

Can Presence of Bacteriophages be Attributed to Subtle Quality of Ganga River Waters?

By



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1. Introduction

All rivers have some natural self-cleansing power i.e. the ability to assimilate certain kinds and amounts of waste substances from other ecosystems by transforming them through physical, chemical, physico-chemical and biochemical processes into products which become part of the river ecosystem. This allows them to maintain river water quality to some extent and have the ability to degrade and dilute the pollutants. The self-cleansing ability depends upon number of factors such as physical, chemical, biological composition of the aquatic system and climatic conditions. River Ganga is one of the important rivers of India and there is notion that Ganga water has some enigmatic property that makes it distinct, like it does not putrefy even after prolonged storages. In ancient India, people used to add Ganga *jal* (water) in other water resources to clean and make water pathogen free. It was observed and reported by English chemist Ernest Hankin in 1896 in his famous two articles published in the French journal *Annales de l'Institut Pasteur*, that Ganga water has some antibacterial property against *Vibrio cholera* (Hankin, 1896 a, b). Although he did not offer any explanation for this but subsequent studies attributed this to a particulate called bacteriophages, based on some partial evidences (Connerton & Connerton, 2006; Deresinski, 2009; Hanlon, 2005; Hudson *et al.*, 2005; Twort, 1915 and d'Herelle, 1917). Some scientists have even stated that world should owe to the river Ganga for discovery of bacteriophages (Nautiyal, 2009). Bacteriophages are the entities which infect and kill bacteria and hence, if present in river water, helps in decreasing the bacterial quantity and microbial pollution in rivers. The question is whether presence of bacteriophages is unique to river Ganga and is this the prime factor for the belief that Ganga river waters have special quality. It is to satisfy this end that the present study was undertaken.

2. Literature Review

It is believed that Ganga water has some subtle properties. For example, antibacterial activity of Ganga water against *Vibrio cholera* was reported by a British Bacteriologist Ernest Hankin in 1896. Although he did not offer any explanation for this but subsequent studies described this inkling, based on some partial scientific evidences, as an attribute due to a particulate called 'Bacteriophages' (Atterbury, 2009; Connerton & Connerton, 2006; Deresinski, 2009; Hanlon, 2005; Hudson *et al.*, 2005; Parfitt, 2005; Wei *et al.*, 2010) and the world should owe to the river Ganga for discovery of bacteriophage. In general, the self-purification capacity of river Ganga is generally considered more than any other river. The processes, such as physical, physico-chemical, chemical and biological that occur in river Ganga, also occur in many other rivers. However, there is belief, and based on some partial scientific evidences it has been speculated that the river Ganga has high self-purification capacity because of the presence of bacteriophages (NEERI, 2004a). Further it is believed, and again based on some incomplete scientific evidences it has been suggested, that quality of Ganga water does not deteriorate on prolonged storage.

Bacteriophages may help in regulating the bacterial population in Ganga river by utilising them as host. Bacteriophages multiply in the host bacterial cell at the expense of the host cell. Whether presence of bacteriophages, particularly coliphages, is unique to the river Ganga is the focus of investigation of the present study. Accordingly, emphasis has been given in reviewing the literature on bacteriophages in river waters to investigate this aspect.

2.1 Bacteriophages

Bacteriophages (or simply phages) are bacterial viruses which infect a specific host bacteria and kills them by lysis of the host cells (Santos *et al.*, 2009). Bacteriophages were independently discovered by Frederick W Twort in England and Felix d'Herelle at the Pasteur Institute in France (Twort, 1915; d'Herelle, 1917). Since then bacteriophages were used for a number of applications such as alternative to antibiotics as it has therapeutic potential for treating diseases (Chhibber *et al.*, 2008; Huff *et al.*, 2005), as an indicator of faecal contamination in water bodies (Araujo *et al.*, 1997; Leon *et al.*, 1990; Havelaar *et al.*, 1986; Sobsey *et al.*, 2006), as an environmental biocontrol agent (Chen *et al.*, 2013), as a biocontrol agent in food (Atterbury, 2009; Hudson *et al.*, 2005), etc. The classification of bacteriophage, into various taxonomic groups like *Order* and *Families*, can be done on the basis of structure or morphology, nature of their genome and envelope composition (refer Table 2.1). The genetic content can be either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) and may be either single stranded or double stranded (Ackermann, 2006).

2.1.1 Life Cycle of Bacteriophage

Bacteriophages are the bacterial viruses and hence they require host bacteria for their growth and remain in dormant state outside host bacteria. Whenever they enter inside bacteria they can multiply themselves with two types of multiplication cycles known as life cycles. First step in the process of completing life cycle of bacteriophages is 'adsorption'. In adsorption bacteriophages attach on the bacterial cell where specific receptors such as proteins on the cell wall, LPS, pili, and lipoprotein are present. This process is reversible. Second step in which nucleic acid from the bacteriophage enters into the bacterial cell through hollow tail is known as 'penetration'. Adsorption and penetration are common for both types of life cycles.

- a) Lytic cycle: In the lytic cycle, the virulent phages use the host biosynthetic machinery to make phage specified mRNA's from viral genome and other phage proteins to package the copied viral genome into viral particles. When enough virus particles are made, the viral particle is released outside by triggering the lysis of host bacteria. The new released viral particles infect new bacterial cells.
- b) Lysogenic cycle: In the lysogenic or temperate phages, the viral genome integrates itself to the host genome by homologous recombination and form a 'prophage'. Therefore the viral genome also gets replicated along with the host chromosome and

passes into daughter cells. An external cue, often a stressor such as UV radiation, subsequently causes the prophage to be excised from the host genome and the virus enters the lytic cycle. (Adams, 1959; Chibani-Chennoufi et al., 2004; Summers, 2005).

Table 2.1: Classification and Basic Properties of Bacteriophages
(Source: Ackermann, 2006)

Symmetry	Nucleic Acid	Order and Families	Genera	Members	Unique features
Binary (Tailed)	dsDNA, L	Caudovirales	15	4950	TC* NCT* ST*
		• Myoviridae	6	1243	
		• Siphoviridae	6	3011	
		• Podoviridae	3	696	
Cubic	ssDNA, C	Microviridae	4	40	Complex capsid, lipids
	dsDNA, C, T	Corticoviridae	1	3	
	dsDNA, L	Tectiviridae	1	18	
	ssRNA, L	Leviviridae	2	39	
	dsRNA, L, S	Cystoviridae	1	1	
Helical	ssDNA, C	Inoviridae	2	57	Filamentous or rods
	dsDNA, L	Lipothrixviridae	1	6	
	dsDNA, L	Rudiviridae	1	2	
	dsDNA, C, T	Plasmoviridae	1	6	
Pleomorphic	dsDNA, C, T	Fuselloviridae	1	8	Spindle shaped, no capsid
	dsDNA, C, T	Fuselloviridae	1	8	

C-Circular, L-Linear, S-Segmented, T-Super helical; ssDNA – Single stranded deoxyribonucleic acid; dsDNA – Double stranded deoxyribonucleic acid; ssRNA – Single stranded ribonucleic acid; dsRNA – Double stranded ribonucleic acid; TC - Tail contractile; NCT - Non contractile tail; ST - Short tail.

2.2 Coliphages

Coliphages are morphologically and genetically diverse group of bacteriophages which infect *Escherichia coli* bacteria and are non-pathogenic to human beings (Snowdon *et al.*, 1989). Coliphages have centrally located nucleic acid and are surrounded by a protein coat known as capsid made up of a protein subunits or capsomeres. There are morphologically different types of capsid such as cubic, filamentous or tailed. Tailed and

some cubic capsid coliphages enter into host cells through adsorption on somatic or cell wall components of the host bacteria while filamentous and other cubic coliphages enter into host cells through adsorption on F-pili of bacteria having F plasmid. The former is known as somatic coliphage whereas latter is male-specific coliphages (Debartolomeis & Cabelli, 1991; Gantzer *et al.*, 1998; Muniesa *et al.*, 1999; Payment & Franco, 1993; Sinton *et al.*, 1996).

Bacteriophages of a particular bacterial species can generally be isolated from the environment having their host bacteria. Thus coliphages are generally found in the gut as a parasite of *E. coli* bacteria, present inside gut and thus they are excreted in the faeces of human and many warm blooded animals in varying densities (Dhillon *et al.*, 1976; Geldreich *et al.*, 1962; Gerba, 1987; Kott, 1981; Mushin & Ashburner, 1964; Scarpino *et al.*, 1972; Smith & Crabb, 1961). Coliphages and *E. coli* are present wherever faecal pollution occurs such as in sewage, wastewater, aquatic sediments and natural waters. These can grow in this environment if they can get suitable physical and chemical factors (Anderson, 1957; Buttiaux & Mossel, 1961; LaLiberte & Grimes, 1982; Parry *et al.*, 1981; Scarpino, 1978; Seeley & Primrose, 1980; Vaughn & Metcalf, 1975). There are some criteria defined for coliphages to be a faecal contamination indicator. These include: (i) it should be found with human enteric virus; (ii) it should have number equal to or greater than human enteric virus recovered; (iii) it should be resistant to adverse environmental conditions and water treatment processes; and (iv) the most important one is that isolation and enumeration should be easier, quicker and cheaper than human enteric viruses (Kott, 1984). A large number of researches have been carried out where coliphages were used to estimate the level of faecal contamination (Araujo *et al.*, 1997), as an indicator for faecal pollution in surface and ground water (O'Keefe & Green, 1989; Snowden *et al.*, 1989), to check the efficiency of drinking water treatment plant (Payment & Franco, 1993) and as an indicator to the overall water quality (Wentsel *et al.*, 1982). Bacteriophages, especially coliphages, have been considered as possible indicators of faecal contamination as well as reliable indicators of enteric viruses because they are relatively similar in origin, structure, size, morphology, composition, release, transport, adverse environmental condition tolerance power such as survival, longer persistence patterns and densities in the aquatic environment (Araujo *et al.*, 1997; Chung & Sobsey, 1993; Cole *et al.*, 2003; Funderburg & Sorber, 1985; Gantzer *et al.*, 1998; Gerba, 1987; Grabow, 2004; Grabow *et al.*, 1995; Grabow *et al.*, 1995; Havelaar *et al.*, 1986; Havelaar *et al.*, 1993; Hot *et al.*, 2003; Hsu *et al.*, 2002; Kott, 1966; Kott *et al.*, 1974; Lucena *et al.*, 2003; Shah & McCamish, 1972). Coliphages' absence in water indicates absence of human enteric viruses and can be assayed at a very low cost compared to assay of pathogenic enteric viruses. In addition to this it was shown that the coliphages can persist longer than enteric viruses in temperature ($<5^{\circ}$ and $\geq 50^{\circ}$ C) and with different matrix (Bertrand *et al.*, 2012), survived comparable to or better than hepatitis A, poliovirus, and rotavirus in seawater when exposed to low (5° C) and high (25° C) temperatures (Chung & Sobsey,

1993). Also coliphages generally require more than 30°C temperature for efficient phage infection and replication in host bacteria (Havelaar & Nieuwstad, 1985; Pillai, 2006). The optimum temperature needs for maximum outgrowth of F-pilli on *E.coli* is 37°C and ceased at below 25°C. Hence, multiplication and inhibition of coliphages production will be at 37°C and at below 25°C respectively. Along with required temperature, phage multiplication needs optimum phage and bacterial densities as well as optimum bacterial physiological condition. These conditions are rarely found in natural water environment that leads to low number of coliphages count (Maite Muniesa & Jofre, 2004; Novotny & Lavin, 1971).

2.3 Occurrence and Distribution of Bacteriophages and Coliphages in Environment

There are a large number of studies carried out which deals with the occurrence and distribution of coliphages in various environments. A number of coliphages, including somatic coliphages, F-specific DNA and F-specific RNA coliphages have been found and reported in various kinds of environment. Most of the studies focused on water bodies such as rivers, lakes, groundwater, seawater, beach sand and water in sewage treatment plant. The main purposes of most studies carried out was to investigate and propose the coliphages as an indicator of faecal contamination (Araujo *et al.*, 1997; Bonilla *et al.*, 2007; Charles *et al.*, 2009; Davies *et al.*, 2003; Gantzer *et al.*, 1998; Grabow *et al.*, 1984; Haramoto *et al.*, 2009; Miernik, 2004; Paul *et al.*, 1997; Søgaaard, 1983; Sun *et al.*, 1997; Wu *et al.*, 2011; Zaiss, 1981). Coliphages were recovered from the faeces of all types of warm blooded animals with highest number reported in pigs and cows. In addition to this, a habitat preference was displayed by various coliphages.

Some serological groups were prevalent in animal faeces and others in human faeces (Osawa *et al.*, 1981). Coliphages have been recovered ubiquitously from raw sewage as well as sewage treated up to various level such as activated sludge, oxidation ponds, trickling filters, aerosols near sewage treatment plant and chlorinated final effluent (Bell, 1976; Chang *et al.*, 1981; Dhillon & Dhillon, 1974; Dias & Bhat, 1965; Durham & Wolf, 1973; Fannin *et al.*, 1977; Glass & O'brien, 1980; Havelaar & Hogeboom, 1983; Ignazzitto *et al.*, 1980; KENARD & VALENTINE, 1974; Kennedy *et al.*, 1985; Kott *et al.*, 1974). The quantitative distribution of coliphages was estimated in the longitudinal profiles of river and it ranged from 0 to 2380 PFU/ mL and from 0 to 2550 PFU/g in water and sediment respectively (Zaiss, 1981). In another quantitative examination of coliphages, the number of phages present per 100 mL of water samples are less than 10 and only 6 samples were having more than 100 phages (Søgaaard, 1983). Some more quantitative studies were carried out where coliphages concentration was found between 50 and 980 MPN/100 mL in the Oldman River in Canada (Bell, 1976), 11.1 and 33.2 MPN/100 mL in Ottawa River (Dutka *et al.*, 1987) and 0 and 18 number of phages per mL in water samples (Miernik, 2004). In an another research in which investigation

was done to estimate the densities of bacteriophages in river waters in different geographical areas, the coliphages concentration found out ranged from 0 to 6.4, 0 to 5.5 and 0 to 4.3 for somatic coliphages, F-specific bacteriophages and phages infecting *Bcat. fragilis* respectively (Lucena *et al.*, 2003). The study carried out on 11 urban rivers and creeks to investigate the viral impacts on coastal waters of southern California reported the concentration of somatic and F-specific coliphages. It ranged from <2 (below detection limit) to 7597 PFU/100 mL and <2 (below detection limit) to 853 PFU/100 mL respectively (Jiang & Chu, 2004). Seasonal variation in the occurrence and distribution of coliphages has been reported and it was shown that bacteriophage populations did vary concurrently with the change of seasons (Huynh & Kory, 1993). In a study conducted to investigate the occurrence of various *E. coli* host- specific coliphages demonstrated that coliphages concentration varied in Tapovan to Rishikesh stretch of the Ganga River. It varied from 8 to 400 PFU/L during pre- monsoon and from 4 to 374 PFU/L during post-monsoon season (NEERI, 2004a). All these studies reported in the literature indicate that the coliphages concentration in river water occurs in low number. Acceptance of coliphages as an indicator for faecal pollution in sewage contaminated river water is rather limited in the developing countries due to the lack of simple, efficient and less expensive detection and enumeration techniques capable of detecting low number of coliphages that can be easily adopted by less sophisticated laboratories.

2.3.1 Methods for Detection and Enumeration of Bacteriophages and Coliphages

There are a number of methods that have been used for detection and enumeration of bacteriophages. These methods can be categorized into ‘**culture based methods**’ and ‘**rapid methods**’, as it takes one day or less, which include immunology and molecular based methods. Each type of method has its own advantages and disadvantages. A large number of phages are usually detected and enumerated by plaque assay methods, a culture based technique, the principles of which were designed by Adams in 1959. Plaques are the zone of clearance made on the lawn of bacteria when bacteriophages lyse it. (APHA, 2001; Cornax *et al.*, 1990; Grabow & Coubrough, 1986; Grabow *et al.*, 2001; ISO, 1995, 2000, 2001, US EPA, 2001a, b; Eaton *et al.*, 2005; Rodríguez *et al.*, 2012a). International Organisation for Standardisation (ISO) also published bacteriophages estimation methods for F-specific RNA bacteriophages, somatic coliphages, and bacteriophages infecting *Bacteroides fragilis* (*B. fragilis*) (ISO, 1995, 2000, 2001). In addition to this a large number of rapid methods have been developed which include immunology based methods, molecular methods and Fast Phage such as culture latex agglutination and typing [CLAT]), multiple types of PCR and a modified rapid version of EPA Method 1601 respectively (Brown *et al.*, 2015; Brussaard, 2004; Leon *et al.*, 1990; Fong & Lipp, 2005; Gentilomi *et al.*, 2008; Haramoto *et al.*, 2009; Jiang & Chu, 2004; Kirs & Smith, 2007; Love & Sobsey, 2007; Salter *et al.*, 2010; Wolf *et al.*, 2008; Rodríguez *et al.*, 2012b).

Biological and physical factors important for bacteriophage and coliphage detection and enumeration: A wide variety of host bacteria has been used for the detection and enumeration of coliphages. In case of somatic coliphages the host strains are susceptible to a different spectrum of phages. Therefore counts of phages are not similar for different hosts. Some strains of host give higher counts than others. It was shown that the wild-type strains of *E.coli* are poor hosts for the detection and enumeration of wide variety of coliphages in wastewaters as they mask the majority of phage receptors sites, present in the R-core of the cell wall lipopolysaccharide, by complete O-antigen. In addition to this, wild type *E.coli* has nuclease enzymes as one more defence mechanism. These nuclease enzymes destroy nucleic acid of phages during penetration and thus replication of the phage is prevented. Rough or semi-rough laboratory strains of *E.coli* are more productive as they lack O-antigen. (Bell, 1976; Dhillon & Dhillon, 1974; Havelaar & Hogeboom, 1983; Hilton & Stotzky, 1973). Most commonly used laboratory strains of *E. coli* for the detection and enumeration of somatic coliphages are:

E. coli B (Ayres, 1977; Bell, 1976; T. Dhillon *et al.*, 1970; Gerba *et al.*, 1978; Ignazzitto *et al.*, 1980; Kott, 1966; Nupen *et al.*, 1981); *E. coli* C (Borrego *et al.*, 1990; Dhillon *et al.*, 1976; KENARD & VALENTINE, 1974; Wentsel *et al.*, 1982); *E. coli* CN13 (Armon & Kott, 1993) or derivatives of *E. coli* K-12 strains like W3110 (Primrose *et al.*, 1982). Host bacteria strain such as *E. coli* strain K12 and *E. coli* HS and *E. coli* HS- RC were used for the detection and enumeration of F-RNA (male-specific) phages as they carry fertility (F) plasmid which produces fertility fimbriae having receptor sites for F-RNA (male-specific) phages (Debartolomeis & Cabelli, 1991; Dhillon *et al.*, 1970; Glass & O'brien, 1980); the strain, known as WG49, produced by transfer of F- plasmid into *Salmonella typhimurium* and then deleting its gene coding for pathogenicity for safe use in laboratory for F-RNA (male-specific) phages detection (Havelaar *et al.*, 1985). It was found that *Bacteroides fragilis* designated HSP40 (ATCC 51477) were infected by phages found in the faeces of human and not in the warm-blooded animals such as cows, pigs, rabbits, mice, poultry or quail faeces. Therefore it may be useful to distinguish between faecal pollution of human and animal origin (Grabow *et al.*, 1995; Jagals *et al.*, 1995; Tartera & Jofre, 1987).

One of the important factors which influence the result of coliphage detection and enumeration is the volume of sample used for the test. It was reported that by using large volume of samples the somatic coliphages, F-RNA coliphages and *B. fragilis* phages can show positive results otherwise it gives negative results by conventional plaque assays using small volumes of water such as 1 to 10 mL. The direct plaque assays on 100 mL volume of water have been developed which resemble double agar-layer plaque assays commonly used on smaller volumes of water but gives higher counts (Grabow, 1998; Grabow & Coubrough, 1986; Hayward, 1999; Uys, 1999). Qualitative presence-absence test for bacteriophage detection was first described in 1948. In this test, a

mixture of nutrients, host culture and sample of water under investigation was incubated overnight for replication of phage (which may be a single phage) if present. After that replicated phages were readily detected by plaque assays or spot tests (Guelin, 1948; Grabow *et al.*, 1993; Hilton & Stotzky, 1973; Kott, 1966). Qualitative test was developed into quantification of coliphages by Most Probable Number (MPN) method in which the volume used were 10 mL, 1 mL and 0.1 mL of water sample (Kott, 1966). The method developed by Kott, 1966 was suitable when concentration was not very low as less volume of water sample lowers the probability of occurrence of coliphages. It has been shown that sensitivity reduces when less volume of water sample is used (Sinton *et al.*, 1996). Rapid detection methods are developed to get the results in short duration of time compared to the plaque assay methods. Although, it has been shown that these methods have merit for certain purposes but at the same time these are complicated, labour-intensive and expensive (Armon & Kott, 1993; Ijzerman *et al.*, 1994). Some of the 'culture based methods' and 'rapid methods' are briefly described as follows:

- (i) **Culture-Based Methods:** There are wide varieties of culture based methods developed for detection and enumeration of bacteriophages. All these methods are based on the principles given by Andre Gratia in 1936 and finally formalized by Mark Adams in 1959.

In *Standard Agar Overlay Plaque Assay Method*, a mixture is prepared by adding phage dilutions with a permissive host bacterium and dispersed evenly onto solid medium. On incubation, a lawn of host bacterium formed and interrupted by a clear or translucent circular area of lysed cells because of phage-infection, phage-multiplication, and phage-liberation chain reaction events termed as plaque formation. Thus, the plaques formed due to infectious phage particles are counted as plaque-forming units (PFU). Although the method permits isolation of phages, their characterization by plaque morphology (clear versus turbid lysis, size of plaque, presence/absence of a halo), and the isolation of phage mutants from the individual plaques but it has some disadvantages such as (a) inaccuracy of coliphages count because some phages produce small and turbid plaques; (b) inefficient to detect low count of phages; and (c) occurrence of false positive results (Kott, 1966; Serwer *et al.*, 2004; Sobsey, 1982).

Most Probable Number (MPN) Method is developed to detect and enumerate the low count of phages. This method was established for evaluation of low levels of coliphages by means of MPN technique well established for estimation of coliform bacteria. The procedure includes agar layer method described by Adams, 1959 for phage assay in conjunction with MPN method used for the enumeration of coliforms. For MPN method to be applied, three sets of tubes in replicates of five were inoculated as follows. In first set, 10 mL of double-strength PAB broth was inoculated with 10 mL of water sample. In second set, 10 mL of single-strength PAB broth was inoculated with 1 mL of water sample. And in

the third set 10 mL of single-strength PAB broth was inoculated with 1 mL of 1:10 dilution water sample. After that 0.1 mL of *E.coli* cells at a concentration of approximately 10^8 per milliliter was added to each tube. Then each tube was incubated, after shaking thoroughly, at 35 degree Celsius for 16 hours. Thereafter, a loopful from each incubated tube was transferred to freshly seed *E. coli* B plates and incubated for 6 hours at 35 degree Celsius. At the end of incubation time, result was observed and taken as positive where plaque had formed and estimation was done by computing the result with MPN table developed for coliform bacteria (Kott, 1966). Although it is widely used for estimation of coliphages, it has some limitations such as (a) need of unique host strains specific for the phage under test; (b) interferences due to endobacterial species present in samples; (c) less sensitivity for samples having very low count of phages.

A large number of modifications have been done in the standard agar overlay phage plaque assay. Most of these focused on recovery of bacteriophages by using membrane filtration techniques such as cartridge filters (electropositive or electronegative), glass fiber filters, glass wool filters, vortex flow filtration, tangential flow filtration, and acid flocculation (Gantzer *et al.*, 1999; Harding *et al.*, 1957; Jiang *et al.*, 2001; Lipp *et al.*, 2001; Pallin *et al.*, 1997). These techniques rely upon the fact that the bacteriophage will adsorb onto a suitable filter media as they carry predominantly negative charge at or near neutral pH. The charge on the phages can be modified to positive by decreasing pH if electronegative filters are used. Otherwise the samples are passed through electropositive filter media. The adsorbed phages from filter media are released into the elution buffer with high pH. After that these phages are used for normal plaque assay method or with some modification. After incubation with suitable host it produces cytopathic changes or plaques depending upon the virus and host in questions (Loehr & Schwegler, 1965; Méndez *et al.*, 2004; Nupen *et al.*, 1981; Reynolds *et al.*, 1993; Sinton *et al.*, 1996; Sobsey *et al.*, 1990). There are some limitations also with filtration method like low phage collection efficiency due to adsorption of phage onto membrane surface. In some studies, enrichment of bacteriophages were done by, first filtering the water samples through 0.22 μm cellulose acetate filter paper to remove endobacterial cells and then filtered water was inoculated with broth of mixed bacterial culture for enrichment of phages (Cornaxet *et al.*, 1990, khairnaret *et al.*, 2014, Petrovskiet *et al.*, 2011).

