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COLIPHAGE INACTIVATION IN SEAWATER

PROCES PRESTANKA AKTIVNOSTI KOLIFAGA U MORSKOJ VODI

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INTRODUCTION

Pathogenic bacteria and viruses can be carried by sewage into the sea. The large volumes of sewage, which increase every year, may overload the seawater self-purification capacity. It is, therefore, important to study the inactivation of non-marine microorganisms which enter the marine environment.

Today some information is available on the physical, chemical and biological factors (Magnusson, Hedstrom and Lycke, 1966; Matossian and Garabedian, 1967, Mitchell, 1968) involved in the die-away of enteric bacteria in marine environment. The fate of enteric viruses is much less known.

Previous studies done in our laboratory (Shuval, Thompson, Fattal, Cymbalista and Winer, 1971) have shown that there is a definite biological marine anti-viral activity (MAVA) which in seven days causes a three to six log reduction of polio-virus type I seeded into seawater, while in heat - treated seawater, used as control, only a one log reduction was detected. The MAVA activity was removed from seawater by filtration through a membrane filter with a 0.45 μm pores ize. These agents were also ether sensitive.

The present report is a further study on the factors involved in coliphage inactivation in seawater. Coliphage T_2 was chosen as a model because working with bacteriophages instead of enteroviruses has the advantage of easy performance of the titration and the short time needed to gain results.

METHODS AND MATERIALS

For the assay of bacteriophage, samples were diluted in nutrient broth. One ml of each dilution was mixed with 1 ml of 1 percent liquified agar at 52°C to which 0.1 ml *E. coli* culture was added in the logarithmic growth phase.

After stirring, the mixture was poured on Petri dishes containing agar. Plaques were counted after 18 hours incubation at 37°C.

To isolate and obtain counts of bacteria from seawater, seawater samples were taken from the Palmachim Beach 30 to 40 m from shore and 80 cm depth. No known source of pollution exists near the sampling point. This location can be considered as characteristic for an unpolluted area of the Eastern Mediterranean. The samples were inoculated on ZoBell medium 2216 and incubated at 20°C. The formula of the medium was as follows: 0.5 g Bacto peptone, 1.0 g Bacto yeast extract, 0.2 g sodium thiosulfate, 1000 ml seawater (pH of the mixture after autoclaving, 7.4). Descrete colonies which grew on the media were picked and subcultured on agar slopes of ZoBell media. All pure cultures were stored at 4°C. Counts of marine bacteria were done by diluting the sample in sterile seawater. A dilution of 0.1 ml of the original sample was spread with a bent glass rod on ZoBell agar. The plates were incubated at 20°C.

For assays for MAVA in seawater, Erlenmyer flasks of 250 ml, containing 50 ml seawater, were inoculated with 2.5×10^8 coliphage particles suspended in 1 ml phosphate buffer pH 7.5 (final bacteriophage concentration 5×10^6 particles/ml). The inoculated seawater was incubated at 20°C. Counts of phage and of marine population were made immediately after inoculation (0 time) and afterwards in intervals of 24—48 hours.

Seawater was sterilized by filtration through a Sartorius membrane filter of 0.45 μm pore size. The bacteria were washed by centrifugation. A bacterial suspension in sterile seawater was centrifugated 10,000 rpm for 10 sec. The sediment was re-suspended in sterile seawater and centrifugated again. This cycle was repeated five times.

RESULTS

Studies of the anticoliphage activity of seawater were carried out under laboratory conditions. An inactivation curve of coliphage in fresh seawater samples is shown graphically in Figure 1. The inactivation process involves two stages. A lag phase lasting 4 to 8 days after which a decrease in the titer occurs.

In experiments in which marine bacteria were suspended in sterile seawater in concentrations of 10^7 bacteria/ml, the decrease in phage titer took place without any lag phase (Figures 3, 4, 5 and 6).

The initial number of marine bacteria in seawater ranged from 10^4 to 10^5 bacteria/ml (Figure 1, bottom). This number decreased during the assay period in 1—2 logs.

