III. Materials And Methods
III. MATERIALS AND METHODS

3.1. Samples

3.1.1. Sampling location, duration and samples collected

Two sampling locations were selected for the present study namely, Nethravathi and Sharavathi rivers and their adjacent estuarine and sea sites. Nethravathi is a non-dammed river (without a major dam), which finally joins the sea at Mangalore where as Sharavathi has a major dam (Linganamakki dam), which joins the sea at Honnavar along the west coast of Karnataka. Water (surface and bottom) and sediment samples were collected from both the locations at different sampling sites. Samples were collected at regular monthly intervals from May 2001 to April 2003 (for a period of 2 years).

3.1.1.1. River Nethravathi

The Nethravathi River is one of the important west flowing rivers of Karnataka. It rises at an elevation of > 1000 m in Western ghats between Kudremukh and Ballalrayan durga in the South Kanara district of Karnataka (13° 10’ N and 75° 20’ E). It flows in North-South direction for the first 40 km, up to Gohattu where it takes a turn towards the west and flows in East-West direction up to its outfall into Arabian Sea near Mangalore. Its length is about 103 km and has a total drainage area of 3,180 km$^2$. A relatively small check dam is constructed in Nethravathi river near Mangalore for drawing drinking water (Karnataka State Gazetteer Part I, 1982).
3.1.1.2. River Sharavathi

The Sharavathi River is one of the important west flowing rivers of Karnataka, which takes its origin in Western Ghats near Ambutirtha in Tirthahalli Taluk of Shimoga district. The river Sharavathi flows in northwest direction and is mainly utilized for generation of hydroelectric power. Its length is about 128 km and has a total drainage area of 2,771 km². The river along with its tributaries flows along the rugged terrain of Western Ghats of southwest Shimoga and southwest Uttar Kannada. The river drops to a vertical fall of about 253 m near Jog and it joins the Arabian Sea at Honnavar in Uttar Kannada. The Karnataka Power Corporation Limited has constructed a dam across the river in 1964 near Linganamakki, which is at present one of the oldest hydroelectric power projects in India. The dam is located at about 14° 41’ 24” N latitude and 74° 50’ 54” E longitude with an altitude of 512 m. The total capacity of the reservoir is 152 TMC (Thousand Million Cubicfeet). It has a catchment area of nearly 1991.71 km². It receives water mainly from rainfall and also from the Chakra and Savahaklu reservoirs, which are linked through Linganamakki through a canal (Karnataka State Gazetteer Part I, 1982).

3.1.2. Sampling sites

Different Sampling sites were selected in both on the basis of water flow and sediment discharge along the river, estuary as well as towards the sea (Fig 1). The sites S1 & R1 are estuarine sites and the site S1 is near the bar mouth where river joins with sea. R2 and R3 are the riverine sites which are fixed towards the riverside away from the bar mouth. R1 site was 1km away from bar mouth and R2 was 5 km from R1 where as R3 is 10 km from R1. S2, S3 are the sites fixed towards the sea. S2 is 1.5km from the bar mouth and S3 is 3 km from the S1 (fig 1). All sites were fixed using GPS (Global Positioning System). Samples of surface water, bottom water and sediment were collected at each site regularly at monthly intervals and
LOCATIONS:

R_3 \leftarrow R_2 \leftarrow R_1 \leftarrow S_1 \rightarrow S_2 \rightarrow S_3

5Km \quad 5Km \quad 1Km \quad 1.5Km \quad 1.5Km

RIVER \quad \uparrow \quad BARMOUTH \quad \downarrow \quad SEA

Fig: 1 Details of sampling locations
analyzed for various bacteriological parameters. Hydrographic instrument was used to collect the water sample whereas Grab was used to collect the sediment sample. The depth at which bottom water and sediment samples collected varied from 2-3 feet in riverine sites and 4-5 feet in the estuarine region and depth was more than 7-9 feet in sea sites. The depth of sample collection varied seasonally based on the sediment and volume of water discharge. Sharavathi River is deeper when compared with the Nethravathi River. This might have been due to the construction of dam across the Sharavathi River and there would have been decrease in the sediment discharge, as the construction of dam will hinder the process of sedimentation in the river as well as in estuarine and coastal waters.

