023	.376**	-.091	.236**	.204*	-.026	.358**	.120	-.031	.428**	1

** Correlation is significant at the 0.01level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed).

Temp= Temperature, pH= Power of hydrogen ion concentration; DO= Dissolved oxygen; FCO_2= Free carbon dioxide, TA= Total alkalinity; CL= Chloride; Hard= Total hardness; PO_4 = Phosphate-phosphorous; NO_3= Nitrate- nitrogen; NO_2= Nitrite-nitrogen; NH_4=Ammonium-nitrogen.

4.3. BACTERIAL ENUMERATION IN DIFFERENT TISSUES OF FISH

4.3.1. Heterotrophic bacteria

As observed from Table 8 the heterotrophic bacterial count was maximum in gut 4.2 X 10^6 cfu g^{-1} and minimum in skin 2.0 x 10^6 cfu g^{-1}. With respect to seasonal variation summer and monsoon showed much higher heterotrophic count than winter season.

4.3.2. Total Coliform

Maximum counts of total coliform 2.3 x 10^6 cfu g^{-1} was found in the gut and a minimum of 0.2 x 10^6 cfu g^{-1} counts was found in skin as represented in Table 4.3-11. Throughout the three years the total coliform counts were highest in monsoon and lowest in the winter season.

4.3.3. *Aeromonas* spp. and *Pseudomonas* spp.

Table 4.3-11 shows that like counts of heterotrophic and coliform bacteria, counts of *Aeromonas* spp. and *Pseudomonas* spp. were maximum in gut (3.0 x 10^6 cfu g^{-1} and 2.0 x 10^6 cfu g^{-1} respectively) and minimum in skin (3.0 x 10^6 cfu g^{-1} and 2.0 x 10^6 cfu g^{-1} respectively).

Table 4.3-11. Mean ± standard deviation values of bacterial count (cfu ml^{-1}) of different tissues (skin, gills and gut) of fish, *Lepidocephalicthys guntea* during the three years of the study period (March, 2009-Feb, 2012).

Tissues	Seasons	HC (10^6)	TC(10^6)	*Aeromonas* spp. (10^6)	*Pseudomonas* spp. (10^6)
Skin	Summer	2.1±1.1	0.4±1.0	1.0±1.0	0.3±0.5
Gills		2.7±1.3	0.5±1.2	1.1±1.4	0.4±1.0
Gut		1.3±1.4	0.5±1.4	1.1±1.2	0.4±1.0
Skin	Monsoon	3.2±1.1	2.0±2.0	2.2±1.1	1.4±1.7
Gills		4.0±1.4	2.1±2.0	3.0±1.4	2.0±2.1
Gut		4.2±5.0	2.3±2.2	3.0±1.6	2.0±2.3
Skin	Winter	2.0±0.5	0.2±0.3	1.0±1.0	0.3±4.1
Gills		2.4±1.0	0.3±0.4	1.0±1.0	0.5±1.0
Gut		2.5±1.0	0.3±0.5	1.0±1.0	0.5±1.0

HC=Heterotrophic Count, TC=Total Coliform

4.4. BACTERIAL ISOLATION

4.4.1. River Water Samples

A total of 97 bacteria were isolated from four different sites of river and 72 of them were successfully identified. The isolates belonged to *Aeromonas* spp., *Pseudomonas* spp., *Salmonella* spp. and *Enterobacteriaceae* spp. and were found to be uniformly distributed amongst the stretch of the river as represented in Table 4.4-12.

4.4.2. Fish Samples

The number of bacterial isolates from skin, gills and gut of fish are shown in Table 4.4-12. A total of 125 bacteria were isolated from different tissues like skin, gills and gut of fish. Among the total isolates, 103 isolates were successfully identified and all belonged to *Aeromonas* spp., *Pseudomonas* spp., *Salmonella* spp. and *Enterobacteriaceae*.

Table 4.4-12. Distribution of Bacterial Isolates in different sites of rive and tissues of fish

Sites & Tissues	***Aeromonas* spp.**	***Pseudomonas* spp.**	***Salmonella* spp.**	***Enterobacteriaceae***
S1	4	5	2	5
S2	4	5	2	5
S3	5	5	3	7
S4	5	5	3	7
Skin	8	6	4	9
Gills	11	7	5	14
Gut	12	7	5	15
Total	49	40	24	62

4.5. BACTERIAL IDENTIFICATION

4.5.1. Initial Identification of *Aeromonas* spp.

Cultural and microscopic characteristics

The bacterial isolates appearing in *Aeromonas* Starch DNA Agar Base medium were round and white coloured colonies. All isolates showed gram-negative, non-spore forming rod shaped under the microscope (Fig 4.5-5).

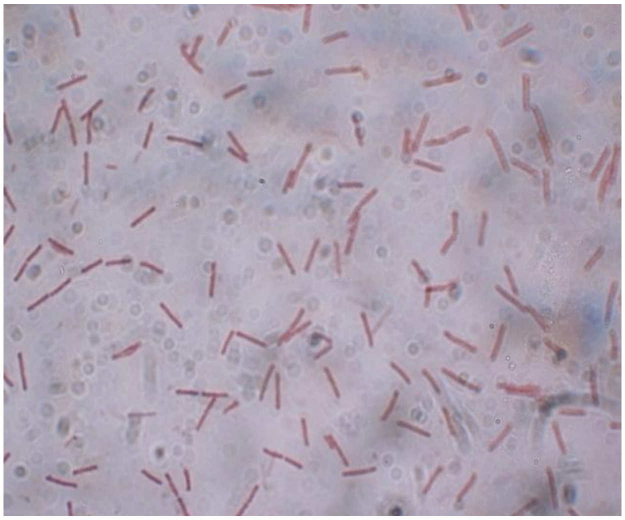


Fig 4.5-5. Microscopic photograph of *Aeromonas* spp.

Biochemical Characterization

As represented in Table 4.5-13, isolates showed positive was against Oxidase, Aesculine and Arginine hydrolysis. In case of Methyl Red (MR) it was negative and in Voges- Proskauer (VP) and Indole positive. Acid and gas was formed in glucose fermentation.

4.5.2. Molecular Identification of *Aeromonas* spp.

16s rRNA *Aeromonas* specific primer was used for Polymerase Chain Reaction (PCR) to determine whether or not the isolates belonged to the *Aeromonas* spp. Forty-nine (49) isolates out of sixty (60) were confirmed as *Aeromonas* spp. The visualization of positive amplicon was approximately 953bp (Fig 4.5-6) and was performed using agarose gel (1.5%) electrophoresis containing ethidium bromide.

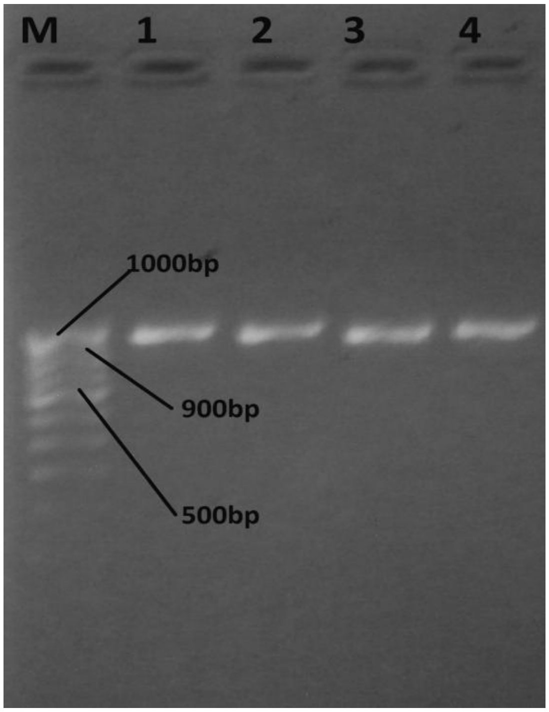


Fig 4.5-6. Positive amplicons of *Aeromonas* spp. specific 16S rDNA PCR amplification

Lane M: 100 bp molecular weight marker, Lane 1- 4: amplicons.

4.5.3. Initial Identification of *Pseudomonas* spp.

Cultural and microscopic characteristics

Isolates produced slight yellowish and white round colonies on Pseudomonas Agar Base medium. Gram stain of selected pure single colonies was performed and confirmed that isolates were gram-negative, non-spore forming rod shaped and motile under microscope (Fig 4.5-7).

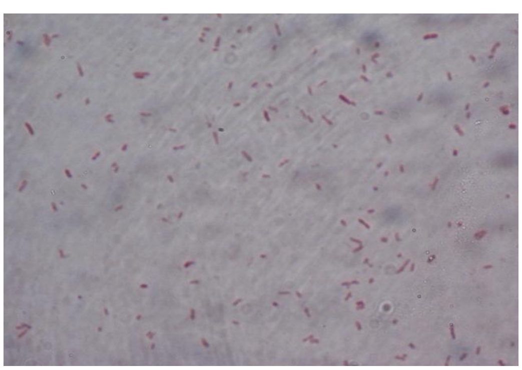


Fig 4.5-7. Microscopic photograph of *Pseudomonas* spp.

Biochemical Characterization

As observed from Table 4.5-13 all isolates utilized citrate but did not produce Catalase and Urease. Formation of acid and gas occurred against glucose fermentation.

4.5.4. Molecular Identification of *Pseudomonas* spp.

For confirmation of identification of *Pseudomonas* spp. Polymerase Chain Reaction (PCR) was performed. A total of 40 isolates amongst 56 isolates gave positive amplification with 16s rRNA *Pseudomonas* spp specific primer. Agarose gel (1.5%) electrophoresis containing ethidium bromide was performed and positive amplicons approximately 990bp were visualized (Fig 4.5-8).

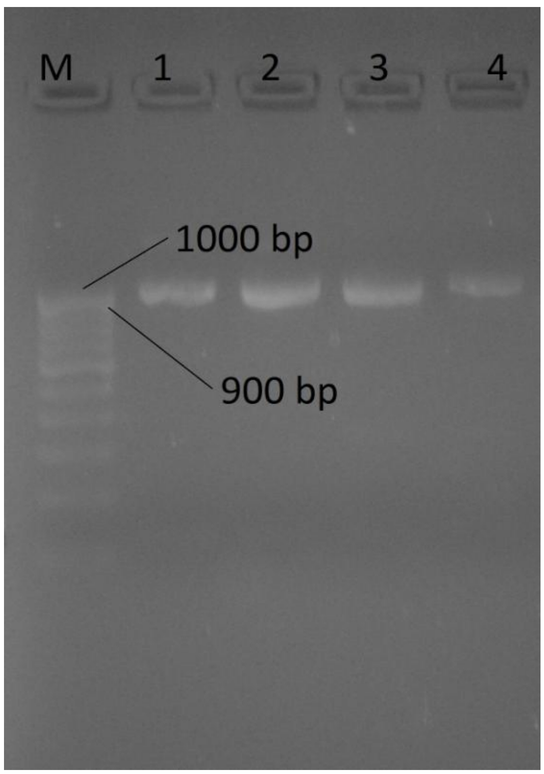


Fig4.5-8: Positive amplicons of *Pseudomonas* spp. specific 16S rDNA PCR amplification.

Lane M: 100 bp molecular weight marker, Lane 1- 4: amplicons.

4.5.5. Initial identification of *Salmonella* spp.

Cultural and microscopic characteristics

Bacterial isolates gave black colonies on Bishmuth Sulphite Agar medium. Selected pure single colonies showed gram-negative, non-spore forming rod shaped and motile under microscope (Fig4.5-9).

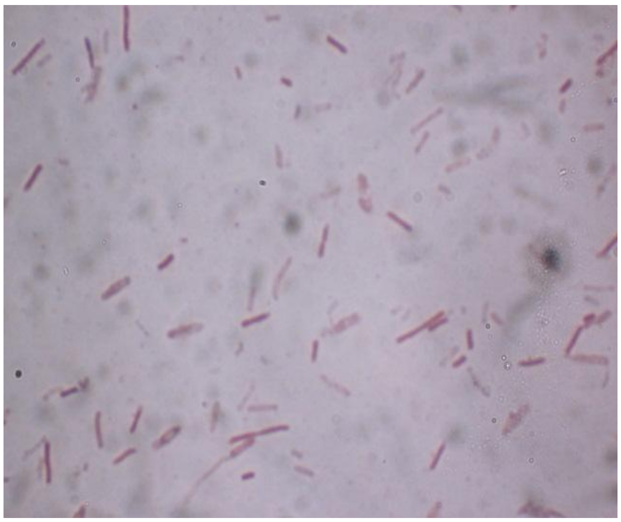


Fig4.5-9. Microscopic photograph of *Salmonella* spp.

Biochemical Characterization

Citrate Utilization test and Methyl Red (MR) gave positive results for all *Salmonella* spp. isolates. Voges- Proskauer (VP) and Indole showed negative results. Glucose fermentation was performed and isolates produced acid and gas.

4.5.6. Molecular Identification of *Salmonella* spp.

16s rRNA *Salmonella* spp. specific primer was used for Polymerase Chain Reaction (PCR) for conformation of identification. Total of 24 isolates amongst 30 isolates gave positive result in PCR. Positive amplicons approximately 396bp were visualized in agarose gel (1.5%) electrophoresis containing ethidium bromide (Fig. 4.5-10).

