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Influence of a Salt Water Regulator on the Survival Response of *Salmonella Paratyphi* in Vembanadu Lake: India

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1. Introduction

Contamination of environmental water by pathogenic microorganisms and subsequent infections originated from such sources during different contact and non- contact recreational activities are a major public health problem worldwide particularly in developing countries. The main pathogen frequently associated with enteric infection in developing countries are *Salmonella* enterica serovar typhi and paratyphi. Although the natural habitat of *Salmonella* is the gastrointestinal tract of animals, it find its way into natural water through faecal contamination and are frequently identified from various aquatic environments (Baudart *et al*., 2000; Dionisio *et al*., 2000; Martinez -Urtaza *et al*., 2004., Abhirosh *et al*., 2008). Typhoid fever caused by *S. enterica* serotype typhi and paratyphi are a common infectious disease occurring in all the parts of the world with its highest endemicity in certain parts of Asia, Africa, Latin America and in the Indian subcontinent with an estimated incidence of 33 million cases each year with significant morbidity and mortality (Threlfall, 2002). In most cases the disease is transmitted by polluted water (Girard *et al*., 2006) because of the poor hygienic conditions, inadequate clean water supplies and sewage treatment facilities. However in developed countries the disease is mainly associated with food (Bell *et al*., 2002) especially shellfish (Heinitz *et al*., 2000).

Salmonella, since being allochthonous to aquatic environments, the potential health hazard is dependent on their period of survival outside the host and retention of critical density levels in the receiving water in a given time frame during transmission via the water route. In general, the major environmental factors influencing the enteric bacterial survival following their exposure to aquatic environments are water temperature (Anderson *et al*., 1983), adsorption and sedimentation processes (Auer & Niehaus, 1993), sunlight action (Sinton *et al*., 1999), lack of nutrients (Sinclair & Alexander, 1984),

predation by bacteria or protozoa (Hahn & Hofle, 2001), bacteriophage lysis (Ricca & Cooney, 1999), competition with autochthonous microbiota (McCambridge & McKeekin, 1981) and antibiosis (Colwell, 1978).

Although *Salmonella* spp. has been isolated from fresh, estuarine and marine waters, they showed differential survival response to those aquatic environments and the results were sometimes contradictory in relation to salinity. For instance, it has been reported that *Salmonella* showed very low survival in sea water (Lee *et al*., 2010) on the contrary Sugumar & Mariappan (2003) found that they exhibited very long survival up to 16 to 48 week in sea water. But it is also documented that it survived for 54 days (Moore *et al*., 2003) and 58 days in freshwater Sugumar & Mariappan (2003). However, when *Salmonella* suspended in stabilization ponds effluent and rapidly mixed with brackish water, survival time was particularly short, whereas it was prolonged when the bacteria was submitted to a gradual increase in salinity (Mezrioui *et al*., 1995). Therefore the survival of pathogenic bacteria in estuarine environments in response to varying saline concentration due to the mixing of salt water with freshwater has of particular health significance especially in locations where contact and non recreation takes place.

Hence the present study has been carried out in Vembanadu Lake that lies 0.6-2.2 m below mean sea level (MSL) along the west coast of India (9°35'N 76°25'E) and has a permanent connection with the Arabian Sea (Fig.1). As the north-east monsoon recedes, the area is exposed to tidal incursion of saline water from the Arabian Sea. In order to prevent the saline incursion during certain periods of the year, a salt water regulator is constructed in the lake. It divides the lake into a freshwater region on the southern part and a saline lagoon on the northern part. As a result, during the closure and opening of the regulator the water quality on both regions of the regulator may change in terms of its salinity and a progressive saline gradient may occur throughout the lake when the regulator is open. On the other hand over 1.6 million people directly or indirectly depend on it for various purposes such as agriculture, fishing, transportation and recreation. As a result water related diseases are very common in this region particularly in young children but none of them were reported officially. Enteric fever caused by *Salmonella* enterica serovars paratyphi *A*, B and C and Newport have been reported in India (Misra *et al*., 2005; Gupta *et al*., 2009).

Since die-off of enteric bacteria in aquatic environment could be attributed to a variety of interacting physical, chemical and biological factors and processes (Rhoder & Kator, 1988), in our previous studies in the Vembanadu lake we have evaluated the effect of sunlight, chemical composition of the estuarine water (Abhirosh & Hatha, 2005) effect of biological factors such as protozoan predation, predation by bacteriophages, autochthonous bacterial competition (Abhirosh *et al*., 2009) on the survival of *Salmonella* and other organisms. However, the effect of salinity, since being important on the survival of enteric bacteria has not been evaluated in Vembanadu lake. As we already reported the presence of different *Salmonella* serotypes such as *Salmonella paratyphi* A, B, C and *Salmonella Newport* in Vembanadu lake (Abhirosh *et al*., 2008), in this study our aim was to evaluate the health risk associated with *S. paratyphi* when released into the water by studying the survival responses to the salinity changes (saline gradient) caused by the saltwater regulator in Vembanadu lake using microcosm experiments at 20°C and 30°C.