3.1.3. Sampling

Sampling has been done in both the locations regularly at monthly intervals from May 2001 to April 2003. During monsoon period sampling was not done in the sea region, as sea was rough due to heavy rains and strong wave and tidal action. Fishing vessels such as trawlers and dugout canoes were used to reach sampling sites and collect samples. Dugout canoe was used in the riverine region and Trawler was used in the sea region. Sterilized water bottles and plastic pouches were used for the collection of water and sediment samples, respectively. A total of 36 samples were collected from both the locations (3 samples at each site and there were 6 sites at each sampling location). The samples were brought aseptically to the laboratory for the analysis of various bacteriological parameters such as total heterotrophs, total coliforms, fecal coliforms, E.coli, presumptive streptococci, fecal streptococci and FC: FS ratio was calculated using MPN values of fecal coliform and fecal streptococci. Also, samples were analyzed for the presence of human pathogens like Salmonella. A molecular method such as PCR was performed for the rapid detection of Salmonella in water and sediment samples.
3.2. Media and Reagents

Total Heterotrophic Bacterial count (TPC)

3.2.1. Physiological Saline

Sodium chloride : 8.5 g
Distilled water : 1000 ml

Sterilization was done by autoclaving at 121°C for 15 min.,

3.2.2. Plate Count Agar (Standard Methods Agar), (Hi Media, Mumbai)

Casein enzymic hydrolysate : 5.0 g
Yeast extract : 2.5 g
Dextrose : 1.0 g
Agar : 15.0 g
Sodium chloride : 5.0 g
Final pH (at 25°C) : 7.0 ± 0.2

The medium was prepared as per the manufacturer’s instructions.

3.2.3. Marine Agar (Zobell Marine Agar), (Hi Media, Mumbai)

Peptic digest of animal tissue : 5.0 g
Yeast extract : 1.0 g
Ferric citrate : 0.1 g
Sodium chloride : 19.45 g
Magnesium chloride : 8.8 g
Sodium sulphate : 3.24 g
Calcium chloride : 1.80 g
Potassium chloride : 0.55 g  
Sodium bicarbonate : 0.16 g  
Potassium bromide : 0.08 g  
Strontium chloride : 0.034 g  
Boric acid : 0.022 g  
Sodium silicate : 0.004 g  
Sodium fluorrate : 0.0024 g  
Ammonium nitrate : 0.0016 g  
Di sodium phosphate : 0.008 g  
Agar : 15.0 g  

Final pH (at 25°C) : 7.6 ± 0.2  

The medium was prepared as per the manufacturer’s instructions.

**Pre-enrichment broth for total coliforms**

3.2.4. **Lauryl Tryptose Broth (Lauryl Sulphate Broth), (Hi Media, Mumbai)**

Tryptone : 20.0 g  
Lactose : 5.0g  
Sodium Chloride : 5.0g  
Dipotassium phosphate : 2.75g  
Mono potassium phosphate : 2.75g  
Sodium Lauryl sulphate : 0.10g  
Distilled water : 1000 ml  
Final pH (at 25°C) : 6.8 ±0.2
The medium was prepared as per the manufacturer’s instructions.

**Selective enrichment broth for *E. coli***

3.2.5. **EC Broth (Escherichia coli Broth)**

- Casein enzymatic hydrolysate : 20.0 g
- Lactose : 5.0 g
- Bile salt mixture : 1.5 g
- Dipotassium phosphate : 4.0 g
- Monopotassium phosphate : 1.5 g
- Sodium chloride : 5.0 g
- Distilled water : 1000 ml

**Final pH (at 25°C) : 6.9 ± 0.2**

The medium was prepared as per the manufacturer’s instructions.

**Selective plating media for *E. coli***

3.2.6. **EMB Agar (Eosin Methylene Blue Agar), (Hi Media, Mumbai)**

- Peptic digest of animal tissue : 10.0 g
- Lactose : 5.0 g
- Sucrose : 5.0 g
- Dipotassium phosphate : 2.0 g
- Eosin Y : 0.4 g
- Methylene blue : 0.065 g
- Agar : 13.5 g
- Distilled water : 1000 ml

**Final pH (at 25°C) : 7.2 ± 0.2**
The medium was prepared as per the manufacturer’s instructions.

**Selective enrichment broth for Presumptive streptococci**

3.2.7. **Azide Dextrose Broth (Hi media, Mumbai)**

- Peptone, special : 15.0 g
- Beef extract : 4.5 g
- Dextrose : 7.5 g
- Sodium chloride : 7.5 g
- Sodium azide : 0.2 g
- Distilled water : 1000 ml

**Final pH (at 25°C)** : 7.2 ± 0.2

The medium was prepared as per the manufacturer’s instructions.