A wide variety of *colorimetric methods* have been developed over the years. The method developed by American Public Health Association (APHA) uses *E. coli* C as a host and 2,3,5-triphenyltetrazolium (TPTZ) in the agar. The intact bacteria show pink colour on growth while plaques shows no colour (APHA, 1992). However, there are three main limitations associated with the APHA method. The first one is the difficulty in accurately determining a coliphage count due to a lack of a significant color contrast on an agar plate (Ijzerman & Hagedorn, 1992). The second one is the inability to detect low numbers of coliphages (Kott, 1966). The APHA recommends its procedure for use only when there are

greater than 5 PFU per 100 mL of sample (APHA, 1992). The last one is the appearance of plaque-like areas in the agar which, when counted, can lead to false-positive results (Sobsey, 1982). To overcome the limitations imposed by APHA (1992) method, two new colorimetric methods were developed for coliphage detection. One was *colorimetric agar based (CAB) method* (Ijzerman & Hagedorn, 1992) and other one was a *liquid colorimetric presence – absence (LCPA) method* (Ijzerman *et al.*, 1993). These methods are based on hydrolysis of β -galactosides coupled to chromogenic molecules resulting into release of chromogen and formation of unique colour product. The enzymes β -galactosidase required for hydrolyses are released from lysed cells and therefore indicates the presence of coliphages in the sample. In laboratory studies, the CAB and LCPA methods proved to be superior, easier to read and interpret, more rapid, simpler to perform, and highly sensitive. Both these methods have some limitations like requirement of *E. coli* C strain as host, higher cost of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) (one of the ingredients used in CAB method), and propagation of false negative results when phages concentration are low (Ijzerman *et al.*, 1993; Ijzerman & Hagedorn, 1992).

Besides these there were more methods developed by various agencies like International Organisation for Standardisation (ISO), United State Environmental Protection Agency (USEPA). The ISO methods for enumeration of somatic coliphages, F-specific RNA phages and phages infecting *B. fragilis* have been published as an ISO 10705-series. The ISO series methods have been evaluated by comparison studies in Europe. On the other hand USEPA developed and standardized a number of methods like EPA Method 1601 (two-step enrichment process) and EPA Method 1602 (single agar layer method). EPA Methods 1601 and 1602 have also undergone multi-laboratory validation (US EPA 2003a, b). These culture-based methods have been applied to rivers, estuaries, drinking water, surface water, storm water, groundwater and wastewater (Ballester *et al.*, 2005; Bonilla *et al.*, 2007; Borchardt *et al.*, 2004; Davies *et al.*, 2003; Francy *et al.*, 2011; Gomila *et al.*, 2008; Havelaar, 1987; Locas *et al.*, 2007; Locas *et al.*, 2008; Lodder & de Roda Husman, 2005; Love *et al.*, 2010; Lucena *et al.*, 2004; Nappier *et al.*, 2006; Rodríguez *et al.*, 2012; M. Sobsey *et al.*, 2004; Stewart-Pullaro *et al.*, 2006).

The ISO methods recommend use of nalidixic acid-resistant *E. coli* CN-13 and *E. coli* C as a host culture for samples having high and low endobacterial concentration respectively. For low number of coliphages detection, ISO recommends *E. coli* K-12 Hfr or *E. coli* HS (pFamp) as a host strain. The agar used for this method should be semi-soft TYG (Tryptone Yeast Extract Glucose) agar having calcium-glucose solution mixed with 1 mL sample volume and then poured over bottom agar plate. This method gives confirmatory result for which RNase (40 μ g/mL) is used in TYG. Only limitation for this method is to have a separate phage specific *E. coli* as host strain (ISO, 1995, 2000, 2001).

EPA Method 1601 describes a qualitative two-step enrichment procedure for coliphages and

developed to determine faecal contamination in groundwater (US EPA, 2001a). However, this validated procedure is used to determine the presence or absence of F-specific and somatic coliphages in groundwater, surface water, and other waters (US EPA, 2003a). This method can be used quantitatively for enumeration of coliphages in most probable number (MPN) format (spot-plating). In this method, a 100 mL or 1 liter groundwater sample is enriched with log-phase host bacteria (*E. coli* Famp for F-specific coliphages and *E. coli* CN-13 for somatic coliphages) for coliphages. After an overnight incubation, samples are inoculated on to a lawn of host bacteria. Then incubated, and examined for plaques, which indicate the presence of coliphages. Control experiments of a coliphage positive reagent (enumerated sewage filtrate or pure cultures of F-specific RNA coliphage MS2 or somatic coliphage ΦX174) water sample and a negative reagent water sample (method blank) are done in parallel to the main experiment for quality control purposes.

The *EPA Method 1602* is a single agar layer method which can be used for quantification of coliphages in a water sample. Procedure can be used to quantify coliphages in a sample. In this method a 100 mL water sample added with the log-phase host bacteria (*E. coli* Famp for F-specific coliphage and *E. coli* CN-13 for somatic coliphage) and 100 mL of double-strength molten tryptic soy agar is prepared. Then mixture is poured, after thoroughly shaken, into multiple plates and incubated for 16 to 24 hours. After incubation plaques are counted and summed for all plates from a single sample. Here also control experiments of a coliphage positive reagent (enumerated sewage filtrate or pure cultures of F-specific RNA coliphage MS2 or somatic coliphage ΦX174) water sample and a negative reagent water sample (method blank) are done in parallel to the main experiment for quality control purposes. EPA method 1601 is considered more sensitive than EPA Method 1602 due to the larger sample volumes used in 1601 (100 mL to 1 L) compared to Method 1602 (100 mL) (Salter *et al.*, 2010).

Rapid Methods: In recent times, a large number of bacteriophages detection methods have been developed which can be used to get results faster than plaque assay methods, although each method has some advantages and disadvantages in terms of speed, accuracy, form of results (i.e., quantitative, qualitative, infectivity of virus), and level of training and equipment required.

Polymerase Chain Reaction Methods: The most common and advanced type of molecular method used to detect coliphages is based on the Polymerase Chain Reaction (PCR). PCR is a cycling process of denaturing, annealing, and extension of new DNA fragments or amplicons. With the help of PCR process DNA of samples is amplified exponentially that can be visualized on an agarose gel (Innis *et al.*, 1990). Depending on quantitative or qualitative information needed, different types of PCR are used. Although there are no universal primers available for the detection of coliphages, but still individual coliphage

strains can be detected with the help of availability of strains specific primers.

(a) *RT-PCR*: Reverse Transcription - Polymerase Chain Reaction has been developed for the detection of F-specific RNA coliphages. The viral RNA is first reverse transcribed into complementary DNA, which is used as a template for the PCR reaction (Fong & Lipp, 2005; Kirs & Smith, 2007; Wolf *et al.*, 2008).

(b) *Quantitative (q) PCR and RT-qPCR*: Both qPCR and RT-qPCR assays have been developed for the quantification of coliphages corresponding to the amount of nucleic acid present. These PCR assays are often used to detect only a subgroup of the total coliphages that would be quantified by plaque assays. Some qPCR methods measure fluorescence generated during each PCR cycle. The underlying idea behind this assay is to establish relation between DNA counts determined by the qPCR and the number of viable phage particles determined by plaque assay. The former will then be used to determine phage concentrations in water samples. A method based on PCR has been performed on digital microfluidic platforms, used to detect bacteriophages and especially coliphages. This method, coliphages Digital PCR, on microfluidic chips has potential to do a fast and accurate high-throughput technique estimate for phage genome quantification (Anderson *et al.*, 2011; Edelman & Barletta, 2003; Gentilomi *et al.*, 2008; Hua *et al.*, 2010; Jebrail & Wheeler, 2010; Kirs & Smith, 2007; Mark *et al.*, 2010; Smith, 2006; Tadmor *et al.*, 2011; Yong *et al.*, 2006; US EPA, 2007, 2010).

(c) *Multiplex PCR*: Another modification of PCR methods is Multiplex PCR such as multiplex qPCR, RT-PCR, and RT-qPCR in which multiple target sequences are detected in the same reaction tube. Therefore it is possible, and generally used, to detect more than one type of phage in one sample (US EPA, 2007, 2010). For example, RT- qPCR only quantitatively detects one type of coliphage per tube (i.e., GII F-specific RNA coliphage) while multiplex RT-qPCR quantitatively detects multiple phage targets per tube (i.e., GI, GII, and GIII F-specific RNA coliphages) (Kirs & Smith, 2007).

Culture Latex Agglutination and Typing (CLAT): CLAT method combines a two-step enrichment process with latex agglutination. Serotyping has been validated and used to monitor the presence of coliphages in faecal contaminated beach waters (Griffith *et al.*, 2009; Love & Sobsey, 2007; Rodríguez *et al.*, 2012; Wade *et al.*, 2010). This rapid antibody-based method indicates samples are positive if visible clumps formation occurred on the agglutination card after 60 seconds and absence of such clumps signifies negative samples (Love & Sobsey, 2007).

Fast Phage Modified Method 1601: A modified EPA Method 1601, called Fast Phage, incorporates the use of shelf-stable, ready-to-use reagents in a simplified format. In this method, β -D-1-thiogalactopyranoside is used as an enrichment medium to induce

transcription of the host *E. coli* lac operon. A large amplification and a rapid extracellular beta-galactosidase enzyme release during lysis of the coliphage infected host cells compared to uninfected host cells because lysis of *E. coli* by coliphages is coupled with lac operon expression. This method is approved for detection of coliphages in groundwater under EPA's Alternative Test Procedure program (Salter & Durbin, 2012; Salter *et al.*, 2010).

Microscopy Method: Bacteriophages can be enumerated by Transmission Electron Microscopy (TEM) after negative staining of or by epifluorescence microscopy after staining with DNA fluorochromes. However, these techniques have limitations such as epifluorescence microscopy suffers from significant background problem and the equipment cannot be used for general purpose as it is very expensive (Carlson, 2005). A nanoparticle tracking analysis (NTA) based approach is used to detect bacteriophages as they are of nano order size. This method utilizes laser illuminated optical microscopy for direct, real time visualization of nanoparticles in a clear liquid. The nanoparticles are detected as light scattering centers moving under Brownian motion, and they are counted in a few seconds or few minutes. But these methods are under initial stages and need to be validated (Anderson *et al.*, 2011; Edelman & Barletta, 2003).

2.4 Summary Remarks

The review presented here suggests that the bacteriophages are the entities which infects and kills bacteria, consequently helps in reducing bacterial load in water bodies. The sources of coliphages reported in literature are faecal matter of human origin and other warm blooded animals. Therefore it was found that coliphages were detected and enumerated in different kinds of water bodies such as river water, sewage water, lake water, seawater, groundwater due to discharge of domestic sewage and other anthropogenic activities. With this knowledge, a number of studies have been carried out to investigate the potential of coliphages as faecal pollution indicator. With this background a study is warranted to investigate if bacteriophages, particularly coliphages, are unique to Ganga river waters, as claimed in some of the studies.

A wide variety of methods, 'Culture based methods' and 'Rapid methods', have been developed for the detection and enumeration of bacteriophages especially coliphages in water samples. However, they have one or more limitations such as requirement of specific host bacteria leading to dependency on cell culture center, less sensitive toward low phage concentration in water samples, inaccuracy in coliphages enumeration due to turbid plaques formation, interferences due to endobacterial growth in culture based methods while rapid methods are very expensive, require sophisticated instruments and demand lots of training and familiarity with the test to be carried out. With all these informations, to overcome the limitations and constraints stated above for earlier

methods, a development of new method or adoption of a method for a particular application is warranted.

3 Objectives

It is believed that Ganga water has some mysterious factors that make it special. Some studies suggest that this mysterious factor is the presence of bacteriophages. Bacteriophages are the entities which infect and kill bacteria and hence, if present in river water, helps in decreasing the bacterial number and microbial pollution in rivers. On the other side literature review suggests that the sources of coliphages are faecal matter of human and animal origin. If this is the case, then presence of coliphages should be associated with contamination from human and animal wastes. Thus, presence of coliphages appear not be an inherent property of river Ganga and should plausibly be associated with human and animal activity in the catchment area. Therefore, investigations are warranted to determine if there is any relationship with coliphages present in water samples and other biological and physico-chemical parameters that determine the degree of pollution of river waters.

Various 'Culture based' and 'Rapid methods' have been developed for the concentration, detection and enumeration of phages in river water, sewage water, lake water, seawater and groundwater. Culture based methods have advantages such as they are less expensive and require less sophisticated instruments. However, they have one or more limitations such as requirement specific host bacteria leading to dependency on cell culture center, less sensitive toward low phage concentration in water samples, inaccuracy in coliphages enumeration due to turbid plaques formation, interferences due to endobacterial growth. MPN method was developed to eliminate the trouble in counting of plaques. But the method has limitation that it is less sensitive for low phage counts in water samples. On the other hand 'Rapid methods', based on colorimetric and molecular biology, give results in less time and with high accuracy. But they are very expensive, require sophisticated instruments and demand lots of training and familiarity with the test to be carried out.

With aforementioned background the main objective of the present study was to investigate whether bacteriophages are unique to Ganga river waters. To answer this question given the state-of-the-art on the subject, the research work was carried out on following lines.

- Selection and adoption of suitable method for enumeration of coliphages in low concentration in water samples.
- Validating the adopted method for enumeration of coliphages using variety of samples collected from different sources.
- Collection of water samples from various locations in river Ganga and from various other rivers

- Assessment of various water quality parameters and enumeration of coliphages on all samples.
- Observing the correlation between selected water quality parameters and coliphages.

4 Materials and Methods

4.1 Materials

4.1.1 Plasticwares, Glasswares and Other Apparatus

Sterile 15 mL and 50 mL Spinwin conical centrifuge tubes (Tarsons, India) were used. Measuring cylinders of 25 mL, 50 mL, 100 mL, 250 mL and 500 mL (Borosil, India) were used to measure required sample volumes. Beaker of volume 50 mL, 100 mL, 250 mL and 500 mL (Borosil, India) were used to store samples during experiment. Round bottom and flat bottom flasks of 250 mL (DURAN, Germany) were used for digestion of water samples for metal analysis. Erlenmeyer flasks of 250 mL and 100 mL form (DURAN, Germany) were used for Phage enrichment process and other parameter analysis. Burette stand and 50 mL burette (Borosil, India) were used for titration. Kjeldahl flask of 100 mL (Borosil, India) was used for digestion of water samples for TKN and phosphates analysis. Erlenmeyer flasks of 250 mL and 100 mL and 20 mL test tubes were used for bacteriophage analysis. Petri Dish of 100 mm from Borosil, India was used for plating of culture. All the glassware were kept in 5% sulphuric acid for 10- 12 hours, and then washed in soap solution. Then they were kept in oven overnight at 180°C. Glassware used for microbiological purpose was sterilized by autoclaving at 121°C and 15 psi pressure for 15 minutes.

4.1.2 Chemicals and Reagents

A number of chemicals were used for a particular study. All the chemicals were analytical reagent grade of more than 99% purity. Chemicals like sodium chloride, magnesium sulphate, manganese sulphate and calcium chloride used for Phage Assay Base (PAB) are from LobaChemie, India.

4.1.3 Pipettes and Membrane Filter Assembly

Micropipette (Accupipet, Tarsons, India) of 20-200 µL and 100-1000 µL were used for culture transfer onto media plates. Tips of 20-200 µL and 100-1000 µL (Tarsons, India) used during microbiological work were autoclaved at 121°C and 15 psi pressure for 15 minutes to sterilize them. Membrane filter assembly having 0.22 µm pore size sterile membrane filter (Diameter: 47 mm, Millipore, USA) was to remove endobacterial species from water samples respectively.

4.1.4 Culture Media

Media used for biological purpose, for each specific purpose, was of high purity. A number of growth and enrichment media were used in the current study. EMB agar media (Merck, USA) was used for the isolation of location specific host coliform bacteria from each water sample. Nutrient broth (HIMEDIA, India) was used for enrichment of isolated host coliform bacteria from EMB agar media. The media used in the MPN method for bacteriophage estimation (Kott, 1966) were agar for plating (Adams, 1959) and Phage Assay Base (PAB) broth having following composition: beef extract, 3.0 g; peptone, 5.0 g; sodium chloride, 5.0 g; magnesium sulphate, 0.2 g; and manganese sulphate, 0.05 g in one liter of distilled water (Kott, 1966). Calcium chloride 0.15 g per liter of PAB broth was added in PAB broth after sterilization at 121°C and 15 psi for 20 minutes. In the proposed method, the amount of water sample used for enrichment of bacteriophage was in large volume (Katiyar, 2012) compared to Kott's MPN method, therefore double strength PAB broth was used. Beef extract, 6.0 g; peptone, 10.0 g; sodium chloride, 10.0 g; magnesium sulphate, 0.4 g, and manganese sulphate, 0.1 g were added in one liter of distilled water to make a double strength PAB broth media. Then calcium chloride 0.30 g per liter of PAB broth was added in PAB broth after sterilization at 121°C and 15 psi for 20 minutes. Soyabean Casein Digest agar (Merck, USA and HIMEDIA, India) plates were made for inoculation and spreading of enriched bacteriophage culture, so that lawn of host bacteria with plaque, if bacteriophage present in water samples, can be formed after incubation. Soyabean Casein Digest agar is generally used for this purpose as it does not have serum, which has bacteriocidal and bacteriolytic activity against gram-negative bacteria like coliforms (Taylor, 1983). Lauryl Tryptose Broth (LTB) (HIMEDIA, India and Merck, USA) was used for the detection and enumeration of coliform in river water sample by multiple tube fermentation techniques.

4.1.5 Instruments, Machines and Equipments

Various instruments, machines and equipment were used to carry out the given research. A refrigerator (REMI, India) at 4°C temperature was used for preservation of samples. Vertical laminar hood (Rescholar, India) was used to provide particle free, bacteria free environment as working station for plating and culturing. To provide the bacteria and phage an optimum environment for growth, incubators at 37°C and 45°C temperature were used. For faecal coliform (FC) growth, an incubator at 45°C temperature was used while other incubations were done at 37°C. An autoclave was used to sterilize the glassware, media and distilled water. TOC-L (Shimadzu, Japan) was used for estimation of total carbon (TC), total organic carbon (TOC) and inorganic carbon (IC). In addition to this pH meter for pH determination, thermometer for temperature measurement, conductivity meter to determine the current carrying potential of water samples, turbidity for estimation of clarity of water and oven (180°C) for total dissolve solid measurement were used during current study.

4.2 Methods

4.2.1 Bacteriophage Detection and Estimation

The earlier methods described by various researchers have some limitations. These methods either need specific host bacteria to form plaques or have less sensitivity as less volume is used for test, which results into less chance or probability of phage occurrence. Some other methods are time consuming as plates having lawn of host bacteria need to be plated and incubated for 24 hours, and also enriched bacteriophage culture needs to be filtered and centrifuged to isolate and concentrate bacteriophage. The advance methods like DNA probe method are expensive and require high skilled personnel as well as sophisticated instruments.

To overcome the above problems, the method used in the present studies was developed by carrying out modifications, research and analysis of results. Modifications like,

- a) Isolation of host bacteria from the sample itself so that there will be no dependency on cell culture center for host bacteria.
- b) Water sample used for inoculation and concentration of phage was 50 mL, 25 mL and 10 mL in replicates of five, so that probability or chances of phage occurrence for plaques formation can be increased. It was found out theoretically that bacteriophage concentration as low as 0.24 phage concentration per 100 mL of water sample can be estimated.
- c) Same phage enriched culture having host bacteria with water sample was used on media plate for lawn formation and plaque formation. Hence, no extra time is required to make plates of lawn of bacteria.
- d) Water sample was filtered through 0.22 μm membrane filter which excluded the prerequisite of centrifugation and filtration steps before addition of concentrated phage solution into host bacteria culture or pouring on bacterial lawn. This saved time and waste generation of centrifuge filter tube was reduced.

As the method also did not require any genetic level studies and detection was possible by visible plaques, therefore skilled technician and high cost instrument dependency was omitted. Hence, the developed method can be used to achieve flexibility in sample size for estimation of low concentration phage and at the same time it is less tedious, time consuming, no separate host dependency, less waste generation and cost efficient compared to other agar layer phage plaque methods.

Developed Protocol for the Detection and Enumeration of Bacteriophages:

1. Prepare EMB agar plates by pouring and solidifying EMB agar in sterilized plate to isolate coliform bacteria. Prepare EMB agar by dissolving 35.95 g in one liter of distilled water and then autoclave at 121°C and 15 psi for 15 minutes.

2. Take 100 μL of water sample (diluted water samples if pollution load is high) with micropipette and pour onto EMB agar plate and spread with spreader.
3. Incubate the plate for growth of coliform bacteria at 37°C for 24 hours.
4. Filter 500 mL water sample through 0.22 micron filter paper with help of filter assembly and keep in refrigerator at 4°C .
5. To enrich coliform bacteria, inoculate 20 mL nutrient broth media with coliform cells by transferring it, from a colony on EMB agar plate, using inoculating loop, and incubate at 37°C for 24 hours.
6. Prepare phage assay base (PAB) broth by dissolving required components in distilled water (as mentioned in Section 4.1.4: Culture Media) and autoclave at 121°C and 15 psi for 15 minutes.
7. To enrich bacteriophage, inoculate PAB broth with water sample and enriched coliform bacteria in three sets of glassware consisting of five replicates. Shake the solution thoroughly and incubate at 37°C for 24 hours:
 - Set 1: 50 mL PAB broth + 50 mL Filtered water samples + 500 μL enriched coliform bacteria
 - Set 2: 25 mL PAB broth + 25 mL filtered water samples + 250 μL enriched coliform bacteria
 - Set 3: 10 mL PAB broth + 10 mL filtered water samples + 100 μL enriched coliform bacteria
8. Prepare soyabean casein digest agar plate for formation of bacterial lawn and plaques. Soyabean Casein Digest agar is made by dissolving 40g of media in one liter of distilled water and then autoclave at 121°C and 15 psi for 15 minutes.
9. After 24 hours of incubation, take 100 μL enriched bacteriophage culture onto Soyabean Casein Digest agar plate and spread properly with spreader.
10. Incubate the plate for formation of lawn and plaque at 37°C for 24 hours.
11. After 24 hours of incubation, observe the plate for plaques formation and record the positive tube combination and compute MPN.

4.2.2 Estimation of Bacteriophage

There are various methods for estimation of Most Probable Number (MPN) reported in literature to arrive at microbe's concentration and bacteriophage concentration. All these methods are derived from the original work on numerical interpretation of fermentation tube results. However, all these methods were derived for Most Probable Number (MPN) value per milliliter or gram of samples. For per 100 milliliter or gram, the final answer is calculated from the value given for per milliliter of sample. The other parameters like Standard Deviation (SD), 95% confidence interval are also calculated for

per 100 mL sample from the value derived for per milliliter sample and therefore, there are chances of occurrence of some error in final value. In this study, equations for number of organisms per 100 mL (λ) has been derived and computations are done using these equations for all the parameters required for describing MPN, including MPN per 100 mL, Standard deviation (SD), 95% confidence interval, Rarity Index and category of results (Jarvis *et al.*, 2010) as follows.

‘S’ is the large volume of water sample, for which MPN is performed, having ‘ λ ’ number of microorganisms present per 100 mL. Let us consider the volume of all microorganisms as ‘a’ and divide the water present into the same unit volume particle. Suppose we select one particle and P is the probability that the selected particle is not any microorganism, then

$$P_{\lambda=0} = \frac{\frac{S}{a} - \frac{S\lambda}{100}}{\frac{S}{a}}$$

Where,

$$\frac{S}{a} = \text{Total number of particles in volume } S, \frac{S\lambda}{100} = \text{Number of bacteria in volume } S,$$

$$\frac{S}{a} - \frac{S\lambda}{100} = \text{Number of particle as water in volume } S$$

On simplification, $P_{\lambda=0} = 1 - \frac{a\lambda}{100}$

Therefore, Probability that selected particle is microorganisms, $P_{\lambda \neq 0} = \frac{a\lambda}{100}$

Suppose 1 mL of total sample is removed then the total probability will be expressed by binomial theorem as $\left[\left(1 - \frac{a\lambda}{100} \right) + \frac{a\lambda}{100} \right]^1$. On solving this by binomial theorem and taking logarithm on both sides and for volume ‘v’ mL instead of removing 1 mL, P and Q will be,

$$P_{\lambda \neq 0} = \left(1 - e^{\left(-\frac{v\lambda}{100} \right)} \right) \text{ and } P_{\lambda=0} = \left(e^{\left(-\frac{v\lambda}{100} \right)} \right)$$

Therefore, total probability will be expressed by binomial distribution for single dilution (actual sample volume) inoculated in 5 tubes as,

$$P_{\lambda} = \frac{(p+q)!}{p!q!} \left(1 - e^{\left(-\frac{v\lambda}{100} \right)} \right)^p \left(e^{\left(-\frac{v\lambda}{100} \right)} \right)^q$$

Where, ‘p’ = Number of tubes showing growth and ‘q’= number of tubes showing no growth.

