Survival of multi-drug resistant enteropathogenic *Escherichia coli* and *Salmonella paratyphi* in Vembanadu lake as a function of saltwater barrier along southwest coast of India

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ABSTRACT

The objective of the study was to evaluate the survival response of multi-drug resistant enteropathogenic *Escherichia coli* and *Salmonella paratyphi* to the salinity fluctuations induced by a saltwater barrier constructed in Vembanadu lake, which separates the lake into a freshwater dominated southern and brackish water dominated northern part. Therefore, microcosms containing freshwater, brackish water and microcosms with different saline concentrations (5, 10, 15, 20, 25 ppt) inoculated with *E. coli/S. paratyphi* were monitored up to 34 days at 20 and 30 °C. *E. coli* and *S. paratyphi* exhibited significantly higher ($p < 0.05$) survival at 20 °C compared to 30 °C in all microcosms. Despite fresh/brackish water, *E. coli* and *S. paratyphi* showed prolonged survival up to 34 days at both temperatures. They also demonstrated better survival potential at all tested saline concentrations except 25 ppt where a significantly higher ($p < 0.0001$) decay was observed. Therefore, enhanced survival exhibited by the multi-drug resistant enteropathogenic *E. coli* and *S. paratyphi* over a wide range of salinity levels suggest that they are able to remain viable for a very long time at higher densities in all seasons of the year in Vembanadu lake irrespective of saline concentrations, and may pose potential public health risks during recreational activities.

Key words | *E. coli*, multi-drug resistance, salinity, *S. paratyphi*, survival, Vembanadu lake

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INTRODUCTION

Contamination of surface water by pathogenic micro-organisms is a major public health issue worldwide, particularly in developing countries where surface water is often used for multiple activities including drinking. Infections originating from such sources, especially diarrhoea and typhoid fever caused by multi-drug resistant pathogenic serotypes of *Escherichia coli*, *Salmonella enterica* typhi and paratyphi are highly endemic to India and remain a major cause of morbidity and mortality among young children in the developing world (Paul *et al.* 1994; Nataro & Kaper 1998; Threlfall 2002; Kahali *et al.* 2004; Misra *et al.* 2005). Although the natural habitat is the intestinal tract of humans and warm blooded animals, *E. coli* and *Salmonella* spp. often find their way into natural waters through various routes, and have been frequently detected from fresh, estuarine and sea waters with significant multi-drug resistance (Martinez-Urtaza *et al.* 2004; Ram *et al.* 2009; Abhirosh *et al.* 2011). However, the potential health risk 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.

A number of survival studies have been conducted in fresh, estuarine and sea waters, however, the results were contradictory, particularly on their survival response in relation to salinity. For instance, very low survival of *E. coli* and *Salmonella* have been reported in sea water (Lee

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et al. 2010); on the contrary, prolonged survival for several days to weeks have been reported by others (Sugumar & Mariappan 2003; Hernroth et al. 2010). It has been documented that they could survive for a very long time in freshwater (Flint 1987; Sugumar & Mariappan 2003) and estuarine water (Rhodes & Kator 1988; Abhirosh & Hatha 2005) whereas a short term survival has also been observed (Winfield & Groisman 2003). Moreover, an extended survival of *E. coli* and *Salmonella* were noticed when they were submitted to a gradual increase in saline concentrations, while the survival was short when they rapidly mixed with brackish water (Mezrioui et al. 1995). Since *E. coli* and *Salmonella* exhibited differential survival response and a wide range of salt tolerance in different environmental water (fresh, estuarine and sea water), the potential public health risks associated with these pathogens cannot be assessed in other aquatic environments based on the earlier survival results. Therefore, it is imperative to evaluate their survival in individual source water in order to assess the potential risk associated with that particular source water where constant human contact and non-contact recreational activities take place.

