Hebrew University - Hadassah Medical School

Department of Medical Ecology

ENVIRONMENTAL HEALTH LABORATORY

PUBLIC HEALTH ASPECTS OF WATER-BORNE ENTERIC VIRUSES

Prof. Hillel I. Shuval
Head, Environmental Health Laboratory

IN COLLABORATION WITH:

N. Biderman, B.Sc.	- Environmental Health Laboratory
J. Cohen, Ph.D.	- Environmental Health Laboratory
S. Cymbalista, M.Sc.	- Department of Virology
B. Fattal, M.A.	- Environmental Health Laboratory
Prof. N. Goldblum, Ph.D.	- Department of Virology
E. Katzenelson, Ph.D.	- Environmental Health Laboratory
M. Nishmi, Ph.D.	- Department of Virology
A. Thompson, B.Sc.	- Environmental Health Laboratory
Y. Wiener, B.Sc.	- Environmental Health Laboratory

FINAL REPORT

Under Research Agreement No. 06-330-2

to the

CENTER FOR DISEASE CONTROL

Public Health Service

Health Services and Mental Health Administration

Department of Health, Education and Welfare

June 1971 Jerusalem

TABLE OF CONTENTS

PREFACE

This monograph has been prepared as the final report to the Center for Disease Control - Health Services and Mental Health Administration under Research Agreement 06-330-2. It includes four chapters dealing with various aspects of the research project carried out during the period 1 July 1968 to 30 June 1970.

The research work carried out under this agreement is actually a second stage of an earlier project sponsored and financed by the Public Health Service under research agreement BSS-CDC-IS-10 which was initiated in July 1965. It was under this first agreement that much of the basic work on quantitative methods for concentrating and detecting viruses in the water environment was completed.

The Phase-Separation (PS) method developed by our group for detecting viruses in water, supplied one of the basic tools in quantitative field studies on problems of viruses in the water environment such as the one reported upon here. It is gratifying to state that this method has gained wide use since our first reports on it were presented some five years ago. Research teams in India, South Africa, U.K. and the United States have applied the method which allows for the detection of as few as 1-2 PFU/liter of water tested.

The material presented here covers the major portion of the work carried out under the project. However, in June 1970, during the last month of the research agreement we initiated a major experiment suggested by Dr. James Moseley, Project Officer for most of the period. This complex experiment was designed to monitor a planned simulated polio epidemic in the town of Qiryat Shemoneh and involved the revaccination of some 600 school children. The field phase continued through September 1970 and due to moving our laboratory to new premises the work-up of the samples has been seriously delayed. The majority of the data collected is presented in this report. The remainder will be presented at a later date.

Another series of field studies involving 61 virus and bacteriological assays of polluted and unpolluted water sources in the Jerusalem area was carried out during 1969. However, since there were no positive virus findings in any of the water samples, the details are not included.

The study of problems of water-borne enteroviruses has been advanced considerably over these past few years but many basic questions still remain unanswered and improved techniques are still needed.

Today it is clearer than ever that a quantitative method for detecting a few viruses in water samples of several hundreds of liters is essential for proper monitoring of water supplies. These studies and others have demonstrated the persistence of enteroviruses in the water environment and the inadequacy of the coliform test as a sole measure of a water's microbiological safety.

Water-borne virus diseases such as infectious hepatitis may well pose an even greater problem in the future as levels of water pollution increase and communities throughout the world are forced to turn to renovated wastewater as a source for domestic supplies.

The study of the problem of viruses in water must continue until all the necessary answers are available to provide adequate protection of the health of the public.

On this occasion I would like to offer my sincere appreciation and thanks to Dr. James Moseley for his warm support and guidance during the years we have worked together on these studies. He was more than just Project Officer, he was both an inspiring scientific collaborator and good friend.

The helpful advice and direction provided by Dr. Mildred Kendrik who served as Project Officer during the last period of the study has also been highly valued. We feel that we have gained much from the opportunity of working with two such fine scientists.

Lastly I wish to acknowledge the constant support and collaboration of Prof. Natan Goldblum who helped initiate these studies within the Department of Virology.

If it had not been for his encouragement and sage guidance we would not have been able to progress as we did.

His devoted cooperation and unstinting assistance is sincerely appreciated.

Jerusalem
June 10, 1971

Hillel Shuval

A REVIEW:

Methods for the Detection of Enteric Viruses in the Water Environment

Hillel I. Shuval and Eliyahu Katzenelson

INTRODUCTION

Water-borne Virus Disease

The possibility that water might serve as the vehicle for the transmission of certain virus diseases, particularly those whose infectious agent is excreted through the enteric tract, has been considered feasible for some time.

Mosely (1) has pointed out that over 50 documented water-borne epidemics of infectious hepatitis have been recorded over the years. Apart from infectious hepatitis, poliomyelitis and viral gastroenteritis were the only other viral infections that caused epidemics suspected of being transmitted by water. In most cases, the epidemiological evidence was inconclusive. An exception is possibly a small polio epidemic in Nebraska in 1952 (2) where strong evidence suggested that it was caused at least partially by a water-borne virus. The possibility that viral gastroenteritis may be water-borne on occasion cannot be ruled out, however.

The massive water-borne epidemic of infectious hepatitis in Delhi, India, in 1955 (3) in which some 30,000 persons became infected by the contaminated municipal drinking water which had undergone what is generally considered complete and adequate treatment, including chlorination, emphasized the need to develop new methods of monitoring water supplies for viruses. Over the years, evidence has

pointed to the fact that the usual bacterial parameters of water purity, particularly the coliform group, may not provide an adequate index as to the safety of water from a virological point of view. The need to monitor water specifically for viruses of enteric origin presents many problems and the developments in this field will be reported upon here.

The Presence of Enteric Viruses in Water

Over 100 virus types are known to be excreted from humans through the enteric tract and may find their way together with the sewage into sources of drinking water. Many of these viruses are known to cause disease in man. However, the critical question is whether these viruses can survive long enough and in high enough concentration to cause disease in man consuming such contaminated water. The concentration of enteric viruses in sewage and polluted water is an important factor to consider. Clark and Kabler (4) calculated a theoretical average number of enteric virus in infectious units in sewage and found it to be about 500/100 ml. In our own studies we have found the enteric virus concentration in the sewage of five communities in Israel to average 100/100 ml. (5). Based on these figures it can be assumed that the virus concentration in polluted river water would range from 0.1 - 1 viral infectious units/100 ml. as a result of physical dilution only. The number is lower during the cold months and somewhat higher in the late summer and early fall due to seasonal variation of enteric virus diseases. It can be assumed that this concentration will be further reduced both by processes of natural die away and by water treatment, imperfect as they may be in the removal and inactivation of viruses. Under normal circumstances, only a relatively small number of infective units will, at worst, penetrate a water supply system which derives its raw water from a heavily contaminated river. Simultaneous infection of a large number of people is therefore rather improbable under normal conditions with modern water treatment methods. Sporadic infections, however, are possible, at least theoretically. The latter becomes true particularly in the light of Plotkin and Katz's (6) claim that "one infective dose of

tissue culture is sufficient to infect men if it is placed in contact with susceptible cells." They were able to reach this conclusion as a result of studies on attenuated polio viruses, respiratory viruses, agents of ocular diseases, viruses of animals, and other agents. This might mean that even when virus concentrations as low as one virus infectious unit per 1000 ml. are present in water, a certain number of individuals might well become infected by consuming the normal daily intake of 1-2 liters per capita.

With this in mind, one may form a picture of water as playing a small, but important role in the spread of viral diseases in man in areas provided with modern treatment facilities. The effect of such slightly contaminated water may lead to sporadic cases of disease, dispersed over a large area. However, these occasional cases may, in turn, act as foci and through food or personal contact cause epidemics which may involve much larger numbers of people. In other cases when heavily contaminated water reaches large population groups without adequate treatment, explosive mass epidemics have occurred and may well occur in the future. It is therefore obvious that the development of methods for the detection of viruses in water are required to allow for an adequate evaluation of the virulogical safety of water supplies and treatment processes. Bacterial evaluation of water as an indicator of contamination cannot replace such methods since it became apparent that viruses are not as sensitive as bacteria to hostile environmental factors or to standard purification procedures, and they may be present in water even when bacterial counts are at acceptable standards (7, 8).

Types of Viruses in Water

Water being used for drinking and bathing can act as a vehicle for the transmission of most of the viruses. The exception is the Arbo virus which are transmitted by insects. The Picorna group of viruses is the one most commonly found in sewage; it includes the polio, coxsackie and echo viruses. Adeno viruses, which causes respiratory and eye infections, and sometimes diarrhea, are commonly found in feces.

Infectious hepatitis is actually the only disease for which a water-borne infection has been proven beyond any doubt. However, its viral characteristics are not yet clear. There are some claims that the responsible virus had been isolated from suspected cases of hepatitis, but most virologists feel that these claims are as yet insufficiently established. It must be remembered, however, that in spite of the latter there is strong evidence supporting the view that this disease is actually caused by a virus (9).

Water may also be polluted by most of the known viruses as a result of bathing. People with viral respiratory or skin diseases may enter a pool or the sea and thus contaminate the water. Healthy people using the same water may be infected. The number of potential water-borne viral diseases is therefore large. However, since the most important are the enteric viruses, we shall deal only with them.

Isolation and Identification of Enteric Viruses

Viruses can only multiply inside living cells and therefore live organisms such as animals, chick embryos or tissue culture must be used for their isolation in the laboratory. For the enteric viruses, tissue cultures which may be of two types are generally used: primary tissue cultures and continuous cell cultures. Primary tissue cultures are usually prepared according to the method of Enders et al (10). This method is based on the fact that 0.25% trypsin acts on small cuts prepared from a tissue (usually a kidney) by separating the cells from each other. When put inside a suitable glass or plastic flask, tube or plate together with a tissue culture nutrient medium, these cells attach to the wall of the vessel and multiply. As a result, a monolayer of cells is formed on the wall. Continuous cultures are very similar, but instead of a tissue from an organ, a tissue culture is used as a source for the cells. Initial isolation of enteric viruses is usually done on primary tissue cultures prepared from monkey kidneys which typically have a higher sensitivity than most cell lines. However, any isolated virus can be adapted to cultures of the continuous type.

After inoculation of a virus into a tissue culture some of the cells become infected.. The virus multiplies within these cells and spreads to the neighbouring cells. At the same time, the infected cell usually undergoes morphological and biochemical changes and dies. The result is a slow process of destruction of cells in the culture, a phenomenon known as the cytopathic effect (C.P.E.). The process of viral spread from cell to cell can be slowed down by adding a layer of agar together with tissue culture medium over the cells. As a result, instead of being rapid and confluent, the cytopathic effect will be limited to a smaller area which looks macroscopically like a hole in the monolayer of cells. These holes are also known as "plaques." A single plaque usually originates from a single infected cell which may be caused by a single virus infectious unit.

This method is used for quantitation of entric viruses in tissue cultures in the same manner as agar plates are used in bacteriology for the determination of bacteria counts. The term "plaque forming unit" or PFU was given to the lowest concentration of viruses that form one plaque on a monolayer of cells.

Different viruses cause cytopathic effects which differ morphologically. Also, plaques may be of different sizes and shapes. However, this phenomenon cannot be used for the final identification of the isolates since some viruses, belonging to different groups, cause identical cytopathic effects.

Final identification can only be achieved with specific antisera. Here the identification is based on the fact that specific antiserum will neutralize the effect of the virus against which it was prepared.

Quantitation of Enteric Viruses

Two methods are available for the quantitative determination of enteric viruses in a given sample of material being assayed, both of which give accurate results. Selection of the method to be used is usually based on the experience and

resources of the laboratory. In the first one, the "tube assay method", serial dilutions of the virus suspension to be tested are prepared. Groups of tissue culture tubes are inoculated with each of the dilutions. Each tube is inoculated with 0.1 - 0.2 ml. of the dilution. After proper incubation at 37°C, the inoculated tubes are examined for cytopathic effect. Quantitation is obtained by finding the lowest dilution of the virus suspension that caused C.P.E. in 50% of the tubes. The figure obtained is known as the $TCID_{50}$ (Tissue Culture Infectious Dose - 50%) value of the virus suspension. Using this same method, it also is possible to calculate the virus concentration as a "most probable number" - M.P.N.

In the second, the "plaque assay method", quantities of 0.3 - 1.0 ml. of virus dilutions are inoculated into plates or bottles, the cells of which are later covered with an agar overlay. After proper incubation, usually at 37°C in a humid atmosphere containing 5% CO_2, the inoculated tissue cultures are examined for the presence of plaques. When plaques are present, they are counted and their number for each of the dilutions is determined. The number of plaque forming units in the original virus suspension is then calculated and the virus concentration is reported as PFU/ml. or other unit of volume.

