Chapter 4

Survival kinetics of indicator bacteria and enteric pathogens in Kumarakom estuary - Role of biological factors
4.1 Introduction

The microbial components of aquatic food webs (bacteria, autotrophic picoplankton, heterotrophic and mixotrophic flagellates and ciliates) can often be an important, and sometimes dominant, part of aquatic ecosystems. Biological interactions between allochthonous bacteria and natural microbiota in aquatic environments are a complex process and the survival and persistence of these bacteria in natural aquatic ecosystems has been of interest to public health and microbial ecology. Sewage pollution in tropical Asian regions is a severe health risk to people that live near rivers and waterways. Usually in urban, peri-urban and adjacent rural areas surface water bodies receive faecally polluted urban discharges that contain pathogenic microorganisms in such high numbers that the assimilation and self purifying capacity of the receiving water body is overcome. This results in an increase in the number of faecal indicator organisms in receiving water, which often becomes unfit for domestic purposes, recreation or irrigation of crops eaten raw (Jagals, 2000) and also pose a potential health risk to consumers of shellfish bred in adjacent waters, or to bathers. Most sanitary indicator organisms as well as the enteric water-borne pathogens are intestinal inhabitant of man and other warm blooded animals. These microorganisms frequently gain entry into the water bodies through faecal discharges.

Various physico-chemical and biological factors involved in the disappearance of pollutant microorganisms in the aquatic environment include water temperature (Anderson et al., 1983), adsorption and sedimentation processes (Auer and Niehaus, 1993), sunlight action (Sinton et al., 1999), lack of nutrients (Sinclair and Alexander, 1984), predation by bacteria or protozoa (Hahn and Hofle, 2001), bacteriophage lysis (Ricca and Cooney, 1999), competition with autochthonous microbiota (McCambridge and McKeekin, 1981) and antibiosis (Colwell, 1978). Among the biological factors grazing by protozoans have been identified as a significant factor modifying bacterial populations in aquatic ecosystems and thus implicated as major trophic links between the microbial loop and the classical food chain.
4.2 Review of Literature

The factors controlling the survival of enteric bacteria in the aquatic environment in relation to public health as well as to understand the bacterial responses to environmental stress, numerous studies have explored the fate of *E. coli* and other enteric bacteria following their exposure to aquatic environments. Many of these reports were motivated by the need to properly evaluate the risk posed by such microorganisms when released into natural water, either to the health of bathers in recreational waters or to the safety of fisheries or marine agriculture.

Several workers have reported the specific role of protozoan grazing as the dominant factor regulating the bacterial population in aquatic environments (McCamberge and McMeekin, 1981; Anderson *et al.*, 1983; Barcina *et al.*, 1992; Hahn and Hofle, 2001; Duhamel *et al.*, 2006). Among protozoan nano-flagellates and ciliates have been identified as a significant factor modifying bacterial populations in aquatic ecosystems (Sanders *et al.*, 1986; Pace, 1998; Alonso *et al.*, 2000; Wcislo and Chrost, 2000). Koton-Czarnecka and Chrost (2003) reported grazing preference of Heterotrophic Nano Flagellates (HNF). The results suggested that HNF prefer medium size and actively metabolizing bacterial cells. They also found that grazing rates on live bacteria were 1.83 times greater than grazing rates on dead bacteria. Size selective grazing of protozoan was also reported (Chrzanowski and Siimek, 1990; Gonzalez *et al.*, 1990; Siimek and Chrzanowski, 1992) with most protists grazing preferentially on medium-sized bacterial cells.

Some phagotrophic flagellates are even able to feed on virus particles (Gonzalez and Suttle, 1993) or high molecular weight polysaccharide (Sherr, 1988). Thus, the smallest bacterial cells are not completely protected from grazing but receive, due to lower grazing efficiency in comparison to larger cells, a relative protection. Pernthaler *et al.* (1996) have suggested functional size fractions within bacterial communities: small cells (<0.4μm) weakly affected by protists grazing, medium-sized 'grazing-vulnerable' (0.4-1.6 μm) and 'grazing-suppressed' (1.6-2.4 μm) bacteria and large 'grazing-resistant' bacteria (>2.4 μm).
Besides size, other traits of bacteria such as motility, shape and cell surface characteristics may influence the selectivity of protistan grazing (Monger et al., 1999). Recent investigations have revealed that protistan grazing impacts both the bacterial standing stock and the morphological and taxonomic structure of bacterial communities (Síimek et al., 1999; Jurgens et al., 1999; Hahn and Hofle, 2001). It has been suggested that bacterial prey persist due to the development of predation resistant mechanisms (Gude, 1979) and the development of avoidance strategies (Boenigk et al., 2002). Altogether, grazing by protistan predators on bacterial populations is a very complex interaction.

