Chapter-1

Isolation of oligotrophic bacteria from river Mahananda and determination of antibiotic susceptibility/Resistance pattern of the isolates

1.1. Background

The ability of oligotrophic bacteria to develop at low concentrations of organic substances and the absence of growth of many of them at high concentrations may be ascribed to a number of reasons. The most important peculiarity of oligotrophic bacteria is their ability to rapidly reproduce at the minimal concentrations of organic matter in the medium, which ensures their advantage in competition for the substrate under natural conditions (Rosjak and Cowell, 1987). The reasons behind growth inhibition of the oligotrophic bacteria on the rich nutrient substrates are not clear at present. It may be due to the action of toxic metabolites, in particular hydrogen peroxide, which forms in a number of metabolic reactions. Earlier authors have shown that the oligotrophic bacteria *Leptothrix pseudoochraceae, Siderocapsa eusphaera,* and *Metallogenium personaturn,* upon cultivation on rich nutrient media begin to lyse due to accumulation of sufficient amounts of hydrogen peroxide in the medium (Kuzenetov *et al.*, 1979). It is known that the catalase activity in microorganisms may sharply decrease in the presence of significant concentrations of glucose or on beef extract media (Kuzenetov *et al.*, 1979). Inhibition of growth in some oligotrophs may also occur in the presence of insignificant quantities of amino acids (Koch, 2001).

Oligotrophic bacteria have been shown to be abundant in a wide variety of habitats. They have been isolated from soil (Hattori, 1984), rivers (Yanagita *et al.*, 1978; Kumar *et al.*, 2010; Kumar *et al.*, 2011; Kumar *et al.*, 2012), lakes (Bahr *et al.*, 1996), oceans (Connon and Giovannoni, 2002; Kuzenetov *et al.*, 1979; Martin and Macleod, 1984, and Yanagita *et al.*, 1978) tap water (Ajitkumar *et al.*, 2003; Baida *et al.*, 2001) and ultra pure water lacking organic substances (Audic *et al.*, 2007). Some oligotrophic isolates can even grow in distilled water (Alexander *et al.*, 2005). Oligotrophic bacteria mostly have been isolated on several fold diluted traditional media or on agar plates without supplementing any extra organic nutrients. However, growth on nutrient rich medium do not exclude the oligotrophic organism since facultative oligotrophs have the capability to grow on nutrient-rich as well as on nutrient-poor media. To differentiate colonies of oligotrophs from copiotrophs, replica plating on nutrient-rich and nutrient-poor plates is preferred. The facultative oligotrophic bacterial colonies will appear on the imprints of both nutrient-poor and nutrient-rich agar medium. The colonies of obligate oligotrophs on nutrient-poor master plates will show growth only on nutrient-poor replica plates. The bacterial colonies on nutrient-rich master plates which would show growth only on nutrient-rich replica plates will be differentiated as copiotroph. The oligotrophic bacteria have received little attention and less is known about them. The diversity and biomass of the oligotrophic bacteria are dominant in biosphere, and thus, play an important role in biogeochemical cycles. The applied perspectives of oligotrophic bacteria in environmental science are gaining importance day by day. A previous study reported the role of oligotrophic bacteria in detection of heavy metals from river water samples by simple turbidimetric method (Tada *et al.*, 2001).

There is scarcity of information on antibiotic resistance in oligotrophic bacteria (Nikitin *et al.*, 1988;, Zlatkin *et al.*, 1991; Oh *et al.*, 2009; Kumar *et al.*, 2011; Bhullar *et al.*, 2012). An antibiotic is a compound or substance produced by micro-organisms that either kill or inhibit the growth of other micro-organisms i.e. by microorganism for microorganism (Levy, 1992). The term "antibiotic" is used to refer to a drug that cures infections caused by bacteria, whereas an antimicrobial agent is a

general term that refers to a group of drugs that includes antibiotics, antifungals, antiprotozoals, and antivirals. Antimicrobial agents may be naturally-occurring, semi-synthetic or synthetic compounds with antimicrobial activity that can be administered orally, parenterally or topically. They are widely used as medicine to treat and prevent infectious disease, and also for growth promotion in food animals (Phillip *et al.*, 2004). The first antibiotic, penicillin, was discovered by Noble laureate, Scottish bacteriologist, Alexander Fleming in 1928 when he observed that a common mold (*Penicillium notatum*) produced a substance that destroyed *Staphylococcus* bacteria in culture (Ligon, 2004).

In 1939 René Dubos, an American microbiologist isolated antibiotic tyrothricin from a soil bacterium, *Bacillus brevis* that was highly toxic to a broad range of bacteria as well as to red blood and reproductive cells in humans. This said antibiotic was more effective when applied topically. The first major development came up when penicillin introduced as ampicillin, this offered a broad spectrum activity than the original penicillins. Later on several new antibiotics with novel properties were discovered, including streptomycin, chloramphenicol, and tetracycline. Within the 18 years of the antibiotic era, approximate 30 antimicrobial agents come up into use (Swartz, 2000). In the following decades the discovery of manufactured antibiotics to control diseases revolutionized the medicine world. It has also greatly reduced the threat of many lethal diseases. The use of these marvelous drugs led to a dramatic drop in deaths from diseases that were previously widespread, untreatable and frequently fatal. Along with control of many infectious diseases, these drugs have also contributed to the major gains in life expectancy experienced during the latter part of the last century. These achievements are now seriously jeopardized by another recent development: the emergence and spread of resistant bacteria. Antibiotic resistance has been called one of the world's most pressing public health problems as it creates complications due to the propensity to distribute multiple antimicrobial resistance genes to susceptible bacterial genera and species. Rivers are important sources of drinking water for human and animals, irrigation, fishery and energy production. The quality of water is described by its physical, chemical and microbiological characteristics (Rajeshwari and Saraswathi, 2009). It is well known that rivers are used as the dumping grounds for the sewage of urban effluents, agricultural wastes, and industrial wastes that contain substances varying from simple nutrients to highly toxic chemicals (including heavy metals). The obvious consequence therefore would be that the river water received different types of chemicals, organic and inorganic compounds as it flowed through human settlements. These dissolved compounds changes the river water quality by inducing quantitative variation in certain minerals. In addition to heavy metals, the contamination of antimicrobial agents in river water bodies has become a major threat to public health. The presence of antibiotic residues and the occurrence of bacteria resistant to them in environment are swiftly changing the nature of commensal and nonclinical bacterial flora. Moreover, investigations on antimicrobial resistance of river microflora have led to a new dimension in water pollution studies. Several studies have demonstrated the wide spread occurrence of antibiotic resistant bacteria in many rivers and streams (Ash *et al.*, 2002). Antibiotic resistance is a property of bacteria being able to survive exposure to antibiotic to which they were once sensitive. Antibiotic resistance is as ancient as antibiotics, shielding antibiotic-producing organisms from their own products, and other originally susceptible organisms from their competitive assault in nature. Resistance may occur as a spontaneous, genetic mutation or due to acquisition of genetic elements like plasmids, transposons, integrons, or gene cassettes (Muto *et al.*, 2003) (Fig. 1.1). However, there is great variation in the development of resistance, some may develop rapid resistance in the individual treated, and others may remain susceptible to the exposed drug/antibiotic. Thus antibiotic resistance can be defined as a microbiological phenomenon, which may or may not have health/clinical implications depending on pharmacokinetic and pharmaco-dynamic parameters as they apply to specific antibiotics. Nevertheless, even low-level resistance is noteworthy since it may be a first sign towards bacterial

resistance (Phillip *et al.*, 2004). Since all bacteria do not possess/ or share same biochemical and physiological pathways hence, all antibiotics are not active against all bacteria and they are intrinsically resistant (resistance without chromosomal mutation or acquisition of plasmid bearing resistance factor) to one or more antibiotics. Inherent features of the bacterial cell prevent antimicrobial action, and these properties are typically species characteristics for example; Gram positive *Mycobacteria* produce an unusual bilayer outside the peptidoglycan layer that function as an efficient barrier (Nikaido, 1994) while the acquired resistance emerges through mutation of existing DNA or acquisition of new DNA by horizontal gene transfer (Thomas and Nielsen, 2005).

The present era of bacterial resistance recognized by multiple antibiotic resistant (MAR) bacterial (bacteria resistant to two or more antibiotics) species. The incidence of MAR bacteria in the environment is undeniably a well-known phenomenon (Cook, 1975; Sizemore and Colwell, 1977; Gonzal *et al.*, 1979). Report of UK House of Lords stated that "Resistance to antibiotics and other anti-infective agents constitutes a major threat to public health and ought to be recognized as such more widely than it is at present" (Kummerer, 2004). The percent occurrence of MAR bacteria in different environmental compartments for example wastewater, surface water, ground water, sediments and soils, has been a growing concern. The antibiotic contamination is the major issue for both medical and environmental components. Antibiotic resistant bacteria were detected in drinking water as early as the 1980s (Armstrong *et al.*, 1981) and later in the 1990s (Kolwzan *et al.*, 1991). They found that the percentage MAR bacteria considerably higher among isolates from treated water samples than that of bacteria in corresponding untreated source waters (Armstrong *et al.*, 1981). MAR gram-positive cocci (Staphylococcus) and MAR gram-negative, nonfermentative rods (*Pseudomonas*, *Alcaligenes*, Moraxella-like group M, and *Acinetobacter*) were dominant in drinking waters. Diab *et al.* (2000) reported the presence of antibiotic resistant gram-negative bacteria in several drinking water samples in Islamia city of Pakistan. In addition to these increased rates of bacterial resistance were also noted in the drinking water from different sampling points by Schwartz *et al.* (2003).

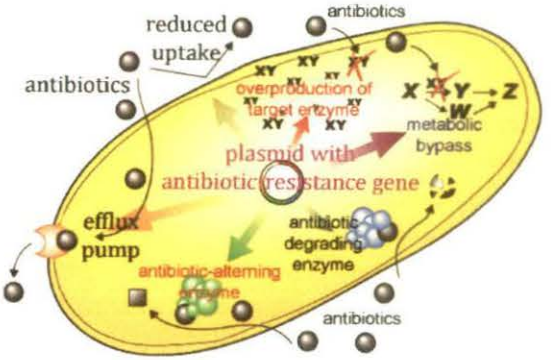

Fig. 1.1: Various mechanism of antibiotic resistance in bacteria. [Fig. adapted from Wicox S. CATIONIC PEPTIDES: A NEW HOPE. The Science Creative *Quarterly.* 2004]

Antibiotics are rarely found in ground water (Kummerer, 2004). However few workers reported antibiotic resistance in bacteria isolated from ground water (McKeon *et al.*, 1995). In India microbial contamination of ground water sources, revealed the presence of coliforms above the acceptable limits, but no attempts have been made to assess the antibiotic resistance profile of the isolates (Dayal, 1992; Sharma and Mathur, 1994; Mitra and Gupta, 1997). Presence of antibiotic resistant bacteria in the aquatic environment has been studied worldwide. The antibiotic resistances in gram-negative bacteria isolated from four tributaries which enter to Tillamook Bay, Oregon and the bay itself have been studied by Kelch and Lee (1978). Distribution of antimicrobial resistance and the pattern of resistance among fecal coliforms in sewage, surface waters and sea water were investigated by Niemi *et al.* (1983). Several other workers demonstrated the wide spread occurrence of such organisms in many rivers and streams (Jones 1986, Sokari *et al.*,. 1988, Magee and Quinn, 1991; Leff *et al.*, 1993; Ogan and Nwiika, 1993). The result of Polluted water samples collected from the River Tigris revealed a high incidence of antibiotic resistant bacteria in natural waters that could be related to the widespread use of antibiotics in that locality (Al-Jebouri, 1985).

Boon and Cattanach (1999) studied the antibiotic resistance of native and faecal bacteria isolated from rivers, reservoirs and sewage treatment facilities in Victoria, southeastern Australia. The occurrence of several representatives from the main group of antibiotics in wastewater treatment plant effluents and in river water was investigated by Hirsch *et al.* (1999). The study conducted by McArthur and Tuckfield (2000) have demonstrated the spatial distribution of antibiotic resistance in natural bacterial communities of two streams. The proportion of resistant bacteria was substantially higher in the mid reaches of an industrially perturbed stream but no such pattern was apparent in an undisturbed reference stream. The results of the said study implied that heavy metal pollution might contribute to increased antibiotic resistance through indirect selection.

According to a report (1999-2000) of the toxic substances hydrology program at the U. S. Geological Survey (USGS), antibiotics were found to be present in many fresh water sources throughout the United States. Four or five different antibiotic residues, out of 22 antibiotics assayed, were present in 139 water samples collected from different streams and rivers. The frequently detected antibiotics were erythromycin-H_2O (22%), lincomycin (19%), trimethoprim (27%) and sulfamethoxazole (19%). The other nine antibiotics detected were: tetracycline, chlortetracycline, oxytetracycline, ciprofloxacin, norfloxacin, roxithromycin, sulfadimethoxine, sulfamethazine and sulfamethizole (Kolpin *et al.,* 2002). It was found that antibiotics used in livestock production have made their way, via animal waste products, into the nation's waterways. Studies performed on 16 United State rivers revealed that rivers turning into the reservoirs of antibiotic resistance genes (Ash *et al.,* 2002). Antimicrobial resistance has also been reported in marine and estuarine bacteria (Cohen *et al.,* 1986; Barkay *et al.,* 1995). Microbiological analyses of coastal waters polluted with sewage showed the presence of gentamicin resistance genes in the members of *Enterobacteriaceae, Acinetobacter* spp., *Pseudomonas* spp. as well as in phylogenetically distant bacterial members of alpha and beta proteobacteria (Heuer *et al.,* 2002). The occurrence of strains that are resistant to oxolinic acid, oxytetracycline, sulfamethoxazole-trimethoprim and nitrofurantoin among heterotrophic bacteria, including human and fish pathogens, in two fresh water eel farms has been reported (Alcaide *et al.,* 2005). A study on an Indian River Mahananda has also revealed the abundance of MAR bacteria (Mukherjee *et al.,* 2005, Kumar *et al.,* 2010). Results of previous studies have shown that river waters are the main receptacle for antibiotic resistant bacteria, since they receive the sewage of urban effluents and the rivers all over the world have started becoming the reservoirs of antibiotic resistance genes to serve as media for the spread of antibiotic resistance genes (Biyela *et al.,* 2004; Kummerer, 2004; Mukherjee *et al.,* 2005).