Hence, the present study has been carried out in Vembanadu lake (~256 km^2, 9°35′N 76°25′E, lies 0.6–2.2 m below mean sea level, and is permanently connected to Arabian Sea) which is being used by over 1.6 million people directly or indirectly for various purposes such as drinking, bathing, cooking, recreation, agriculture, fishing and transportation (Figure 1). As a result, waterborne diseases are very common in this region, especially in young children. The unique feature of the lake is the presence of a saltwater barrier (1,252 m long), which was constructed to prevent incursion of the saline water from the Arabian Sea during certain periods of the year. It separates the lake into a freshwater dominated region on the southern part and a brackish water dominated region on the northern part. As a result, during the closure (December–March) and opening (April–November) of the barrier there would be significant fluctuation in salinity on both regions and a progressive saline gradient may develop throughout the lake which may have considerable effect on the survival of pathogens in the lake.

In our previous studies we have evaluated the effect of sunlight (Abhirosh & Hatha 2005) predation and competition (Abhirosh et al. 2009) on the survival of *E. coli* and *Salmonella* in Vembanadu lake. However, there is a paucity of information pertaining to the survival response of these pathogens in relation to the varying saline concentration in the lake. We already reported the presence of multi-drug resistant serotypes of diarrheagenic *E. coli*, *Salmonella enterica* serotypes such as paratyphi A, B, C and *S. Newport* in Vembanadu lake (Abhirosh et al. 2008, 2011). Therefore, being a potential health hazard to humans exposed to this lake, the period of survival and persistence of these pathogens have greater public health significance. Since the lake has three environmental condition in terms of its salinity (i.e. freshwater on one part, brackish water on the other part and the salinity gradient developed due to the mixing of water from freshwater and brackish water), our aim was to evaluate the survival response of multi-drug resistant enteropathogenic *Escherichia coli* and *Salmonella paratyphi* against these three environmental conditions. Hence, in the present study the survival has been assessed in freshwater, brackish water and at all possible saline gradient that could be developed in the lake using microcosm experiments at 20 and 30 °C. To the best of our knowledge, no published data are available on the effect of salinity on the survival of these drug resistant pathogens in Vembanadu lake.

MATERIALS AND METHODS

Test bacteria

A pure culture of multi-drug resistant enteropathogenic *E. coli* (serotype O114; resistant to amikacin, chloramphenicol, kanamycin, nalidixic acid, oxytetracyline, and tetracycline) *S. paratyphi* (resistant to ampicillin, amikacin, kanamycin, oxytetracycline and tetracycline) isolated from the Vembanadu lake were used for the survival experiments. The isolation of the bacteria and their antibiotic resistance determination is described elsewhere (Abhirosh et al. 2008, 2011).

Microcosms

Freshwater microcosms

In order to study the survival of *E. coli* and *S. paratyphi* in the freshwater part of the lake, sub-surface (0.5 m) water