Over the past 15 years, it has been realized that viruses are an important component of aquatic microbial food webs. They have been shown to be an important controlling agents in planktonic community composition, diversity and succession, playing a key role in cell mortality and nutrient cycles (Weinbauer and Rassoulzadegan, 2004). The role of other biological factors such as bacteriophage, competition, bacterial predation and antibiosis on the removal of bacteria in aquatic environment has also been demonstrated. Hennes and Simon (1995) studied the significance of bacteriophages for controlling bacterioplankton growth in a mesotrophic lake. They estimated the phage-induced mortality of total bacterial population and suggested that phage infection was important in structuring the bacterial host assemblage. Fuhrman and Noble (1995) reported the similar role of viruses and protists in bacterial mortality in coastal waters. Both bacteriophage and flagellates caused significantly higher decrease in the number of bacteria in various aquatic environments (Alonso et al., 2000; Jacquet et al., 2005; Duhamel et al., 2006). It has been estimated that viruses can be responsible for >50% of the bacterial mortality in many ecosystems, thus affecting the flow of energy, carbon and bacterial community composition within the ecosystem (Fuhrman and Noble, 1995; Hennes and Simon, 1995; Mathias et al., 1995; Weinbauer and Höfler, 1998; Wilhelm and Smith, 2000; Fischer and Velimirov, 2002; Jaquet et al., 2005).

The role of competing autochthonous microbiota on the bacterial population has been reported by several workers (McCambridge and McMeekin, 1981; Le Guyader et al., 1991). Jannasch (1968) reported that Enterobacteriaceae have little or no chance to outgrow...
competitors in seawater. It has been demonstrated that bacterial competition, antagonism and even bacterial predation were relatively unimportant in removing coliforms from estuarine water, and that indigenous protozoa were responsible (Enzinger and Cooper, 1976; Mallory et al., 1983). Effect of antibiosis on the bacteria was studied by many workers. Long et al. (2001) observed more than one-half of the isolates from marine pelagic zone expressed antagonistic activity, and this trait was more common with particle-associated bacteria than with free-living bacteria. Robertson et al. (2000) isolated a strain of Corenybacterium spp. that exhibited antagonism against Aeromonas hydrophila, A. salmonicida, Flavobacterium psychrophilum, Photobacterium and hypothesized that bacterium-bacterium antagonistic interactions may contribute to variations in community structure at the micro scale. Marino and Cannon (1991) reported that bacterial antagonists in aquatic system are of minor importance and also suggested that predation assumes a greater role than competition and antagonism in regulating the survival of indicator bacteria.

The review reveals the importance of various biological factors on the survival kinetics of allochthonous bacteria in natural waters. It is also found that virtually no studies have been carried out on this important aspect by Indian researchers. The studies on the prevalence of indicator bacteria and enteric pathogens in the Kumarakom lake revealed considerable prevalence of the above organisms. Hence, the survival of these allochthonous bacteria as a function of various biological factors has been evaluated in this chapter. The specific objectives of the study are as follows.

4.3 Objectives

1. To study the survival of of *E. coli*, *S. paratyphi* and *V. parahaemolyticus* as a function of protozoan bacteriovory alone.

2. To study the survival of *E. coli*, *S. paratyphi* and *V. parahaemolyticus* as a function of competition from autochthonous bacateria
3. To study the survival of *E. coli*, *S. paratyphi* and *V. parahaemolyticus* as a function of competition from autochthonous microbiota (including all components of the microbiota such as protozoans, bacteria and bacteriophages).

4. To find out the antibacterial activity/ antibiosis of the members of autochthonous microbiota on *E. coli*, *S. paratyphi* and *V. parahaemolyticus*.

### 4.4 Materials and Methods

#### 4.4.1 Determination of competing microflora

Water samples were collected from 10 different stations from the Kumarakom estuary in sterile plastic bottles and transported to the laboratory in an icebox and subjected to bacteriological examination within 4 hours of collection (USEPA, 1978). The samples were processed for microbial parameters such as total heterotrophic bacteria and characterisation of protozoan predators.

#### 4.4.2 Enumeration of Total Heterotrophic Bacteria (THB)

Water sample were decimally diluted using sterile distilled water and shaken thoroughly to distribute the microorganisms in the water to the dilution blank. Appropriately diluted samples were plated on Tryptone Soya Agar (TSA) using spread plate technique. The inoculated plates were incubated at 37°C for 24-48 hrs. After incubation plates with countable range (30-300 colonies) were selected for counting using a colony counter and the bacterial load in the sample was expressed as total colony forming units (CFU) per ml of the water sample. Then morphologically different colonies were isolated and identified as various genera as per Bergey’s manual of determinative bacteriology (Buchanan and Gibbons, 1984).
4.4.3 Characterisation of protozoan predators

The protozoans were observed microscopically and identified according to Batish (1992).

