EFFECT OF CONCENTRATION OF ADDED CHELATES AND SALTS UPON SURVIVAL OF *ESCHERICHIA COLI* AND *SHIGELLA SONNEI* IN LAKE MICHIGAN WATER

L. R. HEDRICK
Illinois Institute of Technology, Chicago

Abstract—Sodium thiosulfate in a concentration of 30 mg/100 ml is a protective agent for survival of populations of *Shigella sonnei* and *Escherichia coli* in samples of toxic Lake Michigan water. EDTA is more or less protective according to the concentrations used.

This report is concerned with the effect of tetra-sodium salt of ethylene diamine tetraacetic acid (EDTA), sodium thiosulfate, iodate and phosphate upon the survival of *Shigella sonnei* and *Escherichia coli* in Lake Michigan water. The study of the effect of inorganic ions upon survival of bacteria in lake water is part of an extensive program that has been in progress for about ten years. As the result of this work, we have evidence that Lake Michigan water, with respect to toxicity toward *E. coli* and its relatives, may be characterized by four types, (1) high toxicity and low stability, (2) lower toxicity but greater stability, (3) incipient toxicity which is activated by heat and (4) no toxicity, even when heated at 100° C for ten minutes. In type (1) water, 50 thousand to 100 thousand test bacteria may be killed within one to two hours.

Noble and Gullans (1955) established that coliform bacteria decreased in numbers when the untreated Lake Michigan water was stored at temperatures ranging from 5° C to 30° C. In all months of the year, there was a significant loss in coliform numbers; the losses were especially large in the summer months. On several occasions there was significant loss within one or two hours. They also established that $Na_2S_2O_3$ furnished partial protection when added to the water samples in the concentration of 19 mg per ml. Shipe and Fields (1956) used the chelating agent, versene, EDTA, to eliminate the effects of heavy metal ions in Tennessee River waters. Johannessen (1957) reported that iodate was responsible for the loss of coliform bacteria in New Zealand sea water.

Materials and Methods

Untreated Lake Michigan water collected directly from the incurrent stream at the Chicago 79th Street water filtration plant was brought to the laboratory and sterilized by filtration through Selas, sintered glass or membrane filters. The filtered lake water was placed in chemically clean pyrex bottles. They were washed at least 10 times in tapwater and three times in distilled water. The bottles were covered with aluminum foil during sterilization for three hours at 165°C in an electric oven. Before use, these aluminum films were replaced with cotton stoppers which had been sterilized in an autoclave. This precaution was exercised to prevent the presence in the bottles of any toxic fumes from

heated cotton. The lake water pH was 7.8 to 8.0 and the pH was adjusted with 0.1 N HCl or 0.1 N NaOH after the addition of any agent. Distilled water was thrice distilled in glass from a weak permanganate lake water solution. The distilled water was buffered with 0.001 M potassium phosphate to give a pH of 7.8.

The *S. sonnei* and *E. coli* cells were cultured for 24 hours in heart infusion broth at 37° C. The cells were centrifuged, washed three times and suspended in 0.02 M potassium phosphate buffer at pH 7.0. Suspensions of these cells were brought to an optical density of 1.0 in sterile buffer and 1 ml of the suspension was inoculated into test bottles containing 99ml sterile lake water. One pair of bottles contained a 1 to 100 dilution of the suspension; another pair contained a 1 to 10,000 dilution of the suspension. These latter bottles had about 60 to 80 viable bacteria per ml when a 1-10 dilution was plated at zero time in heart infusion agar. The zero time count was determined for the test organisms in every experiment. The bottles with the higher concentrations of cells were not sampled except for time studies with highly toxic waters. That is, if we suspected that all the bacteria in the bottles with the 10^{-4} dilution would be killed, we would then determine the number of viable cells in the bottle with the 10^{-2} dilution.

