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Survival of *Salmonella typhi* in sea-water

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SUMMARY

The bactericidal activity of natural sea-water on *S. typhi* is due to the combined effect of pH, salinity, toxic ions, presence of competitor and predator marine organisms, unidentified heat-labile toxic substances and competition for the limited food supply. Sea-water subjected to filtration and autoclaving lost the major part of its bactericidal activity. It is concluded that predators and competitors contribute significantly to the rapid death of *S. typhi* in raw sea-water. The addition of peptone decreases the bactericidal activity of sea-water. The fluctuations in the total number of competitors and predators in sea-water together with the fluctuations in the concentration of heavy metal ions may be affected by various factors. Such fluctuations are probably responsible for the variable bactericidal action of different sea-water samples.

Bathing in sewage-polluted sea-water carries with it a health risk in spite of the excessive dilution of pathogenic micro-organisms in the sea and the rapid self-purification process of sea-water. The natural purifying processes are incapable of coping with massive pollution problems and should not be relied on as the sole protection offered to users of sea-water.

INTRODUCTION

The common practice of disposing of untreated sewage and other wastes in the sea is of growing concern nowadays as it may result in the contamination of shellfish areas and bathing beaches with pathogenic agents. These agents may survive sufficiently long in sea-water to be transmitted to man either through the direct use of the water or indirectly through shellfish which have been exposed to it. Oysters and mussels concentrate bacteria suspended in the water so that, unless purified, they offer a risk of infection. The relatively low incidence of disease among swimmers in polluted areas indicates that a process of self-purification, as a result of a combination of physical, chemical and biological factors, takes place in sea-water.

The question thus arises of the fate of the enormous number of enteric bacteria which enter the coastal waters daily by way of sewage outfalls. Extensive studies have been carried out on the survival of *Escherichia coli* in sea-water (Carlucci & Pramer, 1960*a*, *b*; Carlucci, Scarpino & Pramer, 1961), but little work has been done to determine the fate of salmonellas entering bathing areas through sewage

or other means. This present study was conducted to determine the effect of pH, salinity, various chemicals, filtration, autoclaving, organic and inorganic nutrients and other factors on the bactericidal action of sea-water. In view of the prevalence of typhoid fever in the Middle East, *Salmonella typhi* was chosen as the test organism.

MATERIALS AND METHODS

Natural sea-water

Clean sea-water samples were collected, at irregular intervals, in sterile flasks from a sampling point close to the American University of Beirut Beach, about 600 m. from any sewage outfall. Most of the samples were collected in the mornings and all experiments were performed within 1 hr. of their collection. A portion of water from each collection was tested to determine its pH and salinity. The pH was determined electrometrically with a Leeds and Northrup pH meter and salinity was determined by titration with $AgNO_3$ according to the method of Volhard as cited by Vogel (1944).

Artificial sea-water

Sea-water was prepared artificially in distilled water on w/v basis according to the Constan formula for Beirut sea-water as reported by Matossian & Garabedian (1967) and according to the values reported by Tomlinson & MacLeod (1957). Likewise, solutions of single chemicals, at the same concentrations as given in the formula of Constan, were prepared in distilled water; standard analytical reagents were used for all preparations. The solutions were sterilized by autoclaving at 121° C. for 15 min.

The test organism

The test organism that was used throughout this study was a freshly isolated strain of *S. typhi* obtained from the Bacteriology Laboratory of the American University Hospital, Beirut, Lebanon. It was maintained in the lyophylized state at 4° C. until used. Standard inocula were employed routinely throughout this study. They were prepared by suspending an 18–24 hr. old nutrient agar slant culture of the test organism in sterile saline. The cells were collected by centrifugation and washed 3 times. The final cell suspension was adjusted with sterile saline to an optical density of 0·07 (transmittance 0·85) equal to a cell concentration of 10^8/ml. One ml. of this suspension was added to 100 ml. of freshly collected sea-water or other test sample in 200 ml. screw-capped bottles, yielding a final concentration of 10^6 cells/ml.

Index expression of the survival of S. typhi

The inoculated sea-water or other test samples were incubated at room temperature (25° C.) and viable counts were made immediately after inoculation and at daily intervals thereafter. For this purpose duplicate spread plates were prepared from appropriate dilutions of the inoculated sample using SS agar as the plating medium. The percentage survival after 48 hr. incubation was adopted as an index to express the surviving fractions.

RESULTS AND DISCUSSION

Several indices have been used by various investigators to express the survival of bacteria in sea-water. For example, complete survival curves were used by Orlob (1956). Others used the time required to kill 90 % of the bacteria (Vaccaro, Briggs, Carey & Ketchum, 1950), or the percentage survival after a given time (ZoBell, 1936). In our work we adopted the percentage survival after 48 hr. as an index to express the surviving fraction of the test organism and to evaluate the influence of various factors on the survival of *S. typhi* in sea-water.

The bactericidal action of sea-water was established by testing the effect of different concentrations of untreated sea-water on the survival of *S. typhi*. A 25 % concentration favoured the survival of the test organism while higher concentrations reduced it (Table 1). At 25 % concentration the effect of salinity (3·5 %) on survival of organisms is apparently abolished or somewhat reduced. Likewise, the effect of marine bacteria and other factors which normally contribute to the rapid death of the test organism are reduced because of the dilution factor.