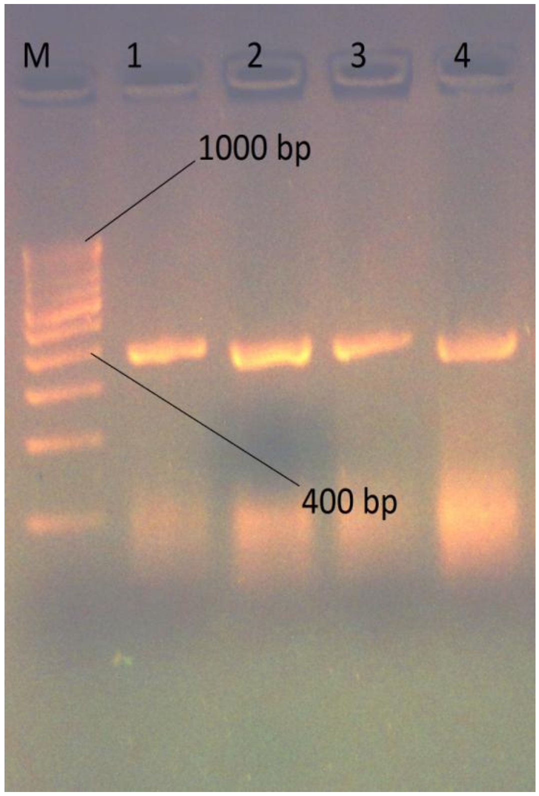


Fig4.5-10. Positive amplicons of *Salmonella* spp. specific 16S rDNA PCR amplification
Lane M: 100 bp molecular weight marker, Lane 1- 4: amplicons

4.5.7. Initial identification of *Enterobacteriaceae*

Cultural and microscopic characteristics

Isolates appeared as red and pink colonies on MacConkey Agar medium. Gram stain and mortality test were performed and selected pure single colonies showed gram-negative, non-spore forming rod shaped (Fig.4.5-11) and motile under microscope.

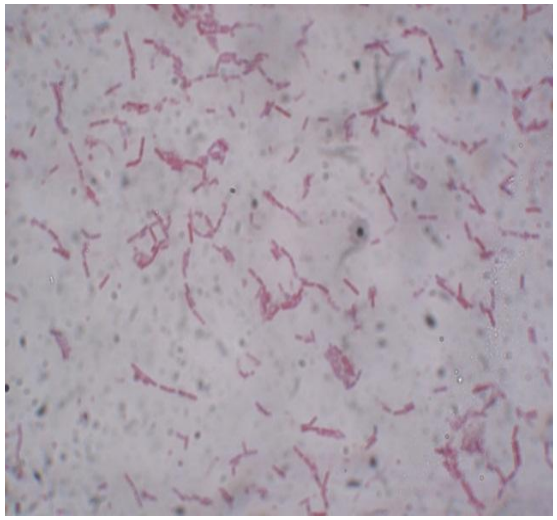


Fig 4.5-11. Microscopic photograph of *Enterobacteriaceae*

Biochemical Characterization

Table 4.5-13 shows that all isolates utilized the citrate. Isolates gave positive results for VP test but for MR and Indole test they were all negative. Acid and gas was formed against glucose fermentation.

4.5.8. Molecular Identification of *Enterobacteriaceae*

For the detection of *Enterobacteriaceae*, Polymerase Chain Reaction (PCR) was performed. A total of 62 isolates amongst 70 isolates were amplified with 'tuf' gene specific primer. The visualization of positive amplicons, approximately 884bp (Fig.4.5-12), was performed by agarose gel (1.5%) electrophoresis containing ethidium bromide.

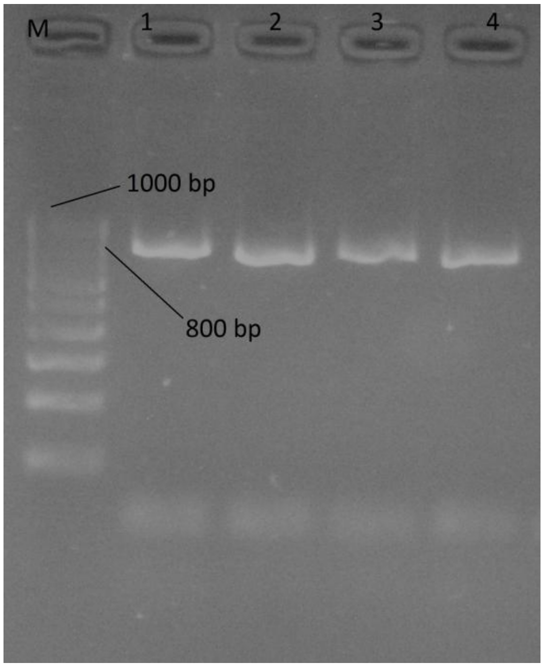


Fig.4.5-12. Positive amplicons of *Enterobacteriaceae* specific 'tuf' gene PCR amplification

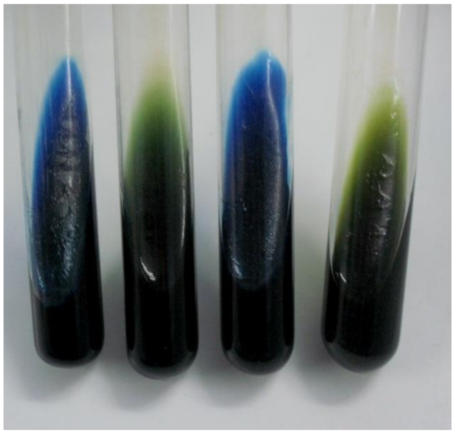
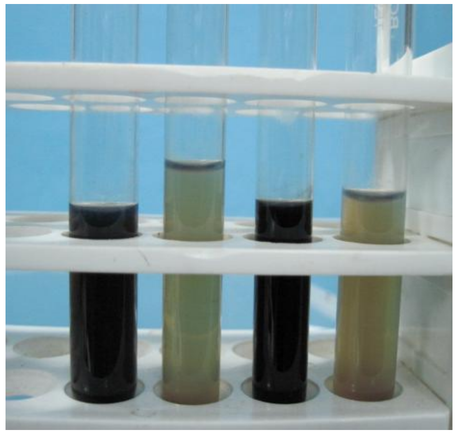
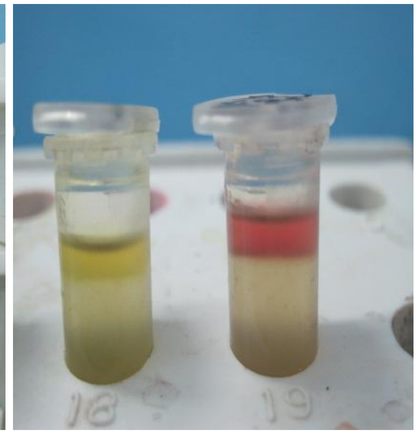
Lane M: 100 bp molecular weight marker, Lane 1- 4: amplicons

Table 4.5-13. Results of biochemical characterization of bacterial isolates from four sites of river Lotchka and tissues of fish, *Lepidocephalichthys guntea*

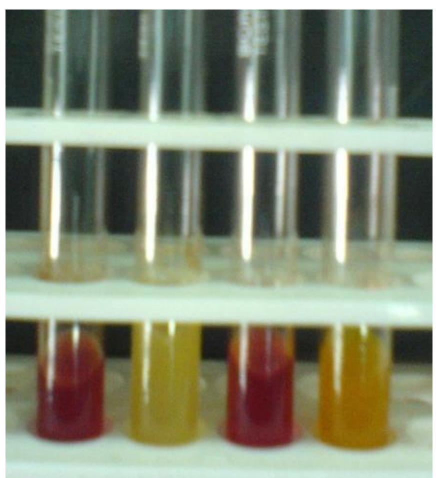
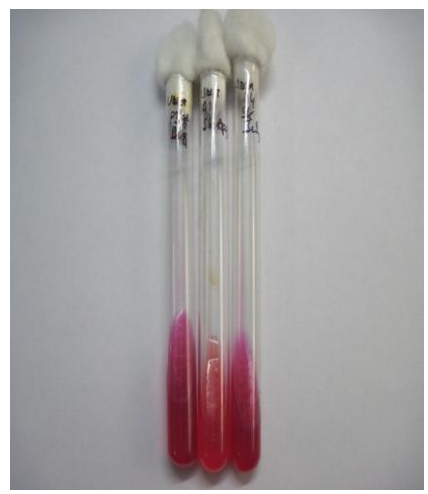
Biochemical Characterization	***Aeromonas* spp.**	***Pseudomonas* spp.**	***Salmonella* spp.**	***Enterobacteriaceae***
Gram stain	-ve	-ve	-ve	-ve
Motility	+ve	+ve	+ve	+ve
MR	-ve	Nd	+ve	-ve
VP	+ve	Nd	-ve	+ve
Indole	+ve	Nd	-ve	-ve
Aesculin Hydrolysis	+ve	Nd	Nd	Nd
Arginine Hydrolysis	+ve	Nd	Nd	Nd
Citrate Utilization	Nd	+ve	+ve	+ve
Catalase Utilization	Nd	-ve	Nd	Nd
Urease	Nd	-ve	Nd	Nd
Glucose	+ve	+ve	+ve	+ve
Lactose	Nd	+ve	-ve	+ve
Sorbitol	Nd	+ve	+ve	+ve
Mannitol	Nd	Nd	+ve	+ve
Raffinose	Nd	Nd	+ve	+ve
Sucrose	+ve	Nd	-ve	+ve
Salicin	+ve	Nd	Nd	Nd
Maltose	+ve	Nd	Nd	+ve
Arabinose	-ve	+ve	+ve	+ve
Inositol	+ve	Nd	Nd	Nd

+ve= Positive; -ve=Negative; Nd= not done

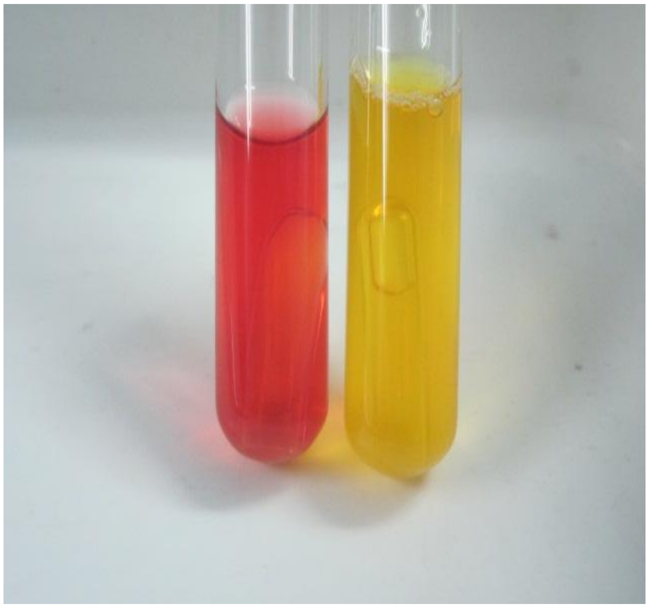
Fig4.5-13. Photographs of different biochemical characterization of bacterial isolates and antibiotic sensitivity test

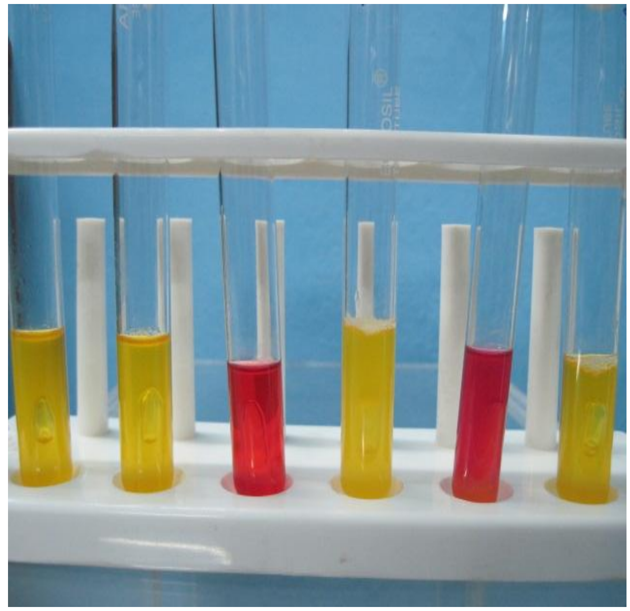




CITRATE TEST **AESCULIN HYDROLYSIS** **INDOLE TEST**

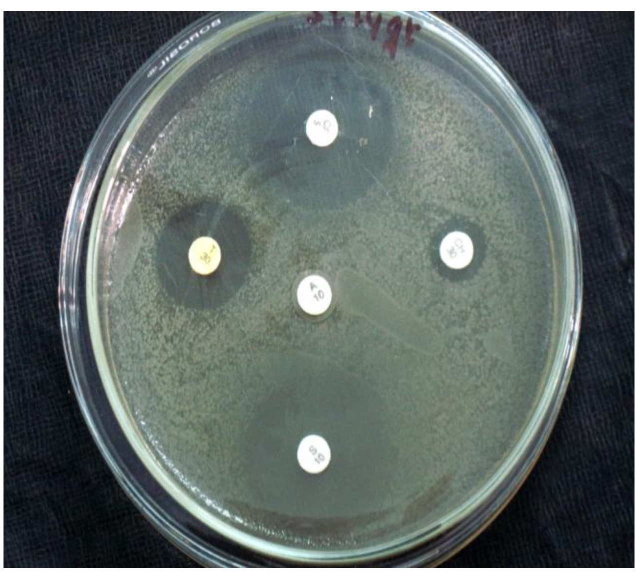




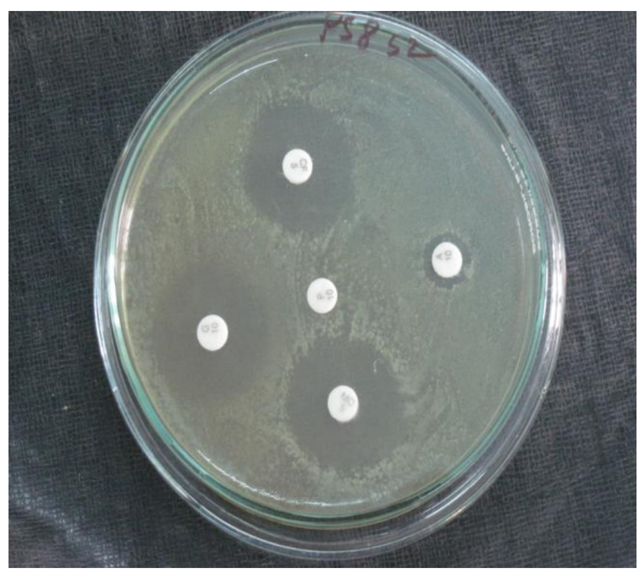
MR-VP TEST **UREASE HYDROLYSE**





SUGAR FERMENTATION





ANTIBIOTIC SENSITIVITY TEST

4.6. ANTIBIOTIC RESISTANCE

The antibiotic resistance of *Pseudomonas* spp., *Aeromonas* spp., *Salmonella* spp. and *Enterobacteriaceae* have shown full range of resistance (0-100%) for ten antibiotics which are commonly used in human beings and in aquaculture. All isolates showed maximum resistance against Ampicillin, Penicillin-G and Cephalothin belonging to the β- lactam class in river water samples.