In studies with sodium thiosulfate, the concentrations tested ranged from 5 mg/100 ml to 1000 mg/ml. Thirty mg/100 ml was the minimum effective concentration. Noble and Gullans (1955) used essentially the same concentration in their study which established that sodium thiosulfate acts as a protective agent for coliform organisms in untreated lake water.

Results and Discussion

In a series of ten experiments, sodium thiosulfate afforded an average protective value of 65% when added in a concentration of 30 mg/100 ml to highly toxic lake water—those waters in which there was over a 95% loss of *Shigella sonnei* within 24 hours. The range of protection varied from 40% to 95%.

When the work of Noble and Gullans (1955) was recalculated on the basis of per cent protection afforded coliforms using the MPN (Most Probable Number) method, the average protective value for 215 experiments was 41%. The amount of protection supplied during each month of the year is given in Table 1. These results are the averages for the effect on toxic and non-toxic lake waters while the average cited in the previ-

Table 1.—Protection Afforded Coliform Bacteria by $Na_2S_2O_3$ in Samples of Lake Michigan Water When Stored for 18 Hours at 5° C. Calculated from Noble (1955), J. Bacteriol. 55: 249.

Month	Number of Samples	Protection Afforded %
January	18	30
February	19	40
March	15	34
April	17	29
May	14	10
June	17	10
July	20	80
August	23	40
September	19	58
October	20	60
November	18	58
December	15	54

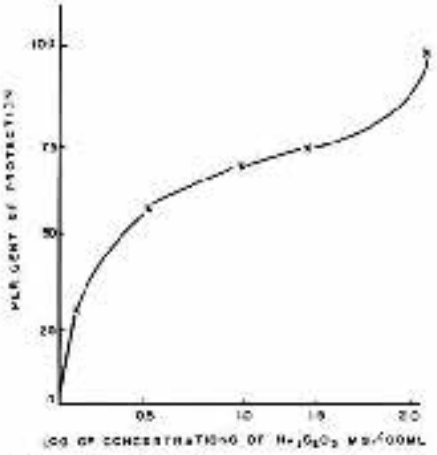

FIGURE 1.—Effect of concentrations of thiosulfate upon protection afforded *Shigella sonnei* when stored in toxic lake water (8-13-57 lake water).

ous paragraphs was for toxic water only in relation to the survival of *S. sonnei*. The separate populations of *E. coli* and *S. sonnei* responded to thiosulfate in essentially the same manner as did the mixed and complex populations of bacteria known as coliforms.

Results of a typical experiment with varying concentrations of sodium thiosulfate are given in Figure 1. In the intermediate range 0.10 mg/ml to 0.30 mg/ml the difference in concentration did not appreciably effect the amount of protection afforded by the salt. However, with higher concentrations the protection afforded by 1.0 mg/ml was nearly 100%.

Shipe and Fields (1956) attributed their protection of coliform organisms in Tennessee River waters to the chelation effect of EDTA upon the toxic heavy metals. In experiments with EDTA in Lake Michigan water the agent was used in concentrations ranging from 10^{-1} to 10^{-8} g/ml. Invariably the 10^{-1} concentration was toxic, but the 10^{-2} concentration was much more protective than were the concentrations of 10^{-3} and 10^{-4}. The 10^{-5} concentration was more protective than the higher concentration of 10^{-5} and 10^{-4} and the lesser concentrations of 10^{-7} and 10^{-8}. Studies with EDTA in triply distilled water gave results similar to those in Lake Michigan water. In these particular experiments both the distilled water and the lake water were highly toxic for the test bacteria without any added agent.

