

Survival of *Salmonella* sp. in Freshwater and Seawater Microcosms Under Starvation

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Abstract

Survival of *Salmonella* in sterile seawater and freshwater microcosms without supplemental nutrition was studied at ambient ($30 \pm 2^\circ\text{C}$) and refrigerated temperature ($5 \pm 1^\circ\text{C}$). When inoculated at high initial concentration ($\log 7.5 \text{ CFU ml}^{-1}$), *Salmonella* survived for up to 16 weeks and 24 weeks respectively in seawater and freshwater at ambient temperature. At low temperature, cells could survive for 48 weeks in seawater and 58 weeks in freshwater microcosms. Trials with low initial cell density ($\log 3.1 \text{ CFU ml}^{-1}$) revealed that the die-off rate was faster and a maximum survival of 13 weeks was recorded at 5°C in both the microcosms. Although, the bacteria survived for 13 weeks without supplemental nutrition, metabolic injury was observed as revealed by their failure to grow on selective media. Phenotypic variations were also observed on colonies recovered after prolonged starvation. These results suggested that *Salmonella* could survive well in nutrient-free seawater and freshwater microcosms. Survival is better supported in freshwater than in seawater microcosms and that low temperature favours longer survival. Starvation induced morphological changes can have identification bias and the survival potential of *Salmonella* sp. could pose potential risk of contaminating the aquatic products not only from polluted waters but also in oligotrophic seawater if they gain entry into such water systems.

Introduction

Salmonella is one of the most important causes of food borne infections, mostly transmitted through poultry and dairy products (Tietjan and Fung, 1995; Xiong et al. 1998). Despite the low incidence of *Salmonella* food poisoning caused by fish and fishery products, considering the health hazards posed by *Salmonella*, a zero tolerance has been prescribed for seafood in export trade. The presence of *Salmonella* in seafood is considered as a contaminant and studies have emphasized that *Salmonella* sp. are not normal inhabitants of either freshwater or seawater environments (Liston et al. 1971). Although a great number of mammals, birds and reptiles are responsible for the maintenance of the chains of infection, other animals including

shellfish and frogs can act as vectors by harbouring or concentrating *Salmonella*. This is of particular significance when fish and shellfish are harvested from coastal polluted waters or from waters fed with animal wastes (Iyer, 2002). Compared to other Gram-negative rods, *Salmonella* are relatively resistant to various environmental factors. They grow at temperatures between 8 and 45°C and reported to survive for longer than *E. coli* in sea and freshwater environments, thereby the relationship between the presence of indicator organisms and enteric pathogens has often been questioned (Sayler et al., 1976; Perales and Audicana, 1989; Morinigo et al., 1990). Further, starvation induced phenotypic alteration of salmonellae in seawater was observed by Bakhrouf et al., (1994) and Joux et al., (1997). This report aimed to study the survival pattern of *Salmonella* in seawater and freshwater microcosms under starvation at ambient ($30 \pm 2^\circ\text{C}$) and at refrigerated temperatures ($5 \pm 1^\circ\text{C}$) to understand the potential risk of contamination of aquatic products by *Salmonella*.

Materials and Methods

Salmonella culture and preparation of inoculum

Salmonella sp used in this study was isolated from a frozen fish fillet. The biochemical characteristics of the isolate are provided in Table 1. Culture stock was maintained on Tryptic Soy agar (TSA) slants at 5°C. Stock culture was subcultured on TSA at 37°C on two successive days and then seeded in 10-ml portions of TSB and incubated at 37°C. 18-h old culture was centrifuged at 5000 rpm for 15 min to harvest the cells. The cells were washed twice with phosphate buffered saline (PBS) and resuspended in PBS to get a final concentration of $\text{ca.}10^9$ CFU ml^{-1} . All microbiological media and chemicals were procured from HiMedia Laboratories, Mumbai, India.

