POLLUTION INDICATOR BACTERIA OF SOME AQUATIC ENVIRONMENTS IN AND AROUND DHAKA.

A

DISSERTATION
SUBMITTED TO THE UNIVERSITY OF DHAKA
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF PHILOSOPHY

IN

MICROBIOLOGY

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REG. NO. 136/80-81
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DHAKA

ACKNOWLEDGEMENT

I wish to express my heartfelt gratitude to my respected supervisor Dr. K.H.S Aziz for his constant supervision, valuable suggestions and encouragement for carrying out this piece of research work. This work could not be initiated in ICDDR,B without his guidance.

I am grateful to my co-supervisors Dr. S. I. Khan, Associate Professor, Department of Microbiology, University of Dhaka for his constructive criticism and suggestion for preparing the draft and checking it carefully. I express my indeptedness to my another co-supervisor Dr. M. R. Khan, Professor, Department of Botany, University of Dhaka for his guidance and valuable suggestions to complete this work.

382951

I would like to express my gratefulness to Dr. Mozammel Hoq, Chairman, Department of Microbiology, University of Dhaka for his encouragement and co-operation.

I would like to thank Dr. W. B. Greenough III, former Director of ICDDR,B; Dr. D. Mahalanabis, Associate Director of Clinical Sciences Division and Moinul Islam, Acting Programme Head of ICDDR,B for kindly allowing me to use different facilities of ICDDR,B while carrying out this work.

I must render my heartfelt gratitude to Drs. M. I. Huq and M. S. Islam for their kind assistance at various phases of the work.

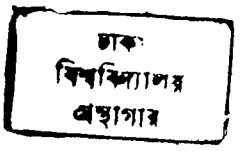
Grateful acknowledgements are due to Ms. L. Unicomb for her constructive criticism of the manuscript, to Mr. Badrul Alam Dewan for helping me with the analysis of data and to Messrs M. S. I. Khan and Forhad Hussain for helping with the literature.

I am grateful to Drs. E. E. Geldreich and Robert Bordner of the U. S. Environmental Protection Agency for kindly supplying me with their valuable publications related to my thesis work.

Thanks are also due to Messrs Ashraf Ali Khan, Sirajul Islam Molla, P. Sukul, Monzurul Haque, G. Rabbani, A. K. Banik and M. A. Malek for their technical assistance in their respective field of expertise.

Finally, I would like to express my utmost gratitude to my wife and uncle for their cooperation and continuous encouragement.

The work described in this dissertation was supported by the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) through protocol No. 82-017.



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ABSTRACT

Prevalence of enterotoxigenic *Aeromonas* spp., faecal coliforms (FC), faecal streptococci (FS) and ratio of FC to FS were studied. Water and sediment samples were collected from the Buriganga river and two ponds nearby. FC counts of water and sediment in the river ranged from 8.0×10^4 to 2.3×10^6/100 ml and from 2.2×10^4 to 8.6×10^7/100 g respectively. Similarly, FS counts of water and sediment in the river respectively ranged from 1.3×10^4 to 3.1×10^5/100 ml and from 4.0×10^3 to 1.8×10^7/100 g. FC counts of water and sediment in the ponds ranged from 1.0×10^3 to 3.7×10^5/100 ml and from 4.0×10^4 to 2.0×10^7/100 g respectively. In the ponds, FS counts of water and sediment ranged from 2.0×10^2 to 7.7×10^6/100 ml and from 9.0×10^4 to 4.2×10^6/100 gm respectively. In most cases, counts of FC and FS were always higher in the sediment samples than water. Seasonal variation in the counts of FC and FS was not apparent either in water or in sediment.

Ratio of FC to FS was always 4.0 or greater which indicated that waters of the river and ponds under investigation were polluted with human faeces. 210 *Escherichia coli* isolates were tested for their ability to produce heat labile (LT) and heat stable (ST) toxin, where 25 (12.0%) of them were toxin positive. Of the 25, five (2.4%) were positive for LT, 10 (4.8%) were positive for ST and the remaining 10 (4.8%) were positive for both LT and ST. Ten isolates producing both LT and ST were tested for antibiotic sensitivity pattern with respect to common

antibiotics. Only three isolates showed resistance to ampicillin while, others were sensitive to chloramphenicol, gentamicin, tetracycline, and trimethoprim-sulfamethoxazole. Ampicillin resistance was mediated by a plasmid having molecular weight of 49 megadalton.

One hundred eighty isolates of *Aeromonas* were isolated during the study period. Of them 120 were *A. hydrophila*, 40 were *A. sobria* and 20 were *A. caviae*. Ten isolates each of *A. hydrophila*, *A. sobria* and *A. caviae* were tested for enterotoxigenicity in rabbit ileal loop (RIL) model. Only five isolates of *A. hydrophila* and seven isolates of *A. sobria* showed enterotoxigenic activity in RIL. All enterotoxigenic strains of *Aeromonas* were haemolytic and thus 100% correlation was found between haemolytic activity on blood agar plate and enterotoxigenicity in RIL. Three RIL negative isolates each of *A. hydrophila*, *A. sobria* and *A. caviae* were subjected to consecutive passage. Within two to three passages, all the isolates showed fluid accumulation in RIL. The enterotoxigenic *Aeromonas* isolates were uniformly sensitive to tetracycline and gentamicin and resistant to ampicillin. It can be concluded that water of the Buriganga river and two ponds are polluted with human faeces and consequently contain enterotoxigenic bacteria. Thus these water sources are considered unsafe for drinking, household and recreational use.

Chapter I

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Population density in Bangladesh is alarmingly very high. As a result large amount of household waste and domestic effluents is generated in the form of pollutants. Disposal of wastes are done close to dwellings and in a disorganised way. Moreover, untreated domestic effluent and sewage are released into the nearby river through drains and sewerage system. Therefore,the land as well as the aquatic environment remain constantly polluted. Such aquatic environment with untreated domestic effluents and sewage may act as reservoir of various enteric pathogens. While using such polluted water for bathing, swimming and other purposes, people are likely to acquire various diseases related to enteric infection. This situation prevails in Bangladesh and other developing countries of the world where diarrhoea and other enteric diseases are endemic. Improvement of sanitation along with improvement of living standard might play a major role in reducing diarrhoea (Wolf *et al.* 1969).

Pathogenic microorganisms that can cause diarrhoeal diseases are transmitted through faecal- oral route mostly through contaminated food and water. Humans are the reservoirs of enteric pathogens which are excreted in faeces and ultimately enter water sources through untreated sewage. Thus the use of such water systems receiving untreated wastes may serve as the vehicle for the transmission of enteric pathogens resulting in humans con-

tracting infection with pathogenic microorganisms. Therefore, one should be aware of the level of pollution of the water before using it. Until today, high coliform count had been used as the measure of water pollution since Eijkman coined the word faecal coliforms (1904). These organisms are the normal flora of gut. Therefore, presence of coliforms in water indicate pollution with intestinal contents. In addition to faecal coliforms (FC), faecal streptococci (FS) are one of the common gut flora of human and other warm-blooded animals. Moreover, FS survive longer than FC. As a result, FS are used as the supplement of FC in determining the source of pollution of an aquatic environment (Bordner and Winter, 1978).

A large number of enteropathogenic microorganisms can be found in faecally polluted waters (Mara, 1978). Yet it is difficult to test such polluted waters for the presence of enteric pathogens, due to a) low concentration of enteric pathogens in water which cannot be detected by the conventional testing procedure; b) pathogens die off or become non- culturable. Therefore, faecal contamination of aquatic ecosystems is ascertained by the presence or absence of faecal pollution indicators: the FC and FS. Both FC and FS are present in higher number in the faeces and can survive longer than enteric pathogens (Bordner and Winter, 1978). *Aeromonas* is also abundant in the polluted aquatic environments (Hazen, *et al.*1978). This organism has attracted particular attention because of its association with

human diseases (von Graevenitz and Mensch, 1968). It is considered to comprise a portion of the normal flora of fish (Trust and Sparrow, 1974) and aquatic plants (Simidu *et al.* 1974). A major proportion of *Aeromonas* strains isolated from iverse aquatic environments are enterotoxigenic and there has been no difference of enterotoxigenicity between *Aeromonas* strains isolated from the environment and clinical sources (Annapurna and Sanyal, 1976). Hence, presence of enterotoxigenic strains in the environment has been considered to be of public health concern. Therefore, the present research was designed to study the abundance and distribution of *Aeromonas*, FC and FS in water and sediment samples of two ponds and three sampling points of the Buriganga river.

1.2 Review of literature

Faecal contamination of food and water is not desirable both for aesthetic reason as well as for avoiding transmission of enteric diseases. Historical accounts of Board Street Well epidemic in 1892 as well as the recovery of *Vibrio cholerae, Salmonella typhi* and entero-viruses from waters document the transmission of enteric pathogens through polluted waters (Hendricks, 1978). Moreover, theoretical basis for using members of coliforms as indicators of pollution can be found in the obscure report of von Fritsch in 1880 who observed *Klebsiella pneumoniae* and *K. rhinoscleromatris* in association with the human faeces (Clark and Kabler, 1964). In the year 1885, Escherich isolated *Bacterium colicommune* from the faeces of a patient suffering from cholera

(Escherich, 1885). Nineteen years later, Eijkman (1904) proposed the concept of faecal coliform (FC) as the indicator of faecal pollution. Over the years, FC have been considered as the normal gut flora of man and other warm-blooded animals. Therefore, presence of FC in water and food indicates direct evidence of faecal contamination .

American Public Health Association (APHA, 1975) has defined coliforms as aerobic, facultatively anaerobic, Gram negative, non-spore forming rods that can ferment lactose with the formation of gas when incubated either at $35\pm0.5^{o}C$ or $44\pm0.5^{o}C$ for 24-48h (APHA, 1975). Clark and Pagel (1977) found similar characteristics in four genera of *Enterobacteriaceae*, namely; *Escherichia*, *Klebsiella*, *Enterobacter* and *Citrobacter*.

Coliform as an indicator of faecal pollution has been used since 1904 (Eijkman 1904) . Several investigations have been carried out on the abundance of coliform in surface water (Bagde *et al*. 1982; Agrawal *et al*. 1976 and Geldreich *et al*. 1975) and in drinking water (Craun, 1981; 1979). In Bangladesh, presence of coliforms in drinking water in the metropolitan Dhaka city has been reported by Khan *et al*. (1978). Moreover, research related to the abundance of coliforms in ponds has been studied by Rashid (1982). Subsequently, abundance of coliforms in river water was carried out by Morshed (1983). But there has been no report of the abundance of faecal coliforms (FC) along with

faecal streptococci (FS) of the surface water available in this subcontinent.

Primarily, faeces are exposed to the nature after defecation. When sanitary latrine is the place of defecation, the excreta go to the safety tank and then contaminate surface water due to leakage or over-flow of the safety tank (Craun, 1981). Several waterborne epidemics of diarrhoeal diseases have been documented in the United States of America from 1971-1978 (Craun, 1981). In most of the instances, faecal contamination of water was detected by coliform counts.

There are a number of reports of epidemic caused by the consumption of contaminated supply of ground water. In Comerio, Puerto Rico, ground water was incriminated as the cause of *Shigella* epidemic (Craun, 1979). During the epidemic, no *Shigella* was isolated from the water sample, whereas the coliform count was very high. Bacteriological investigation revealed total and faecal coliform counts of 4900 and 23 per 100 ml of water respectively.

Prevalence of coliform can be implicated as the epidemic signal of diarrhoeal disease. In New York, in the year 1974 and 1975, there was an outbreak of giardiasis affecting 4,800-5,300 individuals (Shaw *et al.* 1977). Raw water supply of this area showed high faecal coliform counts prior to the outbreak. In another instance in 1974, there was approximately 1200 cases of

acute gastrointestinal illness in Richmond Heights, Fla, U.S.A (Weisman, *et al.* 1976). In this study, epidemiological investigation revealed that tap water consumption was associated with the illness. Further, it was found that one of the two wells providing water to the community had been continuously contaminated with coliforms.

Gelderich (1970) studied the relationship of the occurrence of FC and *Salmonella.* He found that in surface water *Salmonella* occurred in 62.5% of samples having coliform count of 10^3/100 ml. When FC count exceeded this number, *Salmonella* occurrence increased to 93.8% in the samples.

Enteric pathogens and indicators of faecal pollution undergo sedimentation after entering into an aquatic ecosystem (Grimes, 1980). These bacteria have been shown to persist in higher number in bottom sediment than the water column (Goyal *et al.* 1979; Gerba and McLeod, 1976 and Hendrick, 1971). Since, sand adsorb bacteria very loosely (Boyd *et al.* 1969), most of the bacteria including FC would probably be released into the water column during initial sediment disturbance caused by dredge cutter blade. After dredging, sediment bound FC are released into the water column due to which counts of FC increased in the water column but decreased in the soil sediment than those before dredging.

Coliforms survive for a lesser period of time than FS (Davenport *et al* 1976 and McFeter *et al.* 1974). Therefore, FC alone are not sufficient to assess the hygienic status of any aquatic ecosystem related to faecal pollution. For this reason, study of FS should be done along with FC. In addition to faecal pollution, study of FS may provide indication of either recent (Bordner and Winter, 1978 and McFeter*et al.*, 1976) or remote faecal pollution (Davenport *et al.* 1976 and McFeter *et al.* 1974).

In the survey of recent literature, there is a clear indication of the importance of FC as an indicator of the extent of contamination of water and food in the transmission of enteric diseases. In a study to determine the importance of waterborne and water-washed transmission of diarrhoea, Henry and Rahim (1990) made a comparison of the extent of contamination of children's hands and drinking water with their diarrhoeal morbidity. They found significant correlation between the incidence of diarrhoea and contamination of hands as measured by FC counts. Heinan *et al.* (1988), while studying the faecal contamination of drinking water in a rural commercial farming area also used FC counts as the only indication. Using this parameter, they found that borehole water quality (10 or less FC/100 ml) was satisfactory than river water, piped water and piped mountain water. They found increased contamination in household water containers than at the source. This indicated that contamination took place after collection and subsequent

storage. In addition, several accounts of faecal contamination of food (Richter and al-Sheddy, 1990; Imong, *et al.* 1989) and water (Edberg, *et al.* 1990; Mertens, *et al.* 1990; Baveja, *et al.* 1989; Sandiford, *et al.* 1989; Sami *et al.* 1988) have been reported as measured by FC counts.