4.6.1. Antibiotic resistant bacterial pattern in water samples of river Lotchka

4.6.1.1. Antibiotic resistant bacterial pattern of *Aeromonas* spp.

All the isolates of *Aeromonas* spp. showed resistant against Penicillin. 75 to 100% isolates showed resistant against Ampicillin and Cephalothin, respectively. Isolates were also resistant against Erythromycin (25 to 60%) and Kanamycin (20 to 60%). All isolates were sensitive to Ciprofloxacin, Moxifloxacin, Tetracycline and Gentamycin (Table 4.6-14).

Table 4.6-14. Antibiotics resistance (%) among of *Aeromonas* spp. from four different sites of river Lotchka

Sources	AMP R I S	PEN R I S	CEPHA R I S	ERY R I S	KANA R I S	STRE R I S	CIPRO R I S	MOXI R I S	TETRA R I S	GENTA R I S
S1	75 0 25	100 0 0	100 0 0	25 0 75	0 25 75	0 0 100	0 25 75	0 25 75	0 25 75	0 0 100
S2	75 0 25	100 0 0	75 0 25	0 25 75	0 25 75	0 25 75	0 50 50	0 25 75	0 50 50	0 0 100
S3	100 0 0	100 0 0	100 0 0	40 20 40	20 0 80	0 40 60	0 0 100	0 0 100	20 40 40	0 0 100
S4	80 0 20	100 0 0	60 20 20	60 20 20	60 0 40	40 0 60	0 40 60	0 40 60	0 20 80	0 40 60

Abbreviation: R-Resistance, I- Intermediate, S-Sensitive, AMP-Ampicillin, PEN-Penicillin, CEPHA-Cephalothin, ERY-Erythromycin, KANA-Kanamycin, STREPTO-Streptomycin, CIPRO-Ciprofloxacin, MOXI-Moxifloxacin, TETRA-Tetracycline, GENTA-Gentamycin.

4.6.1.2. Antibiotic resistant pattern of *Pseudomonas* spp.

Table 4.6-15 shows that among the total isolates of *Pseudomonas* spp. in four different sites of river there was 100% resistant to Penicillin and Cephalothin. 20 to 100% and 40 to 100% isolates were resistant against Ampicillin and Erythromycin, respectively. But all isolates were sensitive to Streptomycin, Ciprofloxacin, Moxifloxacin, Tetracycline and Gentamycin.

Table 4.6-15. Antibiotics resistance (%) among of *Pseudomonas* spp. from four different sites of river Lotchka

Source	AMP	PEN	CEPHA	ERY	KANA	STRE	CIPRO	MOXI	TETRA	GENTA
	R I S	R I S	R I S	R I S	R I S	R I S	R I S	R I S	R I S	R I S
S1	80 0 20	100 0 0	100 0 0	40 0 60	20 20 60	0 0 100	0 0 100	0 0 100	20 0 80	0 0 100
S2	20 20 60	100 0 0	100 0 0	40 40 20	0 0 100	0 0 100	0 0 100	0 0 100	0 0 100	0 0 100
S3	80 0 20	100 0 0	100 0 0	100 0 0	60 20 20	0 0 100	0 0 100	0 0 100	0 0 100	0 0 100
S4	100 0 0	100 0 0	100 0 0	100 0 0	0 0 100	0 40 60	0 0 100	0 0 100	0 0 100	0 0 100

R-Resistance, I- Intermediate and S-Sensitive

Abbreviation: R-Resistance, I- Intermediate, S-Sensitive, AMP-Ampicillin, PEN-Penicillin, CEPHA-Cephalothin, ERY-Erythromycin, KANA-Kanamycin, STREPTO-Streptomycin, CIPRO-Ciprofloxacin, MOXI-Moxifloxacin, TETRA-Tetracycline, GENTA-Gentamycin.

4.6.1.3. Antibiotic resistant pattern of *Salmonella* spp.

Among isolates of *Salmonella* spp. in the four different sites; 100% resistance to Penicillin and Ampicillin were observed. Isolates were resistant to Cephalothin (33 to 67%), Erythromycin (50 to 60%) and Kanamycin (33 to 67%) respectively. But all isolates showed sensitive against rest of the antibiotics namely Streptomycin, Ciprofloxacin, Moxifloxacin, Tetracycline and Gentamycine, respectively (Table 4.6-16).

Table 4.6-16. Antibiotics resistance (%) among of *Salmonella* spp. from four different sites of river Lotchka

Source	AMP	PEN	CEPHA	ERY	KANA	STRE	CIPRO	MOXI	TETRA	GENTA
	R I S	R I S	R I S	R I S	R I S	R I S	R I S	R I S	R I S	R I S
S1	100 0 0	100 0 0	50 0 50	50 50 0	50 0 50	0 50 50	0 0 100	0 0 100	0 0 100	0 0 100
S2	100 0 0	100 0 0	50 50 0	50 0 50	0 100 0	0 0 100	0 0 100	0 0 100	0 0 100	0 0 100
S3	67 33 0	100 0 0	33 33 33	67 33 0	33 33 33	0 0 100	0 0 100	0 0 100	0 0 100	0 0 100
S4	100 0 0	100 0 0	67 33 0	67 0 33	67 33 0	0 67 33	0 0 100	0 0 100	50 0 50	0 0 100

R-Resistance, I- Intermediate and S-Sensitive

Abbreviation: R-Resistance, I- Intermediate, S-Sensitive, AMP-Ampicillin, PEN-Penicillin, CEPHA-Cephalothin, ERY-Erythromycin, KANA-Kanamycin, STREPTO-Streptomycin, CIPRO-Ciprofloxacin, MOXI-Moxifloxacin, TETRA-Tetracycline, GENTA-Gentamycin.

4.6.1.4. Antibiotic resistant bacterial pattern of *Enterobacteriaceae*.

As represented in Table 4.6-17 all isolates of *Enterobacteriaceae* in four different sites of the river Lotchka exhibited resistance against Ampicillin, Penicillin and Cephalothin. 25 to 70% and 30 to 50% isolates were resistant against Erythromycin and Kanamycine. All isolates were sensitive against rest of the antibiotics namely Streptomycin, Ciprofloxacin, Moxifloxacin, Tetracycline and Gentamycine.

Table 4.6-17. Antibiotics resistance (%) among of *Enterobacteriaceae* from four different sites of river

Source	AMP	PEN	CEPHA	ERY	KANA	STRE	CIPRO	MOXI	TETRA	GENTA
	R I S	R I S	R I S	R I S	R I S	R I S	R I S	R I S	R I S	R I S
S1	100 0 0	100 0 0	100 0 0	60 0 40	30 0 70	0 0 100	0 0 100	0 0 100	0 0 100	0 0 100
S2	100 0 0	100 0 0	100 0 0	70 0 30	50 0 50	0 0 100	0 0 100	0 0 100	0 0 100	0 0 100
S3	100 0 0	100 0 0	100 0 0	60 40 0	40 0 60	0 0 100	0 0 100	0 0 100	0 0 100	0 0 100
S4	100 0 0	100 0 0	100 0 0	25 25 50	40 0 60	0 0 100	0 0 100	0 0 100	0 0 100	0 0 100

R-Resistance, I- Intermediate and S-Sensitive

Abbreviation: R-Resistance, I- Intermediate, S-Sensitive, AMP-Ampicillin, PEN-Penicillin, CEPHA-Cephalothin, ERY-Erythromycin, KANA-Kanamycin, STREPTO-Streptomycin, CIPRO-Ciprofloxacin, MOXI-Moxifloxacin, TETRA-Tetracycline, GENTA-Gentamycin.

4.6.2. Antibiotic resistant bacterial pattern in fish tissues

4.6.2.1. Antibiotic resistant pattern of *Aeromonas* spp.

Among the total isolates of *Aeromonas* spp. in fish tissues namely skin, gills and gut 90 to 100% isolates showed resistant to Penicillin, 76 to 84% resistant to Ampicilin, 64 to 92% resistance against Cephalothin and 8 to 54% resistant to Kanamycin. Isolates found in gills and gut was resistant against Streptomycin (27% and 16%) and Ciprofloxacin (9% and 8%). The resistance patterns of all isolates are represented in the Table 4.6-18.

Table 4.6-18. Antibiotics resistance (%) among of *Aeromonas* spp. from different tissues of fish, *Lepidocephalicthys guntea*.

Source	AMP R I S	PEN R I S	CEPHA R I S	ERY R I S	KANA R I S	STR R I S	CIPRO R I S	MOXI R I S	TETRA R I S	GENTA R I S
Skin	76 7 7	100 0 0	75 0 25	12 38 50	25 0 75	0 12 88	0 0 100	12 13 75	0 0 100	0 12 88
Gills	82 9 9	90 10 0	64 18 18	54 10 36	54 1036	27 9 64	9 19 72	9 28 63	0 0 100	0 18 82
Gut	84 8 8	100 0 0	92 0 8	84 8 8	8 42 50	16 9 75	8 16 74	8 26 66	0 25 75	34 0 66

R-Resistance, I- Intermediate and S-Sensitive

Abbreviation: R-Resistance, I- Intermediate, S-Sensitive, AMP-Ampicillin, PEN-Penicillin, CEPHA-Cephalothin, ERY-Erythromycin, KANA-Kanamycin, STREPTO-Streptomycin, CIPRO-Ciprofloxacin, MOXI-Moxifloxacin, TETRA-Tetracycline, GENTA-Gentamycin.

4.6.2.2. Antibiotic resistant pattern of *Pseudomonas* spp.

All the isolates of *Pseudomonas* spp., showed resistant to Penicillin and Cephalothin, 57 to 100% were resistant against Ampicilin and 28 to 83% to Erythromycin. In the gut, 14% of the isolates were resistant to Kanamycine. All the isolates were sensitive to Streptomycin, Ciprofloxacin, Moxifloxacin, Tetracycline and Gentamycin (Table 4.6-19)

Table 4.6-19. Antibiotics resistance (%) among of *Pseudomonas* spp. from different tissues of fish, *Lepidocephalicthys guntea*.

Source	AMP R I S	PEN R I S	CEPHA R I S	ERY R I S	KANA R I S	STR R I S	CIPRO R I S	MOXI R I S	TETRA R I S	GENTA R I S
Skin	100 0 0	100 0 0	100 0 0	83 17 0	0 0 100	0 0 100	0 0 100	0 0 100	0 0 100	0 0 100
Gills	71 29 0	100 0 0	100 0 0	71 29 0	0 85 15	0 0 100	0 0 100	0 0 100	0 0 100	0 0 100
Gut	57 14 29	100 0 0	100 0 0	28 28 34	14 29 57	0 0 100	0 0 100	0 0 100	0 14 86	0 0 100

R-Resistance, I- Intermediate and S-Sensitive

Abbreviation: R-Resistance, I- Intermediate, S-Sensitive, AMP-Ampicillin, PEN-Penicillin, CEPHA-Cephalothin, ERY-Erythromycin, KANA-Kanamycin, STREPTO-Streptomycin, CIPRO-Ciprofloxacin, MOXI-Moxifloxacin, TETRA-Tetracycline, GENTA-Gentamycin.

4.6.2.3. Antibiotic resistant pattern of *Salmonella* spp.

Data presented in Table 4.6-20 shows that all *Salmonella* spp. isolates were resistant to Penicillin and Cephalothin. 60 to 75% and 75 to 100% isolates showed resistance against Ampicillin and Erythromycin, respectively. Isolates also showed resistant to Kanamycine (25 to 60%) and Streptomycin (25 to 80%). Only isolates of gut showed resistant against Ciprofloxacin (40%), Moxifloxacin (20%) and Gentamycin (60%). However, all the isolates of skin and gill were sensitive to these antibiotics.