To study the effect of added organic matter on the self-purification processes in seawater, assays for anticoliphage activity were performed in seawater containing different amounts of nutrient broth. Figure 2 shows that addition of organic matter to seawater inhibits its anticoliphage activity. Nutrient broth less than 0,01 percent did not inhibit the anticoliphage activity of normal seawater. Figure 3 shows graphically the effect of nutrient broth on the anticoliphage activity of a mixture of serially washed marine microorganisms added to sterile seawater in the concentration of 10^7 bacte-

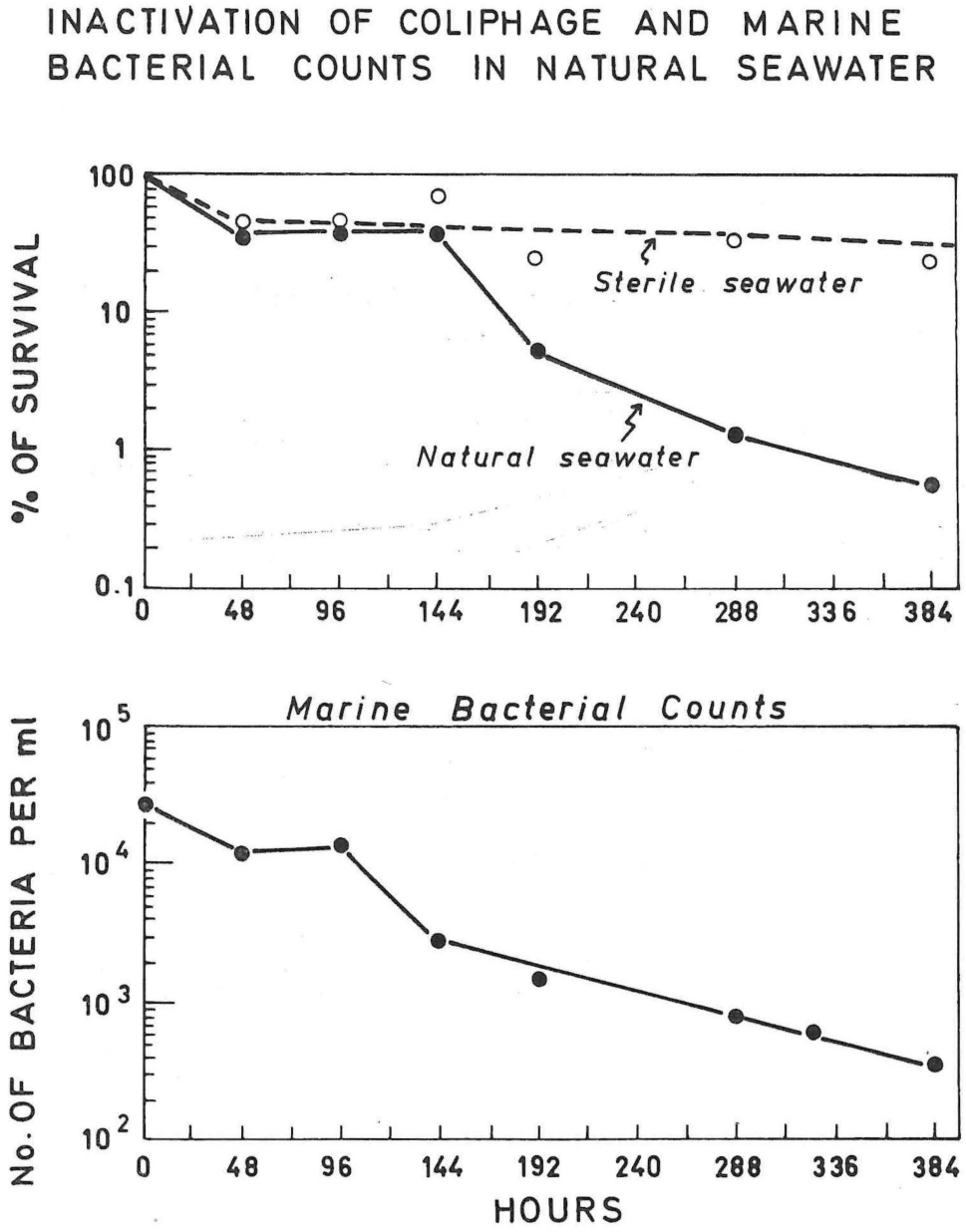

Fig. 1 — Inactivation of coliphage and marine bacterial counts in natural seawater.

ria/ml. Addition of 0.3 percent nutrient broth powder inhibited the anticoliphage activity of the microorganisms.