Detection of Viruses in Water

The isolation of viruses from water is principally the same as from any other source. However, the main difficulty is the usually low concentration of viruses in water. We have already seen that this number may be as low as 1 PFU per 1000 ml. of water or even less. Considering the amount of 0.1 ml. of inoculated material for a tube, 0.3 ml. for a plate and about 1 ml. for a bottle, it is obvious that for the detection of a single virus unit in a 1000 ml. sample at least 1000 bottles or 10,000 tubes should be inoculated. It is therefore not surprising that in the past it has been difficult to detect the presence of viruses in water, even during epidemics.

The story of virus detection in water is therefore a story of the development of methods for virus concentration. The actual inoculation of specimen and the quantitation procedures are essentially the same as in clinical virology.

Methods for the Concentration of Viruses from Water

1. Gauze pad method: One of the first methods used to detect viruses in water was the gauze pad method. Here modification of the "Moore" swab technique (11) for recovery of bacteria from water is exploited. Gauze pads or pads filled with cotton or plastic foam sponges are placed in the water and left for varying periods, usually from one to several days. During the period viruses are absorbed or entrapped in the pad from the flowing stream of water leading to a concentration of viruses. The pads are often treated with dilute sodium hydroxide to increase the pH of the absorbed up water to 8.0. This facilitates the elution of viruses from the pads (12). Liquid expressed from the pads is tested for viruses. It has however been suggested that further concentration of the virus from the expressed liquid increases the sensitivity of the method (39).

The method has been used by many workers for the detection of viruses in water and sewage. Melnick et al. (12) were able to demonstrate its superior sensitivity as compared with a grab sample. In their work, when equal volumes of expressed fluid and of grab sample were compared, more isolates were obtained from the pads. These results were confirmed by others. It appears that pads are able to concentrate viruses from water. In our own studies of this phenomenon, it appears that the liquid eluted from pads suspended for one or two days in sewage or contaminated water contained from 10-50 times as many viruses as detected in parallel grab samples of the same water. However, the mode of action and the degree of concentration have not as yet been determined. Although the pad method is not quantitative, it has proved itself to be a useful and effective procedure for detecting virus in water.

2. Sample Incorporation Method: In this method the conventional culture medium is so concentrated as to allow for the incorporation in it of 10 to 60 ml. of the sample to be assayed (13). According to another approach, large volumes of a maintenance medium, prepared from the water that is being tested are inoculated onto cell cultures (14). No concentration of virus from the water, prior to inoculation is attempted in these cases. Nevertheless, by this method virus detection is enhanced since with the same tissue culture tube or bottle, a volume of sample 10-20 times larger than that normally inoculated can be assayed.

3. Ultra-centrifugation: The centrifuge is mostly used for the concentration of small suspended particles from fluids. Being extremely small particles in the size range of 20-200 mu., viruses are no exception, but because of their small size, relatively high forces of the order of 60,000 x g for 1 hour are required. These are obtained using an ultracentrifuges. In the usual procedure, the water sample is first centrifuged at a relatively low speed to reduce the number of larger particles including bacteria. The supernatant is then centrifuged at high speed. The sediment obtained contains the virus, and is resuspended in a small volume of tissue culture medium. A high concentration factor may thus be reached. When accurate results are required, restrirring of the sediment has to be avoided. A "trap" of 2% gelatin, layered on the bottom of the centrifuge tubes has been used. In this way, the detection of a few virus units in one liter of sample water was possible (15).

Anderson et al (16) described the development of a complex centrifugation system for the isolation and separation of small numbers of virus particles from large volumes of fluid. A high performance continuous-flow centrifuge was constructed which removed over 95% of suspended virus at a flow rate of 2-3 liters/hr. In their rotor the flowing stream moved over a stationary density gradient. Trapped virus particles were banded isopycnically in the gradient and were never pelleted. The recovered material could be further fractionated on the basis of sedimentation rate by zonal centrifugation.

The main disadvantage of ultracentrifugation is that it requires very expensive apparatus, especially when Anderson's technique is used. This may explain why the method has not been used extensively for the detection of viruses in water under actual field conditions.

4. Membrane Filter Adsorption: This technique is based on the fact that viruses are adsorbed onto the matrix of membrane filters of the "Millipore" or "Gelman" type even when the pore diameter of the filter is 10-20 times larger than the virus. Addition of $MgCl_2$ to the virus suspension usually increases the adsorption. A suspension containing virus is passed through a membrane filter, and the viruses are eluted from the membrane with either serum or gelatin (17) (18) or with sodium lauril sulfate, a surface tension reducing agent (19). One of the problems involved with testing large volume of water with this method is clogging of the filter system.

In practice, using coxsackievirus A9 in tap water and membranes of 0.45 u porosity, it appeared that there was at least a 50% probability of detecting virus at a level of two plaque-forming units per liter (20). Berg (21) reports complete recovery of enterovirus and 50-80% of reovirus 1 from distilled water with a 3% solution of dehydrated beef extract after intensive sonication of the filter. The sonication technique was not effective with tap water samples however.

5. Soluble Ultrafilters: The water to be tested is passed through a soluble filter which does not allow the viruses to pass through. The filter is then dissolved in a solvent and the suspension containing the viruses is inoculated onto tissue culture. Filters of aluminum alginate gel and a 3.8% solution of sodium citrate as a solvent have been used (22). With this method it was possible to recover 10 $TCID_{50}$ virus from 10 liters of water.

6. Hydro-extraction: A sample of water is placed in a cellulose dialyzing bag which is then immersed in a hydrophylic agent, polyethylene glycol (23, 24, 25). Water

is then adsorbed from the bag while viruses, like other molecules of high molecular weight, remain inside. A concentration factor of 100 or more may thus be obtained, with an efficiency of recovery somewhat under 50%.

7. The Phase Separation Method: This method is based on the discovery of Albertsson (26) that the rusult of certain mixtures made from two polymers such as dextran sulfate and polyethylene glycol in an aqueous solution leads to the formation of a two-phase system. Introduction of particles and macromolecules into this system will result in the partition of the particles in the two phases, depending on their size and surface properties (27). Viruses show a nearly one-sided distribution and the method may therefore be used for their concentration (28, 29). The concentration is accomplished by adding polymer solutions to a virus suspension in such proportions that almost all the virus particles are collected in a small volume bottom phase and may be drained off separately. In this way, the virus may be concentrated 100-200 times, depending upon the kind of phase system used. Purification of viruses is also obtained, since other substances such as protein and cell fragments distribute in a different way than viruses in the phase system. Dextran sulfate may be easily removed from the virus suspension with barium or potassium ions. This allows for repetition of the concentration procedures (30, 31). Using this relatively simple method, Shuval et al. have shown that a concentration factor of 500 can be achieved using the two-step phase-separation (PS) procedure and as few as 1-2 virus infectious units per liter of sample could be efficiently detected (24, 32). The usefulness of this method in detecting enteric viruses in sewage, water and sea water has been demonstrated. On one occasion during the investigation of a water-borne epidemic, it was possible to detect enteroviruses in contaminated well water with the two-step PS method (5).

8. Electrophoresis: Viruses are usually negatively charged at neutral pH values (33) and will therefore move toward the cathode when a virus suspension is placed in an electric field. This principle was used by Bier for concentrating

bacteriophages in water (34). A simple procedure was developed by which electrophoretic transport was used to bring about adsorption of bacteriophages on dialyzing membranes. With this method a sample of water could be processed in a relatively short time, and a concentration factor of 100 achieved. It seems likely that with some adaption this method could be used for the concentration of enteroviruses too.

9. Adsorption to Particulate Material: Viruses may be adsorbed from water onto a variety of particulate material and then be eluted with a much smaller volume of liquid. Methods that involve the use of precipitates of inorganic salts such as calcium phosphate (35, 36, 37), cobalt chloride (38), aluminium hydroxide (36, 39), and ammonium sulphate (39) or the use of insoluble polyelectrolytes (41, 42, 43), ion exchange resins (44, 45), and passive hemagglutination (23) (46) were described. The adsorbent is first added to the tested water and later separated from it by centrifugation or filtration. Resuspension is carried out in a small volume. Concentration factors of 20-1000 times have thus been obtained, depending on the method used. Due to its low cost, simplicity and relatively high efficiency in recovering viruses, this method holds much promise.

Comparative Studies of Virus Concentration Methods

The great variety of methods which have been described for concentrating viruses in water indicate how different approaches can lead toward the same goal, the detection of a small number of viruses in a large volume of water. However, this variety emphasizes the lack of one general method of proved efficiency, low enough in cost to be acceptable by all workers in the field. It is as yet impossible to say which method is preferable although considerable progress has been made in recent years in improving techniques for the detection of viruses in water. A systematic comparative study of all the available methods is still to be made. However, a limited number of comparative studies have been published which may throw some light on the problem.

Gibbs and Cliver (23) compared three different methods: passive hemagglutination, hydroextraction and ultra-centrifugation. The virus they used was reovirus 3. They found the passive hemagglutination specific for this virus and ineffective for others. The hydroextraction method had a disadvantage as the concentrated fluid resulting from this procedure was somewhat toxic to cell cultures. Ultracentrifugation was superior to the others for the demonstration of viruses in food extracts.

Shuval and his co-workers compared the Phase Separation (PS) method which they had adapted and developed with hydroextraction (24) and found the first to be about 10 times more sensitive in detecting enteroviruses in clarified sewage, normal saline solution, distilled water and phosphate buffer solution. The efficiency of recovery of the PS method approaches 100%, while that of the hydroextraction method is between 40-50%. In latter studies, they demonstrated the effectiveness of the PS method in detecting viruses in sea water as well.

Lund and Hedstrum (40) also compared the Phase Separation (PS) method with a combined ammonium sulphate-ultracentrifugation method. They found both methods to be equally efficient for the detection of enteroviruses in sewage. However, the PS method was by far the simplest.

Moore et al. (47) compared membrane filtration with adsorption on aluminium hydroxide and found the second to be better for the concentration of poliovirus from wastewater. They found very high losses of virus with the membrane method. Aluminium hydroxide was compared also with insoluble polyelectrolytes by Wallis et at. (41). In their study, polyelectrolytes were more efficient in detecting enteroviruses in sewage.

Gravelle and Chin (45) compared three different methods of sewage sample preparation. Their samples were collected by gauze pads which were then inoculated into tissue cultures unconcentrated; concentrated by ultracentrifugation; and concentrated with the resin precipitation method. Evaluation of the methods was based on the number of successful isolates. Their results showed very clearly the superiority of ultracentrifugation over the other two methods. A valuable finding of their study is that a combi-

nation of two methods of concentration can increase the number of virus isolates from sewage.

Evaluation of Virus Concentration Methods

Evaluation of virus concentration methods may be obtained when results from different studies are compared.

Selection of criteria for the comparison is based on the assumption that the best method should enable the quantitative detection of very small numbers of virus infectious units in water. This will become possible only if the method allows for utilization of large quantities of water, has a very high concentration factor and maximum recovery efficiency for as many virus types as possible, i.e. assuming a concentration of viruses in water of 1 virus unit per 10 liters, then the method should enable the processing up to 100 liters of water, have a concentration factor of about 10,000 with recovery efficiency approaching 100% for a broad as possible spectrum of viruses.

TABLE 1

A Comparison of Various Methods for
Detecting Viruses in Water
(based on published data)

	Sample Volume Liters	Concentration Factor	Recovery Efficiency %
Gauze pad method	?	10-50 ?	?
Sample incorporation method	0.06-0.15	10-20	95
Ultra-centrifugation	1-10	30-?	20-100
Membrane filter adsorption	10-100	1000	5- 38
Soluble ultra filters	10	1000	35-100
Hydro extraction	1	100	24- 64
Phase Separation (two steps)	10	1000	35-100
Electrophoresis	0.3	100	100
Adsorption to particulated materials:			
Calcium phosphate	3.8	1000	50-100
Cobalt chloride	1	1000	100
Aluminium hydroxide	3.8-19	1000	50-100
Ion exchange resins	0.1	50-100	20- 50
Passive hemagglutination	?	?	80-100
Insoluble polyelectrolytes	1000	10,000	35- 80

Comparative results from various studies on virus concentration methods are given in Table 1. A rough comparison of some of the methods is thus possible. The given figures were either taken directly from the reports or were calculated on the basis of published data referred to throughout this article.

Information on the specificity of the methods for detecting various types of viruses were not available since different viruses were used by different workers. Such information, however, is very important for an evaluation of the method. An example of the nature of this problem has been reported on in connection with the Phase Separation method.

The PS method was evaluated for its ability to recover seven types of enteroviruses. It was found to be highly effective in the recovery of the 3 types of poliovirus and coxsackie virus types B-3 and A-9. It was, however, found to be inhabitory for coxsackie virus B-2, ECHO virus type 6 and influenza A virus (48).

Another example is the passive hemagglutination technique, which is highly specific for a single type of virus (23, 46). In some of the methods such as electrophoresis, only a single type of virus was tested so that no information is available as yet as to its ability to concentrate a broad range of viruses, although it may well be capable of doing so.