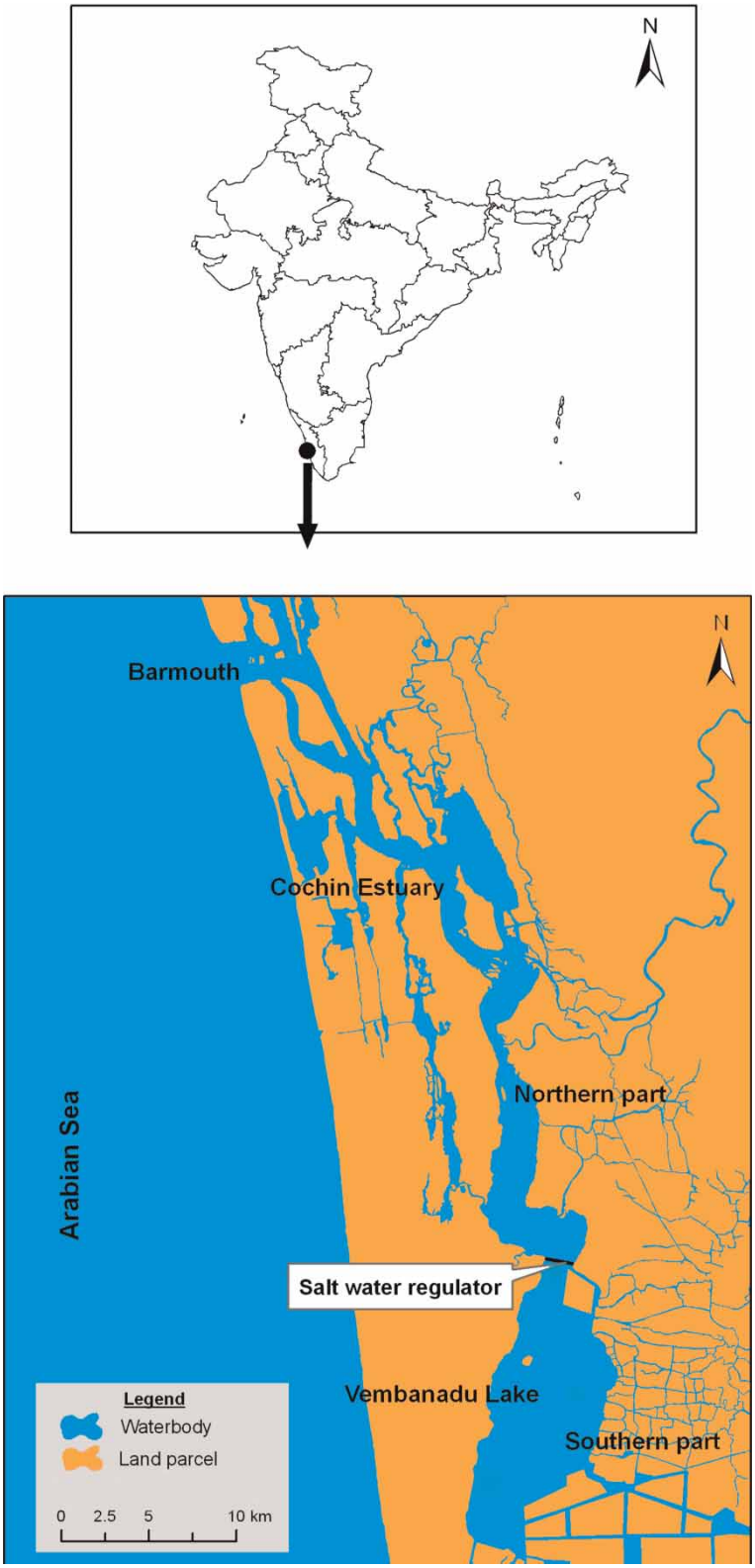

Figure 1 | Map showing Vembanadu lake.

was collected (in 1 L sterile plastic bottles) from the lower southern part when the barrier was closed (in March 2009). It was then filtered (0.2 μm Millipore filter) and 100 mL taken in a 250 mL sterile Erlenmeyer flask was used as microcosms. The salinity was measured with a salinometer (Atago, Japan) and temperature was measured with a mercury thermometer.

Brackish water microcosms

To study the survival of the test organisms during mixing of water from the northern and southern part, sub-surface (0.5 m) water (12.7 ± 0.6 ppt and 30.4 ± 0.5 °C) was collected (1 L in sterile plastic bottles) from the lake when the barrier was open (in May 2009). Then, 100 mL (250 mL Erlenmeyer flask) filter sterilized (0.2 μm Millipore filter) water was used as microcosms. The basic water quality parameters for fresh and brackish water used for the preparation of microcosms are given in Table 1. Nitrate, phosphate, dissolved oxygen (DO), pH were analysed according to APHA (2005).

Microcosms with different saline concentration

Since the maximum salinity reported in the northern part of the lake was around 20 ppt (Abhirosh *et al.* 2008), it was expected that the likely salinity fluctuation in Vembanadu lake would be in the range of 0–20 ppt. Therefore, to assess the survival of *E. coli* and *S. paratyphi* throughout the year in Vembanadu lake at all possible saline gradient that could be developed due to the mixing of water from both parts, microcosms with different saline concentrations (5, 10, 15, 20, 25 ppt) were prepared using fresh lake water collected from the lower southern part of the lake when the lake was closed (in March 2009). The test solutions of desired saline concentrations were prepared using filter sterilized (0.2 μm Millipore filter) fresh lake water with sea salt (Sigma-Aldrich, Bangalore). The saline concentration of 25 ppt was used to find out how it will affect the survival if the salinity increases at any point of time above 20 ppt. Filtration was used to remove the biological factors in order to avoid the effect of predation. Four replicates were used for each microcosm.