In the current study, total three dilutions were used and each dilution is inoculated in five tubes Where, V_1 = Volume of samples removed equal to 50 mL, V_2 = Volume of samples removed equal to 25 mL, V_3 = Volume of samples removed equal to 10 mL, p_1 = number of tubes showing growth in 50 mL samples, q_1 = number of tubes showing no growth in 50 mL samples, p_2 = number of tubes showing growth in 25 mL samples, q_2 = number of tubes

showing no growth in 25 mL samples, p_3 = number of tubes showing growth in 10 mL samples, q_3 = number of tubes showing no growth in 10 mL samples.

Therefore total probability for this given study was estimated using equation derived above for all the three dilutions and 5 inoculated tubes for each dilution.

$$P_{\lambda} = \frac{(p_1 + q_1)!}{p_1! q_1!} \left(1 - e^{\left(-\frac{v_1 \lambda}{100}\right)}\right)^{p_1} \left(e^{\left(-\frac{v_1 \lambda}{100}\right)}\right)^{q_1} \times \frac{(p_2 + q_2)!}{p_2! q_2!} \left(1 - e^{\left(-\frac{v_2 \lambda}{100}\right)}\right)^{p_2} \left(e^{\left(-\frac{v_2 \lambda}{100}\right)}\right)^{q_2} \\ \times \frac{(p_3 + q_3)!}{p_3! q_3!} \left(1 - e^{\left(-\frac{v_3 \lambda}{100}\right)}\right)^{p_3} \left(e^{\left(-\frac{v_3 \lambda}{100}\right)}\right)^{q_3}$$

The above function is the likelihood function of λ for the value of number of positive tubes for each dilution,

$$L = L(\lambda, v_1, v_2, v_3, p_1, p_2, p_3)$$

This likelihood function $L(\lambda)$ will give probability result of the given serial dilutions corresponding to each possible concentration of λ . But MPN will be that value which maximizes the likelihood function. To get MPN, we use the Loglikelihood function as it has also maximum at the same value as function itself.

$$\ln L = \ln \frac{(p_1 + q_1)!}{p_1! q_1!} + \ln \left(1 - e^{\left(-\frac{v_1 \lambda}{100}\right)}\right)^{p_1} + \ln \left(e^{\left(-\frac{v_1 \lambda}{100}\right)}\right)^{q_1} + \ln \frac{(p_2 + q_2)!}{p_2! q_2!} \\ + \ln \left(1 - e^{\left(-\frac{v_2 \lambda}{100}\right)}\right)^{p_2} + \ln \left(e^{\left(-\frac{v_2 \lambda}{100}\right)}\right)^{q_2} \\ + \ln \frac{(p_3 + q_3)!}{p_3! q_3!} + \ln \left(1 - e^{\left(-\frac{v_3 \lambda}{100}\right)}\right)^{p_3} + \ln \left(e^{\left(-\frac{v_3 \lambda}{100}\right)}\right)^{q_3}$$

To Calculate $MPN(\hat{\lambda})$, first derivative of loglikelihood function with respect to λ was done and it is equated to zero and then solving $\frac{dy}{dx} = 0$, the final equation obtained was,

$$\frac{p_1 v_1}{1 - e^{\left(-\frac{v_1 \lambda}{100}\right)}} + \frac{p_2 v_2}{1 - e^{\left(-\frac{v_2 \lambda}{100}\right)}} + \frac{p_3 v_3}{1 - e^{\left(-\frac{v_3 \lambda}{100}\right)}} - (v_1 n_1 + v_2 n_2 + v_3 n_3) = 0$$

To get $Var(\hat{\lambda})$ of the variance $Var(\hat{\lambda})$ of $(\hat{\lambda})$, second derivative of Loglikelihood function was carried out,

$$\frac{d^2 \ln L}{dx^2} = \frac{1}{(100)^2} \times \left\{ \frac{p_1 (v_1)^2 e^{\left(-\frac{v_1 \lambda}{100}\right)}}{\left(1 - e^{\left(-\frac{v_1 \lambda}{100}\right)}\right)^2} + \frac{p_2 (v_2)^2 e^{\left(-\frac{v_2 \lambda}{100}\right)}}{\left(1 - e^{\left(-\frac{v_2 \lambda}{100}\right)}\right)^2} + \frac{p_3 (v_3)^2 e^{\left(-\frac{v_3 \lambda}{100}\right)}}{\left(1 - e^{\left(-\frac{v_3 \lambda}{100}\right)}\right)^2} \right\}$$

And then $Var(\hat{\lambda})$ is estimated as,

$$\begin{aligned} \text{Var}(\hat{\lambda}) &= -\frac{1}{\frac{d^2 \ln L}{dx^2} |_{\lambda = \hat{\lambda}}} \\ &= \frac{1}{\frac{1}{(100)^2} \times \left\{ \frac{p_1(v_1)^2 e^{\left(-\frac{v_1 \lambda}{100}\right)}}{\left(1 - e^{\left(-\frac{v_1 \lambda}{100}\right)}\right)^2} + \frac{p_2(v_2)^2 e^{\left(-\frac{v_2 \lambda}{100}\right)}}{\left(1 - e^{\left(-\frac{v_2 \lambda}{100}\right)}\right)^2} + \frac{p_3(v_3)^2 e^{\left(-\frac{v_3 \lambda}{100}\right)}}{\left(1 - e^{\left(-\frac{v_3 \lambda}{100}\right)}\right)^2} \right\}} \end{aligned}$$

Thereafter, standard deviation of the estimate $\hat{\lambda}$ is calculated as

$$SD = \sqrt{\text{Var}(\hat{\lambda})}$$

There are various methods reported in literature to calculate confidence interval but they all are either tedious or require removal of results which are most unlikely to occur. Here, an approximation of maximum likelihood method is used according to which natural logarithm $\ln \hat{\lambda}$ of $\hat{\lambda}$ will give an approximately normal distribution having calculated variance

$$\sigma_{\ln \hat{\lambda}}^2 = \frac{\text{Var}(\hat{\lambda})}{(\hat{\lambda})^2}$$

Or,

$$\sigma_{\ln \hat{\lambda}}^2 = \frac{1}{\hat{\lambda}^2 \times \frac{1}{(100)^2} \times \left\{ \frac{p_1(v_1)^2 e^{\left(-\frac{v_1 \lambda}{100}\right)}}{\left(1 - e^{\left(-\frac{v_1 \lambda}{100}\right)}\right)^2} + \frac{p_2(v_2)^2 e^{\left(-\frac{v_2 \lambda}{100}\right)}}{\left(1 - e^{\left(-\frac{v_2 \lambda}{100}\right)}\right)^2} + \frac{p_3(v_3)^2 e^{\left(-\frac{v_3 \lambda}{100}\right)}}{\left(1 - e^{\left(-\frac{v_3 \lambda}{100}\right)}\right)^2} \right\}}$$

Therefore approximate 95% confidence interval will be,

$$\text{Lower limit} = \hat{\lambda} \times e^{(-2\sigma_{\ln \hat{\lambda}}^2)} \text{ to } \text{Upper limit} = \hat{\lambda} \times e^{(2\sigma_{\ln \hat{\lambda}}^2)}$$

And,

$$\text{Standard Deviation } \log_{10} \text{MPN} = \log_{10} \times e^{\sqrt{\sigma_{\ln \hat{\lambda}}^2}}$$

Another parameter which is used for the given study is Rarity Index. There are various combinations of positive tubes or outcomes which are most unlikely to occur but still that may occur sometime. For example, $p_1=0$, $p_2=3$ and $p_3=5$ outcomes might occur sometime but they violate the assumptions made under MPN determination (McCrary, 1915). Therefore an index has been introduced by Blodgett (2002, 2008) which categorises the outcomes into various categories. This index is known as Rarity Index and it is calculated as:

$$\text{Rarity Index} = \frac{L(\hat{\lambda})}{L_0(\hat{\lambda})} = \pm \frac{e^{\sum_1^k \left\{ \ln \frac{(p_i+q_i)!}{p_i!q_i!} + p_i \times \ln \left(1 - e^{\left(-\frac{v_i \lambda}{100} \right)} \right)^{p_i} - q_i \frac{v_i \lambda}{100} \right\}}}{\left(\max_{(p_1, \dots, p_k)} \left[e^{\sum_1^k \left\{ \ln \frac{(p_i+q_i)!}{p_i!q_i!} + p_i \times \ln \left(1 - e^{\left(-\frac{v_i \lambda}{100} \right)} \right)^{p_i} - q_i \frac{v_i \lambda}{100} \right\}} \right]} \right)}$$

Here, $L(\hat{\lambda})$ is the likelihood for the result of serial dilutions having positive number of tubes, p_1, p_2, p_3 for estimated MPN($\hat{\lambda}$).

$$L(\hat{\lambda}) = e^{\sum_1^k \left\{ \ln \frac{(p_i+q_i)!}{p_i!q_i!} + p_i \times \ln \left(1 - e^{\left(-\frac{v_i \lambda}{100} \right)} \right)^{p_i} - q_i \frac{v_i \lambda}{100} \right\}}$$

And $L_0(\hat{\lambda})$ is the maximum of the likelihood $L(\hat{\lambda})$

$$L_0(\hat{\lambda}) = \left(\max_{(p_1, \dots, p_k)} \left[e^{\sum_1^k \left\{ \ln \frac{(p_i+q_i)!}{p_i!q_i!} + p_i \times \ln \left(1 - e^{\left(-\frac{v_i \lambda}{100} \right)} \right)^{p_i} - q_i \frac{v_i \lambda}{100} \right\}} \right] \right)$$

This can only be achieved when the condition given below is fulfilled:

$$p_i = (n_i + 1) * \left(1 - e^{\left(-\frac{v_i \lambda}{100} \right)} \right)$$

Where, $i = 1, 2, 3$.

Following categories are defined on the basis of value of Rarity Index (r):

1. Category 1: ($0.05 \leq r \leq 1$)
The MPN value is most likely to occur if “ r ” value falls within this range.
2. Category 2: ($0.01 \leq r < 0.05$)
The MPN value is rare if “ r ” value falls within this range.
3. Category 3: ($0 < r < 0.01$)

The MPN value occurs extremely rarely if “ r ” value falls within this range.

Based on the above derivation and formula, an excel program was developed for the calculation of MPN Index and other MPN parameters. With the help of excel program, a MPN table was generated for different combinations of positive results (See Appendix section, Table: A1, A2).

4.2.3 Assessment of Water Quality Parameters

Current study also aimed at assessment of water quality parameters, such as biological and physico-chemical parameters, for the determination of correlation between selected water

quality parameters and occurrence and densities of coliphages in river water samples. All the selected water quality parameters were determined according to the protocol given in 'Standard Methods for the Examination of Water and Wastewater' (APHA, 1995) (refer Table 4.1).

Table 4.1: Standard Methods used for the Assessment of Selected Water Quality Parameters

S.No.	Water Quality	Standard Methods
1.	Faecal Coliform	Multiple Tube Fermentation Technique (Part - 9221), only presumptive test by using Lauryl Tryptose Broth media
2.	Total Coliform	
3.	pH	Electrometric method (Part - 4500-H ⁺ B) by using potentiometric pH meter
4.	Alkalinity	Titration method (Part - 2320 B)
5.	Dissolve Phosphate	Vanadomolybdophosphoric Acid Colorimetric method (Part - 4500-P C) by using Spectrophotometer in filter (0.45 μ m) samples
6.	Total Phosphate	Vanadomolybdophosphoric Acid Colorimetric Method (Part - 4500-P C) by using Spectrophotometer in sulphuric acid and nitric acid digested samples
7.	Ammonical Nitrogen	Colorimetric Method (Part – 4500)
8.	Total Kjeldahl Nitrogen	Semi-Micro-Kjeldahl method (Part - 4500-N _{org} C)
9.	Chemical Oxygen Demand (COD)	Closed Reflux, Titrimetric method (Part - 5220 C)
10.	Conductivity (μ S/cm)	Conductivity (Laboratory) Method (Part - 2510 B) by using Self-contained conductivity instrument
11.	Total Dissolve Solid (TDS)	Total Dissolved Solids dried at 180 degree Celsius (Part - 2540 C)
12.	Turbidity	Nephelometric method (Part - 2130 B) by using Nephelometer instrument
13.	Total carbon (TC)	Combustion – Infrared method (Part - 5310 B) by using TOC-L Instruments
14.	Inorganic Carbon (IC)	
15.	Total Organic Carbon (TOC)	

4.3 Site Selection and Collection of Water Samples for the Study

The main aim of current study was to corroborate or contradict the 'Bacteriophage Theory' believed to be responsible for special quality of River Ganga. For this a large number of river water samples other than River Ganga and different kinds of water need

to be tested. Therefore rivers selected to carry out research and sites on the river chosen for collection of water samples were based on following criteria.

- Sampling sites should be from different climatic regions of India to determine, if any, climatic effects on coliphages concentration in water samples.
- Perennial and monsoon rivers should be selected to compare the coliphages concentration results obtained for these two rivers system in India.
- Large number of sample should be collected to eliminate biased results due to fewer samples.

Keeping the aforementioned criteria, a total of 40 sampling sites (For locations on map see Figure A1 in Appendix section) were selected: on Bhagirathi river (5), Mandakini river (1), Assi Ganga river (1), Alaknanda river (4), Ganga river (12), Yamuna river (9), Gomati river (1), Ken river (1), Betwa river (1), Ram Ganga river (2), Kali river (1), Sindh river (1) and Chambal river (1). The geographical details of sampling sites with site code are presented in Table 4.2.

Table 4.2 Sampling Locations Selected for the Current Study

Site Code	Sampling Stations	River	North Coordinates	East Coordinates
S-1	Gangotri	Bhagirathi River	30°59'38.7"	78°56'29.7"
S-2	U/S Maneri Bhali	Bhagirathi River	30°44'26.7"	78°32'37.4"
S-3	D/S Maneri Bhali I	Bhagirathi River	30°44'15.5"	78°29'56.7"
S-4	D/S Maneri Bhali II	Bhagirathi River	30°44'19.2"	78°22'29.2"
S-5	Assi Ganga, Uttar Kashi	Assi Ganga	30°45'37.4"	78°27'20.0"
S-6	U/S Mandakni, Rudraprayag	Mandakani River	30°17'18.7"	78°58'46.2"
S-7	U/S Alakananda, Rudraprayag	Alaknanda River	30°17'14.6"	78°59'00.8"
S-8	U/S Srinagar	Alaknanda River	30°13'15.6"	78°48'30.1"
S-9	Kirti Nagar	Alaknanda River	30°12'54.4"	78°44'36.5"
S-10	U/S Alakananda, Devprayag	Alaknanda River	30°08'43.1"	78°35'54.4"
S-11	U/S Bhagirathi, Devprayag	Bhagirathi River	30°08'44.1"	78°35'51.8"
S-12	Sangam, Devprayag	Ganga River	30°08'42.8"	78°35'51.4"
S-13	U/S Pahulok Barrage,	Ganga River	30°07'22.0"	78°18'41.8"
S-14	D/S Pashulok Barrage,	Ganga River	30°04'14.6"	78°17'05.1"
S-15	Haridwar	Ganga River	29°57'31.0"	78°10'32.0"
S-16	Fatehgarh	Ganga River	27°23'56.9"	79°37'39.7"
S-17	Bithoor	Ganga River	26°36'50.2"	80°16'30.5"
S-18	Bhairav Ghat	Ganga River	26°29'42.2"	80°19'34.5"
S-19	Gola Ghat	Ganga River	26°27'59.44"	80°22'30.31"
S-20	Shukla Ganj	Ganga River	26°28'21.98"	80°22'30.52"

S-21	Jajmau Bridge	Ganga River	26°25'44.4"	80°24'47.5"
S-22	Jana Village	Ganga River	26°24'23.32"	80°27'4.12"
S-23	Mehandi Pur, kannauj	Ganga River	27° 0'41.89"	79°59'11.67"
S-24	Hanuman Chatti	Yamuna River	30°55'58.7"	78°23'54.3"
S-25	Barkot	Yamuna River	30°50'23.9"	78°15'50.9"
S-26	Dakpathar	Yamuna River	30°30'06.3"	77°47'41.7"
S-27	Takrupur,	Yamuna River	26° 36' 06.0"	79° 07' 11.6"
S-28	Kanjausa	Yamuna River	26° 25' 58.2"	79° 12' 46.4"
S-29	Auraiya,	Yamuna River	26° 25' 23.6"	79° 28' 35.7"
S-30	Bagariya	Yamuna River	25°57'25.9"	80°09'35.7"
S-31	PateoraDaria,	Yamuna River	25°55'11.5"	80°13'45.4"
S-32	Madanpur	Yamuna River	25°46' 23.6"	80° 31' 58.7"
S-33	Dabri	RamGanga River	27°29'50.0"	79°41'45.9"
S-34	Hullapur	RamGanga River	27°40'59.4"	79°37'18.9"
S-35	Dhakra	Chambal River	26° 32' 39.4"	79° 05' 20.4"
S-36	Bithauli	Sindh River	26° 26' 16.4"	79° 12' 32.3"
S-37	Katri Rampur Nauabad	Kali River	27° 1'15.20"	79°58'27.70"
S-38	Chilla	Ken River	25° 46' 03.6"	80° 31' 32.9"
S-39	Hamirpur	Betwa River	25°56'37.5"	80°09'16.4"
S-40	Lucknow	Gomati River	26°51' 14.2"	80°58' 11.7"

In addition to this different types of water samples were selected for determining the sensitivity, specificity and validation of developed method (Table 4.3)

Table 4.3 Different Types of Water Samples used for Specificity, Sensitivity and Validation of Developed Method

S. No.	Sample Code	Water Sample	S. No.	Sample Code	Water Sample
1.	V-1	River water	8.	V-8	Domestic Sewage
2.	V-2	River water	9.	V-9	Domestic Sewage
3.	V-3	River water	10.	V-10	Domestic Sewage
4.	V-4	River water	11.	V-11	Oxidation Pond
5.	V-5	River water	12.	V-12	Oxidation Pond
6.	V-6	River water	13.	V-13	Drinking Water
7.	V-7	Domestic Sewage	14.	V-14	Autoclaved water

4.4 Statistical Analysis

Statistical analysis for standardization, optimization and validation of developed method was done with the 'OriginPro 9.1' software. In addition to this linear correlation, pearson's coefficient (r_p), of coliphages with biological and physico-chemical parameters was calculated using linear regression and further the significance of the correlation

was determined by student's t-test with 95% confidence limits. Maximum positive correlation and maximum negative correlation corresponding to the Pearson's coefficient equals to +1 and -1 respectively. With t-test, $p < 0.05$ signifies significant correlation while $p > 0.05$ signifies that the correlation is insignificant.

5 Results and Discussion

5.1 General

There is general understanding, because of ancient knowledge, among people that River Ganga has some mystical power which gives it an inscrutable attributes over other rivers. These inscrutable attributes make Ganga water special, and is frequently used for many purposes including worshiping because it is believed that it does not putrefy on prolonged storage. It has been suggested in literature that one of the reasons for arresting the microbial activities could be the presence of Bacteriophages. Some of the studies carried out to decipher the sources indicate that coliphages are the parasites which generally live in the guts of human and animals. Consequently, faecal matter of human and other warm blooded animals could be the main sources of coliphages. Therefore the current study was carried out to investigate if the presence of bacteriophages, especially coliphages, is unique to Ganga river or they are universally distributed. It was found that the coliphages were present in many water samples selected for the study and their numbers were in the range of 0 to 7.98 with mean value of 2.68 MPN/100 mL. It is important to notice that similar results were obtained for river water, sewage, seawater, groundwater, lake waters and marine waters in Europe, South Africa, Israel, and the USA. To confirm this, additional studies were planned as part of this investigation to observe the correlation between various biological and physicochemical water quality parameters that are affected by anthropogenic activities and presence of coliphages.

5.2 Optimization and Standardization of Method Adopted for the Detection and Enumeration of Coliphages

With the help of procedure developed under current study a large number of samples were tested and observed for the plaques formation. A slime lawn was formed on each plate which indicated growth of bacteria. Among them, some of the plates were showing a clear or translucent circular area known as plaques on the bacterial lawn. The plaques indicated that phage-infection and phage-multiplication occurred within bacteria and finally bacteria were lysed during phage-liberation phase. These three phases of phage life (lytic) cycle had been repeated several times which resulted into plaques formation. In addition to this the quality of plaques observed were less turbid and clear enough to be seen by naked eye (Figure 5.1).

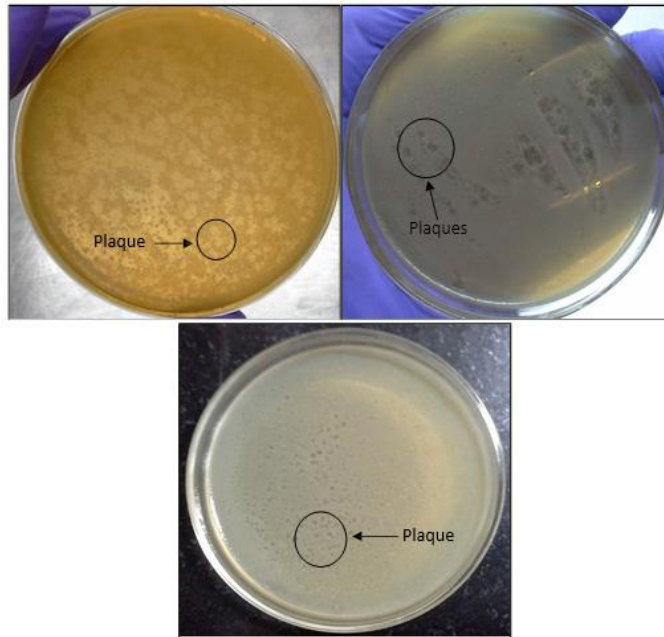


Figure 5.1 Plates Showing Positive Results for Plaques Formation

Some of the plates were also inoculated with different combinations of water samples and host bacteria as control experiments for the optimization and standardization of the method adopted. The results of control experiments are summarized in Table 5.1 and observations can be seen in Figure 5.2 and 5.3. Finally the plates showing plaques were recorded as positive results and coliphage concentration were computed with the excel sheet and MPN table prepared for this purpose based on probability theory.

Table 5.1: Results of the Control Experiments

S.No.	Control Experiments	Observation and Results
1.	Filtered water sample without host bacteria	No plaques
2.	Unfiltered water sample with host bacteria	More number of plaques with endobacterial growth
3.	Unfiltered water sample without host bacteria	Less number of plaques with endobacterial growth
4.	Distilled Water with host bacteria	Bacterial growth only
5.	Distilled water without host bacteria	Neither bacterial growth, no plaques

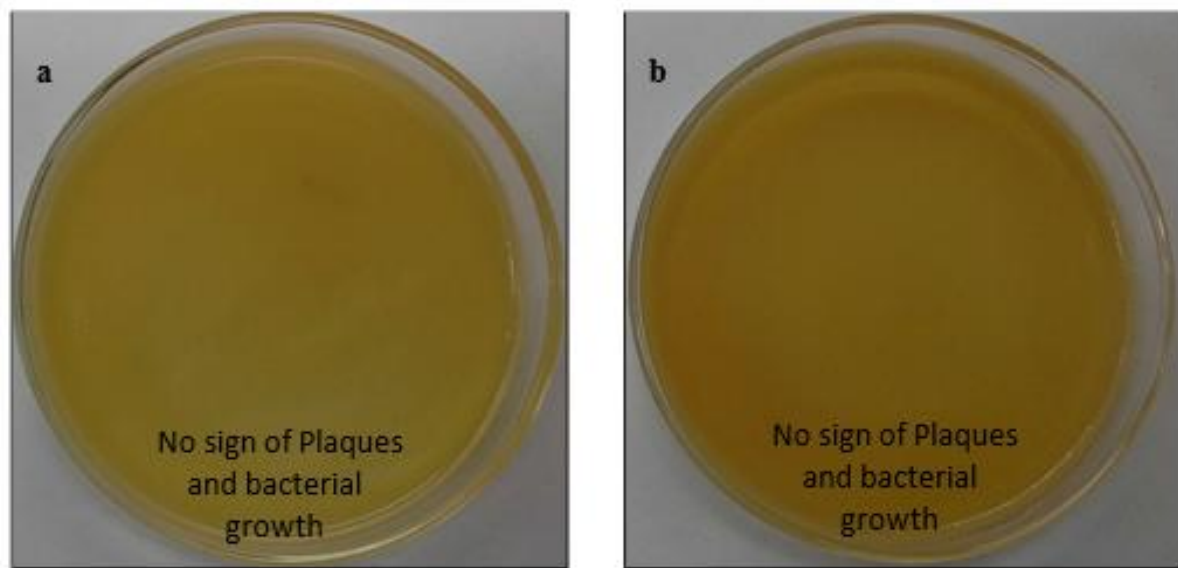


Figure 5.2 Plates inoculated for Control Experiments Showing Different Observations: (a) Distilled water without host bacteria; (b) Filtered water sample without host bacteria

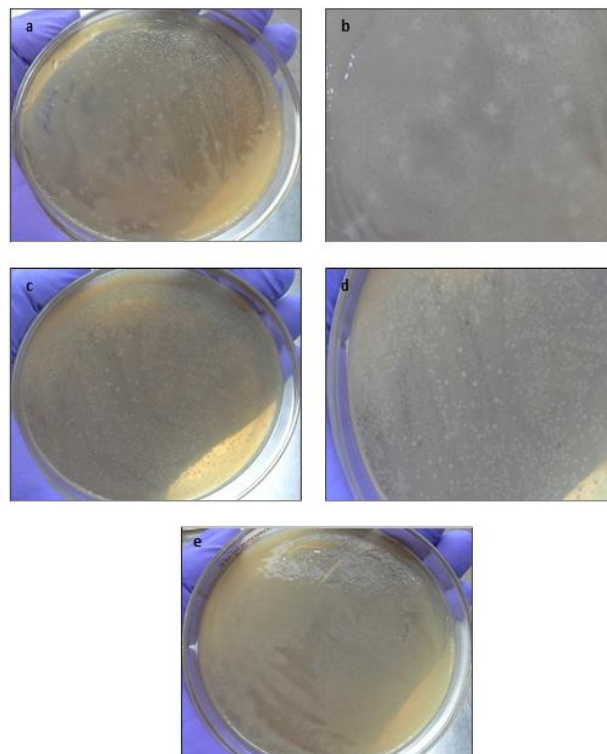


Figure 5.3 Plates Inoculated for Control Experiments Showing Different Observations:

(a) and (b) Unfiltered water sample with host bacteria; (c) and (d) Unfiltered water sample without host bacteria; (e) Distilled water sample with host bacteria

5.3 Derivation and Generation of Excel Spreadsheet and Standard MPN Table

An excel spreadsheet (See Appendix A1) and a set of standard MPN values (See Appendix A2, A3) were derived for bacteriophage estimation as most probable number (MPN) values. In Table A.1, columns 1 to 3 present possible combinations of positive and negative observations for inoculum volumes of 50 mL, 25 mL and 10 mL samples. Column 4 presents the MPN values, rounded to two significant figures per 100 mL of water samples. Values in column 5 and 6 are the estimates of log MPN and standard deviation of log MPN respectively. With these values, it is possible to estimate the uncertainty for the calculated MPN value that can aid in inferences on uncertainties caused by other sources. Column 7 and 8 present the probability of MPN values being lower and higher at 95% confidence level respectively. Values in column 9 indicate the calculated 'rarity value' for MPN values corresponding to each combination of positive and negative observations, and these values are used to determine category and acceptability of MPN results.