River Mahananda is a trans-boundary river that flows through Indian states of West Bengal and Bihar; and Bangladesh. This originates from Himalayas, Mahaldiram Hill near Chimli an eastern part of Kurseong in district Darjeeling of West Bengal at an elevation of 2,100 meters. 11,530 sq. km out of 20,600 sq. Km (total drainage area of the Mahananda river) lies in India (Jain *et al.,* 2011; www.siligurionline.com/info/rivers). Siliguri and Malda are the two important towns situated alongside the Mahananda River. River Mahananda is the prinicipal river flowing through Siliguri. Siliguri, the fastest growing city of West Bengal, with a population of over 5, 00,000, is situated in the foothills of Himalayas on the banks of the River Mahananda. The city with forty seven municipal wards is a serious demographic spot where magnitude of water supply and sanitation problem has reached a critical state in the background of depleting ground water resources and environmental degradation. A huge quantity of community waste water and other kinds of wastes including animal wastes from disorganized cattle farms situated by the side of the river and hospital wastes pass through the estimated 250 km long open drains and ultimately discharged into the main Mahananda River (Fig. 1.2). The unregulated use of antibiotics including therapeutic and prophylactic prescribing in Siliguri was studied indirectly from a random survey conducted on retail medicine sellers at their counters (Mukherjee *et al.,* 2005). The results revealed the presence of fairly high

1.2.3. Study design

Sequence of the microbiological analysis of the river-water samples has been shown in Fig 1.4

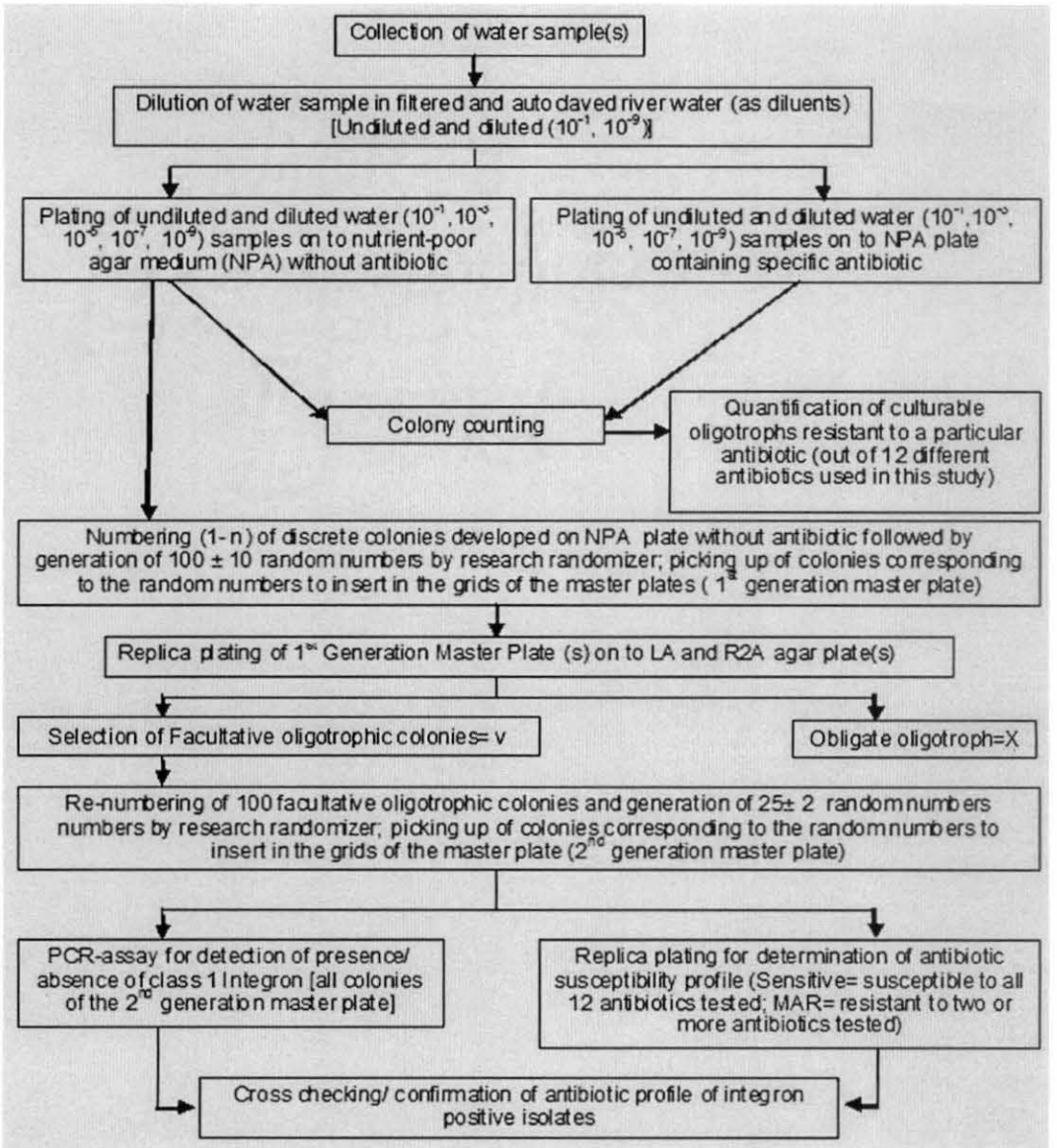

Fig 1.4: Flow diagram describing the sequence of the microbiological analysis of the river-water samples

1.2.4. Sample collection

Samples were obtained either by filling bottles from the river's edge, by wading, or by lowering a sterile glass bottles (lid opened at the time of sampling) from the bridge, depending on the conditions. In case of collecting water samples from the mid-point of the river at all locations, care was taken to obtain the samples following standard technique. From each sampling site, three grab samples were collected from left, right and middle of the river. Sterilized glass bottles (capacity, 500 ml) were used. The bottles were opened under water, rinsed thoroughly with the sample water even it was pre-cleaned and were half filled by opening and closing the bottles underneath flowing water. The samples were transported to the laboratory in icebox and analyses were done within 24 hours.

1.2.5. Preparation of sample and dilution series

Three samples, collected from middle and both banks of a particular sampling site, were mixed in equal proportion under aseptic condition to constitute a composite sample. Each composite water sample was distributed into two sterile containers; one of which was used for microbiological analysis and the other was filtered through 0.2 µm membrane filter. 9.0 mL aliquots of the filtered

composite water in culture tubes were autoclaved at 121 psi for 15 minutes. Sterilized 9 mL blanks were serially diluted (10^{-1} to 10^{-9}) where 1.0 mL of the crude composite water was transferred aseptically to first tube (designated as 10^{-1} dilution) containing 9 mL of filtered-autoclaved river water.

1.2.6. Preliminary isolation of antibiotic sensitive facultative oligotrophic bacterial strains from Mahananda river water and standardization of the protocols for determining antibiotic susceptibility/ resistance patterns of oligotrophic bacteria

Prior to the detailed analyses with water samples collected on monthly basis, a pilot experiment was run to isolate oligotrophic bacteria from a test water sample collected from river Mahananda. The diluted water samples were spread-plated on nutrient-poor agar (NPA) medium [Nutrient-poor broth (NPB) composition (g/L): peptone, 0.01; yeast extract, 0.005; sodium chloride, 0.005 amended with 1.5% agar; pH, 7.0 designated as NPA].

Discrete colonies on NPA plates were numbered serially (1, 2, 3 . . . n). Random numbers comprising a set of n/4 unique numbers were generated using Research Randomizer software (www.randomizer.org). Colonies corresponding to the numbers from the set of unique numbers were picked up with sterilized toothpicks and imprinted on the master plate made of R2A agar (a standard medium, has been used by several workers to isolate oligotrophic bacteria from the environmental samples) [R2A agar composition (g/L): peptone, 0.5; yeast extract, 0.5; casamino acids, 0.5; glucose, 0.5; soluble starch, 0.5; sodium pyruvate, 0.3; K_2HPO_4, 0.3; $MgSO_4.7H_2O$, 0.05; agar 15; pH, 7.2 pH was adjusted with crystalline K_2HPO_4 or KH_2PO_4] . Each master plate was replicated separately on nutrient-rich agar (NRA) [NRA composition (g/L): peptone, 10; yeast extract, 5; sodium chloride, 5 amended with 15 agar; pH, 7.0], NPA and R2A agar. After incubation of these plates for 72 h at 30 °C, colonies that had grown on NPA and R2A agar but not on NRA were termed as "obligate oligotrophs," whereas colonies that were able to grow on all three different plates were termed as "facultative oligotrophs."

Selection of antibiotic-sensitive oligotrophic isolates and determination of LD50 of each of 12 antibiotics used in this study

The master plates constructed with oligotrophic strains were replicated on antibiotic plates. For each antibiotic, three plates were used. The concentration of antibiotics in the plates was 10, 25, and 50 mg/L, respectively. The plates were incubated for 96 h at 30 °C. Colonies that failed to appear in all 12 different antibiotic plates of 25 mg/L concentration were tentatively chosen as sensitive isolates (n=5). For determination of LD_{50} (LD_{50} of each antibiotic is the dose required to kill half the members of the tested bacterial population), a duplicate set of 16 culture tubes each containing sterile 5 ml R2A broth was amended with 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 mg/L of antibiotic, respectively. Each tube was inoculated with pre-grown sensitive bacterial culture to an initial cell density of 10^6 CFU/mL. After 72 hr of incubation at 30 °C, optical density was recorded at 540 nm. The particular tube (with defined antibiotic concentration) wherein OD_{540} was recorded to be half the OD value of the tube without the antibiotic was taken as LD_{50}.

Antibiotic Resistance determination

Obligate oligotrophic isolates were tested for susceptibility (abbreviation: S=sensitive, R=resistant) to a panel of 12 antibiotics, representing 7 different classes. The concentrations of the antibiotics were chosen as five times the calculated LD_{50} values. Antibiotics and their concentrations employed in this investigation were as follows: aminoglycosides (azithromycin, <S/R≥ 5 mg/L; kanamycin, <S/R≥ 5 mg/L; netilmicin, <S/R≥ 3.75 mg/L; and streptomycin, <S/R≥ 2.5 mg/L); antifolates (trimethoprim, <S/R≥ 5 mg/L; co-trimoxazole, <S/R≥ 15 mg/L); cephalosporins (cefepime, <S/R≥ 7.5 mg/L; cefotaxime, <S/R≥ 7.5 mg/L); penicillin (ampicillin, <S/R≥ 25 mg/L); quinolones (ciprofloxacin, <S/R≥ 5.0 mg/L; livofloxacin, <S/R≥ 5.0 mg/L); others (chloramphenicol, <S/R≥

30mg/L; oxytetracycline, <S/R≥ 15mg/L). R2A agar was used as the basal medium. The desired concentrations of the antibiotics were stirred into the melted agar at approximately 45 °C and immediately poured into Petri plates to minimize the exposure of elevated temperatures.

On the other side, sensitivity and resistance of facultative oligotrophic isolates were also determined for above antibiotics following agar dilution method using Mueller Hinton (MH) agar as described in the European Committee on Antimicrobial Susceptibility Testing (EUCAST) definitive document, E. Def 3.1 (2000) [http://www.escmid.org/fileadmin/src/media/PDFs]. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 were taken as the quality control strains. All antibiotics were purchased from HiMedia (Mumbai, India). Antibiotic stock solutions were prepared by dissolving measured amounts of respective antibiotics to its suitable diluents. These concentrated stock solutions were made at least once a month and were stored at
-20 °C.

1.2.7. Detailed microbiological analysis of the water samples collected from three sampling site for three consecutive years

1.2.7.1. Routine Enumeration of oligotrophic bacteria on nutrient-poor agar (NPA) medium and determination of fractions resistant to different antibiotics

0.1 mL of water sample [undiluted and diluted water (10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}, and 10^{-9})] was spread uniformly on NPA and plates were incubated at 30°C for 72 h. Total oligotrophic bacterial population within water sample, was quantified by counting bacterial colony forming units (CFUs) appeared on NPA medium.

Side-by-side an aliquot of 0.1 mL of bacterial suspensions [undiluted and diluted (10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}, and 10^{-9})] of the same water sample were spread uniformly on NPA containing specific concentration of each antibiotic [aminoglycosides (azithromycin, 5 mg/L; kanamycin, 5 mg/L; netilmicin, 3.75 mg/L; and streptomycin, 2.5 mg/L); antifolates (trimethoprim, 5 mg/L; co-trimoxazole, 15 mg/L); cephalosporins (cefepime, 7.5 mg/L; cefotaxime, 7.5 mg/L); penicillin (ampicillin, 25 mg/L); quinolones (ciprofloxacin, 5.0 mg/L; livofloxacin, 5.0 mg/L); others (chloramphenicol, 30 mg/L; oxytetracycline, 15 mg/L)] to quantify total resistant oligotrophic population within water sample. The plates were incubated at 30 °C for 72 h. The bacterial colonies, appeared on NPA containing specific antibiotic, were counted. The fractions of resistant oligotrophic bacterial population were quantified against bacteria developed on NPA without antibiotic.

1.2.7.2. Screening of facultative oligotrophs from the population of oligotrophic bacteria (obligate + facultative oligotrophic bacteria)

Discrete colonies evident on NPA plates (plated with 0.1 mL water samples of different dilutions) were numbered serially (1, 2, 3...n) at the backside of the petri-plates corresponding to the colonies that manifested on the surface of solid medium. Approximately, 100 ±10 random numbers (out of > 300 discrete colonies) were generated using research randomizer tool (www.researchrandomizer.com). Colonies corresponding to the random numbers were then picked up with the help of sterile tooth pick and transferred to respective grids of the first generation master plate composed of R2A agar. The first generation master plates were then replicated separately on NRA, NPA and R2A agar plates and incubated for 72 h at 30 °C. Isolates showing growth on NRA, NPA and R2A agar plates were considered as facultative oligotrophs while colonies showing no growth on NRA but significant growth on NPA and R2A agar plates were considered as obligate oligotrophs. The obligate oligotrophs were not included in this study due to its slow growth and ambiguity in forming colonies. Facultatitve oligotrophic bacterial colonies identified from the master plates were further renumbered and randomized to obtain 25±1 random numbers. The colonies corresponding to each of the 25±1 random numbers were dilution streaked onto R2A agar

plate to get the pure cultures of the isolates. The second generation master plate was prepared on R2A agar with pure cultures of facultatively oligotrophic isolates. All the isolates of second generation master plates were subjected to CS-PCR assay for detection of class 1 integron (will be discussed in next chapter).

1.2.7.3. Determination of antibiotic susceptibility/ resistance of facultatively oligotrophic bacteria

Replica plating method was employed for determining the antibiotic resistance profile of individual isolates. The second generation master plate, prepared with pure cultures of facultatively oligotrophic isolates, was replica plated onto the R2A plate containing antibiotic of defined concentration of each antibiotic as mentioned above. Last impression of 2nd generation master plate was done onto R2A agar plate containing no antibiotic to confirm successful imprinting. All the replicated plates were incubated at 30 °C for 72 hours and drug resistance was determined.