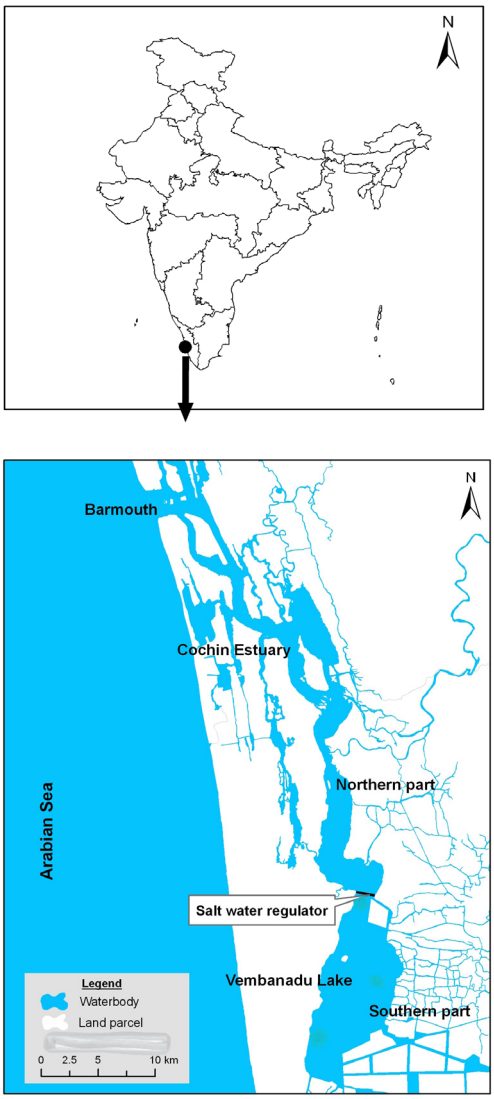


Fig. 1. Map showing Vembanadu Lake

2. Materials and methods

2.1 Test organism and water sample

A pure culture of *S. paratyphi* isolated from the Vembanadu lake was used for the survival experiments. All experiments were conducted in filter sterilized lake water in order to avoid the effect of predation. When saltwater regulator is closed the saline intrusion from northern part is prevented and the water on the southern part becomes freshwater. Therefore, to

imitate the actual condition on the southern part of the lake, experiments were conducted in water collected from the lake when the salinity was 0 ppt (freshwater microcosm). To study the survival of the test organisms during mixing of water from northern and southern part of the Vembanadu lake, experiments were conducted in mixing water samples collected when the regulator was open (mixing water microcosm). Besides, in order to study the survival in all possible saline gradient throughout the year, survival experiments were conducted in lake water with salinity concentration ranged from 0-25 ppt. The test solutions of desired saline concentrations were prepared using fresh lake water with NaCl.