5.4 Evaluation and Validation of the Method

A protocol was adopted under current study for the detection and enumeration of bacteriophage. To validate the method, specificity and sensitivity was determined by experimenting on different types of water samples (Summarised in Table 5.2). The entire test on each water sample was performed according to the procedures developed and standardized under current study (See Section 4.2.1). The purpose of using different types of water sample was to determine its sensitivity towards all kind of water samples such as river water, sewage water and oxidation pond water. The specificity of the method was determined by testing the drinking water sample and autoclaved distilled water sample. Positive results (plaques formation) were observed in river water sample, sewage water sample and oxidation pond samples. This indicated its sensitivity toward coliphages detection. Negative results (no plaques formation) were observed in drinking water and autoclaved distilled water. This demonstrated its specificity toward coliphages detection.

Table 5.2: Summary of Results (Plaques Formation) Obtained for River Water, Sewage Water and Oxidation Pond Water

S. No.	Sample Code	Water Sample	Plaques Formation
1.	V-1	River water	Present
2.	V-2	River water	Present
3.	V-3	River water	Present
4.	V-4	River water	Present
5.	V-5	River water	Present
6.	V-6	River water	Present
7.	V-7	Domestic Sewage	Present
8.	V-8	Domestic	Present

9.	V-9	Domestic	Present
10.	V-10	Domestic	Present
11.	V-11	Oxidation Pond	Present
12.	V-12	Oxidation Pond	Present
13.	V-13	Drinking Water	Absent
14.	V-14	Autoclaved water	Absent

Further validation was carried out by testing two-fold serial dilutions of river water samples, sewage water samples and oxidation pond water samples. A series of two-fold serial dilution from 2^0 to 2^{-3} was prepared from undiluted water samples for this purpose. The results obtained (refer Table 5.2) further strengthen the sensitivity of the adopted protocol. The results also show that the coliphages concentration decreases proportionally to dilution factor in all water samples as shown in Figure 5.4 and 5.5.

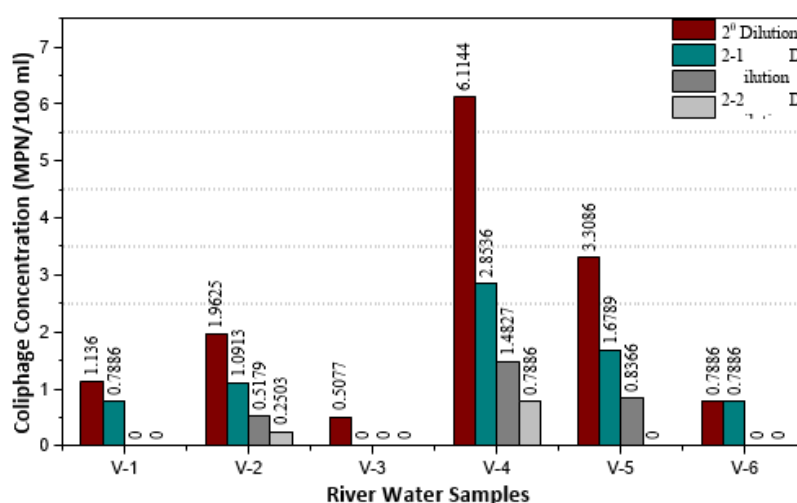


Figure 5.4: Variation Shown in Coliphages Concentration with Two-Fold Serial Dilutions of River Water Samples

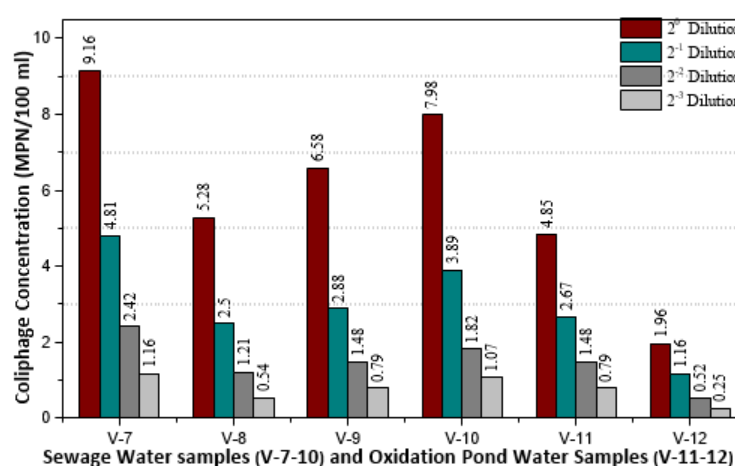


Figure 5.5: Variation Shown in Coliphages Concentration with Two-Fold Serial Dilutions of Sewage Water Samples and Oxidation Pond Water Samples

5.5 Coliphage Detection and Enumeration in River Water Samples

The water samples from different rivers were collected for the detection and enumeration of coliphages. To provide location specific host bacteria, water samples were inoculated on EMB agar media to isolate *E. coli* and number was determined as colony forming unit per mL. No colony of *E. coli* was observed in water sample from sampling site S-4 (Gangotri), a result similar to the earlier result reported by NEERI, 2004. Coliphages were detected and enumerated in water samples of each sampling site by using method adopted under current study. The results are presented in Figure 5.6. To each sample enriched culture of its corresponding isolated *E. coli* was added as host bacteria. The host bacteria used for water sample from S-1 sampling site was provided from *E. coli* isolated from S-2 Sampling site just downstream of S-1 site.

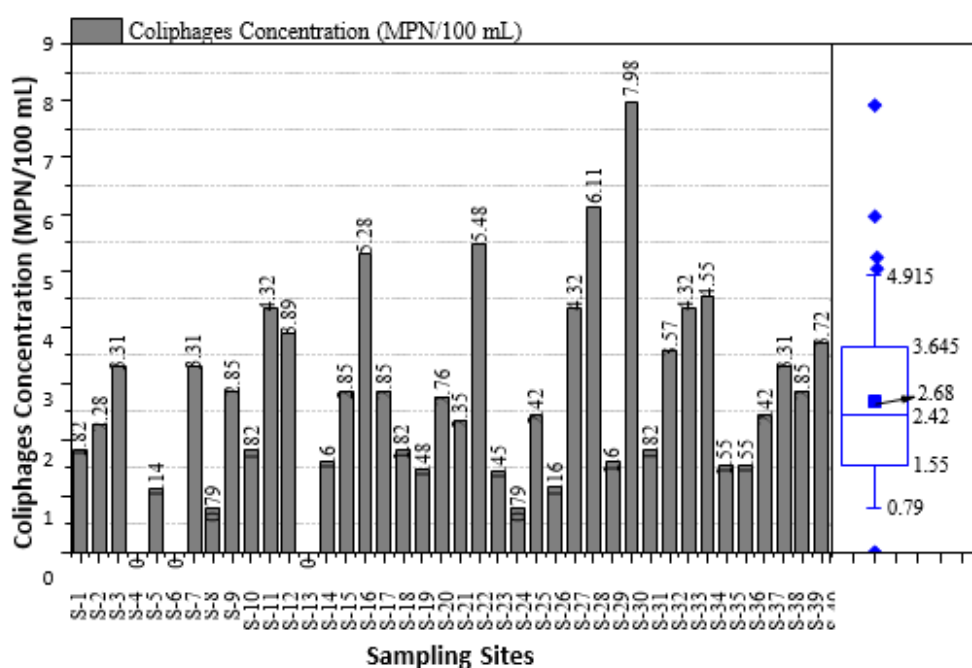


Figure 5.6: Variation in Coliphages Concentration between Different Locations

The finding of current study was very similar to the earlier studies on river waters (Borrego *et al.*, 1990; Borrego *et al.*, 1987; Šimková & Červenka, 1981), lake water (Bergh *et al.*, 1989; Kennedy *et al.*, 1985), sewage water (Adams, 1959; Cornax *et al.*, 1990), seawater (Borrego *et al.*, 1990; Yehuda Kott, 1966), and groundwater (Snowdon *et al.*, 1989) carried out for the detection and enumeration of bacteriophages in different parts of world. Studies carried out in Europe (Jofre *et al.*, 2000), South Africa (Grabow *et al.*, 1993), Israel (Armon 1993) and the USA (Chung *et al.*, 1998) also reported similar results for coliphages detection and enumeration. In a comprehensive study carried out by Lucen *et al.*, 2003 in river waters from Europe and South America reported that the number of bacteriophages were similar in the different geographical areas. The

concentration of bacteriophage found in the current study varied from 0 MPN/100 mL to 7.98 MPN/100 mL with mean value nearly 2.68 MPN/100 mL was very similar to the results reported in Ottawa River (11.1 and 33.2 MPN/100 mL) (Dutka *et al.*, 1987) and in water samples of Vistula River and Zegrze Reservoir (0 to 18 phages per mL) (Miernik, 2004). Among these the study carried out by Lucena *et al.*, (2003) reported phage concentration which was almost similar to what is observed in the current study. They reported phage concentration in the range 0 to 6.4, 0 to 5.5 and 0 to 4.3 for somatic coliphages, F-specific bacteriophages and phages infecting *B. fragilis* respectively. The study carried out by Jiang & Chu (2004) reported coliphages concentration well below 2 PFU/ 100 mL. NEERI (2004) also reported coliphages concentration in the range of 8 to 400 and 4 to 374 PFU/L during pre-monsoon and post-monsoon seasons respectively. Therefore the results found in the current study and all the above cited report suggests that occurrence of coliphages is universal and not limited to specific area and few water bodies. Also the concentration of phages is more or less similar.

5.6 Correlation between Densities of Coliphages and Some Biological and Physico-Chemical Water Quality Parameters

The values of various biological as well as physico-chemical parameters are presented in Table 5.3. Correlation between coliphages and biological as well as various physico-chemical parameters was analysed in order to determine the relationship and to examine the cause and effects of these parameters on coliphages. The results are presented in Figures 5.7 to 5.21.

Table 5.3: Estimated Values of Biological and Physico-Chemical Parameters in Different Samples Collected

S	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI
S-1	1.82	0	4.5	7.33	28	0.50	1.07	7	0.1	0.33	7	79.2	39	11.31	0.57	10.73
S-2	2.28	45	220	8.26	32	0.07	1.14	87.00	0.17	0.35	7	87.8	44	14.10	4.32	9.78
S-3	3.31	78	1100	7.3	24	2.50	4.86	127	0.19	0.38	7	76.8	39	12.48	4.33	8.15
S-4	0	110	790	7.55	16	0.43	3.71	116	0.15	0.98	7	77.2	40	11.13	4.40	6.73
S-5	1.14	170	790	7.65	20	0	2.50	12	0	0.75	7	61.8	31	13.04	5.87	7.17
S-6	0	40	260	7.39	14	0.71	2.57	8	0.08	0.15	7	41.2	21	11.47	4.47	7.00
S-7	3.31	110	790	8.05	36	0.36	2.07	134	0.35	1	7	125	63	14.41	10.98	3.43
S-8	0.79	45	260	8.15	40	1.50	2.86	76	0.02	0.98	7	107.4	54	17.64	8.16	9.48
S-9	2.85	4900	17000	7.57	52	0.57	1.07	149	0.35	0.48	7	168.2	85	17.39	11.80	5.59
S-10	1.82	45	1300	8.15	40	0.43	0.86	84	0.29	0.71	0	116.1	59	21.75	9.19	12.56
S-11	4.32	20	92	7.49	32	0.21	1.50	6	0.06	0.42	0	101.2	50	17.52	7.20	10.32
S-12	3.89	20	400	7.75	40	1.07	3.50	52	0	1.35	7	104.1	54	16.68	8.07	8.61
S-13	0	110	3500	7.92	32	0.07	2.86	38	0.44	0.94	21	123.1	62	15.79	9.86	5.92
S-14	1.6	20	1300	7.35	38	0.50	2.93	52	0.38	0.79	0	127.1	63	16.54	10.54	6.00
S-15	2.85	110	2400	7.99	52	0.14	5.07	51	0.52	1	21	143.4	74	15.29	11.56	3.73
S-16	5.28	170	330	8.5	84	0.36	2.79	12	0.17	0.38	21	222	111	19.50	18.14	1.35
S-17	2.85	790	2800	8.52	176	0.43	1.57	47	1	1.48	44	1342	668	30.05	27.34	2.71
S-18	1.82	22000	92000	8.91	224	0.64	1	49	0.56	1.42	29	712	351	36.46	26.92	9.54
S-19	1.48	11000	54000	7.58	172	0.14	2.64	26	0.42	1.79	53	721	360	38.31	34.69	3.62
S-20	2.76	13000	35000	7.51	168	0.29	1.07	36	1.92	2.71	75	1084	543	24.31	20.61	3.70

S-Sample Code; I- Coliphage concentration (MPN/100 mL); II- Faecal Coliform (MPN/100 mL); III-Total Coliform (MPN/100 mL), IV- pH; V- Alkalinity (mg/L); VI- Dissolved Phosphate (mg/L); VII- Total Phosphate (mg/L); VIII- Turbidity (NTU); XI- Ammonical Nitrogen (mg/L); X- Total Kjeldahl Nitrogen (mg/L); XI- Chemical Oxygen Demand (mg/L); XII- Conductivity ($\mu\text{S}/\text{cm}$); XIII- Total Dissolved Solids (mg/L); XIV- Total Carbon (mg/L); XV- Inorganic Carbon (mg/L); XVI- Total Organic Carbon (mg/L)

Table 5.3 Continued....

S	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI
S-21	2.35	350000	920000	7.28	132	0.36	2.64	0	0.33	1.15	30	612	306	33.69	32.33	1.36
S-22	5.48	240000	920000	8	168	0.07	0.93	28	0.35	1	68	712	351	35.07	32.44	2.64
S-23	1.45	260	3500	7.21	124	0.14	2.86	47	0.31	1.21	29	500	250	35.25	32.65	2.61
S-24	0.79	200	680	7.48	20	0.36	5.57	8	0.06	0.6	7	68	32	13.11	4.54	8.57
S-25	2.42	0	790	7.77	16	0.29	4.64	5.00	0.04	0.48	7	60.8	30	16.05	0.94	15.11
S-26	1.16	0	230	7.35	12	0.21	1.21	35	0	0.25	21	42	21	14.59	3.78	10.80
S-27	4.32	45	78	9.20	216	0.29	2.57	52	1.10	3.21	95	1680	838	39.15	36.55	2.60
S-28	6.11	490	1700	8.58	208	0.07	4	31	0.58	1.06	36	1642	824	35.74	34.14	1.60
S-29	1.6	45	78	8.38	224	0.00	4.29	43	0.88	2.44	36	1565	777	40.92	39.28	1.64
S-30	7.98	490	2200	8.39	244	0.21	2.07	133	0.92	1.33	58	1859	925	38.38	37.75	0.64
S-31	1.82	220	790	8.49	264	0.29	1.79	84	1.42	2.75	22	1868	933	40.23	38.48	1.76
S-32	3.57	490	3500	7.84	224	1.02	6.79	32	1.21	3.65	51	1573	786	38.82	36.83	2.00
S-33	4.32	13000	54000	9.14	196	0.36	3.00	51	1.67	2.46	44	747	372	39.72	31.06	8.66
S-34	4.55	260	3500	7.5	300	0.29	3.29	49	0.67	1.79	83	1084	541	32.34	31.35	0.99
S-35	1.55	110	170	8.34	180	0.07	2.43	35	1.63	1.98	22	1207	603	35.94	31.78	4.16
S-36	1.55	110	700	8.17	280	1.07	6.93	181	1.08	5.94	7	983	491	47.99	47.55	0.44
S-37	2.42	17000	92000	7.91	160	0.57	4	19	0.23	0.79	45	610	305	27.12	22.90	4.22
S-38	3.31	790	2400	8.12	264	0.21	1.43	23	0.46	1.35	36	1308	665	40.59	39.51	1.09
S-39	2.85	1700	9200	8.14	172	0.07	4.50	41	0.94	1.17	15	645	321	29.74	25.31	4.43
S-40	3.72	14000	35000	7.54	316	1.43	3.57	31	1.02	3.19	36	1278	628	61.52	54.78	6.74

S-Sample Code; I- Coliphage concentration (MPN/100 mL); II- Faecal Coliform (MPN/100 mL); III-Total Coliform (MPN/100 mL), IV- pH; V- Alkalinity (mg/L); VI- Dissolved Phosphate (mg/L); VII- Total Phosphate (mg/L); VIII- Turbidity (NTU); XI- Ammonical Nitrogen (mg/L); X- Total Kjeldahl Nitrogen (mg/L); XI- Chemical Oxygen Demand (mg/L); XII- Conductivity ($\mu\text{S}/\text{cm}$); XIII- Total Dissolved Solids (mg/L); XIV- Total Carbon (mg/L); XV- Inorganic Carbon (mg/L); XVI- Total Organic Carbon (mg/L)

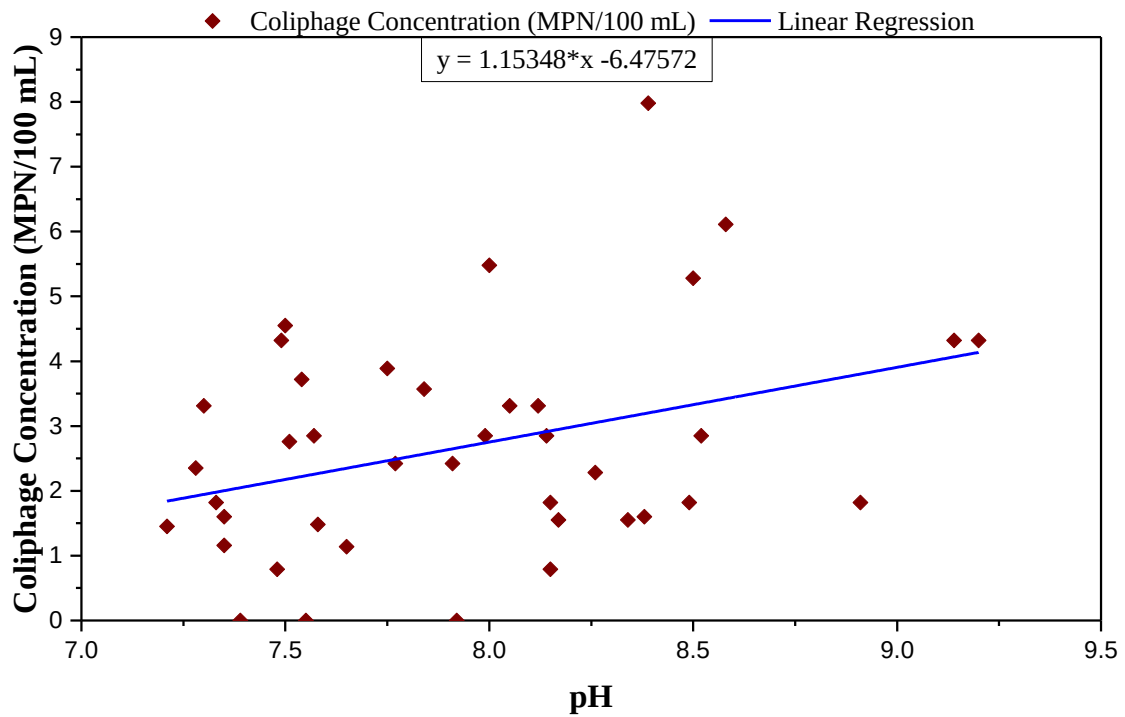


Figure 5.7: Linear Regression Plot between pH and Coliphages Concentration

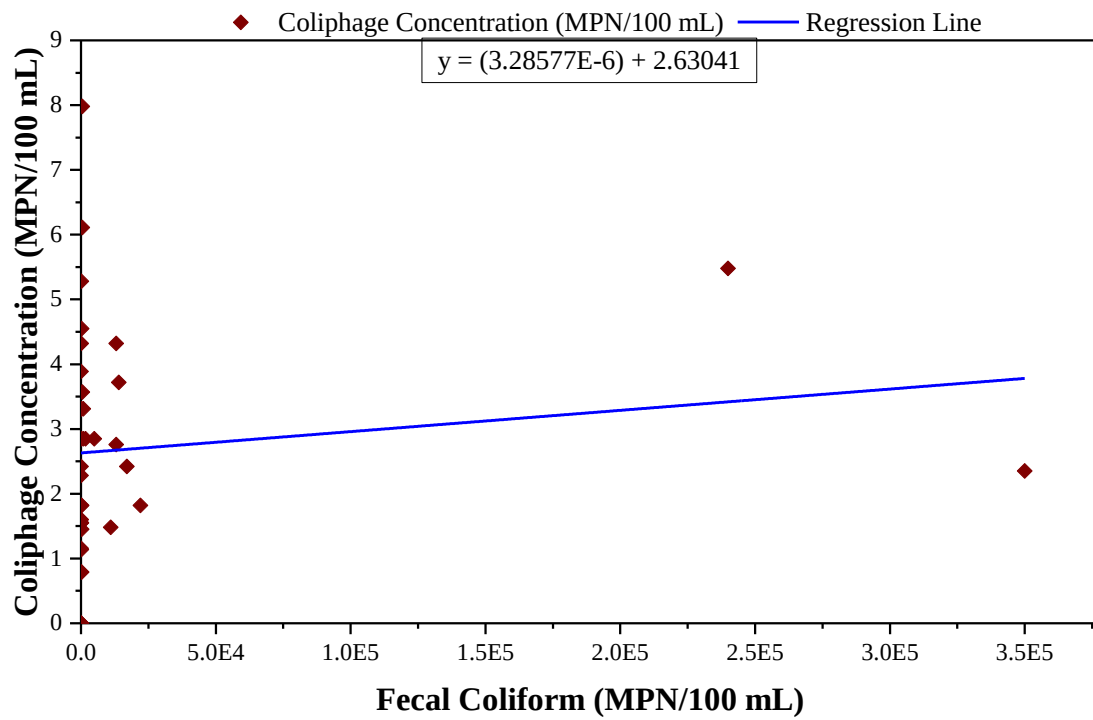


Figure 5.8: Linear Regression Plot between Faecal Coliform Concentration and Coliphages Concentration