The work on the concentration of viruses with aluminium and calcium salts included an evaluation of its efficiency with many types of viruses. Recovery efficiencies of Herpes, Pox, Adeno, Papova, Myxo, Paramyxo, Reo, Entero, Arbo and Rhino viruses were compared. Apart from Reo virus which showed a very poor recovery, the others showed recovery efficiencies in the range of 50 - 100% (37).

Wallis et al. (43) showed that they were able to recover 40% of the poliovirus added to natural water by passing 300 gallons through a filter composed of a thin layer of insoluable polyelectrolyte. The virus concentration in the water was 2-12 PFU per gallon. Even though the efficiency of recovery is somewhat low this method may prove to be particularly useful due to the large valumes of water that can be tested with it.

A very important factor in selecting a method for the detection of viruses in water is the cost involved. Some of the methods such as Anderson's continuous flow ultra-centrifuge technique requires the use of very expensive apparatus and may be feasible for research purposes only. Others such as the Phase Separation method and concentration on aluminium hydroxide and insoluble polyelectrolites are much cheaper, and can be used for the routine monitoring of water without special equipment or expense.

Summary and Conclusion:

The possibility that enteric virus diseases, particularly infectious hepatitis, may be water-borne both in epidemic form and at times as sporadic cases, calls for the development of methods for evaluating the safety of water supplies from a virulogical point of view. The need is especially great in light of even-increasing evidence that coliform bacteria, the classical water pollution indicator organism, are less resistent under certain conditions to both natural hostile environmental factors as well as man-made water and waste water treatment processes. Many of the enterovirus presist longer in water courses and the ocean than do coliforms and are less easily removed or inactivated even by the most rigorous of treatment methods. This may mean that situations can arise where coliform counts are at acceptable low levels either because of natural die away or because of active intervention by man in the form of treatment while pathogenic enteroviruses may still be present in water in concentrations high enough to cause disease in man. This is apparently what happened in Delhi in 1952 and there is presumptive evidence at least suggesting that it can happen more frequently than we might assume.

A specific method to detect viruses in water is required both for the routine monitoring of water supplies and to enable the evaluation of water and wastewater

treatment processes under actual field conditions. Such method should be capable of detecting a few virus infectious units in a relatively large volume of water. The method should ideally be capable of testing at least a 100 liter water sample with the ability to determine with a high degree of reliability whether or not any viruses are present and if so how many. The method should have a virus concentration factor of 1000 - 10,000 with a high efficiency of recovery for very low concentrations of viruses. It also must have the ability to concentrate and detect all enteric virus types with equally high efficiency. Last but not least, the method should be relatively inexpensive so that it can be introduced into most routine water monitoring laboratories. Over a dozen different methods have been developed and studied to date none of them completely meeting all of the ideal requirements outlined above, but some appear quite promising and approach the ideal specification listed.

Adsorption to particulated matter, membrane filters and Phase Separation are some of the most promising methods yet reported upon although others may well prove to be of equal or better performance.

Possible combinations of methods may hold promise as well. For example, it may be attractive to test a sample of 10 liters by the PS method using the two-step procedure which provides a concentration factor of about 500. This would result in a final volume of some 20 ml. to be assayed for viruses. This volume containing essentially all of the viruses present in the sample could be assayed in one tissue culture bottle using the sample incorporation method.

Routine monitoring of water supplies for viruses will most likely not be within the reach of most local water sypply quality control laboratories in the forseeable future. However, the possibility does exist that the routine laboratory concentrate the sample by one of the methods reviewed here which is deemed to be most suitable, and freeze the concentrate for later shipment and assay at a regional water quality or public health laboratory having complete facilities and staff for virus assay and identification work. Frozen water samples containing concentrated virus can be held at $-20^{o}C$ for weeks before testing without an appreciable reduction in virus count. This provides

a decided logistic advantage over bacteriological examination of water which usually must be carried out immediately at a local laboratory.

In conclusion, despite the need for considerable futher development of existing virus detection methods and the need to run carefully controlled comparative studies on the most promising ones, it can be said that a number of effective methods for detecting low concentrations of viruses in large volumes of water, which are relatively inexpensive and easy to operate, are currently available. Some are being used routinely with good effect for that very purpose. Virus assay methods which are currently available could play an important role in protecting the public health in many communities if they were utilized more widely. This could become feasible, particularly in programs where the actual virus assay work is done in one regional laboratory servicing a number of local water quality control laboratories.

REFERENCES

1. Mosley, J.W., (1962) Transmission of Viral Diseases by Drinking Water. In Transmission of Viruses by the Water Route (Edited by Berg, G.) Interscience, London.

2. Dennis, J.M. (1959) Infectious Hepatitis Epidemic in New Delhi, India. J. Am. Wat. Wks. Ass. 51, 1288.

3. Bancroft, P.M., Engelhard, W.E. and Evans, C.A. (1957) Poliomyelitis in Huskerville (lincoln), Nebraska. J. Am. Ass. 164, 836.

4. Clarke, N.A. and Kabler, P.W. (1964) Human Enteric Viruses in Sewage. Hlth. Lab. Sci. 1, 44.

5. Shuval, H. (1970) Detection and Control of Enteroviruses in the Water Environment. Developments in Water Quality Research, Ann Arbor-Humphrey Science Publishers, Ann Arbor, London.

6. Plotkin, S.A. and Katz, M. (1967) Minimal Infective Doses of Virus for Man by the Oral Route. In Transmission of Viruses by the Water Route (Edited by Berg, G.) Interscience, London.

7. Kjellander, J. and Lund, E. (1965) Sensitivity of E. coli and Polio virus to different forms of combined chlorine. J. Am. Wat. Wks. Ass. 57, 893.

8. Shuval, H., Cymbalista, S., Wach, A., Zohar, Y. and Goldblum, N. (1967) The inactivation of enteroviruses in sewage by chlorination. Proc. 3rd Int. Conf. Water Pollution Res. January 1967.

9. Kissling, R.E. (1967) Laboratory status of the infectious hepatitis agent. In Transmission of Viruses by the Water Route (Edited by Berg, G.) Interscience, London.

10. Enders, J.F., Weller, T.H. and Robbin, F.C. (1949) Cultivation of the Lansing strain of Poliomyelitis virus in cultures of various human embryonic tissues. Science 109, 85.

11. Moore, B. (1948) The detection of Paratyphoid carriers in towns by means of sewage examination. Month. Bull. Health Pub. Health Lab. Serv. 7, 241.

12. Melnick, J.L., Emmons, J., Opton, E.M. and Coffey, J.H. (1954) Coxsackie viruses from sewage, Methodology including an evaluation of grab sample and gauze pad collection procedures. Am. J. Hyg. 59, 185.

13. Rawal, B.D. and Godbole, S.H. (1964) A simple tissue culture method for detection of viruses in drinking water: sample incorporation method. Environmental Health 6, 234.

14. Berg, G. Berman, D., Chang, S.L. and Clarke, N.A. (1966) A sensitive quantitative method for detecting small quantities of virus in large volumes of water. Am. J. Epiol. 83, 196.

15. Cliver, D.O. and Yeatman, J. (1965) Ultracentrifugation in the concentration and detection of enteroviruses. Appl. Microbiol. 13, 387.

16. Anderson, N.G., Cline, G.B., Harris, W.W. and Green, J.G. (1967) Isolation of viral particles from large fluid volumes In Transmission of viruses by the water route (Edited by Berg, G.) Interscience, London.

17. Cliver, D.O. (1965) Factors in the Membrane Filtration of Enteroviruses. Appl. Microbiol. 13, 417.

18. Wallis, C. and Melnick, J.L. (1967) Concentration of viruses from sewage by absorption on Millipore membranes. Bull. Wld. Hlth. Org. 36, 219.

19. Wallis, C. and Melnick, J.L. (1967) Concentration of enteroviruses on membrane filters. J. Virol. 1, 472.

20. Cliver, D.O. (1967) Enterovirus detection by membrane chromatography. In Transmission of viruses by the water route (Edited by Berg, G.) Interscience, London.

21. Berg, G. (1970) An integrated approach to the problems of viruses in water. Proc. National Specialty Conference on Disinfection, A.S.C.E., New York.

22. Gartner, H. (1967) Retention and recovery of polioviruses on a soluble ultrafilter. In Transmission of viruses by the water route (Edited by Berg, G.) Interscience, London.

23. Gibbs, T. and Cliver, D.O. (1965) Methods for detecting minimal contamination with reovirus. Hlth. Lab. Sci. 2, 81.

24. Shuval, H.J., Cymbalista, S., Fattal, B. and Goldblum, N. (1967) Concentration of enteric viruses in water by hydro-extraction and two-phase separation. In Transmission of viruses by the water route (Edited by Berg, G.) Interscience, London.

25. Cliver, D.O. (1967) Detection of enteric viruses by concentration with polyethylene glycol. In Transmission of viruses by the water route (Edited by Berg, G.) Interscience, London.

26. Albertson, P.A. (1958) Partical fractionation in liquid two-phase systems. The composition of some phase systems and the behavior of some model particles in them. Application to the isolation of cell walls from micro-organisms. Biochem. et Biophys. Acta. 27, 378.

27. Albertsson, P.A. (1958) Partition of proteins in liquid polymer-polymer two-phase systems. Nature 182, 709.

28. Albertsson, P.A. (1960) Fractionation of cell particles and marcomolecules in aqueous two-phase systems. Almquist and Wilksells, Uppsala.

29. Frick, G. and Albertsson, P.A. (1959) Bacteriophage enrichment in a liquid two-phase system with a subsequent treatment with "Freon" 113. Nature 183, 1070.

30. Wesslin, T., Albertsson, P.A. and Philipson, L. (1959) Concentration of animal viruses using two-phase systems of aqueous polymer solutions. Arch. ges. Virusforsch. 9, 510.

31. Philipson, L., Albertsson, P.A. and Frick, G. (1960) The purification and concentration of viruses by Aqueous polymer phase systems. Virology 11, 553.

32. Shuval, H., Fattal, B., Cymbalista, S. and Goldblum, N. (1969) The phase-separation method for the concentration and detection of viruses in water. "Water Research" pergamon Press 3, 225.

33. Brinton, C.C. and Lauffer, M.A. (1959) in M. Bier, Ed. Electrophoresis, Academic Press, New York.

34. Bier, M., Bruckner, G.C., Casper, F.C. and Roy, H.E., (1967) Concentration of bacteriophage by electrophoresis. In Transmission of viruses by the water route (Edited by Berg, G.) Interscience, London.

35. Salk, J.E. (1941) Partial purification of the virus of epidemic influenza by adsorption on calcium phosphate. Proc. Soc. Exp. Biol. Med. 46, 709.

36. Taverne, J., Marshall, I.H. and Fulton, R. (1957) The prufication and concentration of viruses and virus soluble antigens on calcium phosphate. J. Gen. Microbiol. 19, 451.

37. Wallis, C. and Melnick, J.L. (1966) Concentration of viruses on aluminium and calcium salts. Am. J. Epidemiol. 85, 459.

38. Grossowitz, N., Mercado, A. and Goldblum, N. (1960) A simple method for concentration of live and formaldehyde-inactivated poliovirus. Proc. Soc. exp. Biol. Med. 103, 872.

39. Wallis, C. and Melnick, J.L. (1967) Concentration of viruses on Aluminium Phosphate and Aluminium Hydroxide precipitates. In Transmission of viruses by the water route (Edited by Berg, G.) Interscience, London.

40. Lund, E. and Hedstrom, C.E.. (1967) Recovery of viruses from a sewage treatment plant. In Transmission of viruses by the water route (Edited by Berg, G.) Interscience, London.

41. Wallis, C., Grinstein, S., Melnick, J.L. and Field, J.E., (1969) Concentration of viruses from sewage and excreta on insoluble polyelectrolytes. Appl. Microbiol. 18, 1007.

42. Grinstein, S., Melnick, J.L. and Wallis, C., (1970) Virus isolations from sewage and from a stream receiving effluents of sewage treatment plants. Bull. Wld. Hlth. Org. 42, 291.

43. Wallis, C., Melnick, J.L. and Fields, J.E. (1971) Detection of viruses in large volumes of natural water by concentration on insoluable polyelectrolytes. Water Research (in press).

44. Kelly, S.M. (1953) Detection and occurence of Coxsackie viruses in sewage. Am. J. Pub. Health 43, 1532.

45. Gravelle, C.R. and Chin, T.D.Y. (1967) Enterovirus isolations from sewage: a comparison of three methods. J. Infect. Dis. 109, 205.

46. Smith, J.E. and Courtney, R.J., (1967) Concentration and detection of viral particles by passive hemagglutination. In Transmission of viruses by the waterroute (Edited by Berg, G.), Interscience, London.

47. Moore, M., Ludovici, P.P. and Jetes, W.S. (1970) Quantitative methods for the concentration of viruses in wastewater. J.W.P.C.F. 42, R21.