2.2 Preparation of inocula

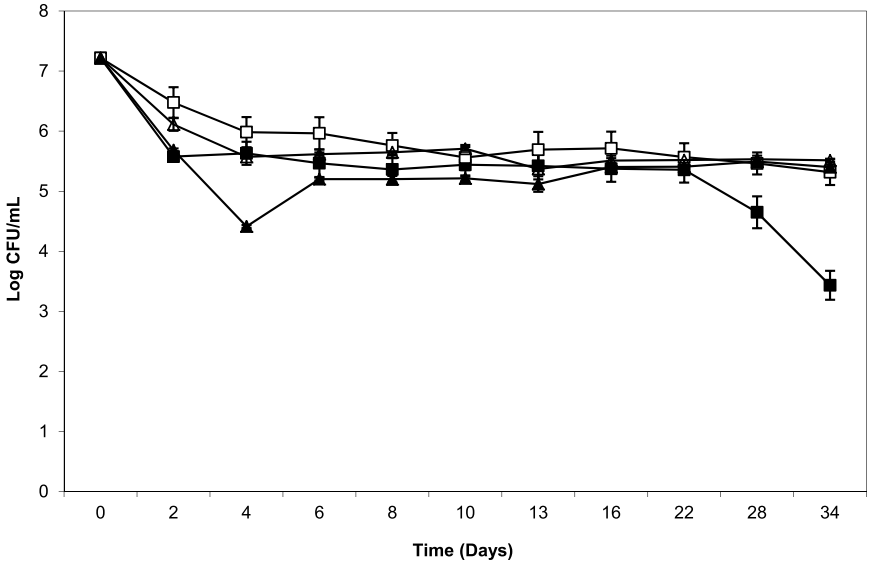
The inocula were prepared as previously described by Abhirosh & Hatha (2005). *S. paratyphi* was grown in Tryptone Soy Broth (TSB) and incubated at 37^{o}C for 24 h. After incubation, the cells were concentrated by centrifugation at 1400 × g for 15 min and washed twice with sterile isotonic saline. After the final wash, the cells were re-suspended in sterile isotonic saline for inoculation into the microcosms. Then 1 ml washed cell suspension of *S. paratyphi* was inoculated into each microcosm containing different test solution (250 ml Erlenmeyer flask with 100 ml) at a concentration of 10^{6-7} CFU/ mL. All inoculated microcosm were incubated at 20^{o}C and 30^{o}C. The microcosms were incubated at 20^{o}C in order to find out the survival at low temperature as the temperature goes down to 20^{o}C in winter as well as at a certain depth. The enumeration of culturable bacteria were done after 2, 4, 6, 8, 10, 13, 16, 22, 28 and 34 days using spread plate technique on TSA agar plates and the colony forming units were counted.

2.3 Decay rate and statistical calculation

The decay rates of culturable *S. paratyphi* cells were calculated as per first order decay model using the following equation $\text{Log } N_t/N_0 = -kt$, where N_t is the number of bacteria at time t, N_0 is number of bacteria at time 0, and t is expressed in days; k is the first-order constant calculated by linear regression technique. T_{99} (time required for 2 log reduction) values were calculated using the decay constant (k) in the following equation, $T_{99} = -2/k$.The difference in the survival at different salinities and temperature was analysed using two way analysis of variance (ANOVA).

3. Results and discussion

The survival curves of *S. paratyphi* in freshwater and mixing water at 20^{o}C and 30^{o}C are given in Fig. 2 and the inactivation rates and T_{99} values are given in Table 1. The results revealed that *S. paratyphi* showed significantly ($p<0.01$) higher survival at 20^{o}C (T_{99}= 25.99) compared to 30^{o}C (T_{99}= 17.68) in freshwater water indicating their better survival capacity at low temperature. However *S. paratyphi* did not show much difference in the survival response in mixing water at both temperature and the T_{99} respectively was 16.37 days at 20^{o}C and 15.12 days at 30^{o}C. The results revealed that *S. paratyphi* cells remained viable until 34 days at a high density of 10^5 CFU/mL. The salinity of the mixing water when it was collected was 12.77 ppt and the average saline concentration of the lake water was 12.5ppt when it was monitored over 2hr interval in a day.



Freshwater 30°C■ and 20°C □; mixing water 30°C ▲and 20°C Δ

Fig. 2. The survival curves of *S. paratyphi* in freshwater and mixing water at 20°C and 30°C

Days	Freshwater 30°C	Freshwater 20°C	Mixing water 30°C	Mixing water 20°C
0	0.00	0.00	0.00	0.00
2	-1.64	-0.74	-2.48	-2.05
4	-1.59	-1.23	-3.75	-2.59
6	-1.75	-1.25	-2.96	-2.54
8	-1.86	-1.46	-2.96	-2.51
10	-1.78	-1.66	-2.95	-2.45
13	-1.80	-1.53	-3.04	-2.79
16	-1.84	-1.50	-2.76	-2.65
22	-1.86	-1.65	-2.75	-2.64
28	-2.57	-1.75	-2.67	-2.63
34	-3.78	-1.90	-2.76	-2.65
K value	-0.11	-0.07	-0.13	-0.12
T99	17.68	25.99	15.20	16.37

Table 1. Inactivation rates of *S. paratyphi* in freshwater and mixing water at 30°C and 20°C

Even though the survival time was longer, in agreement with our results Sugumar & Mariyappan (2003) reported that *Salmonella* survived up to 24 weeks in sterile freshwater microcosm at 30°C but at low temperature it survived for 58 weeks. It is also documented that it survived for 54 days (Moore *et al*., 2003) in freshwater. Since *S. paratyphi* did not show much difference in survival response in mixing water at both temperatures, similar to our results Rhodes and Kator (1988) reported that *Salmonella* populations exhibited significantly less die-off in filtered estuarine water at temperatures of <10°C. In sterile estuarine water virtually unaltered bacterial densities over a 10-day period have also been reported by McCambridge & McMeekin (1980a,b). It has been documented in other studies that low temperature is favorable for the survival of *Salmonella* in (Vasconcelos & Swartz, 1976; Hernroth *et al*., 2010) and other enteric bacteria in aquatic environments (Craig *et al*., 2004; Sampson *et al*., 2006; Silhan *et al*., 2006).