Table 4.6-20. Antibiotics resistance (%) among of *Salmonella* spp. from different tissues of fish, *Lepidocephalicthys guntea*.

Source	AMP	PEN	CEPHA	ERY	KANA	STR	CIPRO	MOXI	TETRA	GENTA
	R I S	R I S	R I S	R I S	R I S	R I S	R I S	R I S	R I S	R I S
Skin	75 25 0	100 0 0	100 0 0	75 25 0	25 75 0	25 25 50	0 0 100	0 0 100	0 25 75	0 50 50
Gills	60 20 20	100 0 0	100 0 0	80 20 0	20 60 20	40 60 0	0 0 100	0 0 100	0 40 60	0 40 60
Gut	60 0 40	100 0 0	100 0 0	100 0 0	60 40 0	80 20 0	40 0 60	20 0 80	0 40 60	60 20 20

Abbreviation: R-Resistance, I- Intermediate, S-Sensitive, AMP-Ampicillin, PEN-Penicillin, CEPHA-Cephalothin, ERY-Erythromycin, KANA-Kanamycin, STR-Streptomycin, CIPRO-Ciprofloxacin, MOXI-Moxifloxacin, TETRA-Tetracycline, GENTA-Gentamycin.

4.6.2.4. Antibiotic resistant pattern of *Enterobacteriaceae*

All isolates belonging to the *Enterobacteriaceae* showed resistance against Ampicillin, Penicillin and Cephalothin. 60 to 100% isolates confirmed resistant to Erythromycin and Kanamycine respectively. In case of Streptomycin, resistant was shown by 20 to 60% of the isolates as represented in Table 4.6-21.

Table 4.6-21. Antibiotics resistance (%) among of *Enterobacteriaceae* from tissues of fish, *Lepidocephalicthys guntea*

Source	AMP	PEN	CEPHA	ERY	KANA	STRE	CIPRO	MOXI	TETRA	GENTA
	R I S	R I S	R I S	R I S	R I S	R I S	R I S	R I S	R I S	R I S
Skin	100 0 0	100 0 0	100 0 0	60 20 20	70 30 0	50 0 50	0 0 100	0 0 100	0 0 100	0 0 100
Gills	100 0 0	100 0 0	100 0 0	75 25 0	60 20 20	20 0 80	0 0 100	0 0 100	0 0 100	0 0 100
Gut	100 0 0	100 0 0	100 0 0	100 0 0	100 0 0	60 0 40	40 0 60	0 0 100	0 0 100	0 0 100

Abbreviation: R-Resistance, I- Intermediate, S-Sensitive, AMP-Ampicillin, PEN-Penicillin, CEPHA-Cephalothin, ERY-Erythromycin, KANA-Kanamycin, STREPTO-Streptomycin, CIPRO-Ciprofloxacin, MOXI-Moxifloxacin, TETRA-Tetracycline, GENTA-Gentamycin.

4.7. Antibiotic Resistant Bacterial Identification up to Species Level

4.7.1. Identification of *Aeromonas veronii* by 16s rDNA gene sequencing

The selected bacterial strain (A30S2) was subjected to PCR with *Aeromonas* genus specific 16S rRNA and the amplified 953 bp product was sequenced. The 16S rRNA sequence (Accession No JN796925) was compared and aligned with sequence deposited in the (NCBI)-Gen Bank database using BLAST (Altschul et al., 1997), a tool for identification of bacteria. The sequences were further aligned in CLUSTAL X (Thompson et al., 1997). The computed alignment was then manually checked and corrected. The comparison of the obtained 16s rRNA sequence of *Aeromonas* spp. with different *Aeromonas* spp. 16s rRNA sequences showed maximum similarity (99%) with *Aeromonas veronii* B565 (NC 015424.1) followed by *Aeromonas hydrophila* subspecies (98%) and *Serratia* sp. (95%). This strongly suggests that the 16s rRNA sequence (AC No JN796925) appears to be *Aeromonas veronii*. The phylogenetic tree based on 16s rRNA of *Aeromonas* spp. (AC No JN796925) and other 16s rRNA sequences (Fig 4.7-14) also indicated that the isolates belonged to *Aeromonas veronii*.

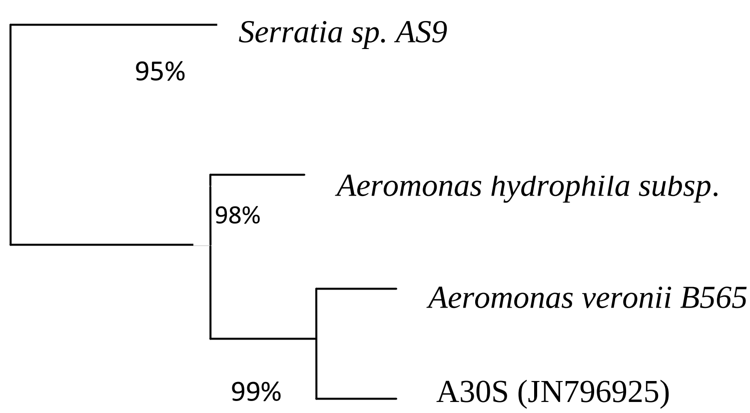


Fig 4.7-14. Phylogenetic tree based on 16S rDNA sequences. The evolutionary distance was inferred using the neighbour-joining method.

4.7.2. Identification of *Enterobacteriaceae* by 'tuf' gene sequencing

After confirmation of genus identification of selected strains ET01, ET02 and ET08, 884 bp, 'tuf' gene PCR product (884 bp) were sequenced. After substracting 714 bp, 774 bp and 741 bp respectively from the three strains, phylogenetic analysis was carried out. The sequences were compared and aligned with sequence deposited in the NCBI-Gen Bank database using BLAST (Altschul et al., 1997) for identification of bacteria and further aligned in CLUSTAL X (Thompson et al., 1997). The computed alignment was then manually checked and corrected.

The comparison of the obtained tuf gene sequence of ET01 (Accession No KF203130), with different *Enterobacteriaceae* family tuf gene sequences showed a similarity of (99%) with *Enterobacter asburiae* (Accession No CP002036), 98% with *Enterobacter cloacae* subsp while *Citrobacter freundii* revealed similarity of 97%. From the result the tuf gene sequence appears to be *Enterobacter asburiae*. The phylogenetic tree (Fig 4.7-15) based on Elongation factor-Tu gene of *Enterobacteriaceae* family (Accession No KF203130), also indicated that the isolates belong to *Enterobacter asburiae*.

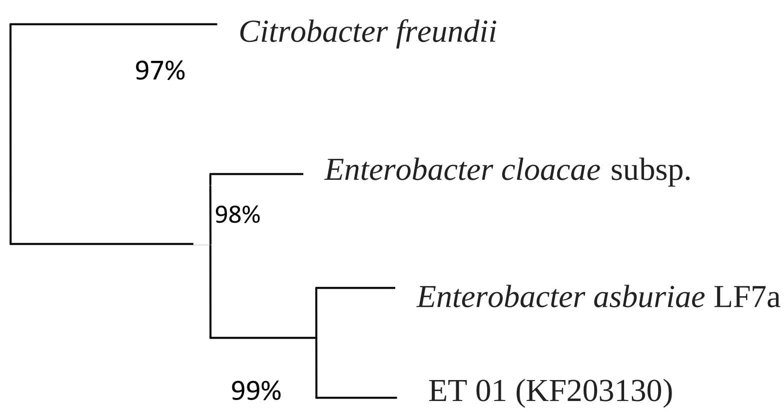


Fig 4.7-15. Phylogenetic tree based on Elongation factor Tu gene sequences of *Enterobacter asburiae* (KF203130). The evolutionary distance was inferred using the neighbour-joining method.

The comparison of Tuf gene sequence of strain ET02 (Accession No KF471081) with different *Enterobacteriacea* family showed a maximum similarity of (99%) with *Enterobacter* sp (Accession No CP001918.1) where as *Citrobacter* sp revealed a similarity of 96%. This suggests that the tuf gene sequence of ET 02 strain belong to the group of *Enterobacter* sp. The phylogenetic tree (Fig 4.7-16) based Elongation factor-Tu gene of *Enterobacteriaceae* family. (Accession No KF471081) also indicated that the isolates belonged to *Enterobacter* sp (Accession No KF471081).

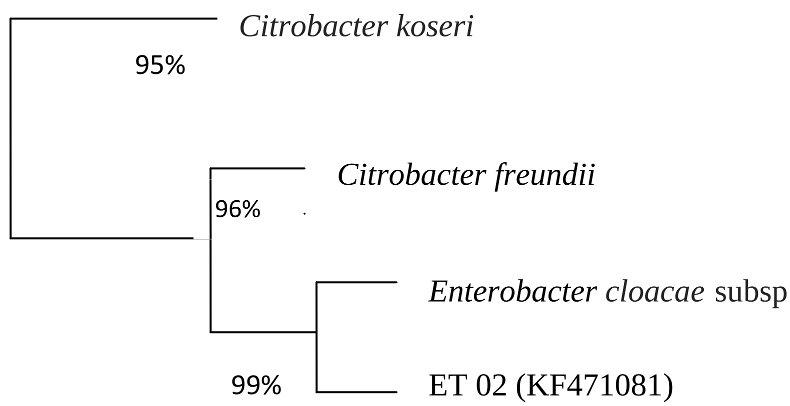


Fig4.7-16 . Phylogenetic tree based on Elongation factor Tu gene sequences of *Enterobacter* sp (KF471081). The evolutionary distance was inferred using the neighbour-joining method.

The comparison of tuf gene sequence obtained from strain ET08 (Accession No KF240806) and different *Enterobacteriacea* family tuf gene sequences revealed a maximum similarity of 98% with *Enterobacteriaceae bacterium* (Accession No CP003938.1) followed by *Klebsiella oxytoca* (98%), while *Salmonella enterica* subsp showed a similarity of 97%. The tuf gene sequence which appeared as out group to *Enterobacteriaceae bacterium*. The phylogenetic tree (Fig 4.7-17) based Elongation factor-Tu gene of *Enterobacteriaceae* family (Accession No KF240806) also indicated that the isolates belong to *Enterobacteriaceae bacterium.*

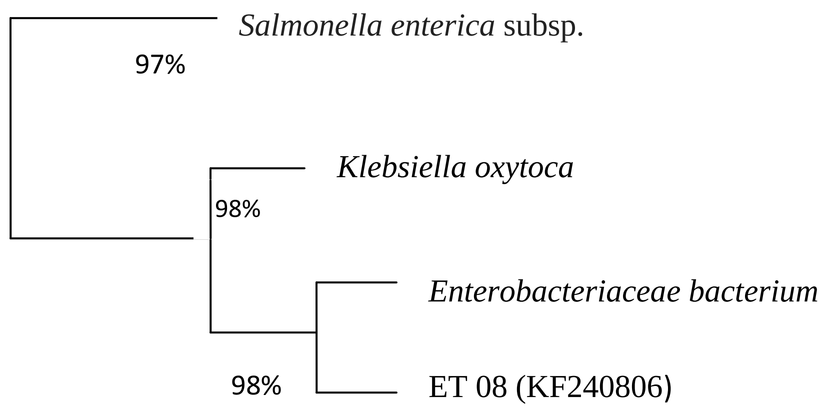


Fig 4.7-17. Phylogenetic tree based on Elongation factor Tu gene sequences of *Enterobacteriaceae bacterium* (KF240806). The evolutionary distance was inferred using the neighbour-joining method.

CHAPTER V

5. DISCUSSION

5.1. LIMNOCHEMISTRY

5.1.1. Temperature

Temperature is the most important factor which influences chemical, physical and biological characteristics of water bodies. In the present study the temperature of the river water varied from 26.5±4.6 to 26.9±4.6 ^{o}C. This range of temperature appeared to be the one of the appropriate for the higher growth of mesophilic bacteria. This finding corroborated the observation of Rheinheimer (1985). A similar water temperature range was noted for water of the river Torsa by Bhadra et al. (2003). In this investigation water temperature was high in summer followed by monsoon and winter season. The fluctuation in river water temperature usually depends on the season, geographic location, sampling time and temperature of effluents entering the stream (Ahipathy, 2006).

5.1.2. pH

pH is an important limnological factor in measuring the water quality, in every aspect of water, such as acid base neutralization, water softening, precipitation, coagulation and acid disinfection, because pH dependent. pH is the scale of intensity of acidity and alkalinity of water and measures the concentration of hydrogen ions. pH is considered as an indicator of overall productivity that causes habitat diversity (Minns, 1989). A pH below 4.0 and above 9.6 is hazardous to most life forms pH gives an idea to the type and increasing of pollution (Mishra and Saksena, 1991). The present studies reveal that the average pH of water ranged between 6.7 ± 0.2 and 6.9 ± 0.3 showing slightly acidic condition. This is in conformity with other water bodies in the Darjeeling district of West Bengal which are acidic in nature (Chakraborty, 1998). Similar observation has also been obtained by Ahmed (2004); Sujitha et al. (2012) and Ahmed et al. (2013). The result for physico-chemical analysis showed that pH of river water was within the permissible limit (pH 6.5 to 8.5) of Indian drinking water standards (2009). These values are typical, a slight seasonal variation would indicate that the water is polluted. The pH values were generally found to be constant due to strong buffering capacity of water. The pH is directly dependent on the amount of CO_2 present and inversely proportional to the activity of photosynthesis (Pandit et al., 2001). pH status showed a significant correlation with the carbon dioxide concentration. pH values in all the sites showed the same seasonal trend with summer minima and winter maxima. This might be due to increasing draining of domestic effluents to the river and microbial activities. According to Chetana and Somashekar, (1997) the winter maxima are due to decreased decomposition rate owing to reduced microbial activity. The summer minima are due to increased decomposition rate, leading to acidification and lowered pH.