48. Grindsod, J. and Cliver, D.O. (1969) Limitations of the polymer two phase system for detection of viruses. Arch. ges. Virusforsch. 28, 337.

STUDIES ON THE DETECTION AND CONTROL OF ENTEROVIRUSES IN THE WATER ENVIRONMENT

Hillel I. Shuval, Badri Fattal, Samuel Cymbalista and Natan Goldblum

1. INTRODUCTION

With the progress in the field of water purification in the more advanced countries of the world, and the resulting reduction or elimination of waterborne diseases, a certain feeling of confidence and security has been achieved. However, the massive waterborne outbreak of viral hepatitis that occurred in Delhi in 1955 (Dennis 1959) gave grounds for some second thoughts as to the effectiveness of standard water purification processes. It appears that the treated water met the accepted coliform bacteria standards but that viruses were able to pass through the treatment plant when challenged by heavily polluted raw water.

An eminent panel of public health experts (U.S. Department of Health, Education and Welfare, 1962), summarized the situation as follows: "More than 70 viruses have been detected in human feces. All may be present in sewage. Viruses

pass through sewage treatment plants, persist in contaminated waters, and may penetrate the water treatment plants. Numerous outbreaks of infectious hepatitus have been traced to contaminated drinking water. The occurrence of such incidents appears to be increasing". A latter report (President's Science Advisory Committee, 1965) stated that "The capability of present water pollution control technology is clearly inadequate as far as viruses are concerned".

This situation has pointed out the need for intensive investigation and study of all aspects concerning the detection and control of pathogenic viruses in the water environment. Engineers and scientists in the field of water quality control and pollution prevention can no longer rely solely on such standard and generally accepted parameters of microbial pollution as coliform bacteria, since there is now ample evidence that these indicator organisms can under certain circumstances be more sensitive to treatment processes or natural inactivation factors than the more resistent pathogenic enteroviruses (Shuval et al., 1967a).

There is a need to develop new and sensitive tools to detect small numbers of enteroviruses in large volumes of water in order to monitor drinking water supplies. Likewise it is imperative to reevaluate the various water and waste water treatment systems to determine their virus removal efficiency.

This paper will present some of the major findings of our studies on the detection and control of enteroviruses in the water environment. Included are a new method developed to concentrate and detect viruses in water; a survey of the levels of enteroviruses in water and waste water in various communities; studies in which various wastewater treatment processes including stabilization ponds, biological filtration and chlorination have been evaluated for their virus removal efficiency; and the results of preliminary investigations of the dispersion and inactivation of viruses in sea water.

2. The Phase-Separation Method for Detecting Viruses in Water and Wastewater

The Phase-Separation (PS) method for concentrating and detecting viruses in water was initially reported on at the Symposium on the Transmission of Viruses by the Water Route in Cincinati in 1965 (Shuval et al., 1967b) while a full evaluation of the method as further developed and refined has subsequently been published (Shuval et. al., 1969a).

The method which is described in full in Appendix I, is simple and inexpensive. It involves adding appropriate chemicals (Sodium dextran sulfate, polyethylene glycol and sodium chloride) to the water sample to be tested which is placed in a separatory funnel and held at 4°C overnight. The small bottom phase formed is drained off together with the interphase. This bottom phase has a volume of about 1:200 of the original water sample and has been shown to contain essentially all of the viruses present in the water sample. For still further concentration, the concentrate is treated with sodium chloride and once again a two phase system is formed after about 12-24 hours at 4°C. This time the viruses are concentrated in the small top phase. By this two-step procedure it is possible to concentrate the viruses in a water sample by a factor of about 500.

The concentrate is then assayed for viruses by the plaque forming technique in disposable plastic petri dishes on primary rhesus kidney cells (RKC) monolayers with the results reported as plaque forming units (PFU) per liter of water tested. An alternative method of assaying the viruses in the concentrate, to be preferred when expected virus concentrations are very low, is to seed 1-2 ml of the concentrate in each of a series of five tissue culture bottles (milk dilution bottles). Bottles showing cytopathogenic effect (CPE) in up to 14 days are considered as presumptive positives for enterovirus and the numerical virus concentration can be reported in a manner similar to the "most probable number" (mpn) commonly used, in testing for coliform organisms.

In many instances water quality control laboratories will not be able to carry out independent virological assay work. But with the PS method it is possible to

freeze the concentrated sample at -20°C for latter shipment to a specialized virus laboratory which can do the quantitative virus assay of the sample as well as identify any virus strains isolated.

Extensive laboratory and field testing of the PS method has shown it can effectivity concentrate viruses in water and waste water samples by a factor of 500 and that as few as one or two virus infectious units can be detected in one liter of water with about 85% reliability. For practical purposes it is now possible to assay a 5 liter water sample and to determine whether viruses are present in it or not.

Although the optimum virus monitoring method should be able to assay tens or even hundreds of liters of water for the presence of viruses, it is felt that the PS method can serve a useful purpose in initiating the routine virus monitoring of potable water supplies, since it is simple and inexpensive, enabling it to be used by most routine water quality control laboratories. This would be particularly true if arrangements can be worked out with regional or national virological laboratories to assay the frozen concentrated samples sent in from local laboratories.

3. Enterovirus Concentrations in Sewage

In the absence of quantitative methods for evaluating the concentration of viruses in water and sewage, a number of authors have made estimates based on information from various experimental sources. In one such estimate it was assumed that the initial concentration of enteroviruses in raw wastewater varies between 20 and 700 PFU/100ml with considerable seasonal variation, while the concentration of viruses in polluted rivers and streams would be expected to be a hundred fold lower (Clarke et al., 1964).

Studies carried out in Israel using the PS method, have provided a preliminary picture of the concentrations of enteroviruses in wastewater from different communities as well as in surface water systems exposed to contamination, which tend to confirm these earlier estimates.

Table 1: Enteroviruses in Raw Sewage Samples from two Neighborhoods in Jerusalem

Date	Katamon PFU/1	Rasco PFU/1
20. 4.67	65	*
7. 5.67	84	*
18. 6.67	1165	9
7. 9.67	109	104
25. 9.67	682	*
12.10.67	119	216
18. 8.68	4185	416
25. 6.68	1213	28
2. 7.68	4035	152
9. 7.68	783	23
25. 7.68	50	91
31. 7.68	3375	101
7. 8.68	101	339
14. 8.68	-	26
27. 8.68	7349	3159
4. 9.68	1844	2977
	Av. 1677	Av. 480

* These samples produced no plaques but did produce CPE on multiple passages.

Table 1 shows the concentration of enteroviruses in raw sewage samples from two neighbourhoods in Jerusalem of differing socioeconomic levels during the period April 1967 - September 1968. It is noted, that the enterovirus concentrations in the raw sewage samples from the Katamon area average 1677 PFU/1 with a high of 7349 PFU/1, while that of the Rassco area for the same period average 480 PFU/1 with a maximum of 3159 PFU/1. The socioeconomic level in the Rassco area is considerably higher than that of the Katamon area.

Table 2 shows the enterovirus levels detected in raw sewage samples taken from the main pumping station in Tel Aviv at Reading during the period April to November 1968. The average enterovirus concentration was 663.5 PFU/1.

Table 3 shows the concentration of enteroviruses in the raw sewage of Kiryat Shmoneh in the upper Galilee. This town with a population of 18,000, disposes of its raw sewage to a drainage ditch which discharges into the Jordan River. During the period under study, from November 1968 to April 1969, the average virus concentration was 1572 PFU/1 with a range of 5 - 11,184 PFU/1.

Table 4 shows the concentration of entroviruses in the raw sewage of the city of Tiberias (population 25,000) on the shores of Lake Kinereth. The average virus concentration was 1135 PFU/1. with a range of 16 - 7546 PFU/1.

From this survey of the enterovirus levels in raw sewage samples in a number of communities in Israel over a period of about one year, it appears that with the PS method it is possible to detect viruses in every case and that the average virus concentration is 1050 PFU/1. No clear seasonal variations in enterovirus levels in raw sewage was evident. Parallel bacteriological tests during the same period indicate that the coliform counts of raw sewage average about 10^9/100ml. This indicates an enterovirus to coliform ratio in Israel in raw sewage roughly of about 1 to 10,000,000.

This ratio which is much larger than the theoretical, and somewhat improbable calculations of 1 to 65,000 made earlier (Clarke et al., 1964), is based on

Table 2: Enteroviruses in Raw Sewage Samples from the Reading Pumping Station, Tel Aviv

Date	Viruses PFU/1
25. 4.68	91
5. 6.68	1094
12. 6.68	280
26. 6.68	371
3. 7.68	476
17. 7.68	367
14. 8.68	1711
21. 8.68	342
28. 8.68	236
10. 9.68	522
16. 9.68	1055
10.10.68	779
17.10.68	392
24.10.68	1493
6.11.68	152
12.11.68	889
19.11.68	457
	Av. 663.5

Table 3: Enteroviruses in Raw Sewage Samples from Kiryat Shmoneh

Date	Viruses PFU/1
11. 2.68	5
3. 4.68	162
29. 4.68	1327
26. 5.68	11184
30.12.68	614
13. 1.69	1175
17. 2.69	135
4. 3.69	183
17. 3.69	1292
31. 3.69	652
21. 4.69	565
Av.	1572

Table 4: Enteriviruses in Raw Sewage Samples from Tiberias

Date	Viruses PFU/1
5. 5.68	114
9. 6.68	1996
18.12.68	268
1. 1.69	7546
13. 1.69	161
3. 2.69	362
17. 2.69	1413
4. 3.69	184
17. 3.69	426
31. 3.69	16
21. 4.69	1269
	Av. 1135

Table 5: Virus Removal Efficiency in the Mefachim Stabilization Ponds - Jerusalem, 1968

Test No.	Virus Concentration Raw Sewage PFU/1	Virus Concentration Effluent PFU/1	Virus Removal Efficiency %
1	1366	529	61.5
2	903	40	95.5
3	12	40	Negative
4	31	71	Negative
5	288	102	47.0
6	132	28	79.0
7	357	572	Negative
8	1185	300	75.0
9	3889	370	91.0
			Av. 67.5%

samples were taken at varying distances down stream from the main source of fecal contamination which is the inflow of raw sewage from the inhabitants of the town of Kiryat Shmoneh. Of the 34 river samples tested, enteroviruses were detected in only three cases (28 PFU/1, 1.4 PFU, and 1 PFU/1). It is interesting to note that the highest concentration of enteroviruses in the Jordan River water, at a point some 25 Kms. down stream from the primary source of contamination, was detected on the same day that the highest concentration of entroviruses was found in the raw sewage of Kiryat Shmoneh (11,184 PFU/1).

In another series of tests, enteroviruses were detected in one out of three samples taken from the Soreq stream. This heavily polluted stream carries the major portion of the untreated sewage of Jerusalem with only minimal dilution. The point the samples were taken was about 30 kms down stream from Jerusalem. The stream is very sluggish and in places flows through large swampy areas and it is estimated roughly that the time of flow from the point that fresh sewage entered the stream to the point of sampling was about two days.

5. The Virus Removal Efficiency of Wastewater Treatment Plants

Two wastewater treatment plants were evaluated as to their virus removal efficiency. The first was the Mefachim stabilization ponds treating the sewage of an area in north-eastern Jerusalem. This plant is made up of a series of four stabilization ponds providing about a 20 day detention period. The Biological Oxigen Demand (B.O.D.) of the raw sewage has been shown to be 450 and the B.O.D. removal efficiency is about 80%. Coliform reductions of about 90% are obtained.

Table 5 shows the virus removal efficiency of the plant on eight different days of sampling in 1968. In 3 out of 9 cases the removal was negative, i.e. the effluent showed higher virus concentrations than the influent. The average virus removal efficiency of the plant was 67.5%, taking the negative efficiencies as zero.

The second plant studied from May 1968 to April 1969 was the Tiberias municipal sewage treatment plant. This plant is a standard biological filter plant. The

Table 6: Virus Removal Efficiency in the Tiberias Biological Filtration Plant 1968 - 1969

Test No.	Virus Concentration in Raw Sewage PFU/1	Virus Concentration in Settled Sewage PFU/1	Efficiency of Primary Sedimentation %	Virus Concentration in final Effluent PFU/1	Total Plant Efficiency %
1	114	275	Negative	103	9
2	1996	-	-	4487	Negative
3	268	1307	Negative	1286	Negative
4	7546	1200	84	1207	84
5	161	653	Negative	400	Negative
6	362	521	Negative	127	65
7	143	157	Negative	259	Negative
8	184	209	Negative	-	-
9	426	538	Negative	230	46
10	16	918	Negative	201	Negative
11	1269	551	56	858	32
				Av.	24

Table 7: Inactivation of Natural Virus Flora in Sewage Effluent with Chlorine (11 mg/1)

Exp. No.	Virus Concn. in effluent PFU/1	Virus Concn. after 30 mins. PFU/1	Inactivation %	Virus Concn. after 4 hrs. PFU/1	Inactivation %
1	12	10	16.5	< 1	> 91.5
1	244	45	81.5	< 1	> 99.5

primary sedimentation is in an Imhoff tank with the effluent passing to two biological filters in parallel. The effluent passes through a final sedimentation tank before being discharged. The B.O.D. removal efficiency of the total plant is about 85% with 92% removal of coliforms. Table 6 shows that the efficiency of virus removal in the Imhoff tank was negative in 8 out of 10 series of tests, while the total plant efficiency for virus removal was negative in 5 out of 10 tests. If the negative series are assumed as showing zero efficiency, the overall average plant efficiency is calculated at 24%. In other words, an insignificant level of virus removal.