To determine anticoliphage activity of isolated marine microorganisms, five different pure cultures of marine bacteria in the stationary growth phase were washed and suspended in sterile seawater in the concentration of 10^7 bacteria/ml. The anticoliphage activity of these isolates is depicted in Figure 4. Three of these isolates showed anticoliphage activity. The isolates having anticoliphage activity differed widely in the morphological characteristics of their colonies. All of them were Gram negative. One isolate was a vibrio and the two others were rods of different sizes.

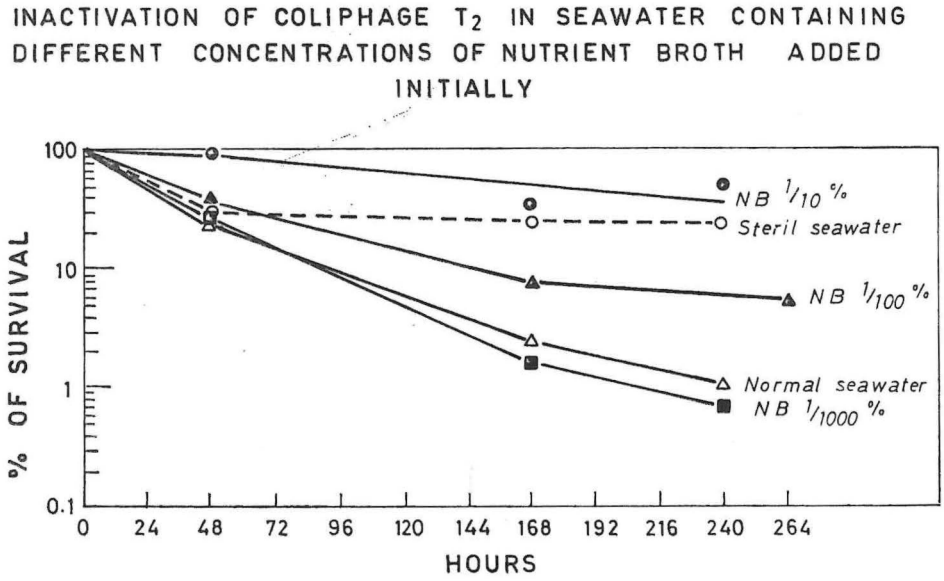

Fig. 2 — Inactivation of coliphage T_2 in seawater containing different concentrations of nutrient broth added initially.

The positive cultures isolated in this study showed reduced activity when assayed again after several transfers on ZoBell medium and stored at 5°C for three months. Figure 5 shows the inactivation activity of the same bacteria (isolate IV) suspended, in the first case, in sterile seawater immediately after isolation and in the second case after several transfers on ZoBell medium and stimulated growth of the bacterium.

To examine some factors in seawater that might reduce the rate of coliphage inactivation by marine bacteria, 0.3 percent nutrient broth powder was added to a suspension of marine bacteria (isolate IV) in concentration of 7×10^6 bacteria/ml in sterile seawater. This suspension was assayed for anticoliphage activity. The same isolate, suspended in sterile seawater without nutrient broth, was used as a control. Figure 6 shows that addition of nutrient broth inhibited the antiphage activity of the marine bacteria, although it stimulated growth of the bacterieum.

