Chapter 7

In situ survival of indicator bacteria and enteric pathogens in estuarine water
7.1 Introduction

Rapid urban and industrial growth has resulted in a vast quantity of potentially harmful waste being released into the environment. Large number of faecal microorganisms, including some pathogenic bacteria, protozoa and viruses are discharged into natural waters through sewage outfall and other types of faecal discharges. Pathogenic organisms found in sewage can adversely affect public health when humans come in contact with water while wading, swimming, fishing, drinking etc.

The behavior of enteric microorganisms in the coastal environment depends on many factors that have been investigated by several workers. These factors 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 and protozoa (Hahn and Hofle, 2001), bacteriophage lysis (Ricca and Cooney, 1999), competition with autochthonous microbiota (McCambridge and McMeekin, 1981) and antibiosis (Colwell, 1978). To better understand the survival and fate of indicator and enteric pathogens in aquatic environments, it is difficult to imitate these various dynamic environmental factors in the laboratory conditions. Hence *in situ* experimental studies using membrane diffusion chamber is a promising option to solve this problem to some extent.

7.2 Review of literature

Studies *in vitro* by Anson and Ware (1975) reported that the fate of enteric microorganisms in aquatic environment is a complex function of interacting physical, chemical and biological factors. Important environmental factors contributing to enteric bacterial mortality in natural waters are sunlight and the indigenous microflora. Several researchers have reported the specific role of protozoan grazing as the dominant factor regulating the bacterial population in aquatic environments (McCambridge and McMeekin, 1981; Anderson et al.,
1983; Barcin et al. 1992; Hahn and Hofle, 2001; Duhamel et al., 2006). Sunlight is also reported to be the most important factor for the inactivation of bacteria in aquatic environments (Davies and Evison, 1991; Sinton et al., 1999, 2002; Abhirosh and Hatha, 2005; Mani et al., 2006).

Several studies have reported faecal coliform, total coliform or E. coli die-off or loss rates in aquatic environments, based on laboratory or in situ experiments (Menon et al., 2003). Strategies to evaluate indicator survival in aquatic environments have included in vitro exposure of batch cultures to ambient water, simultaneous in situ release of coliforms with a conservative tracer, or in situ exposure within dialysis bags or diffusion chambers (McFeters and Stuart, 1972; Davenport et al., 1976; Dawe et al., 1978; Enzinger and Cooper1976; Won and Ross, 1973; Beaudeau et al., 2001; Jacquet et al., 2005).

Survival of bacteria of public health significance in seawater has been studied in situ, using diffusion chamber (Vasconcelos and Swartz, 1976) and reported a greater viability of Salmonella enteritidis than E. coli under similar conditions. Effects of sediments on the survival of bacteria in marine waters were studied by Gerba and McLeod (1976) who observed that a greater content of organic matter present in the sediments resulted in a prolonged survival of bacteria. They concluded that predation by protozoa is responsible for the abrupt fall in frequency of the bacterium in natural soil.

Anderson et al. (1983) observed that E. coli was capable of extended survival during in situ exposure to estuarine water, provided eukaryotes were excluded from diffusion chambers. Survival was directly related to temperature in absence of the eukaryote component of the natural microbiota and the decline coincided with increasing eukaryote densities. In situ diffusion chamber studies done by Carrillo et al. (1985) indicated that E. coli could survive, remain physiologically active, and regrow at rates that were dependent on temperature and nutrient levels of the ambient waters. Perez-Rosas and Hazan (1989) studied the survival of V. cholerae and E. coli using diffusion chamber revealed no significant change in the survival
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during the course of experiment and concluded that *E. coli* must be used for assessing public health risk in tropical waters.

Viability of *Salmonella spp.* and indicator microorganisms in sea water using membrane diffusion chambers were studied by Morinigo *et al.* (1990) who observed a large proportion of cells lost their ability to produce colonies on the selective media but retain the capability on a non-selective medium. It is also observed that *Salmonella spp.* exhibited a similar persistence as *E. coli* in the marine environment.

The previous studies cited in the literature review showed that many workers have employed membrane diffusion chambers to study the survival of enteric bacteria in natural water. It is difficult to imitate all the dynamic environmental factors in the laboratory experiments. Hence in this chapter membrane diffusion chamber was used to study the survival of indicator and enteric pathogens in Vembanad lake itself with the following objectives.

7.3 Objectives

1. To study the *in situ* survival of *E. coli*, *S. paratyphi* and *V. parahaemolyticus* in estuarine water using membrane diffusion chamber.

2. To compare the *in situ* survival of *E. coli*, *S. paratyphi* and *V. parahaemolyticus* in estuarine water using membrane diffusion chamber.

7.4 Materials and Methods

7.4.1 Test microorganisms: *E. coli*, *S. paratyphi* and *V. parahaemolyticus* isolated from the Kumarakom estuary were used for this study.
7.4.2 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 harvested 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 $10^8$ colony-forming units per ml. From this final suspension 1 ml was inoculated into 250 ml Erlenmeyer flask with 100ml of the test solution.