These two series of tests confirm findings of others that certain types of standard wastewater treatment plants have very low efficiencies in removing enteroviruses from sewage.

6. Inactivation of Enteroviruses in Sewage by Chlorination

A number of studies have pointed to the fact that certain of the enteroviruses are more resistent than coliform organisms to chlorination, both in sewage and water (Clarke et al., 1954, Shuval et al., 1967a).

In our studies we have attempted to compare the sensitivity of poliovirus, echovirus and coliforms to chlorination in sewage effluent. In these studies strains of poliovirus type 1 and echovirus type 9 were seeded into samples of effluent from the Haifa municipal high rate biological filter, sewage treatment plant. The effluent has an average B.O.D. of 45mg/1 with an average of 50mg/1 of suspended solids. The pH ranged from 7.7 to 7.8 while the concentration of ammonia varied from 5 to 20mg/1.

Varying doses of chlorine were added to the samples of effluent after an appropriate virus seed was inoculated. The chlorine residuals were determined by the iodometric backtitration method (Standard methods for the examination of water and wastewater, 1967).

Fig. 1.

CHLORINE RESIDUAL IN SEWAGE EFFLUENT AT VARYING CONTACT TIMES

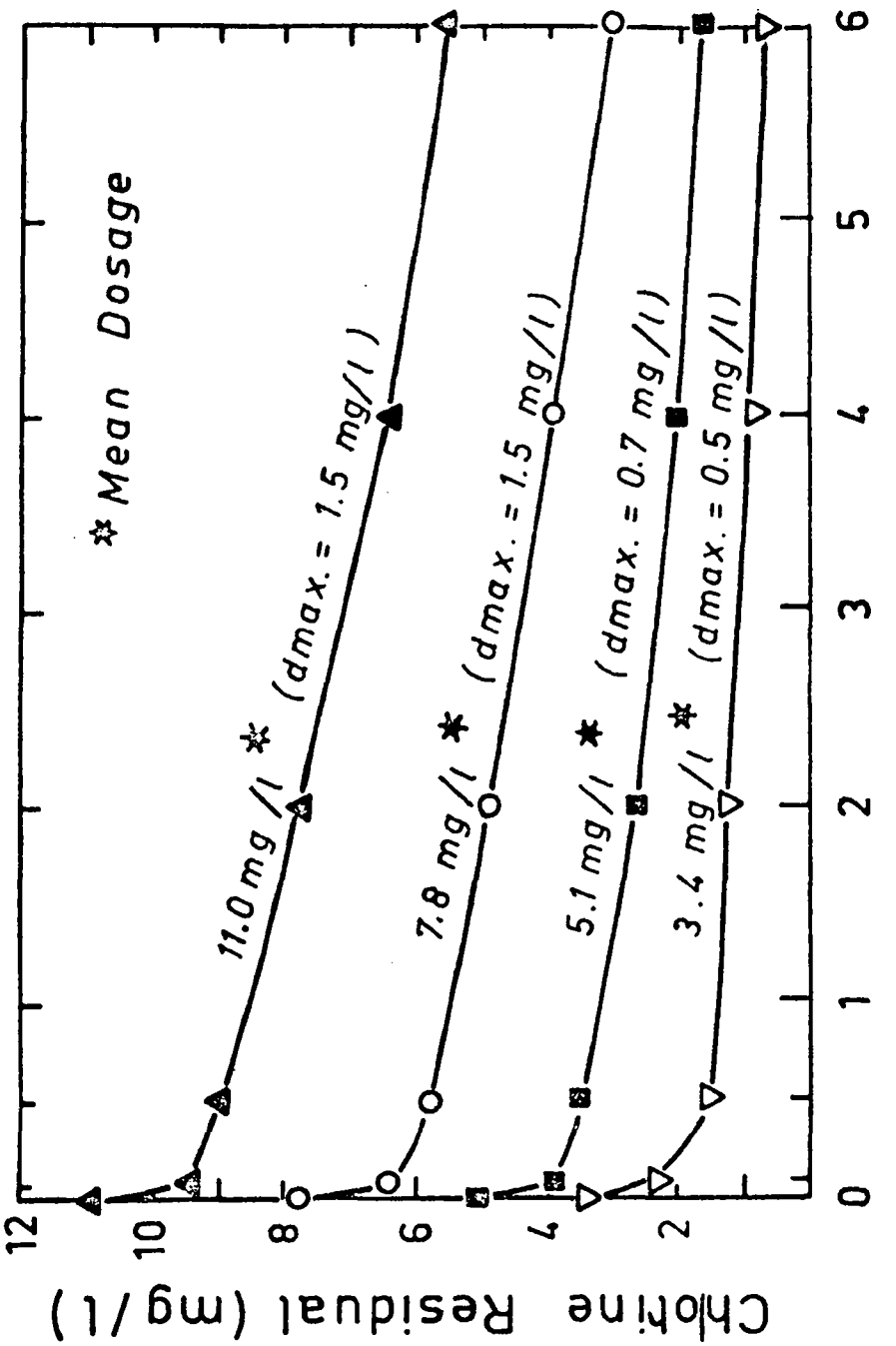


FIG. 2.

INACTIVATION OF COLIFORMS IN SEWAGE EFFLUENT AT VARYING CHLORINE DOSES

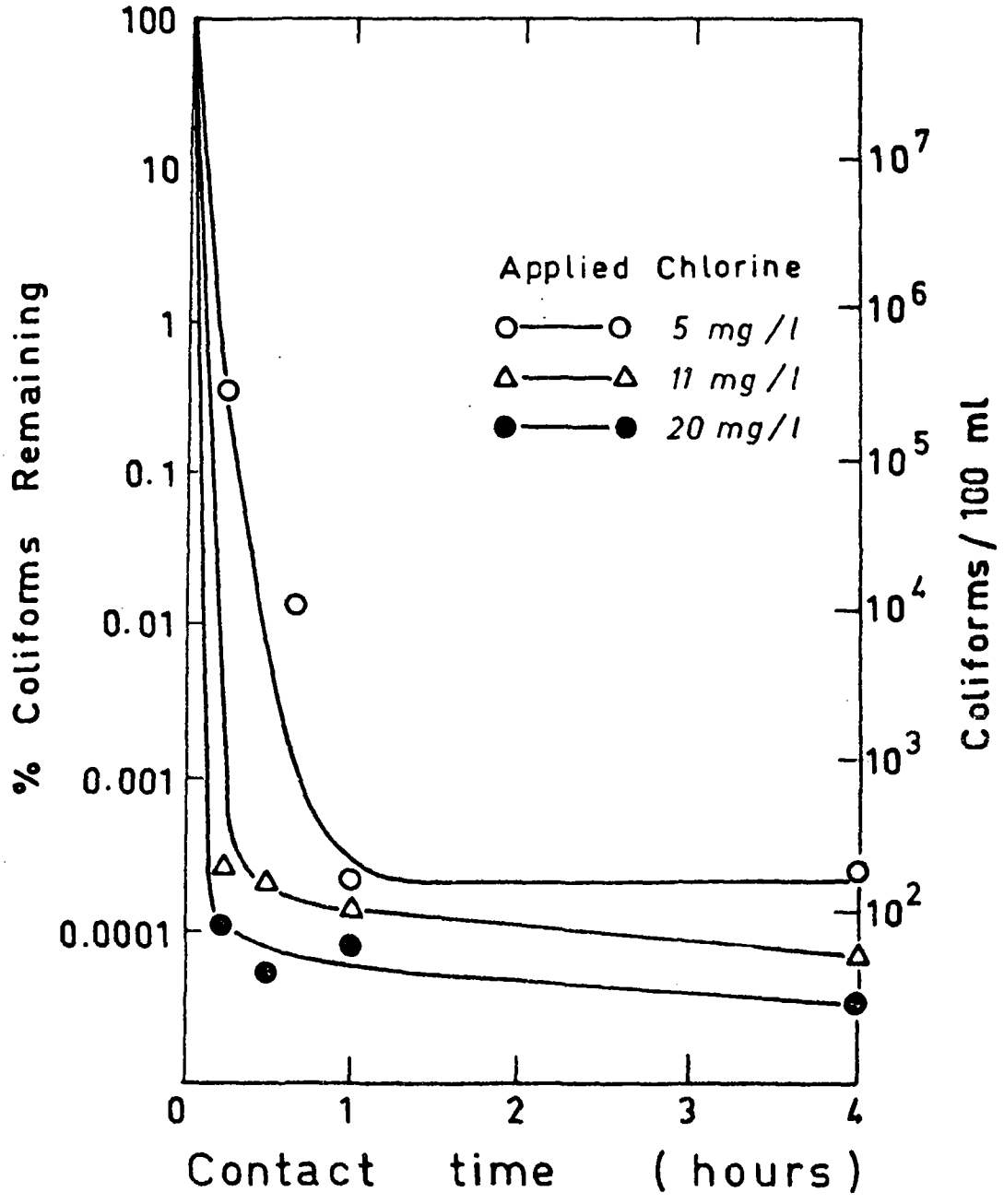
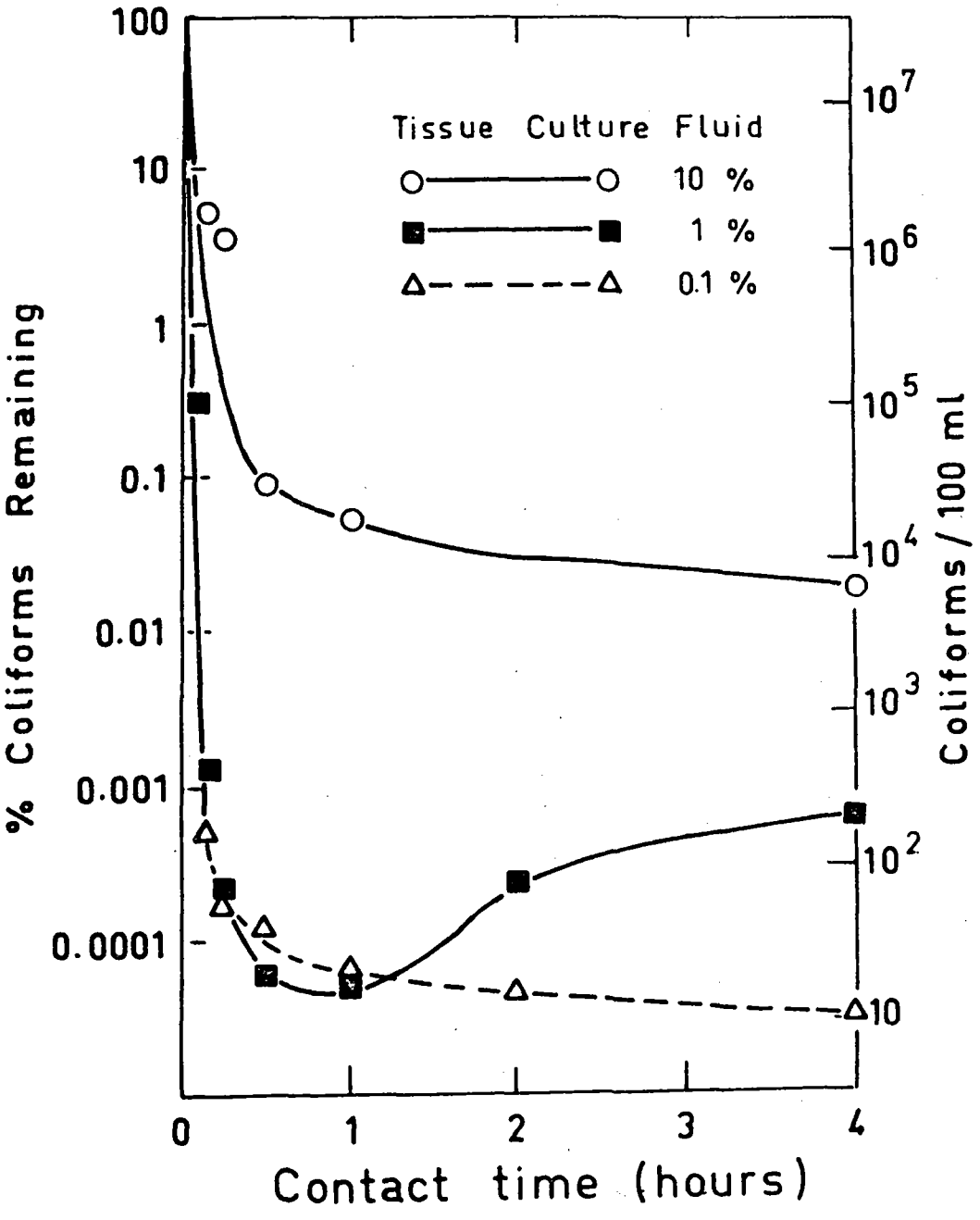


FIG. 3.