The isolates were considered resistant to antibiotic, if their growth on antibiotic containing plate were as good as on the control plate (agar plate having no antibiotic). The isolates were considered MAR (multiple antibiotic resistant) if growth on at least two different antibiotic containing plates was equal to that on the control plate. The isolates which have failed to grow in all the twelve antibiotic plates but have shown growth only on the control plate were considered to be sensitive.

1.3. Results:

1.3.1. Data analyses of total oligotrophic bacteria enumerated on NPA and NPA amended with specific antibiotic used in this study

1.3.1.1. Enumeration of oligotrophic bacterial load in water samples collected from three sampling sites in three consecutive years (2007-2009) from river Mahananda and determination of oligotrophic bacterial fractions resistant to each antibiotic tested

Ninety water samples were collected from three sampling sites in three consecutive years (2007-2009) and studied on monthly basis. The density of oligotrophic bacteria in water samples collected every month (except July and August) from three sampling sites are shown in Table 1.1

Table 1.1: Oligotrophic bacterial density [CFU/mL ($\times 10^3$)] in water samples collected in different months (from Jan 2007-Dec 2009) from three sampling sites (SS).

Months	Sampling sites			Months	Sampling sites			Months	Sampling sites		
	SS I	SS II	SS III		SS I	SS II	SS III		SS I	SS II	SS III
2007 Jan	4	5.1	2.3	2008 Jan	6	8.8	6.7	2009 Jan	1.74	3.2	1.4
2007 Feb	1	7	4	2008 Feb	2.5	3.7	2.8	2009 Feb	5.6	11.84	1.9
2007 Mar	2	4	1.9	2008 Mar	1.86	9.4	1	2009 Mar	3.8	10.9	7.5
2007 Apr	5.6	16.4	3.06	2008 Apr	1.36	2.38	1.51	2009 Apr	2.4	9	7
2007 May	5	2.96	1.64	2008 May	12.4	17	8.4	2009 May	10	27.2	5
2007 Jun	3	10.6	2.2	2008 Jun	31.5	59	38.8	2009 Jun	3.7	9.6	2
2007 Sep	6	8.2	1.3	2008 Sep	9.2	27.6	11	2009 Sep	3.6	9.2	1.15
2007 Oct	1.4	5.3	11.2	2008 Oct	13.2	18.6	6.5	2009 Oct	5.2	8.8	8.4
2007 Nov	4.6	16.6	12.4	2008 Nov	5	14	2.2	2009 Nov	2.89	18.9	4.28
2007 Dec	7.36	8.86	1.22	2008 Dec	6.4	23.2	1.72	2009 Dec	1.76	22.4	6.1

Maximum (31.5×10^3 CFU/mL) and minimum (1×10^3 CFU/mL) occurrences of oligotrophic bacteria were recorded in the month of June 2008 and February 2007 respectively from SS I (Table 1.1). The maximum (59×10^3 CFU/mL) and minimum (2.38×10^3 CFU/mL) oligotrophic bacterial load at SS II

were recorded in June 2008 and April 2008 respectively (Table 1.1). For the SS III, the maximum (38.8×10^3 CFU/mL) occurrence of oligotrophic bacteria was recorded in the month of June 2008 and minimum (1×10^3 CFU/mL) in March 2008 (Table 1.1). The densities of culturable oligotrophic bacteria did not remain constant and have shown considerable variations in water samples collected throughout the successive sampling in three years from 2007-2009. However, high densities of oligotrophs were recorded in the month of June 2008 at each sampling site.

Determination of oligotrophic bacterial fractions resistant to each of the twelve antibiotics tested per sample per month per sampling site

The fraction(s) of oligotrophic bacteria resistant to each of the twelve antibiotics, in water samples collected from three sampling sites on monthly basis are shown in Table 1.2a, 1.2b 1.3a, 1.3b, 1.4a and 1.4b.

Table 1.2a: Fractions (%) of oligotrophic (facultative + obligate) bacteria resistant to each antibiotic per sample per month in three consecutive years (2007-2009) from SS I.

	Antibiotics[a]											
Months	**Amp**	**Azi**	**Cef**	**Cft**	**Chl**	**Cip**	**Cot**	**Kan**	**Lev**	**Net**	**Str**	**Tet**
2007 Jan	13.3	1.4	3.3	12.6	3.3	2.05	2.1	2.3	2.33	1.5	0.8	13.12
2007 Feb	3.6	0.21	0.5	1.3	8.6	1.15	3.43	2	6.25	6.2	2.25	9.31
2007 Mar	4.6	0.43	7.4	5.6	4.45	10.75	9.87	1	1.4	9.12	2.3	1.59
2007 Apr	12.8	2.5	0.17	3.1	19.1	1.44	32.6	0.12	1.47	4.3	1.7	23
2007 May	46	0	8.23	34	8.02	4	38	3.6	0.41	60	0	3
2007 Jun	53.33	0.52	13.33	17.33	31	0.14	9.33	1.5	1.35	3.2	0.66	2.42
2007 Sep	35.5	3.8	22.2	19.4	14	8.8	45.33	0.25	3.77	4.44	3.7	24.8
2007 Oct	80	1.4	8.57	4.28	78.57	3.04	6	1.88	24.28	7.85	34.28	2.2
2007 Nov	26.6	1.33	12	6	7.33	9.33	12	2.5	0	22.6	1.33	4.6
2007 Dec	3.47	0.33	0.67	1.42	2.45	4	3	1.75	0.06	0.57	0.51	12.28
2008 Jan	32.33	0	1.33	1.26	33	2.05	2.1	2.43	0.033	15.8	0.8	3.2
2008 Feb	16.65	0.21	0.25	11.53	8.46	11.15	3.3	0	1.23	6.92	6.65	9.1
2008 Mar	18.6	4.3	7.74	5.26	4.5	1.075	9.67	1	1.34	8.92	2.79	0.59
2008 Apr	28	0.25	1.7	3.35	19.1	0.44	32.6	5.12	14.7	48.3	2.147	2.35
2008 May	42.35	0.14	1.59	1.41	1.07	0.3	11.7	1.51	22	32.9	2.06	0.41
2008 Jun	73.33	5.2	1.33	7.3	43.1	24	93.3	7.15	15.3	23.05	6.6	37.2
2008 Sep	28.4	1.9	2.62	5.04	21.47	0.79	6.73	4	0.85	15.9	1.97	11.25
2008 Oct	66.66	0.37	20.6	34.84	2.06	2.65	8.33	1.66	0.22	5.45	30.3	23.1
2008 Nov	76	0.4	6.4	22	10.9	1.6	3	3.33	1.4	6	19	27
2008 Dec	15	20.45	9.5	21.87	0.57	7.84	1.71	1.81	3.6	26.25	12.9	4.37
2009 Jan	85	3.63	4	5.29	1.5	7.03	3.18	2.17	3.2	5.37	4.04	5.6
2009 Feb	29.4	2.21	6.5	7.43	6.9	3.04	5.53	1.25	8.6	9.1	6	2.3
2009 Mar	26.02	1.92	39.27	11.57	8.02	2.65	8.83	1.47	44.3	26.74	90.6	14.45
2009 Apr	22.83	6.87	12.33	10	0.84	5	12.33	4.37	15.83	27.66	8.66	3.41
2009 May	54.4	0.35	5.08	4.4	0.23	0.58	5.11	1.34	2.23	3.88	3.86	0.68
2009 Jun	37.51	3.35	10.91	11.67	5.4	0.48	8.86	4.1	8.34	34.05	29.72	4.1
2009 Sep	23.55	9.38	22.22	32.4	10.14	5.88	45.33	0.25	43.77	44.44	23.77	4.88
2009 Oct	14.12	17.77	20.63	17.46	7.7	15.55	10.31	3.78	33.96	26.34	15.87	25.39
2009 Nov	33.77	20.2	44.01	21.68	9.68	15.77	10.65	1.88	3.14	31.55	22.69	27.68
2009 Dec	47.27	8.65	23.87	12.27	16.32	6.13	22.04	0.98	4.82	30.9	27.5	22.27

[a]antibiotics: Amp, ampicillin; Azi, azithromycin; Cef, cefepime; Cft, cefotaxime; Chl, chloramphenicol; Cip, ciprofloxacin; Cot, cotrimoxazole; Kan, kanamycin; Lev, levofloxacin; Net, netilmicin; Str, streptomycin; Tet, oxytetracycline.

Table 1.2b: The average, maximum and minimum percentage(s) of oligotrophic (facultative+obligate) bacteria resistant to each antibiotic in water samples of SS I.

	Antibiotics[a]											
%	**Amp**	**Azi**	**Cef**	**Cft**	**Chl**	**Cip**	**Cot**	**Kan**	**Lev**	**Net**	**Str**	**Tet**
Average	35.01	3.98	10.60	11.76	12.92	5.29	15.54	2.21	9	18.31	12.18	10.85
Max	85	20.45	44.01	34.84	78.57	24	93.3	7.15	44.3	60	90.6	37.2
Min	3.47	0	0.17	1.26	0.23	0.14	1.71	0	0	0.57	0	0.41

[a]antibiotics: same described in Table 1.2a; Max, maximum; Min, minimum

The incidence of oligotrophic bacteria resistant to ampicillin in SS I was highest (85%) in January 2009, and the lowest occurrence (3.47%) of the said population was recorded in December 2007 (Table 1.2a). The average of all the values obtained from 30 samples (spread for three consecutive years) for the fraction of ampicillin-resistant oligotrophic bacteria in SSI was 35.01%; the maximum average value amongst all the resistant-fractions corresponding individually to twelve different antibiotics, while the least average value (2.22%) was obtained for kanamycin-resistant fraction followed by azithromycin-resistant fraction (3.98%), ciprofloxacin-resistant fraction (5.29%), and levofloxacin (9.0%). The average values corresponding to fractions resistant to rest of the antibiotics tested (cefepime, oxytetracycline, cefotaxime, streptomycin, chloramphenicol, cotrimoxazole, and netilmicin) fell in the range of 10.6 – 18.3% (Table 1.2b). The maximum occurance of azithromycin-resistant oligotrophic bacteria was documented in December 2008. Equal percentage (0.21%) of azithromycin resistant oligotrophic bacteria were observed in months of February 2007 and 2008 while no antibiotic-resistance were found in the samples collected in the months of May 2007 and January 2008. Similarly kanamycin-resistant, levofloxacin-resistant, and streptomycin- resistant oligotrophic bacteria were absent in the water sample collected in the month of February 2008, November 2007, May 2007 respectively. Data revealed that resistance to cefepime cefotaxime, and ciprofloxacin of the oligotrophic bacterial population was highest in the month of November 2009 (44%), October 2008 (38.84%), and June 2008 (24%) respectively. The lowest frequencies of resistance were documented in April 2007 (0.17%) for cefepime, January 2008 (1.26%) for cefotaxime and June 2007 (0.14%) for ciprofloxacin. The chloramphenicol-resistant bacteria occurred maximally (78.57%) in the water sample collected in the month of October 2007. Also in June 2008, the occurrence of chloramphenicol resistant population was recorded quite high (43%). Lowest occurrence (0.23%) for chloramphenicol resistant population was recorded in May 2009. Cotrimoxazole-resistant oligotrophic bacteria were found maximum in June 2008 and lowest in December 2008 (1.71%). At SS I, the percentage occurrence of cotrimoxazole-resistant oligotrophic bacteria was documented highest (93.3%) among all the antibiotics tested. The maximum occurrence (7.15%) of kanamycin resistance was observed in June 2008. The resistance towards levofloxacin and netilmicin was high (44.3% and 60%) in the month of March 2009 and May 2007 respectively. The lowest (0.57%) resistance of netilmicin was noticed in December 2007. The fraction of bacterial population exhibiting resistance to streptomycin was highest (90.6%) in March 2009. Maximum occurrences (37.2%) of oxytetracycline-resistant oligotrophic bacteria were recorded in June 2008 while minimum (0.41%) in the month of May 2008. Average percentages of oligotrophic isolates, resistant to each antibiotic at SSI in three consecutive years (2007-2009) are summarized in Fig. 1.5.

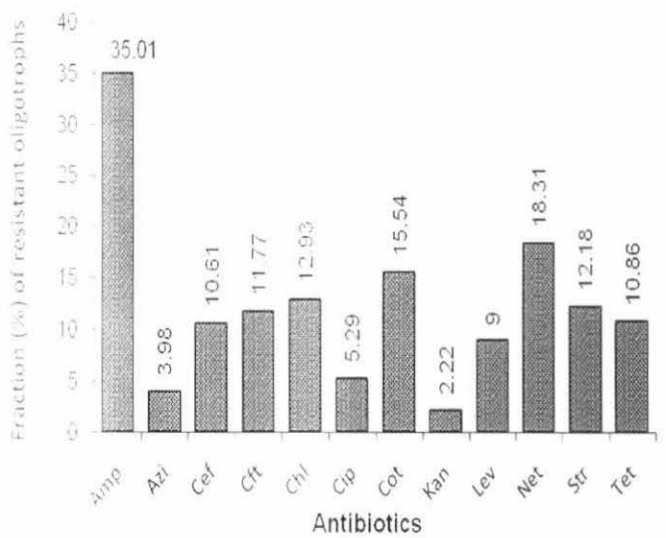

Fig. 1.5: Three years mean of fractions of oligotrophic bacteria resistant to each antibiotic in water samples of sampling site I.

The incidence of oligotrophic bacteria resistant to ampicillin in SS II was highest (70.45%) in February 2009, and the lowest occurrence (6.9%) of the said population was recorded in the same month of year 2007 (Table 1.3a).

Table 1.3a: Table 1.2a: Fractions (%) of oligotrophic (facultative + obligate) bacteria resistant to each antibiotic per sample per month in three consecutive years (2007-2009) from SS II.