4.4.4 Enumeration of bacteriophages

Bacteriophages were enumerated by plaque assay using double-layer agar method (Kennedy et al., 1986) and is carried out as follows. Forty-five millilitre of the sample and 5ml of E. coli/ S. paratyphi/V. parahaemolyticus culture was inoculated into 45 ml of deca strength phage broth (DSPB) and incubated at 37°C for 24hrs. After incubation, the cells were centrifuged at 2500 rpm for 10 minutes and the supernatant was filtered through 0.45 µm filter. Then 0.1 ml of the filtrate was mixed with 1ml of E. coli / S. paratyphi/ V. parahaemolyticus culture and 5ml of 0.6% nutrient agar (used as top agar) and poured over nutrient agar plates with 1.2% agar concentration (basal agar). The plates were then incubated at 37°C for 24 hrs and the plaques were counted and expressed as plaques forming units (PFU) per millilitre.

4.4.5 Test organisms: E. coli, S. paratyphi and V. parahaemolyticus isolated from the Kumarakom estuary were used for survival studies.

4.4.5.1 Preparation of inocula: E. coli, S. paratyphi and V. parahaemolyticus were grown separately in 10ml sterile Tryptone Soya Broth (TSB) and incubated at 37°C for 24 hours. After incubation the cells were concentrated by centrifugation at 3000 rpm for 15 minutes and washed twice with sterile isotonic saline. After the final wash the cells were resuspended in 10ml sterile isotonic saline at a concentration of 10^8 CFU per ml. From this final suspension 1 ml was inoculated into 250 ml Erlenmeyer flask with 100 ml of the test solution so as to give an initial inoculum density of 10^6 CFU per ml of test solution.

4.4.6 Test solutions: To find out the effect of various self-purifying biological factors such as protozoan predation, competition from autochthonous microorganisms and antagonism on test organisms, survival experiments were conducted in the following test solutions inoculated with test organisms.
4.4.6.1 **Raw estuarine water**: This test solution was used to determine the combined effect of all the self contained biological factors such as protozoan, bacterial and bacteriophage predators on the test organisms. Estuarine water from different stations was collected, pooled and then a subsample of 100 ml was taken to suspend the test organisms. The test organisms were suspended at a final concentration and survival and injury were estimated at 3, 5 and 7 days intervals.

4.4.6.2 **Raw estuarine water supplemented with cycloheximide**: This test solution was used to determine the effect of bacterial predator and bacteriophage alone on the test organisms. Protozoan was excluded from the test solution by the addition of prokaryotic inhibitor, cycloheximide at a concentration of 500 mg/l (Davis et al., 1995).

4.4.6.3 **Filter sterilized estuarine water inoculated with autochthonous bacteria**: This test solution was used to study the effect of competing autochthonous bacteria isolated from estuarine water acting together on the survival of test organisms. All the 8 bacterial genera isolated from the estuarine water were grown separately in TSB overnight at 37°C for 24 hrs. From each of the enriched culture 1 ml was added into the test solution containing *E. coli*, *S. paratyphi* and *V. parahaemolyticus* in separate bottles. One set of the test solutions was incubated at 30°C and another set at 20°C. Survival at low temperature was carried out considering the reduction in temperature to 20°C in winter and at certain depth.

4.4.6.4 **Enumeration Techniques**: The samples from the test solution were taken aseptically and assayed after 3, 5, and 7 days using spread plate technique. The enumerations and quantification of survived and injured cells were done using two plating media in parallel, one non selective and the other one is selective. TSA was employed as non selective medium while Eosin Methylene Blue (EMB) agar was used as selective medium for *E. coli*, Xylose Lysine Deoxycholate (XLD) agar for *S. paratyphi* and Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar for *V. parahaemolyticus*. All the samples were replicated two-fold. The percentage of survivors and injured cells at time ‘t’ were calculated according the formula:
Percentage of survival of *E. coli* at time ‘t’

\[
\text{Percentage} = \left( \frac{\text{Count on TSA plates at time ‘t’}}{\text{Count on TSA plates at time ‘0’}} \right) \times 100
\]

Percentage of injury of *E. coli* at time ‘t’

\[
\text{Percentage} = \left( 1 - \frac{\text{Count on EMB plates at time ‘t’}}{\text{Count on TSA plates at time ‘t’}} \right) \times 100
\]

Percentage of survival of *S. paratyphi* at time ‘t’

\[
\text{Percentage} = \left( \frac{\text{Count on TSA plates at time ‘t’}}{\text{Count on TSA plates at time ‘0’}} \right) \times 100
\]

Percentage of injury of *S. paratyphi* at time ‘t’

\[
\text{Percentage} = \left( 1 - \frac{\text{Count on TSA plates at time ‘t’}}{\text{Count on TSA plates at time ‘0’}} \right) \times 100
\]

Percentage of survival of *V. parahaemolyticus* at time ‘t’

\[
\text{Percentage} = \left( \frac{\text{Count on TSA plates at time ‘t’}}{\text{Count on TSA plates at time ‘0’}} \right) \times 100
\]

Percentage of injury of *V. parahaemolyticus* at time ‘t’

\[
\text{Percentage} = \left( 1 - \frac{\text{Count on TCBS plates at time ‘t’}}{\text{Count on TSA plates at time ‘t’}} \right) \times 100
\]

4.4.6.5 Antagonistic activity of THB

The antagonistic activities of the isolated total heterotrophic bacteria on *E. coli*, *S. paratyphi* and *V. parahaemolyticus* were studied using cross streak assay. Overnight grown cultures of each heterotrophic bacteria were streaked on one side of sterile TSA plates and the test organisms were streaked perpendicular to it and incubated at 37°C for 24 hours. After incubation the plates were examined for the zone of inhibition of *E. coli* and *V. parahaemolyticus* cultures nearest to the streak of heterotrophic bacterial genera.
4.4.7 Statistical analysis

The difference in the survival of the test organisms in cycloheximide treated and non-treated and THB added samples were analysed using two way analysis of variance (ANOVA).