Faecal streptococci (FS) are present in the faeces of human and other warm-blooded animals (Clausen *et al.* 1977). Therefore, their presence in the aquatic ecosystem indicates pollution with faeces of either human or warm-blooded animals. They can survive longer than coliforms and other bacterial pathogens (Clausen *et al.* 1977; Davenport, *et al.* 1976 and McFeter, *et al.* 1974), although some of the species of FS die off rapidly (McFeter, *et al.* 1974). Moreover, FS cannot multiply in nature or in faecally polluted waters. Therefore, either they die-off or their number remains unchanged for a considerable period of time in aquatic ecosystem receiving faecal pollution (Kenner *et al.* 1980).

Faeces of livestock are distinguished by the presence of significant proportions of *S. bovis* and *S equinus* (Clausen *et al.* 1977). In aquatic ecosystem, presence of these two species in higher number indicates pollution from meat processing plants, run-off and from feed-lot and farmlands (Bordner and Winter, 1978). On the other hand, faeces of human beings are characterized by the presence of enterococci, but the exact speciation of enterococci has not yet been ascertained. *S. faecalis* subsp. *liquefaciens* constitutes about 25 percent, *S. faecalis* of about

40 percent and *S. duran* constitutes about 4 percent of the total streptococcal population (Clausen *et al.* 1977). Little is known about the importance of the remaining 31 percent of the 8 streptococci in relation to faecal pollution indicator. Percentage of these two species (*S. bovis* and *S. equinus*) together ranged from 18.9 for pigs to 66.2 for cattle (Kenner *et al.* 1980). The remaining population is primarily enterococci, of which 18 percent have been identified as *S. faecalis* subsp. *liquefaciens*.

Faeces of human origin may be specified not only by the speciation of enteric bacteria but more simply by the determination of the ratio of FC to FS (Clausen *et al.* 1977). Human faeces are characterized by higher number of coliforms than streptococci whereas animal faeces are characterised by higher number of streptococci than coliforms. Therefore, the source of faecal pollution of a aquatic ecosystem can be determined by the ratio of FC to FS. Pollution with human faeces are characterized by the ratio of 4.0 or above and that with animal faeces are characterised by the ratio of 0.7 or less (Bordner and Winter, 1978).

Escherichia coli is a normal flora of gut but few strains of *E. coli* produce enterotoxin and are responsible for causing diarrhoea. Such *E. coli* strains are known as enterotoxigenic *E. coli* (ETEC). Smith and Halls first isolated ETEC from diarrhoeal stools of pig in the year 1967. Subsequently, it has been reported that ETEC was responsible for causing cholera-like rice-

watery diarrhoea. In an urban situation, ETEC has been reported as one of the etiological agents of diarrhoea among 55.0% of the hospitalised diarrhoeal cases studied in Bangladesh (Merson *et al.* 1976). In rural Bangladesh, ETEC was the most frequently isolated pathogen in all age groups and its isolation rate was higher than all other etiological agents of diarrhoea (Black *et al.* 1980). ETEC has been reported to be associated with childhood diarrhoea in the developing countries (Black *et al.* 1981) and with 70.0% of travellers' diarrhoea in Mexico (Merson *et al.* 1976). In an epidemiological study, Tailor *et al.* (1982) have identified contaminated food and water as the main vehicle of the transmission of ETEC. Their findings gave a clue that ETEC could be isolated from the environment. In the year 1983, 10 strains of ETEC were isolated from the Buriganga river (Morshed, 1983).

The emergence of antibiotic resistant strains of bacteria is closely linked with the use of antibiotics for the treatment of human infection (Ayliffe, 1976, Mouton *et al.* 1976). Resistance may develop rapidly or slowly depending on the organism concerned, the volume and type of antibiotic used, and the method of application. From human beings and animals, antibiotic resistant bacterial strains are released into the sewerage and surface water through excretion, death and decay. Ultimately, sewerage and surface waters contribute to the distribution and circulation of resistant organisms. Sewerage and surface waters represent a natural medium in which R-plasmid transfer can occur under cer-

tain physical, chemical and biological conditions (Bell *et al* 1980). From sewerage and surface waters, antibiotic resistant bacteria enter food and drinking water under some circumstances which leads to recycling of antibiotic resistant bacteria to vulnerable man and animals (Richardson, 1972 and Smith, 1970).

The distribution of antibiotic resistant strains of *Enterobacteriaceae* in aquatic environment has been studied in recent years in different parts of the world. For example, river and sewerage waters in South Africa (Grabow and Prozesky, 1973); surface waters, sea water and shell-fish in New Zealand (Cooke, 1976); sewerage in Canada (Bell *et al.* 1980); and surface water, sea water and sediment in the United States of America (Goyal *et al.* 1979 and Kelch and Lee, 1978) have been studied for the occurrence of antibiotic resistant *Enterobacteriaceae.* In majority of the studies, interest was mostly focused on transferable drug resistance because of its practical implication. In Bangladesh, incidence of drug resistant coliform bacteria has been reported (Aziz *et al.* 1986) but the transferability of drug resistance has not been studied so far.

Sanarelli (1891) reported the isolation of a bacterium from frog and named it *Bacillus hydrophillus fucus*. He reported the potentiality of this bacterium for causing bacteremia in frog and pathogenicity in various lizards, sunfish and warm-blooded laboratory animals such as rodents, young dogs, cats, pigeons and fowl. Later, he also gave some taxonomical description of this

bacterium. In 1898, Russel reported isolation of the same bacterium of Sanarelli (1891) and described its polar flagellation. Subsequently, Weildin and Levine (1923) placed *Bacillus hydrophillus* in the genus *Proteus* and this microorganism was classified as *Proteus hydrophilus* by Bergey *et al.* (1923). However, Rustigian and Stuart (1941, 1943, 1945) listed a number of reasons and argued in favor of putting this bacterium away from the genus *Proteus*. Kulp and Borden (1942) reconfirmed polar, monotrichous flagella of *B. hydrophillus*. Their (Kulp and Borden, 1942) observation helped microbiologists to take *B. hydhophillus* away from *Proteus* which bears peritrichus flagella. In the sixth edition of the Bergey's manual (Breed *et al.* 1948), *B. hydrophillus* Chester was correctly described as a monotrichous bacterium and placed in the genus *Pseudomonas* as *P. hydrophila*. In the seventh edition of the Bergey's Manual (Breed *et al.* 1957), the generic term *Aeromonas* (Kluyver and van Niel, 1936) was adopted. Thus the microorganism that has been discussed in the foregoing paragraph was named *Aeromonas*.

The genus *Aeromonas* is a facultatively anaerobic, asporogenous, polarly (occasionally atrichus) flagellated, Gram negative, rod shaped bacterium that can ferment carbohydrates with or without acid and gas and produce positive cytochrome oxidase reaction. This bacterium has been regarded as an autochthononus inhabitants of aquatic environments where it shows depth distribution gradient (Hazen, 1976). It has been isolated from fresh

water (Rippey and Cabelli, 1979) as well as from brackish water (Kaper *et al.* 1981). This bacterium is also important as one of the bacterial agents causing diarrhoea (Wadstrom and Ljungh, 1977 and Sanyal *et al.* 1976). *Aeromonas* secretes enterotoxin which induces cyclic adenosine monophosphate mediated diarrhoea. Moreover, it has been found that enterotoxigenicity is similar irrespective of their source of isolation (Annapurna and Sanyal, 1976). Since *Aeromonas* is an environmental organism having potentiality to induce enterotoxigenicity, its abundance in the water should be considered while judging water quality.

1.3 Objective

From the foregoing review of literature, it is clear that FC and FS are one of the important components of pollution indicator. Suitability of water use for various purposes has been conditioned on the counts of FC and FS by the W.H.O. (1971). Moreover, the source of a number of diarrhoeal epidemics has been traced using FC and FS as the only indicator which helped to reduce diarrhoeal mortality and morbidity through necessary precautionary measures. FC and FS are mostly non-toxigenic but some of them have been proven to be enterotoxigenic for humans. Besides, some strains of environmental *Aeromonas* are potentially enterotoxigenic and can cause human diarrhoea. Considering the implication of pollution due to FC, FS and *Aeromonas* spp., the following objectives were set for this investigation:

i) to determine the prevalence of faecal coliforms (FC) and faecal streptococci (FS) and to establish possible correlation between them

ii) to test the potentiality of the *Escherichia coli* isolates to produce heat labile (LT) and heat stable (ST) toxin and to determine the drug sensitivity pattern of enterotoxigenic *E. coli* isolates

iii) to test the enterotoxin producing capability of *Aeromonas* spp. and to assess their drug sensitivity pattern

iv) to determine the enzyme profile of enterotoxigenic *Aeromonas hydrophila* isolates

Chapter II

MATERIALS AND METHODS

2.1 Sampling Sites:

Samples were collected from the Buriganga river and from two ponds of the village Chunkuthi para (Fig. 1)

2.1.1 Sampling sites in the Buriganga river: The river Buriganga flows along the southern side of the Dhaka city at a latitude and longitude of 20^{o} 43.20"N and 90^{o} 26.10"E respectively. It is a branch of the river Dhaleswari and receives domestic effluents and sewerage discharge of the Greater Dhaka city. Samples were collected from three points of the Buriganga river: Babubazar ghat, Sadar ghat and Farashganj ghat.

2.1.1.a Babubazar ghat : It is the first sampling site in the river which receives sewerage and domestic wastes from the Salimullah Medical College and old part of the Dhaka city. Water of this ghat is being used for bathing by the people living in the Babubazar area. Water from this ghat is also being used for washing of utensils and other household purposes.

2.1.1.b Sadar ghat: It is the second sampling site in the river. Sadar ghat connects the capital city Dhaka with other important districts through riverways. Steamer and launch passengers coming to Dhaka or going out usually defecate at the terminal. Therefore, this sampling point receives faecal pollution from the

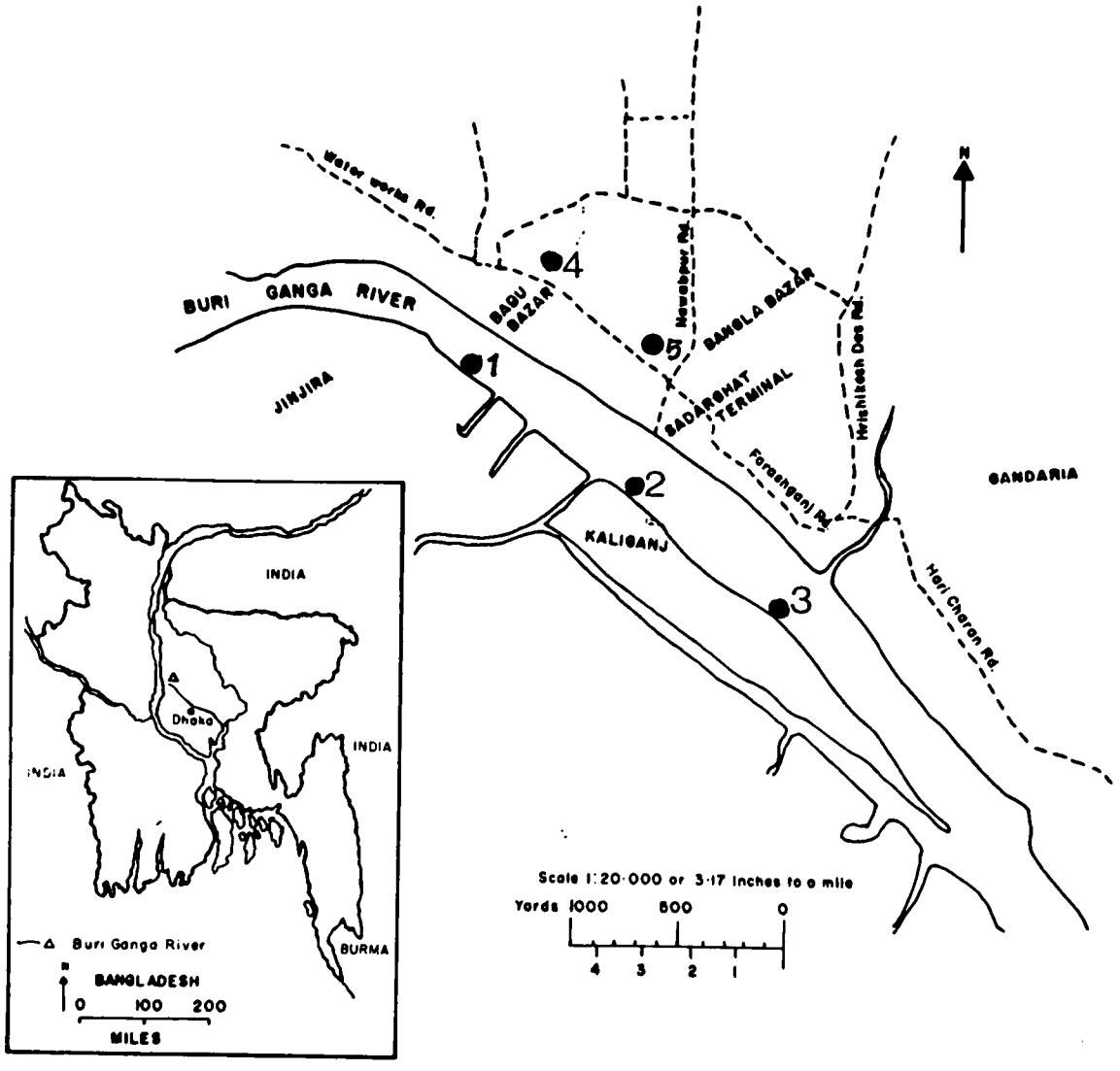

Fig. 1. Map showing sampling points (●). 1= Babubazar ghat 2= Sadar ghat, 3= Farashganj ghat, 4= pond No. 1 and 4= pond No. 2

passengers in addition to the domestic wastes and sewerage from the city. This ghat is also being used as a bathing place. Water from this ghat is also used by the people for various household purposes.

2.1.1.c Farashganj ghat: It is river sampling point No. 3. It is used for bathing of hundreds of people living nearby. This sampling point apparently seemed clean except for one fly latrine.

2.1.2 Sampling from ponds: Ponds were selected in the village Chunkuthipara which is situated on the southern bank of the river Buriganga.

2.1.2.a Pond No. 1: Water of this pond is being used daily by a large number of people living nearby. Two fly latrines are constructed near this pond. There is no embankment surrounding this pond to protect its water from being drained out or in during monsoon.

2.1.2.b Pond No. 2: Water of this pond is used by hundreds of people for bathing, washing of utensils and soiled clothes of sick children and adults. There is no latrine constructed nearby. Moreover, there is a high embankment surrounding this pond to prevent inflow and outflow of water during monsoon.