	Antibiotics[a]											
Months	**Amp**	**Azi**	**Cef**	**Cft**	**Chl**	**Cip**	**Cot**	**Kan**	**Lev**	**Net**	**Str**	**Tet**
2007 Jan	9.5	1.7	2.32	10.2	9	9.93	2.17	3.34	7.61	12	3.7	3.1
2007 Feb	6.9	1.31	1.75	1.9	2.9	10.6	4.41	1.1	0.19	7	7.25	8.2
2007 Mar	54	3.7	27.2	17.66	3.1	2.02	15.37	3.7	7.9	7.47	4.95	33.88
2007 Apr	21	1.4	8.97	11.7	12	5.1	2.03	9.37	42.31	26.37	0.46	43.2
2007 May	15.4	0	24.47	36.97	43.75	16.66	52.6	6.2	11.97	38.54	0.52	78.12
2007 Jun	11.32	0.5	2.73	5.09	1.79	0.56	8.3	2.5	1.13	4.71	0.188	4.05
2007 Sep	53.2	11.2	0.45	0.77	27	0.762	8.77	5.4	8.5	3.7	7.07	1.73
2007 Oct	12.07	0.11	0.77	3.77	1.5	2.16	4.98	2.48	0.98	1.92	1.33	3.77
2007 Nov	17	5	21.6	2.17	36.45	28.38	32.25	4.5	6.9	29.6	13.46	50.32
2007 Dec	11.91	0.112	0.76	0.73	0.53	0.41	1.41	2.85	0.29	0.03	0.88	50.32
2008 Jan	51.95	1.75	0.22	42	90.9	1.93	22.57	1.43	7.1	1.27	2.27	23.17
2008 Feb	36.69	2.43	1.5	4.19	10.29	11.06	5.51	1	9.19	3.97	1.25	7.72
2008 Mar	18.4	1.8	11.06	17.6	11.1	0.57	1.32	2.1	6.21	1.01	0.79	1.29
2008 Apr	69.08	0.97	5.6	3.35	22.3	21.46	31.57	9.21	5.1	2.31	13.67	13.57
2008 May	31.02	0.44	6.2	4.23	6.8	4.6	35.51	3.15	0.25	6.24	3.26	0.76
2008 Jun	25	14	7.9	14.5	37.7	4.1	29.1	3.8	27.9	10.9	7.052	17.55
2008 Sep	9.3	1.25	3.5	20.77	41.22	7.6	18.57	7	0.45	0.47	0.77	1.69
2008 Oct	38.7	0.21	17.2	19.08	7.2	6.02	30.1	2.65	3.76	3.76	2.247	13.44
2008 Nov	31.42	1.21	7.85	4.4	3.1	2.14	22.42	4.36	1.92	8.57	3.57	16.42
2008 Dec	31.3	2.41	1.55	41.8	5.07	32	24.68	3.81	58	79.7	9.65	3.56
2009 Jan	65.46	2.41	2.72	4.6	0.05	37.7	3.6	2.27	2.65	3.75	5.07	3.6
2009 Feb	70.45	0.86	0.25	0.36	2.6	0.47	0.74	2.35	0.9	0.75	0.83	0.7
2009 Mar	54.31	13.57	27.52	37.6	3.11	13.02	51.37	3.47	71.19	47.7	45.5	31.83
2009 Apr	54.21	2.48	0.97	1.76	2.12	1.41	2.33	10.37	2.1	2.67	2.46	3.2
2009 May	13.66	1.61	2.35	6.07	1.21	20.5	0.22	0.74	11.1	13.77	0.66	1.05
2009 Jun	45.09	3.04	47.91	5.45	17.72	24.1	2.91	1.38	0.47	10.49	5.52	1.75
2009 Sep	36.41	4.49	3.93	5.95	13.1	4.54	13.93	4.02	9.34	15.65	11.31	6.23
2009 Oct	24.1	4.16	2.16	14.1	5.1	3.61	24.23	1.33	24.3	16.2	0.36	4.11
2009 Nov	11.42	59.35	9.87	33.86	0.35	14.39	32.16	3.88	53.69	24.97	19.04	35.97
2009 Dec	56.78	38.21	4.7	42.85	12.6	10.17	53.57	1.44	10.71	50.71	35.08	38.03

[a] antibiotics: same as described in Table 1.2a.

Table 1.3b: The average, maximum and minimum percentage(s) of oligotrophic (facultative+obligate) bacteria resistant to each antibiotic in water samples of SS II.

	Antibiotics[a]											
%	**Amp**	**Azi**	**Cef**	**Cft**	**Chl**	**Cip**	**Cot**	**Kan**	**Lev**	**Net**	**Str**	**Tet**
Average	32.9	6.05	8.53	14.11	14.38	9.93	17.95	3.70	13.13	14.54	7	16.74
Max	70.45	59.35	47.91	42.85	90.9	37.7	53.57	10.37	71.19	79.7	45.5	78.12
Min	6.9	0	0.22	0.36	0.05	0.41	0.22	0.74	0.19	0.03	0.188	0.7

[a]antibiotics, same as described in Table 1.2a; Max, maximum; Min, minimum

The average of all the values obtained from 30 samples (spread for three consecutive years) for the fraction of ampicillin-resistant oligotrophic bacteria in SSI was 32.9%.;the maximum average value amongst all the resistant-fractions corresponding individually to twelve different antibiotics, while the least average value (3.7%) was obtained for kanamycin-resistant fraction followed by azithromycin-resistant fraction (6.05%), streptomycin-resistant fraction (7%), cefepime (8.5%), and ciprofloxacin-resistant fraction (9.93%). The average values corresponding to fractions resistant to rest of the antibiotics tested (levofloxacin, cefotaxime,

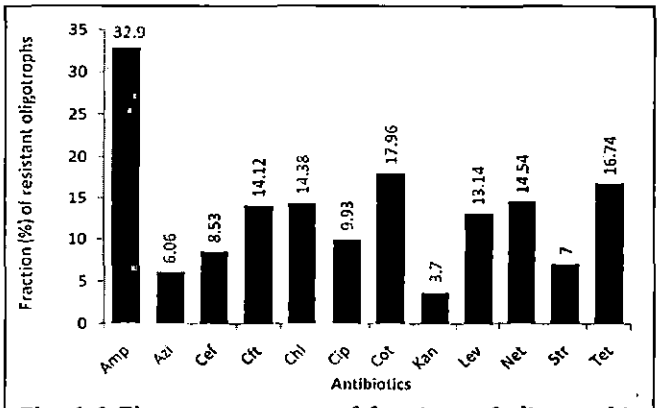

Fig. 1.6 Three years mean of fractions of oligotrophic bacteria resistant to each antibiotic in water samples of sampling site II.

chloramphenicol, netilmycin, oxytetracycline, and cotrimoxazole) fell in the range of 13.13 – 17.95% (Table 1.3b). Among all the antibiotics tested, the incidence of chloramphenicol-resistant oligotrophic bacteria was highest (90.9%). The maximum occurance of chloramphenicol-resistance was observed in the month of January 2008, and the lowest (3.47%) of the said population was recorded in January 2009 (Table 1.3a). In October and December 2007, equal frequency of azithromycin-resistance was found at SS II while no resistance was observed in the water sample collected in the month of May 2007 (Table 1.3a). The azithromycin resistance was highest (59.35%) in November 2009. Among the recovered oligotrophic bacterial population, the highest fraction resistant to Cefepime (47.9%), cefotaxime (42.85%) and ciprofloxacin (37.7%) appeared in June 2009, December 2009 and January 2009 correspondingly. Highest percentage of the kanamycin (10.37%), levofloxacin (71.2%) and netilmicin (79.7%) resistant oligotrophic bacterial population was found in April 2009, March 2009 and December 2008 respectively; and lowest were found in the month of May 2009 for Kanamycin, February 2007 for levofloxacin, and December 2007 for netilmicin. The highest occurrence of streptomycin (45.5%), co-trimoxazole (53.57%) and tetracycline (78.2%) resisting population were recorded in March 2009, December 2009, and May 2007respectively. In order to above said antibiotics the lowest resistant percentage were documented during June 2007, May 2009 and February 2009. The percentage of oxytetracycline resistant bacteria remained same in November and December 2007. Average frequency of isolates, resistant to each antibiotic at SS II in three consecutive years are reviewed in Fig. 1.6

The incidence of oligotrophic bacteria resistant to ampicillin in SS III was highest (92.45%) in December 2009 followed by November 2008 (86.36%). The lowest occurrence (0.98%) of the said population was recorded in May 2008 (Table 1.4a).

Table 1.4a: Fractions (%) of oligotrophic (facultative + obligate) bacteria resistant to each antibiotic per sample per month in three consecutive years (2007-2009) from SS III

	Antibiotics[a]											
Months	**Amp**	**Azi**	**Cef**	**Cft**	**Chl**	**Cip**	**Cot**	**Kan**	**Lev**	**Net**	**Str**	**Tet**
2007 Jan	9	3.5	8.6	3.3	25.1	7.9	15.16	3.21	23.12	7.1	5.15	7.44
2007 Feb	14	1.81	2.71	4.47	7	1.25	3.15	5.215	10.78	4.32	0.53	2.37
2007 Mar	6.74	0.4	2.23	4.1	2.5	7.13	11.2	1.9	10.3	1.57	2.03	10.4
2007 Apr	43.6	12.4	13.2	3.53	4.16	3.15	12.05	8.02	22.7	8.27	24.12	3.72
2007 May	10.36	0	0.87	10.9	2.6	0.4	2.43	1.16	0.03	4.26	0	0.6
2007 Jun	40.9	1.81	5.45	11.7	10.45	0.45	35.9	0.95	1.36	13.63	1.81	6.81
2007 Sep	7.2	10	2.4	1.74	13.13	6.05	5.09	4.435	7.6	15.4	1.91	4.32
2007 Oct	30.2	0.3	2.08	4.54	3.9	1.86	2.35	1.11	0.29	5.6	0.9	5.6
2007 Nov	4.93	0.23	1.024	1.2	0.48	0.54	3.37	2.3	0.12	2.71	0.96	1.8
2007 Dec	6.8	1	3.36	10.24	3.27	0.98	2.37	0.75	0.4	0.24	2.95	29.5
2008 Jan	7.89	0.5	7.67	1.34	5.12	1.31	5.6	1.23	3.2	0.71	2.35	0.74
2008 Feb	2.14	0.81	0.71	1.42	27	0.5	0.35	0.25	0.78	1.42	0	0
2008 Mar	6	2.4	2.3	1	2.5	1.3	1.4	1.1	0.3	1.7	2.4	0.4
2008 Apr	2.36	2.4	3.2	2.33	48.6	3.5	2.5	3.02	2.75	8.33	23.2	7.2
2008 May	0.98	0.1	2.06	1.31	4.3	0.29	5.29	0.51	0.07	0.29	1.37	0.35
2008 Jun	41.6	11.16	15.2	31	17.2	9.12	5.72	6.12	3.71	4.28	0.92	3.51
2008 Sep	1.72	1.03	1.41	1.45	13.63	0.65	8	3	0.6	0.54	12.21	0.42
2008 Oct	8	1.57	56.61	1.23	8.13	0.3	56.61	2.16	8.3	8.61	8.46	98.46
2008 Nov	86.36	13.63	54.54	45.4	0.45	0	40.9	1.31	13.63	27.27	0	36.36
2008 Dec	12.2	7.09	4.65	7.9	17.2	7.67	5.34	0.17	8.13	22.32	15.34	1.02
2009 Jan	1.07	1.32	0.71	0.89	1.4	0.51	0.37	1.61	0.45	2.58	1.94	0.04
2009 Feb	5.91	12.09	12.47	12.25	2.5	4.95	8.92	0.25	34.94	27.09	28.49	8.65
2009 Mar	20.26	3.46	12	3.86	5.29	1.6	5.6	2.047	57.33	18.4	14.66	4.8
2009 Apr	7.37	1.27	2.48	1.86	12.21	3.63	2.35	3.327	8	7.77	3.87	1.1
2009 May	32	0.6	0.8	12.6	11.45	1.6	3.8	0.31	11.9	19.33	7.4	1.8
2009 Jun	10.6	1.6	15.8	10	7.2	0.95	5.2	0.12	5.31	24.8	10.2	4.15
2009 Sept	13.56	19.04	18.95	66.08	11.4	6	16.17	1.01	71.65	46.6	30.26	10.69
2009 Oct	11.73	9.23	6.52	18.47	21.5	5.1	20.87	1.421	31.3	16.95	15.65	7.33
2009 Nov	27.11	26.16	89.71	24.29	15.29	24.53	30.37	7.047	83.17	97.19	53.73	39.25
2009 Dec	92.45	72.13	14.42	70.81	2.23	15.57	59.01	1.307	10.32	85.24	65.57	34.42

[a]antibiotics, same as described in Table 1.2a

Table 1.4b: The average, maximum and minimum percentage(s) of oligotrophic (facultative+obligate) bacteria resistant to each antibiotic in water samples of SS III.

	Antibiotics[a]											
%	Amp	Azi	Cef	Cft	Chl	Cip	Cot	Kan	Lev	Net	Str	Tet
Average	18.83	7.3	12.14	12.37	10.24	3.96	12.58	2.21	14.42	16.15	11.28	11.1
Max	92.45	72.13	89.71	70.81	48.6	24.53	59.01	8.02	83.17	97.19	65.57	98.46
Min	0.98	0	0.71	0.89	0.45	0	0.35	0.12	0.03	0.24	0	0

[a]antibiotics, same as described in Table 1.2a; Max, maximum; Min, minimum

The average of all the values obtained from 30 samples (spread for three consecutive years) for the fraction of ampicillin-resistant oligotrophic bacteria in SS III was 18.83%.;the maximum average value amongst all the resistant-fractions corresponding individually to twelve different antibiotics, while the least average value (2.2%) was obtained for kanamycin-resistant fraction followed by ciprofloxacin-resistant fraction (3.96%), and azithromycin (7.3%). The average values corresponding to fractions resistant to rest of the antibiotics tested (chloramphenicol, oxytetracycline, streptomycin, cefepime, cefotaxime, cotrimoxazole, levofloxacin, and netilmycin) fell in the range of 10.24 - 16.15% (Table 1.4b). There were no azithromycin-resistant oligotrophic bacteria in the water sample collected in the month of May 2007. Similarly no resistance towards ciprofloxacin and oxytetracycline were observed in the month of November 2008 and February 2008 respectively. Streptomycin-resistant oligotrophic bacteria were absent in the water sample collected in the months of May 2007, February 2008, and November 2008. The occurance of oligotrophic bacteria resistant to azithromycin was maximum (72.13%) in the month of December 2009 while lowest (0.1%) were observed in May 2008. The proportion of chloramphenicol-resistant oligotrophic bacteria was highest (48.6%) in month of April 2008 while lowest occurrence was observed in November 2008. Maximum occurances of antibiotic-resistant-oligotrophic bacteria were documented in November 2009 for cefepime (89.7%) and ciprofloxacin (24.53%); and in December 2009 for cefotaxime (70.81%). The percentage resistance of ciprofloxacin in compare to cefepime and cefotaxime was quite low throughout the sampling. The highest percentage of kanamycin-resistant (8.02%), levofloxacin-resistant (83.17%), and netilmicin-resistant (97.2%) oligotrophic bacterial population were recorded in the month April 2007 and November 2009 respectively. The lowest occurrences of resistance for the said antibiotics were recorded in June 2009, May 2007 and December 2007 correspondingly. The highest occurrence (65.57%) of streptomycin and cotrimoxazole-resistance among oligotrophic bacteria were recorded in December 2009 and lowest were recorded in October 2007 and February 2008 respectively.