The aim of conducting this survival experiments in freshwater and mixing water was to evaluate the public health risk associated with *S. paratyphi* in Vembanadul lake during the closure and subsequent opening of the regulator. While addressing this issue it has been noticed that similar to other studies *S. paratyphi* could survive very long time in freshwater and mixing water until the end of the experimental period. Therefore the log term survival potential *S. paratyphi* in freshwater may pose health risk since people use this region for their freshwater needs and we have already recorded high abundance of indicator bacteria and enteric pathogens (*Salmonella* serotypes such as *S. paratyphi* A, B, C and S. Newport) on the southern part during the closure of the saltwater regulator (Abhirosh *et al*., 2008). During the closure of the saltwater regulator the water on southern part of the lake become fresh and the natural flow is prevented which results in the accumulation of organic load in the southern part of the lake, giving proper environmental conditions for the multiplication of bacteria. Besides, the high survival capacity noticed at low temperature further increases the health risk during monsoon season because of the drop down of the water temperature to nearly 20°C and we already reported high prevalence of indicator and pathogenic bacteria in southern part of the lake during monsoon season (Abhirosh *et al*., 2008) and every year waterborne disease outbreaks occur during monsoon season. Prolonged survival of *S. paratyphi* in mixing water suggests that it can remain viable in water at high concentration (10^5CFU/ml) when the saltwater is open. It was almost similar to the results we obtained for *S. typhimurium* in Cochin estuary where we found it remained viable at even higher density (10^6 CFU/mL) until the end of experiment (Abhirosh & Hatha . 2005) at 20°C and 30°C. Our results are also in agreement with other studies that better survival of enteric bacteria in estuarine and other aquatic environments (Rhodes and Kator, 1988; Placha *et al*., 2001).

It has been reported that *Salmonella* may be of prolonged public health significance once it is introduced into tropical surface waters than *E. coli* (Jimenez *et al.,* 1989). Sporadic outbreaks of enteric fever due to *S. enterica* serovars paratyphi A, have been reported in India with an annual incidence of 3 million cases (Threlfall,2002; Misra *et al*., 2005). *S. enterica* serovar paratyphi A has emerged as an important cause of enteric fever in India Gupta *et al*. (2009). These reports suggest that the high survival of *S. paratyphi* in Vembanadu lake could be a public health concern.

In order to assess the survival in all possible saline concentrations on both sides of the salt water regulator, survival experiment were conducted in lake water at 5, 10, 15, 20, and

25ppt at 20°C and 30°C and the results are represented in Fig 3-8 and the inactivation rates are given in Table 2 and 3. When the saltwater is closed the saline concentration on Northern part was reported to a maximum of 20ppt. Even though no significant variation in the survival response of *S. paratyphi* was noticed at 0, 5, 10, 15 and 20 ppt ($p>0.05$), they exhibited an extended survival for 34 days at 20°C and 30°C. They showed enhanced survival in water at 0 ppt at both temperatures as evident from T_{99} values and it was 25.99 days at 20°C and 17.68 days at 30°C (Table 2 and 3). However as time goes depending on the increasing saline concentration from 5to 25 ppt it showed gradual decrease in the T_{99} values at both temperatures. The lowest T_{99} was observed at 25 ppt (8.61 and 7.25) and showed a significant ($p<0.0001$) decline of cultural cells at both temperature indicating the deleterious effect of high saline concentration. However the most suitable condition for their growth was found to be at 0 and 5 ppt and suggests that they can survive well at low salinity levels in Vembanadu lake. The results indicate that *Salmonella* can survive well in water weakly diluted or with gradually increasing saline concentrations. In agreement with our results Mezrioui *et al*. (1995) reported that when *Salmonella* suspended in stabilization ponds effluent and rapidly mixed with brackish water survival time was particularly short as we found at 25 ppt where it showed a sudden decline at both temperature, whereas it was prolonged when the bacteria was submitted to a gradual increase in salinity.