5.1.3. Dissolved Oxygen

The dissolved oxygen content at the four sites of the river water showed almost a uniform trend, irrespective of the site of sample collection. In the present study the mean values of the dissolved oxygen which were quite low ranged between 3.6±1.2 to 4.1±1.5 mg l^{-1}. The value of dissolved oxygen content is a remarkable criterion for determining the water quality of an aquatic system. With dissolved oxygen content ranging between 1.0 - 5.0 mg l^{-1} fishes can survive, but their reproduction is poor and growth is slow if exposure to such condition is continued (Swingle, 1967). However, when dissolved oxygen concentration is less than 5.0 mg l^{-1}, it is not considered conducive for fish growth (Mondal and Barat, 2004). The lower value of dissolved oxygen content in the river Lotchka as recorded by Roy and Barat (2011) indicated that the river water received discharge of sewage effluent from surrounding localities. Higher dissolved oxygen concentration observed during the monsoon might be due to the cumulative effect of heavy rainfall and the resultant freshwater mixing. Similar observation was made by Saravanakumar et al. (2008) and Adeyemo et al. (2008). The adverse condition of DO was observed during winter. These low values may be due to lesser level of river water which is natural phenomena of river of North Bengal during winter and enhancement of decomposition of organic materials. The low dissolved oxygen level could also lead to eutrophication. Positive correlation was found between dissolved oxygen and heterotrophic bacterial load. The addition of a variety of biodegradable pollutants from domestic and industrial sources also stimulates the growth of microorganisms, which consumes the dissolved oxygen (Moundiotiya et al. 2004).

5.1.4. Free carbon dioxide

The amount of CO_2 present in simple water medium phase that in the form of carbonic acid is called free carbon dioxide. Carbon dioxide dissolved in water more readily dissolution depends on temperature, pressure and mineral content of the water. The average free carbon dioxide in the water ranged between 15.0±5.8 and 16.7±6.1 and was present throughout the study period as the pH remained acidic. Ahmed et al. (2013) reported that the water is slightly acidic due to higher amount of dissolved CO_2. This high concentration of free carbon dioxide may be attributed to the inflow of organic wastes in the water body from surrounding areas of the river and respiration of organisms. In the present study free CO_2 showed a negatively significant correlation with DO and total hardness in the river. Carbon dioxide is not appreciably toxic to most fish but it antagonizes the uptake of dissolved oxygen (Tucker and Boyd, 1985). Rawat et al. (1993) and Mondal et al. (2012) also found negative correlation between free CO_2 and DO in Deoria Tal Lake and Mirik Lake.

5.1.6. Total Alkalinity

The total alkalinity of natural waters is due primarily to the salts of weak acids, even through weak or strong bases may also contribute. It is measure the basic substances of water. Bicarbonate represents the major form of alkalinity along with carbonate and hydroxide alkalinity. Total alkalinity in the form of bicarbonate alkalinity was only recorded and ranged between 19.8±7.6 and 21.4±7.4 mg l^{-1}indicating that the carbonate alkalinity was absent in the water body. pH with values greater than 8.2 contain carbonate alkalinity (Trivedy and Goel, 1984). In the present study pH remained much below 8.2. Bicarbonate alkalinity was also recorded in the River Mahanada near Siliguri city (Barat and Jha, 2002). Total alkalinities recorded during the present study are comparable to the studied by others (Dobriyal and Singh, 1988; Goutam, 1990; Mukhopadhyay, 1996 and Haque et al., 2010). Total alkalinity in stream and rivers fluctuate in a wide range and mainly depends on influx of effluents, evaporation and precipitation. Seasonal variations of total alkalinity in the present study showed that higher value in summer and monsoon than in winter season. This may be attributed to the increased rate of organic decomposition during which CO_2 is liberated, which reacts with water to form HCO_3, thereby increasing the total alkalinity during summer (Shinde et al., 2011). Shiddamallayya and Pratima (2008) and Pawar and Shembekar (2012) also reported similar kind of results. In the present study, total alkalinity showed positively significant correlation with total hardness. Barat and Jha (2002) and Thapa-Chhetry and Pal (2011) reported positive significant correlation of total alkalinity with hardness.

5.1.7. Chloride

The chloride concentration in river water plays a good indicator ion for pollution sources as well as organic waste of animal origin (Kegley and Andreus, 1997). There is a relation between pollution level and chloride concentration in river water (Munawar, 1970). In the present study, chloride concentration in the water samples averaged between 20.0±4.4 and 23.1±5.3 mg l^{-1} thereby indicating that the concentration falls within the permissible limit of 250 mg l^{-1} (WHO, 2003). Further, maximum chloride concentration was found in winter and a minimum in summer. Barat and Jha, (2002) reported a value of 39.4 mg l^{-1}in the river Mahananda located in the foot-hills of Darjeeling Himalaya. Maximum amount of chloride in winter and post monsoon months may be due to loading of organic material, high rate of decomposition of organic matter, and low volume of water. Thapa-Chhetry and Pal (2011) and Mondal (2009) observed almost similar seasonal variations.

5.1.8. Total Hardness

Total Hardness of water is an important component to determine the suitability of water for domestic and industrial uses. Hardness is caused by multivalent metallic cations and with certain anions present in the water. The principal hardness-causing cations are the divalent calcium, magnesium, strontium, ferrous iron and mangnous ions. Total hardness varied from 62.6±8.6 and 68.5±9.7 mg l^{-1}. Water with hardness value less than 60 mg l^{-1} is soft (Kannan, 1991), as such the river water may be considered as moderately hard. During the study period the maximum total hardness of water was recorded in summer season and minimum in winter season. Sawant et al. (2013) and Sing et al. (2013) also found similar results.

5.1.9. Ammonium-Nitrogen (NH_4-N)

Presence of ammonium–nitrogen (NH_4-N) acts as an evidence of sewage inflow to a water body (Saksena at al., 2008). Moreover, Jana and Chakrabarti (1993) opined that the metabolism of the water body can be considered an index of environmental stress. Concentration of ammonium-N varied from 0.08±0.13 to 0.10±0.18 mg l^{-1}.The most important source of ammonia is the ammonification of organic matter. Sewage has large quantities of nitrogen matter, thus its disposal tends to increase the ammonia content of the water (Trivedy and Goel, 1984). Roy and Barat (2011) opined that many heterotrophic bacteria utilize organic-N rich substrate to release ammonium and ammonium salts. Hence, it is apparent that the rate of ammonium in the environment is determined by the heterotrophic bacterial flora.

5.1.10. Nitrite-nitrogen (NO_2-N)

Nitrite-N (NO_2-N) varied from 0.07±0.07 to 0.12±0.09 mg l^{-1} and showed higher value than 0.05 mg l^{-1} permissible limit (WHO, 1992). Wetzel (1983) stated that nitrite level of natural lake water are generally very low in the range of 0-0.01 mg l^{-1}, although up to 1.0 mg l^{-1} has been found in the interstitial water of deep sediments of Lake Mendota (Konrad et al., 1970). Nitrite is a very unstable ion and gets converted into either ammonia or nitrate depending upon the conditions prevailing in the water (Trivedy and Goel, 1984). However, in the present study Nitrite-N concentration was highest in monsoon and lowest in winter. Similarly Imnatoshi and Aahmed (2012) observed that maximum values of nitrite during the monsoon period which may be attributed to the seasonal rainfall. The low value of nitrite recorded in post monsoon / pre monsoon maybe due to the lesser amount of freshwater inflow. Similar trend of nitrite level during different seasons were also observed by previous workers (Satpathy, 1996; Bhadra et al., 2003; Barat and Jha, 2002; Prabhu et al., 2008 and Damotharan et al., 2010).

5.1.11. Nitrate-nitrogen (NO_3-N)

The nitrate-N is one of the most oxidisable forms of nitrogen and is essential for plant nutrient and also associated with sewage and sullage discharge (Barat and Jha, 2002). In the present study the range varied from 0.17±0.08 to 0.24±0.20 mg l^{-1}. Usually Nitrate-N levels in excess of 0.2 mg l^{-1} show possible eutrophic conditions (WHO, 1992). Thus, the water of the river Lotchka may be considered to be slightly eutrophic. Bhadra et al. (2003) and Barat and Jha, (2002) reported that the range of NO_3-N in the two rivers of North Bengal (Torsha and Mahananda) were 0.090-2.200 mg $l^{-1}$and 0.080- 0.042 mg l^{-1}respectively. The maximum concentrations of nitrate were found in monsoon and minimum in winter. Jhingran (1991) also recorded maximum nitrate-N in monsoon than winter in the river Brahmaputra. The increase in level of NO_3-N is therefore attributed to the precipitation and terrestrial runoff during the monsoon period (Karuppasamy and Perumal, 2000 and Manikannan et al., 2011). The most important source of NO_3-N in the natural water is domestic and industrial sewage which contains very high amount of nitrogenous compounds. Runoff from agricultural fields also contain high concentration nitrate. Katavkar et al. (1989) observed that sewage entry into the water body is the major factor responsible for the enrichment of nitrate.

5.1.12. Phosphate-phosphorus (PO_4-P)

Phosphate-P averaged between 0.23±0.11 and 0.33±0.24 mg l^{-1} in the four different sites and the low level of phosphate may be due to the discharge of human excreta, use of fertilizers in cropland, washing of clothes through detergents. According to CPAB (2008), the maximum permissible limit of phosphate is 5.0 mg l^{-1} for inland surface water. The environmental significance of phosphorus arises out of its role as a major nutrient for both plants and microorganisms (Vanloon and Duffy, 2000). Nutrients that contribute to eutrophication are most commonly phosphorus and this can be considered as a pollutant if it is present at a higher concentration than the normal limit (Saad, 1973). This study showed that highest PO_4-P value was recorded in the monsoon and lowest in the winter. This finding is supported by that of Imnatoshi and Aahmed (2012). The probable reasons may the high rate of decomposition of waste material or due to surface runoff from the surrounding crop fields fertilized with phosphate during the monsoon period, which increases the level of phosphate. In selection to our findings similar observations were also made by Nagarathna and Leelavathi (2008) and Manikannan et al. (2011).

Roy and Barat (2011) showed, that the high concentration of nutrients in the four sites of river Lotchka was due to the river receiving huge amount of sewage and waste from the surface run-off and effluent discharged by the inhabitants and from agriculture fields along the course of the river.

5.2. BACTERIAL ENUMERATION OF RIVER WATER

5.2.1. Heterotrophic bacteria

Heterotrophic bacteria play an important role in altering water quality and leads to the transformation of organic matter in the water column (Jana and Barat, 1983). In all the sites of the river, heterotrophic bacterial counts was maximum during monsoon season due to the mixing of some domestic sewage during the overflowing of runoff water in monsoon which brings rich organic matter and nutrients facilitating the growth of heterotrophic bacteria. Chandra et al. (2006) and Rajee and Palaniappan (2006) also found similar results in Gola River. Minimum count was found in winter season due to low temperature and low input of organic matter.

Similar results were also observed by Mishra et al. (2009). Positive correlation of temperature with heterotrophic bacteria indicated that water temperature plays a major role for the higher growth of heterotrophic bacteria. Heterotrophic bacterial population showed positive correlation with dissolved oxygen content, nitrite-N, nitrate-N and phosphate-P. Low dissolved oxygen value and high concentration of nutrients also provide favourable conditions for the higher heterotrophic bacterial growth of river water. Roy and Barat (2011) reported that deteriorating water quality can enhance the bacterial growth in the river Lotchka.

5.2.2. Total Coliform

Presence of coliform organisms in water is regarded as evidence of pathogenic bacterial contamination, faecal and organic pollution of water body (Hiraishi et al., 1987 and Figueras and Borrego, 2010). Primary routes of coliform pollution of natural water sources comprise vegetation, flood runoffs and untreated or partly treaded water including domestic agricultural and industrial waste. Microbial contamination of the soil and natural water occurs from land application of sludge which results from sewage and waste water treatment plants (Sidhu and Toze, 2009). The present investigation exhibits maximum total coliform count in monsoon season. This may be due to the result of indiscriminate dumping of waste and human faeces passed around the river bank and being washed into the river during rains. It is natural that the incoming nutrient load finds its way first to the surface, thereby encouraging bacterial proliferation during monsoon. Similar kind of observation was found by Maitera and Sudi (2011). Collins (1963) had suggested that the rains bring in particulate matter, which serves as sites for adsorption of bacteria, thereby increasing the bacterial load. Minimum total coliform count found in winter may be due to cold climatic condition which is not conductive to bacterial multiplication to a greater extent (Venkatesharaju et al., 2010). Total Coliform count was positively correlated with temperature, dissolved oxygen and nitrite-N. Chatterjee et al. (2010) also reported that physico-chemical constituents of the river Damodar provided favourable microhabitat for the growth of coliform bacteria.

5.2.3. *Aeromonas* spp. and *Pseudomonas* spp.

Allied to the counts of heterotrophic and coliform bacteria the maximum count of *Aeromonas* spp. and *Pseudomonas* spp. was found in monsoon and minimum in winter. This indicates that in monsoon season river water receives indiscriminate discharges of domestic effluents from surrounding areas.