2.2 Method of Sample Collection:

2.2.1 Kinds of sample: Water and sediment samples were collected monthly from each sampling point in the morning.

2.2.1.a Collection of water sample: Water samples were collected from a depth of one meter by Hach dissolve oxygen sampler and taken into a sterile 4 ounce glass bottle.

2.2.1.b Collection of sediment sample: Sediment samples were collected with the help of a ICDDR,B constructed core sampler and taken into a sterile 4 ounce glass bottle. Both water and sediment samples were collected 5-6 yards away from the bank.

2.2.2 Transportation of sample: After collection, water and sediment samples were transported to the laboratory in an insulating foam box with ice to maintain a temperature ranging from 4^{o} to $6^{o}C$.

2.3 Processing of Sample

2.3.1 Serial dilution of water sample: Before making any dilution, each of the water samples was shaken vigorously. One ml water sample was added to 9.0 ml of sterile normal saline to give a 10^{-1} dilution. Then one

ml sample from 10^{-1} dilution was transferred to 9.0 ml of sterile normal saline to give a 10^{-2} dilution. In this way, ten fold serial dilution of water sample was made from 10^{-1} to 10^{-5} in sterile normal saline. Each dilution was thoroughly mixed using vortex mixture (Vortex Genie, Scientific Industries, Inc. Bohemia, N.Y. 11716, USA).

2.3.2 **Serial dilutin of sediment sample:** One gram sediment was added to 9.0 ml of sterile normal saline and shaken vigorously to give a homogenous suspension of 10^{-1} dilution. Then 1.0 ml mixture from 10^{-1} dilution was transferred to another tube containing 9.0 ml of sterile normal saline to give 10^{-2} dilutin. In this way ten fold serial dilution of sediment samples was made from 10^{-1} to 10^{-5}.

2.3.3 **Membrane filtration method:** From each dilution, one ml water sample was passed through grided membrane filter (Gelman GM6, 47 mm diameter, 0.45 um pore size) using vacuum pump and Millipore filtration apparatus. Then the filter with retained bacteria was placed either on mFC or on K.F. streptococcus agar plate (65 x 15 mm style, Falcon: 1950 Williams Dr. Oxnard, CA 93030 USA). Membrane filter with retained bacteria was placed onto mFC agar plate and incubated at 44.0 $^{+}$- 0.5^{o}C for

18 to 24 h for growth of FC. Similarly, membrane filter with retained bacteria was placed on K.F. streptococcus agar plate and incubated at 37.0 ^{o}C for 48 h for growth of FS.

2.3.4 Spread plate method: From each dilution, 0.1 ml of serially diluted sediment sample was plated either on mFC or on K.F. streptococcus agar plate (15 x 100 mm) and spread all over the surface with the help of a sterile bent glass rod (APHA, 1975). Inoculated plates were incubated at 44.0^{o}C $\pm$ 0.5^{o}C for 18 - 24 h for growth of FC or at 37.0^{o}C for 48 h for growth of FS.

2.4 Enumeration of Faecal Coliforms (FC):

2.4.1 Medium: Membrane faecal coliform (mFC)(Difco Chemical Company) is one of the best selective media for isolation of FC. Therefore, mFC agar was used throughout the study.

2.4.2 Enumeration of FC from water sample: From water sample, FC was counted following membrane filtration method as described in Section 2.3.3. After overnight incubation, typical FC colonies (greenish blue to bluish black) were counted over the membrane filter (APHA, 1975) and the count was expressed as FC/100 ml water.

2.4.3 **Enumeration of FC from sediment sample:** From sediment sample, FC was counted following spread plate method as described in Section 2.3.4. After overnight incubation, typical FC colonies (greenish blue to bluish black) were counted on the mFC agar plate (APHA, 1975) and the count was expressed as FC/100 g sediment.

2.5 **Enumeration of FS:**

2.5.1 **Medium:** K.F. streptococcus agar medium was used for enumeration of FS. The medium was prepared according to the manufacturer's instruction (Difco Chemical Company).

2.5.2 **Enumeration of FS from Water sample:** From water sample, FS was counted following membrane filtration method as described in Section 2.3.3. After 48 h of incubation, typical FS colonies (dark red to pink coloured colony) were counted (Bordner and Winter, 1978) and the count was expressed as FS/100 ml water.

2.5.3 **Enumeration of FS from sediment sample:** From sediment sample, FS was counted following spread plate method as described in Section 2.3.4. After 48 h of incubation, typical FS colonies (dark red to pink coloured colony) were counted on K.F. streptococcus agar plate (Bordner

and Winter, 1978) and the count was expressed as FS/100 g sediment.

2.6 Ratio of FC to FS: Density of FC was divided by the density of FS to have a FC/FS ratio. This value was calculated for all the water and sediment samples collected from all the sampling sites throughout the study period.

2.7 Correlation between FC and FS: Correlation between FC and FS of water and sediment samples was calculated for all the sampling sites throughout the atudy period.

2.8 Identification of FC:

2.8.1 Preparation of pure culture FC: Two to five morphologically different typical FC colonies were picked up from over the membrane filter placed on the mFC agar (in case of water sample) or directly from the mFC agar plate (in case of sediment sample) and streaked onto Eosin Methylene Blue (EMB) agar plate for pure culture as well as for confirmation. Typical FC colonies appears greenish black with metallic sheen on the surface of the colony on EMB agar plate.

2.8.2 Biochemical reaction for identification of FC: Confirmed FC isolates were identified by their characteristic reaction in kliglar iron agar; motility

indole urea; simmon's citrate, arginine dihydrolase, lysine and ornithine decarboxylase and fermentation of malonate, rhamnose and sorbitol. Isolates were identified by matching the reaction of the test isolates with standard reaction (Edward and Ewing, 1982)

2.9 Identification of FS:

2.9.1 Preparation of pure culture of FS: Two to five morphologically different typical FS colonies (dark red to pink colored colony) were picked up from over the membrane filter placed on K.F. streptococcus agar (in case of water sample) as well as directly from K.F. streptococcus agar plate (in case of sediment sample) and streaked onto brain heart infusion agar plate (Difco Chemical Company) which was incubated aerobically at 37^{o}C for 18-24 h. After incubation, well-isolated colonies were selected for identification following procedure as described below.

2.9.2 Biochemical reaction for identification of FS: Isolates of FS were identified through a series of biochemical tests involving catalase; Gram staining; ability to grow at different temperatures (10^{o} and 45^{o}C) and at pH 9.6; ability to reduce potassium tellurite (0.04%) and triphenyltetrazolium chloride (1.0%);

fermentation of glycerol (1.0%), D-sorbitol (1.0%) and L-arabinose (1.0)%); haemolytic activity on sheep blood agar (10.0%) and liquefaction of gelatin (12.0%) (Borddner and Winter, 1978)

2.10 Test for Detection of Heat Labile (LT) and Heat Stable (ST) Toxin of *Escherichia coli* Isolates:

2.10.1 Preparation of crude toxin: Culture filtrate containing crude toxins of both LT and ST was prepared in the same culture tube. *E. coli* isolates to be tested were inoculated into tube containing 2.5 ml of Trypticase soy broth with 0.6% yeast extract. After inoculation, tubes were incubated in roller drum at 37^{O}C having 22-25 circles per minutes for 20-22 h.

After incubation, tubes were taken out and the cultures were centrifuged at 22,000 x g for 20 minutes at 4.0^{O}C. The supernatant was divided equally into two one drum vials. One drop (5 ug) gentamicin was added into one of the two vials which was stored in room temperature for detection of ST. The other vial was kept frozen at - 20^{O}C and was used for detection of LT.

2.10.2 Detection of ST by suckling mice model: In this investigation, 2-4 day old new born Swiss albino suckling mice were used. Before use, suckling mice

were separated from their mothers and were divided into groups of two. Each mouse was inoculated (by peritoneal injection) with 0.1 ml of crude toxin containing two drops of 2.0% Evans Blue per ml. Then the inoculated mice were incubated at 25^{o}C for 4 h. After incubation, the mice were killed by ether inhalation. Abdomens of the killed mice were opened by a pair of forceps and the entire intestine was removed. The weight of the gut to the remaining carcass was taken. The ratio of the gut weight to remaining carcass weight was calculated for each mouse and the results averaged. A ratio less than 0.070 is considered negative, 0.070 to 0.085 is considered border line and a ratio over 0.085 was considered positive (Giannella *et al.* 1976).

2.10.3 Detection of LT by tissue culture assay: Chinese hamster ovary (CHO) cell assay is a standard procedure for detection of LT. Guerrant *et al.* (1974) found this assay to be reproducible and simple.

2.10.3.a Preparation of CHO cell monolayer: New tissue culture flask (Falcon, 25 CM^2) containing 4.5 ml of F-12 medium with 10.0% foetal calf serum and antibiotics (100 ug/ml of streptomycin, and 100 unit/ml of penicillin G) was inoculated with one or two drops of trypsinized cell

suspension. A monolayer of CHO cell formed all over the floor of the tissue culture flask within 3-4 days.

2.10.3.b Trypsinization of CHO cell: F-12 medium was decanted after the monolayer had formed and 2.0 ml of trypsin-EDTA (Sigma Cell Culture Reagent, x1) was added into flask which was tilted 10-12 times within 5-6 seconds. Then the trypsin was decanted and the flask was then incubated at 37^{o}C for 20 minutes with monolayer side of the flask up. Trypsinization helps to dissociate monolayer into component cells.

2.10.3.c Preparation of CHO cell for LT assay: After incubation, trypsin was completely decanted and 2.0 ml of F-12 medium with 10.0% foetal calf serum was added into the culture. Flask was agitated to get the cells released from the flask wall as well as to break off any clot present.

2.10.3.d CHO cell assay: An aliquot of 0.02 ml of prepared crude toxin was added in each well of the microtitre plate in duplicate. 0.2 ml of cell suspension (as prepared in Section 2.10.3.b) was added in each well of the plate and incubated at 37^{o}C. After 20-24 h of incubation, number of elongated cells/100 cells in each well was recorded. Equal or less than 9.3 elongated

cells/100 cell is considered negative and a positive is equal to or more than 13.6 elongated cells/100 cell.

2.11 Isolation and Identification of *Aeromonas* spp.: Rose coloured, non-faecal coliforms were picked up from the mFC agar and streaked onto mFC agar for pure culture. One discrete colony was tested for cytochrome oxidase. Cytochrome oxidase positive colonies were tested for resistance to 0/129 (2, 4-diamino 6,7-diisopropylpteridine), decarboxylation of lysine and ornithine; dehydrogenation of arginine; fermentation of glucose (with or without gas) and salicine; utilization of arabinose; hydrolysis of esculine; Voges-Proskauer reaction; growth in KCN and peptone water containing 0 and 3.0% NaCl (Popoff, 1984).

2.12 Test of Enterotoxigenicity of *Aeromonas* Isolates:

2.12.1 Preparation of live culture: The stock culture was subcultured onto nutrient agar plates. One isolated colony from the nutrient agar plate was inoculated into T_1N_1 broth (1.0% NaCl + 1.0% Trypticase, pH 7.2) for 4 h. One ml of such culture was used for testing toxigenicity . Live cultures of positive (*Vibrio cholerae* 569B) and negative (*E. coli* K-12) controls were prepared similarly.

2.12.2 **Preparation of cell-free culture filtrate:** Ten ml of Richardson's medium (Richardson *et al.* 1964) was inoculated with one ml of fresh *Aeromonas* culture (4 h growth in T_1N_1 broth at 37°C) in 50.0 ml conical flask. The inoculated flasks were incubated at 37°C in a shaking waterbath with 80 oscillations per minutes for 18 - 24 h. After incubation, cultures were centrifuged at 22,000 x g for 20 minutes at 4°C and the supernatants were sterilized by passing through millipore membrane filter (0.22 um pore size). Sterilized culture filtrates were stored at -20°C until further use. Culture filtrates from control (positive and negative) strains were prepared similarly.

2.12.3 **Rabbit ileal loop (RIL) assay:** The RIL test was performed in adult rabbit following the method of De and Chatterjee (1953). In short, rabbit weighing 1.0-2.0 kg was starved for 48 h. During starvation, only water was provided to the starved rabbits. Before operation, the rabbit was anasthesized with intravenous injection of sodium pentothanate (0.5 ml per kg body weight). Then the small intestine was taken out of the abdomen by laperotomy and loops of approximately 10 cm length were tied beginning near the ileoscaecal junction with intervals of about 5.0 cm in between. Four to six loops were made in each rabbit. One ml

each of 4 h culture of test strains in T_1N_1 broth and cell-free culture filtrates (as prepared in Section 2.12.2) were inoculated into ligated loops. Live cultures and cell-free culture filtrates prepared from the positive and negative controls were also injected into the loops as positive and negative controls respectively. The first loop was always used as positive control and the last loop was used as negative control. Live cutures and cell-free culture filtrates were tested separately in duplicate rabbits. Animals were sacrificed after 18- 20 h and the abdomen of killed rabbit was punctured to take out the inoculated ileum. Reaction in each loop and inter-loop was noted. Results showing fluid accumulation in the inter-loop was repeated. Then the length of loop and quantity of fluid accumulation in each loop were measured. From the measurement, volume of fluid accumulation per cm of gut was calculated. Fluid less than 0.5 ml per cm of gut is considered negative (DE and Chatterjee, 1953)

2.12.4 Passage of *Aeromonas* isolates into RIL: Live cultures of those strains which failed to induce fluid in RIL were subjected to repeated passage. One ml of fresh live culture (4 h growth in T_1N_1 broth at 37^oC) was inoculated into ligated ileal loop of the rabbit which was sacrificed after 18 - 20 h. Content of each loop

was cultured on MacConkey's agar following aseptic technic and incubated overnight for bacterial growth. After incubation, one well-isolated typical aeromonad colony was inoculated into T_1N_1 broth for 4 h and one ml of such culture was injected into the loop of another rabbit. The inoculated rabbit was sacrificed after 18 - 20 h and the reaction in the loop was noted. The process was repeated until the isolate could induce fluid in the loop (Annapurna and Sanyal, 1977). In a rabbit, two controls (one positive and one negative) along with six test isolates are passaged in one rabbit like normal RIL assay.

2.13 Comparison of the Enzyme Profile of Enterotoxigenic *Aeromonas hydrophila* Isolated from Surface Water (pond and river) and Ulcerated Fish

2.13.1 Test isolates: Enzyme profile of enterotoxigenic *Aeromonas hydrophila* isolated form surface water (N=7) (river and pond water) and ulcerated fish were compared. Fish isolates were obtained from the Environmental Microbiology Laboratory of ICDDR,B.