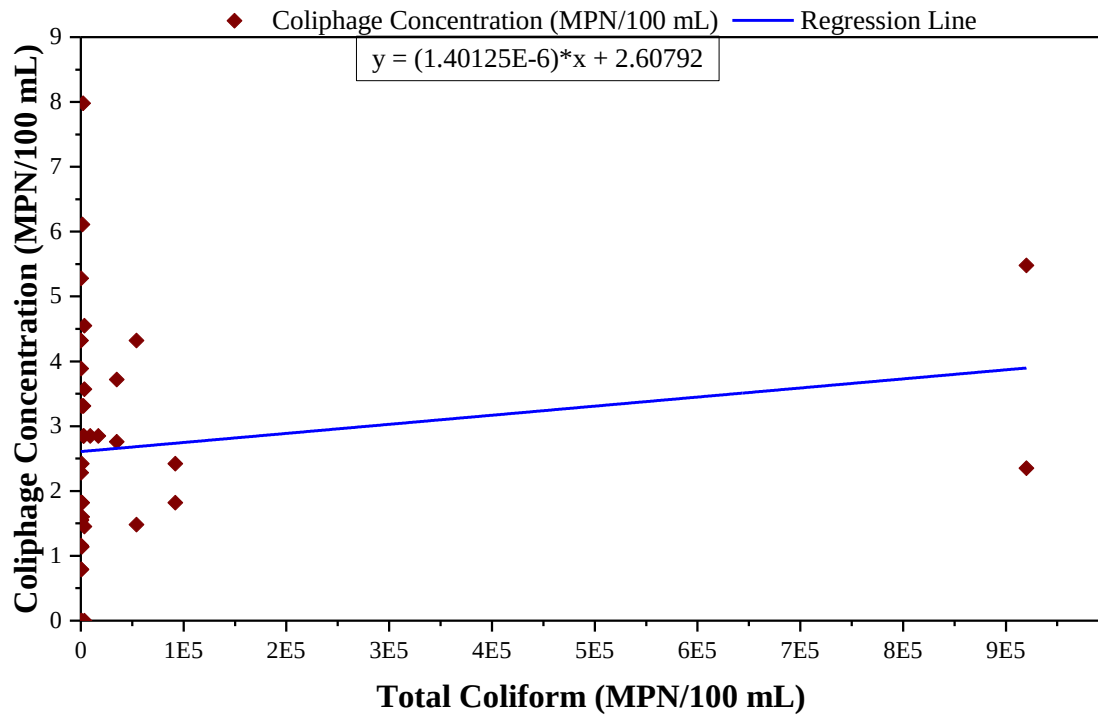


Figure 5.9: Linear Regression Plot between Total Coliform Concentration and Coliphages Concentration

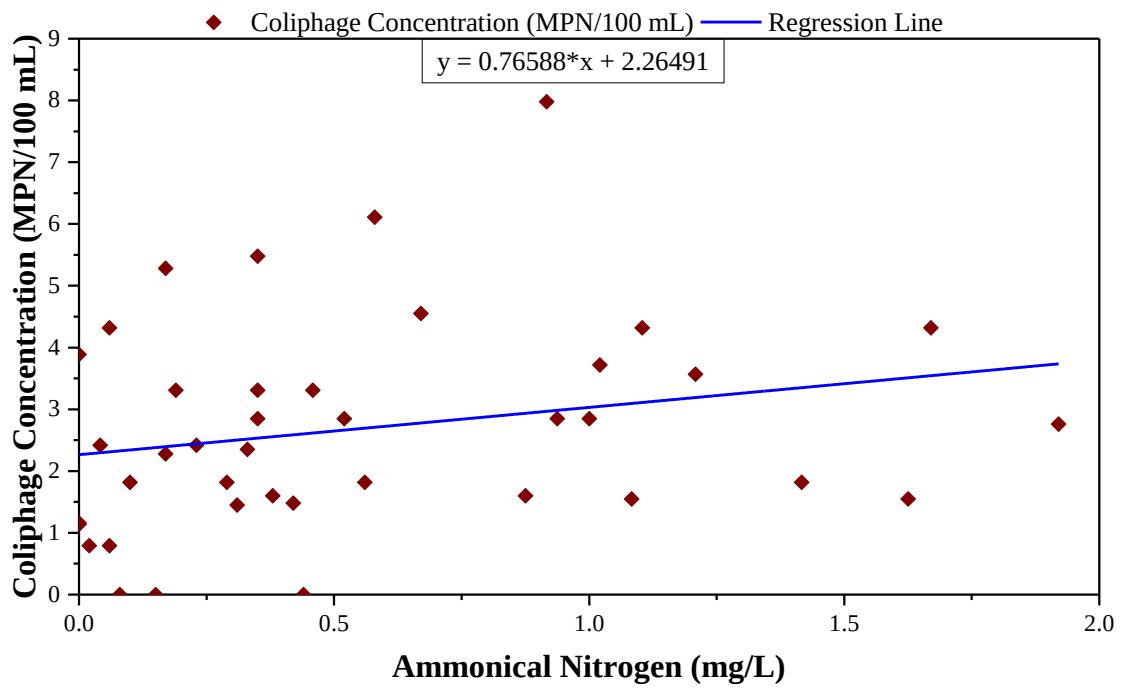


Figure 5.10: Linear Regression Plot between Ammonical Nitrogen Concentration and Coliphages Concentration

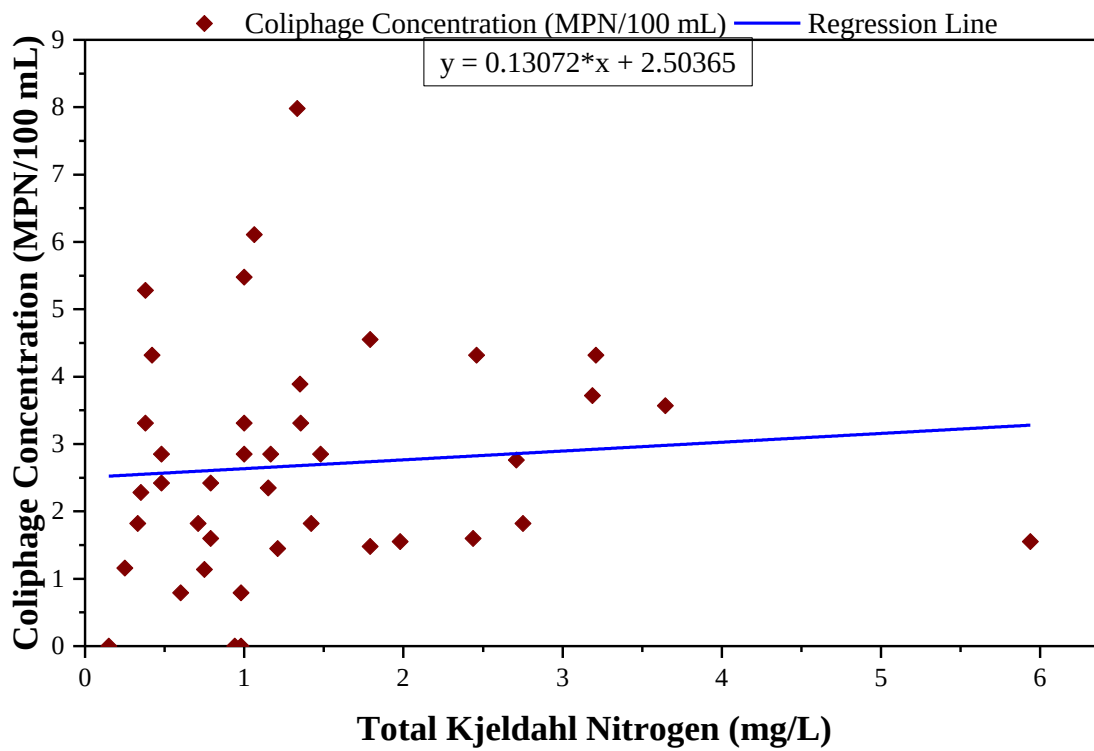


Figure 5.11: Linear Regression Plot between Total Kjeldahl Concentration and Coliphages Concentration

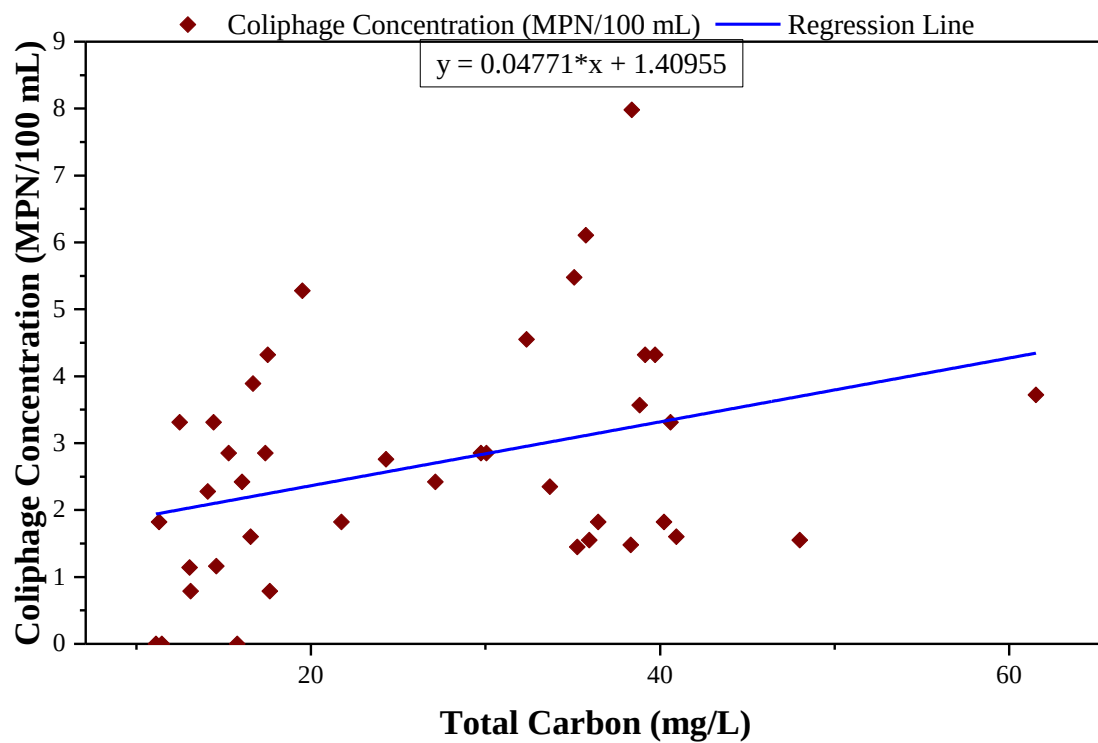


Figure 5.12: Linear Regression Plot between Total Carbon Concentration and Coliphages Concentration

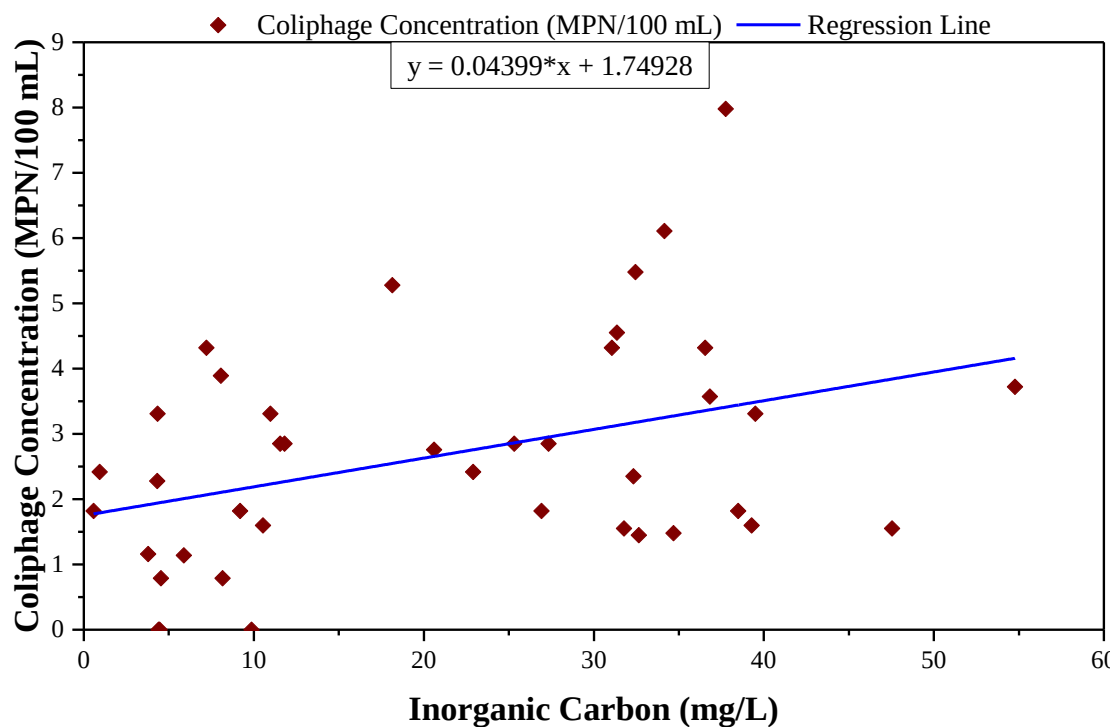


Figure 5.13: Linear Regression Plot between Inorganic Carbon Concentration and Coliphages Concentration

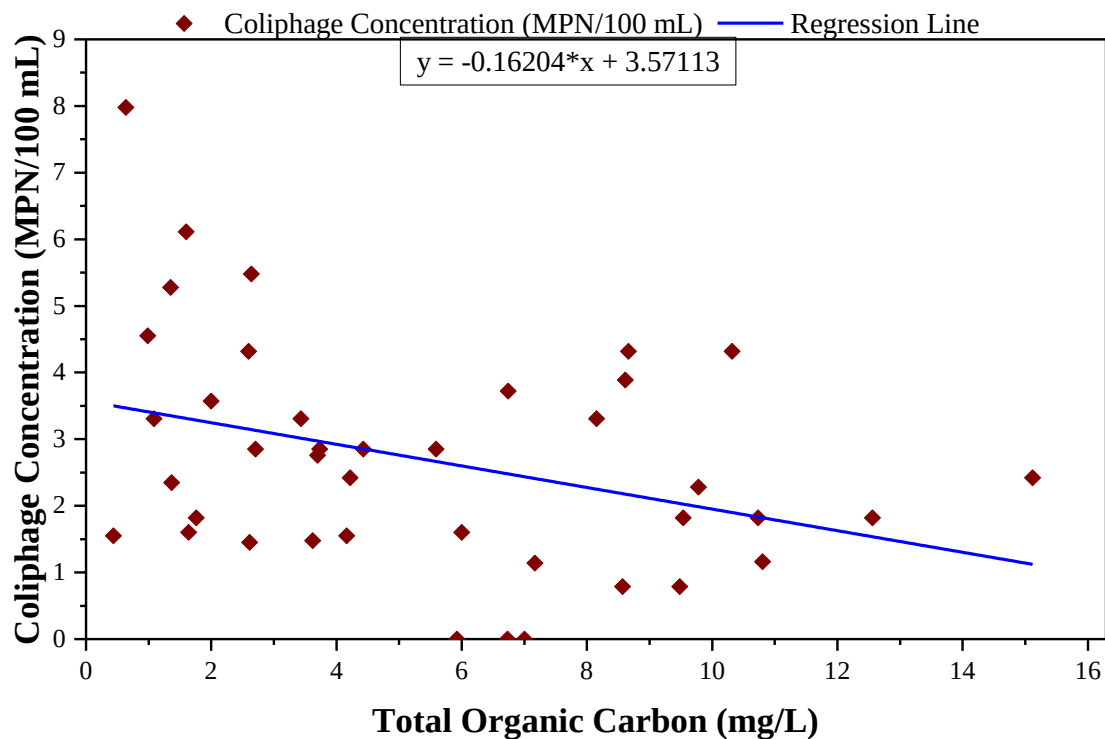


Figure 5.14: Linear Regression Plot between Total Organic Carbon Concentration and Coliphages Concentration

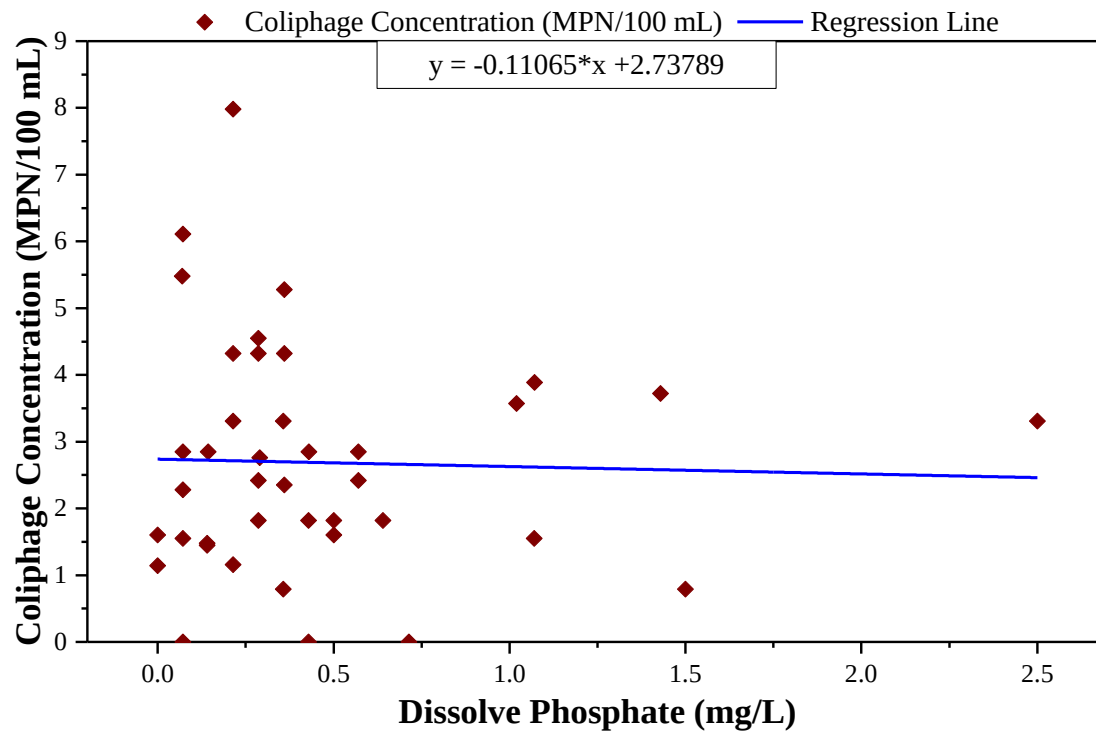


Figure 5.15: Linear Regression Plot between Dissolve Phosphate Concentration and Coliphages Concentration

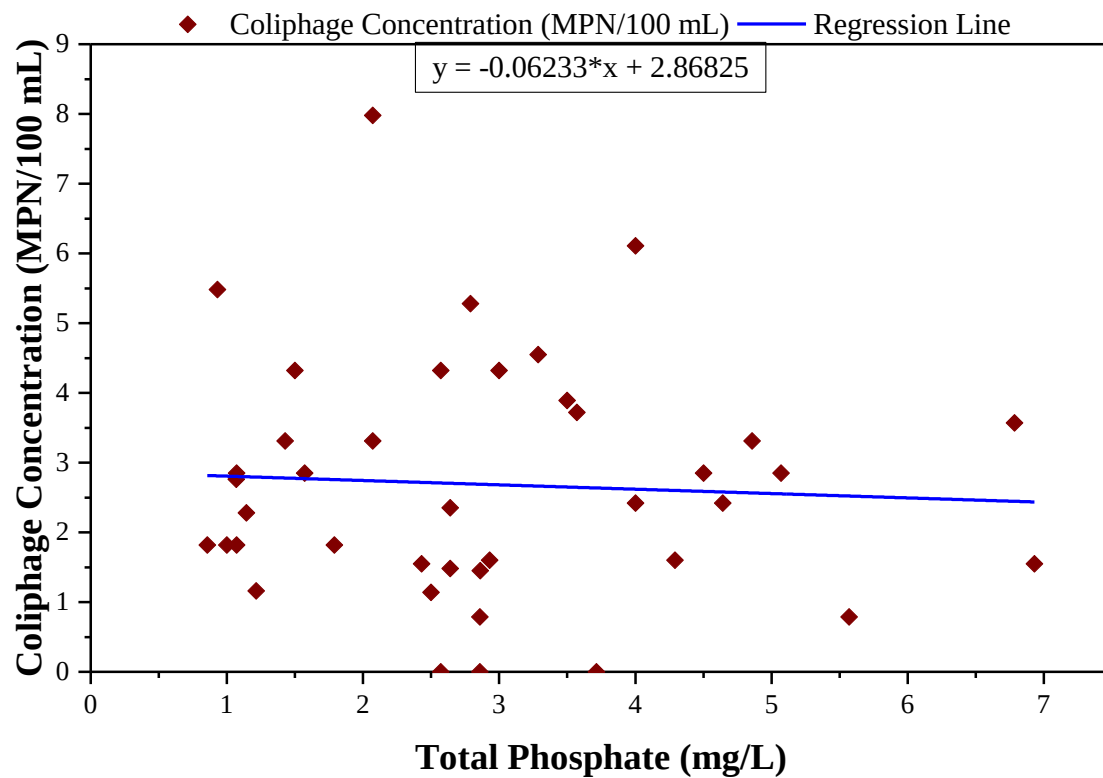


Figure 5.16: Linear Regression Plot between Total Phosphate Concentration and Coliphages Concentration

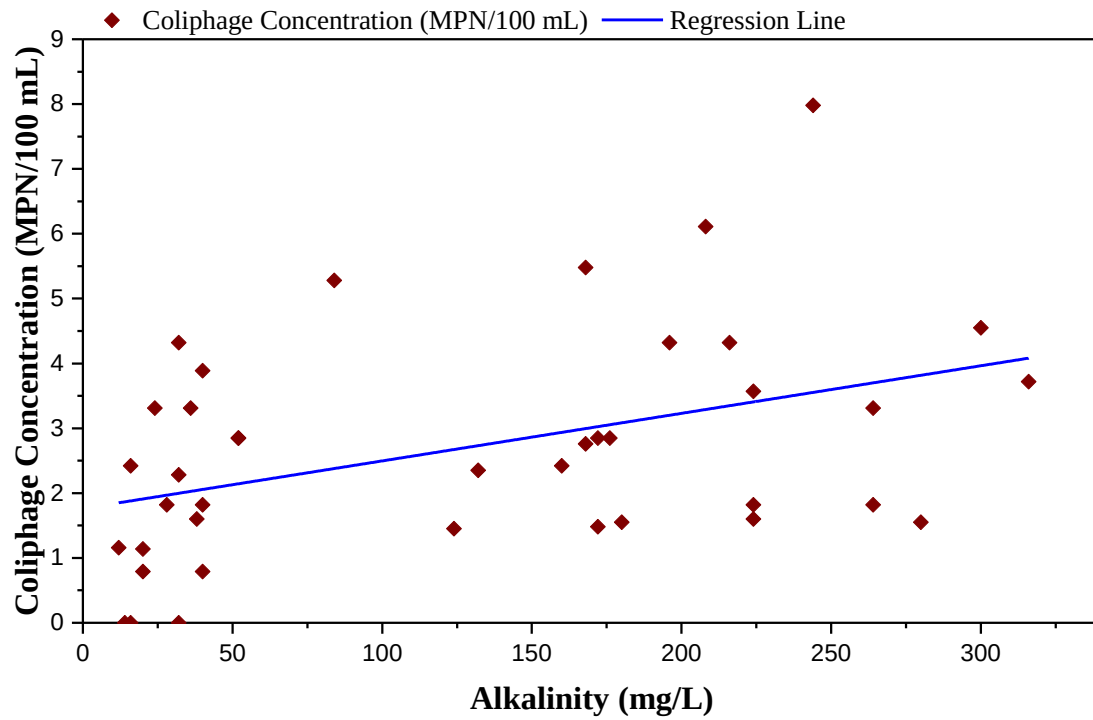


Figure 5.17: Linear Regression Plot between Alkalinity Concentration and Coliphages Concentration

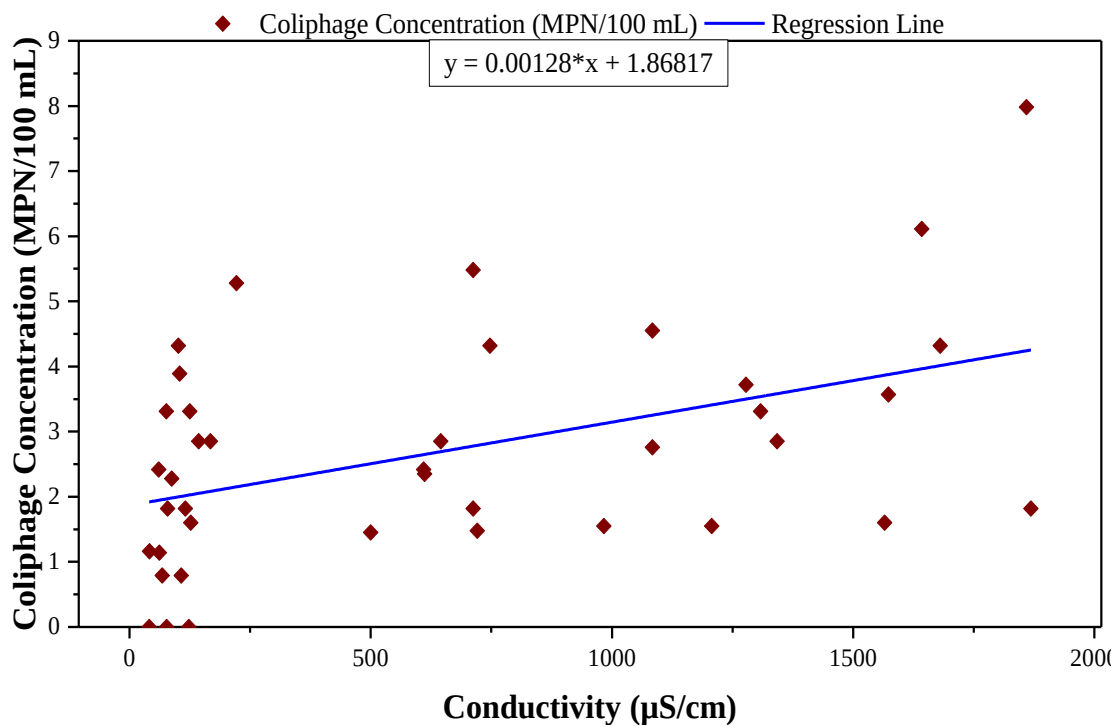


Figure 5.18: Linear Regression Plot between Conductivity and Coliphages Concentration