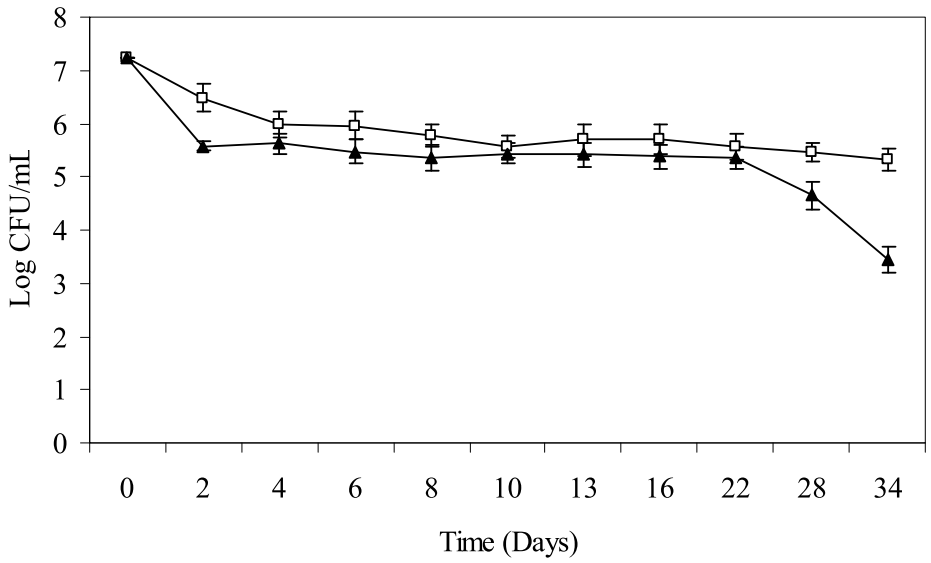


Fig. 3. Survival curves of *S. paratyphi* in fresh sterile water at 0 ppt at 20°C (□)and 30°C (▲) (Mean ±SD, n = 4).

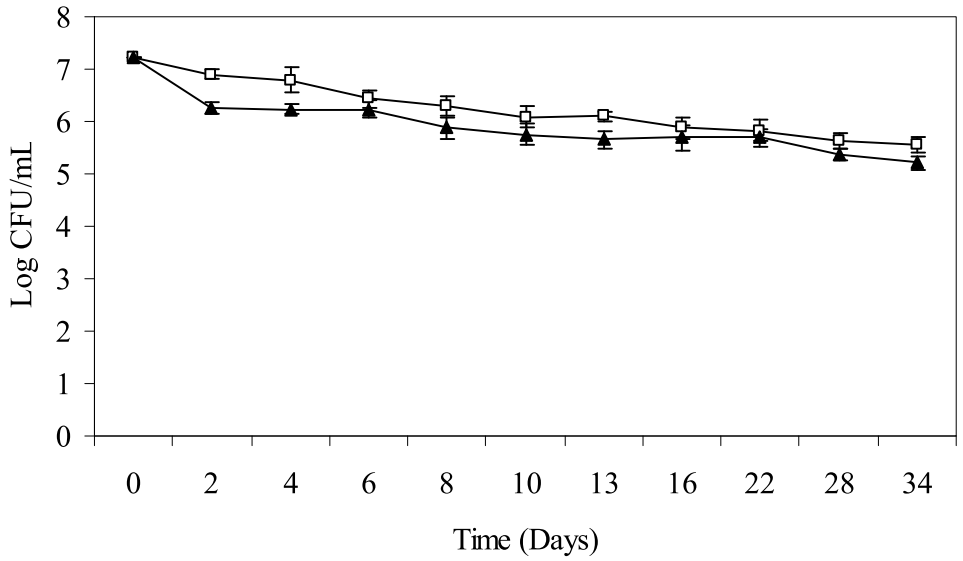


Fig. 4. Survival curves of *S. paratyphi* in sterile water at 5 ppt at 20°C (□)and 30°C (▲) (Mean ±SD, n = 4).