INACTIVATION OF COLIFORMS BY 11 mg / l APPLIED CHLORINE IN SEWAGE EFFLUENT IN THE PRESENCE OF VARYING CONCENTRATIONS OF TISSUE CULTURE FLUID



In figure 1 the chlorine residuals remaining in the effluent at varying contact times up to 6 hrs are shown. In most cases the chlorine residual remaining at the end of 6 hrs. was between half and one-third of the original dose added. Under the conditions obtaining in these tests, it is assumed that essentially all the chlorine applied was transformed rapidly to chloramines because of the high concentration of ammonia and organic nitrogenous materials present.

Fig. 2 shows the percent of coliforms remaining in the sewage effluent at varying chlorine doses. It can be seen that a 5 to 6 log reduction is achieved in one hour with applied chlorine doses of from 5 - 20 mg/1, with the coliform levels being reduced to about 100/100 ml.

In our earlier experiments, the tissue culture fluids added together with the virus inoculum apparently interferred with the inactivation processes. In Fig. 3 it can be seen that only a 3 log reduction of coliforms takes place when 11 mg/1 of chlorine is applied to effluent containing a 10% tissue culture fluid inoculum, while a six log reduction is obtained in one hour with 1% and 0.1% of inoculum with the same chlorine dose. The same interferring effect on poliovirus inactivation is shown in Fig. 4.

However, when a 0.1% of concentrated poliovirus suspension was inoculated into the sewage effluent, poliovirus reductions in one hour were very much less than the coliform reductions obtained under equivalent conditions. (See Fig. 5). Only about a 1 log reduction was obtained with 5 and 11 mg/1 of chlorine, while a three log reduction was obtained with 20 mg/1 of applied chlorine. Inactivation continued throughout the four hour period of the test with somewhat improved reduction being obtained with time.

In Fig. 6 it can be seen that the ECHO 9 strain tested was inactivated more effectively than the poliovirus strain tested.

In Fig. 7 a comparison of the chlorine concentrations and contact times required to achieve a 99.9% inactivation of poliovirus, echovirus and coliforms is

FIG 4

INACTIVATION OF VARIOUS CONCENTRATIONS OF POLIOVIRUS SUSPENSIONS IN SEWAGE EFFLUENT TREATED WITH 11 mg/l CHLORINE

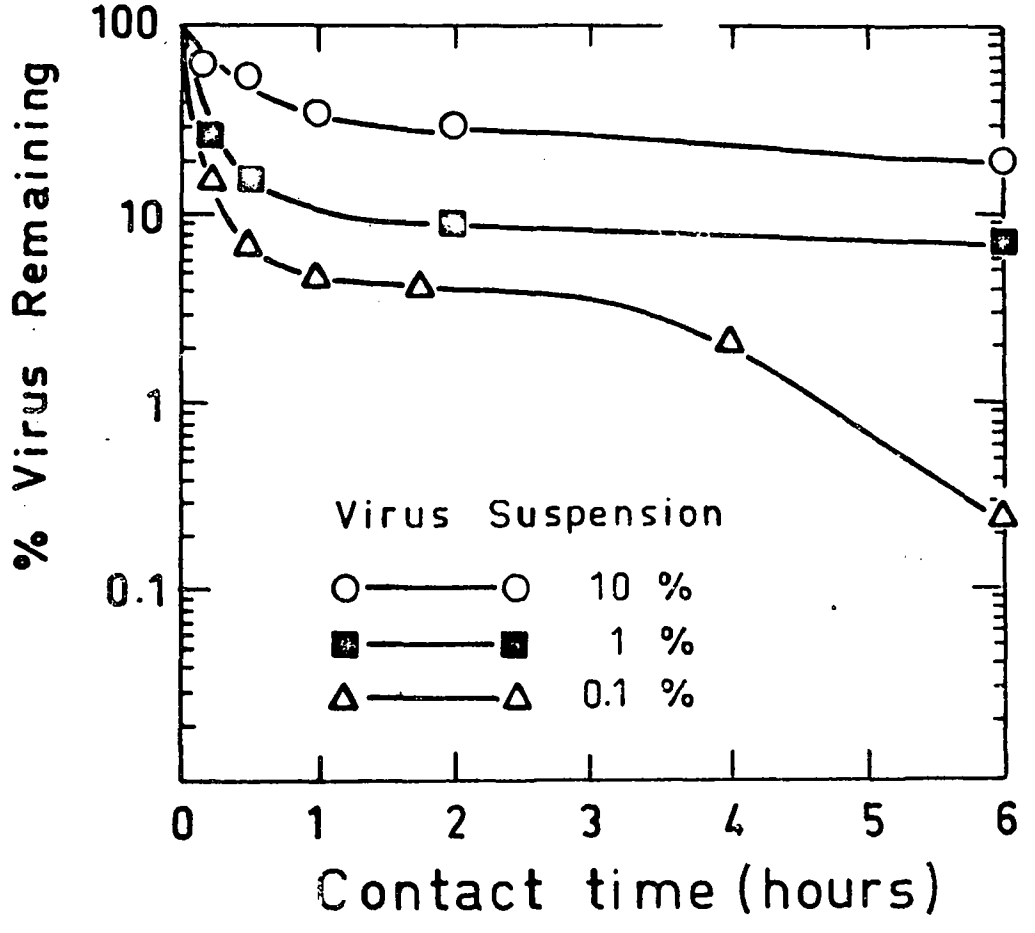


FIG. 5

INACTIVATION OF 0.1 % POLIOVIRUS SUSPENSION IN SEWAGE EFFLUENT BY VARIOUS CONCENTRATIONS OF APPLIED CHLORINE

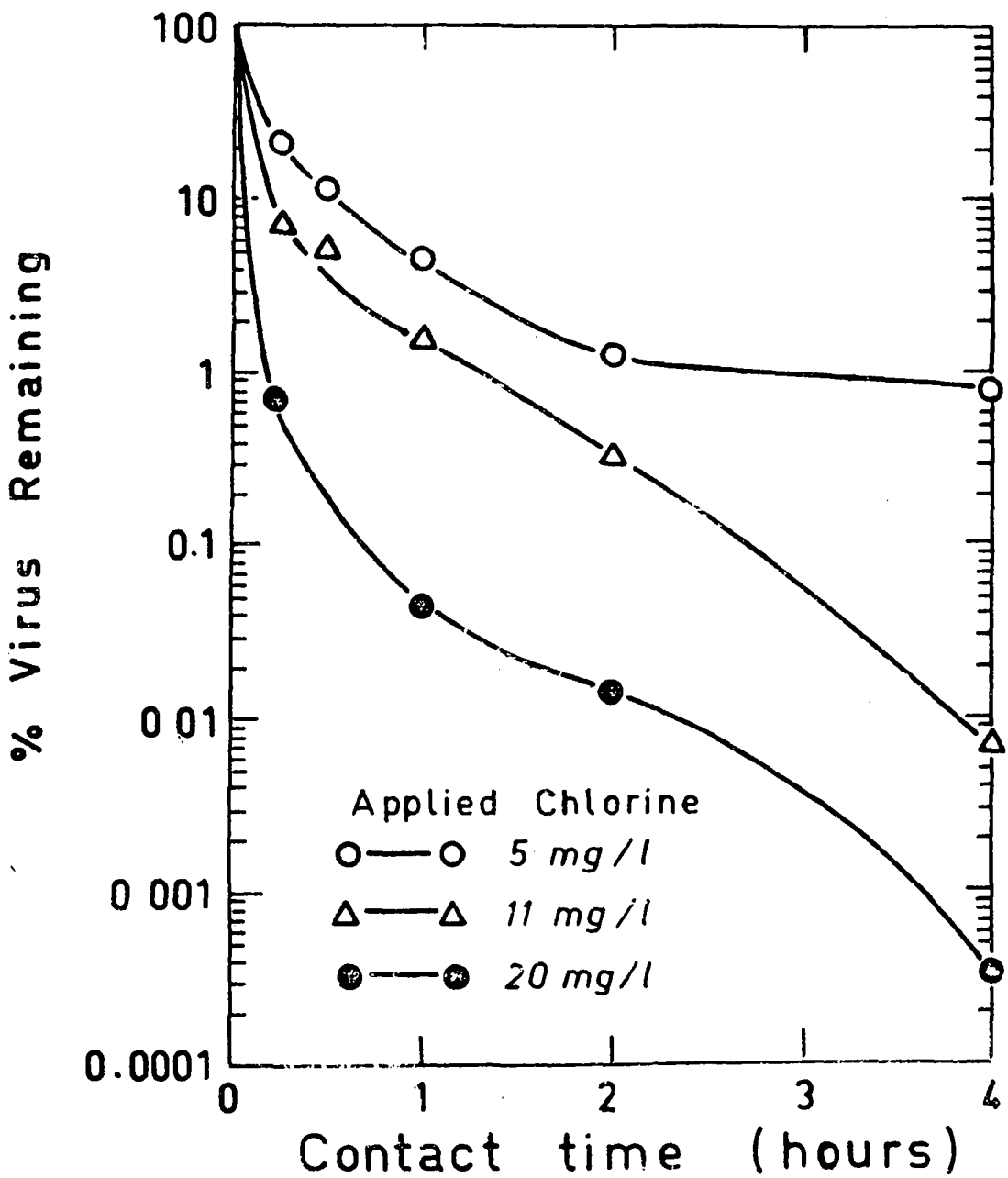


Fig. 6.