7.4.3 Enumeration techniques

Enumeration and the percentage survivors and injured cells at time ‘t’ was calculated as described in chapter 4: 4.4.5. The samples from the test solution were taken and assayed after 1, 2 and 3 days with spread plate technique.

7.4.4 Membrane diffusion chamber

The membrane diffusion chambers were constructed of Plexiglass of 12mm thickness. The lumen in the central spacer was 3 cm in diameter, which accommodated 9-ml sample when the chamber was assembled. One stopper system was fitted into the top of the central spacer to allow filling and withdrawal of samples. The Plexiglass parts of the chambers were sterilized by autoclaving, and the membranes (0.4µm) were irradiated with ultraviolet light. The chambers were assembled aseptically by placing the membranes between the central spacer and the retainers and securing them with stainless-steel bolts and nuts. In this chamber, porous membranes retain a viable suspension of bacteria in a natural or artificial aqueous environment for study or enumeration. The membrane allows the water and solutes in that environment to diffuse readily through the chamber and to interact with the bacterial suspension. A significant advantage of this system over procedures in which bacteria are studied in a limited and unchanging water sample is that a continuous exchange of water and...
Plate - 6: Membrane diffusion chamber: a. lateral view; b. dorsal view.
solutes come in contact with the bacteria under investigation. This characteristic allows the system to be responsive to physical and chemical changes that may occur in the surrounding water.

Individual membrane diffusion chambers loaded separately with 1 ml of washed cells of *E. coli, S. paratyphi, V. parahaemolyticus* were placed in lake, at sites near the vicinity of the Thanneermukkam barrage. To study the effect of biological factors on the test organisms raw estuarine water collected from the lake were introduced into the chamber along with washed test organisms. In another chamber test organisms suspended in sterile estuarine water was used as a control. Heavy string was used to suspend the chambers from an overhead support. This allowed some rotational movement of the chambers within the lake. One ml of sample was removed daily in between 7 -9 a.m with sterile syringe for bacterial enumeration for a period of three days. At the same time, the water samples were collected from the lake to study the physicochemical properties of the lake water. The main physico-chemical characteristics studied were temperature, pH, conductivity, acidity, alkalinity, hardness, chloride and salinity.

### 7.4.5 Determination of Physico-chemical characteristics

Physicochemical factors such as pH, conductivity, Alkalinity, Acidity, chloride, Hardness were studied as per American Public Health Association (APHA, 1998).

### 7.5 Results

In the present study the *in situ* the survival of *E. coli, S. paratyphi* and *V. parahaemolyticus* cells in estuarine water using membrane diffusion chambers was analysed. The mean values of physiochemical parameters of estuarine water during the study periods is given in Table 7.1
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Table 7.1  The mean values of physiochemical parameters of estuarine water during the study period

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values (Mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water temperature (°C)</td>
<td>29°C</td>
</tr>
<tr>
<td>pH</td>
<td>6.5</td>
</tr>
<tr>
<td>Conductivity</td>
<td>1.73 mS</td>
</tr>
<tr>
<td>Acidity</td>
<td>30 mg CaCO₃/l</td>
</tr>
<tr>
<td>Alkalinity</td>
<td>19 mg CaCO₃/l</td>
</tr>
<tr>
<td>Hardness</td>
<td>7600 mg/l</td>
</tr>
<tr>
<td>Salinity</td>
<td>10.1 ppt</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>6.7 mgO₂/l</td>
</tr>
</tbody>
</table>

Percentage of survival and injury of *E. coli*, *S. paratyphi* and *V. parahaemolyticus* under *in situ* condition is presented in Table 7.2. The percentage survival was found to be decreasing as the time progressed. *V. parahaemolyticus* showed better survival followed by *E. coli* and *S. paratyphi*. However, the percentage of injury showed temporal variations for each organism. For *E. coli* the level of injury was constant during the second and third day while *S. paratyphi* exhibited relatively lower injury at the beginning which was found to be increasing as the time progressed. But in the case of *V. parahaemolyticus* the fractions of injured cells were lower on 2<sup>nd</sup> day when compared to 3<sup>rd</sup> day.

Table 7.2. Percentage of survival and injury of *E. coli*, *S. paratyphi* and *V. parahaemolyticus* in raw estuarine water under field conditions (*in situ*)

<table>
<thead>
<tr>
<th>Test microorganisms</th>
<th>Percentage of survival</th>
<th>Percentage of injury</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>6.83</td>
<td>3.1</td>
</tr>
<tr>
<td><em>S. paratyphi</em></td>
<td>22.5</td>
<td>9.1</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em></td>
<td>20.2</td>
<td>8</td>
</tr>
</tbody>
</table>
Relative survival curves of *E. coli*, *S. paratyphi* and *V. parahaemolyticus* in raw and sterile estuarine water under *in situ* condition is shown in Fig. 7.1 and 7.2. The results indicated that the cells suspended in raw estuarine water declined rapidly compared to cells suspended in sterile estuarine water. The experiment started with around $10^8$ cells of the test organisms, which reduced almost 2 logs in 3 days in raw estuarine water and almost 1 log in sterile estuarine water. In sterile estuarine water when the biological factors removed by sterilization the test organisms showed enhanced survival. Hence the chemical composition of the estuarine water was found to be suitable for the growth of the test organisms. Among the bacteria *S. paratyphi* showed slightly enhanced survival capacity followed by *V. parahaemolyticus* and *E. coli* in raw estuarine water and in sterile estuarine water.