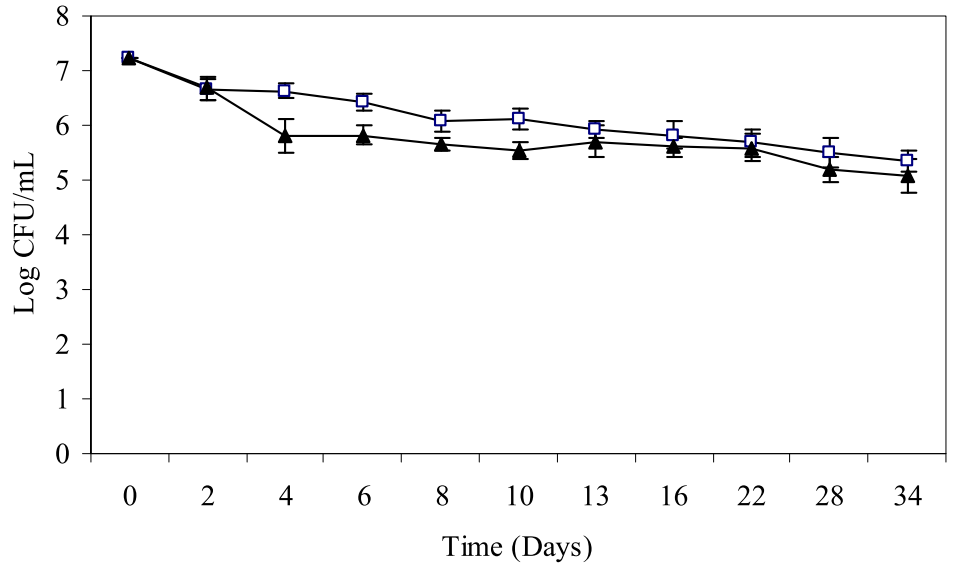


Fig. 5. Survival curves of *S. paratyphi* in sterile water at 10 ppt at 20°C (□)and 30°C (▲) (Mean ±SD, n = 4).

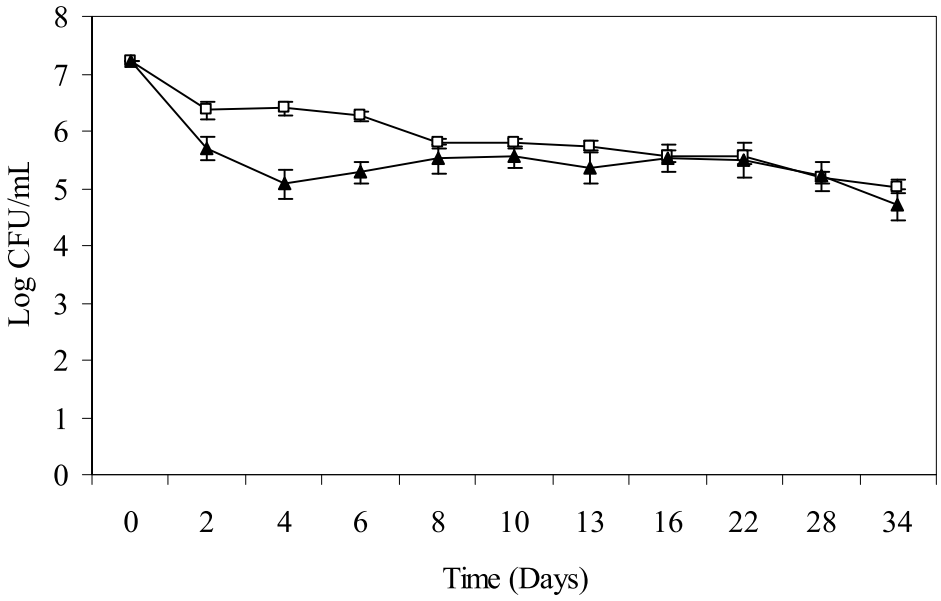


Fig. 6. Survival curves of *S. paratyphi* in sterile water at 15 ppt at 20°C (□)and 30°C (▲) (Mean ±SD, n = 4).

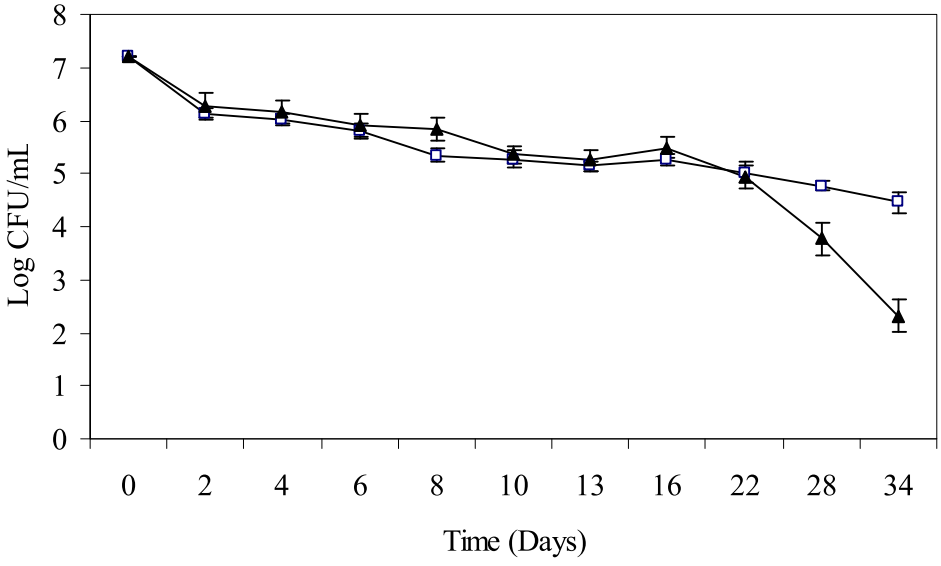


Fig. 7. Survival curves of *S. paratyphi* in sterile water at 20 ppt at 20°C (□)and 30°C (▲) (Mean ±SD, n = 4).