Preparation of microcosms

Freshly collected seawater (33.4 ppt) and freshwater were filtered through coarse filter paper and were sterilized by autoclaving after transferring 300 ml portions into Erlenmeyer flasks (500 ml). Microcosms were divided into two sets. One set

Table 1. Characteristics of *Salmonella* isolate used in the study

Test/Substrate	Reaction
TSI reaction	Acid batt / Alk slant
LIA reaction	Alk butt
H ₂ S production	+
Urease test	-
Lysine decarboxylase broth	+
Phenol red dulcitol broth	+
Malonate broth	-
Indole test	-
Methyl red test	+
Voges - Proskauer test	-
Simmons citrate	+
Polyvalent 'H' antisera	+
Polyvalent 'O' antisera	+

was inoculated with washed *Salmonella* cells to obtain a high initial cell density of $\text{ca.}10^7$ CFU ml^{-1} and the other set was inoculated appropriately to get a low cell density of $\text{ca.}10^3$ CFU ml^{-1} . All microcosms were incubated in a static state at two temperatures $30 \pm 2^\circ\text{C}$ (ambient) and $5 \pm 1^\circ\text{C}$ (refrigerated).

Enumeration of survivors

All microcosms (with varying cell numbers at both the temperatures) were sampled thrice during the first week, weekly during the first month and then twice a month in the case of microcosms with high initial cell numbers. Microcosms with low initial cell density were sampled weekly after the first week. Time zero (inoculation time) samples were taken for plate counts both on non selective (Tryptic soy agar) and selective (*Salmonella* - *Shigella* agar and Xylose Lysine Deoxycholate agar) agar plates.

Colony forming units of culturable cells were determined by plating techniques on both selective and non-selective agar in the case of microcosms with low initial cell density and only on selective agar in the case of microcosms with high initial cell density. Counts on both selective and non-selective agar plates were taken to analyze injury to starved cells. Most Probable Number (MPN) technique was used for estimating the survivors when plate counts were under 10 CFU ml^{-1} . MPN determinations were performed by the 3-tube series test with single strength lactose broth. Volumes of 1, 0.1 and 0.01 ml of microcosms were inoculated and the tubes were examined for growth after 24 h of incubation at 37°C , followed by streaking on SSA and XLD agar plates. To confirm their identity, colonies were randomly picked and subjected to urease test, reaction on triple sugar iron agar (TSI) and lysine Iron agar (LIA) tubes. Biochemically typical isolates were serologically confirmed by slide agglutination test using polyvalent 'H' phase 1 and phase 2 antiserum (Murex, UK). All methods were followed according to standard procedures (Speck, 1976; FDA, 1998). All tests including the experimental set up were made in duplicate and the data reported are mean values of the replicates.

Results

The survival pattern of *Salmonella* sp in both fresh and seawater microcosms was slightly different at both the temperatures. Results of survival of *Salmonella* in freshwater and seawater microcosms with high initial densities are shown in figures 1 and 2. As shown in Fig. 1, freshwater microcosms supported viability of cells for up to 24 weeks at ambient temperature. In seawater microcosms the die-off rate was faster and the cells were detectable for only up to 16 weeks. However, at refrigerated temperature (Fig. 2), the reduction in cell numbers was very slow in freshwater, where viable cells of *Salmonella* were recovered for more than one year (58 weeks), while in the seawater microcosm, cell numbers diminished to less than 10

after 48 weeks of incubation. The die-off rate was very slow, almost similar both in freshwater and seawater microcosms initially up to three weeks after which, the rate of reduction in viable cells was faster in seawater than in freshwater microcosms. In both freshwater and seawater microcosms incubated at ambient temperature, there was a sharp decline in cell numbers during the first day and the reduction was about 1 log unit, followed by a period characterized by stable or slow declining densities. The die-off rate was relatively faster in seawater microcosms than those in freshwater microcosms after three weeks.

In the cases of microcosms inoculated with low initial cell density (ca. $3.1 \log \text{CFU ml}^{-1}$) also, the survival pattern was different at different incubation temperatures (Figs. 3 and 4). At ambient temperature, cells could be recovered by plating techniques for only up to six weeks in the case of seawater microcosms. However, *Salmonella* survived at low numbers ($\text{MPN } 2 \text{ ml}^{-10}$) for up to eight weeks as determined by MPN technique (Fig. 3-A). At refrigerated temperature, cells could be recovered by plating for up to seven weeks and viability was observed up to 13 weeks by MPN technique (Fig. 3-B). In freshwater microcosms, as shown in fig. 4, *Salmonella* cells were recovered for up to 13 weeks at both the temperatures. It was interesting to note that the cell numbers increased at ambient temperature by about 2 log units early in the storage period, which started declining after 10 days of incubation and reached below detection limit of after 13 weeks. (Fig. 4-A). No such rise in cell numbers was observed at 5°C (Fig. 4-B) although fluctuations were recorded. However, when incubated at 5°C cell densities decreased more gradually in freshwater than in seawater microcosms. In seawater, the cell numbers decreased to $< 100 \text{ CFU ml}^{-1}$ in two weeks while in