Table 1 | Physico-chemical characteristics of fresh and brackish water used for the preparation of microcosms

Parameters	Freshwater	Brackish water
Salinity (ppt)	0.1 ± 0.01	12.7 ± 0.6
Temperature (°C)	30.9 ± 0.7	30.4 ± 0.5
pH	7.12 ± 0.19	7.48 ± 0.12
DO (mg/L)	7.09 ± 0.34	7.54 ± 0.6
Nitrate (μmol/L)	0.05 ± 0.01	0.01 ± 0.009
Phosphate (μmol/L)	0.3 ± 0.4	0.7 ± 0.3

Preparation of inocula

The inocula were prepared as previously described by Abhirosh & Hatha (2005). *E. coli*/*S. paratyphi* was grown in Tryptone Soy Broth (TSB, Hi-Media Mumbai) and incubated at 37 °C for 24 h. After incubation, the cells were concentrated by centrifugation at 1,400 × *g* for 15 minutes and washed twice with sterile distilled water. After the final wash, the cells were re-suspended in sterile distilled water for inoculation into the microcosms. Then 1 mL washed cell suspension of *E. coli*/*S. paratyphi* were inoculated separately into each microcosm containing freshwater/brackish water/water with different salinity (100 mL water in 250 mL Erlenmeyer flask) at a concentration of 10^{6-7} CFU/mL. The enumeration of culturable bacteria was carried out after 2, 4, 6, 8, 10, 13, 16, 22, 28 and 34 days using a spread plate technique on TSA (Hi-Media Mumbai) agar plates and the colony forming units were counted. Ten randomly picked colonies from TSA plates were subjected to biochemical screening in order to confirm the colonies as *E. coli*/*S. paratyphi* as described elsewhere (Abhirosh *et al.* 2008). Dilutions of the samples were carried out whenever necessary using sterile distilled water.

Decay rates and statistical calculations

The decay rates of culturable *E. coli* and *S. paratyphi* cells were calculated as per first order decay model using the following equation $\mathrm{Log}N_t/N_0 = -kt$ (Pommepuy *et al.* 2006), 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 logs reduction) values were calculated using the decay constant (k) in the following equation, $T_{99} = -2/k$ (Pommepuy *et al.* 2006). The difference in the survival at different salinities and temperature was analysed using two-way analysis of variance (ANOVA) using statistical package in Microsoft excel (2003).

RESULTS AND DISCUSSION

The decay curves of *E. coli* and *S. paratyphi* in fresh and brackish water microcosms at 20 and 30 °C are given in Figure 2 and the T_{99} values (in days) are given in Table 2(a). The results revealed that *E. coli* exhibited significantly higher ($p < 0.0001$) survival at 20 °C respectively in freshwater ($T_{99} = 20$) and brackish water ($T_{99} = 22$) compared to 30 °C ($T_{99} = 14$; 16). Similarly, *S. paratyphi* also showed higher ($p < 0.01$) survival at 20 °C ($T_{99} = 25$) compared to 30 °C ($T_{99} = 17$) in freshwater. However, it did not show much difference in brackish water at both temperatures and the T_{99} values were 16 days at 20 °C and 15 days at 30 °C. Comparatively, *E. coli* showed slightly better survival in brackish water whereas *S. paratyphi* survived better in freshwater. This was contrary to the results reported by other researchers who observed enhanced survival of *Salmonella* in brackish water than *E. coli* (Rhodes & Kator 1988; Abhirosh & Hatha 2005). Nevertheless, irrespective of fresh/brackish water, *E. coli* and *S. paratyphi* remained viable for 34 days at higher density indicating their longer persistence and better survival capacity in both waters, especially at lower temperature with no salinity or moderate salinity.