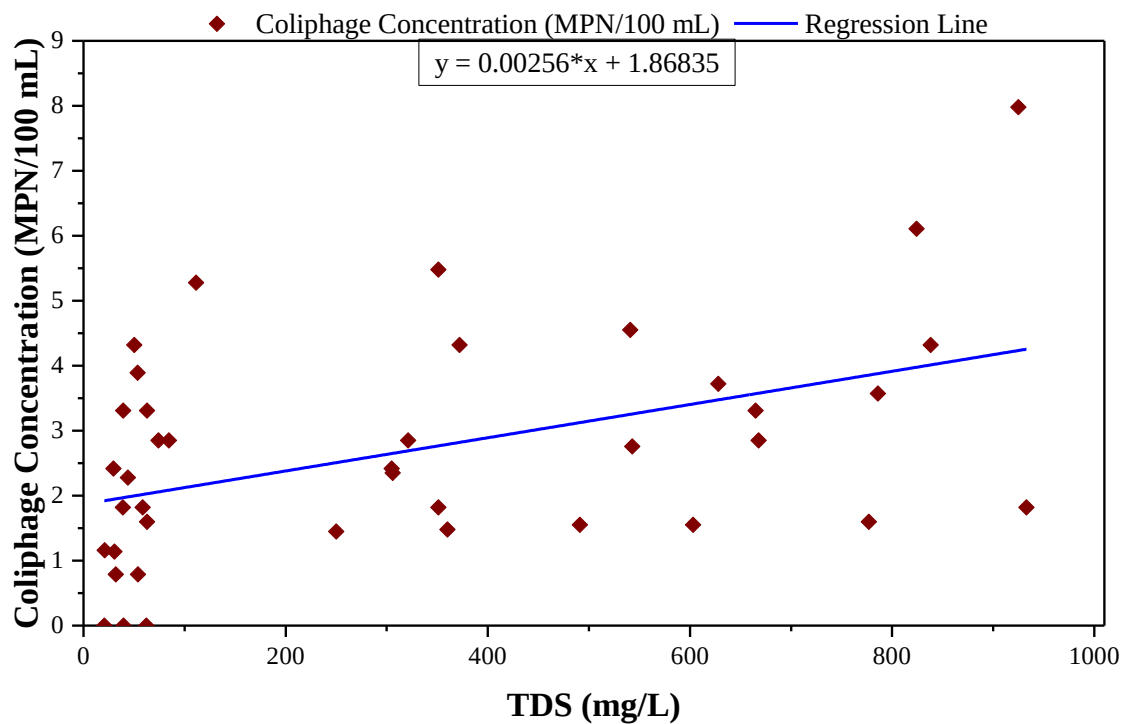


Figure 5.19: Linear Regression Plot between Total Dissolve Solid (TDS) and Coliphages Concentration

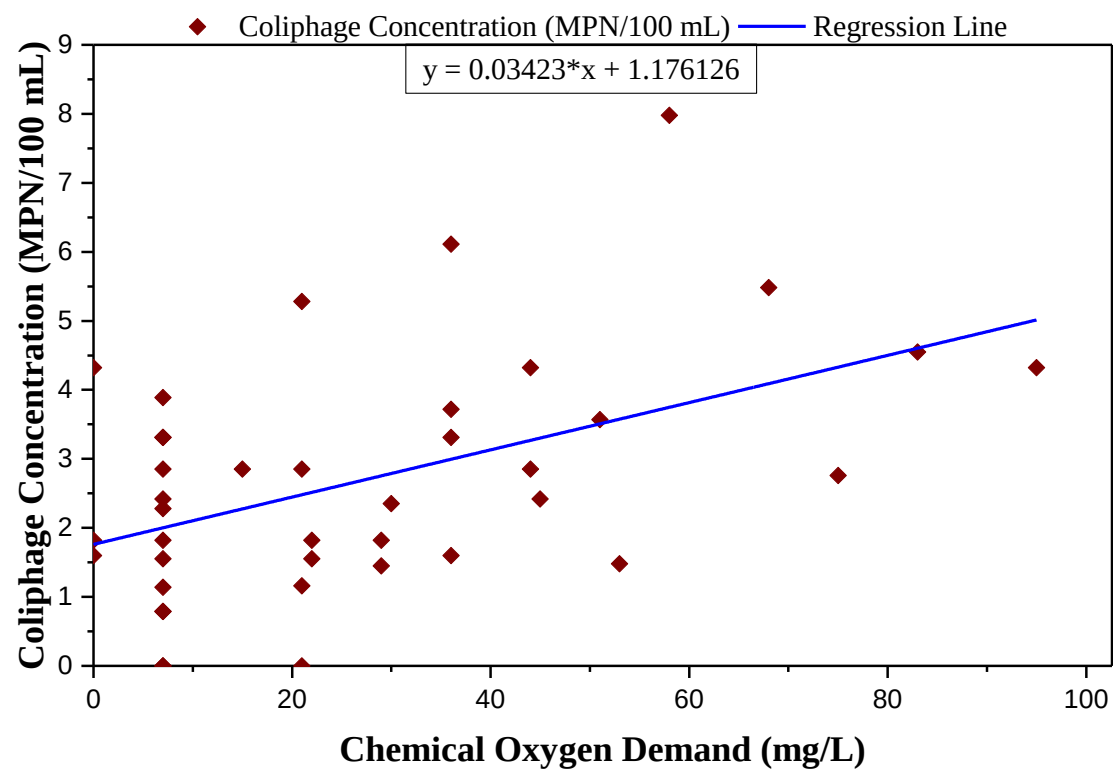


Figure 5.20: Linear Regression Plot between Total Dissolve Solid (TDS) and Coliphages Concentration

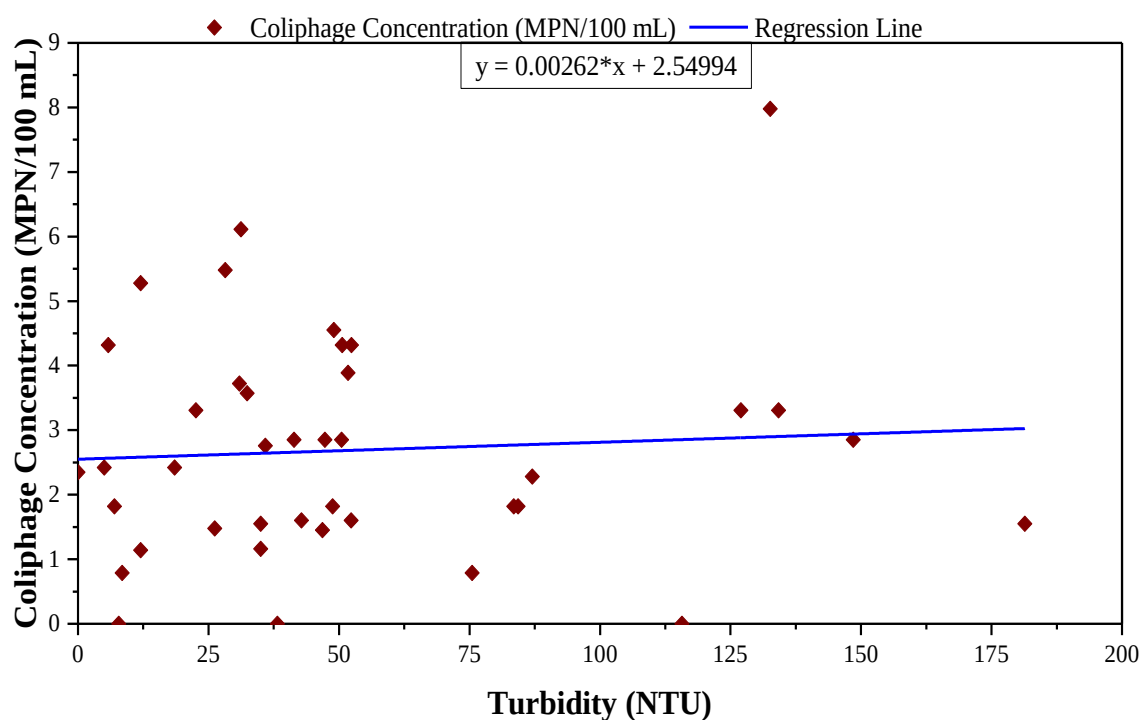


Figure 5.21: Linear Regression Plot between Turbidity (TDS) and Coliphages Concentration

The various statistical estimates were made on correlation of coliphages with biological parameters such as faecal coliform (FC) and total coliform (TCol), and physico- chemical parameters such as pH, alkalinity, dissolve phosphate (DP), total phosphate (TP), ammonical nitrogen (AN), total Kjeldahl nitrogen (TKN), chemical oxygen demand (COD), total carbon, inorganic carbon (IC), total organic carbon (TOC), conductivity and total dissolve solid (TDS). The results are presented in Table 5.4.

It is evident from the statistical analysis that coliphages show significantly weak negative correlation with physico-chemical parameters such as total organic carbon ($r_p = -0.35$, $p < 0.050$), dissolved phosphate (DP) ($r_p = -0.03$, $p < 0.05$) and total phosphate (TP) ($r_p = -0.05$, $p < 0.05$). However, the physico-chemical parameters such as pH ($r_p = 0.35$, $p < 0.05$), total carbon ($r_p = 0.35$, $p < 0.05$), inorganic carbon (IC) ($r_p = 0.41$, $p < 0.05$), chemical oxygen demand (COD) ($r_p = 0.48$, $p < 0.05$), conductivity ($r_p = 0.45$, $p < 0.05$), total dissolved solids ($r_p = 0.45$, $p < 0.05$) and alkalinity ($r_p = 0.41$, $p < 0.05$) show significantly a moderate correlation with occurrence of coliphages in river water. With respect to the biological parameters like faecal coliform ($r_p = 0.12$, $p > 0.05$) and total coliform ($r_p = 0.16$, $p < 0.05$), coliphages show a non-significant weak positive correlation and a very low significant weak positive correlation respectively. The other physico-chemical parameters such as ammonical nitrogen ($r_p = 0.23$, $p < 0.05$), total Kjeldahl nitrogen ($r_p = 0.09$, $p < 0.05$), and turbidity ($r_p = 0.07$, $p < 0.05$) show a significantly weak positive correlation with coliphages.

Table 5.4: Results Obtained by Linear Regression Analysis and t-test

S.No.	Parameters	Pearson's Coefficient (r_p)	df	t-Statistic	P - Value
1	Total Organic Carbon	-0.35	39	-3.77	0.00
2	Inorganic Carbon	0.42	39	8.02	0.00
3	Total Carbon	0.35	39	-12.64	0.00
4	Faecal Coliform (MPN/100 mL)	0.13	39	-1.66	0.05
5	Total Coliform (MPN/100 mL)	0.16	39	-1.77	0.04
6	pH	0.35	39	-20.61	0.00
7	Alkalinity (mg/L)	0.42	39	-8.02	0.00
8	Dissolve Phosphate (mg/L)	-0.03	39	7.82	0.00
9	Total Phosphate (mg/L)	-0.06	39	-0.57	0.28
10	Ammonical Nitrogen (mg/L)	0.23	39	8.05	0.00
11	Total Kjeldahl Nitrogen (mg/L)	0.09	39	4.09	0.00
12	COD (mg/L)	0.48	39	-6.57	0.00
13	Conductivity (μ S/cm)	0.45	39	-6.62	0.00
14	TDS (mg/L)	0.45	39	-6.60	0.00
15	Turbidity (NTU)	0.07	39	-7.20	0.00

6 Conclusions

Based on the observations, results obtained and synthesis of information available in literature, following conclusion may be drawn.

- The method presented in the current study is a simple modification of Culture based agar overlay phage assay that produces phage plaques, improving their visibility and can be used without requirement of sophisticated instruments.
- The developed method uses host bacteria isolated from sample itself therefore eliminating the dependency on cell culture center for host bacteria and provide location specific host bacteria.
- The developed method is highly sensitive; it gives positive results with river water, sewage water and oxidation pond water and shows variation in phage concentration proportionally to dilution factor. Also it can detect phages concentration as low as 0.24 MPN/100 mL. Similarly the method is highly specific as it can show true negative results for phage less sample shown for drinking water and autoclaved water.
- Coliphages are found in all water samples of River Ganga and also in water samples of other rivers tested under current study and previously reported for different geographic areas. Therefore it can be concluded that presence of bacteriophages especially coliphages are universal and more or less same in concentration. Therefore, occurrence of coliphages in Ganga river is not unique property of Ganga waters.
- Coliphages show significantly weak negative correlation with the physico-chemical parameters such as total organic carbon, dissolve phosphate and total phosphate. However, the physico-chemical parameters such as pH, total carbon, inorganic carbon, chemical oxygen demand, conductivity, total dissolve solids and alkalinity show significantly moderate correlation with occurrence of coliphages in river water.
- The biological parameters like faecal coliform and total coliform did not show a significant correlation with coliphages as a pollution indicator contrary to some previous results reported by various scientists.

7 Scope for Future Work

It was evident from the statistical analysis that coliphages show a mixed of a significant weak negative correlation, moderate positive correlation and a significantly weak positive correlation with physico-chemical parameters while with biological parameters a non-significant weak positive correlation and a very low significant weak positive correlation was observed. With the findings of current study and the information reviewed in the literature, it was concluded that the bacteriophages especially coliphages are present in various river water, sewage waters, seawater, marine water, lake water and groundwater, and hence the special quality of Ganga river waters may be due to some other unknown factors

Based on the observations and limitations of the present study and synthesis of information given in literature, further work may be carried out on the following aspects as a logical continuation of research presented in this thesis.

- Further investigations, by taking other phages or any other inorganic and organic compounds or biochemical compounds for study, can be done to decipher the mystery behind subtle quality of Ganga river waters.
- The developed method can be further validated on large number of water samples, sediments, soils and vermicompost for the improvisation and comprehensiveness of sensitivity and specificity for the detection and enumeration of bacteriophages in general, and coliphages in particular.
- The source(s) of coliphages can be further investigated by analysing water samples from different locations categorised on the basis of degree of anthropogenic activities.
- Microscopy Study (TEM) can be done know the exact nature and types of phages.

References

- Ackermann, H.W. (2006). Classification of bacteriophages. *The bacteriophages*, 637, 8- 16.
- Adams, M. H. (1959). Bacteriophages. *Bacteriophages*.
- Anderson, B., Rashid, M. H., Carter, C., Pasternack, G., Rajanna, C., Revazishvili, T., . . . Sulakvelidze, A. (2011). Enumeration of bacteriophage particles: Comparative analysis of the traditional plaque assay and real-time QPCR-and nanosight-based assays. *Bacteriophage*, 1(2), 86-93.
- Anderson, E. (1957). *The relations of bacteriophages to bacterial ecology*. Paper presented at the Symp. Soc. Gen. Microbiol.
- Araujo, R., Puig, A., Lasobras, J., Lucena, F., & Jofre, J. (1997). Phages of enteric bacteria in fresh water with different levels of faecal pollution. *Journal of Applied Microbiology*, 82(3), 281-286.
- Armon, R., & Kott, Y. (1993). A simple, rapid and sensitive presence/absence detection test for bacteriophage in drinking water. *Journal of applied bacteriology*, 74(4), 490-496.
- Association, A. P. H. (2001). Method 1602: Male-specific (F+) and somatic coliphage in water by single agar layer (SAL) procedure. *Washington, DC: United States Environmental Protection Agency*, 20460.
- Atterbury, R. (2009). Bacteriophage biocontrol in animals and meat products. *Microbial biotechnology*, 2(6), 601-612.
- Ayres, P. (1977). Coliphages in sewage and the marine environment. *Aquatic microbiology*. Academic Press, Inc., New York, 275-298.
- Ballester, N., Fontaine, J., & Margolin, A. (2005). Occurrence and correlations between coliphages and anthropogenic viruses in the Massachusetts Bay using enrichment and ICC-nPCR. *J Water Health*, 3, 59-68.
- Bell, R. (1976). The limitation of the ratio of faecal coliforms to total coliphage as a water pollution index. *Water Research*, 10(8), 745-748.
- Bergh, Ø., Børshheim, K. Y., Bratbak, G., & Haldal, M. (1989). High abundance of viruses found in aquatic environments. *Nature*, 340(6233), 467-468.
- Bertrand, I., Schijven, J., Sanchez, G., Wyn-Jones, P., Ottoson, J., Morin, T., . . . de Roda Husman, A. (2012). The impact of temperature on the inactivation of enteric viruses in food and water: a review. *Journal of Applied Microbiology*, 112(6), 1059-1074.
- Bonilla, T. D., Nowosielski, K., Cuvelier, M., Hartz, A., Green, M., Esiobu, N., . . . Rogerson, A. (2007). Prevalence and distribution of faecal indicator organisms in South Florida beach sand and preliminary assessment of health effects associated with beach sand exposure. *Marine pollution bulletin*, 54(9), 1472-1482.

- Borchardt, M. A., Haas, N. L., & Hunt, R. J. (2004). Vulnerability of drinking-water wells in La Crosse, Wisconsin, to enteric-virus contamination from surface water contributions. *Applied and environmental microbiology*, 70(10), 5937-5946.
- Borrego, J. J., Córna, R., Morinigo, M. A., Martínez-Manzanares, E., & Romero, P. (1990). Coliphages as an indicator of faecal pollution in water. Their survival and productive infectivity in natural aquatic environments. *Water Research*, 24(1), 111-116.
- Borrego, J. J., Morinigo, M. A., de Vicente, A., Córna, R., & Romero, P. (1987). Coliphages as an indicator of faecal pollution in water. Its relationship with indicator and pathogenic microorganisms. *Water Research*, 21(12), 1473-1480.
- Brown, M., Camézuli, S., Davenport, R., Petelenz-Kurdziel, E., Øvreås, L., & Curtis, T. (2015). Flow cytometric quantification of viruses in activated sludge. *Water Research*, 68, 414-422.
- Brussaard, C. P. (2004). Optimization of procedures for counting viruses by flow cytometry. *Applied and environmental microbiology*, 70(3), 1506-1513.
- Buttiaux, R., & Mossel, D. (1961). The significance of various organisms of faecal origin in foods and drinking water. *Journal of applied bacteriology*, 24(3), 353- 364.
- Carlson, K. (2005). Appendix: working with bacteriophages: common techniques and methodological approaches. *Bacteriophages: Biology and applications*, 437-494.
- Chang, L., Farrah, S., & Bitton, G. (1981). Positively charged filters for virus recovery from wastewater treatment plant effluents. *Applied and environmental microbiology*, 42(5), 921-924.
- Charles, K., Shore, J., Sellwood, J., Laverick, M., Hart, A., & Pedley, S. (2009). Assessment of the stability of human viruses and coliphage in groundwater by PCR and infectivity methods. *Journal of Applied Microbiology*, 106(6), 1827- 1837.
- Chen, L.-K., Liu, Y.-L., Hu, A., Chang, K.-C., Lin, N.-T., Lai, M.-J., & Tseng, C.-C. (2013). Potential of bacteriophage Φ AB2 as an environmental biocontrol agent for the control of multidrug-resistant *Acinetobacter baumannii*. *BMC microbiology*, 13(1), 154.
- Chhibber, S., Kaur, S., & Kumari, S. (2008). Therapeutic potential of bacteriophage in treating *Klebsiella pneumoniae* B5055-mediated lobar pneumonia in mice. *Journal of medical microbiology*, 57(12), 1508-1513.
- Chibani-Chennoufi, S., Bruttin, A., Dillmann, M.-L., & Brüssow, H. (2004). Phage-host interaction: an ecological perspective. *Journal of bacteriology*, 186(12), 3677- 3686.
- Chung, H., & Sobsey, M. (1993). Comparative survival of indicator viruses and enteric viruses in seawater and sediment. *Water Science & Technology*, 27(3-4), 425-428.
- Cole, D., Long, S. C., & Sobsey, M. D. (2003). Evaluation of F+ RNA and DNA coliphages as source-specific indicators of faecal contamination in surface waters. *Applied and environmental microbiology*, 69(11), 6507-6514.
- Connerton, P., & Connerton, I. (2006). *Campylobacter* and their bacteriophage in poultry.

Avian gut function in health and disease, 311-321.

Cornax, R., Moriñigo, M. A., Paez, I. G., Muñoz, M. A., & Borrego, J. J. (1990). Application of direct plaque assay for detection and enumeration of bacteriophages of *Bacteroides fragilis* from contaminated-water samples. *Applied and environmental microbiology*, 56(10), 3170-3173.

Cornax, R., Moriñigo, M. A., Paez, I. G., Muñoz, M. A., & Borrego, J. J. (1990). Application of direct plaque assay for detection and enumeration of bacteriophages of *Bacteroides fragilis* from contaminated-water samples. *Applied and environmental microbiology*, 56(10), 3170-3173

d'Hérelle, F. (1917). On an invisible microbe antagonistic to dysentery bacilli

Comptes Rendus Academie des Sciences, 165:373-5.

Davies, C., Yousefi, Z., & Bavor, H. (2003). Occurrence of coliphages in urban stormwater and their fate in stormwater management systems. *Letters in applied microbiology*, 37(4), 299-303.

De Leon, R., Shieh, C., Baric, R., & Sobsey, M. (1990). *Detection of enteroviruses and hepatitis A virus in environmental samples by gene probes and polymerase chain reaction*. Paper presented at the Proceedings of the 1990 Water Quality Technology Conference.

Debartolomeis, J., & Cabelli, V. J. (1991). Evaluation of an *Escherichia coli* host strain for enumeration of F male-specific bacteriophages. *Applied and environmental microbiology*, 57(5), 1301-1305.

Deresinski, S. (2009). Bacteriophage therapy: exploiting smaller fleas. *Clinical Infectious Diseases*, 48(8), 1096-1101.

Dhillon, E. K., & Dhillon, T. (1974). Synthesis of indicator strains and density of ribonucleic acid-containing coliphages in sewage. *Applied microbiology*, 27(4), 640-647.

Dhillon, T., Chan, Y., Sun, S., & Chau, W. (1970). Distribution of coliphages in Hong Kong sewage. *Applied microbiology*, 20(2), 187-191.

Dhillon, T., Dhillon, E., Chau, H., Li, W., & Tsang, A. (1976). Studies on bacteriophage distribution: virulent and temperate bacteriophage content of mammalian feces. *Applied and environmental microbiology*, 32(1), 68-74.

Dias, F., & Bhat, J. (1965). Microbial ecology of activated sludge II. Bacteriophages, bdellovibrio, coliforms, and other organisms. *Applied and environmental microbiology*, 13(2), 257-261.

Durham, D., & Wolf, H. (1973). Wastewater chlorination: panacea or placebo? *Wat. Sewage Wk*, 120, 67-70.

Dutka, B., El Shaarawi, A., Martins, M. T., & Sanchez, P. S. (1987). North and South American studies on the potential of coliphage as a water quality indicator. *Water Research*, 21(9), 1127-1134.

Edelman, D. C., & Barletta, J. (2003). Real-time PCR provides improved detection and titer determination of bacteriophage. *Biotechniques*, 35(2), 368-375.

Fannin, K., Gannon, J. J., Cochran, K. W., & Spendlove, J. (1977). Field studies on coliphages and coliforms as indicators of airborne animal viral contamination from wastewater treatment facilities. *Water Research*, 11(2), 181-188.

Fong, T.-T., & Lipp, E. K. (2005). Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools. *Microbiology and Molecular Biology Reviews*, 69(2), 357-371.

Francy, D. S., Stelzer, E. A., Bushon, R. N., Brady, A. M., Mailot, B. E., Spencer, S. K., . . . Gellner, T. M. (2011). *Quantifying Viruses and Bacteria in Wastewater: Results, Interpretation Methods, and Quality Control*: US Department of the Interior, US Geological Survey.

Funderburg, S. W., & Sorber, C. A. (1985). Coliphages as indicators of enteric viruses in activated sludge. *Water Research*, 19(5), 547-555.

Gantzer, C., Maul, A., Audic, J., & Schwartzbrod, L. (1998). Detection of infectious enteroviruses, enterovirus genomes, somatic coliphages, and *Bacteroides fragilis* phages in treated wastewater. *Applied and environmental microbiology*, 64(11), 4307-4312.

Gantzer, C., Senouci, S., Maul, A., Levi, Y., & Schwartzbrod, L. (1999). Enterovirus detection from wastewater by RT-PCR and cell culture. *Water Science and Technology*, 40(2), 105-109.

Geldreich, E., Bordner, R., Huff, C., Clark, H., & Kabler, P. (1962). Type distribution of coliform bacteria in the feces of warm-blooded animals. *Journal (Water Pollution Control Federation)*, 295-301.