**Selective plating media for fecal streptococci**

3.2.8. **KF Streptococcal Agar Base (Hi Media, Mumbai)**

- Peptone, special : 10.0 g
- Yeast extract : 10.0 g
- Sodium chloride : 5.0 g
- Sodium glycerophosphate : 10.0 g
- Maltose : 1.0 g
- Lactose : 0.4 g
- Sodium azide : 20.0 g
- Agar : 20.0 g
- Distilled water : 1000 ml
Final pH (at 25°C) : 7.2 ± 0.2

The medium was prepared as per the manufacturer’s instructions.

After sterilization, the media was cooled to 50°C and 10 ml of 1% Triphenyl Tetrazolium chloride (TTC) was added aseptically to the sterile medium. The medium was mixed well before pouring into sterile petriplates.

**Pre-enrichment Broth for Salmonella**

3.2.9. **Lactose Broth**

- Beef extract : 3.0 g
- Peptone : 5.0 g
- Lactose : 5.0 g
- Distilled water : 1000 ml
- PH : 6.9 ± 0.2

The medium was sterilized by autoclaving at 121°C for 15 min.

**Selective Enrichment Broths for Salmonella**

3.2.10. **Fluid Selenite Cystine medium (Hi Media, Mumbai)**

- Casein enzymatic hydrolysate : 5.0 g
- Lactose : 4.0 g
- Di sodium phosphate : 10.0 g
- L-Cystine : 0.01 g
- Sodium hydrogen selenite : 4.0 g
- Distilled water : 1000 ml
- Final pH (at 25°C) : 7.0 ± 0.2
The medium was prepared as per the manufacturer’s instructions.

3.2.11. **Tetrathionate CV Enrichment Broth (Hi Media, Mumbai)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein enzymatic hydrolysate</td>
<td>4.3 g</td>
</tr>
<tr>
<td>Peptic digest of animal tissue</td>
<td>4.3 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>6.4 g</td>
</tr>
<tr>
<td>Potassium tetrathionate</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Crystal violet</td>
<td>0.005 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td><strong>Final pH (at 25°C)</strong></td>
<td>6.5 ± 0.2</td>
</tr>
</tbody>
</table>

The medium was prepared as per the manufacturer’s instructions.

**Selective plating media for *Salmonella***

3.2.12. **Bismuth Sulfide Agar (Hi Media, Mumbai)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptic digest of animal tissue</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Di sodium phosphate</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>0.3 g</td>
</tr>
<tr>
<td>Bismuth sulphite indicator</td>
<td>8.0 g</td>
</tr>
<tr>
<td>Brilliant green</td>
<td>0.016 g</td>
</tr>
<tr>
<td>Agar</td>
<td>12.7 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td><strong>Final pH (at 25°C)</strong></td>
<td>7.6 ± 0.2</td>
</tr>
</tbody>
</table>
The medium was prepared as per the manufacturer’s instructions.

3.2.13. **Hektoen Enteric Agar (Hi Media, Mumbai)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteose peptone</td>
<td>12.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Lactose</td>
<td>12.0 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>12.0 g</td>
</tr>
<tr>
<td>Salicin</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Bile Salts mixture</td>
<td>9.0 g</td>
</tr>
<tr>
<td>Sodium thiosulphate</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Ammonium ferric citrate</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Acid fuscsin</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Bromo thymol blue</td>
<td>0.065 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
</tbody>
</table>

Final pH (at 25°C) : 7.5 ± 0.2

The medium was prepared as per the manufacturer’s instructions.

3.2.14. **Trypticase Soya Agar (TSA), (Hi Media, Mumbai)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Soya peptone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
</tbody>
</table>

The medium was prepared as per the manufacturer’s instructions.
3.2.15. **Triple Sugar Iron Agar (TSI), (Hi Media, Mumbai)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Tryptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Lactose</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Sodium thio sulphate</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Phenol red</td>
<td>0.0024 g</td>
</tr>
<tr>
<td>Agar</td>
<td>12.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Final pH (at 25°C): 7.2 ± 0.2

The medium was dissolved by boiling, distributed to tubes, and autoclaved at 110°C for 15 min.,

3.2.16. **Oxidase reagent**

Oxidase reagent (N,N,N,N’-tetra methyl p-phenylene diamine dihydro chloride): 10.0 g

Distilled water: 1000 ml

Whatman filter paper No.1 was cut into stripes of 2.5X 1.0 cm, dipped in the reagent, dried and stored in dark at 4°C.
3.2.17. **Urea broth base (Hi Media, Mumbai)**

Mono potassium phosphate : 91.0 g  
Di potassium phosphate : 95.0 g  
Yeast extract : 1.0 g  
Phenol red : 0.1 g  
Distilled water : 1000 ml  

Final pH (at 25°C) : 6.8 ± 0.2  

The medium was dissolved, distributed to tubes, and autoclaved at 121°C for 15 min., at around 50°C after sterilization 1.5-3.0 ml of sterile 40% urea solution was added to 13X100 sterile test tubes aseptically and mixed well.