Although the survival time was longer, similar to our results, Flint (1987) reported prolonged survival of *E. coli* in freshwater for up to 260 days at low temperature. Similarly, Czajkowska *et al.* (2005) observed higher survival of *E. coli* O157:H7 up to 50 days in various freshwater microcosms at low temperature ($T_{99} = 4$–11 days), though at higher temperature the survival was relatively lower ($T_{99} = 2$–8 days). Likewise, Sugumar & Mariappan (2003) documented that *Salmonella* survived up to 24 weeks in sterile freshwater microcosm at 30 °C but at low temperature it survived for 58 weeks. Conversely, the long term survival shown by *E. coli* and *S. paratyphi* in brackish water indicate that they are well adapted to water with moderate salinity (12.7 ± 0.6 ppt) and the condition is similar to an estuarine environment where significantly less die-off has been reported at temperatures of <10 °C (Rhodes & Kator 1988) and 20 °C (Abhirosh & Hatha 2005). Virtually unaltered bacterial densities over a 10-day period have also been observed in estuarine water by McCambridge & McMeekin (1980a, b).

The aim of conducting the survival experiments in freshwater and brackish water was to evaluate the potential public health risk associated with *E. coli* and *S. paratyphi* in Vembanadu lake during the closure and subsequent opening of the barrier. While addressing this issue, it has been noticed that *E. coli* and *S. paratyphi* showed extended survival in both waters until the end of the experimental period.

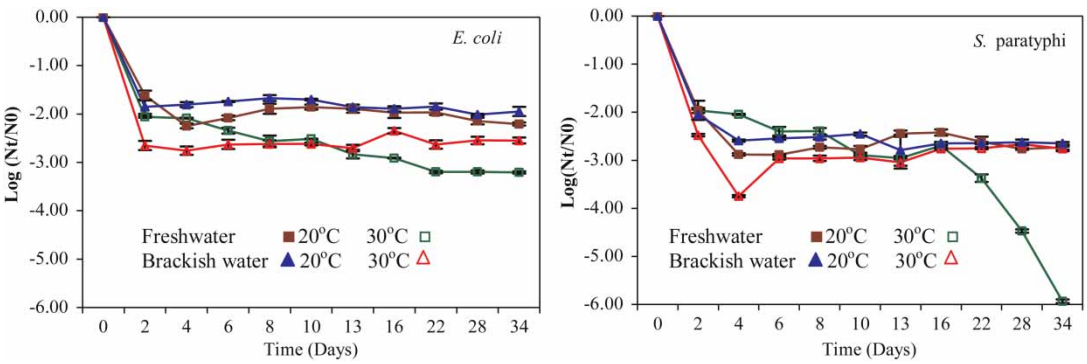

Figure 2 | Decay curves of *E. coli* and *S. paratyphi* in freshwater and brackish water at 20 and 30 °C. Error bars showing standard deviation ($n = 4$).

Table 2 | (a) T_{99} values (in days) of *E. coli* and *S. paratyphi* in fresh and brackish water at 20 and 30 °C. (b) T_{99} values (in days) of *E. coli* and *S. paratyphi* at different saline concentration at 20 and 30 °C

	Freshwater		Brackish water		
	20 °C	30 °C	20 °C	30 °C	
(a)					
E. coli	20	14	22	16	
S. paratyphi	25	17	16	15	
	5 ppt	10 ppt	15 ppt	20 ppt	25 ppt
(b)					
E. coli 20 °C	28	24	18	15	8
E. coli 30 °C	18	16	15	14	7
S. paratyphi 20 °C	31	28	24	19	8
S. paratyphi 30 °C	26	24	21	14	7

In fact, a large population of the area is facing severe drinking water scarcity due to the saline nature of the ground water and the lake being serves as the only source of freshwater. Thus, the extended survival potential particularly exhibited in freshwater could be a public health concern since people use the southern freshwater region for their freshwater needs. The higher abundance of *E. coli* and enteric pathogens (*S. paratyphi* A, B, C and *S. Newport*) recorded from the southern freshwater region (Abhirosh *et al.* 2008) further increase the concern over using this part of water for various purposes. Most significantly, all of them showed multi-drug resistance to more than four antibiotics (Abhirosh *et al.* 2011). In addition, the higher survival noticed at lower temperature augment the health risk during the monsoon season because of the drop down of the water temperature to nearly 20 °C, and also by reducing the salinity with rain water which in turn enhances the survival of the pathogens. These results could be linked to the higher density of indicator and pathogenic bacteria we recorded on the southern part of the lake during the monsoon season, along with high bacterial input through surface runoff (Abhirosh *et al.* 2008), and every year the frequency of waterborne outbreaks are very high during this season.