All types of bacterial counts showed higher values in monsoon season followed by summer and winter. But the luxuriant growth of all bacterial types in all the sites of the river was observed throughout the three seasons (summer, monsoon and winter). This clearly suggests that the bacterial contamination in the water body of Lotchka is chiefly caused by surrounding effluents (Roy and Barat, 2011). The major water quality parameters were highly correlated with heterotrophic bacterial load and total coliform, indicating that the water quality parameters provided favourable condition for bacterial growth in the river water.

5.3. BACTERIAL ISOLATION AND IDENTIFICATION FROM RIVER WATER

All the 97 gram negative rod-shaped bacteria isolated and examined from the water sample collected of different sites of the river were classifiable into four main genera.

Initially a total of thirty *Aeromonas* spp. were identified among the all sites of the river Lotchka. These isolates were characterized with physiological and conventional biochemical tests. All were Gram-negative, glucose fermenting, rod-shaped bacteria of the family *Aeromonadaceae* (ICMSF, 1996). *Aeromonas* spp. are common aquatic microorganisms that occur in irrigation water, river water, brackish water, freshwater, spring water, surface water, groundwater, estuarine and sea water, chlorinated and non-chlorinated drinking water (Kaper et al., 1981; Fiorentini et al., 1998; Gavriel et al., 1998 and Soler et al., 2002). The prevalence and distribution of *Aeromonas* in aquatic environments are potential for pathogenicity mediated by mesophilic *Aeromonas* and are all of great public health concern (Dumontet et al., 2000 and Gavriel et al., 1998).

A total of 22 *Pseudomonas* spp. were isolated from the four sites of the river. Initial identification was done by different biochemical procedures. All the isolates were Gram negative, motile and rod shaped. Their, citrate utilization, H_2S and urease showed negative, producing acid and gas from glucose (Roy et al., 2014). The genus *Pseudomonas* is the most heterogeneous and ecologically significant group of known bacteria and includes Gram-negative motile aerobic rods that are widespread throughout nature (Franzetti and Scarpellin, 2007). *Pseudomonas* spp. are commonly found in natural sources of water and associated with septicaemia in aquatic animals (Roberts, 1978). *Pseudomonas* was also reported as a pathogen in fishes and shellfishes in aquatic ecosystem (Kamat, 1982). These bacteria are considered opportunistic pathogens, causing disease when the host is subjected to stress. A number of aquatic animals including fish, frogs and soft-shelled turtles are reported to be susceptible to *Pseudomonas* spp. with moderate to high losses (Somsiri and Soontornvit, 2002). So, the occurrence of this opportunistic pathogen in the sites may lead to diseases in humans and other animals too.

A total of thirty bacteria isolated from water sample of four different sites of river mainly belong to *Enterobacteriaceae* family. All isolates utilized the citrate and gave positive results for VP test but for MR and Indole tests the results were all negative. Acid and gas was formed against glucose fermentation. *Enterobacteriaceae* family are not considered as specific pathogen but regarded as opportunistic pathogens (Purdy et al., 2010). These bacteria were defined as a sign of faecal contamination in areas polluted by urban, industrial and domestic waste by other workers (Alonso et al., 1999 and Toroglu et al., 2005b). All these organisms are potential pathogens of man capable of causing a variety of diseases. When the aquatic system is contaminated with pathogenic bacteria, these bacteria too become part of microflora of fish and the fish pose a serious threat to public health.

In this investigation a total of fifteen *Salmonella* spp. were isolated from the water samples of four sites of the river. Initially bacteria were identified by morphological and biochemical procedures (Roy et al., 2013). *Salmonella* are pathogens that are distributed worldwide and transmitted mainly through food and water ingestion. Their presence in water including recreational water, render that water unfit for human use (Elmanama et al., 1995). It has been frequently defined, that *Salmonella* spp. are opportunistic and potential pathogenic bacteria of water bodies in warm climatic zones and poses a great risk for human health (Heinitz et al., 2000). *Salmonella* spp. infections can be life-threatening, especially for the very young, the elderly and for persons with impaired immune systems. These enteric bacterial pathogens are variously incriminated for diarrhoea, which accounts for a substantial degree of morbidity and mortality in different age groups worldwide (Black, 1993; Prado and O'ryan, 1994, Obi et al., 1997 and El-Sheikh and El-Assouli, 2001).

Presence of opportunistic pathogenic bacterial population in river water along with deteriorating water quality, point to the degree of human activity and anthropogenic pressures which contribute to pollution of Lotchka river.

5.4. BACTERIAL ENUMERATION IN DIFFERENT TISSUES OF FISH

The heterotrophic bacterial count, total coliform count and counts of total *Aeromonas* spp. and *Pseudomonas* spp. were found higher in monsoon followed by summer and winter. In addition to seasonal variation, during the entire study period counts of these bacterial groups were luxuriant. An interesting observation was that seasonal trend of bacterial loads were almost found to be parallel in fish tissues and in river water. Results also showed that bacterial counts in both river water and in the fish were relatively higher. These observation indicated that bacterial load in fish is the reflection of the quality of environment (Roy and Barat, 2011). Higher bacterial counts were also observed by earlier workers in river water and fish organs (Sugita et al., 1983). Roy et al. (2014) observed, that bacterial population of *Pseudomonas* spp. was equally dominant in both the fish tissues and water samples. The maximum counts of bacteria in the above category; heterotrophic bacteria 4.2 X 10^6 cfu g^{-1} and total coliform 2.3 x 10^6 cfu g^{-1} has been found to be present in the gut followed by gills and skin in *Lepidocephalichthys guntea*. Highest count of *Aeromonas* spp. and *Pseudomonas* spp. were also observed in the gut than gill and skin. Latha and Mohon (2013) also reported that different bacterial loads were maximum in the intestine followed by gills and skin of contaminated and fresh water fishes. Al-Harbi and Uddin, (2004) reported identically, that the intestinal bacterial load of fresh water healthy Tilapia in Saudi Arabia was higher than in the gill filaments. The presence of a high bacterial load in intestine and gill of fish might be due to high metabolic activity of fish associated with increased feeding rates of organic matter at higher temperatures.

Growth of total coliform bacteria in tissues revealed the existence of pathogenic bacteria in fish tissues. Coliform are not the normal bacterial flora in fish (Cohen and Shuval, 1973). Fish take a large number of bacteria into their gut from water, sediment and food (Sugita et al., 1988). It is well known, that both fresh and brackish water fishes can harbour human pathogenic bacteria, particularly the coliform group (Leung et al., 1990 and Ramos and Lyon, 2000). This is further justified by the findings of Apun et al. (1999) that the type of bacteria carried in the gastro-intestinal tract of fishes is related to a certain degree on the level of contamination of water by enteric bacteria.

5.5. BACTERIAL ISOLATION AND IDENTIFICATION FROM FISH

From different tissues namely skin, gills and gut of fish, *Lepidocephalichthys guntea*, a total of 30 *Aeromonas* spp., 34 *Pseudomonas* spp., 40 *Enterobacteriaceae* and 15 *Salmonella* spp. were isolated and identified. These bacterial genera were present in the aquatic environment and were often observed also in the internal organs of fishes (Sugita et al., 1985; Sakata, 1989; Toranzo et al., 1985 and Cahill, 1990). Earlier workers such as Sugita et al., (1983) had also noticed the presence of these organisms in the river and intestine of different river water fishes. Sausa and Sauza (2001) in a similar study had also observed that the leading genus belonged to *Pseudomonas, Aeromonas, Enterobacteriaceae* and *Streptococcus* in the internal organ of fourteen different fishes of the River Congonhas in Brazil. Several of these bacteria are opportunistic pathogens and may cause diseases when the fish are under unfavourable condition. Apun et al. (1999) and Al-Harbi and Uddin (2005) reported, similar bacterial groups were dominant in the intestine of pond cultured fishes and pond water.

Massa et al. (2001) reported, *Aeromonas* spp. are ubiquitous inhabitants of aquatic ecosystems such as freshwater, coastal water and sewage. These bacteria are usually natural microbiota as well as primary or secondary pathogens of fish (Cahill, 1990 and Janda and Abbott, 1998). In this study, *Aeromonas* spp. was uniformly isolated from skin, gills and gut of the fish and such occurrence remarkably supports several previous observations (Kaper et al., 1981; Nieto et al., 1984; Austin and Al-Zahrani, 1998 and Kasing et al., 1999). According to Noga, (1996), motile aeromonad infection (MAI) is likely the most common bacterial disease of fresh water fishes, all of which are probably susceptible. Many researchers reported, that *Aeromonas* is a dominating causal organism for disease particular in fresh water fish (Pal and Pradhan, 1990; Kasing et al., 1999 and Goldschmidt et al., 2008).

Pseudomonas spp. are naturally present in aquatic environments and therefore may be expected to occur in fish (Cahill, 1990). *Pseudomonas* spp. is commonly associated with fish tissues namely skin, gills, and gut. Chen and Kou (1987) and Hnadi (2008) reported the presence of *Pseudomonas* spp. in skin, gills and intestines in number of fishes. However, secondary occurrence of pseudomonads was found to be rather occasional in several culture and wild fish species of Southeast Asia (Boonyaratpalin, 1989). Tripathy et al. (2007) also detected *Pseudomonas* sp. in healthy and diseased fishes.

According to Alonso et al. (1999) and Toroglu et al. (2005b), presence of *Enterobacteriaceae* is a sign of faecal contamination in areas polluted by urban, industrial and domestical wastes. All these organisms are potential pathogens of man capable of causing a variety of diseases. When the aquatic system is contaminated with pathogenic bacteria, these bacteria too become part of microflora of fish. In the case of being contaminated with pathogenic bacteria, fishes pose a serious threat to public health (Toroglu et al., 2009). *Enterobacteraceae* in fish are considered as an indicator to sewage pollution and has been reported as an opportunistic pathogen in fish (Rajasekaran, 2008). Lotfy et al. (2011) from a similar study have the reported the presence of *Salmonella* spp. in different organs of fish. It has been frequently defined, that *Salmonella* spp. are opportunistic and potential pathogenic bacteria of water bodies in warm climatic zones and pose a great threat to human health (Heinitz et al., 2000). *Salmonella* spp. infections can be life-threatening, especially for the very young, the elderly and for persons with impaired immune systems. They cause a wide range of human diseases such as enteric fever, gastroenteritis and bacteremia (Anonymous, 1995). Isolated *Enterobacteriaceae* and *Salmonella* spp. from different tissues of *Lepidocephalichthys guntea* reveal that river water may be polluted by faecal contamination, waste disposal and agricultural runoff which is very dangerous to public health as the condition affects human who consume such fish. Presence of other enteric bacteria like *Aeromonas* spp. and *Pseudomonas* spp. in resident fish of Lotchka river may also risk aquatic animal health and human beings. Since the genera *Pseudomonas*, *Aeromonas* and *Vibrio* are commonly isolated from normal healthy fish, only certain strains of these bacteria possess the virulence factors necessary to induce disease. The incidence of virulence factors in strains associated with populations of healthy fish could be determined for epidemiological purposes (Cahill, 1990). This study, therefore, clearly indicates that the river water fish can act as bioindicator of transmission and contamination of enteric pathogenic bacteria.

5.6. MOLECULAR IDENTIFICATION OF BACTERIAL ISOLATES FROM WATER AND FISH SAMPLES

Morphological and biochemical characteristic are not sufficient enough for bacterial identification. The methods are time consuming. For the accuracy of identification Polymerase

Chain Reaction (PCR) based identification is more reliable. According to Harmsen et al. (2000), recent advances in PCR based molecular techniques make it possible to identify different bacterial populations in environmental samples without cultivation. Therefore, in this investigation all isolates from river water and fish tissues samples were identified by PCR based techniques using genus specific 16s rDNA and 'tuf' primers for strengthening the reliability of identification. This is because, positive amplification products for specific primers signal only for the appropriate target nucleic acid sequences present in the sample. A total of 97 bacteria were isolated from four different sites of the river and 72 of them were successfully identified. A total of 119 bacteria were isolated from different tissues like skin, gills and gut of fish. Among the total isolates, 103 isolates were successfully identified. All the isolates belonged to *Aeromonas* spp., *Pseudomonas* spp., *Salmonella* spp. and *Enterobacteriaceae*.

Genus specific 16s rDNA primers were used mainly for identification of *Aeromonas* spp, *Pseudomonas* spp. and *Salmonella* spp. Forty-nine (49) isolates out of sixty (60) *Aeromonas* spp., forty (40) isolates amongst fifty-six (56) *Pseudomonas* spp. and twenty-four (24) isolates amongst thirty (30) *Salmonella* spp. gave positive results in PCR. These results thus clearly show that PCR based technique is more authentic than cultivation method. Polymerase chain reaction (PCR) is more advanced approaches to identification have been developed (De Vos et al., 1997). The rRNA-coding regions (16S rDNA) have been used extensively to analyse phylogenetic relationships at the species level or above (Woese, 1987). However, intergenic spacer regions (ISRs), especially those located between the 16S and 23S rDNAs have been shown to be under less evolutionary pressure (Rijpens et al., 1996; Smart et al., 1996; Berridge et al.. 1998 and Chun et al., 1999). For the detection of *Enterobacteriaceae* in river water and fish tissue samples genus specific 'tuf' gene based primer used in PCR. Total of sixty-two (62) amongst seventy (70) *Enterobacteriaceae* showed positive results in PCR based technique using tuf gene primers. tuf is the gene encoding elongation factor Tu. Elongation factor of Tu is involved in peptide chain formation (Ludwig et al., 1990). The two copies of the tuf gene (tufA and tufB) found in *Enterobacteria* (Sela et al., 1989) share high levels of identity (99%) in *Salmonella typhimurium* and in *Escherichia coli*. A recombination phenomenon could explain sequence homogenization between the two copies (Abdulkarim and Hughes, 1996 and Grunberg-Manago, 1996). Elongation factor Tu has been highly conserved throughout evolution and show functional constancy (Ludwig et al., 1990). Phylogenies based on protein sequences from elongation factor Tu have shown good agreement with each other and with the rRNA gene sequence data (Ludwig et al., 1993).