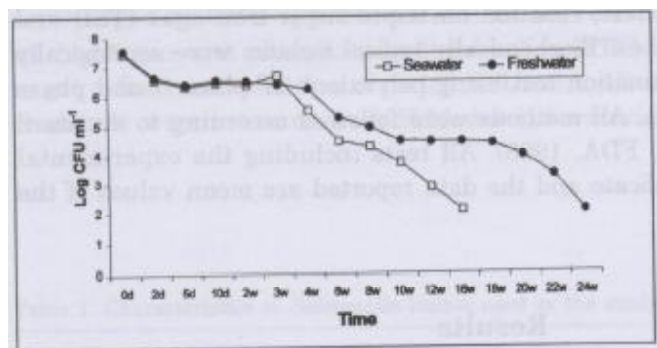


Fig. 1. Survival of *Salmonella* in seawater and freshwater microcosms at ambient temperature ($30 \pm 2^\circ\text{C}$); d = days; w = weeks

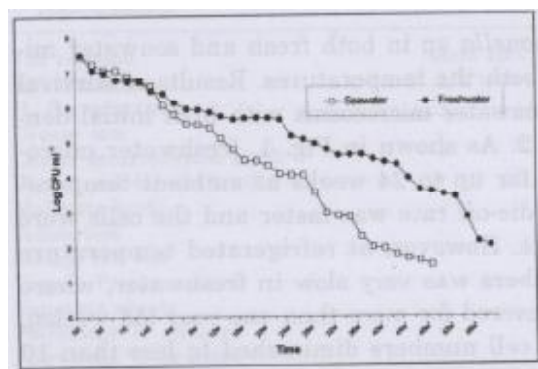


Fig. 2. Survival of *Salmonella* in seawater and freshwater microcosms at $5 \pm 1^\circ\text{C}$; d = days; w = weeks

freshwater the same level was reached after six weeks. However, low-level survival was seen in seawater microcosms also. Although the cells could be recovered, variation in colony morphology was observed upon starvation. Varying morphotypes were seen. Variants appeared as small, rough, dense or more opaque colonies while the normal colonies were smooth and large. Starvation related injury was also evidenced by reduction in cell numbers on selective media than on non-selective media (Figs. 3 and 4). The percentage recovery of *Salmonella* cells on selective media compared to their recovery on non-selective medium upon starvation-survival in both seawater and freshwater microcosms is shown in Table 2. The reduction was more pronounced in *Salmonella* starved in seawater microcosms, where the number was less on selective media than those on TSA. The cells were not

Table 2. Percentage recovery of *Salmonella* cells in selective medium compared to non-selective medium upon starvation in seawater and freshwater microcosms

Time (days)	Seawater		Freshwater	
	5°C	30°C	5°C	30°C
0	98.7	98.7	100	100
1	99.3	98.42	95.69	98.88
3	83.65	97.63	94.96	99.23
10	86.63	99.21	96.57	98.86
14	74.22	97.81	86.85	99.73
21	67.53	93.60	90.85	95.33
28	56.18	89.67	87.15	94.63
35	0	40.14	47.30	98.67
42	—	0	35.41	98.34
49	—	—	0	92.21
56	—	—	—	53.80
63	—	—	—	0

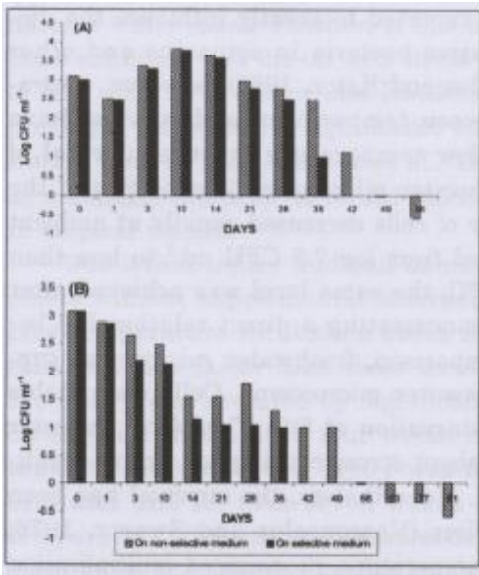


Fig. 3. Survival of *Salmonella* in seawater microcosms at (A) ambient temperature (B) 5°C