2.13.2.a Detection of enzyme profile of enterotoxigenic *Aeromonas hyrophila* isolates: Enzyme profile of enterotoxigenic *Aeromonas hydrophila* isolates was determined by the API 20 zym kit (from API System, S.A.

Montalieu, Vercieu, France). This kit is a semiquantitative micromethod system in which 19 different enzymes can be measured by matching the intensity of colour in the chart (provided by the company) with the colour that develops in the kit during the process. In the chart, each colour intensity corresponds with a particular amount of enzyme which ranges from 0 to $\geq$ 40 nanomoles (nm). The amount of enzyme between zero and less than 5 nm has been described as trace. The 19 enzymes assayed were: phosphatase alkaline, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, phosphatase acid, phosphoamidase, β -galactosidase, α - galactosidase, α-glucuronidase, α-glucosidase, β-glucosidase, N - acetyl - β - glucosaminidase, α-mannosidase, and α- fucosidase.

2.13.2.b Preparation of culture for detection of enzyme by API 20 zym: Culture of test isolates from an agar slant was suspended in 2.0 ml of sterile normal saline to give a turbidity between McFarland No. 5 and 6 standard. In order to obtain a reproducible results, the organisms to be compared were grown on the same isolation medium. The dilution and the turbidity were kept uniform.

2.13.2.c Preparation of API gallaries: A set of tray and lid was prepared for each test isolate. Isolate number or any other identifying remarks of the test isolates was written on the elongation flap of the tray. For providing a humid atmosphere during incubation, 5.0 ml of distilled water was dispensed into the tray.

2.13.2.d Inoculation and incubation of API gallaries: API zym gallaries were taken out of the sealed envelope and placed into the incubation tray. With the help of a Pasteur pipette, two drops of culture were added in each cupule of the gallary. The lid was replaced after inoculation. Then the inoculated API gallaries within the incubation tray (with lid) was wrapped with alluminium foil (to avoid light exposure) and incubated for 4 h at 37^{o}C. After incubation, one drop each of API zym A and API zym B was added in each cupule. Then each of the gallery was allowed to stand for five minutes and exposed to bright sun light for 10 seconds. After light exposure, negative cupules appeared colourless whereas, cupules with enzyme developed colour of different shades depending on the concentration of enzyme.

2.13.2.e Quantitation of enzyme: Quantity of enzyme in each cupule was determined by matching the intensity of colour in the chart (provided by the manufacturer) with the colour developed in the kit during the process.

2.14 Test of Drug Sensitivity:

Test of drug sensitivity was carried out by standard single disk agar diffusion method of Bauer *et al.* (1966). In short; test isolates were grown in T_1N_1 broth for 4 h at 37^oC. After incubation, cultures having uniform turbidity equal to that of a turbidity standard prepared by adding 0.5 ml of 1.0% $BaCl_2$ to 99.5 ml of 1.0% H_2SO_4 (0.36 N) was incubated onto Mueller-Hinton agar plate with the help of a sterile cotton swab.

2.14.1 Antibiotic disk: Antibiotic disks were obtained from BBL Microbiology System (Becton Dickinson and Co., USA). The antibiotics and chemotherapeutic agents and their concentrations were expressed in microgram (except where indicated otherwise) per disk: ampicillin, 10; cefamandol, 30; chloramphenicol, 30; colistin, 10; getamicin, 10; tetracycline, 30 and trimethoprim + sulfamethoxazole, 1.25 + 23.75.

2.14.2 Test isolates: Ten isolates of enterotoxigenic *E. coli* and 12 isolates of enterotoxigenic aeromonads were tested for antibiotic sensitivity.

2.14.3 Inoculation of antibiotic disk: Antibiotic disks were placed on the inoculated Mueller-Hinton agar plate with the help of a pair of cool flamed forces. Inoculated plates were incubated overnight at $37^{o}C$.

2.14.4 Determination of resistance or sensitivity pattern of a bacterial strain: After overnight incubation, diameter of the zone of inhibition that appeared around each antibiotic disk was measured in mm with a ruler on the under surface of the Petri dish. Then the diameter of the zone of inhibition was compared with standard chart to characterize a strain either resistant or sensitive.

2.15 Plasmid Profile of Enterotoxigenic *E. coli* Isolates:

2.15.1 Control bacterial strain: 14R525 is a nalidixic acid resistant mutant of *E. coli* K -12 and is devoid of fertility factor (F^{-}). This strain was obtained from Enteric Reference Laboratory, Colindale Avenue, London, UK.

2.15.2 Transfer of plasmid by conjugation: Transfer of drug resistance was performed by conjugation described by Smith and Guild (1980). Strains were conjugated in

Mueller - Hintion broth as described by Anderson and Lewis (1965). The donor (enterotoxigenic *E. coli* isolates) and recipient 14R525 (nalidixic acid resistant mutant of *E. coli* K-12) were separately grown in Mueller-Hinton broth at 37°C for 4 h with constant shaking. One ml culture of each of donor and recipient was mixed and incubated at 37°C for 18-24 h.

After incubation, 0.1 ml of aliquot from the mixed culture was plated onto MacConkey's agar plate containing nalidixic acid only and nalidixic acid in addition to antibiotics to which the bacteria showed resistance. The concentration of antibiotics used were: ampicillin 5 ug/ml; chloramphenicol, 30 ug/ml and tetracycline, 30 ug/ml. Isolate acquiring resistance to these antibiotics will only grow on these plates containing respective antibiotic.

2.15.3 Isolation of plasmid DNA: The method of isolation of plasmid DNA described by Birnboin and Doly (1979) was followed. The detailed methodology is as follows:

The recipient *E. coli* K-12 strains was inoculated into screw cap tube containing 5.0 ml of Trypticase Soy broth (TSB) with 0.3% yeast extract. Cultures were grown overnight at 37°C in a roller drum (Belco,

Biotechnology, Vineland, USA). After incubation, 0.3 - 0.5 ml culture was transferred to an eppendorf centrifuge tube (1.5 ml capacity) and sedimented using an eppendorf microcentrifuge (Eppendorf, Model No. 5412, West Germany). The pellet was then washed in 0.5 ml of a solution containing glucose (50 mM), Na_2EDTA (10 mM) in Tris HCl (25 mM), pH 8.0 and centrifuged. The cells were then resuspended in 100 ul of the same solution containing lysozyme (4 mg/l). After incubation for 30 minutes at 0^oC, 200 ul of NaOH (0.2N) containing SDS (1.0%) was added and then mixed gently. The suspension was incubated for further 5 minutes at 0^oC. Immediately after 5 minutes, 150 ul of sodium acetate (3M, pH 4.8) was added and incubated for at least 1 h at 0^oC. The mixture was then centrifuged for 5 minutes The resultant lysate (400 ul) was decanted into a fresh eppendorf tube. One ml of cold (- 20^oC) ethanol (99.9%) was added, and the tube was kept at -20^oC or 30 minutes to precipitate DNA. The precipitated DNA was recovered by centrifugation for 5 minutes. Ethanol was decnted from the tube and the pallet was dried. The dried DNA samples were finally dissolved in 25-30 ul TE buffer containing tris (50 mM, pH 8.0) and Na_2 EDTA (1.0 mM). A 10 ul of ribonuclease (1 mg/l) was added in each sample and incubated for 10-15 minutes at room temperature. The DNA samples

were analysed by agarose gel electrophoresis. Plasmid DNA isolated from *E. coli* V-157 with known molecular weight was loaded in each gel as standard.

2.15.4 Characterization of plasmid DNA by agarose gel electrophoresis: Electrophoresis was carried out in a vertical lucid gel apparatus (Model 100, Aqueboque Machine Shop, N.Y., USA). The extracted DNA was dissolved in TE, was subjected to electrophoresis in 0.7% agarose dissolved in Tris borate buffer (89 mM Tris - base, 2.5 mM Na_2EDTA and 8.9 mM boric acid) (Meyer *et al.* 1976). A 5 ul dye consisting of bromothymol blue (0.07%), SDS 0.7%) and glycerol (33.0%) in water was added per 25 ul DNA samples prior to electrophoresis. Agarose was dissolved by boiling in a water bath for 15 minutes. When cooled to 55 - $60^{o}C$, melted agarose was poured into the chamber made by assembling glass plates and spacers. The dimension of the gel was 15 x 12x 0.3 cm. Well in which samples were applied, were prepared with combs containing 10 or 15 teeth. After pouring, gel was allowed to solidify within the glass plate chamber for at least 1 h at room temperature. The power source (Bucharest, Instruments, Fortlee, USA) supplied regulated current and electrophoresis was carried out at 50 mA, 100 A for 2 h or until the dye reached near the bottom of the

gel. After completing electrophoresis, the gel was stained in ethidium bromide solution (0.5 ug/ml) for 30 minutes and destained for another 30 minutes or overnight at $4^{o}C$ in distilled water. The gel was placed on an ultraviolet light box (short wave ultraviolet Products, Inc., Sam Gabriel, California, USA) and photographed by using Polaroid MP-4 Land Camera, (USA) with wratten orange gelatin filter (Kodak No. 22). The films used were of Polaroid type 55, 4 x 5 inch land film. The negatives were treated with sodium sulfite (12.0%), washed in water thoroughly, dried and stored in plastic envelope.

Chapter III

RESULTS

3.1 Prevalence of FC and FS : Prevalence of FC and FS in water and sediment samples of the Buriganga river and two ponds were assessed throughout the stuy period. During this time, slight variation in density of FC and FS was observed in different locations described below.

3.1.1 FC counts in river: In the river, FC counts in water and sediment samples ranged from 8.0×10^4 to 2.3×10^6/100 ml and 2.2×10^4 to 8.6×10^7/100 ml respectively. In the water samples, the highest FC count was found in the month of December, 1982 in river sampling point No. 2 (Table 2) and the lowest count was found in the month of October, 1983 in river sampling point No. 1 (Table 1) as well as in February, 1983 in river sampling point No. 3 (Table 3). In the sediment samples, the highest FC count was noted in the month of April, 1983 in river sampling point No. 1 (Table 1) and the lowest count was noted in the month of August 1982 in river sampling point No. 3 (Table 3).

3.1.2 FS counts in river: FS counts in water and sediment samples of the river ranged from 1.3×10^4 to 3.1×10^5/100 ml and from 4.0×10^3 to 1.8×10^7/100 g respectively. In the water samples, the highest FS count was noted in the month of December, 1982 in river sampling point No. 2 (Table 2) and the lowest count was noted in the month of September, 1982 in river sampling point No. 3

(Table 3). In the sediment samples, the highest FS count was noted in the month of April, 1983 in river sampling point No. 1 (Table 1) and the lowest count was noted in the month of August, 1982 in river sampling point No. 3 (Table 3).

Table 1

Counts of faecal coliforms (FC) and faecal streptococci (FS) of the Buriganga River *river sampling point No. 1

Parameters	Counts by month											
	Aug 1982	Sep 1982	Oct 1982	Nov 1982	Dec 1982	Jan 1983	Feb 1983	Mar 1983	Apr 1983	May 1983	Jun 1983	Jul 1983
FC counts (No/100 ml) in water	$1.6x10^6$	$2.4x10^5$	$8.0x10^4$	$1.1x10^5$	$3.3x10^5$	$9.3x10^4$	$1.0x10^5$	$1.8x10^5$	$6.9x10^5$	$1.4x10^5$	$3.3x10^5$	$1.3x10^5$
FS counts (No/100 ml) in water	$2.6x10^5$	$6.0x10^4$	$1.5x10^4$	$2.0x10^4$	$6.5x10^4$	$2.2x10^4$	$2.5x10^4$	$1.4x10^5$	$7.9x10^4$	$3.7x10^4$	$6.9x10^4$	$3.1x10^4$
FC counts (No/100 g) in sediment	$1.1x10^7$	$1.7x10^6$	$2.0x10^7$	$1.0x10^5$	$9.3x10^6$	$8.6x10^6$	$1.2x10^6$	$2.5x10^6$	$8.6x10^7$	$1.1x10^7$	$4.8x10^6$	$4.8x10^7$
FS counts (No/100 g) in sediment	$2.5x10^6$	$3.8x10^5$	$3.2x10^6$	$2.0x10^4$	$1.4x10^6$	$2.1x10^6$	$3.0x10^5$	$6.2x10^5$	$1.8x10^7$	$2.2x10^6$	$9.7x10^5$	$9.2x10^6$

*Point No. 1 = Babubazar Ghat

Table 2

Counts of faecal coliforms (FC) and faecal streptococci (FS) of the Buriganga *river sampling point No. 2

Parameters	Counts by month											
	Aug 1982	Sep 1982	Oct 1982	Nov 1982	Dec 1982	Jan 1983	Feb 1983	Mar 1983	Apr 1983	May 1983	Jun 1983	Jul 1983
FC counts (No/100 ml) in water	$4.6x10^5$	$3.0x10^5$	$3.3x10^5$	$7.4x10^5$	$2.3x10^6$	$8.7x10^5$	$3.7x10^5$	$9.7x10^5$	$6.7x10^5$	$1.3x10^6$	$2.3x10^5$	$1.8x10^5$
FS counts (No/100 ml) in water	$1.0x10^5$	$7.0x10^4$	$6.0x10^4$	$3.0x10^4$	$3.1x10^5$	$1.9x10^5$	$4.8x10^4$	$2.3x10^5$	$1.5x10^5$	$3.0x10^5$	$5.2x10^4$	$4.1x10^4$
FC counts (No/100 g) in sediment	$1.1x10^5$	$1.3x10^5$	$1.1x10^7$	$6.0x10^6$	$2.2x10^7$	$8.6x10^6$	$5.9x10^7$	$3.4x10^6$	$1.2x10^7$	$6.6x10^6$	$1.1x10^7$	$2.5x10^6$
FS counts (No/100 g) in sediment	$2.3x10^4$	$3.0x10^4$	$2.5x10^6$	$8.6x10^5$	$5.3x10^6$	$2.1x10^6$	$1.3x10^7$	$8.0x10^5$	$2.6x10^6$	$1.5x10^6$	$2.5x10^6$	$5.2x10^5$

*Point No. 2 = Sadar Ghat

Table 3

Counts of faecal coliforms (FC) and faecal streptococci (FS) of the Buriganga *river sampling point No. 3

Parameters	Counts by month											
	Aug 1982	Sep 1982	Oct 1982	Nov 1982	Dec 1982	Jan 1983	Feb 1983	Mar 1983	Apr 1983	May 1983	Jun 1983	Jul 1983
FC counts (No/100 ml) in water	$4.5x10^5$	$9.0x10^4$	$3.8x10^5$	$4.4x10^5$	$1.3x10^6$	$6.2x10^5$	$8.0x10^4$	$2.6x10^5$	$5.3x10^5$	$1.4x10^5$	$2.5x10^5$	$2.3x10^5$
FS counts (No/100 ml) in water	$1.0x10^5$	$1.3x10^4$	$4.8x10^4$	$1.0x10^5$	$2.0x10^5$	$1.3x10^5$	$2.0x10^4$	$5.3x10^4$	$9.1x10^4$	$3.3x10^4$	$5.2x10^4$	$5.2x10^4$
FC counts (No/100 g) in sediment	$2.2x10^4$	$2.0x10^5$	$1.0x10^6$	$2.6x10^6$	$1.7x10^6$	$8.3x10^5$	$1.9x10^6$	$7.3x10^6$	$5.2x10^6$	$1.1x10^7$	$4.0x10^4$	$1.0x10^7$
FS counts No/100 g) in sediment	$4.0x10^3$	$2.8x10^4$	$2.2x10^5$	$5.2x10^5$	$3.0x10^5$	$2.0x10^5$	$4.5x10^5$	$1.8x10^6$	$1.1x10^6$	$2.6x10^6$	$8.5x10^3$	$2.4x10^6$

*Point No. 3 : Farashganj Ghat

3.1.3 FC counts in ponds: In the ponds, FC counts in water and sediment samples ranged from $1.0x10^3$ to $3.7x10^5$/100 ml and $4.0x10^4$ to $2.0x10^7$/100 g respectively. In the water samples, the highest FC count was recorded in pond No. 2 in the month of April, 1983 (Table 5) and the lowest count was noted in pond No. 1 in the month of January, 1983 (Table 4). In the sediment samples, the highest FC count was recorded in pond No. 1 in the month of April,

1983 (Table 4) and the lowest count was noted in the month of November, 1982 in pond No. 2 (Table 5).