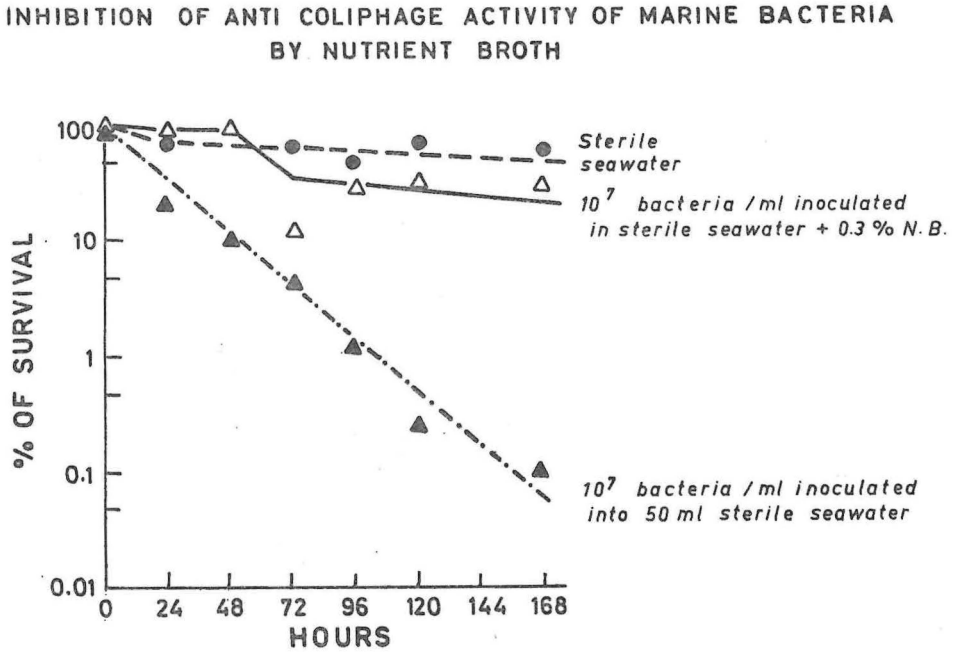

Fig. 3 — Inhibition of anticoliphage activity of marine bacteria by nutrient broth.

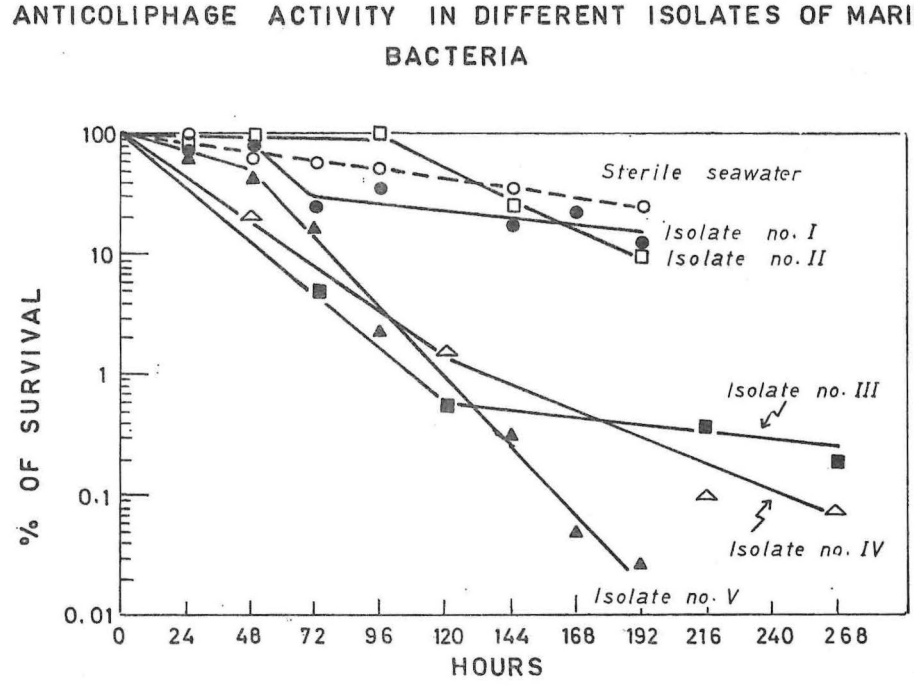

Fig. 4 — Anticoliphage activity of different isolates of marine bacteria.