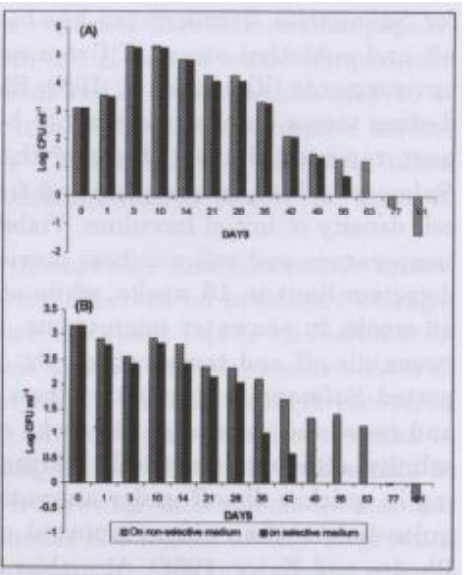


Fig. 4. Survival of *Salmonella* in freshwater microcosms at (A) ambient temperature (B) 5°C

recoverable on both SSA and XLD agar after five weeks upon direct plating. In the case of freshwater microcosms stored at ambient temperature, the cells were recoverable on selective media for up to eight weeks. At low temperature incubation also, the *Salmonella* cells showed variation in number on selective and non-selective media. In seawater microcosms viable cells were recoverable on selective media up to five weeks where as on non-selective media cells were recoverable on direct plating up to seven weeks. In freshwater microcosms, the recovery of cell numbers was very low on selective media especially after four weeks and viable cells were recoverable only up to six weeks compared to over nine weeks on non-selective medium. Appearance of colonies with morphological variations could be related to cell injury with the progression of storage time and was more evident with the reduction in colony numbers on selective media.

Discussion

Die-off of enteric bacteria in aquatic environment could be attributed to a variety of interacting physical, chemical and biological factors and processes (Rhoder and Kator, 1988). Although, all these factors have not been attempted in this study, the results clearly demonstrated a prolonged survival potential of *Salmonella*, not only in freshwater microcosms but also in seawater microcosms. Survival capacity of enteric bacteria in seawater depends on osmoregulatory processes induced by salts, which possibly involve the intracellular accumulation of organic or inorganic components and experimental observations have emphasized the possible adaptation of these bacteria to stress in nutrient-free seawater after previous growth on a salt-medium (Munro et al., 1987; 1989). Although there was reduction in cell numbers, seawater salinity and low temperature incubation were not lethal for *Salmonella*. Temperature has been reported to greatly influence the die-off and sublethal stress of Gram negative bacteria in estuarine and other environments (Rhodes et al; 1983; Rhodes and Kator, 1988), however, contradicting views on the relationship between temperature and survival have been reported. It was observed that low temperature favours survival of *Salmonella* both in seawater and freshwater microcosms irrespective of the cell density of initial inoculum. Viability of cells decreased rapidly at ambient temperature and cell numbers decreased from log 7.5 CFU ml⁻¹ to less than detection limit in 16 weeks, while at 5°C, the same level was achieved after 46 weeks in seawater microcosms, demonstrating a direct relationship between die-off and temperature. On comparison, freshwater microcosms supported *Salmonella* cells better than seawater microcosms. Cells were viable and recovered even after 58 weeks of starvation at 5°C. Therefore, seawater salinity, although is not lethal, brings about stress causing cell injury resulting in quicker die-off under starvation. Nevertheless, the survival has been quite longer than those reported earlier (Vasconcelos and Swartz, 1976; Rhodes and Kator, 1988). At ambient temperature, pronounced multiplication of *Salmonella* cells occurred initially in freshwater microcosms inoculated