4.5 Results

In the present study an attempt has been made to understand the role of biological parameters on the survival of *E. coli*, *S. paratyphi* and *V. parahaemolyticus* in Kumarakom estuary. Biological factors studied included competition from autochthonous heterotrophic microflora, antibiosis/inhibition of growth due to the production of antibacterial substances by the heterotrophic bacterial genera and predation by protozoans. Different microcosms inoculated with various test solutions were designed to study each of the above parameters. The competing autochthonous flora was assessed in terms of the total load as well as various genera involved.

4.5.1 Load and composition of the competing autochthonous microflora

The load of competing autochthonous microflora and the various genera encountered is given in Table 4.1. The load of competing heterotrophic bacteria was $3.8 \times 10^6$ CFU/ml. Percentage occurrence of different bacterial genera isolated from the estuarine water revealed that majority of the genera were Gram-negative bacteria (85.72%) and the remaining 14.28% were Gram-positive bacteria. The bacterial genus identified includes *Alcaligenes*, *Aeromonas*, *Bacillus*, *Microoccus*, *Enterobacteriaceae*, *Pseudomonas*, *Actinomycetes* and *Moraxella*. *Alcaligenes* (29.76%), members of the family *Enterobacteriaceae* (20.23%) and *Aeromonas* (15.48%) were found to be predominant in the estuarine water examined.

Diversity and survival of diarrhegenic *E. coli* and enteric pathogens in Vembanad lake with special reference to Kumarakom area
Table 4.1. Load and composition of the competing autochthonous microflora

<table>
<thead>
<tr>
<th>Coliphage PFU/ml</th>
<th>Load of total heterotrophic bacteria (CFU/ml)</th>
<th>Composition of autochthonous heterotrophic flora</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gram-positive genera (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gram-negative genera (%)</td>
</tr>
<tr>
<td>2.8 × 10^6</td>
<td>3.8 × 10^6</td>
<td>Bacillus (10.71)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alcaligenes (29.76)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Micrococcus (10.71)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enterobacteriaceae (20.23)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Actinomyces (7.14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aeromonas (15.48)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pseudomonas (3.57)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moraxella (1.19)</td>
</tr>
</tbody>
</table>

4.5.2 Protozoans encountered in the Kumarakom estuary

The protozoans encountered from the estuarine water sample is represented in plate 4., which included Coleps hirutus, Amoeba radiosa, A. proteus, Chylomonas, Arcella spp., Difflugia pyriformis, D. lobostoma, Vorticella campanula, Lionotus spp., Phacus pleuronectes, Oxytricha spp., Trachelomonas, Actinophrys sol, Paramoecium spp., Stylonicha spp., Euplotes spp. and Euglena spp. Among these the ciliates and flagellates exhibited the greater diversity and abundance. The protozoan cell count in the raw estuarine water samples ranged from 8-20 individual/ml.

4.5.3 Survival in raw and cycloheximide treated estuarine water

Percentage survival and injury of E. coli S. paratyphi and V. parahaemolyticus cells inoculated in raw estuarine water and cycloheximide treated estuarine water are presented in Table 4.2, 4.3, 4.4, 4.5, 4.6 and 4.7 The survival of both organisms was low when they inoculated into raw estuarine water at 30°C and 20°C. But the survival was enhanced when the protozoan inhibitor was introduced into the test solution. The survival rate was decreased with time in all the test solution at both the temperatures but the injury (loss of ability to form colonies on selective media) was increased with time.
Plate  - 5: Antibacterial activity of Autochthonous bacteria:
(a) Bacillus and (b) Actinomycetes against E. coli and V. parahaemolyticus.
Table 4.2 Percentage survival and injury of *E. coli* in raw estuarine water

<table>
<thead>
<tr>
<th>Test solution</th>
<th>Percentage of survival</th>
<th>Percentage of injury</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days</td>
<td>Days</td>
</tr>
<tr>
<td>Raw estuarine water at 30°C</td>
<td>3.39</td>
<td>0.36</td>
</tr>
<tr>
<td>Raw estuarine water at 20°C</td>
<td>0.36</td>
<td>0.0599</td>
</tr>
</tbody>
</table>

Table 4.3 Percentage survival and injury of *E. coli* in raw estuarine water supplemented with cycloheximide