SURVIVAL OF ECHOVIRUS IN SEWAGE AT VARYING CHLORINE DOSES

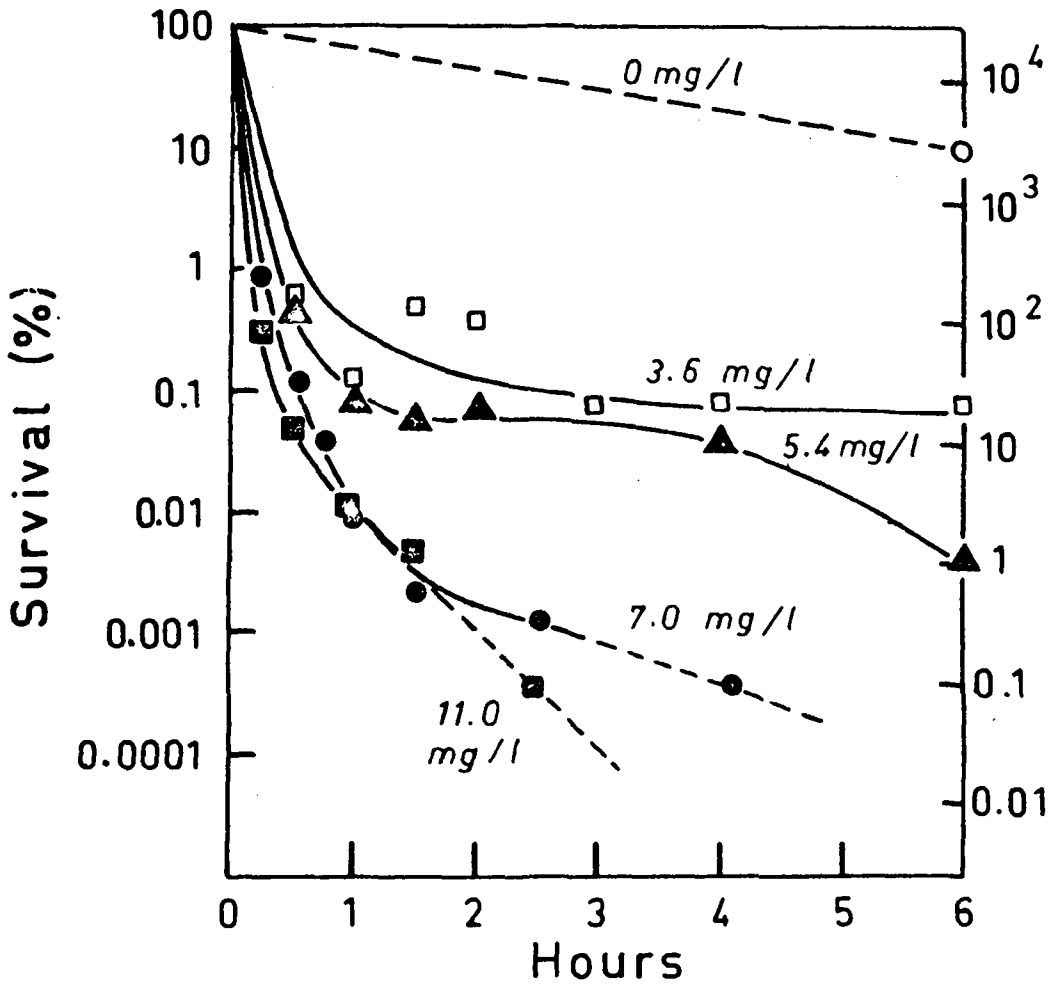
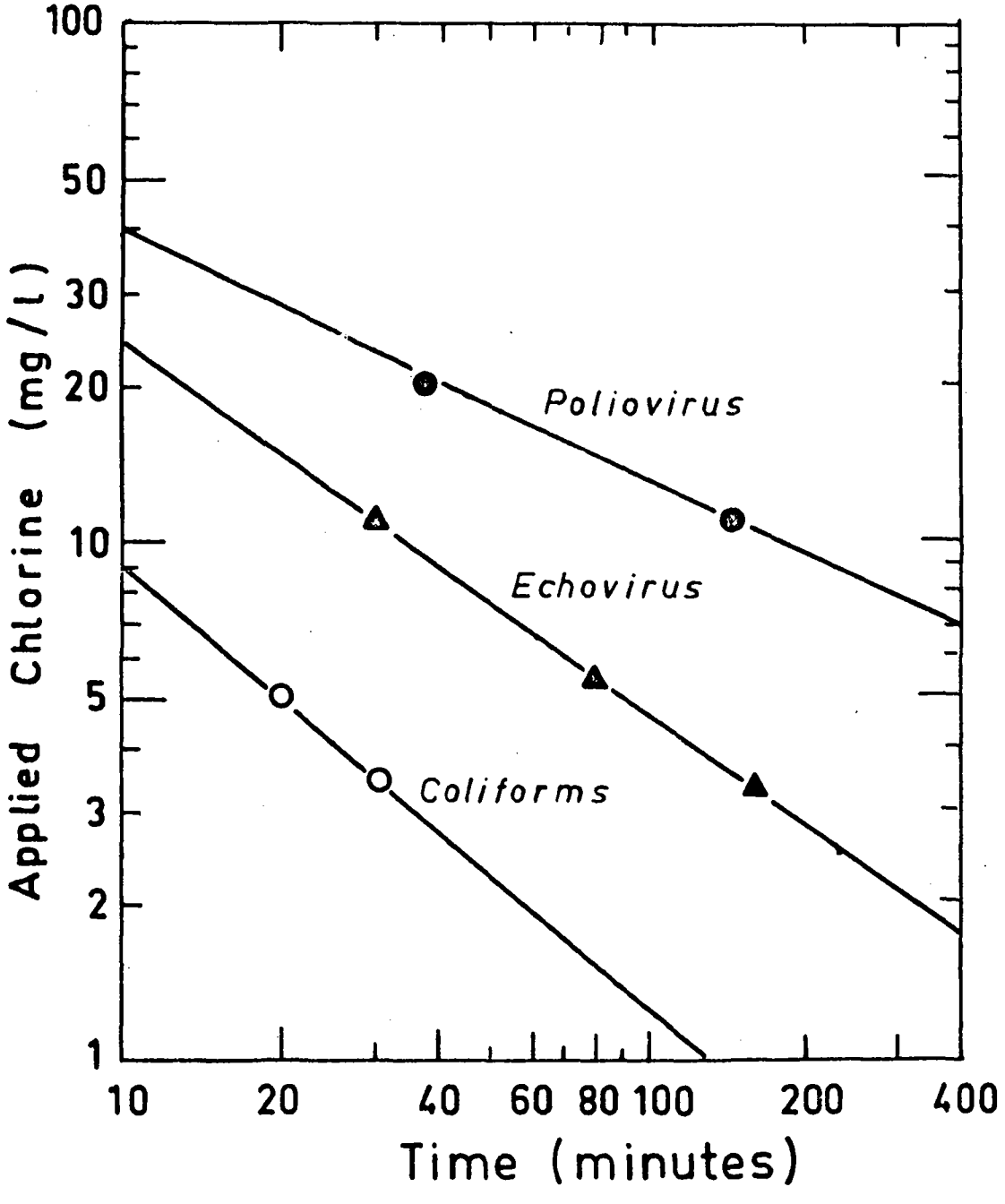


FIG. 7.

CONCENTRATION - TIME RELATIONSHIP FOR 99.9 % INACTIVATION OF POLIOVIRUS ECHOVIRUS AND COLIFORMS IN SEWAGE EFFLUENT BY CHLORINE AT 20°C



given. From this figure it can be seen that with a one hour contact time 2 mg/1 of applied chlorine is sufficient for coliforms, about 8 mg/1 is required for the echovirus, while about 20 mg/1 of chlorine is required to achieve an equivalent inactivation of poliovirus. Under the conditions of this study, a chlorine dose, some ten times greater than that required for coliforms was required for the inactivation of the poliovirus in sewage effluent.

It can be clearly seen that usual wastewater chlorination practices are insufficient to provide effective inactivation of poliovirus which can be considered as a model of the more resistant forms of enteric viruses. This also points out the possible dangers of a situation where coliform levels have been greatly reduced by chlorination but still leaving significant levels of enteroviruses.

In addition to the experiments in which laboratory strains of viruses were inoculated into sewage effluent, a number of experiments were carried out in which the viruses naturally found in the effluent were tested by the PS method, before and after chlorination. These tests, more closely simulated true field conditions and avoided the many problems associated with inoculating higher concentrations of laboratory strains of viruses.

In these tests, with 11 mg/1 of chlorine applied, (Table 7) it can be seen that in one case little virus inactivation occurred in 30 minutes while in the other an 81.5% reduction was achieved. However, after 4 hours of contact the viruses were reduced to an undetectable level. A conservative estimate of the virucidal efficiency of 11 mg/1 of chlorine with 4 hours of contact is based on the assumption that the limit of detection of the phase separation method is 1 PFU/1. This would mean a virus reduction of something greater than 91.5% and 99.5% respectively.

7. The Dispersion and Inactivation of Enteroviruses in Coastal Waters

The coliform index has served as an accepted parameter of pollution of coastal water. The design of sewage outfall sewers are often based on achieving

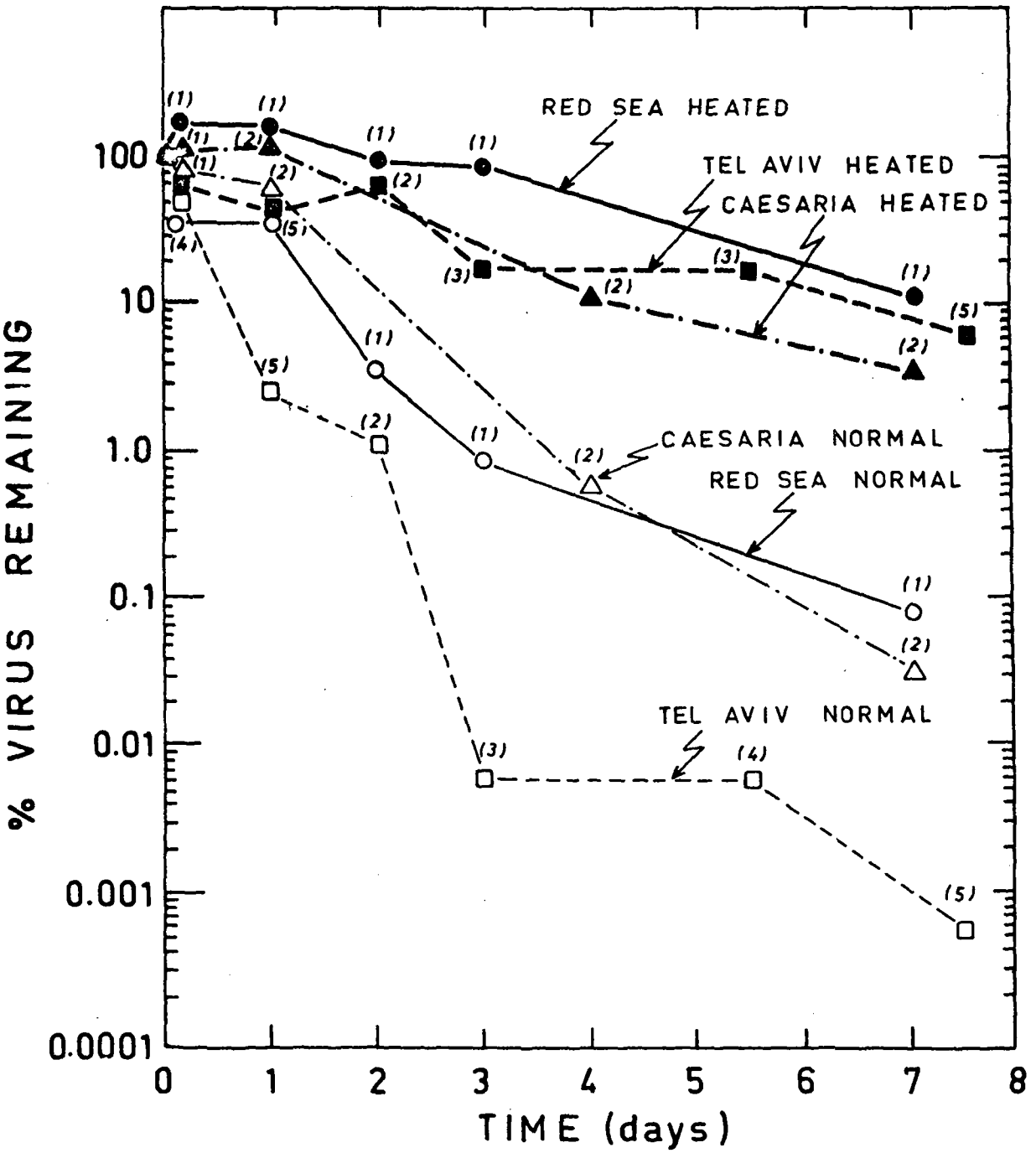
certain coliform standards along bathing beaches (Pomeroy, 1960, Harremoes, 1966). Little attention has been paid in the past to the possible role of enterovirus in the pollution of bathing beaches, although studies concerning the contamination of shellfish have pointed out that a satisfactory coliform index in seawater does not necessarily mean that the area is free of enterovirus (Metcalf et al., 1968).

A study of the dispersion and inactivation of enteroviruses in seawater was undertaken by our group in cooperation with the staff of the Ministry of Health and the Soreq Nuclear Research Center. Our earlier efforts had been limited to the study of bacterial parameters of pollution (Shuval et al., 1968). With the adaptation of the PS method for detecting entroviruses in seawater samples, it was felt that a quantitative field study could be effectively carried out.

Preliminary studies initiated in the summer of 1968 demonstrated that enteroviruses could be detected routinely in sea water samples collected from a number of fixed stations marked by bouys placed at varying distances from the 880 meter long Reading outfall sewer off the coast of Tel Aviv. Some 100,000 cu m of sewage is discharged daily into the sea through this outfall. As shown previously in Table 2, the average virus concentration in the raw sewage at Reading is 663 PFU/1. The average virus concentration detected in the sea surface immediately above the diffusers at the end of the outfall based on 15 samples was 8 PFU/1. Tests using ^{82}Br as a radioactive tracer tended to confirm this finding (Gilat et al., 1969). Six out of 48 samples of seawater tested for viruses from more distant sampling stations were positive. It is interesting to note that two of these positive samples were taken from a bouy opposite the Tel Baruch bathing beach, some 1.5 km. north of the point of sewage discharge. One of these samples showed a virus concentration of 32 PFU/1. on that same day the seawater sample taken over the end of the outfall in the "boil" showed a virus concentration of 60PFU/1. The radiosotope tracer studies indicated that the physical dilution between these two points might be as low as 1 to 10. These results can be considered only of a qualitative nature but do indicate that with virus detection methods now available it is possible to initiate quantitative evaluation of virus contamination in coastal waters.

Fig. 8.

INACTIVATION OF POLIOVIRUS IN NORMAL AND HEAT-TREATED SEA WATER SAMPLES FROM THE MEDITERRANIAN AND RED SEA



(n) = number of experiments

Antibacterial factors in seawater have been studied for a number of years (Mitchel, 1968) but there is little information concerning the antiviral activity of seawater, therefore, simultaneously with the field work, laboratory studies were initiated to determine the nature and extent of any antiviral factors in seawater.