Prolonged survival of *E. coli* and *S. paratyphi* in brackish water with moderate (12.7 ± 0.6 ppt) salinity suggests that the health risk is not only confined to the closure period but also exists during the opening period because of the gradual mixing of saline water with freshwater, which further reduces the salinity and enhances the bacterial survival. In an experiment, fairly similar to the above conditions in the Vembanadu lake, Mezrioui *et al.* (1995) reported that the survival time of *E. coli* and *Salmonella* were longer when they were exposed to a gradual increase in salinity by mixing brackish water with pond water, similarly less of a decline of *E. coli* cells were reported at low salinities (Faust *et al.* 1975; Bordalo *et al.* 2002). We also reported higher survival of *E. coli* and *S. typhimurium* in estuarine water in the Cochin region of Vembanadu lake (Abhirosh & Hatha 2005).

The decay curves of *E. coli* and *S. paratyphi* at different saline concentrations at 20 and 30 °C are represented in Figures 3 and 4 and the T_{99} values are given in Table 2(b). They showed enhanced survival at all saline concentrations ranging from 5 to 20 ppt. However, a significant ($p < 0.0001$) decay was observed at 25 ppt at both temperatures with the lowest T_{99} values (7–8 days) indicating the deleterious effect of high saline concentration. Similarly, the negative effect of high saline concentrations on the survival of enteric bacteria

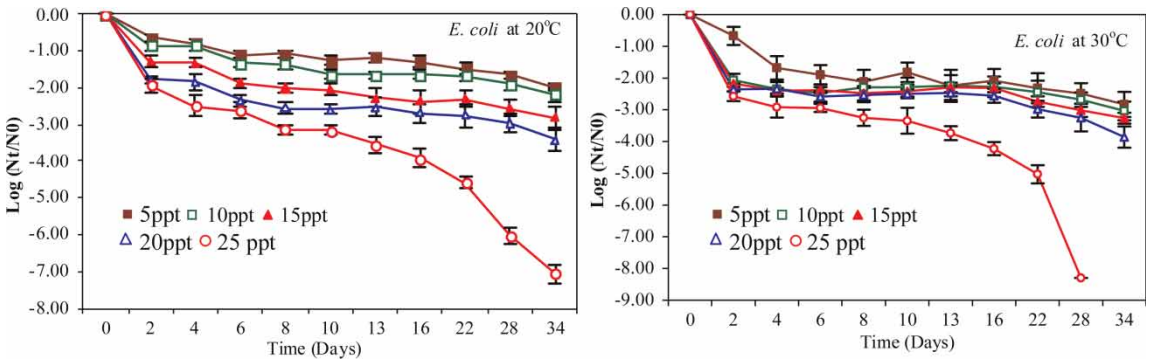

Figure 3 | Decay curves of *E. coli* in different saline concentrations at 20 and 30 °C. Error bars showing standard deviation ($n = 4$).

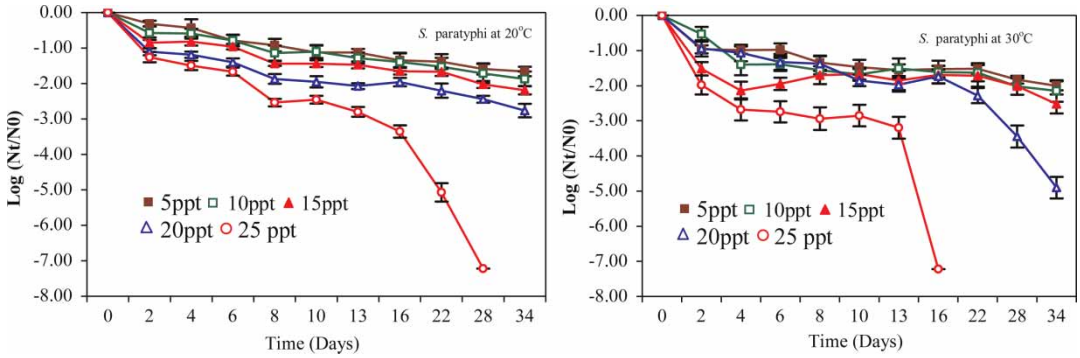

Figure 4 | Decay curves of *S. paratyphi* in different saline concentrations at 20 and 30 °C. Error bars showing standard deviation ($n = 4$).