<table>
<thead>
<tr>
<th>Test solution</th>
<th>Percentage of survival</th>
<th>Percentage of injury</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days</td>
<td>Days</td>
</tr>
<tr>
<td>Raw estuarine water with cycloheximide at 30°C</td>
<td>0.625</td>
<td>0.0031</td>
</tr>
<tr>
<td>Raw estuarine water with cycloheximide at 20°C</td>
<td>0.625</td>
<td>0.078</td>
</tr>
</tbody>
</table>

Table 4.4 Percentage survival and injury of *S. paratyphi* in raw estuarine water

<table>
<thead>
<tr>
<th>Test solution</th>
<th>Percentage of survival</th>
<th>Percentage of injury</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days</td>
<td>Days</td>
</tr>
<tr>
<td>Raw estuarine water at 30°C</td>
<td>0.108</td>
<td>0.016</td>
</tr>
<tr>
<td>Raw estuarine water at 20°C</td>
<td>79.83</td>
<td>0.47</td>
</tr>
</tbody>
</table>
Table 4.5 Percentage of survival and injury of *S. paratyphi* in raw estuarine water supplemented with cycloheximide

<table>
<thead>
<tr>
<th>Test solution</th>
<th>Percentage of survival</th>
<th>Percentage of injury</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days</td>
<td>Days</td>
</tr>
<tr>
<td></td>
<td>3 5 7</td>
<td>3 5 7</td>
</tr>
<tr>
<td>Raw estuarine water with cycloheximide at 30°C</td>
<td>10.94 0.59 1.11</td>
<td>3.64 26.88 80</td>
</tr>
<tr>
<td>Raw estuarine water with cycloheximide at 20°C</td>
<td>35.29 0.588 0.694</td>
<td>3.88 35.29 0.59</td>
</tr>
</tbody>
</table>

Table 4.6 Percentage of survival and injury of *V. parahaemolyticus* in raw estuarine water

<table>
<thead>
<tr>
<th>Test solution</th>
<th>Percentage of survival</th>
<th>Percentage of injury</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days</td>
<td>Days</td>
</tr>
<tr>
<td></td>
<td>3 5 7</td>
<td>3 5 7</td>
</tr>
<tr>
<td>Raw estuarine water at 30°C</td>
<td>1.2 0.033 0.032</td>
<td>93.33 97.56 97.46</td>
</tr>
<tr>
<td>Raw estuarine water at 20°C</td>
<td>2.4 0.544 0.421</td>
<td>96.67 98.53 98.10</td>
</tr>
</tbody>
</table>

Table 4.7 Percentage of survival and injury of *V. parahaemolyticus* in raw estuarine water supplemented with cycloheximide

<table>
<thead>
<tr>
<th>Test solution</th>
<th>Percentage of survival</th>
<th>Percentage of injury</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days</td>
<td>Days</td>
</tr>
<tr>
<td></td>
<td>3 5 7</td>
<td>3 5 7</td>
</tr>
<tr>
<td>Raw estuarine water with cycloheximide 30°C</td>
<td>0.5 0.575 0.055</td>
<td>90 99.13 90.9</td>
</tr>
<tr>
<td>Raw estuarine water with cycloheximide at 20°C</td>
<td>195.0 5.05 0.13</td>
<td>97.44 99.00 99.15</td>
</tr>
</tbody>
</table>
Effect of protozoan predation and predacious microorganisms other than protozoan on the survival of *E. coli*, *S. paratyphi* and *V. parahaemolyticus* is represented in Fig. 4.1, 4.2 and 4.3 respectively. In raw estuarine water, *E. coli* cells reduced nearly 5 logs at 30°C and almost 4 logs at 20°C after 7 days of exposure. *E. coli* cells showed better survival at 20°C than at 30°C. However in cycloheximide treated estuarine water *E. coli* showed enhanced survival at 30°C than 20°C.

**Fig. 4.1 Survival curves of *E. coli* in raw and cycloheximide treated estuarine water at 20°C and 30°C**

*S. paratyphi* demonstrated high die-off in raw estuarine water at 30°C and 20°C. It showed a continuous steep reduction throughout the exposure time in estuarine water. The initial inoculum density was around $10^9$ / ml and it declined almost 7 logs at 30°C and nearly 6 logs at 20°C. While *S. paratyphi* exhibited improved survival at 20°C. However in the cycloheximide treated water at 30°C, *S. paratyphi* showed relatively high reduction than at 20°C. At 30°C it showed almost 3 log reduction in 7 days but at 20°C it exhibited some kind of acclimatization towards the end of the experiment.
Fig. 4.2 Survival curves of *S. paratyphi* in raw and cycloheximide treated estuarine water at 20°C and 30°C