3.2.18. **Tryptone broth**

Tryptone : 10.0 g  
Yeast extract : 5.0 g  
Sodium chloride : 5.0 g  
Distilled water : 1000 ml  

PH : 7.1 ± 0.2  

The medium was sterilized by autoclaving at 121°C for 15 min.,

3.2.19. **Kovac’s reagent (p-DMAB)**

p-Dimethyl amine benzaldehyde : 5.0 g  
Iso amyl alcohol : 750 ml  
Conc. Hcl : 250 ml
3.2.20. **Lysine Iron Agar (LIA), (Hi Media, Mumbai)**

- Gelysate peptone : 5.0 g
- Yeast extract : 3.0 g
- Dextrose : 1.0 g
- L-Lysine : 10.0 g
- Ferric ammonium citrate : 0.5 g
- Sodium thio sulphate : 0.04 g
- Bromo cresol purple : 0.02 g
- Agar : 15.0 g
- Distilled water : 1000 ml
- Final pH (at 25°C) : 6.7 ± 0.2

Boiling, distributed to tubes, dissolved the medium and autoclaved at 110°C for 15 min., the medium was then allowed to set in a sloped condition with a butt of 1”.

3.2.21. **Methyl Red- Voges Proskauer broth (MR-VP), (Hi Media, Mumbai)**

- Glucose : 5.0 g
- Peptone : 7.0 g
- K2HPO4 : 5.0 g
- Sodium chloride : 5.0 g
- Distilled water : 1000 ml

The medium was dispensed in 5 ml proportions into tubes and autoclaved at 110°C for 15 min.,
3.2.22. **Methyl Red reagent**

Methyl Red : 0.2 g  
Ethyl alcohol : 600 ml  

The reagent made up to 1000 ml with distilled water.

3.2.23. **Voges Proskauer’s reagent**

Solution A : L-naphthol : 50.0 g  
Absolute alcohol : 1000 ml  

Solution B: 40% KoH

3.2.24. **Simmon’s citrate Agar (Hi Media, Mumbai)**

Sodium citrate : 2.0 g  
Sodium chloride : 5.0 g  
K2HPO4 : 1.0 g  
(NH4)2H2PO4 : 1.0 g  
Mg SO4 : 2.0 g  
Bromothymol blue : 0.08 g  
Agar : 15.0 g  
Distilled water : 1000 ml  

Final pH (at 25°C) : 6.8  

The medium was dissolved by boiling, dispensed into tubes, and sterilized by autoclaving at 121°C for 15 min., After cooling to 50-55°C, allowed solidifying in slanted position.
Maintenance media

3.2.25. **T₃ N₀.₅ medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Final pH</td>
<td>7.1 ± 0.2</td>
</tr>
</tbody>
</table>

The medium was sterilized by autoclaving at 121°C for 15 min.,

3.2.26. **Liquid paraffin**

Sterilized in hot air oven at 180°C for 2 h.

**PCR (Polymerase Chain Reaction)**

3.2.27. **1X PCR Buffer (Bangalore Genei, Bangalore)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 m mol 1-1</td>
<td>Tris-HCl pH 8.8)</td>
</tr>
<tr>
<td>1.5 m mol 1-1</td>
<td>MgCl</td>
</tr>
<tr>
<td>50 m mol 1-1</td>
<td>KCl</td>
</tr>
<tr>
<td>0.1%</td>
<td>Triten X-100</td>
</tr>
</tbody>
</table>

3.2.28. **10 X TAE Buffer (Tank Buffer)**

per liter:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>48.4 g</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>11.42 ml</td>
</tr>
<tr>
<td>0.5 M EDTA (pH 8.0)</td>
<td>20 ml</td>
</tr>
</tbody>
</table>
3.2.29. Taq DNA polymerase (3 units/ul, Bangalore Genei, Bangalore)

3.2.30. DNTPS (deoxyribo nucleoside tri phosphates), (200µM, Pharmacia Biotech, USA)

3.2.30. Primers (Bangalore Genei, Bangalore)

A set of HNS primers such as LHNS 531 and RHNS 682 were used in PCR amplification (Daniel et al., 1993)

3.2.32. DNA Molecular weight Marker (Bangalore Genei, Bangalore)

Agarose gel electrophoresis

3.2.33. Agarose (Hi Media, Mumbai)

3.2.34. Ethidium bromide (Sigma chemicals co., USA)

A stock solution of ethidium bromide was prepared by dissolving 10mg/ml in distilled water and stored in dark bottle at 4°C.