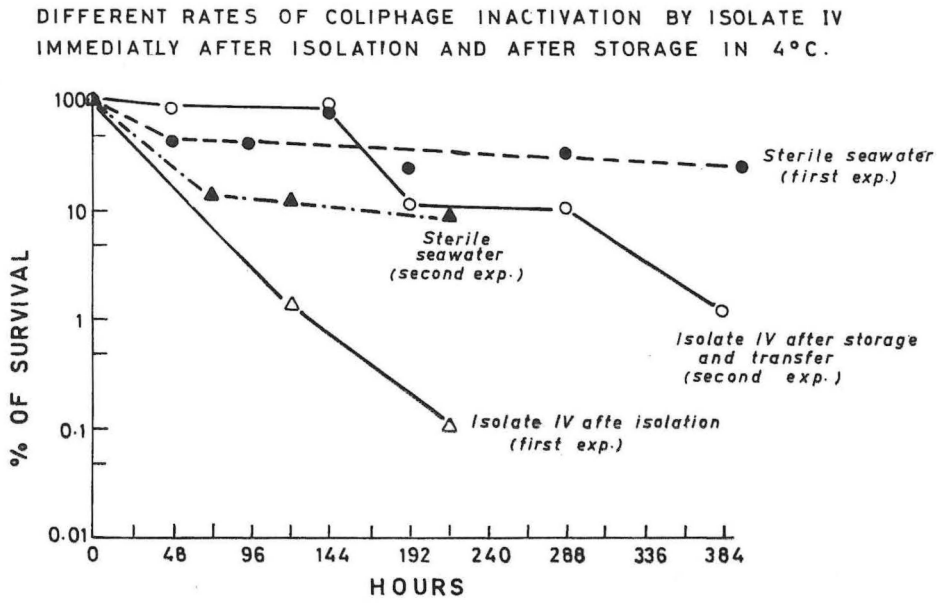

Fig. 5 — Different rates of coliphage inactivation by isolate IV immediately after isolation and after storage in 4°C.

Bacterial counts in seawater to which coliphage were added showed that the coliphage inactivation was associated with a decrease in the marine bacterial population (see Figure 1) and it usually occurred after a lag period. In order to study the effect of this phenomena on coliphage inactivation in seawater, the following experiments were performed. Several Erlenmyer flasks containing seawater were incubated at 20°C in the dark. Each flask was inoculated with coliphage at a different time interval after incubation had begun. The rates of coliphage inactivation in the pre-incubated seawater are summarized in Figure 7. The inactivation curve of the coliphage, inoculated in seawater pre-incubated for 360 hours, lacks the lag period which characterized the inactivation in fresh seawater. In that suspension the bacterial population was already in the decline phase at the time the phage was introduced.

DISCUSSION

In this report some factors involved in the self-purifying capacity of vitro seawater were studied. The coliphage inactivation model was chosen because T bacteriophages, like enteroviruses, are found in domestic sewage and are discharged into seawater.

Mitchell and Jannasch (1969) noted that the antiviral activity of natural seawater was stronger than that of seawater polluted with organic matter. Lycke, Magnusson and Lund (1956) described a heat sen-