*V. parahaemolyticus* showed almost 4 log reduction at 30°C at the end of fifth day and after that it demonstrated a steady growth till the end of the experiment but at 20°C it reduced only 2 logs indicating their better survival at low temperature. However in cycloheximide treated water it exhibited variation in the survival pattern. At 30°C it demonstrated an initial reduction until 3rd day and after that a slight growth and again showed a reduction. On the other hand at 20°C an initial growth was noticed, after that it showed a steep reduction till the end of the experiment. The results revealed that *E. coli*, *S. paratyphi* and *V. parahaemolyticus* showed better survival in cycloheximide treated water indicating the specific role protozoan predators on their survival. Besides, they also showed enhanced survival at 20°C. However no significant variation was observed in the survival of *E. coli*, *S. paratyphi* and *V. parahaemolyticus* in cycloheximide treated and non treated samples at both the temperatures.
Fig. 4.3 Survival curves of *V. parahaemolyticus* in raw and cycloheximide treated estuarine water at 20°C and 30°C

![Survival curves of *V. parahaemolyticus*](image)

- Raw estuarine water at 30°C
- Raw estuarine water at 20°C
- Raw estuarine water + cycloheximide at 30°C
- Raw estuarine water + cycloheximide at 20°C

### 4.5.4 Effect of competing heterotrophic bacteria

Relative survival curves of *E. coli*, *S. paratyphi* and *V. parahaemolyticus* in autoclaved estuarine water supplemented with THB bacteria isolated from estuarine water at different temperatures is represented in Fig. 4.4. In order to elucidate the effect of competing heterotrophic bacteria, the test organisms were inoculated separately into the test solution containing all of the autochthonous THB genera together. The results revealed that *E. coli* showed a slight reduction initially at both the temperatures and after that it exhibited little growth and continue to decline towards the end of the experiment at 30°C, whereas it showed a slight growth at the end of the experiment at 20°C. *S. paratyphi* demonstrated a slight reduction during the initial stage of the experiment at 30°C, whereas at 20°C it showed an initial growth. The survival of *S. paratyphi* was slightly higher at 20°C than at 30°C. After that the cells did not changed much in their number. *V. parahaemolyticus* demonstrated a steady growth till 5th day of the experiment and after that started to decline gradually at...
both the temperatures. No significant difference was observed on the survival of \textit{E. coli}, \textit{S. paratyphi} and \textit{V. parahaemolyticus} by the addition of THB population at both temperatures.

\textbf{Fig. 4.4} Relative survival curves of \textit{E. coli}, \textit{S. paratyphi} and \textit{V. parahaemolyticus} in sterile estuarine water supplemented with competing heterotrophic bacteria at 20°C and 30°C.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\end{figure}

\begin{enumerate}
\item[$\times$] \textit{E. coli} + THB at 30°C;
\item[$\blacktriangle$] \textit{E. coli} + THB 20°C;
\item[$\bigcirc$] \textit{S. paratyphi} + THB at 30°C;
\item[$\bullet$] \textit{S. paratyphi} + THB at 20°C;
\item[$\blacksquare$] \textit{V. parahaemolyticus} + THB at 30°C;
\item[$\square$] \textit{V. parahaemolyticus} + THB at 20°C.
\end{enumerate}

\subsection*{4.6 Discussion}

\subsection*{4.6.1 Survival in raw estuarine water}

The mortality rate of \textit{E. coli}, \textit{S. paratyphi} and \textit{V. parahaemolyticus} was high when they introduced into the raw estuarine water incubated at 30°C and at 20°C compared to cycloheximide treated samples indicating the effective role of protozoa in the removal of test microorganisms. It has been well documented that protozoan grazing was the significant factor responsible for the removal of bacterial population in aquatic environments (Hahn and Hofle, 2001). The observations of the present study are in agreement with the findings of the above results that the destruction of \textit{E. coli}, \textit{S. paratyphi}
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and *V. parahaemolyticus* was very high in natural estuarine water compared to cycloheximide treated and autoclaved estuarine water in the absence of predators. The results support the previous observation in Cochin estuary that the biological factors such as protozoan predation and bacteriophage exerts a high inactivation on *E. coli* and *Salmonella typhimurium* in estuarine water followed by sunlight (Abhirosh and Hatha, 2005).

It has also been demonstrated that grazing by phagotrophic protists, especially bacterivorous nanoflagellates has been identified as the significant factor modifying bacterial populations in aquatic ecosystems (Sherr and Sherr, 2002). However, Amblard *et al.* (1995) observed a lack of correlation between bacteria and nanoflagellates in humic lakes and Caron *et al.* (1983) reported that only part of heterotrophic nanoflagellates is really bacterivorous. But Kalinowska (2004) found a significant correlation between bacteria and ciliates and suggested that the density of bacteria were determined by ciliates in mesotrophic, humic and in eutrophic lakes. Simek *et al.* (1990) demonstrated that ciliates were important consumers of bacteria and the role of heterotrophic nanoflagellates in grazing on bacteria decreases and that of ciliates increases with the increasing trophic status of lakes. Epstein and Shiaris (1992) found that those ciliates consumed bacteria 17 times faster on average than did flagellate and thus ciliates and nanoflagellates may play a similar role in controlling bacteria.