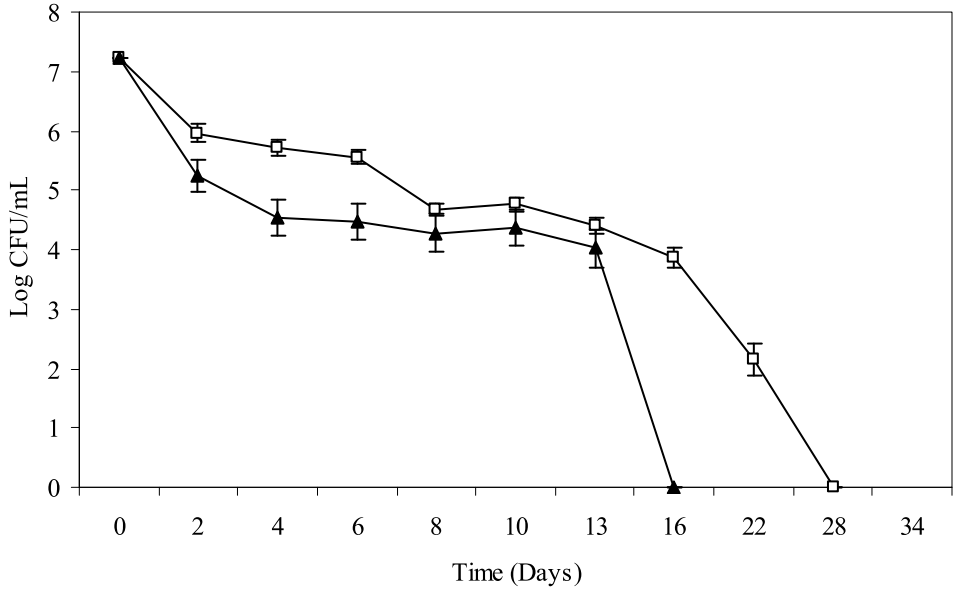


Fig. 8. Survival curves of *S. paratyphi* in sterile water at 25 ppt at at 20°C (□)and 30°C (▲) (Mean ±SD, n = 4).

Days	Saline concentration					
	0 ppt	5 ppt	10ppt	15ppt	20ppt	25 ppt
0	0.00	0.00	0.00	0.00	0.00	0.00
2	-0.74	-0.31	-0.58	-0.85	-1.10	-1.26
4	-1.23	-0.43	-0.59	-0.82	-1.19	-1.49
6	-1.25	-0.78	-0.79	-0.96	-1.40	-1.67
8	-1.46	-0.92	-1.14	-1.44	-1.87	-2.54
10	-1.66	-1.13	-1.10	-1.44	-1.95	-2.46
13	-1.53	-1.12	-1.28	-1.47	-2.07	-2.80
16	-1.50	-1.34	-1.39	-1.65	-1.96	-3.35
22	-1.65	-1.38	-1.53	-1.67	-2.20	-5.07
28	-1.75	-1.59	-1.72	-2.03	-2.44	-7.22
34	-1.90	-1.66	-1.87	-2.19	-2.76	-
k	-0.07	-0.06	-0.06	-0.08	-0.10	-0.23
T99	25.99	31.74	28.85	24.54	19.23	8.61

Table 2. Inactivation rates of *S. paratyphi* in water at different saline concentration at 20°C

	Saline concentration					
Days	0 ppt	5 ppt	10ppt	15ppt	20ppt	25 ppt
0	0.00	0.00	0.00	0.00	0.00	0.00
2	-1.64	-0.97	-0.53	-1.52	-0.94	-1.98
4	-1.59	-0.98	-1.40	-2.14	-1.07	-2.68
6	-1.75	-0.98	-1.39	-1.95	-1.32	-2.74
8	-1.86	-1.35	-1.56	-1.70	-1.37	-2.94
10	-1.78	-1.47	-1.68	-1.67	-1.85	-2.85
13	-1.80	-1.56	-1.51	-1.85	-1.97	-3.20
16	-1.84	-1.53	-1.61	-1.69	-1.74	-7.22
22	-1.86	-1.52	-1.63	-1.72	-2.28	-
28	-2.57	-1.83	-2.02	-2.01	-3.45	-
34	-3.78	-2.00	-2.15	-2.52	-4.90	-
K	-0.11	-0.07	-0.08	-0.09	-0.13	-0.27
T99	17.68	26.14	24.03	21.56	14.95	7.25