3.1.4 **FS counts in ponds:** FS counts in water and sediment samples of the ponds ranged from $2.0x10^2$ to $6.8x10^4$/100 ml and from $7.0x10^3$ to $4.2x10^6$/100 g respectively. In the water samples, the highest FS count was noted in the month of April, 1983 in pond No. 2 (Table 5) and the lowest count was noted in pond No. 1 in the month of January 1983 (Table 4). In the sediment samples, the highest FS count was noted in pond No. 1 in the month of April 1983 (Table 4) and the lowest count was noted in pond No. 2 in the month of November, 1982 (Table 5).

Table 4

Counts of faecal coliforms (FC) and faecal streptococci (FS) of Pond No. 1

Parameters	Counts by months											
	Aug 1982	Sep 1982	Oct 1982	Nov 1982	Dec 1982	Jan 1983	Feb 1983	Mar 1983	Apr 1983	May 1983	Jun 1983	Jul 183
FC counts (No/100 ml) in water	$1.1x10^4$	$2.3x10^4$	$1.9x10^4$	$5.0x10^4$	$2.1x10^5$	$1.0x10^3$	$1.7x10^3$	$2.0x10^3$	$3.2x10^4$	$4.2x10^4$	$2.6x10^4$	$4.0x10^3$
FS counts (No/100 ml) in water	$2.0x10^3$	$5.0x10^3$	$4.0x10^3$	$9.0x10^3$	$5.0x10^4$	$2.0x10^2$	$2.8x10^2$	$5.0x10^2$	$4.7x10^3$	$9.8x10^3$	$5.2x10^3$	$8.7x10^2$
FC counts (No/100 g) in sediment	$8.4x10^5$	$5.6x10^5$	$2.3x10^6$	$7.5x10^5$	$4.5x10^5$	$3.7x10^5$	$5.9x10^5$	$8.3x10^5$	$2.0x10^7$	$2.4x10^6$	$1.0x10^6$	$6.2x10^5$
FS counts (No/100 g) in sediment	$1.8x10^5$	$1.1x10^5$	$3.5x10^5$	$1.6x10^5$	$1.1x10^5$	$9.0x10^4$	$7.0x10^4$	$1.8x10^5$	$4.2x10^6$	$5.8x10^5$	$2.3x10^5$	$1.3x10^5$

Table 5

Counts of faecal coliforms (FC) and faecal streptococci (FS) of Pond No. 2

Parameters	Counts by month											
	Aug 1982	Sep 1982	Oct 1982	Nov 1982	Dec 1982	Jan 1983	Feb 1983	Mar 1983	Apr 1983	May 1983	Jun 1983	Jul 1983
FC counts (No/100 ml) in water	$1.5x10^4$	$3.0x10^4$	$1.6x10^4$	$9.5x10^4$	$4.4x10^4$	$5.0x10^4$	$3.5x10^3$	$1.3x10^4$	$3.7x10^5$	$6.3x10^4$	$1.0x10^4$	$4.0x10^3$
FS counts (No/100 ml) in water	$3.5x10^3$	$7.0x10^3$	$3.0x10^3$	$1.5x10^4$	$1.1x10^4$	$1.0x10^4$	$8.0x10^2$	$3.0x10^3$	$6.8x10^4$	$1.4x10^4$	$2.2x10^3$	$8.5x10^2$
FC counts (No/100 g) in sediment	$3.4x10^5$	$3.8x10^5$	$4.0x10^5$	$4.0x10^4$	$9.4x10^5$	$4.3x10^5$	$3.7x10^5$	$2.7x10^5$	$7.7x10^6$	$4.7x10^6$	$6.5x10^4$	$6.2x10^5$
FS counts (No/100 g) in sediment	$7.5x10^4$	$8.0x10^4$	$6.0x10^4$	$7.0x10^3$	$2.2x10^5$	$9.0x10^4$	$9.0x10^4$	$5.0x10^4$	$1.2x10^6$	$1.1x10^6$	$1.4x10^4$	$1.4x10^5$

3.2 Comparative counts of FC and FS in water and sediment samples of the Buriganga river and two ponds: The lowest and the highest counts of FC and FS in water and sediment samples of the Buriganga river were higher than the lowest and the highest counts of FC and FS in water and sediment samples of ponds.

3.3 Ratio of FC to FS in the Buriganga river and two ponds: FC to FS ratio in water and sediment samples in the river ranged from 4.0 to 8.6 and from 4.0 to 8.5 respectively (Tables 6,7 & 8). In ponds, FC to FS ratio in water and sediment samples ranged from 4.0 to 6.8 and from 4.1 to 8.4 respectively (Tables 9 & 10).

Table 6

Ratio of faecal coliforms (FC) to faecal streptococci (FS) of the Buriganga *river sampling point No. 1

Parameters	FC/FS ratio in the month of											
	Aug 1982	Sep 1982	Oct 1982	Nov 1982	Dec 1982	Jan 1983	Feb 1983	Mar 1983	Apr 1983	May 1983	Jun 1983	Jul 1983
FC/FS ratio in water	6.2	4.0	5.3	5.5	5.1	4.2	4.0	4.3	8.6	4.3	4.8	4.2
FC/FS ratio in sediment	4.3	4.3	5.9	5.0	6.6	4.1	4.1	4.0	4.8	5.2	4.9	5.2

*Point No. 1 = Babubazar Ghat

Table 7

Ratio of faecal coliforms (FC) to faecal streptococci (FS) of the Buriganga *river sampling point No. 2

Parameters	FC/FS ratio for the month of											
	Aug 1982	Sep 1982	Oct 1982	Nov 1982	Dec 1982	Jan 1983	Feb 1983	Mar 1983	Apr 1983	May 1983	Jun 1983	Jul 1983
FC/FS ratio in water	4.6	7.3	5.5	4.7	7.3	4.6	7.7	4.3	4.5	4.1	4.3	4.4
FC/FS ratio in sediment	4.9	4.3	4.2	7.2	4.2	4.1	4.5	8.5	4.5	4.4	4.2	4.8

*Point No. 2 = Sadar Ghat

Table 8

Ratio of faecal coliforms (FC) and faecal streptococci (FS) of the Buriganga *river sampling point No. 3

Parameters	FC/FS ratio for the month of											
	Aug 1982	Sep 1982	Oct 1982	Nov 1982	Dec 1982	Jan 1983	Feb 1983	Mar 1983	Apr 1983	May 1983	Jun 1983	Jul 1983
FC/FS ratio in water	4.5	6.9	7.9	4.4	6.5	4.8	4.0	4.8	5.8	4.3	4.8	4.4
FC/FS ratio in sediment	5.5	7.1	4.5	5.0	5.7	4.1	4.2	4.2	4.8	4.2	4.7	4.2

*Point No. 3 : Farashganj Ghat

Table 9

Ratio of faecal coliforms (FC) to faecal streptococci (FS) of pond No. 1

Parameters	FC/FS ratio in the month of											
	Aug 1982	Sep 1982	Oct 1982	Nov 1982	Dec 1982	Jan 1983	Feb 1983	Mar 1983	Apr 1983	May 1983	Jun 1983	Jul 1983
FC/FS ratio in water	5.5	4.6	4.8	5.6	4.1	5.1	6.1	4.0	6.8	4.3	4.8	4.6
FC/FS ratio in sediment	4.7	5.9	6.5	4.7	4.1	4.1	8.4	4.6	4.9	4.1	4.3	4.8

Table 10

Ratio of faecal coliforms (FC) to faecal streptococci (FS) in pond No. 2

Parameters	FC/FS ratio in the month of											
	Aug 1982	Sep 1982	Oct 1982	Nov 1982	Dec 1982	Jan 1983	Feb 1983	Mar 1983	Apr 1983	May 1983	Jun 1983	Jul 1983
FC/FS ratio in water	4.3	4.3	5.3	6.3	4.0	5.0	4.4	4.2	5.4	4.5	4.5	4.7
FC/FS ratio in sediment	4.5	4.8	6.7	5.7	4.3	4.8	4.6	5.4	6.3	4.1	4.6	4.4

3.4 Correlation between FC and FS in water and sediment samples of the Buriganga river and two ponds: Significant correlation was found between FC and FS in water and sediment samples of all the sampling points (Tables 11,12,13,14 & 15). However lack of correlation was found between FC and FS of sediment samples of the river sampling poit No. 1 (Table 11).

Table 11

Correlation coefficient (r) values between faecal coliforms (FC) and faecal streptococci (FS) of the Buriganga river sampling point No. 1*

	FC in water	FS in water	FC in sediment	FS in sediment
FC in water		0.887**	0.201	0.229
FS in water			- 0.028	0.00096
FC in sediment				0.671
FS in sediment				

*Sampling point No. 1 = Babubazar ghat
**1% level of significance

Table 12

Correlation coefficient (r) values between faecal coliforms (FC) and faecal streptococci (FS) of the Buriganga river sampling point No. 2*

	FC in water	FS in water	FC in sediment	FS in sediment
FC in water		0.870**	0.071	0.101
FS in water			-0.076	-0.400
FC in sediment				0.998**
FS in sediment				

*Sampling point No. 2 = Sadar ghat
**1% level of significance

Table 13

Correlation coefficient (r) values between faecal coliforms (FS) and Faecal streptococci (FS) of the Buriganga river sampling point No. 1*

	FC in water	FS in water	FC in sediment	FS in sediment
FC in water		0.965**	-0.225	-0.286
FS in water			-0.244	-0.272
FC in sediment				0.998**
FC in sediment				

* Sampling point 3 = Farashganj ghat
**1% level significance

Tabble 14

Correlation coefficient (r) between faecal coliforms (FC) and faecal streptococci (FS) in pond No. 1

	FC in water	FS in water	FC in sediment	FS in sediment
FC in water		0.997*	-0.0519	-0.0427
FS in water			-0.0887	-0.0909
FC in sediment				0.999*
FS in sediment				

*1% level of significance

Table 15

Correlation coefficient (r) values between faecal coliforms (FC) and faecal streptococci (FS) of pond No. 2

	FC in water	FS in water	FC in sediment	FS in sediment
FC in water		0.997**	0.862**	0.743*
FS in water			0.883**	0.771*
FC in sediment				0.997**
FS in sediment				

* 5% level of significance
**1% level of significance

3.5 Abundance of different species of FC: During this study period, 410 suspected coliform isolates were identified. Of them, 210 isolates were identified into *E. coli*, 100 were identified into *Klebsiella* spp., 20 were identified into *Enterobacter cloacae* and 80 were identified as *Citrobacter frundii*.

3.6 Abundance of different species of FS: A total of 306 FS isolats were identified into species level. Of them, 155 were *S. faecalis*, 103 were *S. faecium*, 23 were *S. faecalis* subspp. *zymogenes*, 23 were *S. bovis* and two were *S. equinus*.

3.7 Prevalence of differen species of aeromonads: One hundred eighty aeromonad strains were tested for different biochemical reactions for identification. Of them, 120 strains were identified as *A. hydrophila*, 40 were identified as *A. sobria* and 20 were identified as *A. caviae*.

3.8 Enterotoxigenicity of *E. coli* isolates : During the study period, 210 *E. coli* isolates were tested for LT and ST. Out of 25 (12.0%) isolates positive for toxin production, five (2.4%) isotates were positive for LT, isolates were toxin negative (Table 16).

Table 16

Incidence of enterotoxigenic *E. coli* in different months.

Months	Total strains tested	Total positive	LT positive	ST positive	LT/ST positive
Aug. 1982	15	1	1	0	0
Sep. 1982	20	0	0	0	0
Oct. 1982	20	1	0	0	1
Nov. 1982	25	6	0	3	3
Dec. 1982	15	6	0	6	0
Jan. 1983	20	1	0	1	0
Feb. 1983	10	0	0	0	0
Mar. 1983	25	3	0	0	3
Apr. 1983	20	2	0	0	2
May. 1983	15	1	0	0	1
Jun. 1983	15	1	1	0	0
Jul. 1983	10	3	3	0	0
Total	210	25 (12%)	5 (2.4%)	10 (4.8%)	10 (4.%)

3.9.1 Enterotoxigenicity of aeromonads : Ten isolates each of *Aeromonas hydrophila*, *A. sobria* and *A. caviae* were tested for their enterotoxigenic activity. Live culture of five isolates of *A. hydrophila* and seven

isolates of *A. sobria* induced fluid accumulation ranged from 0.5 to 2.3 ml/cm of gut. Culture filtrate induced fluid accumulation of each of three *A. hydrophila* and *A. sobria* isolates which ranged from 0.5 to 2.7 ml/cm of gut (Table 17).