3.2.35. Sample loading buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromocresol purple</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>40.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Statistical analysis of data

3.2.36. SPSS software package (SPSS Inc., Chicago)
3.3. METHODS

Bacteriological analysis of samples

3.3.1. Total plate count (TPC)

Water and sediment samples collected from two sampling locations (Nethravathi and sharavathi rivers and their adjacent estuarine and sea sites) were brought to the laboratory aseptically for the enumeration of total heterotrophic bacterial load. Initially PCA (Plate Count Agar) was used for the plating of samples collected from riverine sites and ZMA (Zobell Marine Agar) for estuarine and sea site samples. Later, both PCA and ZMA media were used for the riverine, estuarine as well as sea site samples to study the salt tolerance characteristic of bacteria of fresh water origin in various habitats mentioned above.

Spread plate technique was followed for the enumeration of TPC. Samples were plated on PCA (0.5%) and ZMA after serial ten fold dilutions with physiological saline. For the water samples, useful counts were usually observed under dilutions $10^{-1}$ to $10^{-3}$ while for sediment samples it was $10^{-3}$ to $10^{-5}$. All the colonies developing in the plates were observed after 48 hr of incubation at room temperature and counted and expressed as cfu per ml or g of water or sediment sample.

3.3.2. Bacterial identification

A series of biochemical reactions (as described by Mac Faddin, 1980) were performed to identify Bacteria such as *Salmonella spp*, and *E.coli*. The characteristic colonies of *Salmonella* and *E.coli* colonies were picked from respective selective media such as BSA (Bismuth Sulfide Agar) and HEA (Hektoen Enteric Agar) for *Salmonella* and EMB (Eosin Methylene Blue agar) for *E. coli*. The colonies were maintained on TSA (Trypticase Soya
FLOW CHART 1: IDENTIFICATION OF GRAM NEGATIVE MICROORGANISMS

O/F Glucose
+/-(Fermentative)
Oxidase test

Sensitivity to 0/129

Vibrio Aeromonas Plesiomonas

Escherichia Klebsiella Enterobacter Salmonella Hafnia Serratia Proteus

Arginine - + +
Lisine + - +
Ornithine + - +
Catalase + + + + + + +
Oxidase - - - - - - -
Motility + - + + + + +
Indole + - - - - D D
M.R. + - - + - D D
V.P. - - + - D + -
Citrate - + + + + + + D
Urease - + D - - - +

D. Some strains positive and some negative

Agar) (0.5%) and preserved at refrigerated temperature for further identification by conventional and molecular methods such as PCR.

Bacterial identification was done following the identification scheme for Gram-negative bacteria (Le chavallie et al., 1980). The scheme is indicated in Flow chart 1. In the case of Streptococcus, no biochemical test was done, since the colonies on the KF Streptococcal agar were considered as fecal streptococci. Typical pinkish-red colonies were considered as fecal streptococci and expressed as MPN/100 ml/g of water or sediment.

3.3.3. Enumeration of fecal coliforms by 3-tube MPN method

LSTB (Lauryl Sulphate Tryptose Broth) media was prepared according to the manufacturer’s instruction. Ten milliliter of water sample each was transferred to set of 3 DS (Double Strength) and 1 ml and 0.1 ml sample each to set of 3 tubes containing Single Strength. The media was incubated for 48 h at 37°C. The tubes showing turbidity and gas production were considered as positive for total coliforms. Results were recorded and expressed as MPN/100ml using Mc Crady MPN Table.

From the positive tubes of LSTB, a loopful of culture was inoculated to EC broth and incubated for 48h in water bath set at 44.5°C. The tubes showing turbidity with gas production were considered positive for fecal coliforms and results were recorded as MPN/100ml (Flow chart 2).

In the case of sediment samples, 10 g of the sample weighed and diluted in 90 ml of sterilized physiological saline (0.85% NaCl w/v) and mixed well and kept for 30 min., to settle. Then the supernatant was processed as described above.
3.3.4. **Enumeration and identification of E.coli**

From the positive tubes of EC broth, a loopful of culture was taken and streaked on EMB plates (Selective media for *E.coli*). Plates were kept at 37°C in incubator for 24-48h. Typical colonies having pink, purple, greenish metallic sheen (with or without black centers) and black colonies were picked from the plates and aseptically transferred to TSA (0.5%) plates or slants and kept for incubation at 37°C for 24-48h for further confirmation.