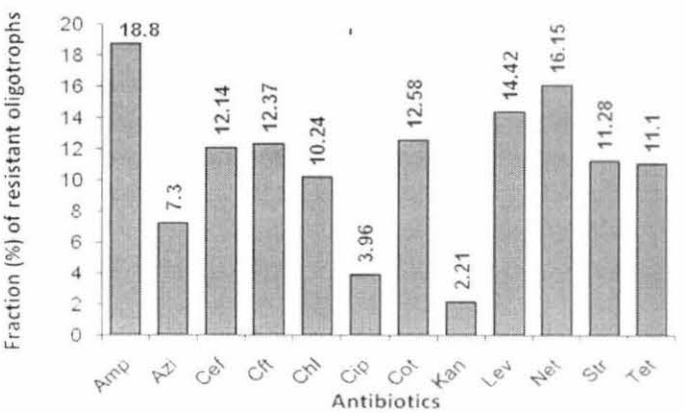

Fig. 1.7: Three years mean of fractions of oligotrophic bacteria resistant to each antibiotic in water samples of sampling site III.

The occurrence of oligotrophic bacteria resistant to oxytetracyline was found highest (98.46%) amongst all the tested antibiotics in the water samples collected in all three years. The lowest incidence (0.04%) of the said population was recorded in May 2008 (Table 1.4a). Average frequencies of antibiotic-resistant oligotrophic bacteria collected from three sampling sites in three consecutive years are shown in Fig. 1.7.

1.3.1.2. Comparative analyses of annual average percentages of twelve different antibiotic-resistant oligotrophic bacteria and their trends of increase or decrease

Comparative analyses of annual average percentages of twelve different antibiotic-resistant oligotrophic bacteria and their trends of increase or decrease have been represented in the Fig. 1.8 (a, b, c) and 1.9 (a, b, c). The average percentage occurrences of azithromycin, cefotaxime, ciprofloxacin, levofloxacin netilmicin, and streptomycin resistant bacteria in water samples collected during three consecutive years (January 2007 to December 2009) from SS I, have shown an upward trend but downward trend was noted for chloramphenicol resistant bacteria. Variable trend of occurrences of chloramphenicol, cefepime, cefotaxime netilmicin cotrimoxazole and tetracycline resistant bacteria were recorded from January 2007 to December 2009 at SS II. Analysis of Site II and Site III samples revealed an upward trend for ampicillin, azithromycin, ciprofloxacin, levofloxacin, streptomycin resistance while uneven trend of chloramphenicol and tetracycline resistance were observed at both sampling sites in all three years. An upward trend of cefipime resistance was found in water samples collected month-wise from all three years at SS III but was variable at SS II. During January 2007 to December 2009 oligotrophic bacteria, resistant to cefotaxime, and netilmicin have shown to be fluctuating at sampling site SS II. Kanamycin resistant bacteria showed an approximate constant level of occurrences in all sampling sites.

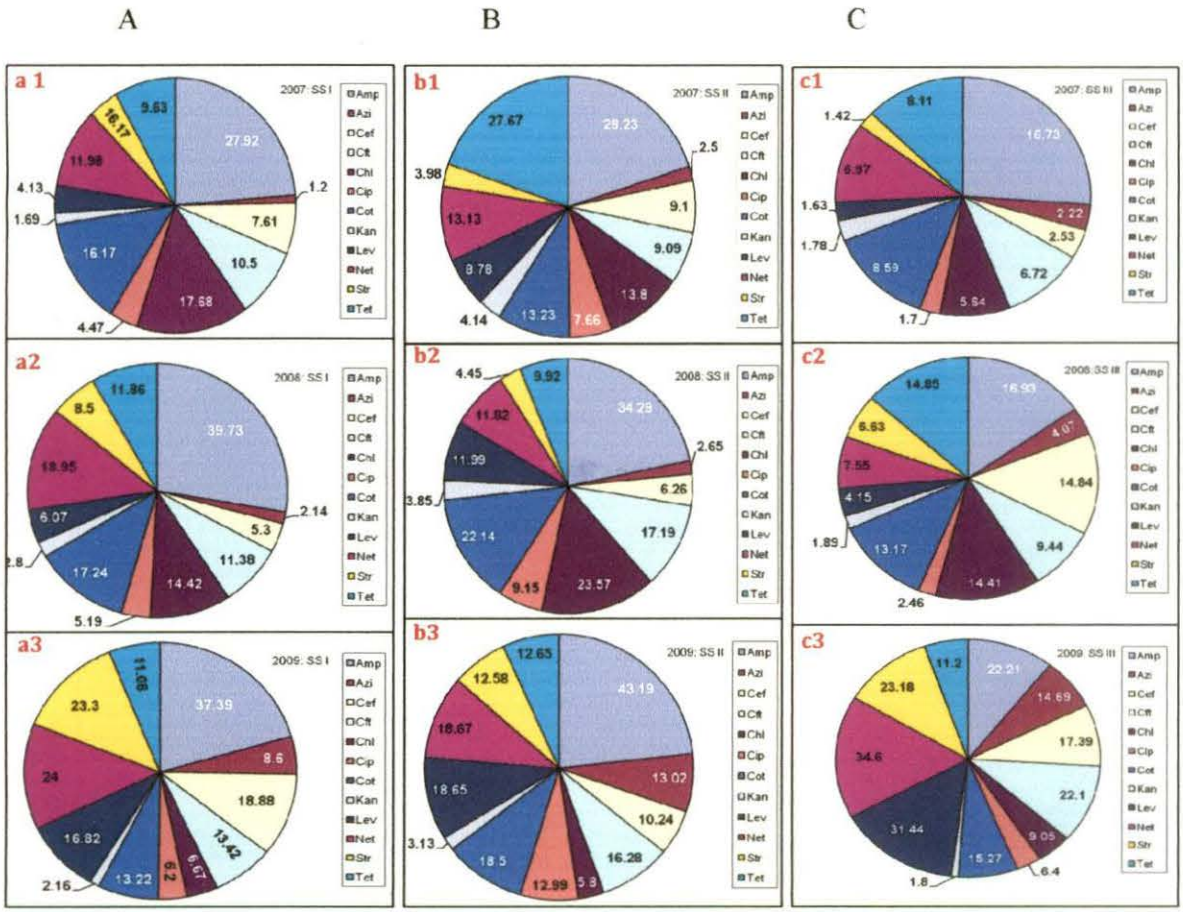

Fig. 1.8: Pie diagram showing fraction(s) of oligotrophic bacteria resistant to each antibiotic in water samples collected from three different sampling sites in three consecutive years (Jan 2007- Dec 2009).
A, sampling site I (a1, 2007; a2, 2008; a3, 2009); B, sampling site II (b1- b3: same as a1-3); and C, sampling site III (c1-c3: same as a1-3).

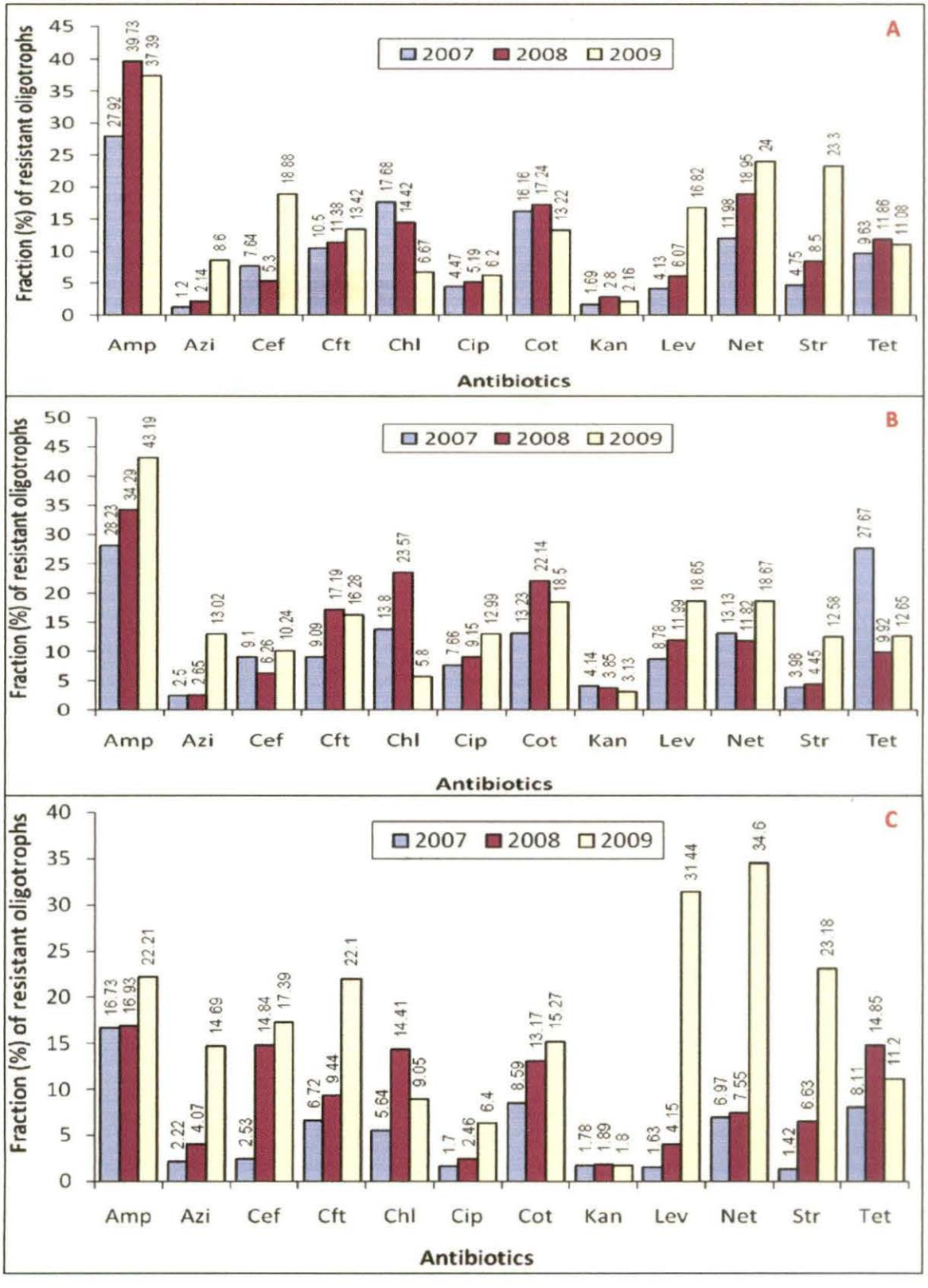

Fig. 1.9: Bar diagram showing comparative analyses of the fraction(s) of oligotrophic bacteria (present in water samples) resistant to each of the 12 different antibiotics at a particular sampling site in three consecutive years (2007-2009). A, sampling site I; B, sampling site II; C, sampling site III.

1.3.2 Occurrence(s) of sensitive, single and multiple-antibiotic-resistant bacteria among randomly selected facultatively oligotrophic (from second generation master plates) population

25±1 randomized colonies of facultatively oligotrophic isolates of first generation master plate (for detail please see section 1.2.7.2) were picked and transferred to R2A plate which were designated as second generation master plates. A total of 2188 isolates were picked in three consecutive years (2007-2009) from all three sampling sites and screening was performed by the replica-plating method (described in the section 1.2.7.2) for differentiation of sensitive, single-antibiotic-resistant, and multiple-antibiotic-resistant isolates.

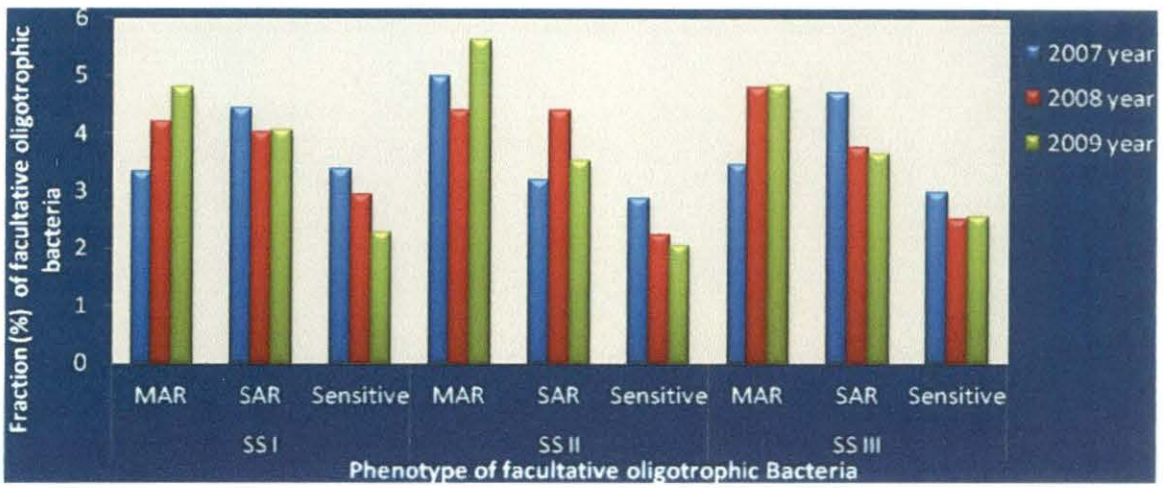

Fig 1.10: Comparative analyses of fraction(s) of MAR, SAR and sensitive facultative oligotrophic isolates out of entire oligotrophic (facultative + obligate) bacterial population in water samples from three different sampling sites (SS) in three consecutive years

The percentage occurrences of multiple-antibiotic-resistant facultatively oligotrophic bacteria have shown increasing trends in SS I and SS III, while fluctuation has been observed among SS II isolates. The occurrences of single-antibiotic-resistant facultatively oligotrophic bacteria were found to be decreasing from 2007 to 2009 at SS III. Approximately similar frequencies of single-antibiotic-resistant ones were observed during year 2008 and 2009 at SS I. Maximum frequency of single-antibiotic-resistant facultatively oligotrophic bacteria was observed in isolates collected from samples of the year 2008 at SS II. A gradual decrease in sensitive and concomitant increase in multiple-antibiotic- bacteria was observed from 2007- 2009 in randomly selected facultatively oligotrophic isolates from SS I and SS III. However for SS II isolates, multiple-antibiotic-resistant bacteria were most frequent ones than sensitive or single-antibiotic-resistant isolates; but gradual decrease in sensitive isolates has been noted (Fig. 1.10).

Table 1.5: Annual distribution of sensitive, SAR and MAR isolates at different sampling sites in different years.