Table 17

Haemolytic and enterotoxigenic activities of *Aeromonas* isolates

isolate No.	Haemolytic activity	Range of fluid accumulation (ml/cm) in rabbit gut induced by	
		Live culture	Cell-free culture filtrate
2	α	0.8 - 1.7	0.6 - 1.3
5	α	0.7 - 2.2	-
9	α	0.8 - 1.5	-
13	β	0.5 - 1.2	0.6 - 1.0
14	α	1.6 - 2.3	-
15	α	0.7 - 1.2	-
16	α	0.7 - 0.9	-
23	α	0.6 - 0.8	0.5 - 0.7
24	α	0.8 - 0.9	-
29	β	0.8 - 1.5	0.6 - 1.6
30	α	0.7 - 1.3	0.5 - 2.7
31	α	0.6 - 1.2	0.8 - 1.0
Vibrio cholerae 569B		1.0 - 1.2	0.9 - 1.5
E. coli k- 12		0	0

3.9.2 Haemolytic activity of aeromonads on blood agar plate: Those aeromonad istolates which induced fluid in rabbit ileal loop, produced either alpha or beta-haemolytic zone around the colony on blood agar plate (Table 17).

3.10 Effect of repeated passage of aeromonads: Three isolates each of *A. hydrophila*, *A. sobria* and *A. caviae* out of those that failed to induce fluid in RIL model were subjected to repeated passage. Within two to three consecutive passages, all the isolates could induced fluid in RIL which ranged from 0.5 to 2.6 ml/cm of gut (Table 18).

Table 18

Aeromonas induced fluid accumulation in RIL after repeated passage

Isolate No.	Fluid accumulation in number of passage	Live culture induced fluid (ml/cm of gut)
6	3rd	2.3
10	2nd	1.5
17	3rd	2.4
18	2nd	1.0
20	3rd	2.6
21	2nd	1.5
24	2nd	0.8
25	2nd	0.5
27	2nd	2.0

3.11 Enzyme profile of enterotoxigenic *A. hydrophila* isolated from surface water (river and pond): Of seven isolates tested for nineteen different enzymes, eleven were detected; such as phosphatase alkaline, esterase lipase, leucine arylamidase, valine arylamidase, trypsin, phosphatase acid, phosphoamidase, β-galactosidase, α-glucosidase, N-acetyl β-glucosamidase. Phosphatase alkaline was deficient in one isolate (9E). Lipase and valine arylamidase were also deficient in isolate (26E) (Table 19).

3.12 Enzyme profile of enterotoxigenic *A. hydrophila* isolated from ulcerated fish : Of nineteen enzymes, fourteen were detected : phosphatase alkaline, esterase lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, phosphatase acid, phosphoamidase, β-galactosidase, α-glucosidase, β-glucosidase, and N-acetyl β-glucosamidase. However, isolate No. 19F and 16F were deficient in esterase and valine arylamidase. Cystine arylamidase was undetected in isolate No. 6F, 16F, 19F and 25F. Five Isolates, such as 15F, 16F, 19F, 21F and 25F were deficient in phosphoamidase. β-galactosidase was not detected in four isolates (15F, 16F, 17F and 25F) (Table 19).

Table 19

Amount of enzymes detected in Aeromonas hydrophila isolated from fish and surface water

Enzymes	Amounts of enzyme (Nanomoles) in Fish isolate No										Surface water isolate No						
	2F	4F	6F	9F	15F	16F	17F	19F	21F	25F	9E	10E	11E	13E	20E	26E	38E
Phosphatase alkaline	30	30	//40	30	10	20	30	//40	//40	30	0	30	10	30	20	20	//40
Esterase	30	20	30	20	10	10	20	0	20	10	20	20	10	20	10	30	10
Esterase lipase	30	30	//40	30	30	30	//40	//40	30	20	10	//40	20	//40	20	//40	20
Lipase	10	5	5	5	5	5	5	10	10	5	20	5	5	5	5	0	5
Leucine arylamidase	30	20	30	30	20	10	30	//40	//40	30	20	30	20	20	30	30	20
Valine arylamidase	5	5	Tr	5	Tr	0	5	5	5	5	5	5	Tr	Tr	5	0	10
Cystine arylamidase	Tr	5	0	Tr	Tr	0	Tr	0	Tr	0	0	0	0	0	0	0	0
Trypsin	5	5	5	30	Tr	Tr	10	30	10	20	20	30	20	20	20	30	20
Phosphatase acid	30	10	30	30	20	10	30	30	20	30	30	30	20	//40	20	//40	//40
Phosphoamidase	Tr	Tr	Tr	Tr	0	0	Tr	0	0	0	0	Tr	0	0	0	0	0
β-galactosidase	30	30	10	20	20	10	30	20	//40	20	20	10	30	20	10	30	30
α-glucosidase	10	5	5	5	5	Tr	5	20	20	5	0	0	Tr	0	0	0	Tr
β-glucosidase	20	5	10	10	0	0	0	5	10	0	0	0	0	0	0	0	0
N-acetylβ-glucosamidase	//40	30	20	20	20	20	30	20	20	20	20	20	20	20	20	30	20

Tr = trace (between 0 and 5 nanomoles)

3.13 **Difference of enzyme profile between *Aeromonas hydrophila* isolated from surface water (river and pond) and ulcerated fish :** Fish isolates differed from surface water isolates with respect to cystine arylamidase, phosphoamidase, α- glucosidase and β-glucosidase (Table 20).

Table 20

Enzymatic differences between *Aeromonas hydrophila* isolated from ulcerated fish and surface water

Enzymes	Number of positive isolates among		level of significance*
	Fish isolates (N=10)	Surface water isolates (N=7)	
Cystine arylamidase	6	0	0.05
Phosphoamidase	5	1	insignificant
α- glucosidase	10	2	0.01
β- glucosidase	6	0	0.05

*Fisher's exact test

3.14 Drug sensitivity pattern of enterotoxigenic *E. coli* isolates : Ten enterotoxigenic *E. coli* isolates from which both LT and ST activity was found were tested for their sensitivity to ampicillin, cefamandol, chloramphenicol, colistin, gentamicin, tetracycline and trimethoprim + sulfamethoxazole. Of them, only three isolates were resistant to ampicillin and rest were sensitive to all the antibiotics tested.

3.15 Drug sensitivity pattern of enterotoxigenic aeromonads : All isolates (n=12) that initially showed enterotoxigenic activity were tested for their sensitivity to seven antibiotics (Table 21). All the isolates were uniformly sensitive to gentamicin and tetracycline and resistant to ampicillin. In addition, eight isolates were sensitive to cefamandole, ten to chloramphenicol, three to colistin and one to trimethoprim + sulfamethoxazole (Table 21).

Table 21

Antibiotic sensitivity pattern of enterotoxigenic *Aeromonas* isolates

Antibiotics	Response	
	No. of isolates sensitive (N=12)	resistant
Ampicillin	0	12
Cefamandol	8	4
Chloramphenicol	10	2
Colistin	3	9
Gentamicin	12	0
Tetracycline	12	0
Trimethoprim + sulfamethoxazole	1	11

3.16 Plasmid profile of enterotoxigenic *E. coli* isolates: Plasmid profile of three ampicillin resistant enterotoxigenic *E. coli* isolates were analysed. Ampicillin resistance was transferred to *E. coli* K-12. Agarose gel electrophoresis analysis showed that the ampicillin resistance is located on a plasmid having molecular weight of 49 megadalton.

Chapter IV

DISCUSSION

Coliforms have been considered as indicator of faecal pollution. Since these bacteria are always present in the intestinal contents of human and other warm blooded animals, presence of coliforms in water is an indication of polluted water and absence of coliforms is therefore an indication of bacteriologically safe water. However, different members of coliforms have origin other than faecal matter (e.g. some coliforms are of soil origin) which creates confusion in the assessment of faecal pollution of water. Hence, faecal coliforms (FC), are tested for assessing faecal pollution. Other than FC, faecal streptococci (FS) are also present in the intestinal contents of warm blooded animals. Moreover, FS survive longer than FC. Therefore, considering the direct implications, both FC and FS were counted as pollution indicator throughout the study period to assess the level of faecal pollution in the sampling sites.

FC and FS counts were always higher in sediment as compared to that in water. These findings are in agreement with the observation of Carney *et al.* (1975), Rashid (1982) and Morshed (1983). This is because, enteric bacteria undergo sedimentation immediately after entering an aquatic ecosystem. As a result, they are shown to persist in higher number in the sediment than that in the water column. Moreover, sediment having nutrients in

the form of organic matter, can support the growth of various micro-organisms (Grimes, 1980).

Ponds are closed ecosystem. Unlike rivers, ponds have limited sources of pollution as they are used by a limited number of people. On the other hand, various types of pollutants such as sewage, domestic effluent and faeces coming from direct defecation of passengers of different waterways have free access into the river. As a result, level of faecal pollution in the river is higher than that in the ponds. This is also evident in the present study where faecal pollution as measured in terms of FC and FS counts in river water and sediment were always higher than in the ponds.

There is a sharp contrast between the density of FC and FS in the faeces of man vs the faeces of other warm blooded animals. Geldreich and Kenner (1969) were the first to utilize this concept to identify the sources of faecal pollution (Geldreich and Kenner 1969). They found that in case of pollution with human faeces, FC/FS ratio was 4.0 or above whereas ratio of 0.7 or less was found in the case of pollution with faeces of other warm blooded animals. Geldreich and Kenner's (1969) observation was supported by Feachem (1974) who found that pollution with human and pig faeces could be separated by applying the concept of FC/FS ratio. In the present investigation, FC/FS ratio in water and sediment was always 4.0 or above in the sampling sites of the Buriganga river and two ponds which suggests pollution with

human faeces. Before this study, nobody could tell the source of faecal pollution of the Buriganga river and two ponds studied in this investigation. This is the first report in the subcontinent to document the source of pollution of water bodies with clear scientific data.

FC and FS are not the autochthonous inhabitants of natural aquatic environment. Therefore they have originated from the faeces of either human or of other warm-blooded animals. In the faeces of human beings, FC counts are higher than FS whereas the reverse applies in the case of faeces of warm-blooded animals. Thus there exists some correlation between the counts of FC and FS. Petrilli *et al.* (1980) found positive correlation between *E. coli* and total coliforms and between *E. coli* and FS. In the present study, significant correlation between FC and FS in water and sediment samples was observed in all the sampling points except river sampling point No.1 (Table 2). This lack of correlation in one sampling site may be due to small sample size as observed by Sayler *al et.* (1975).

In the present investigation, counts of FC and FS in water and sediment did not show any apparent seasonal variation (Fig 2,3,4,5). However, when the counts of FC and FS in water samples of the two ponds were combined, FS and FC counts showed depression in the month of February, 1983 and February and March, 1983 respectively whereas FC and FS counts in sediment showed a rise in the month of April, 1983. In other months, both FC and FS

counts showed slight fluctuation (i.e. one log count higher in one month and one log count lower in the following month)(Table. 22). In the river, FC counts in water was uniform throughout the year except December, 1983 when one log higher count was observed than other months (Table. 23). These results are in partial agreement with that of Panicker *et al.* (1976) who did not find any seasonal variation of FC and FS in a raw water supply.