When the residual population numbers are plotted again EDTA concentrations, a bimodal curve is obtained (Fig. 2). Since the distilled water came from chemically clean glass, the concentration of heavy metals in this water is very low. The lake water with a pH of 8.0 would likewise have a low concentration of heavy metal ions. Hence the protective effect of EDTA is due to some

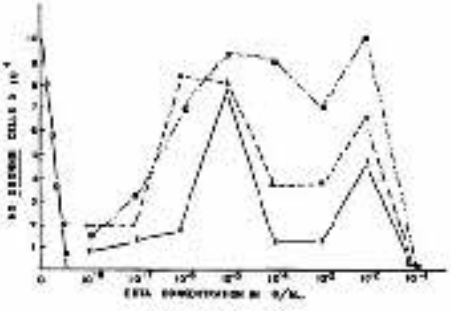

FIGURE 2.—Effect of incubation time and EDTA concentration upon survival of *S. sonnei* in lake water and in distilled water. Symbols: circle, dot and dashed line, 5 hours incubation; triangle and dashed line, 10 hours incubation; X-mark and line, 24 hours incubation; and square and line, distilled water 24 hours

mechanism other than the chelation of heavy metal ions inherent in the water.

What is the explanation for the bimodal curve with differing concentrations of EDTA? The answers are not known, but some tentative hypotheses will be presented.

The 10^{-1} concentration is obviously toxic to the bacterial cells. The protection provided by the 10^{-2} concentration of EDTA, may be explained by assuming that: (a) the agent in some manner acts upon the surface of the cells by binding some inhibitory ions and/or (b) sufficient adsorption of EDTA by cells to block the entrance of the lake water toxic factor. We have no proof for either hypothesis. Why the concentrations of 10^{-3} and 10^{-4} are less protective than either of the 10^{-2} or the 10^{-5} concentrations is not known. The nearly equal protection provided by the 10^{-2} and 10^{-3} concentrations may be attributed to the chelation of different types of toxic substances. Shipe and Fields reported that optimum concentrations of EDTA for chelation of the heavy metals, zinc and copper, were 10^{-3} and 10^{-5} respectively. This chelation of the heavy metals prevented the loss of the bacteria in the Tennessee River waters containing these toxic heavy metal ions.

In an experiment with the survival of *S. sonnei* in lake water, the concentration 10^{-3} was as protective as 10^{-5} for the first 12 hours. For longer periods of time it was much less effective (Fig. 3). The absence of protection in both distilled and lake water for the weaker concentrations 10^{-7} and 10^{-8} is obviously due to the low concentration of EDTA.

As indicated in Table 2, there was protection against the toxic factor

FIGURE 3.—Effect of EDTA concentration upon survival of *S. sonnei*.

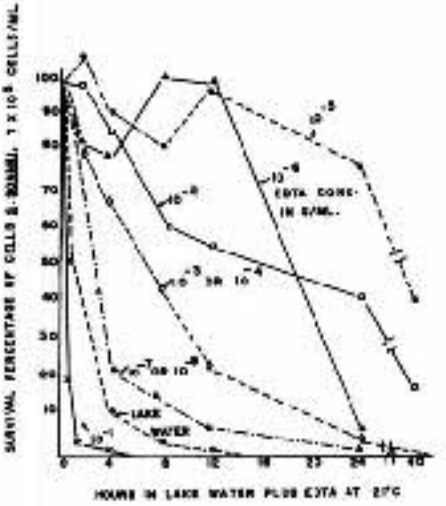

TABLE 2.—Protective Effect of Iodate Upon Toxic Factor in Lake Michigan Water.

Suspending Medium	Number of *Shigella sonnei* x 10^3 per/ml of Lakewater or Lakewater Plus Iodate.	
	Zero Time	After 24 hr Storage at 21°C.
Lake Water (10-15-57)	85, 100	0, 0
Lake Water Plus Iodate 0.01 ug/ml	106, 88	60, 70
0.02 ug/ml	150, 73	52, 42
0.03 ug/ml	92, 100	30, 24
0.04 ug/ml	128, 85	43, 23

in Lake Michigan water when the iodate concentration was as low as 0.01 ug/ml. The results given in this table are the averages of four carefully controlled experiments. However, Johannessen (1957) reported that iodate in the concentrations of .02 ug to .04 ug/ml was toxic to coliform in sea water. The attempts to detect any iodate in Lake Michigan water using the sulfamic acid titration procedure employed by Johannessen were negative.