has been reported earlier (Anderson *et al.* 1979; Troussellier *et al.* 2004). The survival was significantly higher ($p < 0.05$) at 20 °C compared to 30 °C at all saline concentrations.

Prolonged survival observed at all saline concentration (5–20 ppt) indicates that these drug resistant bacteria are able to remain viable for longer periods of time in Vembanadu lake at all possible saline concentration in all seasons throughout the year at higher (10^{-6} CFU/mL) density and may pose a considerable public health threat since their infective dose can be as low as 10–100 CFU (Kuhnert *et al.* 2000; Cobbold *et al.* 2006). Therefore, the presence of multi-drug resistant serotypes of diarrheagenic *E. coli*, *Salmonella* serotypes in Vembanadu lake (*S. paratyphi* A, B, C, *S. Newport*) detected in our previous investigation (Abhirosh *et al.* 2008, 2011) have greater public health significance in view of the current survival results for protecting public health among the local population. Furthermore, considering the frequent detection of multi-drug resistant *E. coli* and *Salmonella* in Indian seafood (Kumar *et al.* 2005; Shabarinath *et al.* 2007), especially from shellfish, outbreaks due to the consumption of contaminated shellfish cannot be ignored because of their filter feeding nature that can concentrate pathogens on their body.

The majority of the previous survival experiments have been performed using reference strains of *E. coli* and *Salmonella* spp., which might have been isolated from different hosts or locations. However, in the present study the bacterial strains isolated from the Vembanadu lake itself have been used, which would give a more meaningful understanding regarding the bacterial behaviour to the salinity changes and related public health risks in Vembanadu lake. It has been reported that the presence of pathogenic *E. coli* and *Salmonella* may be of prolonged public health significance once it is introduced into surface waters, especially the multi-drug resistant strains (Ram *et al.* 2009; Abhirosh *et al.* 2011) and contact with bathing water subject to faecal contamination increases the risk of disease (Fleisher *et al.* 1993; Kay *et al.* 1994). Since enteric fever is highly endemic to India, sporadic outbreaks due to multi-drug resistant *E. coli* (Kahali *et al.* 2004) and *S. enterica* serovars typhi and paratyphi have been reported with an estimated incidence of 3,000,000 cases each year (Threlfall 2002; Misra *et al.* 2005; Gupta *et al.* 2009). In fact, regarding the survival of antibiotic resistant enteric bacteria in the aquatic environment, an enhanced survival of multiple antibiotic resistant *E. coli* has been reported compared to its antibiotic sensitive counterparts indicating the R^+ plasmid mediated survival capacity (McDermott *et al.* 1997). Therefore, the long term survival shown by the multi-drug resistant *E. coli* and *S. paratyphi*, regardless of saline concentration, in Vembanadu lake may pose a severe public health risk since the lake is being used by a large population for various recreational activities including drinking. The results give information on the survival response of these pathogens to the salinity fluctuations in Vembanadu lake and could be used for the proper evaluation and protection of potential public health risks.

CONCLUSIONS

In conclusion, the results of this study demonstrated that multi-drug resistant *E. coli* and *S. paratyphi* exhibited better survival potential and adaptability over a wide range

of saline concentration in Vembanadu lake and suggests that these pathogens can survive and remain viable for a very long time at higher concentrations in all seasons of the year throughout Vembanadu lake, irrespective of the saline concentration. It may pose serious public health risks since the lake is being used for various contact and non-contact recreational activities. The results give information on the behaviour of these pathogens to the salinity changes in Vambanadu lake and could be used for the proper evaluation of potential public health risks.

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