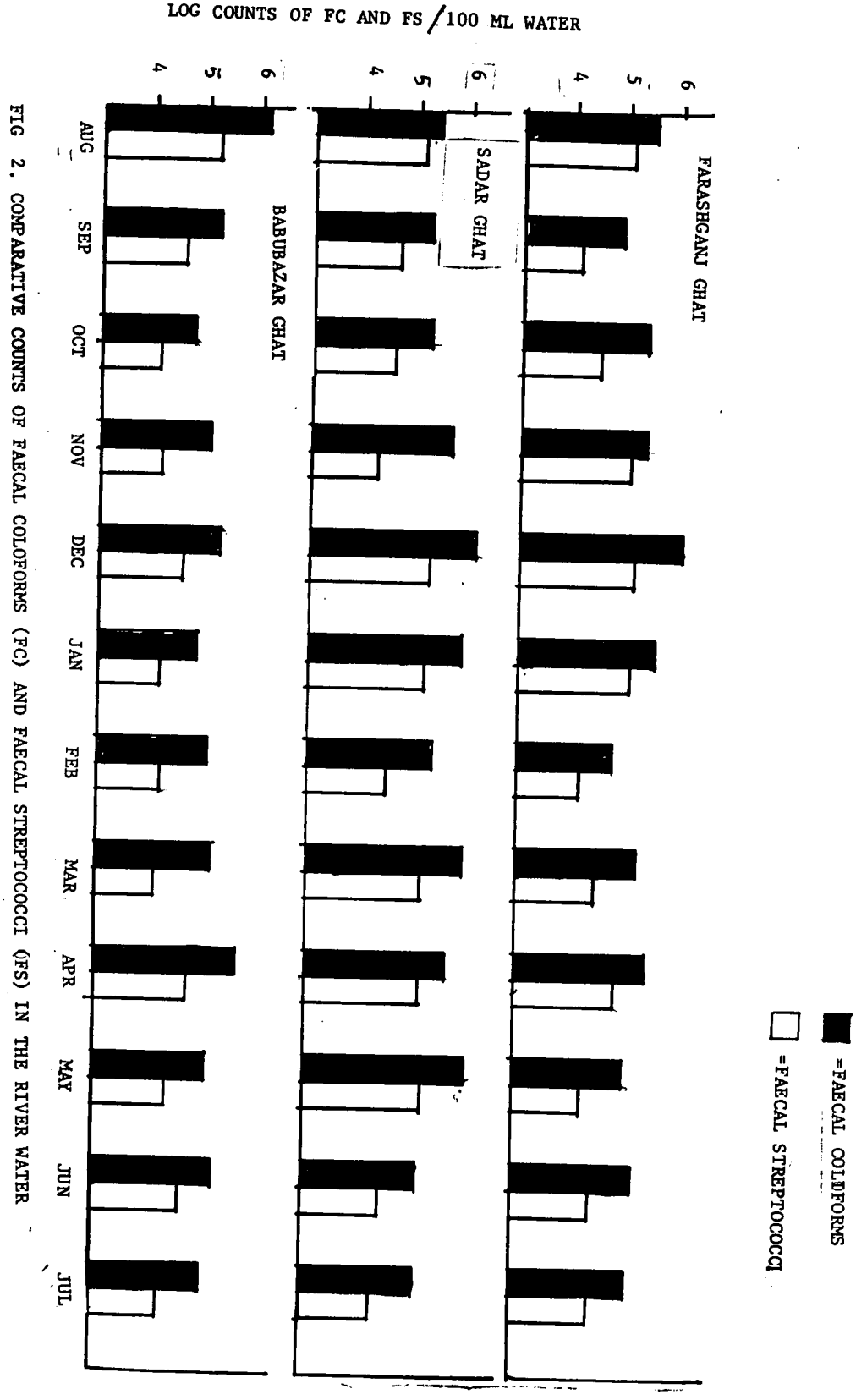

FIG 2. COMPARATIVE COUNTS OF FAECAL COLOFORMS (FC) AND FAECAL STREPTOCOCCI (FS) IN THE RIVER WATER

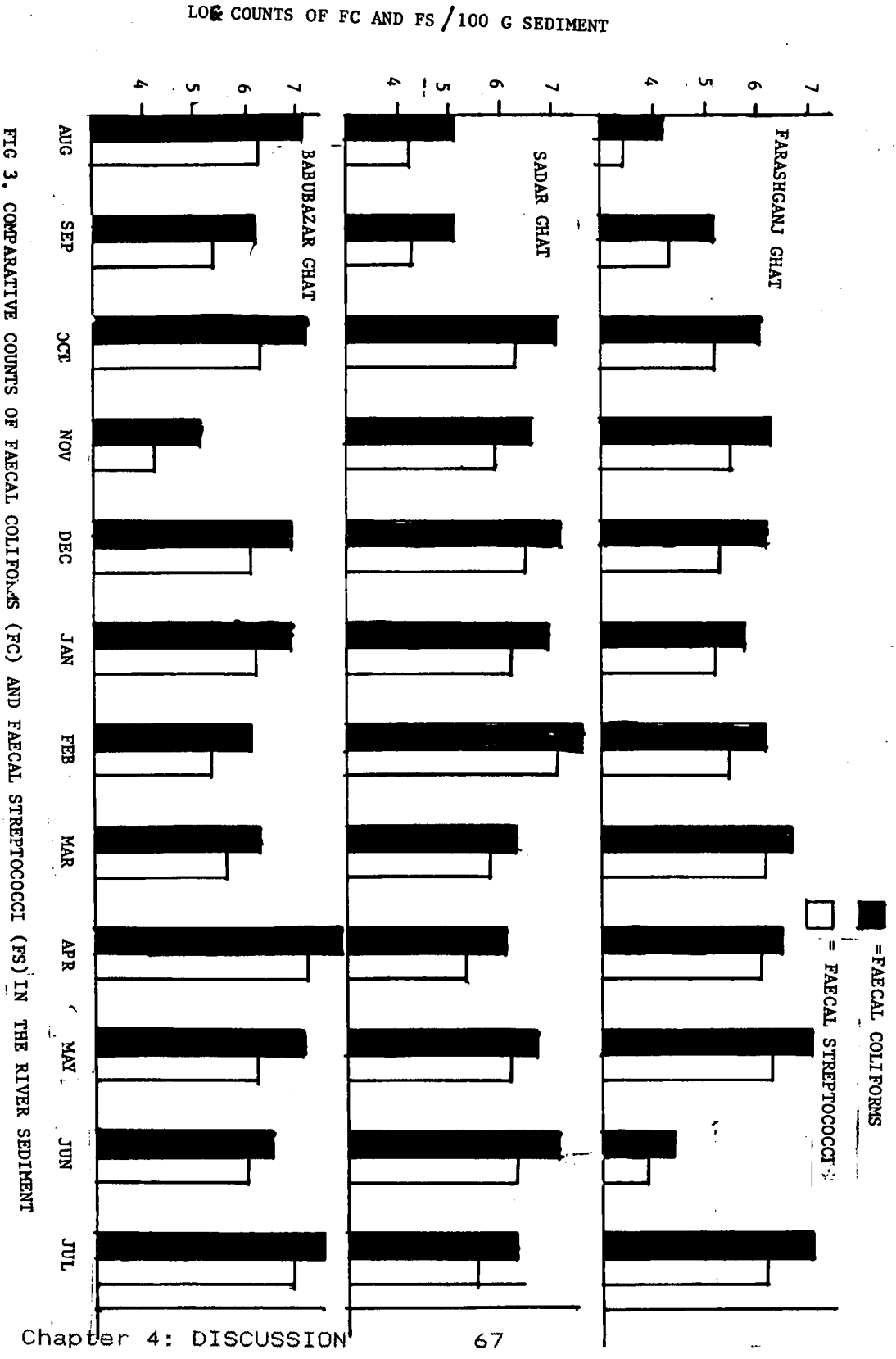

FIG 3. COMPARATIVE COUNTS OF FAECAL COLIFORMS (FC) AND FAECAL STREPTOCOCCI (FS) IN THE RIVER SEDIMENT

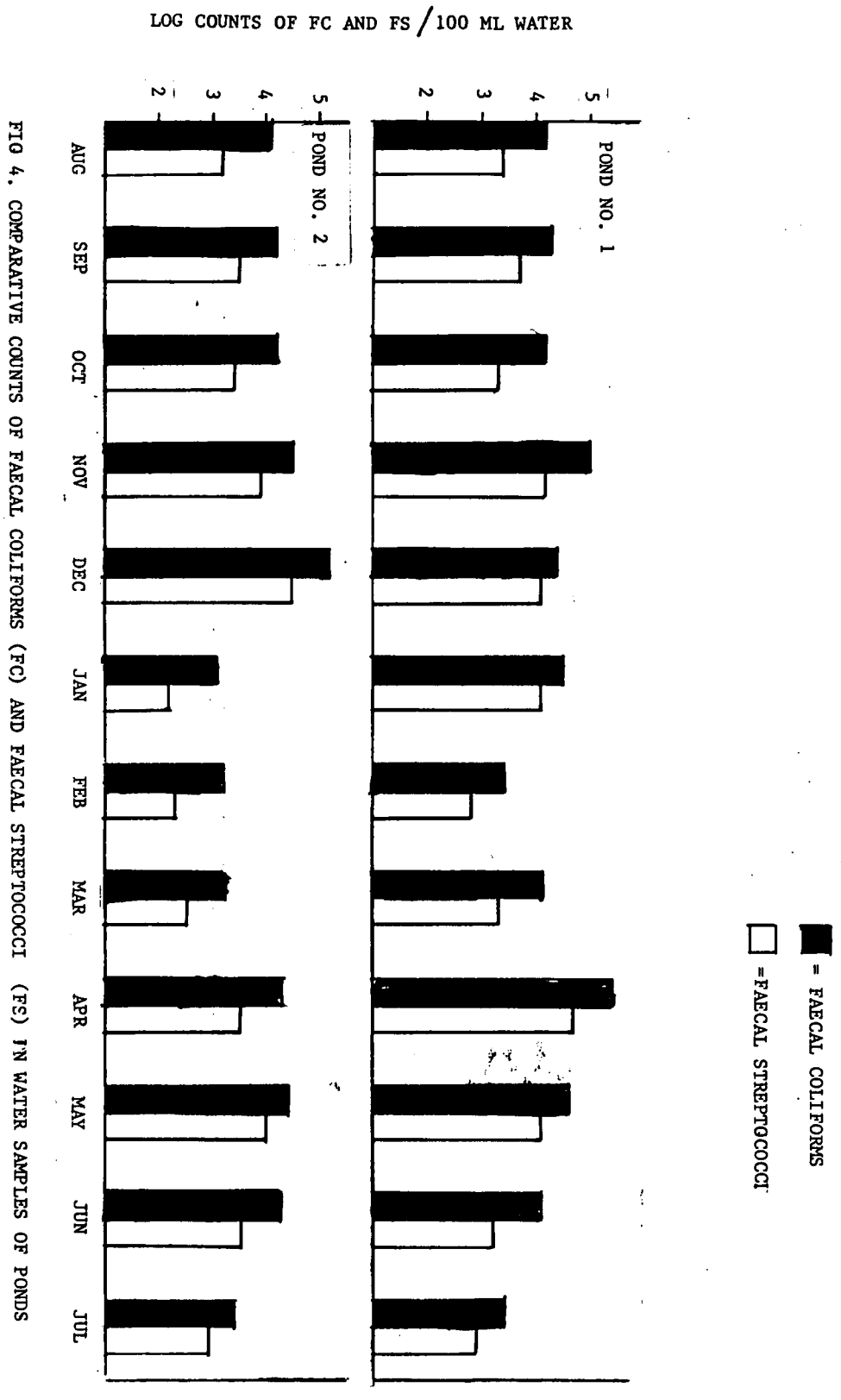

FIG 4. COMPARATIVE COUNTS OF FAECAL COLIFORMS (FC) AND FAECAL STREPTOCOCCI (FS) IN WATER SAMPLES OF PONDS

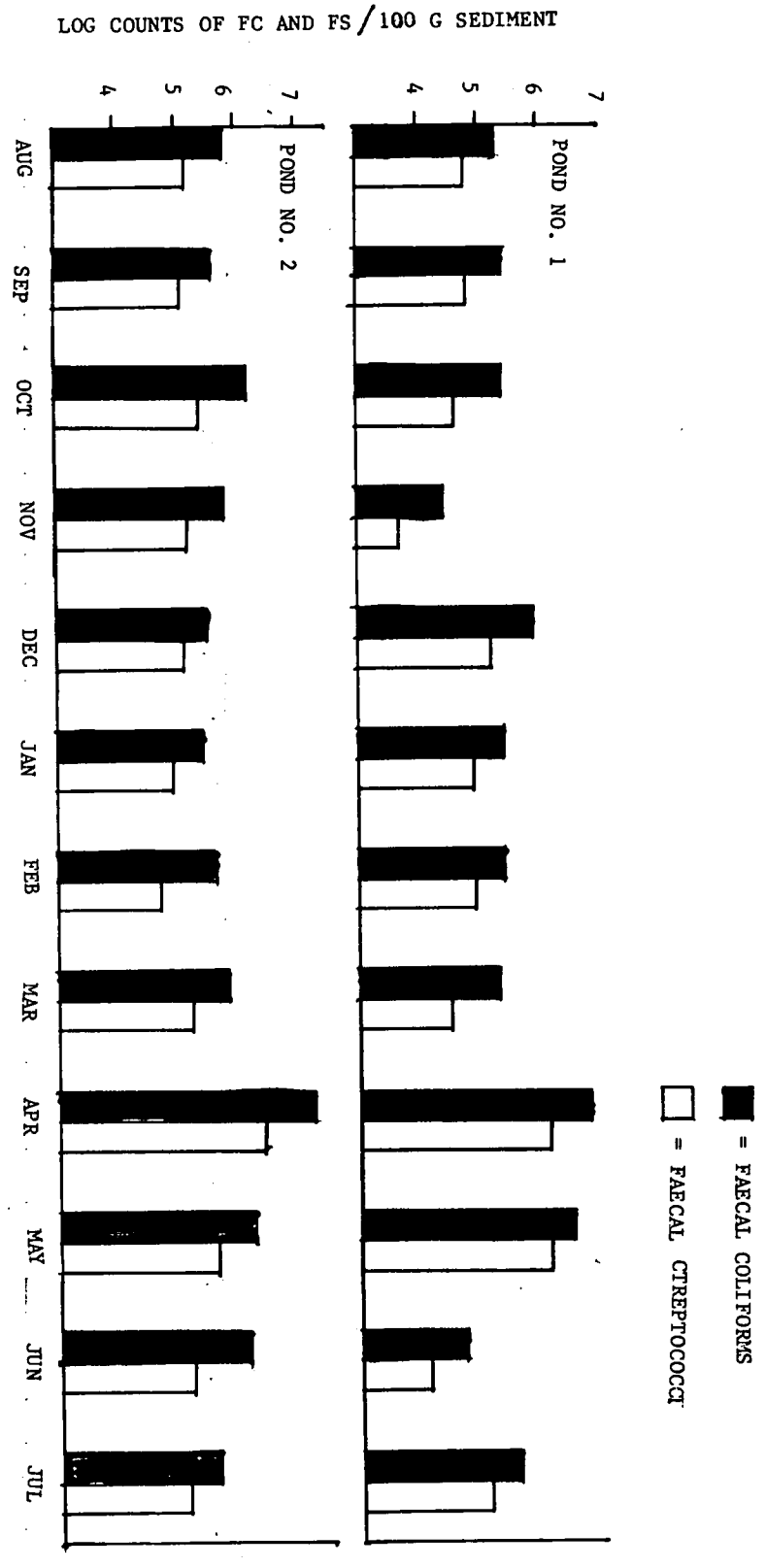

FIG 5. COMPARATIVE COUNTS OF FAECAL COLIFORMS (FC) AND FAECAL STREPTOCOCCI (FS) IN POND SEDIMENT SAMPLES

Table 22

Counts of faecal coliforms (FC) and faecal streptococci (FS) of the Buriganga river (three points combined) in different month

Parameters	Counts by month											
	Aug 1982	Sep 1982	Oct 1982	Nov 1982	Dec 1982	Jan 1983	Feb 1983	Mar 1983	Apr 1983	May 1983	Jun 1983	Jul 1983
FC counts (No/100 ml) in water	$8.3x10^5$	$2.1x10^5$	$2.6x10^5$	$4.3x10^5$	$1.3x10^6$	$5.2x10^5$	$1.8x10^5$	$4.7x10^5$	$6.3x10^5$	$5.2x10^5$	$2.7x10^5$	$1.8x10^7$
FS counts (No/100 ml) in water	$1.5x10^5$	$4.1x10^4$	$4.1x10^4$	$5.0x10^4$	$1.9x10^5$	$1.1x10^5$	$3.1x10^4$	$1.4x10^5$	$1.1x10^5$	$1.2x10^5$	$5.8x10^4$	$5.8x10^4$
FC counts (No/100 g) in sediment	$3.7x10^6$	$6.8x10^5$	$1.1x10^7$	$2.9x10^6$	$1.1x10^7$	$6.0x10^6$	$2.0x10^7$	$4.4x10^6$	$3.4x10^7$	$9.5x10^6$	$5.2x10^6$	$1.7x10^7$
FS counts (No/100 g) in sediment	$4.8x10^5$	$1.5x10^5$	$1.9x10^6$	$4.7x10^5$	$2.3x10^6$	$1.5x10^6$	$4.5x10^6$	$1.0x10^6$	$7.2x10^6$	$2.1x10^6$	$1.2x10^6$	$4.0x10^6$