Allen, Pasley and Pierce (1952) reported that 0.003 M phosphate was the most desirable concentration for the storage of *E. coli* and related organisms. Straka and Stokes (1957) indicated that phosphate buffer used as diluent in dilution bottles permitted the loss in some instances of as much as 20% to 30% of the bacteria within 20 minutes and as much as 80% loss within an hour.

In the lake water studies it was desirable to compare the survival of numbers of bacteria in well water with those in lake water. The pH of the well water was adjusted to that of the lake water with 0.003 M phosphate. The effect of the same concentration of phosphate in lake water was determined on several different sampling days. In general, the results in lake water for *S. sonnei* and *E. coli* were similar. In tests with phosphate (0.001 M and 0.005 M) in lake water afforded some protection; there was about a 50% loss in 24 hours. In the weaker phosphate concentration, less than 0.001 M, there was a 66% loss. However, this is probably not a significant difference. In the studies with triply distilled water from glass, the survival for *S. sonnei* with or without 0.001 M phosphate were essentially the same, that is, about a 50% loss within 24 hours. In both of these conditions there was more loss than with the parent lake water from which the distilled water was prepared. *Escherichia coli* appears to be more sensitive to distilled water than does *S. sonnei*, and phosphate is protective for *E. coli* in distilled water. These data are given in Table 3. They are at variance with the work reported by Straka and Stokes with the effect of phosphate in dilution solutions. In experiments with lake water, the water was sterilized by filtration after the addition of the phosphate. In the Straka and Stokes studies, the phosphate was added to the dilnent prior to sterilization in an autoclave. Finkelstein and Lankford (1957) have indicated that phosphate at pH 8.0 induces toxicity upon being autoclaved, especially in the presence of sugars.

Summary

In the studies of the survival of populations of *Shigella sonnei* and *Escherichia coli* in Lake Michigan water, it was determined that sodium thiosulfate in a concentration of 30 mg/100 ml was a protective agent in samples of toxic lake water. In nontoxic lake water, it produced no effect. Iodate in the concentrations used was not toxic to these test bacteria nor could iodate be detected in Lake Michigan water.

The chelating agent EDTA, was highly protective at concentrations of 10^{-3}, 10^{-5}, and 10^{-6} g/ml, but it was less protective at concentrations of 10^{-2}, 10^{-4}, 10^{-7} and 10^{-8}. At the 10^{-1} concentration, EDTA was highly

TABLE 3.—Influence of Phosphate Upon Survival of *E. coli* and *S. sonnei* in Lake Water and Triply Distilled Water. Temperature of Storage 21° C; pH of Lake Water and Distilled Water 7.8. Multiply Colony Counts by 10^4.

	Lake Water		Lake Water plus Phosphate						Lake Water		Triply Distilled Water		Triply Distilled Water Plus 0.001 M. PO_4	
			0.001 M PO_4		0.0035 M PO_4		0.0001 M PO_4							
	Time 0	Hr 24	Time 0	Hr 24	Time 0	Hr 24	Time 0	Hr 24	Time 0	Hr 24	Time 0	Hr 24	Time 0	Hr 24
E. coli														
Avg. No. of Colonies	64	39	71	67	76	29	64	16	52	37	62	2	50	67
No. of Tests	40		36		16		16		14		14		14	
S. sonnei														
Avg. No. of Colonies	59	40	70	91	68	32	71	24	58	42	59	20	56	33
No. of Tests	46		36		18		16		14		14		12	

toxic to the bacteria. Since the results were obtained in both triply distilled and lake water, its action is other than the chelation of the heavy metals inherent in the waters. Potassium phosphate, 0.001 M, is somewhat protective to *S. sonnei* and *E. coli* in lake water and is protective to *E. coli* in distilled water. Phosphate in 0.005 M and 0.0001 M concentrations is neither protective nor especially toxic.

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Manuscript accepted March 2, 1962.