Gentilomi, G. A., Cricca, M., Luca, G. D., Sacchetti, R., & Zanetti, F. (2008). Rapid and sensitive detection of MS2 coliphages in wastewater samples by quantitative reverse transcriptase PCR. *The new microbiologica*, 31(2), 273.

Gerba, C. P. (1987). Phage as indicators of faecal pollution. *Phage ecology*, 197-209. Gerba,

C. P., Stagg, C. H., & Abadie, M. G. (1978). Characterization of sewage solid-associated viruses and behavior in natural waters. *Water Research*, 12(10), 805-812.

Glass, J., & O'brien, R. (1980). Enterovirus and coliphage inactivation during activated sludge treatment. *Water Research*, 14(7), 877-882.

Gomila, M., Solis, J., David, Z., Ramon, C., & Lalucat, J. (2008). Comparative reductions of bacterial indicators, bacteriophage-infecting enteric bacteria and enteroviruses in wastewater tertiary treatments by lagooning and UV-radiation.

Grabow, W. (1998). *Evaluation of the application of bacteriophages as indicators of water quality*: Water Research Commission.

Grabow, W. (2004). Bacteriophages: update on application as models for viruses in water. *Water Sa*, 27(2), 251-268.

- Grabow, W., & Coubrough, P. (1986). Practical direct plaque assay for coliphages in 100-mL samples of drinking water. *Applied and environmental microbiology*, 52(3), 430-433.
- Grabow, W., Coubrough, P., Nupen, E., & Bateman, B. (1984). Evaluation of coliphages as indicators of the virological quality of sewage-polluted water. *Water Sa*, 10(1), 7-14.
- Grabow, W., Holtzhausen, C., & De Villiers, J. (1993). Research on bacteriophages as indicators of water quality. *Water Research Commission report*, 321(1), 93.
- Grabow, W., Neubrech, T., Holtzhausen, C., & Jofre, J. (1995). Bacteroides fragilis and Escherichia coli bacteriophages: excretion by humans and animals. *Water Science and Technology*, 31(5), 223-230.
- Grabow, W., Taylor, M., & de Villiers, J. (2001). New methods for the detection of viruses: call for review of drinking water quality guidelines. *Water Science & Technology*, 43(12), 1-8.
- Grabow, W. O. K., Neubrech, T. E., Holtzhausen, C. S., & Jofre, J. (1995). Bacteroides fragilis and Escherichia coli bacteriophages: excretion by humans and animals. *Water Science and Technology*, 31(5), 223-230.
- Griffith, J. F., Cao, Y., McGee, C. D., & Weisberg, S. B. (2009). Evaluation of rapid methods and novel indicators for assessing microbiological beach water quality. *Water Research*, 43(19), 4900-4907.
- Hankin, E. (1896a). L'action bactericide des eaux de la Jumna et du Gange sur le vibron du cholera. *Ann. Inst. Pasteur*, 10, 511.
- Hankin, M. (1896b). Les microbes des rivières de l'Inde. *Ann Inst Pasteur (Paris)*, 10, 175-176.
- Hanlon, G. (2005). Bacteriophage therapy. A once and future solution? *BIOMEDICAL SCIENTIST*, 49(10), 1048.
- Haramoto, E., Kitajima, M., Katayama, H., Asami, M., Akiba, M., & Kunikane, S. (2009). Application of real-time PCR assays to genotyping of F-specific phages in river water and sediments in Japan. *Water Research*, 43(15), 3759-3764.
- Harding, J., Done, J., & Kershaw, G. (1957). A transmissible polio-encephalomyelitis of pigs (Talfan disease). *Vet. Rec*, 69, 824-832.
- Havelaar, A. (1987). Bacteriophages as model organisms in water treatment. *Microbiological sciences*, 4(12), 362-364.
- Havelaar, A., Furuse, K., & Hogeboom, W. (1986). Bacteriophages and indicator bacteria in human and animal faeces. *Journal of applied bacteriology*, 60(3), 255- 262.
- Havelaar, A., & Hogeboom, W. (1983). Factors affecting the enumeration of coliphages in sewage and sewage-polluted waters. *Antonie van Leeuwenhoek*, 49(4-5), 387- 397.
- Havelaar, A., Hogeboom, W., & Pot, R. (1985). F specific RNA bacteriophages in

sewage: methodology and occurrence. *Water Science & Technology*, 17(4-5), 645- 655.

Havelaar, A., & Nieuwstad, T. J. (1985). Bacteriophages and faecal bacteria as indicators of chlorination efficiency of biologically treated wastewater. *Journal (Water Pollution Control Federation)*, 1084-1088.

Havelaar, A., Van Olphen, M., & Drost, Y. (1993). F-specific RNA bacteriophages are adequate model organisms for enteric viruses in fresh water. *Applied and environmental microbiology*, 59(9), 2956-2962.

Hayward, K. (1999). Phages gain ground as water quality indicators. *Water* 21, 36-37. Hilton,

M. C., & Stotzky, G. (1973). Use of coliphages as indicators of water pollution. *Canadian journal of microbiology*, 19(6), 747-751.

Hot, D., Legeay, O., Jacques, J., Gantzer, C., Caudrelier, Y., Guyard, K., . . . Andreoletti, L. (2003). Detection of somatic phages, infectious enteroviruses and enterovirus genomes as indicators of human enteric viral pollution in surface water. *Water Research*, 37(19), 4703-4710.

Hsu, F.-C., Shieh, Y.-S. C., & Sobsey, M. (2002). Enteric bacteriophages as potential faecal indicators in ground beef and poultry meat. *Journal of Food Protection*®, 65(1), 93-99.

Hua, Z., Rouse, J. L., Eckhardt, A. E., Srinivasan, V., Pamula, V. K., Schell, W. A., . . . Pollack, M. G. (2010). Multiplexed real-time polymerase chain reaction on a digital microfluidic platform. *Analytical chemistry*, 82(6), 2310-2316.

Hudson, J., Billington, C., Carey-Smith, G., & Greening, G. (2005). Bacteriophages as biocontrol agents in food. *Journal of Food Protection*®, 68(2), 426-437.

Huff, W., Huff, G., Rath, N., Balog, J., & Donoghue, A. (2005). Alternatives to antibiotics: utilization of bacteriophage to treat colibacillosis and prevent foodborne pathogens. *Poultry science*, 84(4), 655-659.

Huynh, T. D., & Kory, M. M. (1993). The Seasonal Distribution of Some Bacteriophages in the Akron Sewage Treatment Plant.

Ignazzitto, G., Volterra, L., Aulicino, F. A., & D'Angelo, A. M. (1980). Coliphages as indicators in a treatment plant. *Water, Air, and Soil Pollution*, 13(4), 391-398.

Ijzerman, M. M., Falkinham, J. O., & Hagedorn, C. (1993). A liquid, colorimetric presence-absence coliphage detection method. *Journal of virological methods*, 45(2), 229-234.

Ijzerman, M. M., Falkinham, J. O., Reneau, R. B., & Hagedorn, C. (1994). Field evaluation of two colorimetric coliphage detection methods. *Applied and environmental microbiology*, 60(3), 826-830.

Ijzerman, M. M., & Hagedorn, C. (1992). Improved method for coliphage detection based on β -galactosidase induction. *Journal of virological methods*, 40(1), 31-36.

ISO. (1995). Water quality-Detection and Enumeration of Bacteriophages-Part 1:

Enumeration of F-specific RNA Bacteriophages: International Organization for Standardization Geneva.

ISO. (2000). Water quality-Detection and Enumeration of Bacteriophages-Part 2: Enumeration of Somatic Bacteriophages: International Organization for Standardization Geneva.

ISO. (2001). Water quality -- Detection and enumeration of bacteriophages -- Part 4: Enumeration of bacteriophages infecting *Bacteroides fragilis*.

Jagals, P., Grabow, W., & De Villiers, J. (1995). Evaluation of indicators for assessment of human and animal faecal pollution of surface run-off. *Water Science and Technology*, 31(5), 235-241.

Jarvis, B., Wilrich, C., & Wilrich, P. T. (2010). Reconsideration of the derivation of Most Probable Numbers, their standard deviations, confidence bounds and rarity values. *Journal of Applied Microbiology*, 109(5), 1660-1667.

Jebrail, M. J., & Wheeler, A. R. (2010). Let's get digital: digitizing chemical biology with microfluidics. *Current opinion in chemical biology*, 14(5), 574-581.

Jiang, S., Noble, R., & Chu, W. (2001). Human adenoviruses and coliphages in urban runoff-impacted coastal waters of Southern California. *Applied and environmental microbiology*, 67(1), 179-184.

Jiang, S. C., & Chu, W. (2004). PCR detection of pathogenic viruses in southern California urban rivers. *Journal of Applied Microbiology*, 97(1), 17-28.

KENARD, R. P., & VALENTINE, R. S. (Mar. 1974). Rapid Determination of the Presence of Enteric Bacteria in Water. *American Society for Microbiology*, 27(3), 484-487.

Kennedy, J., Bitton, G., & Oblinger, J. (1985). Comparison of selective media for assay of coliphages in sewage effluent and lake water. *Applied and environmental microbiology*, 49(1), 33-36.

Khairnar, K., Pal, P., Chandekar, R. H., & Paunikar, W. N. (2014). Isolation and Characterization of Bacteriophages Infecting Nocardioforms in Wastewater Treatment Plant. *Biotechnology research international*, 2014.

Kirs, M., & Smith, D. C. (2007). Multiplex quantitative real-time reverse transcriptase PCR for F+-specific RNA coliphages: a method for use in microbial source tracking. *Applied and environmental microbiology*, 73(3), 808-814.

Kott, Y. (1966). Estimation of low numbers of *Escherichia coli* bacteriophage by use of the most probable number method. *Applied microbiology*, 14(2), 141-144.

Kott, Y. (1966). Survival of T bacteriophages and coliform bacteria in sea water. *Bull. Inst. Mar. Sci*, 11, 1-6.

Kott, Y. (1981). Viruses and bacteriophages. *Science of the Total Environment*, 18, 13- 23.

Kott, Y. (1984). Coliphages as reliable enteric virus indicators.

- Kott, Y., Roze, N., Sperber, S., & Betzer, N. (1974). Bacteriophages as viral pollution indicators. *Water Research*, 8(3), 165-171.
- LaLiberte, P., & Grimes, D. J. (1982). Survival of *Escherichia coli* in lake bottom sediment. *Applied and environmental microbiology*, 43(3), 623-628.
- Lipp, E. K., Lukasik, J., & Rose, J. B. (2001). 28 Human Enteric Viruses and Parasites in the Marine Environment.
- Locas, A., Barthe, C., Barbeau, B., Carriere, A., & Payment, P. (2007). Virus occurrence in municipal groundwater sources in Quebec, Canada. *Canadian journal of microbiology*, 53(6), 688-694.
- Locas, A., Barthe, C., Margolin, A. B., & Payment, P. (2008). Groundwater microbiological quality in Canadian drinking water municipal wells. *Canadian journal of microbiology*, 54(6), 472-478.
- Lodder, W., & de Roda Husman, A. (2005). Presence of noroviruses and other enteric viruses in sewage and surface waters in The Netherlands. *Applied and environmental microbiology*, 71(3), 1453-1461.
- Loehr, R. C., & Schwegler, D. T. (1965). Filtration method for bacteriophage detection. *Applied microbiology*, 13(6), 1005-1009.
- Love, D. C., Silverman, A., & Nelson, K. L. (2010). Human virus and bacteriophage inactivation in clear water by simulated sunlight compared to bacteriophage inactivation at a southern California beach. *Environmental Science & Technology*, 44(18), 6965-6970.
- Love, D. C., & Sobsey, M. D. (2007). Simple and rapid F+ coliphage culture, latex agglutination, and typing assay to detect and source track faecal contamination. *Applied and environmental microbiology*, 73(13), 4110-4118.
- Lucena, F., Duran, A., Moron, A., Calderon, E., Campos, C., Gantzer, C., . . . Jofre, J. (2004). Reduction of bacterial indicators and bacteriophages infecting faecal bacteria in primary and secondary wastewater treatments. *Journal of Applied Microbiology*, 97(5), 1069-1076.
- Lucena, F., Mendez, X., Morón, A., Calderón, E., Campos, C., Guerrero, A., . . . Skrabber, S. (2003). Occurrence and densities of bacteriophages proposed as indicators and bacterial indicators in river waters from Europe and South America. *Journal of Applied Microbiology*, 94(5), 808-815.
- Mark, D., Haeberle, S., Roth, G., von Stetten, F., & Zengerle, R. (2010). Microfluidic lab-on-a-chip platforms: requirements, characteristics and applications. *Chemical Society Reviews*, 39(3), 1153-1182.
- Méndez, J., Audicana, A., Isern, A., Llaneza, J., Moreno, B., Tarancón, M. a. L., . . . Lucena, F. (2004). Standardised evaluation of the performance of a simple membrane filtration-elution method to concentrate bacteriophages from drinking water. *Journal of virological methods*, 117(1), 19-25.

Miernik, A. (2004). Occurrence of bacteria and coli bacteriophages as potential indicators of faecal pollution of Vistula River and Zegrze Reservoir. *Polish Journal of Environmental Studies*, 13(1), 79-84.

Muniesa, M., & Jofre, J. (2004). Factors influencing the replication of somatic coliphages in the water environment. *Antonie van Leeuwenhoek*, 86(1), 65-76.

Muniesa, M., Lucena, F., & Jofre, J. (1999). Study of the potential relationship between the morphology of infectious somatic coliphages and their persistence in the environment. *Journal of Applied Microbiology*, 87(3), 402-409.

Mushin, R., & Ashburner, F. (1964). ECOLOGY AND EPIDEMIOLOGY OF COLIFORM INFECTIONS: I. THE INCIDENCE OF ENTEROPATHOGENIC AND OTHER SPECIFIC SEROTYPES OF ESCHERICHIA COLI. *The Medical journal of Australia*, 1, 257.

Nappier, S. P., Aitken, M. D., & Sobsey, M. D. (2006). Male-specific coliphages as indicators of thermal inactivation of pathogens in biosolids. *Applied and environmental microbiology*, 72(4), 2471-2475.

Nautiyal, C. S. (2009). Self-purificatory Ganga water facilitates death of pathogenic *Escherichia coli* O157: H7. *Current microbiology*, 58(1), 25-29.

NEERI. (2004a). Guidance Manual for Drinking Water Quality Monitoring and Assessment: Second Edition.

NEERI. (2004b). Self-Purification Capacity of River Bhagirathi: Impact of Tehri Dam.

Novotny, C. P., & Lavin, K. (1971). Some effects of temperature on the growth of F pili. *Journal of bacteriology*, 107(3), 671-682.

Nupen, E., Basson, N., & Grabow, W. (1981). Efficiency of ultrafiltration for the isolation of enteric viruses and coliphages from large volumes of water in studies on wastewater reclamation. *WATER SCI. & TECH.*, 13(2), 851-863.

O'Keefe, B., & Green, J. (1989). Coliphages as indicators of faecal pollution at three recreational beaches on the firth of forth. *Water Research*, 23(8), 1027-1030.

Osawa, S., Furuse, K., & Watanabe, I. (1981). Distribution of ribonucleic acid coliphages in animals. *Applied and environmental microbiology*, 41(1), 164-168.

Pallin, R., Wyn-Jones, A., Place, B., & Lightfoot, N. (1997). The detection of enteroviruses in large volume concentrates of recreational waters by the polymerase chain reaction. *Journal of virological methods*, 67(1), 57-67.

Parfitt, T. (2005). Georgia: an unlikely stronghold for bacteriophage therapy. *The Lancet*, 365(9478), 2166-2167.

Parry, O., Whitehead, J., & Dowling, L. (1981). *Temperature sensitive coliphage in the aquatic environment*. Paper presented at the Viruses and Wastewater Treatment: Proceedings of the International Symposium on Viruses and Wastewater Treatment, Held at the University of Surrey, Guildford, 15-17 September 1980.

Paul, J. H., Rose, J. B., Jiang, S. C., London, P., Xhou, X., & Kellogg, C. (1997). Coliphage and indigenous phage in Mamala Bay, Oahu, Hawaii. *Applied and environmental microbiology*, 63(1), 133-138.

Payment, P., & Franco, E. (1993). Clostridium perfringens and somatic coliphages as indicators of the efficiency of drinking water treatment for viruses and protozoan cysts. *Applied and environmental microbiology*, 59(8), 2418-2424.

Petrovski, S., Seviour, R. J., & Tillett, D. (2011). Genome sequence and characterization of the Tsukamurella bacteriophage TPA2. *Applied and environmental microbiology*, 77(4), 1389-1398.

Pillai, S. D. (2006). Bacteriophages as faecal indicator organisms *Viruses in Foods* (pp. 205-222): Springer.

Primrose, S. B., Seeley, N. D., Logan, K. B., & Nicolson, J. W. (1982). Methods for studying aquatic bacteriophage ecology. *Applied and environmental microbiology*, 43(3), 694-701.

Reynolds, K. A., Rose, J. B., & Giordano, A. T. (1993). Comparison of methods for the recovery and quantitation of coliphage and indigenous bacteriophage from marine waters and sediments. *Water Science & Technology*, 27(3-4), 115-117.

Rodríguez, R. A., Thie, L., Gibbons, C. D., & Sobsey, M. D. (2012). Reducing the effects of environmental inhibition in quantitative real-time PCR detection of adenovirus and norovirus in recreational seawaters. *Journal of virological methods*, 181(1), 43-50.

SALTER, R. S., & Durbin, G. W. (2012). Modified USEPA method 1601 to indicate viral contamination of groundwater. *Journal: American Water Works Association*, 104(8).

Salter, R. S., Durbin, G. W., Conklin, E., Rosen, J., & Clancy, J. (2010). Proposed modifications of environmental protection agency method 1601 for detection of Coliphages in drinking water, with same-day fluorescence-based detection and evaluation by the performance-based measurement System and alternative test protocol validation approaches. *Applied and environmental microbiology*, 76(23), 7803-7810.

Santos, S. B., Carvalho, C. M., Sillankorva, S., Nicolau, A., Ferreira, E. C., & Azeredo, J. (2009). The use of antibiotics to improve phage detection and enumeration by the double-layer agar technique. *BMC microbiology*, 9(1), 148.

Scarpino, P. V. (1978). Bacteriophage indicators. *Indicators of Viruses in Water and Food*, 201-227.

Scarpino, P. V., Berg, G., Chang, S. L., Dahling, D., & Lucas, M. (1972). A comparative study of the inactivation of viruses in water by chlorine. *Water Research*, 6(8), 959-965.

Seeley, N., & Primrose, S. (1980). The effect of temperature on the ecology of aquatic bacteriophages. *Journal of General Virology*, 46(1), 87-95.

Serwer, P., Hayes, S. J., Zaman, S., Lieman, K., Rolando, M., & Hardies, S. C. (2004). Improved isolation of undersampled bacteriophages: finding of distant terminase genes.

Virology, 329(2), 412-424.

Shah, P. C., & McCamish, J. (1972). Relative chlorine resistance of poliovirus I and coliphages f2 and T2 in water. *Applied microbiology*, 24(4), 658.

Šimková, A., & Červenka, J. (1981). Coliphages as ecological indicators of enteroviruses in various water systems. *Bulletin of the World Health Organization*, 59(4), 611.

Sinton, L., Finlay, R., & Reid, A. (1996). A simple membrane filtration-elution method for the enumeration of F-RNA, F-DNA and somatic coliphages in 100-mL water samples. *Journal of microbiological methods*, 25(3), 257-269.

Smith, D. C. (2006). *Microbial source tracking using F-specific coliphages and quantitative PCR*. University of Rhode Island.

Smith, H. W., & Crabb, W. (1961). The faecal bacterial flora of animals and man: its development in the young. *The Journal of Pathology and Bacteriology*, 82(1), 53- 66.

Snowdon, J. A., Cliver, D. O., & Hurst, C. J. (1989). Coliphages as indicators of human enteric viruses in groundwater. *Critical Reviews in Environmental Science and Technology*, 19(3), 231-249.

Sobsey, M., Yates, M., Hsu, F., Lovelace, G., Battigelli, D., Margolin, A., . . . Nwachuku, N. (2004). Development and evaluation of methods to detect coliphages in large volumes of water. *Water Science & Technology*, 50(1), 211- 217.

Sobsey, M. D. (1982). Quality of currently available methodology for monitoring viruses in the environment. *Environment International*, 7(1), 39-51.

Sobsey, M. D., Love, D. C., & Lovelace, G. L. (2006). F+ RNA coliphages as source tracking viral indicators of faecal contamination. *Project report for the NOAA/UNH Cooperative Institute for Coastal and Estuarine Environmental Technology (CICEET)*.

Sobsey, M. D., Schwab, K. J., & Handzel, T. R. (1990). A simple membrane filter method to concentrate and enumerate male-specific RNA coliphages. *Journal (American Water Works Association)*, 52-59.

Søgaard, H. (1983). Relationship between the occurrence of coliphages and E. coli in Danish marine bathing areas. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene. 1. Abt. Originale B, Hygiene*, 177(5), 394-401.

Stewart-Pullaro, J., Daugomah, J., Chestnut, D., Graves, D., Sobsey, M., & Scott, G. (2006). F+ RNA coliphage typing for microbial source tracking in surface waters. *Journal of Applied Microbiology*, 101(5), 1015-1026.

Summers, W. C. (2005). Bacteriophage research: early history. *Bacteriophages: Biology and applications*, 5-27.

Sun, Z., Levi, Y., Kiene, L., Dumoutier, N., & Lucena, F. (1997). Quantification of bacteriophages of *Bacteroides fragilis* in environmental water samples of Seine River. *Water, Air, and Soil Pollution*, 96(1-4), 175-183.

- Tadmor, A. D., Ottesen, E. A., Leadbetter, J. R., & Phillips, R. (2011). Probing individual environmental bacteria for viruses by using microfluidic digital PCR. *Science*, 333(6038), 58-62.
- Tartera, C., & Jofre, J. (1987). Bacteriophages active against *Bacteroides fragilis* in sewage-polluted waters. *Applied and environmental microbiology*, 53(7), 1632- 1637.
- Taylor, P. W. (1983). Bactericidal and bacteriolytic activity of serum against gram-negative bacteria. *Microbiological reviews*, 47(1), 46.
- Uys, M. (1999). *Molecular Characterisation of F-Specific RNA Phages in South Africa*: Department of Medical Virology, Faculty of Medicine, University of Pretoria.
- Vaughn, J. M., & Metcalf, T. G. (1975). Coliphages as indicators of enteric viruses in shellfish and shellfish raising estuarine waters. *Water Research*, 9(7), 613-616.
- Wade, T. J., Sams, E., Brenner, K. P., Haugland, R., Chern, E., Beach, M., . . . Li, Q. (2010). Rapidly measured indicators of recreational water quality and swimming- associated illness at marine beaches: a prospective cohort study. *Environmental Health*, 9(66), 1-14.
- Wei, Y., Ocampo, P., & Levin, B. R. (2010). An experimental study of the population and evolutionary dynamics of *Vibrio cholerae* O1 and the bacteriophage JSF4. *Proceedings of the Royal Society of London B: Biological Sciences*, 277(1698), 3247-3254.
- Wentzel, R. S., O'Neill, P. E., & Kitchens, J. F. (1982). Evaluation of coliphage detection as a rapid indicator of water quality. *Applied and environmental microbiology*, 43(2), 430-434.
- Wolf, S., Hewitt, J., Rivera-Aban, M., & Greening, G. E. (2008). Detection and characterization of F+ RNA bacteriophages in water and shellfish: application of a multiplex real-time reverse transcription PCR. *Journal of virological methods*, 149(1), 123-128.
- Wu, J., Long, S., Das, D., & Dorner, S. (2011). Are microbial indicators and pathogens correlated? A statistical analysis of 40 years of research. *Journal of water and health*, 9(2), 265-278.
- Yong, S., Ngeow, Y., Tong, Y., & Ong, J. (2006). Real-time PCR detection of male- specific coliphages. *Malaysian Journal of Pathology*, 28(2), 79.
- Zaiss, U. (1981). Dispersal and fate of coliphages in the River Saar. *Zbl. Bakt. Hyg., I. Abt. Orig. B*, 1974, 160-173.

APPENDIX

Table A1: MPN Index, Log₁₀ MPN, Standard Deviation (SD) Log₁₀MPN, 95% Confidence Intervals, Rarity Index and Category for various Combinations of Positive Results when Five Replicates are used per Dilution (50 mL, 25 mL, 10 mL).