3.3.5. **Identification of *E.coli* by biochemical tests**

Colonies suggestive of *E.coli* appearing on selective media were subjected to series of biochemical tests for confirmation. The cultures showing positive reactions for indole and methyl red and negative for Voges proskauer’s and citrate utilization test were considered as *E. coli* (Flow chart 2).

3.3.6. **Enumeration of Fecal Streptococci by 3-tube MPN method**

Using Azide dextrose broth (ADB), enumeration of presumptive streptococci was done following 3-tube MPN method as described earlier. The inoculated media was incubated for 48h at 37°C. The tubes showing the turbidity were considered as positive and counts expressed as MPN/100ml/g by referring to the Mc Crady Table.

From the positive tubes of ADB, a loopful of inoculum was streaked to KF Streptococcal agar plate and incubated for 48 h at 37°C. Appearance of pink to reddish colonies was considered as fecal Streptococci. The fecal streptococcal counts were expressed as MPN/100 ml/g by referring to the Mc Crady table (Flow chart 3).
FLOW CHART 2: ILLUSTRATION OF THE STEPS IN ENUMERATION AND IDENTIFICATION OF FECAL COLIFORMS AND E.coli

Sample
↓
Inoculated to LSTB (3-tube MPN method)
↓
Transfer of inoculum from positive tubes (turbidity and gas production) to EC broth
↓
A loopful of culture from positive tubes (turbidity and gas production) of EC broth and streaked on selective plate EMB (selective for E.coli)
↓
Typical colonies with morphology of pink, purple to greenish with or without metallic sheen were picked and maintained on TSA 0.5%
↓
Biochemical tests such as IMViC and oxidase will be performed to confirm the presence of E.coli (Typical reaction for E.coli-IMViC: +:+:-:- and oxidase negative)
↓
If the colony from the respective positive tube of EC broth answers to the standard typical biochemical reaction then that colony is considered as typical E.coli and expressed as MPN/100ml/g.
↓
Maintainance of typical E.coli on T1N0.5 butt overlaid with sterilized liquid paraffin
FLOW CHART 3: ILLUSTRATION OF THE STEPS IN ENUMERATION AND IDENTIFICATION OF FECAL STREPTOCOCCI

Inoculating to ADB (3 tube MPN method)

Presumptive streptococcal
Count (MPN/100ml/g) → Incubated for 48h at 37°C

Transfer of inoculum from positive tubes (turbidity) on KF Streptococcal
agar plate by streaking method

Fecal streptococcal count
(MPN/100ml/g) → Incubated for 48h at 37°C

Typical colonies with morphology of pink to reddish with or without yellow
halo and size ranging from pinpoint, small to medium were regarded as fecal
Streptococci and expressed as MPN/100ml/g
3.3.7. FC: FS ratio

It is the ratio between MPN count of fecal coliform and fecal Streptococci enumerated for different sampling habitats. This ratio helps to determine whether the sample is contaminated by human fecal source or non-human fecal source. The ratio of more than 4 infers that the sample is contaminated by human fecal source whereas less than 0.7 indicates that the contamination is by non-human fecal source.

3.3.8. Isolation and Identification of Salmonella

The method described by Andrews et al., (1992) was followed for identification of Salmonella. The flow diagram illustrating the steps followed in the identification of Salmonella is given in Flow chart 4.

3.3.9. Isolation of Salmonella

For the isolation of Salmonella from water sample, two enrichment broths were used. Ten ml of water sample each was inoculated to 10 ml of double strength enrichment broth namely Selenite cystein broth (SCB) and Tetrathionate broth (TTB) aseptically and kept at 37°C for 24 to 48 hr of incubation.

A loopful of culture from SCB and TTB was streaked on to BSA (Bismuth Sulfide Agar) and HEA (Hektoen Enteric Agar), respectively. Plates were incubated for 24 to 48 hr at 37°C and were examined for presence of colonies suggestive of Salmonella.

On HEA plates, typical Salmonella colonies appear yellow to light green with black centers and also blue-green to blue with or without black centers. Rarely, few colonies look like blue with or without black centers. Many cultures of Salmonella may produce colonies with large, glossy black
Flow Chart 4: Illustration of the steps in identification of *Salmonella*

Gram-negative bacilli

↓

Oxidase test (if positive, discard the culture)

↓

Urease test (if positive, discard the culture)

↓

Indole test (if positive, discard the culture)

↓

MR-VP test (+: -, otherwise discard the culture)

↓

Triple Sugar Iron test (A/K, A/A, with or without H2S or gas)

↓

Lysine Iron Agar test (Positive: purple butt)

↓

Citrate test (Variable)

↓

Maintenance of culture

↓

Reconfirmation by PCR
centers or may appear as almost completely black colonies (depending on the amount of H2S produced by the colony).