Year	Fraction (actual number in parentheses) of sensitive and antibiotic-resistant (SAR and MAR) facultative oligotrophic isolates out of entire oligotrophic bacteria (obligate + facultative) at different sampling sites								
	SS I			SS II			SS III		
	MAR	SAR	Sensitive	MAR	SAR	Sensitive	MAR	SAR	Sensitive
2007	3.33 (73)	4.43 (97)	3.38 (74)	4.98 (109)	3.2 (70)	2.88 (63)	3.47 (76)	4.7 (103)	2.97 (65)
2008	4.2 (92)	4.02 (88)	2.93 (64)	4.39 (96)	4.39 (96)	2.24 (49)	4.8 (105)	3.75 (82)	2.51 (55)
2009	4.79 (105)	4.06 (89)	2.29 (50)	5.62 (123)	3.52 (77)	2.06 (45)	4.84 (106)	3.65 (80)	2.56 (56)

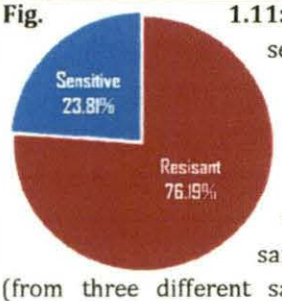

Fig. 1.11: Over all fraction(s) of sensitive and antibiotic-resistant Facultative oligotrophic bacterial population of Mahananda river water [data of 90 water samples taken together (from three different sampling sites in three years)].

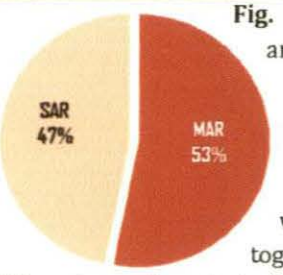

Fig. 1.12: Distribution of MAR and SAR population within antibiotic-resistant facultative oligotrophic bacteria of Mahananda river water [data of 90 water samples taken together (from three different sampling site in three years)].

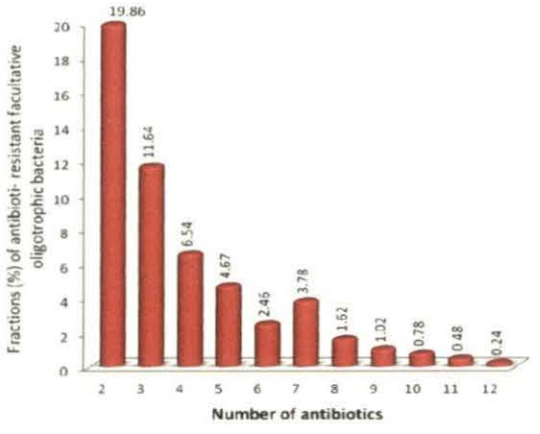

Fig. 1.13: Fraction of antibiotic resistant isolates, resisting 2, 3, 4, 5, 6, 7, 8, 9 , 10, 11, and 12 number of antibiotics within MAR facultative oligotrophic bacteria

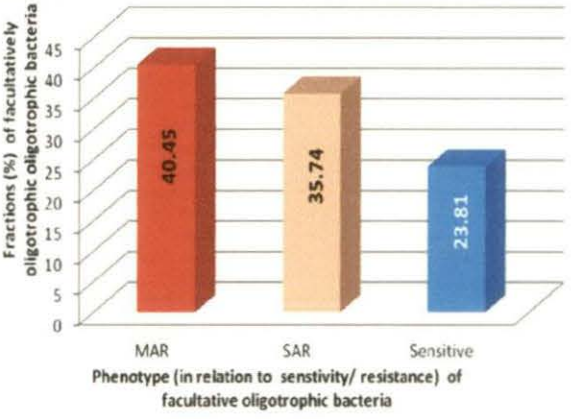

Fig. 1.14: Over all distribution of isolates exhibiting different phenotypes [in relation to antibiotic sensitivity/resistance] among facultative oligotrophic bacteria [data of 90 water samples taken together (from three different sampling site in three years)].

Out of 2188 facultatively oligotrophic bacterial isolates, 76.19% (1667) were antibiotic-resistant and 23.81% (521) were sensitive to all antibiotics used in this study (Fig. 1.11). Annual distributions of sensitive, single and multiple-antibiotic-resistant isolates at different sampling sites are presented in Table 1.5. Amongst antibiotic-resistant isolates, 47% (782) exhibited resistance to single antibiotic, designated as SAR (single-antibiotic-resistant) and 53% (885) were resistant to two or more than two antibiotics, designated as MAR (multiple-antibiotic-resistant) (Fig. 1.12). Among the multiple-antibiotic-resistant group, 19.86% (331) were resistant to two, 11.64% (194) to three, 6.54% (109) to four, 4.67% (78) to five, 2.46% (41) to six, 3.78% (63) to seven, 1.62% (27) to eight, 1.02% (17) to nine, 0.78% (13) to ten, 0.48% (8) and 0.24%(4) to all the 12 antibiotics tested(Fig. 1.13). Fig. 1.14 showed an average percentage of fractions of multiple-antibiotic-resistant, single-antibiotic-resistant and the bacteria sensitive to all antibiotic used. A gradual drop was observed in the numbers of sensitive facultative bacteria from year 2007 to 2009 in the pool of randomly selected isolates.

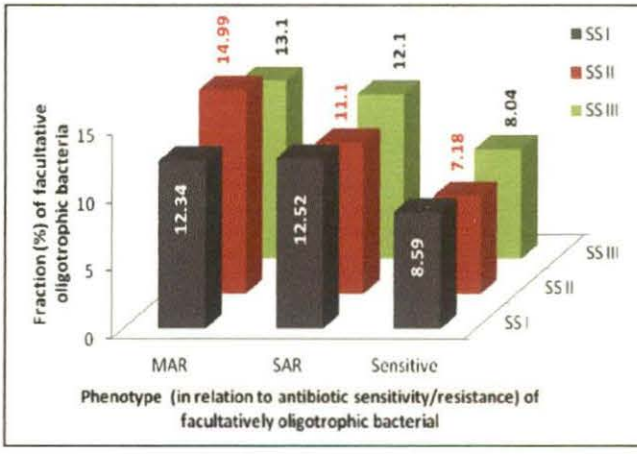

Fig. 1.15: Relative abundance (%) of sensitive, single-antibiotic (SAR) and multiple-antibiotic resistant (MAR) among facultative oligotrophic bacteria [data of 90 water samples taken together (from three different sampling site in three years)].

The relative abundance of sensitive, SAR and MAR bacteria in the pool of selected facultative oligotrophic bacterial population in three successive years, 2007, 2008 and 2009, has been depicted in Fig. 1.15. It was found that MAR facultatively oligotrophic bacteria (in total) were dominant in SS II samples compared to SS I and SS III. The SAR ones were more frequent amongst isolates from SSI and SSIII; similar observation was also noted in case of sensitive oligotrophic bacteria.

1.4 Discussion

For successful monitoring of river water quality it is important to have sufficient knowledge about the morphometric details of the river to be investigated, selection of particular sampling site(s), sample collection methods, and preservation & maintenance of samples for the types of analyses to be made. The study of morphometry, measurement of morphological features of the river basin, always provides valuable information in selecting sample collection site(s). Water quality of the river water also depends on physiographical factors, such as basin, bank, catchments area, and settlement around the river, as well as annual sedimentation load, water volume, width, and depth of the river.

Water samples collected from the river are of two main types depending on the collection principle: grab-samples, and composite samples. Grab samples are collected at a specific spot in a site over a short period of time, on the other hand when multiple grab samples are combined and treated as a single sample, it is called composite sample. Samplers and containers should always be thoroughly cleaned before use, and should be rinsed with the sample water before collection. Preferably the amber colored glass containers with polypropylene cap should be used for the collection and preservation of samples. For microbiological study collected samples should be transported and kept until use in ice container (4°C) and should be processed within 24 hrs.

Much of the current concern with regards to environmental quality is focused on water because of its importance in maintaining the human health and health of the ecosystem. The earth is almost flooded with water reservoirs but most of them are oceanic i.e salty which cannot be used in drinking or in other purposes e.g. irrigation etc. Freshwater is the fundamental requirement to sustain terrestrial life. It is finite resource, essential for agriculture, industry and even human existence; without fresh water of adequate quantity and quality, sustainable development will not be possible. The amount of fresh water is large as well but its distribution over the globe is uneven. With the increase in population, there is growing demand for fresh water supplies all over the world. In an effort to spur action to meet the impending crisis, the UN General Assembly has proclaimed the period from 2005 to 2015 as the International Decade for Action, "Water for Life" (www.peopleandplanet.net). In theory, some 34,000 cubic kilometres of freshwater are available globally for human use every year. If evenly distributed this would provide each person with roughly 8,000 cubic metres of water per year. Even this amount would be enough to meet human needs, if fresh water resources were evenly distributed. But available fresh water supplies are not distributed evenly around the globe throughout the seasons or from year to year. Throughout much of the developing world the freshwater supply comes in the form of seasonal rains. Such rains run off too quickly for efficient use, as during the monsoons in Asia. India, for example, gets 90 per cent of its annual rainfall during the summer monsoon season, which lasts from June to September. For the other eight months the country gets barely a drop (www.peopleandplanet.net).

Rivers are the main sources of fresh water. There is an extensive literature, which stresses deterioration of water quality (Tiwari and Mishra, 1986; Reddy and Venkateswar, 1987). The addition of various kinds of pollutants and nutrients through the agency sewage, industrial effluents, agricultural runoff etc. in to the water bodies brings a series of changes in the physicochemical and other characteristics of water, which have been the subject of several investigations (Vollenweidre, 1986; Milway, 1987; Olimax and Sikorska, 1975; Piecznska *et al.*, 1975). Deterioration of the fresh water quality is now a global problem (Mahananda *et al.*, 2005). Discharge of toxic chemicals, over pumping of aquifer and contamination of water bodies, demographic explosion, urbanization,

unplanned development, land degradation and lack of infrastructure for waste disposal are leading to a rapid deterioration in water quality in the majority of rivers all over the world. This poses a threat both to the environment and to the health of the people in the region. Pollution of rivers and lakes reduces accessible fresh water supplies. In developing countries, 90% of the sewage is being discharged directly into rivers, lakes, coastal waters without any treatment (World Resource Institute, 1996) (www.wri.org). Each year roughly 450 cubic meters of wastewater are discharged into rivers, streams and lakes. To dilute and transport this dirty water before it can be used again, another 6000 cubic kilometers of clean water are needed – an amount equal to about two thirds of the world's total annual usable fresh water runoff (www.peopleandplanet.net).

The trends, in the contamination of river water worldwide, have changed greatly over time. The fecal and organic pollution from untreated wastewater was the major contamination problem 100 years ago. In most industrialized countries, fecal contamination of water has been largely eliminated, however, in much of the world especially in cities in developing countries, organic pollution is still a problem. New pollution problems, particularly from agricultural runoff and industrial effluents are increasing in both industrialized and developing countries. In rapidly industrializing countries like China, India, Mexico, Brazil untreated sewage and industrial waste create substantial pressures on water quality that are much greater than the problems of the past.

In the recent years, emphasis is on the so-called emerging contaminants including pharmaceuticals such as antibiotics, endocrine disrupters and on various additives. Human use pharmaceuticals enter sewage effluents through improper disposal from private households and from hospitals. Direct inputs into natural waters are also possible during rain events and this normally occurs in less industrialized countries. In wastewater treatment plants the antibiotics are only partially eliminated and residual amounts can reach ambient waters and ground water. Antibiotics are of main interest because we do not know currently whether their presence in natural waters contributes to the spread of antibiotic resistance in organisms. Successive knowledge regarding the effect of sub-inhibitory concentrations of the antimicrobials on the survival of bacteria in environment is insufficient and contradictory. But on the other face voluminous evidences are there which revealed the existence of antibiotic resistant bacteria in nature and horizontal transfer of antibiotic resistance determinants between them.

In environmental settings polluted by human and animal waste or both, high frequencies of MAR isolates exist in the coliform and fecal coliform population. These environments include surface waters receiving runoff from lands occupied by livestock, polluted estuaries and contaminated water supplies. Fluvial waters receive human and animal wastewater discharges, which are expected to contain antimicrobial agents likely to exert a selective pressure, and commensal resistant bacteria, capable of transferring their resistances to autochthonous bacteria. Consequently, the fresh water bacteria may become a reservoir for antimicrobial resistance genes, and the reuse of these waters for humans and animals may contribute to the limitation of antimicrobials efficiency. Any body of water that receives human waste products can be studied for its content of antibiotic resistant bacteria. Beyond human use of antibiotics, there are a number of other sources that may shoulder part of the blame for high resistance levels. Resistance can come from the natural production of antibiotics by organisms in the soil. It may also result from antibiotic-contaminated runoff from animal feed or crops, or wastes from farm animals (Ash *et al.,* 2002). It was shown in Greece that some resistant bacteria came from the feces of seabirds or warm-blooded mammals that live near the coastal waters (Arvanitidou *et al.,* 2001; Bennani *et al.,* 2012). Studies have also been performed within the animal production industry to show the impact of antibiotic resistance. For example, fish farms routinely treat bacterial infections in the fish with the use of antibiotics. These antibiotics are released into the water. They then can move downstream, unfiltered and untreated by the fish farms (Schmidt *et al.,* 2000). Occurrences such as this allow for an increased ability of bacteria to develop a resistance.

The distribution of antibiotic-resistant strains in the aquatic environment has been studied in different parts of the world. Majority of the investigations focused on the antibiotic resistance patterns of the fecal coliform bacteria because of their use as pollution indicators and association with disease causing genera of importance to public health and hygiene. However, in many freshwater systems, fecal bacteria are of little numerical significance in spite of the fact that they are released into almost all inland waters. It is also not uncommon to find standard plate count bacteria (SPC) in drinking water at frequencies more than 10,000 times the frequency of coliforms. Earlier studies have reported the occurrence of high frequencies of antibiotic resistant organisms within the SPC populations (Armstrong *et al.*,. 1982). There is evidence that SPC bacteria in marine and freshwater environments can possess the same kinds of antibiotic resistance patterns as total and fecal coliform populations.