with low cell density, where the cell numbers increased considerably. In seawater microcosms with a low-density inoculum, there was a slight decrease in numbers on day one followed by an increase in the subsequent period for about 10 days. It was not ascertained whether this multiplication was due to the utilization of endogenous reserves. However, the considerable increase recorded in freshwater microcosms suggests that the multiplication occurred at the expense of ambient nutrients, especially when the inoculated bacterial numbers were less. This phenomenon was not recorded in freshwater microcosms inoculated with high cell density indicating that the available nutrients could only sustain the viability but not support multiplication. Although the bacterial attrition was evident at both warm and cold temperatures, the process was slow at 5°C, and even slower in freshwater microcosms, than in seawater microcosms. Perceivable difference was recorded in the rate of die off of *Salmonella* cells in both the microcosms with high and low initial inoculum at low temperature incubation. High numbers although have been known to favour better survival in adverse conditions, the factors that permit long term starvation-survival could not be ascertained. Better survival of enteric bacteria at temperatures less than 10°C has already been recorded in estuarine and other environments (Metcalf et al., 1973; Rhodes and Kator, 1988; Placha et al., 2001). Cells of *Salmonella* have been shown to produce enzymes capable of inducing protection against osmotic stress thus enabling the organism to resist the high salinity of oligotrophic seawater (Dupray et al., 1995). Contrarily, elimination of *Salmonella* up to 99% within 24 h in seawater was also documented (Huanca et al., 1996).

Studies by Morinigo et al. (1989) on the viability of *Salmonella* in natural waters revealed that although several biotic and luminous factors were responsible for the inactivation and disappearance of *Salmonella* in seawater and freshwater microcosms under laboratory conditions, physical dilution was the main factor implicated in the disappearance of *Salmonella* from the natural water mass. Further, it has been reported that salmonellae populations exhibited less die-off and stress than did *E. coli* at water temperature of less than 10°C (Kator and Rhodes, 1988) and *S. typhimurium* may be of prolonged public health significance once it is introduced into tropical surface waters than *E. coli* (Jimenez et al., 1989). Therefore, *E. coli* cannot be taken as a reliable indicator of recent faecal contamination or pathogenic bacteria in tropical waters.

Sub-lethal injury and cell damage in seawater and freshwater microcosms without supplemental nutrition were observed on prolonged storage. Low temperature incubation could induce metabolic injury on *Salmonella* cells at a rate faster than those incubated at ambient temperature. At 5°C, the injury was revealed by significant reduction in cell numbers recovered on selective media after four weeks in freshwater and within two weeks in seawater. However, cell injury was not significant for about four weeks in seawater and for over seven weeks in freshwater microcosms at ambient temperature. Growth on non-selective media but loss of ability to produce colonies on the selective media on short term exposure to seawater was recorded by Morinigo et al. (1990), as observed in the present study. Although

metabolic injury was inflicted on cells of *Salmonella* quite early at low temperature incubation, the cells survived at low numbers for longer at low temperature than at ambient temperature in the case of seawater microcosm.

Phenotypic alterations of *Salmonella* sp. as revealed by changes in colony morphology on both selective and non-selective media under starvation-survival conditions in seawater and freshwater microcosms can have serious identification bias. This can have serious implications in public health safety and quality assurance of aquatic food products, besides epidemiological and medical implications. Starvation induced changes in salmonellae have been experimentally demonstrated. Joux et al., (1997) have revealed an important heterogeneity within *S. typhimurium* population based on measurement of substrate responsiveness, cell permeability and DNA content and a progressive physiological cell alteration throughout the starvation process in artificial seawater microcosms. Most *Salmonella* serotypes isolated from healthy carriers, when incubated in seawater lost characteristics that are generally used to identify salmonellae and cells become progressively smaller and filterable on filters of 0.45mm porosity (Bakhrouf et al., 1994). Such phenotypic alteration has also been reported in *Salmonella* sp. upon low temperature storage along with fish mince (Mariappan, 2002). These factors potentialize *Salmonella* to contaminate seafood harvested not only from the contaminated coastal waters but also from the oligotrophic off shore seawater once the cells find entry into such waters.

Based on these observations, it is concluded that *Salmonella* has a good potential to survive in freshwater and seawater microcosms without supplemental nutrition. With such a starvation- survival potential, *Salmonella* poses a great risk of contaminating seafood, which have a zero tolerance for the organism in export trade. This can have serious implications in the quality assurance of aquatic food products and have a bearing on the public health safety. However, it remains to be seen whether salmonellae could survive under such nutrient depleted conditions in competition with other bacteria occurring naturally in seawater and freshwater systems

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