Combination of Positives			MPN/100mL	Log ₁₀ MPN	SD Log ₁₀ MPN	95% Confidence Interval		Rarity Index	Category
						Lower	Upper		
0	0	0	0.00	-	-	0.00	0.87	1.00	1
0	0	1	0.24	-0.62	0.43	0.03	1.76	0.12	1
0	0	2	0.48	-0.32	0.31	0.12	1.98	0.02	2
0	0	3	0.73	-0.14	0.25	0.23	2.32	0.00	3
0	0	4	0.99	-0.01	0.22	0.36	2.69	0.00	3
0	0	5	1.25	0.10	0.19	0.51	3.06	0.00	3
0	1	0	0.24	-0.62	0.43	0.03	1.79	0.31	1
0	1	1	0.49	-0.31	0.31	0.12	2.02	0.12	1
0	1	2	0.75	-0.13	0.25	0.23	2.37	0.03	2
0	1	3	1.01	0.00	0.22	0.37	2.74	0.00	3
0	1	4	1.28	0.11	0.19	0.52	3.13	0.00	3
0	1	5	1.55	0.19	0.18	0.69	3.52	0.00	3
0	2	0	0.50	-0.30	0.31	0.12	2.06	0.12	1
0	2	1	0.76	-0.12	0.25	0.24	2.42	0.07	1
0	2	2	1.03	0.01	0.22	0.38	2.80	0.02	2
0	2	3	1.30	0.12	0.19	0.53	3.20	0.00	3
0	2	4	1.59	0.20	0.18	0.70	3.60	0.00	3
0	2	5	1.88	0.27	0.16	0.88	4.02	0.00	3
0	3	0	0.78	-0.11	0.25	0.24	2.47	0.04	2
0	3	1	1.05	0.02	0.22	0.39	2.86	0.02	2
0	3	2	1.33	0.12	0.19	0.54	3.27	0.01	3
0	3	3	1.62	0.21	0.18	0.72	3.69	0.00	3
0	3	4	1.92	0.28	0.16	0.90	4.11	0.00	3
0	3	5	2.23	0.35	0.15	1.10	4.55	0.00	3
0	4	0	1.07	0.03	0.22	0.39	2.93	0.01	3
0	4	1	1.36	0.13	0.20	0.55	3.34	0.01	3
0	4	2	1.66	0.22	0.18	0.73	3.77	0.00	3
0	4	3	1.97	0.29	0.17	0.92	4.21	0.00	3
0	4	4	2.29	0.36	0.15	1.12	4.67	0.00	3
0	4	5	2.62	0.42	0.15	1.34	5.14	0.00	3
0	5	0	1.39	0.14	0.20	0.57	3.42	0.00	3
0	5	1	1.70	0.23	0.18	0.75	3.87	0.00	3
0	5	2	2.02	0.30	0.17	0.94	4.32	0.00	3
0	5	3	2.35	0.37	0.16	1.15	4.79	0.00	3
0	5	4	2.69	0.43	0.15	1.37	5.28	0.00	3
0	5	5	3.05	0.48	0.14	1.60	5.78	0.00	3
1	0	0	0.25	-0.60	0.43	0.03	1.85	0.67	1

1	0	1	0.51	-0.29	0.31	0.12	2.09	0.26	1
1	0	2	0.77	-0.11	0.25	0.24	2.46	0.06	1
1	0	3	1.05	0.02	0.22	0.38	2.85	0.01	3
1	0	4	1.33	0.12	0.20	0.54	3.26	0.00	3
1	0	5	1.62	0.21	0.18	0.71	3.68	0.00	3
1	1	0	0.52	-0.29	0.31	0.13	2.14	0.69	1
1	1	1	0.79	-0.10	0.25	0.25	2.51	0.41	1
1	1	2	1.07	0.03	0.22	0.39	2.91	0.09	1
1	1	3	1.36	0.13	0.20	0.55	3.33	0.02	2
1	1	4	1.65	0.22	0.18	0.73	3.76	0.00	3
1	1	5	1.96	0.29	0.17	0.92	4.21	0.00	3
1	2	0	0.81	-0.09	0.25	0.25	2.57	0.45	1
1	2	1	1.09	0.04	0.22	0.40	2.98	0.25	1
1	2	2	1.39	0.14	0.20	0.56	3.41	0.09	1
1	2	3	1.69	0.23	0.18	0.74	3.86	0.02	2
1	2	4	2.01	0.30	0.17	0.94	4.31	0.00	3
1	2	5	2.34	0.37	0.16	1.14	4.79	0.00	3
1	3	0	1.12	0.05	0.22	0.41	3.05	0.14	1
1	3	1	1.42	0.15	0.20	0.58	3.50	0.13	1
1	3	2	1.73	0.24	0.18	0.76	3.95	0.05	1
1	3	3	2.06	0.31	0.17	0.96	4.43	0.01	2
1	3	4	2.40	0.38	0.16	1.17	4.92	0.00	3
1	3	5	2.76	0.44	0.15	1.40	5.43	0.00	3
1	4	0	1.45	0.16	0.20	0.59	3.58	0.04	2
1	4	1	1.78	0.25	0.18	0.78	4.06	0.04	2
1	4	2	2.12	0.33	0.17	0.98	4.55	0.02	2
1	4	3	2.47	0.39	0.16	1.20	5.06	0.00	3
1	4	4	2.83	0.45	0.15	1.44	5.59	0.00	3
1	4	5	3.22	0.51	0.14	1.69	6.15	0.00	3
1	5	0	1.82	0.26	0.18	0.80	4.17	0.00	3
1	5	1	2.17	0.34	0.17	1.01	4.68	0.00	3
1	5	2	2.54	0.40	0.16	1.23	5.22	0.00	3
1	5	3	2.92	0.47	0.15	1.48	5.77	0.00	3
1	5	4	3.32	0.52	0.14	1.73	6.36	0.00	3
1	5	5	3.74	0.57	0.14	2.01	6.97	0.00	3
2	0	0	0.54	-0.27	0.31	0.13	2.22	0.62	1
2	0	1	0.82	-0.09	0.25	0.26	2.61	0.38	1
2	0	2	1.11	0.05	0.22	0.41	3.04	0.09	1
2	0	3	1.41	0.15	0.20	0.57	3.48	0.02	2
2	0	4	1.73	0.24	0.18	0.76	3.94	0.00	3
2	0	5	2.05	0.31	0.17	0.95	4.42	0.00	3
2	1	0	0.84	-0.08	0.25	0.26	2.67	1.00	1
2	1	1	1.14	0.06	0.22	0.41	3.11	0.60	1
2	1	2	1.45	0.16	0.20	0.59	3.57	0.23	1
2	1	3	1.77	0.25	0.18	0.77	4.05	0.05	2
2	1	4	2.11	0.32	0.17	0.98	4.54	0.00	3
2	1	5	2.46	0.39	0.16	1.20	5.06	0.00	3
2	2	0	1.16	0.07	0.22	0.42	3.19	0.67	1

2	2	1	1.48	0.17	0.20	0.60	3.66	0.65	1
2	2	2	1.82	0.26	0.18	0.79	4.16	0.27	1
2	2	3	2.16	0.34	0.17	1.00	4.67	0.06	1
2	2	4	2.53	0.40	0.16	1.23	5.21	0.01	3
2	2	5	2.91	0.46	0.15	1.47	5.77	0.00	3
2	3	0	1.52	0.18	0.20	0.61	3.76	0.38	1
2	3	1	1.86	0.27	0.18	0.81	4.28	0.39	1
2	3	2	2.23	0.35	0.17	1.03	4.81	0.18	1
2	3	3	2.60	0.42	0.16	1.26	5.37	0.05	2
2	3	4	3.00	0.48	0.15	1.51	5.96	0.01	3
2	3	5	3.42	0.53	0.14	1.78	6.58	0.00	3
2	4	0	1.92	0.28	0.18	0.83	4.40	0.11	1
2	4	1	2.29	0.36	0.17	1.06	4.96	0.13	1
2	4	2	2.68	0.43	0.16	1.30	5.55	0.07	1
2	4	3	3.10	0.49	0.15	1.56	6.17	0.02	2
2	4	4	3.54	0.55	0.14	1.83	6.83	0.00	3
2	4	5	4.01	0.60	0.14	2.13	7.53	0.00	3
2	5	0	2.36	0.37	0.17	1.09	5.13	0.02	2
2	5	1	2.77	0.44	0.16	1.33	5.75	0.02	2
2	5	2	3.20	0.51	0.15	1.60	6.40	0.01	2
2	5	3	3.67	0.56	0.14	1.89	7.10	0.01	3
2	5	4	4.16	0.62	0.14	2.21	7.85	0.00	3
2	5	5	4.69	0.67	0.13	2.54	8.66	0.00	3
3	0	0	0.87	-0.06	0.25	0.27	2.79	0.45	1
3	0	1	1.19	0.07	0.22	0.43	3.26	0.29	1
3	0	2	1.51	0.18	0.20	0.61	3.75	0.12	1
3	0	3	1.86	0.27	0.18	0.81	4.27	0.02	2
3	0	4	2.22	0.35	0.17	1.02	4.80	0.00	3
3	0	5	2.60	0.41	0.16	1.26	5.37	0.00	3
3	1	0	1.21	0.08	0.22	0.44	3.34	0.84	1
3	1	1	1.55	0.19	0.20	0.63	3.85	0.84	1
3	1	2	1.91	0.28	0.18	0.83	4.39	0.34	1
3	1	3	2.28	0.36	0.17	1.05	4.95	0.08	1
3	1	4	2.68	0.43	0.16	1.29	5.55	0.01	2
3	1	5	3.09	0.49	0.15	1.55	6.17	0.00	3
3	2	0	1.60	0.20	0.20	0.64	3.97	0.98	1
3	2	1	1.96	0.29	0.18	0.85	4.53	1.00	1
3	2	2	2.35	0.37	0.17	1.08	5.12	0.47	1
3	2	3	2.76	0.44	0.16	1.33	5.74	0.14	1
3	2	4	3.20	0.50	0.15	1.60	6.40	0.02	2
3	2	5	3.66	0.56	0.14	1.89	7.11	0.00	3
3	3	0	2.02	0.31	0.18	0.87	4.67	0.59	1
3	3	1	2.42	0.38	0.17	1.11	5.29	0.71	1
3	3	2	2.85	0.46	0.16	1.37	5.95	0.42	1
3	3	3	3.31	0.52	0.15	1.65	6.66	0.15	1
3	3	4	3.80	0.58	0.14	1.95	7.41	0.03	2
3	3	5	4.33	0.64	0.14	2.28	8.23	0.00	3
3	4	0	2.50	0.40	0.17	1.14	5.48	0.21	1

3	4	1	2.95	0.47	0.16	1.41	6.18	0.32	1
3	4	2	3.44	0.54	0.15	1.70	6.94	0.24	1
3	4	3	3.96	0.60	0.15	2.02	7.76	0.10	1
3	4	4	4.52	0.66	0.14	2.37	8.65	0.02	2
3	4	5	5.14	0.71	0.14	2.75	9.63	0.00	3
3	5	0	3.06	0.49	0.16	1.46	6.44	0.04	2
3	5	1	3.57	0.55	0.15	1.76	7.25	0.08	1
3	5	2	4.13	0.62	0.15	2.09	8.14	0.07	1
3	5	3	4.74	0.68	0.14	2.46	9.13	0.03	2
3	5	4	5.41	0.73	0.14	2.87	10.22	0.01	3
3	5	5	6.16	0.79	0.13	3.31	11.46	0.00	3
4	0	0	1.27	0.10	0.22	0.46	3.52	0.21	1
4	0	1	1.63	0.21	0.20	0.65	4.07	0.22	1
4	0	2	2.01	0.30	0.18	0.87	4.66	0.09	1
4	0	3	2.42	0.38	0.17	1.10	5.28	0.02	2
4	0	4	2.85	0.45	0.16	1.36	5.95	0.00	3
4	0	5	3.31	0.52	0.15	1.64	6.66	0.00	3
4	1	0	1.68	0.23	0.20	0.67	4.20	0.63	1
4	1	1	2.07	0.32	0.18	0.89	4.82	0.67	1
4	1	2	2.50	0.40	0.17	1.14	5.48	0.33	1
4	1	3	2.95	0.47	0.16	1.40	6.18	0.10	1
4	1	4	3.43	0.54	0.15	1.70	6.94	0.02	2
4	1	5	3.96	0.60	0.15	2.01	7.77	0.00	3
4	2	0	2.14	0.33	0.18	0.92	4.99	0.80	1
4	2	1	2.58	0.41	0.17	1.17	5.69	1.00	1
4	2	2	3.06	0.49	0.16	1.45	6.44	0.62	1
4	2	3	3.57	0.55	0.15	1.75	7.26	0.26	1
4	2	4	4.13	0.62	0.15	2.09	8.16	0.05	1
4	2	5	4.74	0.68	0.14	2.46	9.16	0.00	2
4	3	0	2.67	0.43	0.17	1.21	5.92	0.62	1
4	3	1	3.18	0.50	0.16	1.50	6.73	1.00	1
4	3	2	3.72	0.57	0.16	1.82	7.62	0.83	1
4	3	3	4.32	0.64	0.15	2.17	8.61	0.35	1
4	3	4	4.99	0.70	0.14	2.56	9.72	0.08	1
4	3	5	5.74	0.76	0.14	3.00	10.98	0.01	3
4	4	0	3.31	0.52	0.16	1.55	7.05	0.33	1
4	4	1	3.89	0.59	0.16	1.89	8.03	0.69	1
4	4	2	4.55	0.66	0.15	2.26	9.14	0.57	1
4	4	3	5.28	0.72	0.15	2.68	10.40	0.27	1
4	4	4	6.11	0.79	0.14	3.15	11.86	0.09	1
4	4	5	7.08	0.85	0.14	3.69	13.58	0.02	2
4	5	0	4.09	0.61	0.16	1.96	8.51	0.09	1
4	5	1	4.80	0.68	0.15	2.36	9.76	0.19	1
4	5	2	5.62	0.75	0.15	2.81	11.22	0.20	1
4	5	3	6.57	0.82	0.15	3.33	12.98	0.15	1
4	5	4	7.71	0.89	0.15	3.93	15.12	0.06	1
4	5	5	9.11	0.96	0.15	4.65	17.84	0.01	2
5	0	0	1.77	0.25	0.20	0.70	4.47	0.07	1

5	0	1	2.20	0.34	0.18	0.94	5.16	0.07	1
5	0	2	2.67	0.43	0.17	1.20	5.91	0.04	2
5	0	3	3.17	0.50	0.16	1.49	6.73	0.01	2
5	0	4	3.72	0.57	0.16	1.81	7.63	0.00	3
5	0	5	4.32	0.64	0.15	2.16	8.63	0.00	3
5	1	0	2.28	0.36	0.19	0.97	5.37	0.22	1
5	1	1	2.77	0.44	0.17	1.24	6.17	0.30	1
5	1	2	3.30	0.52	0.16	1.54	7.06	0.20	1
5	1	3	3.89	0.59	0.16	1.88	8.05	0.08	1
5	1	4	4.55	0.66	0.15	2.26	9.17	0.02	2
5	1	5	5.29	0.72	0.15	2.68	10.44	0.00	3
5	2	0	2.88	0.46	0.18	1.28	6.46	0.37	1
5	2	1	3.45	0.54	0.17	1.60	7.43	0.67	1
5	2	2	4.09	0.61	0.16	1.96	8.53	0.56	1
5	2	3	4.81	0.68	0.15	2.36	9.80	0.23	1
5	2	4	5.63	0.75	0.15	2.81	11.28	0.06	1
5	2	5	6.60	0.82	0.15	3.33	13.05	0.01	3
5	3	0	3.62	0.56	0.17	1.66	7.86	0.46	1
5	3	1	4.31	0.63	0.16	2.04	9.11	0.93	1
5	3	2	5.11	0.71	0.16	2.47	10.58	0.77	1
5	3	3	6.05	0.78	0.15	2.96	12.36	0.47	1
5	3	4	7.19	0.86	0.15	3.55	14.57	0.21	1
5	3	5	8.60	0.93	0.15	4.25	17.40	0.03	2
5	4	0	4.58	0.66	0.17	2.13	9.81	0.30	1
5	4	1	5.48	0.74	0.16	2.60	11.57	0.68	1
5	4	2	6.58	0.82	0.16	3.14	13.80	1.00	1
5	4	3	7.98	0.90	0.16	3.80	16.76	0.79	1
5	4	4	9.85	0.99	0.16	4.64	20.91	0.39	1
5	4	5	12.54	1.10	0.17	5.77	27.25	0.11	1
5	5	0	5.95	0.77	0.17	2.74	12.89	0.10	1
5	5	1	7.30	0.86	0.17	3.35	15.90	0.43	1
5	5	2	9.16	0.96	0.17	4.12	20.39	0.67	1
5	5	3	12.03	1.08	0.18	5.16	28.04	0.86	1
5	5	4	17.53	1.24	0.21	6.76	45.46	1.00	1
5	5	5	26.44	1.42	0.24	8.61	81.24	1.00	1

Table A2: MPN Index and 95% Confidence Limits for Various Combinations of Positive Results when Five Replicates per Dilution (50 mL, 25 mL, 10 mL) are Inoculated

Combination of Positives Plates			MPN/100 mL	Confidence Limits		Category
				Lower	Upper	
0	0	0	<0.2381	N/A	1.76	1
0	0	1	0.24	0.03	1.76	1
0	1	0	0.24	0.03	1.79	1
0	1	1	0.49	0.12	2.02	1
0	2	0	0.50	0.12	2.06	1
0	2	1	0.76	0.24	2.42	1
1	0	0	0.25	0.03	1.85	1
1	0	1	0.51	0.12	2.09	1
1	0	2	0.77	0.24	2.46	1
1	1	0	0.52	0.13	2.14	1
1	1	1	0.79	0.25	2.51	1
1	1	2	1.07	0.39	2.91	1
1	2	0	0.81	0.25	2.57	1
1	2	1	1.09	0.40	2.98	1
1	2	2	1.39	0.56	3.41	1
1	3	0	1.12	0.41	3.05	1
1	3	1	1.42	0.58	3.50	1
1	3	2	1.73	0.76	3.95	1
2	0	0	0.54	0.13	2.22	1
2	0	1	0.82	0.26	2.61	1
2	0	2	1.11	0.41	3.04	1
2	1	0	0.84	0.26	2.67	1
2	1	1	1.14	0.41	3.11	1
2	1	2	1.45	0.59	3.57	1
2	2	0	1.16	0.42	3.19	1
2	2	1	1.48	0.60	3.66	1
2	2	2	1.82	0.79	4.16	1
2	2	3	2.16	1.00	4.67	1
2	3	0	1.52	0.61	3.76	1
2	3	1	1.86	0.81	4.28	1
2	3	2	2.23	1.03	4.81	1
2	4	0	1.92	0.83	4.40	1
2	4	1	2.29	1.06	4.96	1
2	4	2	2.68	1.30	5.55	1
3	0	0	0.87	0.27	2.79	1
3	0	1	1.19	0.43	3.26	1
3	0	2	1.51	0.61	3.75	1
3	1	0	1.21	0.44	3.34	1

3	1	1	1.55	0.63	3.85	1
3	1	2	1.91	0.83	4.39	1
3	1	3	2.28	1.05	4.95	1
3	2	0	1.60	0.64	3.97	1
3	2	1	1.96	0.85	4.53	1
3	2	2	2.35	1.08	5.12	1
3	2	3	2.76	1.33	5.74	1
3	3	0	2.02	0.87	4.67	1
3	3	1	2.42	1.11	5.29	1
3	3	2	2.85	1.37	5.95	1
3	3	3	3.31	1.65	6.66	1
3	4	0	2.50	1.14	5.48	1
3	4	1	2.95	1.41	6.18	1
3	4	2	3.44	1.70	6.94	1
3	4	3	3.96	2.02	7.76	1
3	5	1	3.57	1.76	7.25	1
3	5	2	4.13	2.09	8.14	1
4	0	0	1.27	0.46	3.52	1
4	0	1	1.63	0.65	4.07	1
4	0	2	2.01	0.87	4.66	1
4	1	0	1.68	0.67	4.20	1
4	1	1	2.07	0.89	4.82	1
4	1	2	2.50	1.14	5.48	1
4	1	3	2.95	1.40	6.18	1
4	2	0	2.14	0.92	4.99	1
4	2	1	2.58	1.17	5.69	1
4	2	2	3.06	1.45	6.44	1
4	2	3	3.57	1.75	7.26	1
4	2	4	4.13	2.09	8.16	1
4	3	0	2.67	1.21	5.92	1
4	3	1	3.18	1.50	6.73	1
4	3	2	3.72	1.82	7.62	1
4	3	3	4.32	2.17	8.61	1
4	3	4	4.99	2.56	9.72	1
4	4	0	3.31	1.55	7.05	1
4	4	1	3.89	1.89	8.03	1
4	4	2	4.55	2.26	9.14	1
4	4	3	5.28	2.68	10.40	1
4	4	4	6.11	3.15	11.86	1
4	5	0	4.09	1.96	8.51	1
4	5	1	4.80	2.36	9.76	1
4	5	2	5.62	2.81	11.22	1
4	5	3	6.57	3.33	12.98	1
4	5	4	7.71	3.93	15.12	1
5	0	0	1.77	0.70	4.47	1
5	0	1	2.20	0.94	5.16	1

5	1	0	2.28	0.97	5.37	1
5	1	1	2.77	1.24	6.17	1
5	1	2	3.30	1.54	7.06	1
5	1	3	3.89	1.88	8.05	1
5	2	0	2.88	1.28	6.46	1
5	2	1	3.45	1.60	7.43	1
5	2	2	4.09	1.96	8.53	1
5	2	3	4.81	2.36	9.80	1
5	2	4	5.63	2.81	11.28	1
5	3	0	3.62	1.66	7.86	1
5	3	1	4.31	2.04	9.11	1
5	3	2	5.11	2.47	10.58	1
5	3	3	6.05	2.96	12.36	1
5	3	4	7.19	3.55	14.57	1
5	4	0	4.58	2.13	9.81	1
5	4	1	5.48	2.60	11.57	1
5	4	2	6.58	3.14	13.80	1
5	4	3	7.98	3.80	16.76	1
5	4	4	9.85	4.64	20.91	1
5	4	5	12.54	5.77	27.25	1
5	5	0	5.95	2.74	12.89	1
5	5	1	7.30	3.35	15.90	1
5	5	2	9.16	4.12	20.39	1
5	5	3	12.03	5.16	28.04	1
5	5	4	17.53	6.76	45.46	1
5	5	5	26.44	8.61	81.24	1

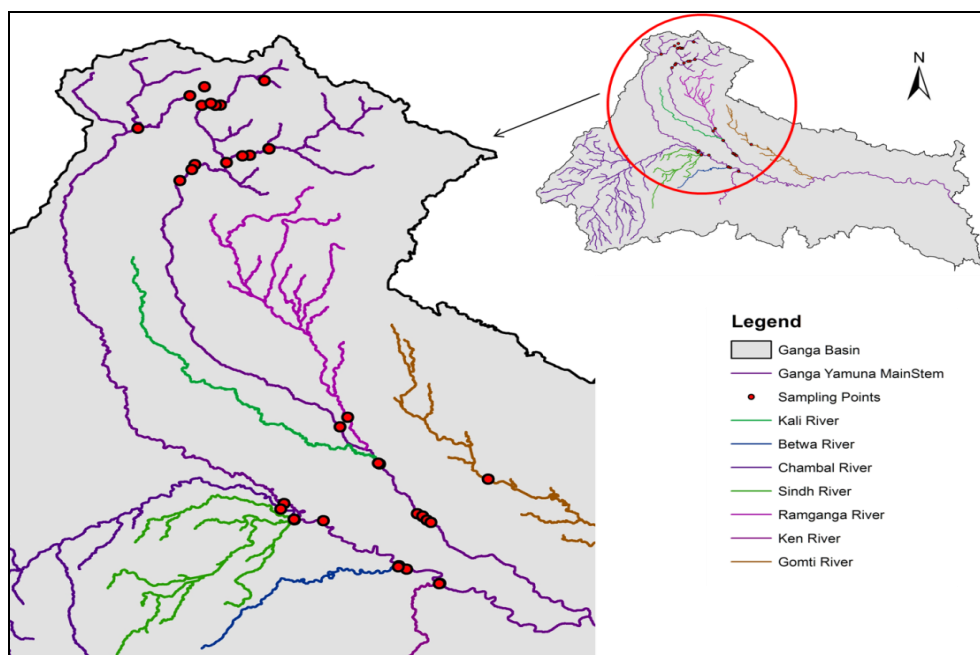


Figure A1: Sampling Locations on Various Rivers in Different Climatic Zone

Abbreviations

APHA	American Public Health Association
ATCC	American Type Culture Collection
CAB	Colorimetric agar based
CFU	Colony Forming Unit
CLAT	Culture latex agglutination and typing
COD	Chemical Oxygen Demand
D/S	Downstream
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EMB	Eosin - Methylene Blue
FC	Faecal Coliform
ISO	International Organization for Standardization
LCPA	Liquid colorimetric presence - absence
LPS	Lipopolysaccharides
MPN	Most Probable Number
mRNA	Messenger ribonucleic acid
NEERI	National Environmental Engineering Research Institute
PAB	Phage assay Base
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Unit
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
RT - PCR	Reverse Transcription - Polymerase chain reaction
SD	Standard Deviation
TCol	Total Coliform
TDS	Total Dissolve Solid
TOC	Total Organic Carbon
TPTZ	2,3,5-triphenyltetrazolium
TYG	Tryptone Yeast Extract Glucose
U/S	Upstream
US EPA	United State Environment Protection Agency
UV	Ultra Violet