THE EFFECT OF NUTRIENT BROTH ON THE COLIPHAGE INACTIVATION CAPACITY OF AN ACTIVE MARINE BACTERIA

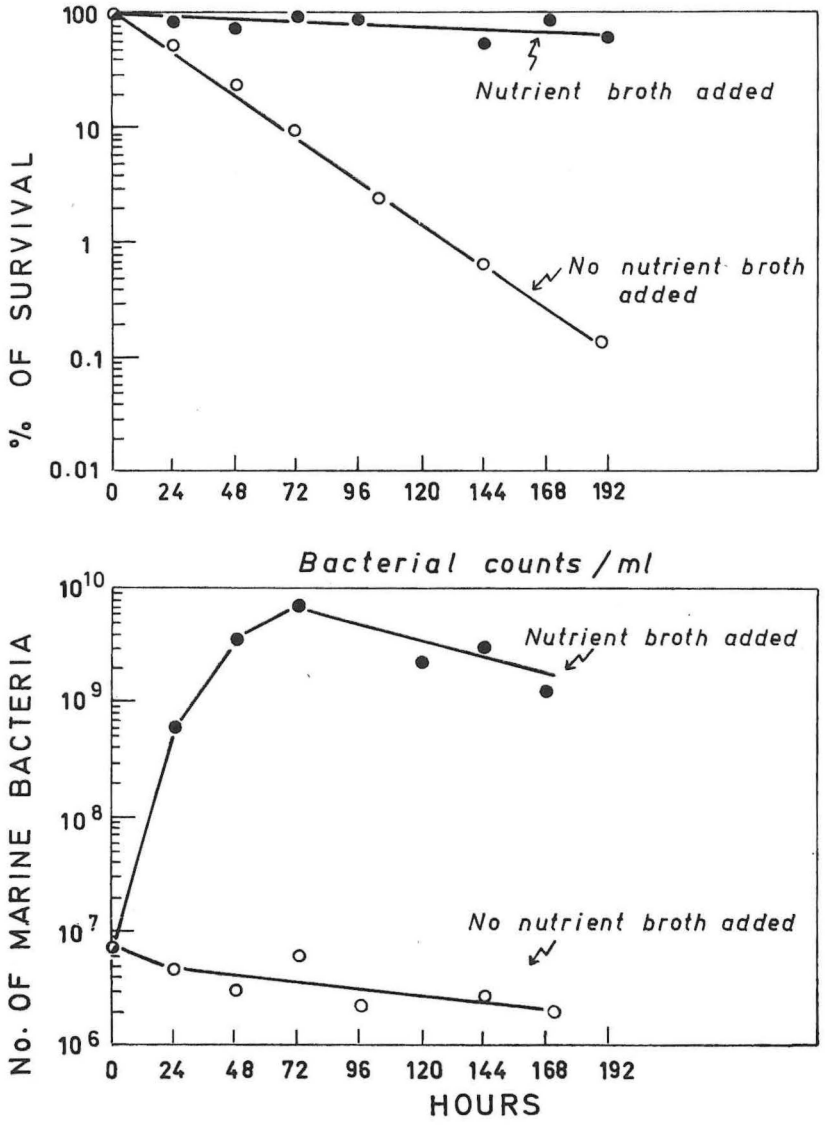

Fig. 6 — The effect of nutrient broth on the coliphage inactivation capacity of an active marine bacteria.