Table 23

Counts of faecal coliforms (FC) and faecal streptococci (FS) of the ponds (two ponds combined) in different months

Parameters	Counts by month											
	Aug 1982	Sep 1982	Oct 1982	Nov 1982	Dec 1982	Jan 1983	Feb 1983	Mar 1983	Apr 1983	May 1983	Jun 1983	Jul 1983
FC counts (No/100 ml) in water	$1.4x10^4$	$2.6x10^4$	$1.7x10^4$	$7.2x10^4$	$1.3x10^5$	$2.6x10^4$	$2.6x10^4$	$7.5x10^3$	$2.0x10^5$	$5.3x10^3$	$1.8x10^4$	$4.0x10^3$
FS counts (No/100 ml) in water	$2.7x10^3$	$6.0x10^3$	$3.5x10^3$	$1.2x10^4$	$3.0x10^4$	$5.1x10^3$	$5.4x10^2$	$1.7x10^3$	$3.6x10^4$	$1.1x10^4$	$3.7x10^3$	$8.6x10^2$
FC counts (No/100)	$5.9x10^5$	$4.7x10^5$	$1.4x10^6$	$4.0x10^5$	$7.0x10^5$	$4.0x10^5$	$4.8x10^5$	$5.5x10^5$	$4.9x10^7$	$2.8x10^6$	$5.3x10^5$	$6.2x10^5$
FS counts (No/100 g) in sediment	$1.2x10^5$	$9.5x10^4$	$2.1x10^5$	$8.4x10^4$	$1.7x10^5$	$9.0x10^4$	$8.0x10^4$	$1.2x10^5$	$2.7x10^6$	$8.4x10^5$	$1.9x10^5$	$1.4x10^5$

In the Buriganga river, probable sources of faecal pollution are: defecation from destitute persons directly into the river, fly latrines constructed over the river, domestic effluents and direct defecation from passengers of various boatcraft. Due to drainage of all types of pollutants into the river during monsoon, levels of faecal pollution rise and are subsequently diluted by the increased volume of water due to rainfall. As a result, counts of FC and FS do not increase. In the dry months, load of faecal pollution is lower as faeces coming from direct defecation of destitutes and from fly latrines remain dried on the bank and do not mix with river water due to lower water level. Moreover, movement of boatcraft is reduced during winter (dry season) due to the low water level in the river. As a result, defecation of passengers in the river is reduced. However, depletion of water in the dry months results in lower water consumption resulting in lower domestic effluents output. Thus water and other pollutants concentrate in the dry season but level of faecal pollution does not increase. Hence, seasonality was not reflected in the present study.

Various microbiological standards have been postulated to provide guidelines for safe use of drinking water. These standards indicate a predetermined number of microorganisms that denotes no health risk to the people using the water. According to the drinking water standards of WHO (1971), FC counts per 100 ml should be zero . In a study of bathing water quality and

health, Stevenson (1953) indicated that the total coliform value of $2.3X10^3/100$ ml is an appropriate standard for bathing water. A second phase of the same study reported the value indicating safe use for recreational purpose for the Ohio river as $2.7 \times 10^3/100$ ml. With counts reaching and exceeding this value, a significant increase in the incidence of various illnesses was noted among swimmers and recreational users. In this subcontinent, there has been no enforcement of water quality standards for drinking, bathing and recreation. Comments, therefore, has been made on the water quality of these aquatic ecosystems (ponds and river) by comparing results of the present study with the above standards. The lowest counts of FC in this study was always higher than the above standards. Therefore, it can be concluded that waters of the study points are not safe for drinking, swimming, bathing as well as for other recreational purposes. At this state of pollution, the users may suffer from infection of skin, eyes and nose (Mujeriogo and Murias, 1980).

Since the discovery of *Aeromonas* by Sanarelli (1891), speciation has undergone several taxonomical modification. Finally, Popoff and Veron (1976) proposed species designation of *Aeromonas* into *A. hydrophila*, *A. sobria* and *A. caviae* for the motile aeromonads. In the present study, all the three species

as described by Popff and Veron (1976) were isolated, although *A. sobria* isolation was more abundant than the remaining two species. Sukroongreung *et al.* (1983) reported similar findings from Thailand.

Rosenberg *et al.* (1977) reported diarrhoeal epidemic caused by waterborne enterotoxigenic *E. coli* (ETEC). They isolated a single serotype of *E. coli* (O6:K15:H16) from water samples as well as from stool of diarrhoeal patients. This was possibly the first report indicating waterborne transmission of ETEC. This information prompted the fellow researchers to investigate the incidence of ETEC in the aquatic environments of Bagladesh. From Banglaedsh, ETEC was first detected by an M. Sc. thesis student of the Department of Microbiology, University of Dhaka from pond water (Rashid, 1982). He detected one ETEC out of 27 *E. coli*. Next year, Morshed (1983) detected ten out of 144 *E. coli* to be toxigenic. In the present investigation, 25 ETEC was detected out of 210 *E. coli* isolates during a year long study. This refutes earlier ideas that ETEC cannot be isolated from the environment.

High FC counts in the aquatic environment (river and ponds) is a threatening situation for the dwellers. People using such polluted waters for bathing, swimming as well as for ablution may harbour significant numbers of faecal *E. coli* on the inner lining of their buccal cavity. These *E. coli* ultimately enter the gut and are passaged. Repeated passage enhance their pathogenicity

(Vasil, 1975). Such micro-organisms are subsequently released into the environment by excretion. Constant passage can convert non-toxigenic *E. coli* into toxigenic one. Persistence of such toxigenic *E. coli* in the environment may induce community outbreaks of diarrhoea. This study provides supportive evidence towards recurrence of diarrhoea in Bangladesh.

ETEC has been recognized as an enteric pathogen with a world-wide distribution with higher frequency in developing countries and has been identified as one of the common causes of travellers' diarrhoea (Merson *et al.* 1976). ETEC strains produce one or both types of enterotoxins: heat labile (LT) and heat stable (ST). LT is immunologically related to cholera toxin whereas ST is a non- antigenic enterotoxin. Genetic material that controls the production of these enterotoxins is located on plasmid and can be easily transferred into other bacteria under laboratory conditions (Anonymous, 1980).

ETEC isolated from clinical specimens showed geographical difference in the type of enterotoxin production. For example, in Mexico and Morocco, LT/ST strains are predominant and in Kenya LT strains are most common whereas in Bangladesh, ST strains are more prevalent than LT/ST with LT strains being less common (Anonymous, 1980). In the present investigation, incidence of LT/ST and ST strains was found to be similar but higher than LT strains.

Aeromonas was thought to be pathogen since it's discovery by Sanarelli (1891), but until 1974, it was not clearly demonstrated as a pathogen. In the year 1975, Annapurna and Sanyal first demonstrated fluid accumulation in the rabbit ileal loop (RIL) model after inoculation with live cultures of *A. hydrophila*. In the following year Annapurna and Sanyal (1977) reported fluid accumulation in RIL following inoculation with live cultures as well as cell free culture filtrates. Moreover, increased level of cyclic adenosine monophosphate (cAMP) in the epithelial cells by the partially purified enterotoxin from *A. hydrophila* was also demonstrated by Sanyal *et al.* (1978). Thus enteropathogenicity of *Aeromonas* was proven in an *in vivo* model (Ljungh *et al.* 1978; Wadstrom *et al.* 1976; Annapurna and Sanyal, 1977 and 1975). *Aeromonas* spp. examined in this study demonstrated enterotoxigenicity induced by both live cultures as well as by the cell-free culture filtrates. Of the three species, enterotoxigenicity was demonstrated by *A. hydrophila* (five out of ten isolates) and *A. sobria* (seven out of ten isolates). None of the *A. caviae* strains could induce fluid accumulation in RIL. Therefore, it was concluded that *A. caviae* may be non-pathogenic. Janda *et al.* (1985) reported similar results while studying pathogenicity of *Aeromonas* in mouse model.

It has been observed that pathogenic microorganisms can lose their pathogenicity when preserved in the laboratory for long period of time. It is assumed that some sort of repression or depression occurs in the toxin gene during preservation in

artificial medium (Vasil *et al.* 1975). Pathogenic microorganisms isolated from the environment that are unable to express virulence, may be avirulent for the same reason. After repeated passage in an animal model, such pathogenic microorganisms may regain virulence. As demonstrated by Annapurna and Sanyal (1977), fluid accumulation could be induced in *Aeromonas* strains after repeated passage. This was also shown in the present investigation. Other than *Aeromonas*, enterotoxigenicity was enhanced in case of NAG vibrios after repeated passage in animal model (Shanker *et al.* 1978; Singh and Sanyal, 1978).

Waltman *et al.* (1982) performed enzymatic characterization of *A. hydrophila*. In addition to the enzymes detected in this study (Table. 20), they reported caprylate esterase lipase and leucine aminopeptidase. But they were unable to detect chymotrypsin, α-galactosidase, α-glucosidase, α-mannosidase and α-fucosidase. Likewise, Ljungh *et al.* (1977) were unable to detect chymotrypsin, α-glucuronidase, α-mannosidase, and α-fucosidase. Absence of these enzymes may be a uniform and persistence characteristics of *A. hydrophila*.

During characterization of the *A. hydrophila* complex, Waltman *et al.* (1982) were unable to show significant enzymatic differences among isolates from various sources. In the present study, enzymatic differences were observed with respect to cystine arylamidase, phosphoamidase, α-glucosidase, β-glucosi-

dase. These enzymes were detected in the fish isolates but not in those from surface water. A statistically significant difference was observed with respect to cystine arylamidase ($P < 0.05$), α-glucosidase ($P < 0.01$) and β-glucosidase ($P < 0.05$) but the difference was not significant with respect to phosphoamidase (Table 21).

In the environment, as a result of constant interaction between humans and a polluted aquatic environment during bathing, swimming and ablution, aeromonad strains can be passaged in the gut of humans and are subsequently released into the environment by excretion. Thus non-toxigenic strains can acquire enterotoxigenicity and counts of enterotoxigenic strains in the environment can therefore increase which may result in aeromonad induced community outbreaks of diarrhoea.

Haemolytic activity of *Aeromonas* isolates on blood agar plate showed 100% correlation with enterotoxigenicity in RIL model. Therefore, test of enterotoxigenicity in RIL could be replaced with a simple test of haemolytic activity on blood agar plate. Test of haemolytic activities on blood agar plate is simple, cheap and can be performed easily in any laboratory having minimum facilities. Therefore, this should be considered as one of the most significant findings in this study. In the past, *Aeromonas* enterotoxigenicity has been tested (Ljungh *et*

al. 1978; Annapurna and Sanyal, 1977; Wadstrom *et al*. 1976) but none of the workers tried to correlate these two pathogenic factors.

In the present study, Haemoltic activity was detected in *Aeromonas hydrophil* and *A. sobria*. Isolates of *A. cavie* were non-haemolytic. Recently, Monfort and Baleux (1990) have reported haemolytic activity in all the three species of *Aeromonas*.

Aeromonas has been considered as an autochthonous inhabitants of aquatic environment. It is abundant in surface water (Hazen, *et al*. 1978) and as well as in drinking water (Pittock and Donovan 1986). In some instances, density of *Aeromonas* exceeded faecal coliform counts (Rahim, *et al*. 1989). A considerable number of *Aeromonas* are enterotoxigenic. In the present study, 40% (12 out of 30) of the aeromonad strains were enterotoxigenic which were higher than the isolation rate of environmental enterotoxigenic *E. coli* previously reported by Rashid (1982; one enterotoxigenic strain out of 27 *E. coli*) and Morshed (1983; 10 enterotoxigenic strains out of 144 *E. coli*). *Aeromonas* could be simply isolated on blood agar plate (Kay, *et al*. 1985). Since it has been shown in this study that haemolytic activity correlates 100% with enterotoxigenicity, therefore, enterotoxigenic property of aeromonads could be assessed by simply examining their haemolytic activity on the same plate. From this study, it is

hypothesized that enterotoxigenic *Aeromonas* could be an indicator of water pollution. Further work is needed to confirm the potentiality of *Aeromonas* as water pollution indicator.

Treatment of diseases with antibiotics and chemotherapeutic agents is responsible for the occurrence of drug resistance among enteric pathogens and the majority of drug resistant bacteria carry transferable drug resistance (R) factor (Anderson, 1968). Through discharge of human and animal waste, drug resistant bacteria are distributed in the sewage and surface water where exchange of R plasmids can occur under certain physical, chemical and bacteriological condition (Anonymous 1978). Thus drug resistant bacteria can spread in the environment where man and animal acquire infection with bacteria carying drug resistant plasmids (Joseph *et al.* 1979). In Bangladesh, there is clear evidence of abuse of antibiotics (Hussain *et al.* 1982) which is responsible for occurrence of drug resistance of the pathogenic bacteria. Isolation of drug resistant enterotoxigenic *E. coli* and *Aeromonas* in the present study also supports the observation of Hussain *et al.* (1982).

Aeromonas shows variable sensitivity patterns with respect to certain antibiotics. Ampicillin resistant *Aeromonas* have been reported universally (Palfreeman *et al.* 1983; Joseph *et al.* 1979 and von Graevenitz and Mensch, 1968). Similar result was observed in the present investigation. However, ampicillin sensitive environmental *Aeromonas* strains has also been reported from

Bangladesh (Rahim *et al.* 1984). As was found with clinical isolates (Palfreeman *et al.* 1983; von Graevenitz and Mensch, 1968), environmental *Aeromonas* strains in the present investigation were sensitive to tetracycline. Present investigaton shows uniform sensitivity pattern *Aeromonas* with respect to gentamicin and variable sensitivity pattern with respect to chloramphenicol as compared to clinical isolates from Bangladesh (Aziz *et al.* 1986).

It can be concluded that the Buriganga river and two ponds showed high counts of FC and FS. Counts of FC and FS in water and sediment samples of the river are higher than that in the ponds. In all the sampling sites, counts of FC and FS in sediment are higher than that in water. In most of the instances, FC counts correlats with FS in water and sediment. It has been demonstrated that both the ponds and the river are polluted with human faeces and hence not suitable for swimming, bathing ablution etc. Haemolytic activity correlates well with enterotoxigenicity in rabbit ileal loop (RIL) model. Therefore, RIL could be replaced with haemolytic activity on blood agar plate. Moreover, prevalence of enterotoxigenic *E. coli* and enterotoxigenic and drug resistant *Aeromonas* spp. indicates that the use of water of the Buriganga river and two ponds is not safe.

Chapter V

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