The present study has identified 19 protozoan species from the estuarine water and the most predominant groups were ciliates and flagellates. The abundance of protozoan cells encountered from the sample during the test period ranges from 8-20 individual/ml. Hence the reduction of the test organisms in raw estuarine water was explained by the grazing effect of these flagellates and ciliates. Kalinowska (2004) found ciliates were the dominant taxa in different lakes followed by heterotrophic flagellates. According to Simek *et al.* (1995) the grazing rate on bacteria by a single individual of *Vorticella* (4200 bacteria per hour) is much higher than by *Cyclidium* (470 bacteria per hour) or by Oligotrichida (380–2130 bacteria per hour). The occurrence of *Vorticella* in the estuarine water was also observed in the present study area.
Hahn et al. (1998) reported *Vibrio* strain CB5 dominated the total bacterial cell numbers during the flagellate-free phase of the experiments with a relative abundance of 93%, but this declined to 33% after inoculation with the flagellates. Jurgens et al. (1999) observed in enclosure studies, after experimentally increasing the protozoan grazing pressure, there was a rapid and strong change in the morphological structure of the bacterial community. Although there is different opinion about the role of ciliates and flagellates on the control of bacteria in different aquatic systems, in general, all these results are in agreement with the present observation that predation by protozoans exerts a major role in the removal of bacteria in aquatic environments.

However, in raw estuarine water *E. coli* showed enhanced survival at 30°C than at 20°C but *S. paratyphi* and *V. parahaemolyticus* survived well at 20°C than at 30°C. This may be due to the preference of *E. coli* to temperature around 30°C. Temperature stress in such conditions will be less on the bacterial cell forming an enhanced survival. The grazing of bacteria is size selective with most protists grazing preferentially on medium-sized bacterial cells (Síimek et al., 1992). Anderson et al. (1983) demonstrated that *E. coli* showed an extended survival during *in situ* exposure to estuarine water, provided eukaryotes were excluded from the diffusion chamber and also noted that the *E. coli* disappearance was most pronounced in the presence of natural microbiota at warm water.

### 4.6.2 Survival in cycloheximide treated estuarine water

In order to find out the role of predacious bacteria and bacteriophages, the protozoans were inhibited by the addition of cycloheximide. Eventhough the predators were eliminated by the addition of cycloheximide, *E. coli* showed 4 log reductions within 7 days while *S. paratybi* and *V. parahaemolyticus* exhibited almost 3-4 log reduction at 30°C. But *E. coli*, *S. paratybi* and *V. parahaemolyticus* demonstrated better survival at 20°C. These results indicated that even in the absence of protozoan predators a greater reduction in the number of *E. coli*, *S. paratybi* and *V. parahaemolyticus* were observed at 30°C suggesting the deleterious effect of other biological factors (predacious bacteria and bacteriophages) other than protozoan on the survival of test organisms at 30°C. However, these predatory

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bacteria and bacteriophage had very little effect at 20°C. The statistical analysis revealed that there was no significant difference in the survival of the test organisms in cycloheximide treated and nontreated microcosms. Test microorganisms showed a constant growth in autoclaved estuarine water (control), which is devoid of any life forms. Similarly the direct effect of cycloheximide on the test microorganisms in autoclaved estuarine water was negligible.

It is assumed that the high mortality rate in the absence of predacious protozoa may be due to the action of bacteriophage and 
_{Bdellovibrio}. Several workers stated that bacteriophages have been considered as a factor in the removal of coliforms from natural environments (Alonso et al., 2000; Jacquet et al., 2005). In marine ecosystems, for example, Suttle and Chen (1992) estimated that 4-13% of the bacterial community would have to be infected daily and that 8-26% of the bacterial mortality could be explained by viral lysis in marine ecosystems. In freshwater ecosystems, viruses have also been shown to play a crucial role in bacterial mortality, with daily bacterial removal reaching up to 97% (Weinbauer and Hofle, 1998). The mean coliphage count encountered in the present study was $2.8 \times 10^9$ PFU/ml (Table 1). A typical viral abundance of $10^9$ to $10^{10}$ viral particles per litre in seawater and lake water were reported (Paul et al., 1991). Henceforth, high viral abundances have been observed worldwide in seawaters, coastal waters and freshwaters (Paul et al., 1991; Bergh et al., 1989).

There has been considerable speculation on the role of _Bdellovibrio bacteriovorus_ in controlling bacterial population in natural waters. Based on the observation that _Salmonella_ die-off was associated with the presence of _Bdellovibrios_ in untreated river water, Guelin et al. (1967) suggested that _Bdellovibrios_ might be important in the auto purification of polluted natural waters. Incidence of marine _Bdellovibrios_ lytic against _V. parahaemolyticus_ was reported by Williams et al. (1980) and the utilisation of lipopolysaccharides and membrane proteins from _E. coli_ by _Bdellovibrio bacteriovorus_ was also reported (Murry et al., 1992). On the contrary Mallory et al. (1983) suggested that the bacterial decline was not due to bacteriophages and _Bdellovibrios_, but because of protozoan predation, and also the impact
of viruses on the changes in the structure of bacterioplankton were found negligible (Hornak et al., 2005).