On BSA plates, typical *Salmonella* colonies may appear brown, gray or black often with metallic sheen. Grey to black with or without black centers or completely black colonies was also observed depending on the amount of H$_2$S production. Atypically few *Salmonella* species produces pale greenish colonies with or without black centers.

Spreaders appearing completely green on BSA, yellow or red on HEA (sometimes colonies picked from selective plates show spreading nature on maintenance media such as TSA) were considered as non-*Salmonella*. On BSA, surrounding medium is usually brown at first, but may turn black in time with increased incubation, producing the so-called halo-effect. Some strains may produce green colonies with little or no darkening of surrounding medium (Andrewa et al., 1992)

### 3.3.10. Biochemical identification tests for *Salmonella*

Colonies suggestive of *Salmonella* appearing in selective media were subjective to battery of biochemical tests to confirm as *Salmonella*. The typical biochemical reactions for *Salmonella* are present in the Table: A.

#### 3.3.10.1. Oxidase test

Test cultures were grown on TSA plates and were taken by a glass rod and transferred onto the pre-moistened filter paper strip with the oxidase reagent. Oxidase positive colonies showed a dark purple color with in 10 sec., *salmonellae* are strictly oxidase negative.
**TABLE: A. BIOCHEMICAL AND SEROLOGICAL REACTIONS OF SALMONELLA (ANDREWS ET AL., 1992)**

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Test or substrate</th>
<th>Results</th>
<th>Salmonella Reaction&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>1.</td>
<td>TSI</td>
<td>Yellow</td>
<td>Red</td>
</tr>
<tr>
<td>2.</td>
<td>Lysine decarboxylase (LIA)</td>
<td>Purple</td>
<td>Yellow butt</td>
</tr>
<tr>
<td>3.</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;S (TSI and LIA)</td>
<td>Blackening</td>
<td>No blackening</td>
</tr>
<tr>
<td>4.</td>
<td>Urease</td>
<td>Purple</td>
<td>No color change</td>
</tr>
<tr>
<td>5.</td>
<td>Phenolred dulcitol broth</td>
<td>Yellow and/or gas</td>
<td>No gas: no color change</td>
</tr>
<tr>
<td>6.</td>
<td>KCN broth</td>
<td>Growth</td>
<td>No growth</td>
</tr>
<tr>
<td>7.</td>
<td>Malonate broth</td>
<td>Blue</td>
<td>No blue</td>
</tr>
<tr>
<td>8.</td>
<td>Indole test</td>
<td>Deep red at surface</td>
<td>Yellow at surface</td>
</tr>
<tr>
<td>9.</td>
<td>Polyvalent somatic antibody test</td>
<td>Agglutination</td>
<td>No agglutination</td>
</tr>
<tr>
<td>10.</td>
<td>Polyvalent flagellar antibody test</td>
<td>Agglutination</td>
<td>No agglutination</td>
</tr>
<tr>
<td>11.</td>
<td>Phenolred lactose broth</td>
<td>Yellow and/or gas</td>
<td>No gas; no color change</td>
</tr>
<tr>
<td>12.</td>
<td>Phenolred sucrose broth</td>
<td>Yellow and/or gas</td>
<td>No gas; no color change</td>
</tr>
<tr>
<td>13.</td>
<td>Voges-Proskauer test</td>
<td>Pink-to-red color</td>
<td>No color change</td>
</tr>
<tr>
<td>14.</td>
<td>Methyl red test</td>
<td>Diffuse red</td>
<td>Diffuse yellow</td>
</tr>
<tr>
<td>15.</td>
<td>Simmon’s citrate test</td>
<td>Growth; blue</td>
<td>No growth; no color change</td>
</tr>
</tbody>
</table>

<sup>a</sup> +, 90% or more positive in 1 or 2 days; 90% or more negative in 1 or 2 days

<sup>b</sup> Majority of *S. arizonae* are negative

<sup>c</sup> Majority of *S. arizonae* are positive

V, variable
3.3.10.2. Urease test

With a inoculation loop, growth from test culture (oxidase negative) was inoculated into tubes of urea broth and incubated at 37°C for 24hr. An uninoculated tube of this broth was kept as control. Since *Salmonella* are urease negative, all positive cultures were discarded. An uninoculated urea broth tube and an inoculated urea less broth (basal medium) tubes were used as controls for each isolate.