5.7. ANTIBIOTIC RESISTANCE PATTERN OF BACTERIAL ISOLATES

5.7.1 River Water Samples

Aeromonas spp., *Pseudomonas* spp., *Enterobacteriaceae* and *Salmonella* spp. have shown a full range of resistance (0-100%) for ten (10) antibiotics which are commonly used by human being. Maximum resistance of all bacterial isolates was exhibited for Ampicillin, Penicillin-G, and Cephalothin belonging to the β- lactam class in river water sample. Whereas, resistance against other antibiotics namely ciprofloxacin, gentamicin, tetracycline and streptomycin have shown a very lower range of resistance in river water. Significant differences of resistance pattern of total isolates ($P \leq 0.01$) using Z (Standard Normal) test were observed between antibiotics of β-lactam class and others. Several molecules belonging to the same chemical family are usually affected by a single mechanism of resistance (cross-resistance). Both ampicillin and cephalothin, for example, are inactivated by chromosomal β- lactamases produced by many *Aeromonas* species (Walsh et al., 1995). Most studies on bacterial antibiotic resistances in sewage (Al-Jebouri, 1985 and Fernandez et al., 1990) and freshwater (Pathak et al., 1993) did not take into account these elements, leading to unexpected conclusions. Jones et al. (1986) have found a higher incidence of resistance in the bacteria isolated from remote upland tarns than in those isolated from a polluted lake or sewage, with the highest values being observed for Pseudomonads, which are naturally multi resistant organisms. Similarly, McKeon et al. (1995) have reported, that 100% of *A. hydrophila* strains isolated from rural groundwater supplies were resistant to at least two antibiotics, but among the tested antibiotics as Ampicillin and Cephalothin, were found to be naturally resistant to *A. hydrophila* (Walsh et al. 1995). β-lactamase producing *Pseudomonas* spp. and *Enterobacteriaceae* was also found in Asku River by Toroglu et al. (2005 a and b). Multiple antibiotic resistances were also found in strains of *Enterobacteriaceae* isolated from rivers of Bangladesh (Zahid et al., 2009). Based on the results of several studies one major conclusion can be drawn that antibiotic-resistant bacteria were widely distributed in water bodies of India (Mukherjee and Chakraborty, 2006; Malik et al., 1995, 2008 and Ansari et al., 2008). As pointed out by Hsu et al. (1992), difference in percentage of bacterial resistance to various antibiotics may reflect the history of antibiotic application, and hence, there is possibility of using bacterial resistance as an indicator of antibiotic application. Tao et al. (2010) have suggested that wastewater is a possible source of antibiotic resistant bacteria. Human activities, especially discharge of wastewater, are probably the main contributor to the multi-drug resistance patterns since rivers are the primary receptacle of sewage effluents.

Isolates resistant against antibiotics are also used to determine the sources of faecal contamination in water. Resistance against antibiotics is found in bacteria species isolated from humans or animals (Toroglu et al., 2005b). Presence of resistance against man made antibiotics such as β- lactamase producing bacteria is assumed to have an anthropogenic origin (Mensink and Montforts, 2007). This has also been reported earlier by Roy and Barat (2011) where, the river Lotchka received sewage and waste from the surface run-offs and effluents discharged from surrounding inhabitants and agriculture fields. Antibiotics are predominantly water soluble and enter the aquatic environment through sewage systems following consumption and excretion by humans and via effluent from farms, abattoirs and landfills (Daughton and Ternes, 1999). The occurrence of resistance for common antibiotics is an indication of indiscriminate use of these antibiotics leading to constraint in antimicrobial therapy for infectious diseases. Antibiotics released into the aquatic environment are of concern for the following reasons: (i) contamination of raw, treated and recycled water used for drinking, irrigation and recreation, (ii) potential to accelerate widespread bacterial resistance to antibiotics and (iii) negative effect on important ecosystem bacteria (through death or inhibition). Between 30% and 90% of an administered dose of most antibiotics to humans and animals are excreted in the urine as the active substance (Rang et al., 1999). The loss of antibiotic susceptibility among the aquatic bacteria has been observed to be affected to a considerable extent by the physico-chemical qualities of water (Pathak et al., 1993). This investigation, therefore, signifies that antibiotics and resistant bacteria are entering our local waterways and have potential to influence biotic processes. The occurrence of antibiotic-resistant pathogenic bacteria in surface waters and aquaculture environments is also a well-known phenomenon that carries a negative impact for public health and for the safety of the fish supply (González et al., 1999 and Toroglu et al., 2005a and b).

5.7.2. Fish Tissue Samples

The percentage patterns of resistant bacterial isolates were similar in the fish as that in the river water. Bacterial isolates like *Aeromonas* spp., *Pseudomonas* spp., *Enterobacteriaceae* and *Salmonella* spp. of fish tissues namely skin, gills and gut also showed higher range of resistance (57 to 100%) against Ampicillin, Penicillin-G, and Cephalothin belonging to the β- lactam class. These isolates showed lower range of resistance (0 to 84%) against erythromycin, kanamycin, streptomycin, ciprofloxacin, gentamicin, tetracycline, respectively. Like in case of river water, significant differences of resistant pattern of total isolates of fish tissues ($P \leq 0.01$) using Z (Standard Normal) test were observed between antibiotics of β-lactam class and others. This also indicated that dominance of β-lactam producing bacteria mainly occurred in the fish tissues. Results clearly indicated that river water was contaminated with antibiotic resistant bacteria due to anthropogenic pressure, which made direct effect on resident fishes. Similar observation was also reported by Roy et al. (2013) and (2014). With these observations it appears, that the source of the problem of antibiotic resistance in riverine ecosystems is due to faecally contaminated water and fish population in them (Pathak and Gopal, 2005). Karki (2013) have worked on antibiotic resistant *Aeromonas* spp. and *Enterobacteriaceae* in wild fresh water fish intestine. Akinbowale et al. (2007) also found resistant *Aeromonas* spp. and *Pseudomonas* spp. against amoxicillin and cephalothin which mainly belong to β -lactam class and all isolates were sensitive to ciprofloxacin and gentamicin in rainbow trout (*Oncorhynchus mykiss*). Similar to the present study, antibiotic resistant motile aeromonads were also found in fresh water fishes (Hatha et al., 2005) and in Danish rainbow trout fish (Schmidt et al., 2000). Sarter et al. (2007) studied on isolation and identification of the multiple antibiotic resistant *Enterobacteriaceae* and *Pseudomonas* spp. from the fresh water catfish. Pradhan and Pal (1993), Saha and Pal (2002) and Das et al. (2009) have reported the multiple antibiotic resistant *Aeromonas hydrophilla* isolated from Epizootic Ulcerative Syndrome (EUS) affecting fishes in India.

This also indicated that dominance of β-lactam producing bacteria mainly occurred in the fish tissues. Results clearly indicated that river water was contaminated with antibiotic resistant bacteria due to anthropogenic pressure, which made direct effect on resident fishes. (Sugita et al., 1983 and 85; Sakata et al., 1989 and Cahill, 1990). According to Karki (2013) the presence of antibiotic resistant bacteria in wild fish populations suggests that antibiotic resistant bacteria or antibiotics are present in the water. This is thus another point of view, where concluding remark is, contamination of antibiotic resistant bacteria in gut of fish was influenced by their surrounding environment. Because, intestinal bacterial composition of fish act as indicator of contamination level of surrounding water body (Apun et al., 1999).

5.8. IDENTIFICATION OF ANTIBIOTIC RESISTANT BACTERIA UP TO SPECIES LEVEL

Among all the isolates, one strain belonged to *Aeromonas* spp. and three strains belonged to *Enterobacteriaceae* from the gut of fish, *Lepidocephalichthys guntea* based on their antibiotic resistance ability, were identified up to the species level. For the identification purpose genus specific 16s rDNA and tuf gene based sequencing was performed. The strain (A30S2) was amplified by 16s rDNA *Aeromonas* specific primers and 953 bp positive amplicon product was sequenced. The sequences were compared and aligned with sequence deposited in the NCBI-Gen Bank database. After the comparison with other sequence data base, maximum similarities of the strain (A30S2) was found with *Aeromonas veronii*. The comparison of the 16S rRNA gene sequences allows differentiation between organisms at the genus level across all major phyla of bacteria, in addition to classifying strains at multiple levels, including the species and subspecies level. The occasional exceptions to the usefulness of 16S rRNA gene sequencing usually relate to more than one well-known species having the same or very similar sequences (Clarridge, 2004).

Aeromonas veronii is present widely in aquatic environment and known to pose an aquatic animal health risk. *Aeromonas veronii* may spread in one environment to other by infections and causes serious problems as it is also one of the most important pathogen of humans as well. *Aeromonas veronii*, is a food borne bacterial pathogen causing infections in fishes, animals and humans. It is Gram-negative, rod-shaped bacteria found in fresh water and have been associated with animal diseases. *Aeromonas veronii* is an opportunistic human pathogen that causes diarrhoea and extra intestinal infections, that is, wound infection and septicaemia in immune compromised patients (Janda and Abbott, 1996). Rahman et al. (2002) detected *Aeromonas veronii* as a causative agent of EUS associated infected fish.

Aeromonas veronii has shown resistance to eight antibiotics namely Penicillin-G, Ampicillin, Cephalothin, Streptomycin, Gentamicin, Kanamycin, Ciprofloxacin, Erythromycin and sensitive to Moxifloxacin and Tetracycline. Orozova et al. (2010) reported on antibiotic resistant *Aeromonas veronii* which was isolated from fish.

Strains ET01, ET02 and ET08 belong to *Enterobacteriaceae* family isolated from the gut of the fish and amplified with *Enterobacteriaceae* specific tuf gene based primers. 884 bp 'tuf' gene PCR products were sequenced and aligned with sequence deposited in the NCBI-Gen Bank database. The comparison with other deposited sequences showed maximum similarity with *Enterobacter asburiae, Enterobacter* sp and *Enterobacteriaceae bacterium,* respectively. Hassan et al. (2012) using this technique has isolated various members of *Enterobacteriaceae* from cultured freshwater fishes. The highly conserved function and ubiquitous distribution of the gene encoding elongation factor Tu (EF-Tu) may render this gene a valuable phylogenetic marker for eubacteria (Boissinot et al., 2000). The *tuf* gene product brings aminoacylated tRNA molecules to the ribosome. This gene represents an ideal target candidate for diagnostic purposes because it is highly conserved and ubiquitous in bacteria (Ludwig et al., 1990 and 1993). Many researchers demonstrated that the use of *tuf* genes as an alternative or complement to the 16S rRNA marker mainly supports the phylogenetic relationships revealed by the 16S rRNA. It provides more detailed insight to distinguish closely related species that can be helpful for inferring their phylogeny (Ventura et al., 2003 and Paradis et al., 2005). *Enterobacteraceae* in fish is considered as an indicator to sewage pollution and has been reported as opportunistic pathogen in fish (Rajasekaran, 2008). These three isolated strains showed resistance to Penicillin-G, Ampicillin, Cephalothin, Streptomycin, Gentamycine, Kanamycine, Erythromycin, respectively and sensitive to Ciprofloxacin, Moxifloxacin and Tetracycline, respectively.

Presence of *Aeromonas veronii*, *Enterobacter asburiae, Enterobacter* sp and *Enterobacteriaceae bacterium* and its resistance to multiple antibiotics therefore indicates, that the fish is contaminated with multi-antibiotic resistant enteric pathogenic bacteria.