It has been observed that bacteria survive to a greater extent in heat-sterilized than in untreated sea-water (Carlucci *et al.* 1961; Vaccaro *et al.* 1950). Our results (Table 2) confirm these findings and show that survival of the test organism in untreated sea-water was much less than in the autoclaved samples. In five of the six experiments the percentage survival was much greater than in untreated sea-water. The decreased survival of *S. typhi* in the filtered sample No. 2 may be explained by the assumption that a beneficial substance or organism was removed by filtration. It is assumed that the favourable effect of filtration and autoclaving on survival resulted from the removal or destruction of predators and competitors. These vary in number and activities from one sample to the other. This partly explains the fluctuation in the percentage survival in the six samples tested. The beneficial effect of autoclaving over filtration may be due to the presence of unidentified heat-labile toxic substances in sea-water which are destroyed by autoclaving and to the destruction of Bdellovibrio predators which are not retained by filters. Autoclaving may also disturb the ionic balance in sea-water, thus rendering these ions less bactericidal. The survival time of *S. typhi* in untreated, filtered and autoclaved sea-water samples ranged from 3 to 6, 4 to 6 and 7 to 12 days respectively.

The survival of *S. typhi* was greater in autoclaved sea-water supplemented with peptone than in untreated sea-water similarly supplemented (Table 3). In the supplemented and untreated samples peptone influenced the native population to a greater extent than the test organism. It appeared that *S. typhi* had difficulty competing successfully with the natural microflora. This indicates that competitors and predators play a primary role in limiting the utilization of peptone by *S. typhi*.

Artificial sea-water prepared according to the Constan formulation (Table 4) is less bactericidal than untreated sea-water, presumably because the former is free from predators and competitors and the latter possesses certain unidentified factors which have bactericidal properties. The bactericidal effect of artificial sea-water prepared according to the data of Tomlinson & MacLeod (1957) was more

Table 1. *Effect of various concentrations of sea-water on the survival of* S. typhi*

Concentration of sea-water (%)	Survival after 48 hr. (%)
0†	24·8
25	34·9
50	16·2
75	10·1
100	6·7

* Number of cells in the suspensions at zero time = 10^6 cells/ml.
† Deionized water.

Table 2. *Survival of* S. typhi *in untreated, filtered and autoclaved sea-water samples**

(Percentage survival after 48 hr.)

	Number of samples					
Treatment	1	2	3	4	5	6
Untreated	0·04	11·40	0·17	1·20	0·16	0·16
Filtered	14·00	1·00	12·00	5·20	1·36	6·40
Autoclaved	13·70	19·20	11·40	11·20	8·80	6·60

* Sea-water samples represent six different collections.

Table 3. *Effect of peptone on the survival of* S. typhi *in untreated and autoclaved sea-water*

	Peptone (p.p.m.)	Percentage survival after (days)			
		1	2	3	4
Untreated	0	19	10	0·012	0·006
	1	27	13	0·022	0·006
	10	30	20	0·002	0·017
	50	46·6	27	0·05	0·02
Autoclaved	0	38	21	6·9	6·3
	1	37·7	23	16	14·6
	10	39	23·5	16·7	15·0
	50	62·6	37·0	18·2	15·3

pronounced when compared with that described by Constan (Table 5). This could possibly be attributed to the additional Br^-, HCO_3^-, Sr^{+++} and H_3BO_3 present in the sea-water constituents described by Tomlinson & MacLeod (Table 4). Inorganic salts are the most potentially toxic substances in the sea. They may adversely influence the survival of bacteria by a general osmotic effect or by specific ion toxicity.

Single salts showed a bactericidal action with great difference in magnitude. Sodium chloride, potassium chloride and calcium sulphate showed a more marked

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Table 4. *Composition of two artificial sea-waters*

Constituent	Constan* (%)	Tomlinson & MacLeod (%)
H_2O	96·38	95·993
NaCl	2·80	2·4
KCl	0·08	0·07
$MgCl_2$	0·37	—
$MgSO_4$	0·23	—
$CaSO_4$	0·14	—
$CaCO_3$	0·013	—
$MgCl_2.6H_2O$	—†	1·1
Na_2SO_4	—	0·4
$NaHCO_3$	—	0·02
KBr	—	0·01
$SrCl_2.6H_2O$	—	0·004
H_3BO_3	—	0·003

* As cited by Matossian. † Not included.

Table 5. *Effect of artificial sea-water and single salts on the survival of* S. typhi

Sample	Survival after 48 hr. (%)
Untreated sea-water	1·5
Artificial sea-water (Constan)	15·0
Artificial sea-water (Tomlinson & MacLeod)	0·7
Single salts	
NaCl 2·8 %	16·5
$MgCl_2$ 0·37 %	44·0
$MgSO_4$ 0·23 %	44·5
KCl 0·08 %	17·2
$CaSO_4$ 0·14%	16·0
$CaCO_3$ 0·013 %	00·0

Table 6. *Effect of pH on the survival of* S. typhi *in sea-water and in a solution of NaCl of equal salinity*

pH	Percentage survival after 48 hr. NaCl	Sea-water
5	25·60	33·50
6	4·95	4·10
7	0·67	3·30
8	0·70	1·79
9	0·00	0·35

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bactericidal effect than magnesium sulphate and magnesium chloride (Table 5) Calcium carbonate solution completely abolished the survival owing to its alkaline pH (9·1). These toxic ions seemingly play an important role as bactericidal agents. The variation in the bactericidal action of natural sea-water is partly due to the fluctuation in the concentrations of toxic ions.

The pH value of sea-water as given by Harvey (1955) ranged between 7·5 and 8·5. In the present study the pH values of the sea-water samples studied were $7{\cdot}9 \pm 0{\cdot}2$ and the salinity was $3{\cdot}5 \pm 0{\cdot}1$ %. Table 6 shows that acidic pH favours the survival of *S. typhi* in contrast to alkaline pH. This suggests that acidic pH limits the survival of marine bacteria more than the test organism. The survival of *S. typhi* was higher in sea-water than in sodium chloride solution of equal salinity and pH. This may be due to the ionic balance in sea-water. A similar protective action of the balance of salts in sea-water was described by Spencer (1957).

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