Table 3. Inactivation rates of *S. paratyphi* in water at different saline concentration at 30^{o}C

We clearly observed that the decline of cell density with increasing saline concentration. Similar results were reported previously when a freshwater bacteria was exposed to brakish water, Painchaud *et al*. (1987, 1995; Painchaud and Therriault 1989). Similar gradients were reported in other estuaries (Albright, 1983) Rivers (Prieur, 1987). Painchaud *et al*.(1995) reported that no mortality resulted from exposure to water with a salinity of >10ppt and high bacterial count at saline concentration between 0-5. He also reported drastic decline of bacteria at higher salinity (20ppt). This is in agreement with our results that we observed high survival rate at 0 and 5 ppt which was found to be the most suitable condition for the growth whereas at 25 ppt a drastic decline was noticed indicates the deleterious effect of high saline concentration.

At higher saline concentration, for example in sea water, enteric bacteria are subjected to an immediate osmotic upshock, and their ability to overcome this by means of several osmoregulatory systems could largely influence their subsequent survival in the marine environment (Gauthier *et al*., 1987; Davies *et al*., 1995). This osmotic shock might be the reason for the sudden decline of cells at 25ppt. However there are contradicting reportes related to enteric bacterial survival in sea water. Lee *et al* (2010) Gerba and McLeod, (1976) reported that noh halophilic bacterial like *Salmonella* and *E. coli* do not survive well in seas water whereas Sugumar & Mariappan (2003) reported very long survival up to 16 to 48 week in sea water. Upon an osmotic upshift, bacterial cells accumulate or synthesize specific osmoprotectant molecules, in order to equalize osmotic pressure and avoid drastic loss of water from the cytoplasm (Csonka & Epstein, 1996). Although the accumulation or synthesis

of such molecules (trehalose, glycine betaine, glutamic acid) has been reported in *Salmonella* spp. in estuarine waters, in the present study *S. paratyphi* might not overcome the stress caused by the high saline concentration at 25ppt whereas all other saline concentration tested were not found to be lethal.

The maximum saline concentration during the closure of the regulator on the Northern part of the Vembanadu lake is 20 ppt and minimum is 0ppt. Therefore in a year the possible seasonal salinity changes in Vembanadu lake could be between 0-20ppt. It has been generally assumed that when the regulator is closed the bacterial density on the Northern part would be very low because of the increasing saline concentration compared to Southern part. But it has been clearly observed that *S. paratyphi* exhibited high survival capacity in all possible saline gradients from 0 to 20ppt. The result indicates that *S. paratyphi* could survive very long time throughout Vembanadu lake irrespective of the saline concentration. Since the opening and closing of the regulator related to water quality and recreational activities has always been a topic of endless debate, the results indicates that the opening and closing of the salt water regulator does not have any significant impact on the survival (reduction in survival) of the enteric pathogens in relation to saline concentration in Vembanadu lake. However, if the saline concentration reaches 25 ppt it will negatively affect their survival ($p<0.0001$) but the maximum salinity so far reported is 20ppt. Since the lake is being used for various recreational activities the long term survival of *S. paratyphi* in all season regardless of saline concentration in Vembanadu lake could be a public health concern.

4. Conclusions

The results of the microcosm experiment revealed that *S. paratyphi* has a better survival capacity over a wide range of saline concentration from 0 to 20 ppt in Vembanadu lake. It exhibited significantly higher survival at 20^{o}C compared to 30^{o}C. It also showed prolonged survival in all other saline concentration at a higher density at both the temperature and the most suitable saline concentration was found to be 5 ppt. The result indicates that *S. paratyphi* could survive very long time throughout Vembanadu lake irrespective of the saline concentration. The opening and closing of the salt water regulator does not have any significant impact on the survival (reduction in survival) of the enteric pathogens in relation to saline concentration in Vembanadu lake. However, if the saline concentration reaches 25 ppt it will negatively affect their survival but the maximum salinity so far reported is 20ppt. Since the lake is being used for various recreational activities the long term survival of *S. paratyphi* in all season regardless of saline concentration in Vembanadu lake could be a public health concern.

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