A substantial portion of bacteria previously thought to be unculturable were recently shown to be oligotrophic. Oligotrophic bacteria have been isolated from diverse sources including clinical sample and distilled water (Mallory *et al.*, 1977; Kuznetsov *et al.*, 1979; Tada *et al.*, 1995; Watve *et al.*, 2000; Nagarkar *et al.*, 2001; Miyake *et al.*, 2003; Pramanik *et al.*, 2003; Alexander *et al.*, 2005; Katsunori and Masafumi, 2006; Hu *et al.*, 2007; Ishii *et al.*, 2011, Bhullar *et al.*, 2012). Studies showed that a number of oligotrophic bacteria exhibit antibiotic resistance (Nikitin *et al.*, 1988; Zlatkin *et al.*, 1991; Kimura *et al.*, 1995; Oh *et al.*, 1995, Tada *et al.*, 1995; Miyake *et al.*, 2003; Kumar *et al.*, 2010, Kumar *et al.*, 2011, Bhullar *et al.*, 2012; Kumar *et al.*, 2012, Mandal *et al.*, 2012), most of them were plasmid borne (Tada *et al.*, 1995, Zlatkin *et al.*, 2012). On the basis of existing data it was hypothesized that oligotrophic bacteria can therefore be a potential reservoir of novel antibiotic resistance genes that can be acquired by pathogens through plasmid transfer. The nutrient content in river water is indeed far less than that in microbiological media conventionally used for the cultivation of bacteria from a natural water environment. Nutrients in free flowing river water system are heterogeneously distributed in an environment; therefore, based on this consideration, we could assume that there must be bacteria that are able to grow in the presence of low level of nutrients (oligotrophs). Oligotrophic bacteria are defined (though no uniformly acceptable definition formulated yet) as heterotrophic bacteria that able to multiply in very low nutrient condition (media containing a minimal content of organic matter of either 1 mg or 1 to 15 mg C/L) usually supplied as complex mixtures of peptone, trypticase, and other nutrients (Ishida and Kadota, 1981; Kuzenetsov *et al.*, 1979). Eutrophic or copiotrophic bacteria, on contrary, have been considered as the bacteria able to grow on similar nutrients but at levels supplying 2 or more than 2 g C/L (Akagi *et al.*, 1977). In India there are few reports available on oligotrophic bacteria (Watve *et al.*, 2000; Pramanik *et al.*, 2003) but not on incidences of antibiotic-resistant oligotrophs. Recently class-I integrons bearing MAR oligotrophic bacteria from an Indian River was reported by Kumar *et al.* (2010, 2011).

The present study validated the capability of nutrient-poor medium (NPA/NPB) and its amendment with antibiotics to quantify proportions of antibiotic-resistant oligotrophic bacteria in river water. In fact, standardization of the assay conditions for assessing fractions of antibiotic resistant oligotrophic bacteria, especially the type and concentration of antibiotic exposure by determining LD_{50} concentration for each antibiotic using sensitive oligotrophic strains, marked a major advancement in this area of water research. With the set of standards for determining susceptibility/ resistance, bacteriological investigations on the prevalence and abundance of antibiotic-resistant oligotrophic bacteria in water samples of the river Mahananda (collected month-wise from three sampling site for three consecutive years) at Siliguri city of the northern West Bengal, were done. Ninety water samples were collected and analyzed in between January 2007 to December 2009 from the three sampling sites from River Mahananda. The SS I is situated at the entry point of city Siliguri near Champasari (26°44′22.62″ N, 88° 25′21.92″E) (Fig. 1.3a). The location of SS II is under Mahananda bridge (26°44'23.20"N, 88°25'22.89"E) at the heart of city Siliguri (Fig. 1.3b).The SS III is chosen at the exit point of river from city Siliguri near Fulbari dam (26°38′42.44″

N, 88°24'19.67"E) [this sampling site supposedly has weak anthropogenic activity](Fig. 1.3c). Plate count on nutrient-poor medium was made to assess the oligotrophic bacterial content of the water samples collected from the three sampling sites. For the cultivation of oligotrophic bacteria, NPA agar plates were used. The recovered bacteria may not represent all the bacterium present in water but only those able to grow and form visible colonies on the NPA media under given condition of temperature and incubation were considered to assess the situation. The cultivable oligotrophic bacteria developed on agar plate did not reflect the actual number; the number that can be enumerated may be higher than the cultivated. It was observed that the numbers of colonies developed on low nutrient Luria agar were quite high in compared to the numbers that grew on rich nutrient Luria agar (this study).

Maximum recovery of the culturable oligotrophic bacterial populations recorded in month of June 2008 at all three sampling sites. The oligotrophic bacterial density in collected water samples of river Mahananda ranged 1×10^3 to 5.9×10^4 CFU/mL. In an earlier study, those bacteria which grew at the lower nutrient level but failed to grow when transferred to the higher nutrient concentration were considered to be oligotrophic bacteria (Yanagita, *et al.*, 1978); however report supports that on subsequent subculture of the isolates, many of them regained their ability to grow on rich medium (Kuznetsov *et al.*, 1979). Somehow a clear-cut distinction has been accepted that bacteria having the ability to grow only at lower concentration of nutrients were called obligate oligotrophs, whereas those which grew at both low and high concentrations of nutrients were termed facultative oligotrophs (Ishida *et al.*, 1980; Ishida *et al.*, 1982). In this study the obligate oligotrophs were omitted due to their slow growth and ambiguity in forming colonies on NPA medium used. Facultatitve oligotrophic bacterial colonies identified from the first generation master plates were further renumbered and randomized to obtain 25±1 random numbers. The colonies corresponding to each of the 25 random numbers were purified onto R2A agar plate and pure colonies were then imprinted onto the fresh R2A plate, designated as second generation master plate. The second generation master plate was replica plated on to the R2A plate containing antibiotic of defined concentration to determine the susceptibility profile (antibiogram) of the isolates.

The overall distribution of resistant oligotrophs was achieved by plating 0.1 mL aliquot of composite water sample on NPA agar amended with defined concentration of each antibiotic. The percentages of resistant oligotrophic bacteria were estimated throughout the study during each sampling in the same manner. The antibiotic resistance profile (ARP)/antibiogram/susceptibility profile/resistance profile of the facultative oligotrophic bacteria (obtained from first generation master plate through replica plating method) were determined by replica plate method as described elsewhere in this chapter. All the colonies picked up randomly to screen sensitive and resistant bacterial strains were used for the screening of class 1 integron (detail description in chapter 2).

The quantification of antibiotic-resistant oligotrophic bacteria showed that the maximum average-percentage of oligotrophic bacteria resistant to an antibiotic, in all three consecutive years (2007-2009), was documented for ampicillin and minimum for kanamycin amongst the 12 different antibiotics tested (Fig. 1.5, 1.6, and 1.7). In addition to ampicillin, the annual average percent of resistant-oligotrophic bacteria in the year 2009 at SS I was recorded maximum for netilmicin (24%) (Fig. 1.9a). The average annual frequency resistant oligotrophs at SS II were observed quite high for oxyteteracycline (27.67%) in the year 2007 (Fig. 1.9b). Similarly maximum frequency of antibiotic-resistance at SS III, were observed for netilmicin (34.6%) in year 2009 (Fig. 1.9c).

It has been a general observation that SS II [compared to SS I (upstream) and SS III (downstream)] receives high influx of household, hospital effluents, cattle slurry, and industrial wastes and have high anthropogenic activities on both banks. Since this site was highly polluted with different kinds of wastes, it was assumed that probability of resistant bacteria will be high. In a previous study conducted on Yarra river of Australia, it was predicted that the incidence of antibiotic resistance in bacteria isolated from polluted sites would be greater than the incidence in bacteria

isolated from pristine sites and upstream reaches or flowing through areas of low intensity agriculture (Boon and Cattanach, 1999). The results of this study supported the hypothesis of Boon and Cattanach (1999); i.e high probability of finding resistant bacteria in the sampling site II. Since, oligotrophic bacteria constitutes a sub-set of the universal set of bacteria present in an ecological niche, high occurrences of oligotrophic bacteria resistant to single and multiple antibiotics were obtained from samples of SS II [the profile(s) of MAR facultatively oligotrophic isolates from SS II having class 1 integrons have been detailed in chapter 2] . The study on facultatively oligotrophic bacteria showed that the average percentage of multiple-antibiotic resistant (MAR) was greater than the single-antibiotic resistant (SAR) at SS II, however approximate similar load of MAR and SAR facultatively oligotrophic bacteria were observed at SS I and SS II (Fig. 1.9, 1.10 and 1.15). In an anthropogenically affected site, like sites where the river flows through a populous city, the chances of horizontal transfer of antibiotic resistance genes amongst diverse bacteria following dissemination into the environment are often very high. Many antibiotics of industrial origin also circulate in aquatic-environments which also act as the selective agents for amplification of antibiotic-resistance genes vis-à-vis enrichment of antibiotic-resistance gene pool of the microbial ecosystems. Previous studies concerned with rivers of northern West Bengal, dealt with copiotrophic bacteria (Mukherjee *et al.*, 2005). As such, the studies on antibiotic-resistance phenomenon in oligotrophic bacterial isolates are very limited. The results of the present study on facultatively oligotrophic bacteria have shown that 76.2% of them were antibiotic resistant and 23.8% were sensitive (subject to the limitation that the study was of random type) of an Indian river is resistant to one or more than one antibiotics. Among resistant facultatively oligotrophic bacteria 47% were SAR and 53% were MAR. The frequency of multiple-antibiotic-resistant facultatively oligotrophic bacteria was 6% greater than the bacteria resistant to one antibiotic. Within the MAR population of facultatively oligotrophic bacteria, 19.86% were resistant to two, 11.64% to three, 6.54% to four, 4.67% to five, 2.46% to six, 3.78% to seven, 1.62% to eight, 1.02% to nine, 0.78% to ten, 0.48% and 0.24% to all the 12 antibiotics tested (Fig. 1.13). An inverse relation was observed in average frequency of resistant and sensitive facultatively oligotrophic bacteria examined in three consecutive years (2007-2009) or in other words we can say that with progress of time (2007 to 2009), there is a gradual decrease observed in susceptible oligotrophic population in Mahananda River water. Furthermore a progressive decrease in facultatively oligotrophic bacterial population resisting single antibiotics associated with increase in MAR oligotrophic bacteria resisting more than two antibiotics was noted. Hence, the trend analyses (responses to antibiotics according to time) of total oligotrophic (obligate + facultative) and selected facultative oligotrophic bacterial population showed that proportion of oligotrophic bacteria was found to be increasing and percentage of sensitive oligotrophic bacteria decreasing with passage of time. This is an alarming situation; where immediate intervention is necessary. These bacteria were found to carry several antibiotic resistance genes as cassettes in their class 1 integron platforms; many of these cassettes are yet to be predicted for a functional protein (codes for hypothetical protein). These hypothetical proteins may in course of time develop into resistance genes against yet-to-develop chemotherapeutic agents. Hence it was really thought-provoking and tempting to assume that the oligotrophic bacteria may serve as potential reservoir of novel genes (antibiotic resistance and other stress-combating genes).

1.5 Conclusion

The antibiotic resistance in bacteria is a world-wide problem. It is therefore essential to assess the gravity of the problem and also to concentrate on types of resistances not only in the commensal and pathogenic copiotrophic bacteria but also in oligotrophic bacteria which are largely uncultured; and we are ignorant about the resistance-gene carriage within oligotrophic bacterial genomes. Earlier studies have shown that rivers are major reservoirs of antibiotic resistance genes (Park *et al.*, 2003; Biyela *et al.*, 2004; Xi *et al.*, 2012). Finding antibiotic resistance in copiotrophic bacteria is not novel

but probing antibiotic-resistance in oligotrophic bacteria of a dynamic fluvial system certainly deserves an element of novelty. Most of the reports on antibiotic resistance have been done on copiotrophic bacteria (standard plate count bacteria; retrieved on nutrient-rich media) but not on oligotrophic ones. Since oligotrophic bacteria (which can sustain long period of low nutrient conditions) have been reported from clinical sample (Tada *et al.*, 1995), hospital tap water (Katsunori and Masafumi, 2006), and filtered water used for patients in the hospital (Audic *et al.*, 2007) which may cause opportunistic infections in susceptible patients, the present study on antibiotic-resistance in oligotrophic bacteria present in environmental samples has become immensely important. A noteworthy paper by Chee *et al.* (2001) addressed the critical area of antibiotic resistance and dissemination of resistance genes in the environment. Surface waters, like lakes and rivers, could promote mobilization of resistance genes through the aquatic food chain with consequent exposures to humans. Therefore surveillance programmes are needed to understand the size of the problem and also for the better control of antibiotic resistance. The results of this study revealing high frequency of antibiotic-resistant oligotrophic bacteria, in a major river of northern West Bengal draining one of most populous cities, have broadened the complexity of the problem.

REFERENCES

Ajithkumar B, Ajithkumar VP, Iriye R *et al.* Spore-forming *Serratia marcescens* subsp. *sakuensis* subsp. nov., isolated from a domestic wastewater treatment tank. *Int J Syst Evol Microbiol* 2003; 53: 253–8.

Akagi Y, Taga N, Simidu U. Isolation and distribution of oligotrophic marine bacteria. *Can J Microbiol* 1977; 23: 981-7.

Alcaide E, Blasco MD, Esteve C. Occurrence of drug-resistant bacteria in two European eel farms. *Appl Environ Microbiol* 2005; 71: 3348-50.

Alexander L, Wolfgang B, Harald M. Diversity of bacteria growing in natural mineral water after bottling. *Appl Environ Microbiol* 2005; 71: 3624-35.

Al-Jebouri MM. A note on antibiotic resistance in the bacterial flora of raw sewage and sewage polluted River Tigris in Mosul, Iraq. *J Appl Bacteriol* 1985; 58 : 401-6.

Armstrong J, Shigeno DS, Calomiris JJ *et al.* Antibiotic-resistant bacteria in drinking water. *Appl Environ Microbiol* 1981; 42: 277-83.

Armstrong JL, Calomiris JJ, Seidler RJ. Selection of antibiotic-resistant standard plate count bacteria during water treatment. *Appl Environ Microbiol* 1982; 44: 308-16.

Arvanitidou M., Katsouyannopoulos V, Tsakris A. Antibiotic resistance patterns of enterococci isolated from coastal bathing waters. *J Med Microbiol* 2001; 50: 1001–5.

Ash RJ, Mauck B, Morgan M. Antibiotic resistance of gram-negative bacteria in rivers, United States. *Emerg Infect Dis* 2002; 8: 713-6.

Audic S, Robert C, Campagna B *et al.* Genome analysis of Minibacterium massiliensis highlights the convergent evolution of waterliving bacteria. *PLoS Genet* 2007; 3: e138.

Bahr M, Hobbie JE, Sogin ML. Bacterial diversity in an arctic lake: Fresh water AR cluster. *Aquat Microb Ecol* 1996; 11: 271-7.

Baida N, Yazourh A, Singer E *et al. Pseudomonas brenneri* sp. nov., a new species isolated from natural mineral waters. *Res Microbiol* 2001; 152: 493-502.

Barkay T, Kroer N, Rasmussen LD *et al.* Conjugal transfer at natural population densities in a microcosm simulating an estuarine environment. *FEMS Microbiol Ecol* 1995; 16: 43-53.

Bennani M, Amarouch H, Oubrim N *et al.* Identification and Antimicrobial Resistance of Fecal Enterococci Isolated In Coastal Mediterranean Environments of Morocco. *Eur J Sci Res* 2012; 70:266-75.