CHAPTER VI

6. SUMMARY AND CONCLUSIONS

The present thesis entitled "**Investigation of pathogenic bacteria of a resident fish, *Lepidocephalichthys guntea* (Hamilton Buchanan), in relation to limnochemistry of a Terai River Lotchka in the Darjeeling foothills of West Bengal, India"** which was therefore executed for a period of three years (2009 March-2012 February) and embodies a work to determine the abundance of opportunistic pathogenic bacteria with their antibiotic resistance ability in resident fish, *Lepidocephalichthys guntea* and Terai river Lotchka water in relation to limnochemistry parameters. The thesis is consisting of seven chapters *viz,* Introduction, Review of Literature, Materials and Methods, Results, Discussion, Summary and conclusion and Bibliography. The important findings of the thesis are as follows:

- Variation study of physic-chemical parameters and different bacterial counts between the four different sites (S1, S2, S3 and S4) of river water was performed by ANOVA (Analysis of Variation) and no significant variation was found between the four sites. Temperature of water at the four different sampling sites throughout the study period averaged between 26.5±4.6 and 26.9±4.6 °C and more found to be uniformly distribution at all four sites. Water temperature of the four sites of the river ranges from a maximum summer temperature of 30.3 ^{0}C to a minimum of winter temperature of 21^{0}C.The average pH of water ranged between 6.7 ± 0.2 and 6.9 ± 0.3 showing slightly acidic condition. Minimum pH 6.7 was recorded in summer at Site 1 and maximum 7.0 in winter at Site 3. The Dissolved Oxygen (DO) content at the four sites of river water showed similar trend, irrespective of the site of sample collection. Dissolved oxygen at the four different sites ranged between 3.6±1.2 and 4.1±1.5 mg l^{-1}. Maximum value of DO was observed in monsoon 5.3±1.3 mg l^{-1} at Site 1 and minimum in winter 3.0±1.0 mg l^{-1} at Site 3. The average Free Carbon dioxide in the water ranged between 15.0±5.8 and 16.7±6.1 mg l^{-1} and was present throughout the period of study as the pH remained acidic. The free carbon dioxide value was minimum in summer (11.6± 4.8 mg l^{-1}) at Site 4 and maximum in winter (19.0±6.0 mg l^{-1}) at Site 1. Total alkalinity in the form of bicarbonate alkalinity was only recorded and ranged between 19.8±7.6 and 21.4±7.4 mg l^{-1} indicating carbonate alkalinity was absent in the water body. Total alkalinity was maximum in monsoon

(22±6.0 mg l^{-1}) at Site 4 and minimum in winter (18.5± 9.1 mg l^{-1}) at Site 1. In the present study, chloride concentration in the water samples averaged between 20.0±4.4 and 23.1±5.3 mg l^{-1}. Chloride values was highest (24.7±5.2 mg l^{-1}) in winter at site 4 and lowest (19.1± 5.0 mg l^{-1}) in the summer at Site 1 of the river. The maximum concentration of total hardness was observed in the summer (73±4.4 mg l^{-1}) at Site 4 and minimum in winter (56±7.8 mg l^{-1}) at Site 1. Total hardness varied from 62.6±8.6 to 68.5±9.7 mg l^{-1}. Concentration of ammonium-N varied from 0.08±0.13 to 0.10±0.18 mg l^{-1}. A maximum value of ammonium-N (0.15± 0.3 mg l^{-1}) was found in winter at Site 2 and minimum (0.06±0.04 mg l^{-1}) in summer at S1. Nitrite-N (NO_2-N) averaged between 0.07±0.07 to 0.12±0.09 mg l^{-1}. Nitrite-N concentration was highest in monsoon (0.20±0.11 mg l^{-1}) at Site 4 and minimum (0.00±0.0 mg l^{-1}) in winter at Site 2. Nitrate-N (NO_3-N) concentration in the present study varied from 0.17±0.08 to 0.24±0.20 mg l^{-1}. The Nitrate -N concentration was distinctly higher in the monsoon (0.3±0.03 mg l^{-1}) at site 4 and minimum in the winter (0.14±0.2 mg l^{-1}) at site 1. Phosphate-P averaged between 0.23±0.11 and 0.33±0.24 mg l^{-1} in the four different sites. Phosphate-P concentration was maximum in summer (0.47±0.33 mg l^{-1}) at Site 3 and minimum (0.19±0.11 mg l^{-1}) at Site 2 in winter.

- The heterotrophic bacterial count in the four sites of river average ranged from 3.3±1.1 x 10^6 and 4.6±1.4 x 10^6 cfu ml l^{-1} throughout the study period. Heterotrophic bacterial population showed positive correlation with Dissolved oxygen content, Nitrite-N, Nitrate-N and Phosphate-P. The total coliform counts in water recorded ranged from 1.1±1.3 x 10^6 and 1.6±2.6 x 10^6 cfu ml^{-1}. Total Coliform Count was positively correlated with Temperature, Dissolved oxygen and Nitrite-N.
- All types of bacterial counts including major water quality parameters namely Dissolved oxygen, Total alkalinity, Nitrite-N, Nitrate-N and Phosphate-P showed higher trend in monsoon season followed by summer and winter. But, the luxuriant growth of all bacterial types in all the sites of the river was observed throughout the three seasons (summer, monsoon and winter). Nitrite-N, Nitrate-N and Phosphate-P showed higher values than permissible limit (WHO, 1992), thereby, indicating eutrophication of the river.

- The maximum counts of bacteria in the above category; heterotrophic bacteria 4.2 X 10^6 cfu g^{-1} and total coliform 2.3 x 10^6 cfu g^{-1} has been found to be present in the gut followed by gill and skin in *Lepidocephalichthys guntea*. The heterotrophic bacterial counts, total coliform counts and counts of total *Aeromonas* spp. and *Pseudomonas* spp. were found higher in monsoon followed by summer and winter. In addition to seasonal variation, during the entire study period counts of these bacterial groups were luxuriant. An interesting observation was that seasonal trend of bacterial loads were almost found to be parallel in fish tissues and in river water. Results also showed that bacterial counts in both river water and in the fish were relatively higher.
- All the 97 gram negative rod-shaped bacteria examined in this study were classifiable into four main genera. These were isolated from the water samples collected from different sites of the river. All isolates belonged to the genera of *Aeromonas*, *Pseudomonas*, *Salmonella* and *Enterobacteriacea*. A total of 30 *Aeromonas* spp., 22 *Pseudomonas* spp., 30 *Enterobacteriaceae* and 15 *Salmonella* spp. were isolated and initial identification was done by different biochemical procedures. From different tissues namely skin, gills and gut of fish, *Lepidocephalichthys guntea* a total of 119 bacteria were isolated. Among the total isolates 30 *Aeromonas* spp., 34 *Pseudomonas* spp., 40 *Enterobacteriaceae* and 15 *Salmonella* spp. respectively were isolated and identified by morphological and biochemical procedures.
- All isolates from river water and fish tissues samples in this investigation were identified by Polymerase Chain Reaction (PCR) based techniques using genus specific 16s rDNA and 'tuf' primers for strengthening the reliability of identification. This is because, positive amplification products for specific primers signal only for the appropriate target nucleic acid sequences present in the sample. A total of 97 bacteria were isolated from four different sites of the river and 72 of them were successfully identified. A total of 119 bacteria were isolated from different tissues like skin, gills and gut of fish. Among the total isolates, 103 isolates were successfully identified. All the isolates belonged to *Aeromonas* spp., *Pseudomonas* spp., *Salmonella* spp. and *Enterobacteriaceae*.
- Among the total isolates of *Aeromonas* spp., *Pseudomonas* spp., *Enterobacteriaceae* and *Salmonella* spp. in four different sites of river there was 75 to 100% resistant to Penicillin

and Cephalothin and Ampicillin respectively. 25 to 70% isolates were resistant against Erythromycin and Kanamycine respectively. But all isolates showed almost 100% sensitive to Streptomycin, Ciprofloxacin, Moxifloxacin, Tetracycline and Gentamycin respectively.

- Bacterial isolates belonging to *Aeromonas* spp., *Pseudomonas* spp., *Enterobacteriaceae* spp. and *Salmonella* spp. of fish tissues namely skin, gills and gut also showed higher range of resistance (57 to 100%) against Ampicillin, Penicillin-G, and Cephalothin and belong to the β- lactam class. These isolates showed lower range of resistance (0 to 84%) against erythromycin, kanamycin, streptomycin, ciprofloxacin, gentamicin, tetracycline respectively. The percentage patterns of resistant bacterial isolates were similar in the fish and river water.
- All isolates among the four strains belong to *Aeromonas* spp. and *Enterobacteriaceae* spp. from the gut of fish, *Lepidocephalichthys guntea,* based on their antibiotic resistance ability were identified up to the species level. For the identification purpose genus specific 16s rDNA and tuf gene based sequencing was performed. The strain (A30S2) was amplified by 16s rDNA *Aeromonas* specific primers and 953 bp positive amplicon product was sequenced. The sequences were compared and aligned with sequence deposited in the NCBI-Gen Bank database. After the comparison with other sequence data base maximum similarities of the strain (A30S2) was found with *Aeromonas veronii*. *Aeromonas veronii* had shown resistance to eight antibiotics namely Penicillin-G, Ampicillin, Cephalothin, Streptomycin, Gentamycine, Kanamycine, Ciprofloxacin and Erythromycin and sensitive to Moxifloxacin and Tetracycline.
- Strains ET01, ET02 and ET08 belong to *Enterobacteriaceae* family isolated from the gut of the fish, *Lepidocephalichthys guntea,* and amplified with *Enterobacteriaceae* specific tuf gene based primers. 884 bp 'tuf' gene PCR products were sequenced and aligned with sequence deposited in the NCBI-Gen Bank database. The comparison with other deposited sequences showed maximum similarity with *Enterobacter asburiae, Enterobacter* sp and *Enterobacteriaceae bacterium*. These three isolated strain showed resistance to Penicillin-G, Ampicillin, Cephalothin, Streptomycin, Gentamycine,

Kanamycine, Erythromycin respectively and sensitive to Ciprofloxacin, Moxifloxacin and Tetracycline.

CONCLUSIONS

It may be concluded from the study that deteriorating water quality of river Lotchka provide a favourable condition for heavy growth of different types of opportunistic pathogenic bacteria including coliform group and potential enteric pathogens like *Enterobacteriaceae* spp., *Salmonella* spp., *Aeromonas* spp. and *Pseudomonas* spp. Another important finding is that isolation of antibiotic resistant bacteria. These resistant bacteria mainly belong to the β- lactamase producing bacterial group because, highly resistant antibiotics are penicillin, ampicillin and cephalothin. All of these antibiotics are particularly from β- lactam class. Deteriorating water quality of river water and highly presence of opportunistic pathogens *Enterobacteriaceae* spp., *Salmonella* spp., *Aeromonas* spp. and *Pseudomonas* spp. with antibiotic resistant property mainly for antibiotics of β- lactam class in river water clearly suggests that river Lotchka received huge amount of runoffs which may be come from surrounding localities, domestic sewage and agricultural fields. Another interesting point of this investigation, was that, different tissues of *Lepidocephalichthys guntea* were composed of opportunistic pathogens namely *Enterobacteriaceae* spp., *Salmonella* spp., *Aeromonas* spp. and *Pseudomonas* spp. respectively. These bacteria also have highly antibiotic resistant ability to antibiotics of β-lactam group. Resistance patterns of fish tissue isolates are almost similar to the isolates of river water. Crucial part of this study is that occurrence of multi-antibiotic resistant *Aeromonas veronii*, *Enterobacter asburiae*, *Enterobacter* sp and *Enterobacteriaceae bacterium* strain in the gut of fish, *Lepidocephalichthys guntea*, thereby indicating the fish is also contaminated with multi-antibiotic resistant enteric pathogenic bacteria. This finding again supported that river Lotchka may be highly contaminated with antibiotic resistant bacteria due to the human activity and anthropogenic pressure. Because, presence of bacterial population in fish is the reflection of their surrounding environment.

Finally, it could be said that the present investigation revealed that the river Lotchka was probably in eutrophic condition due to loss of essential nutrients and abundance of antibiotic resistant entero-pathogenic bacteria in water which had ultimately influenced greater harbouring of antibiotic resistant entero-pathogenic bacteria in resident fish, *Lepidocephalichthys guntea.*

CHAPTER VII

7. ANNEXURE

I. **SYSTEMATIC POSITION OF *LEPIDOCEPHALICTHYS GUNTEA***

Phylum: Chordata

Class: Actinopterygii (Ray-finned fishes)

Order: Cypriniformes (Carps)

Family: Cobitidae (Loaches)

Genus: *Lepidocephalichthys*

Species: *Lepidocephalichthys guntea*

II. **Common names:** Poa, Puiya, Gutum, Gutia, Gorkun (West Bengal, India and Bangladesh); Guntea loach (Fishbase).

III. **Distribution:** Northern India; Bangladesh; Nepal; Pakistan; Myanmar and Thailand (Talwar and Jhingran, 1991).

IV. **Maximum length:** Maximum length reported 15 cm (Shafi and Quddus, 1982 and Talwar and Jhingran, 1991).

V. **Habitat:** Found in freshwater bodies (Galib *et al.*, 2009).

Photograph of *Lepidocephalichthys guntea*

CHAPTER VIII

8. BIBLIOGRAPHY

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CHAPTER IX

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PUBLICATIONS

- Rudra Prasad Roy and Sudip Barat (2011): Influence of water quality on the bacterial contamination of resident loach, *Lepidocephalichthys guntea* (Hamilton Buchanan) and on a Terai River Lotchka of Darjeeling District, West Bengal, India. *Arch. Environ. Sci.* 5: 116-123.

- Rudra Prasad Roy, Min Bahadur and Sudip Barat (2013): Isolation, identification and antibioticresistance of *Aeromonas* spp. and *Salmonella* spp. from fresh water loach, *Lepidocephalichthys guntea* (Hamilton Buchanan) and water of Terai river Lotchka, Darjeeling District, West Bengal, India *Journal of Zoologicae Poloniae*. 58:1-13. DOI: 10.2478/zoop-2013-0001.

- Rudra Prasad Roy, Min Bahadur and Sudip Barat (2013): Isolation and Identification of Multi-antibiotic Resistant Aeromonas veronii by 16s rDNA Gene Sequencing from the Gut of Fresh Water Loach, *Lepidocephalycthys guntea* (Hamilton Buchanan). *International Journal of Bio-resource and Stress Management.* 4(4):496-499.

- Rudra Prasad Roy, Min Bahadur and Sudip Barat (2014): Studies on antibiotic resistant activity of *Pseudomonas* sp., isolated from fresh water loach, *Lepidocephalichthys guntea* and water sample of river Lotchka, Darjeeling, India *Journal of Environmental Biology*. 35(1):237-241.

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