3.3.10.3. Indole test

Small amount of growth of test culture (urease negative) was inoculated into tryptone broth and incubated for 24h at 37 ºC. Production of indole from tryptophan was tested by adding 0.5 ml of Kovac’s reagent to the culture tubes. Since *Salmonella* gives negative test (lack of deep red color at surface of broth), all positive cultures were discarded.

3.3.10.4. Triple Sugar Iron test (TSI)

Colonies suspected to be *Salmonella* were inoculated into TSI agar tube by streaking on the slant and then stabbing the butt. The tubes were incubated at 37°C for 24h. *Salmonella* typically produces alkaline (pink) slant and acid (yellow) butt, with or without production of H₂S (blackening of butt). Atypically few *Salmonella* species produce reactions like A/A, A/A gas or A/A with or without production of H₂S.

3.3.10.5. Lysine Iron Agar test (LIA test)

LIA slants were inoculated with a small amount of culture grown on TSA plate by stabbing the butt twice and streaking the slant. Since lysine decarboxylation reaction is strictly anaerobic, the LIA slants were prepared with deep butt (4 cm). Inoculated tubes were incubated for 48h at 37°C.
*Salmonella* typically produce alkaline (purple) reaction in butt. Only distinct yellow in butt of tube considered as acidic (negative) reaction. But most *Salmonella* cultures were not eliminated solely on this basis. Cultures that gave an acid butt and an alkaline slant in LIA and an alkaline slant and acid butt in TSI were also been considered as presumptive *Salmonella* isolates. But cultures that gave an acid butt and an acid slant in LIA and acid butt and acid slant in TSI were discarded as not *Salmonella*.

### 3.3.10.6. Methyl Red-Voges Proskauer test (MR-VP)

Test cultures were inoculated to MR-VP broth and incubated for 72h at 37°C. About 2.5 ml of 48h culture in MR-VP broth was taken in a clean test tube and 5-6 drops of Methyl red indicator was added. *Salmonella* cultures give positive test indicated by diffuse red color in medium. A distinct yellow color was considered negative test and such cultures were discarded as not *Salmonella*.

Voges-Proskauer test was performed as follows: 0.6 ml of α-naphthol was added to the tube containing 48h incubated MR-VP broth culture and shaken well. Then 0.2 ml of 40% KoH solution was added and mixed and results were read after 4h. Development of pink-to-ruby red color throughout the medium was considered as positive test. *Salmonella* are VP negative.

### 3.3.10.7. Simmon’s citrate test

Test cultures were inoculated on citrate agar by streaking the slant and stabbing the butt and incubated for 48h at 37°C. Presence of growth usually accompanied by color change from green to blue was considered positive reaction. Most cultures of *Salmonella* are citrate positive. But few are citrate negative also.
3.3.11. Maintenance of cultures

Isolates, which gave typical biochemical reactions, were referred to be typical *Salmonellas* and those, which gave variable reactions in tests such as in MR-VP, TSI and LIA tests were considered to be atypical. They were further confirmed subjected to PCR. The isolates, which were confirmed by both conventional as well as PCR, were maintained at refrigerated temperature (-80°C) and also at room temperature in stabs (T1N0.5 medium) overlaid with liquid paraffin.

Statistical analysis of data obtained from the bacteriological analysis of the samples collected from various sampling sites of Rivers Nethravathy and Sharavathy

3.3.12. One-way ANOVA and Calculation of mean

Using software package SPSS, statistical analysis done for the data obtained from the bacteriological processing of the samples. One-way ANOVA statistical analysis was done in order to find the significant difference, if any, between the rivers and stations in relation to season, depth and tide with respect of various bacteriological parameters analysed for the water and sediment samples collected from riverine, estuarine and sea sites adjacent to Rivers Nethravathy and Sharavathy. Simultaneously, mean was calculated for all the parameters. The voluminous data collected during the period of study was subjected to statistical analysis to determine the difference, if any, between the two rivers with respect to several parameters studied. The statistical analysis was performed in the following pattern;
a. River wise
b. River wise and station wise
c. River, station and season wise
d. River, station, season and depth wise
e. River, station and tide wise

3.3.13. Correlation

Using software package SPSS, correlation was determined between water discharge and bacteriological parameters and also with in the bacteriological parameters analyzed for the samples collected from both the rivers and their estuarine as well as adjacent coastal sites.