Several workers reported an enhanced bacterial survival in different cycloheximide treated samples with non-treated raw samples such as water (McCambridge and McMeekin, 1981), sewage (Mallory et al., 1983) and sediment (Marino et al., 1991; Davies et al., 1995) and suggested that protozoan grazing plays an important role as a bacterial removal mechanism in the various aquatic environments. These results support the present observation that the mortality rates of the test organisms were high in raw estuarine water containing protozoan predators compared with cycloheximide treated estuarine water in which protozoan were inhibited.

4.6.3 Effect of competing heterotrophic bacteria

The THB load encountered in the present study was $3.8 \times 10^6$/ml. Bacterial genera such as *Alcaligenes* (29.76%), members of the family *Enterobacteriaceae* (20.23%) and *Aeromonas* (15.48%) were predominant in the estuarine. When the test organisms were inoculated into test solution containing all these genera together in order to elucidate their competitive effect on the test organisms revealed that the competing bacterial population had no or very little effect on the test organisms. The statistical results also revealed that the difference in the survival was not significant. The enhanced survival of these organisms may be due to compounds produced by the bacterial population or by cryptic growth (when cells die, they generally produce compounds such as energy nutrient substrates through cell lysis. These compounds serve as a nutrient source for viable cells in the same population, Ryan, 1959).

It has been reported that bacterial competition, antagonism and even bacterial predation were relatively unimportant in removing coliforms from estuarine water. (Mallory et al., 1983). But the role of competing autochthonous microbiota has also been reported (McCambridge and McMeekin, 1981) Jannasch (1968) noted that *Enterobacteriaceae* have little or no chance to outgrow competitors in seawater.
Production of antibiotic substance by the isolated genera of the THB bacteria on growth of *E. coli*, *S. paratyphi* and *V. parahaemolyticus* has also been analysed. Among the different genera *Bacillus* and *Actinomycetes* exhibited antagonistic activity against the test microorganisms but it did not show any antibacterial activity against *S. paratyphi*. Diameter of the inhibition zone produced by *Bacillus* found to be more than that of *Actinomycetes*. Although *V. parahaemolyticus* was found to be more sensitive than *E. coli* towards *Bacillus* and *Actinomycetes* (Plate 2). Long et al. (2001) observed that more than one-half of the isolates from marine pelagic zone had antagonistic activity, and this trait was more common with particle-associated bacteria than with free-living bacteria. Marino et al. (1991) reported that bacterial antagonists in aquatic system are of minor importance and also suggested that predation assumes a greater role than competition and antagonism in regulating the survival of indicator bacteria.

### 4.6.4 Cellular injury

In the present study the injury levels caused by different test solutions on the test organisms was examined. Among the test solutions the injury level was high in raw estuarine water and cycloheximide treated estuarine water when compared to the injury level in THB supplemented test solution. Clesceri et al. (1998) reported that bacteria may undergo variable amounts of growth inhibition, stress or injury in environmental waters. It has been observed that the sub-lethal physiological damage results from exposure to chemical, physical and/or biological factors which may cause the organisms to lose the ability to grow on those routine selective media that are otherwise satisfactory for the cultivation of healthy cells (Calabrese and Bissonnette, 1990; Kang and Siragusa, 1999) and injured pathogens may retain their pathogenicity following injury (Singh and McFeters, 1987). It is obvious that low temperature plays an important role in the formation of nonculturable *V. parahaemolyticus* cells (Jiang et al., 1996).

### 4.7 Conclusions

It is concluded that the natural water has the inherent capacity to purify themselves involving various environmental factors. Different biological factor exerts...
different magnitude of elimination of *E. coli*, *S. paratyphi* and *V. parahaemolyticus* from the Kumarakom estuary. Among the different biological factors protozoan predation is the major biotic factor influencing the survival of *E. coli*, *S. paratyphi* and *V. parahaemolyticus*. Native predacious microorganisms other than protozoan also cause considerable inactivation effect on *E. coli*, *S. paratyphi* and *V. parahaemolyticus*. The survival of *E. coli* was comparatively higher than *S. paratyphi* and *V. parahaemolyticus*. However, in raw estuarine water *E. coli* showed enhanced survival at 30°C than at 20°C but *S. paratyphi* and *V. parahaemolyticus* survived well at 20°C. In cycloheximide treated samples, *E. coli*, *S. paratyphi* and *V. parahaemolyticus* demonstrated better survival at 20°C. However no significant variation was observed on the survival of *E. coli*, *paratyphi* and *V. parahaemolyticus* in cycloheximide treated, non treated and THB added microcosms. Although understanding these factors and their relative importance is justifiable purely on ecological basis, in particular, such studies are necessary to assess the validity of faecal coliform group or other microbial indicators which may be used as quantitative measures of faecal pollution in shellfish-growing waters.