Bhullar K, Waglechner N, Pawlowski A *et al.* Antibiotic Resistance Is Prevalent in an Isolated Cave Microbiome. *PLoS ONE* 2012; 7: e34953.

Biyela PT, Lin J, Bezuidenhout CC. The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. *Water Sci technol* 2004; 50: 45-50.

Boon PI, Cattanach M. Antibiotic resistance of native and faecal bacteria isolated from rivers, reservoirs and sewage treatment facilities in Victoria, south-eastern Australia. *Lett Appl Microbiol* 1999; 28: 164-8.

Chee-Sanford JC, Aminov RI, Krapac IJ *et al.* Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl Environ Microbiol* 2001; 67: 1494-502.

Cohen E, Colwell R. Coincident plasmids and antimicrobial resistance in marine bacteria isolated from polluted and unpolluted Atlantic Ocean samples. *Appl Environ Microbiol* 1986; 51: 1285-92.

Connon SA, Giovannoni SJ. High throughput methods for culturing micro- organisms in very low-nutrient media yield diverse new marine isolates. *Appl Environ Microbiol* 2002; 68: 3878-85.

Cooke MD. Antibiotic resistance in coliform and fecal coliform bacteria from natural waters and effluents. *NZJ Mar Freshwater Res* 1975; 10:391-7.

Dayal G. Groundwater qualities of rural and urban settlements at Agra. *J Nature Conserv* 1992; 4:89-93.

Diab AM, Aziz MHA, Salim SA. Antibiotic-resistant gram-negative bacteria isolated from drinking water in Ismalia city. The Tenth Conference on Microbiology. Cairo, Egypt. November 12-14. 2000. p.1339-153.

Gonzal SM, Gerba CP, Melnick JL. Transferable drug resistance in bacteria of coastal canal water and sediment. *Water Res* 1979; 13:349-56.

Hattori T. Physiology of Soil oligotrophic bacteria. *Microbiol Sci* 1984; 1: 102-4.

Heuer H, Krögerrecklenfort E, Wellington EMH *et al.* Gentamicin resistance genes in environmental bacteria: prevalence and transfer. *FEMS Microbiol Ecol* 2002; 42: 289-302.

Hirsch R, Ternes T, Harber K *et al.* Occurrence of antibiotics in the environment. *Sci Total Environ* 1999; 225: 109-18.

Horowitz A, Krichevsky MI, Atlas RM. Charaterestics and diversity of subarctic marine oligotrophic, stenohetertrophic, and euryheterotrophic bacterial population. *Can J Microbiol* 1983; 29: 527-35.

Hu YS, Li CX, Sun FL *et al.* Improved culturability of soil bacteria using proper combination with various culturing medium. (Article in Chinese). *Wei Sheng Wu Xue Bao* 2007; 47: 882-7.

Ishida Y, Kadota H. Growth patterns and substrate requirements of naturally occurring obligate oligotrophs. *Microbiol Ecol* 1981; 7: 123–30.

Ishii S, Ashida N, Otsuka S *et al.* Isolation of oligotrophic denitrifiers carrying previously uncharacterized functional gene sequences. *Appl Environ Microb* 2011; 77: 338-42.

Jain SK, Agrawal PK, Singh VP. Hydrology and water resources of India. Google Books. Data retrieved 22nd October 2011.

Jones JG. Antibiotic resistance in aquatic bacteria. *J Antimicrob Chemother* 1986; 18: 149-154.

Katsunori F, Masafumi F. Isolation of Oligotrophic Bacteria from Hospital Tap Water. (article in Japanese). *J Antibact Antifungal Agents* 2006; 34: 323-8.

Kelch WJ, Lee JS. Antibiotic resistance patterns of gram negative bacteria isolated from environmental sources. *Appl Environ Microbiol* 1978; 36 : 450-6.

Kimura Y, Nakamura T, Kajikawa K *et al.* Antibiotic sensitivity of oligotrophic and eutrophic bacteria. Technical Bulletin of Faculty of Agriculture-Kagawa University 1995; 47: 121-6.

Koch L. Oligotrophs versus copiotrophs. *BioEssays* 2001; 23: 657-61.

Kolpin DW, Furlong ET, Meyer MT *et al.* Pharmaceuticals, hormones and other organic waste water contaminants in U. S. streams, 1999-2000: A national reconnaissance. *Environ Sci Technol* 2002; 36: 1202-11.

Kolwzan B, Traczewska T, Pawlaczyk-Szipilowa M. Examination of resistance bacteria isolated from drinking water to antibacterial agents. *Environ Prot Eng* 1991; 17: 53-60.

Kumar A, Chakraborti S, Joshi P *et al.* A multiple antibiotic and serum resistant oligotrophic strain, *Klebsiella pneumoniae* MB45 having novel *dfrA30*, is sensitive to ZnO QDs. *Ann Clin Microbiol Antimicrob* 2011; 10, 19.

Kumar A, Mukherjee S, Chakraborty R. Characterization of a Novel Trimethoprim Resistance Gene, *dfrA28*, in Class 1 Integron of an Oligotrophic *Acinetobacter johnsonii* Strain, MB52, Isolated from River Mahananda, India. *Microb Drug Resist* 2010; 16: 29-37.

Kumar A, İnce İA, Kati A *et al. Brevibacterium siliguriense* sp. nov., a novel facultatively oligotrophic bacterium isolated from river water. *Int J Syst Evol Microbiol* 2012; doi:10.1099/ijs.0.038281-0.

Kummerer K. Resistance in the environment. *J Antimicrob Chemother* 2004; 54: 311-20.

Kuzenetov SI, Dubinina GA, Lapteva NA. Biolgy of oligotrophic bacteria. *Annu Rev Microbiol* 1979; 33: 377-87.

Leff LG, McArthur JV, Shimkets LJ. Spatial and temporal variability of antibiotic resistance in freshwater bacterial assemblages. *FEMS Microbiol Ecol* 1993; 13: 135-44.

Levy SB. The antibiotic paradox: how miracle drugs are destroying the miracle. Plenum Press, New York. 1992.

Ligon BL. Sir Alexander Fleming: Scottish researcher who discovered penicillin. Source. Semin. *Pediatr Infect Dis* 2004; 15: 58-64.

MacDonell MT, Hood MA. Isolation and characterization of ultramicrobacteria from Gulf Coast estuary. *Appl Environ Microbiol.* 1982. 43: 566-71.

Magee AM, Quinn JP. Antibiotic resistance in the bacteria of a remote upland river catchment. *Lett Appl Microbiol* 1991; 13: 145-9.

Mahananda HB, Mahananda MR, Mohanty BP. "Studies on the Physico-chemical and Biological Parameters of a Fresh Water Pond Ecosystem as an Indicator of Water Pollution". *Ecol Env Cons* 2005; 11:537-41.

Mallory LM, Austin B, Colwell RR. Numerical taxonomy and ecology of oligotrophic bacteria isolated from the estuarine environment. *Can J Microbiol* 1977; 23:733-50.

Mandal AK, Yadav KK, Sen IK *et al.* Partial characterization and flocculating behavior of an exopolysaccharide produced in nutrient-poor medium by a facultative oligotroph *Klebsiella* sp. PB12. *J Biosci Bioeng* 2012. doi: 10.1016/j.jbiosc.2012.08.006.

Martin P, Macleod RA. Observations on the distinction between oligotrophic and eutrophic marine bacteria. *Appl Environ Microbiol* 1984; 47:1017-22.

McArthur JV, Tuckfield RC. Spatial patterns in antibiotic resistance among stream bacteria: effects of industrial pollution. *Appl Environ Microbiol* 2000; 66: 3722-6.

McKeon DM, Calabrese JP, Bissonnette GK. Antibiotic resistant gram-negative bacteria in rural ground water supplies. *Water Res* 1995; 29: 1902-8.

Milway CP. "Educational in large lakes and impoundments". Proc.Upplasale Symp. OECD Paris. 1969.

Mitra A, Gupta SK. Assessment of groundwater quality from sewage fed farming area of east Calcutta. Indian. *J Environ Prot* 1997; 17: 447.

Miyake D, Kasahara Y, Morisaki H. Distribution and Characterization of Antibiotic Resistant Bacteria in the sediment of Southern Basin of Lake Biwa. *Microb Environ* 2003; 18: 24-31.

Mukherjee S, Bhadra B, Gurung A *et al.* Unregulated use of antibiotics in Siliguri city vis-à-vis occurrence of MAR bacteria in community waste water and river Mahananda, and their potential for resistance gene transfer. *J Environ Biol* 2005; 26: 229-38.

Muto CA, Jernigan JA, Ostrowsky BE *et al.* SHEA Guidelines for preventive Nosocomial transmission of multi resistant strains of *Staphylococcus aureus* and *Enterococcus.* Infec. Control. *Hosp Epidemiol* 2003; 24: 362-8.

Nagarkar P, Ravetkar SD, Watve MG. Oligophilic Bacteria as Tools To Monitor Aseptic Pharmaceutical Production Units. *Appl Environ Microbiol* 2001; 67: 1371–4.

Niemi M, Sibakov M, Niemala S. Antibiotic resistance among different species of fecal coliforms isolated from water samples. *Appl Environ Microbiol* 1983; 45: 79-83.

Nikaido H. Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* 1994; 264: 382-8.

Nikitin DI, Vishnevetskaia O, Zlatkin IV. Clustering of oligotrophic microorganisms on the basis of their antibiotic resistance and dynamic membrane characteristics. *Mikrobiologiia*; 1988; 57:262-7.

Ogan MT, Nwiika DE. Studies on the ecology of aquatic bacteria of the Lower Niger Delta: multiple antibiotic resistance among the standard plate count organisms. *J Appl Bacteriol* 1993; 74: 595-602.

Oh H, Lee J, Kim K *et al.* A novel laboratory cultivation method to examine antibiotic-resistance-related microbial risks in urban water environments . *Water Sci Technol* 2009; 59: 346-52.

Olimax T, Sikorska U. "Field experiment on the effect of municipal sewage on macrophytes and epifauna in the lake littoral". *Bull Acad Pol Sc Clii* 1975; 23: 445-7.

Park JC, Lee JC, Oh JY *et al.* Antibiotic selective pressure for the maintenance of antibiotic resistant genes in coliform bacteria isolated from the aquatic environment. *Water Sci Technol* 2003; 47: 249-53.

Phillips I, Casewell M, Cox T *et al.* Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J Antimicrob Chemother* 2004; 53:28–52.

Piecznska E, Sikorna U, Olimak T. "The influence of domestic sewage on the littoral zone of lakes". *Pol Arch Hydrobiol* 1975; 22: 141-56.

Pramanik A, Gaur R, Sehgal M, *et al.* Oligotrophic bacterial diversity of Leh soils and its characterization employing ARDRA. *Curr Sci* 2003; 84:1550–5.

Rajeshwari CV, Saraswathi B. "Assessment of water quality of Rivers Tungabhadra & Hundri, India". *Pollution Research* 2009; 28: 499-505.

Reddy PM, Venkateswar V. "Assessment of water quality in the river Tungabhadra near Kurnol", A.P. *J Environ Biol* 1987; 8:109-19.

Rivers in Siliguri. Mahananda river. Siliguri online (www.siligurionline.com/info/rivers.htm). Data retrieved 22nd October 2011.

Rosjak D B, Colwell RR. Survival strategies of bacteria in the natural environment. *Microbiol Rev* 1987; 51: 365-79.

Schmidt AS, Bruun MS, Dalsgaard I *et al.* Occurrence of antimicrobial resistance in fish pathogenic and environmental bacteria associated with four Danish rainbow trout farms. *Appl Environ Microbiol* 2000; 66: 4908-15.

Schwartz T, Kohnen W, Jansen B *et al.* Detection of antibiotic-resistant bacteria and their resistance genes in waste water, surface water and drinking water biofilms. *FEMS Microbiol Ecol* 2003; 43: 325-35.

Sharma S, Mathur R. Bacteriological quality of groundwater in Gwalior. *Indian J Environ Prot* 1994; 14:905-907.

Sizemore, RK, Colwell RR. Plasmids carried by antibiotic-resistant marine bacteria. *Antimicrob Agents Chemother* 1977; 12: 373-82.

Sokari TG, Ibiebele DD, Ottih RM. Antibiotic resistance among coliforms and *Pseudomonas* spp. from bodies of water around Port Harcourt, Nigeria. *J Appl Bacteriol* 1988; 64: 355- 9.

Swartz M N. Impact of antimicrobial agents and chemotherapy from 1972 to 1998. *Antimicrob Agents Chemother* 2000; 44: 2009-16.

Tada Y, Ihmori M, Yamaguchi J. Oligotrophic bacteria isolated from clinical materials. *J Clin Microbiol* 1995; 33: 493–4.

Tada Y, Kobata T, Nakaoka C. A simple and easy method for the monitoring of environmental pollutants using oligotrophic bacteria. *Lett Appl Microbiol* 2001; 32: 12-5.

Thomas CM, Nielsen KM. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nature Rev Microbiol* 2005; 3:711-21.

Tiwari TN, Mishra M. "Pollution in the river Ganga at Varanashi". *Life Sc Advances* 1986; 5: 130-37.

Vollenweidre RA. "Scientific fundamental of the eutrophication of lakes and flowing waters with special reference to nitrogen and phosphorus, as factoring eutrophication". OECD Paris. 1986.

Watve MG, Shejwal V, Sonawane C, *et al.* The 'K' selected oligophilic bacteria: a key to uncultured diversity? *Curr Sci* 2000; 78: 1535–42.

Wilcox S. CATIONIC PEPTIDES: A NEW HOPE. *The Science Creative Quarterly.* 2004. *[Online.]* http://www.scq.ubc.ca/cationic-peptides-a-new-hope/

Xi C, Zhang Y, Marrs CF. Prevalence of Antibiotic Resistance in Drinking Water Treatment and Distribution Systems. *Appl Environ Microbiol* 2009; 75: 5714–8.

Yanagita T, Ichikawa T, Tsuji T *et al.* Two trophic groups of bacteria, oligotrophs and eutrophs: their distributions in fresh and sea water areas in the central northern Japan. *J Gen Appl Microbial* 1978; 24: 59-88.

Zlatkin IV, Vishnevestskaia O, Nikitin DI. Some aspects of antibiotic resistance of oligotrophic bacteria. *Antibiot Khimioter* 1991; 36 (3): 34-7.

Zlatkin IV, Nikitin DI, Sigalevich PA. Investigation of unusual growth and phenotypic characteristics of plasmid-containing and plasmid-free strains of oligotrophic bacterium Ancylobacter vacuolatus. *Mikrobiologiia* 2012; 81:39-47.