**Fig. 7.1 In situ survival curves of *E. coli*, *V. parahaemolyticus* and *S. paratyphi* in raw estuarine water**

![Graph showing survival curves of bacterial species in estuarine water](image-url)
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Fig. 7.2 In situ survival curves of *E. coli*, *V. parahaemolyticus* and *S. paratyphi* in sterile estuarine water

![Survival Curves](image)

7.6 Discussion

Die-off of enteric bacteria in estuarine environments has been broadly attributed to a variety of interacting physical, chemical, and biological factors and processes. Although understanding these factors and their relative importance is justifiable purely on an ecological basis, in practical such studies are necessary to assess the validity and suitability of the fecal coliform group or other microbial indicators which may be used as quantitative measures of fecal pollution in shellfish-growing waters.

In the present investigation experiments have been conducted to evaluate the survival of *E. coli*, *S. paratyphi* and *V. parahaemolyticus* as a function of biotic and chemical factors in estuarine water under *in situ* condition using membrane diffusion chamber. Survival capacity of *S. paratyphi* and *V. parahaemolyticus* in relation to *E. coli* has been worked out in order to see whether *E. coli* can be considered as valid indicator for *Salmonella* and *V. parahaemolyticus* in estuarine waters.

In membrane diffusion chamber the bacteria under investigation come in contact with a continuous exchange of water and solutes and the membrane prevents the entry of native
bacteria into the diffusion chamber. Test microorganisms inoculated into raw estuarine water with all of its biological factors showed a rapid reduction in the number of cells, while they showed an enhanced survival in sterile estuarine water. This indicates that the protozoan and other autochthonous microorganisms present in the raw estuarine water cause the inactivation of test microorganisms. The enhanced survival in sterile water also revealed that the chemical composition of the estuarine water was found to be suitable for the survival of the above microorganisms in estuarine water. Important physical factors such as dilution and mixing of the estuarine water were also allowed to operate by keeping the test microorganisms in the membrane diffusion chamber, which permitted the movement of water.

Several workers have reported the role of protozoan grazing on the removal of enteric bacteria from natural waters. Enzinger and Cooper (1976) reported that the survival of *E. coli* was dependent on the presence of protozoan predators and when the indigenous protozoa were removed by filtration the destruction of the coliforms population was negligible. Similar observation was also made by McCambridge and McMeekin (1981), that predacious protozoa exerted their major influence on *E. coli* destruction in natural water, when the protozoans were inhibited, it had little effect on survival of *E. coli*. Several researchers have reported the specific role of protozoan grazing as the dominant factor regulating the bacterial population in aquatic environments (Barcina *et al.* 1992; Hahn and Hofle, 2001; Duhamel *et al.*, 2006).

Other biological factors involved in the removal of bacteria includes bacteriophage lysis (Ricca and Cooney, 1999), competition with autochthonous microbiota (McCambridge and McKeekin, 1981) and antibiosis (Colwell, 1978). These results are in agreement with the present observation that the destruction of *E. coli*, *V. parahaemolyticus* and *S. paratyphi* was very high in natural estuarine water than in sterile estuarine water. The results are also reconfirmed the previous observation in Cochin estuary that the biological factors such as protozoan predation and bacteriophage exerted a high inactivation of *E. coli* and *Salmonella typhimurium* in estuarine water (Abhirosh and Hatha, 2005).
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 (Rhodes *et al.*, 1988). Gonzalez *et al.* (1990) reported the size selective grazing of bacteria by protozoa. Hahn and Hofle (2001) documented that predation by bacteriovorus protists in aquatic habitat can influence the morphological structure, taxonomic composition and physiological status of bacterial communities. It has been suggested that bacterial prey persists due to the development of predation resistant organisms (Gude, 1979) and the development of avoidance strategies (Boenigk *et al.*, 2002).

In the present studies with laboratory microcosms (chapter 4) also revealed the role of protozoan predators, bacteriophages and competing flora on the survival of test microorganisms. Results of the *in situ* studies using membrane diffusion chambers reiterated the previous findings as well as demonstrated the effect of chemical and physical factors, which were found to be negligible, on survival of the test microorganisms.

### 7.7 Conclusions

*In situ* experiments using membrane diffusion chamber revealed the effect of biological factors especially protozoan and other predators on the removal of the test organisms in aquatic environments. The results also showed that the chemical composition of the estuarine water was suitable for their survival. The comparative survival experiment revealed that the survival of *E. coli* was low compared to *S. paratyphi* and *V. parahaemolyticus* in water. Hence the use of *E. coli* as an indicator for the presence of pathogenic bacteria such as *V. parahaemolyticus* and *Salmonella* in aquatic environments needs to be reconsidered at least in the present study area.