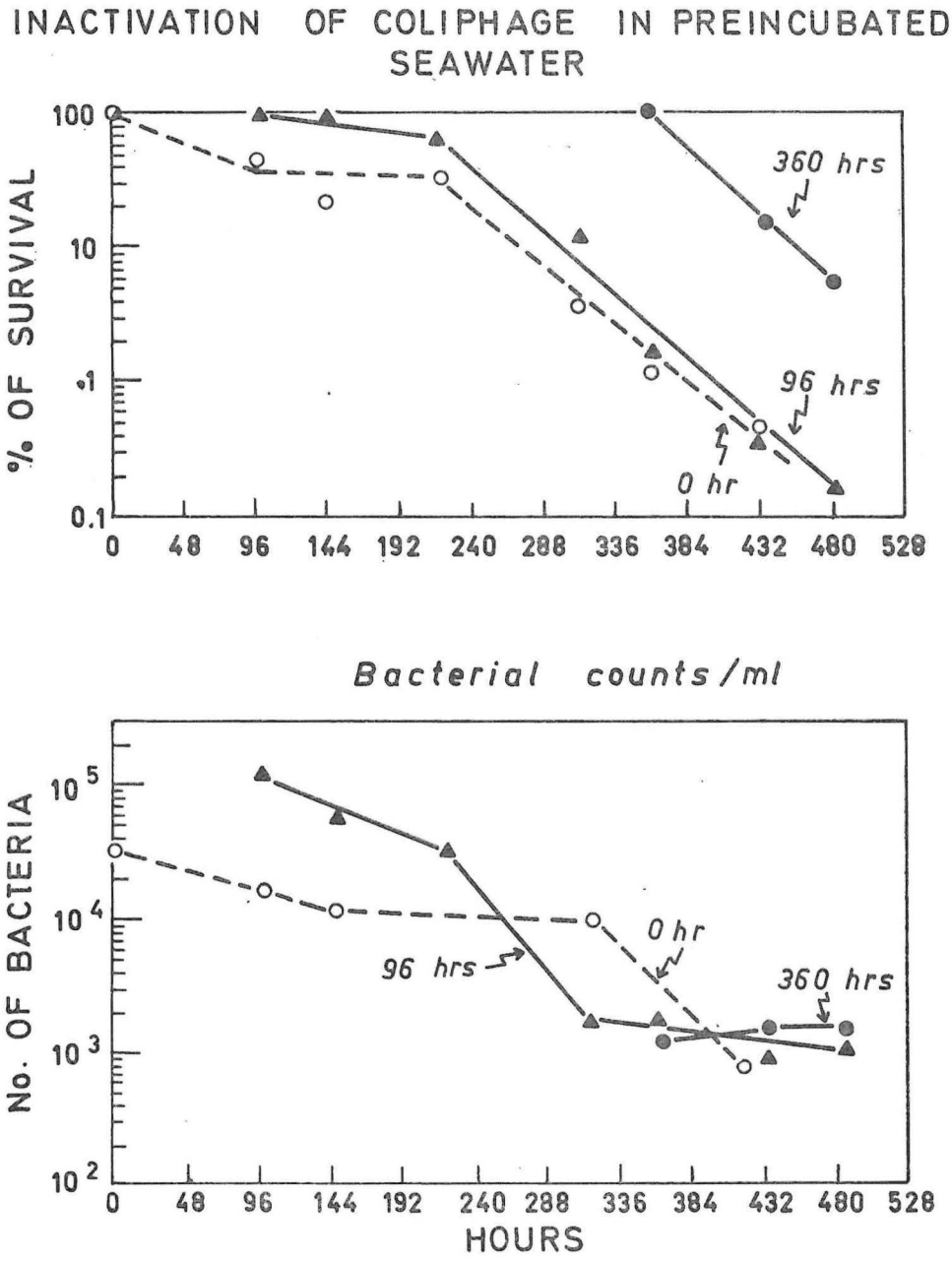

Fig. 7 — Inactivation of coliphage in preincubated seawater.

sitive antiviral factor in seawater. Proteins and amino acids were found to inhibit this factor. They suggested that the inhibition was caused by reaction of the protein material with the antiviral factor.

The observations in the present report correlate quite well with those mentioned above. Addition of nutrient broth inhibited the antiviral activity of seawater. The inhibitory effect of nutrient broth could be caused by its action on either the marine microorganisms or on their products.

Phages can be used as a source of food for bacteria when they are digested by proteolytic enzymes producted by the bacteria. Such a possibility was described by Cliver and Herrmann (1972) who showed that proteolytic enzymes of *Pseudomonas aeruginosa* can be involved in nutrition or autolysis. Nutrient broth may protect the bacteriophages by competitive inhibition.

The loss of the antiviral activity by marine isolates after several transfers and storage was also observed by other authors. Magnusson, Gunderson, Brandberg and Lycke (1967) isolated a marine microorganism with a virus inactivating capacity. This capacity was lost after several transfers on artificial media and storage at 25°C. The authors of this report suspect that nutrients, which had been stored in the bacteria during the passages on rich media, caused the loss of phage inactivating activity. This may also be the reason for the reduced antiphage activity in our experiments.

Studies on the kinetics of coliphage inactivation in pre-incubated seawater showed that the lag phase was lost after a long pre-incubation of natural seawater at 20°C. During that time, the number of marine bacteria decreased at least in one lag. It is possible that during this period, bacterial exocellular polymers were produced and these caused phage flocculation or absorption, thus decreasing the number of phages in the suspension. Such exocellular polymers were shown by Pavoni, Tenney and Echelberger (1972).

SUMMARY

Seawater has been shown to possess a self-purifying capacity which enables it to inactivate foreign microorganisms. The present report deals with biological and chemical factors involved in the inactivation of coliphage T_2 in seawater. It was found that a typical inactivation curve of T_2 phage in fresh seawater involved a lag phase after which a decrease in the titer occurs. When marine bacteria were added to sterile seawater, the decrease in phage titer occurred without a lag phase. Nutrient broth in low concentration was found to inhibit the antiviral activity of seawater. Pre-incubated seawater exhibits the anticoliphage activity without any lag period. Two alternative explanations for the anticoliphage activity in seawater are suggested: a) phage are digested by proteolitic enzymes of marine bacteria b) bacterial exocellular polymers cause biological flocculation.

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KRATAK SADRŽAJ

Morska voda posjeduje sposobnost samoočišćenja, koja omogućava inaktiviranje mikroorganizama stranog porijekla. U ovom referatu tretirani su biološki i kemijski faktori koji su uključeni u proces inaktiviranja kolifaga T_2 u morskoj vodi. Nađeno je da tipična krivulja inaktiviranja T_2 faga u svježoj morskoj vodi uključuje fazu zaostajanja (lag phase) nakon koje slijedi opadanje titra. Kada su morske bakterije dodadne sterilnoj morskoj vodi, neposredno slijedi opadanje titra faga, bez pojave faze zaostajanja. Hranjivi bujon male koncentracije spriječava antivirusnu aktivnost morske vode. Morska voda prije inkubacije ispoljava antikolifagnu aktivnost bez prisustva faze zaostajanja. Autori sugeriraju dva alternativna tumaćenja za antikolifagnu aktivnost u morskoj vodi:

a) fagi su razgrađeni pomoću proteolitičkih enzima morskih bakterija;

b) izvanstanični polimeri bakterija prouzrokuju biološku flokulaciju.