Our studies, reported on in full elsewhere, (Shuval et al., 1969b) have shown that there is a definite antiviral factor in seawater which appears to be of a biological nature. When poliovirus type 1 was seeded into seawater samples, taken from various points along the Mediterranean and Red Sea coast, a 3 to 6 log reducation was obtained in 7 days, at $22^{o}C \pm 3^{o}$. Parallel samples heated to $90^{o}C$ for 1 hour and then seeded with an equal inoculum of poliovirus showed only about a one log reduction at the end of the seven day period (See Fig. 8). These findings parallel the findings of a group in Sweden studying this question, who feel that the microorganism, Vibrio marinus, is the responsibile agent (Magnusson et al., 1967). Our studies have shown that the antiviral factor in seawater is heat labile and ether sensitive. It is also removed from seawater samples by filtration through a membrane filter with a 0.45 micron pore size. Interestingly enough, the factor is not completely removed from the supernatant of a seawater sample centrifuged at 60,000 x g., however heat treated seawater samples to which the centrifuged pellet of the above seawater sample was added showed a similar degree of antiviral activity as natural seawater samples. It is also noted that the antiviral factor in samples of Tel Aviv coastal waters appears stronger than that of sea samples taken at the relatively unpolluted areas of Caesaria on the Mediterranean or Elath on the Red Sea. One preliminary explanation is that the factor may be stimulated by the higher nutrient levels resulting from the disposal of large volumes of sewage into the sea at Tel Aviv. There seems to be evidence that the antiviral factor is of a complex nature not completely explained by any reported findings to date.

Our preliminary laboratory findings indicate that the time required for a 90% decrease of poliovirus in seawater (T_{90}) may be somewhere between 12 and 48 hours. In light of the relatively rapid travel of sewage disposed of into the sea

which may range from one half to several km/hr, it can be assumed that pollution will reach nearby beaches in a few hours time at most. Thus, it may be difficult to depend on the natural virus inactivation in seawater as a safeguard above and beyond actual physical dilution. A conservative estimate of the T_{90} for coliform organisms off the Tel Aviv coast based on the radioisotope tracer experiments is from one to three hours. This appears to be much more rapid than that found for poliovirus. Again this points to the possibility of enteroviruses being present in water of low coliform count.

Further intensive studies are being carried out to isolate and identify the antiviral factor or factors in seawater and to study their mode of action. Such studies may be of importance above and beyond its ecological role in the self-purification capacity of seawater.

8. SUMMARY AND CONCLUSIONS

a. It has been felt for some time that there is a need to develop methods for the monitoring of water supplies for enterovirus contamination. The Phase-Separation (PS) method which has been developed and extensively tested both in the laboratory and in the field, is both simple and inexpensive and should now provide local water quality control laboratories with a tool for carrying out such tests in collaboration with regional or national virus laboratories. It has been shown that this method can concentrate the viruses in a water sample by a factor of 500 and is capable of detecting as few as one or two virus infectious units per liter of water.

b. A survey of the enterovirus concentration in raw sewage in four cities in Israel, based on regular tests carried out over a period of about one year has shown that with the PS method it is possible to detect enterovirus in all samples regardless of season. The average enterovirus concentrations of raw sewage from cities in Israel is 1050 PFU/1 while the average enterovirus to coliform ratio is about one to 10,000,000. In the course of this survey, all types of polio-virus as well as types of echovirus and coxsackievirus were detected. As many as four different types of virus have been isolated from one sewage sample.

c. In the course of examining some 70 samples of drinking water, enteroviruses were detected in two samples by the PS method. In this case the water came from a polluted municipal well suspected of being involved in a common source epidemic of infectious hepatitis. This is felt to be the first reported isolation of viruses in a domestic water supply involved in a waterborne epidemic.

d. A survey of surface water sources contaminated to varying degrees, produced five samples positive for enteroviruses out of 37 tested. In three cases positive samples were detected in the Jordan River, one at a point some 25 km downstream from the main point of sewage inflow.

e. Standard methods of wastewater purification may be very ineffective in removing enterovirus contamination. This was supported in a study of a municipal biological filtration plant and a stabilization pond system. The virus removal in the primary sedimentation stage (Imhoff tank) was essentially zero while only in 5 out of 10 tests was there any virus reduction through the whole biological filtration plant. The average virus removal in the plant under study was negligable, although the plant was considered to be operating efficiently by the usual standard parameters. B.O.B. reduction was 85% and coliform counts were reduced by 92%. The oxidation pond system with about 20 days detention was somewhat more effective. In three out of nine cases, there was no removal, with the average virus removal efficiency calculated as 67%. B.O.D. and coliform reductions were 80% and 90% respectively.

f. Chlorination of wastewater effluent by standard procedures was also shown to have a low degree of effectiveness in activating enteroviruses. While chlorine doses of 11 mg/l added to treated sewage effluent resulted in a 5 log reduction of coliforms in 1 hr, only a 1 log reduction was achieved with a poliovirus. In order to achieve a 99.9% inactivation of poliovirus in the effluent, a chlorine dose had to be applied that was 10 times larger than the one required to achieve the equivalent degree of inactivation for coliforms.

g. The PS method has been adapted and tested for the detection of enteroviruses in seawater and has been found effective. Enteroviruses were detected in

14 out of 48 samples of seawater taken from points at varying distances from an outfall sewer. In two cases enteroviruses were detected at a point in the sea 1.5 km from the source of pollution, opposite a main bathing beach. Radioisotope tracer tests indicated that at that point the degree of physical dilution of the raw sewage in the sea would be at least 1:100. This indicates both the sensitivity of the PS method and the degree of persistence of enteroviruses in the marine environment.

h. In laboratory studies an apparently biological antiviral factor in seawater was detected in samples from both the Mediterranean and the Red Sea. Poliovirus type 1 inoculated into natural seawater samples was reduced by 3-6 logs in seven days, while in heat treated seawater samples, used as controls, only a one log reduction was detected in this period. The T_{90} of poliovirus in seawater as determined from these laboratory experiments was between 12 to 48 hours. This is considerably greater than that of coliforms in the same environment. Although the antiviral activity of seawater may provide little safety factor in preventing the contamination of bathing beaches, it is now under intensive study. This marine antiviral factor may prove of considerable interest in other areas of application when isolated, identified and its mode of action established.

i. In conclusion, a major reevaluation of water quality control and water pollution prevention, based on the use of enterovirus parameters as illustrated in this paper, must be made. This is essential in light of current knowledge concerning the risk of waterborne virus diseases particularly infectious hepatitis, and the recognized inadequacy of the standard parameters such as the coliform index.

REFERENCES

CLARK, N.A. and KABLER, P.W. (1954) The inactivation of purified Coxsackie Virus in water by chlorine. - Amer. Jour. Hyg. 59,119.

CLARKE, N.A., BERG, G., KABLER, P.W. and CHANG, S.L. (1964) Human enteric viruses in water: source, survival and removability. In Advances in Water Pollution Research, Vol. 2, p. 523-524. Pergamon Press, London.

DENNIS, J.M. (1959) 1955-56 infectious hepatitis epidemic in New Delhi, India, J. Am. Wat. Wks Ass. 51, 1288-1296.

GILAT, C., SHUVAL, H.I., YOSHPR-PURER, Y. and COHEN N. (1969) The use of radioisotope tracers for the study of the dispersion and inactivation of bacteria in coastal pollution - Proc. Jerusalem Conf. on Water Quality Research - Ann Arbor-Humphry Science Press, Ann Arbor.

HARREMOES, P. (1966) Prediction of pollution from planned waste water outfalls. J.W.P.C. Fed., 38, 12,23.

MAGNUSSON, S., GUNDERSEN, K., BRANDBERG, A. and LYCKE, E. (1967) Marine Bacteria and their possible relation to virus inactivation capacity of seawater. - Acta Path. et Microbiol. Scandinav. 71. 274-280.

METCALF, T.G. and STILES, W.C. (1968) Survival of enteric viruses in estuary waters and shellfish. - Jour. A.S.C.E. San. Eng. Div. 40: 439-447.

MITCHELL, R. (1968) Factors affecting the decline of non-marine microorganisms in seawater. - Water Research, 2, 535-534

POMEROY, R. (1960) The empirical approach for determining the required length of an ocean outfall. - Proc. 1st Conf. on Waste Disposal in the Marine Environment, Pergamon Press, London.

PRESIDENT'S SCIENCE ADVISORY COMMITTEE (1955) Restoring the quality of our environment. Report of the Environmental Pollution Panel, Washington.

SHUVAL, H., CYMBALISTA, S., FATTAL, B. and GOLDBLUM, N. (1967a) The concentration of enteric viruses in water by hydroextraction and two-phase separation. In Transmission of Viruses by the Water Route (Edited by Berg, G.) Interscience.

SHUVAL, H., CYMBALISTA, S., WACH, A., ZOHAR, Y. and GOLDBLUM, N. (1967b) - The inactivation of enteroviruses in sewage by chlorination. - Proc. 3rd Int. Conf. Water Pollution Res., January 1967.

SHUVAL, H.I., COHEN, N. and YOSHPE-PURER, Y. (1968) The dispersion of bacterial pollution along the Tel Aviv shore. - Rev. Intern. Oceanogr. Med. 9:107-121.

SHUVAL, H.I., FATTAL, B., CYMBALISTA, S., and GOLDBLUM, N. (1969a) The Phase-Separation method for the concentration and detection of viruses in water. - Water Research, 3:225-240.

SHUVAL, H.I., FATTAL, B., CYMBALISTA, S. and GOLDBLUM, N. (1969b) Antiviral Factor in Seawater. Proc. 4th Conf. on Medical Oceanography, (in press).

STANDARD METHODS FOR THE EXAMINATION OF WATER, SEWAGE AND INDUSTRIAL WASTES. (1965) Amer. Pub. Health Assn., New York.

U.S. DEPT. OF HEALTH, EDUCATION AND WELFARE. (1962) Report to the Surgeon General of the Committee on Environmental Health Problems, Washington.

APPENDIX 1

PROPOSED PROCEDURE FOR DETERMINING THE PRESENCE OF ENERTIC VIRUSES IN WATER BY THE PHASE-SEPARATION (PS) METHOD

1. General Discussion

1.1 Principle: The "enteric virus" group in the broad sense includes all viruses known to be excreted in quantity in the feces of man. The group includes Polioviruses, the Coxsackie viruses, the Echo (enteric cytophathogenic human orphan) viruses, the adenoviruses, and the viruse of infectious hepatitis. The presence of members of this group of viruses in water can be considered as evidence of fecal pollution of human origin and as presumptive evidence that the consumption of such water by large non-immune population groups might lead to human infections. In the PS method, water samples of several liters in volume are concentrated by a phase-separation technique which produces a concentrate of about 1:500 of the original sample volume which contains essentially all of the viruses present in the original sample. This concentrate is assayed for enteric virus by standard quantitative tissue culture methods.

1.2 Minimum Detectable Contamination: This method should be able to detect as few as one or two virus infectious units of enteric viruses per liter of sample tested.

2. Apparatus

2.1 Separatory funnels, pear shaped, of one to five liters capacity.

2.1 Centrifuge, capable of centrifuging at 15,000 x g. (this unit is not required if the virus assay is done in another laboratory).

2.3 Magnetic stirrer.

3. Reagents

3.1 Sodium dextran sulfate 2000 (A.B. Pharmacia, Sweden)

3.2 Polyethylene glycol (Carbowax 4000)

3.3 Sodium chloride

3.4 Streptomycin

3.5 Penicillin

3.6 Fungizone (Amphototeriein, Squibb.)

4. Procedure

4.1 Place water sample to be tested in a sterile flask of suitable volume to allow for mixing of added chemicals.

4.2 To each liter of sample, the following chemicals are added: 2 g sodium dextran sulfate, 64.50 g. polyethylene glycol and 17.50 g. NaCl. Mix on a magnetic stirrer until the chemicals are dissolved.

4.3 Transfer solution to a sterile separatory funnel and hold at 4°C for 12 - 24 hours after which time the bottom and interphase is drained off into a sterile test tube.

4.4 Add NaCl to the concentrate to a final concentration of 1.0 M (50 mg NaCl per ml concentrate) and hold for an additional 12 - 24 hours at 4°C. Withdraw upper phase in sterile pipette for virus assay. Concentrate can also be frozen and held at -20°C for assay at a later date, or sent to a specialized virus laboratory for quantitative assay.

4.5 Centrifuge final concentrate at 15,000 g for 30 minutes and then add streptomycin 20,000 µg/ml, penicillin 20,000 µ/ml and Fungizone 60 µg/ml.

4.6 Assay concentrate on primary rhesus kidney cell monolayers by the plaque forming method or in tissue culture tubes or bottles using standard virological procedures not to be covered here.

5. Calculation

When the plaque forming technique for assaying viruses in the concentrate is used:

$$\text{PFU/liter} = \frac{A \times C}{B \times D}$$

where A = total number of plaque forming units (PFU) on all plates assayed, B = ml of concentrate assayed, C = total ml of concentrate produced in final upper phase and D = liters of original sample tested.

THE DISPERSION AND DISAPPEARANCE OF ENTEROVIRUSES AND BACTERIAL POLLUTION INDICATORS IN THE JORDAN RIVER—LAKE KINNERET WATERSHED

Hillel I. Shuval, Badri Fattal, Judith Cohen
and Eliyahu Katzenelson

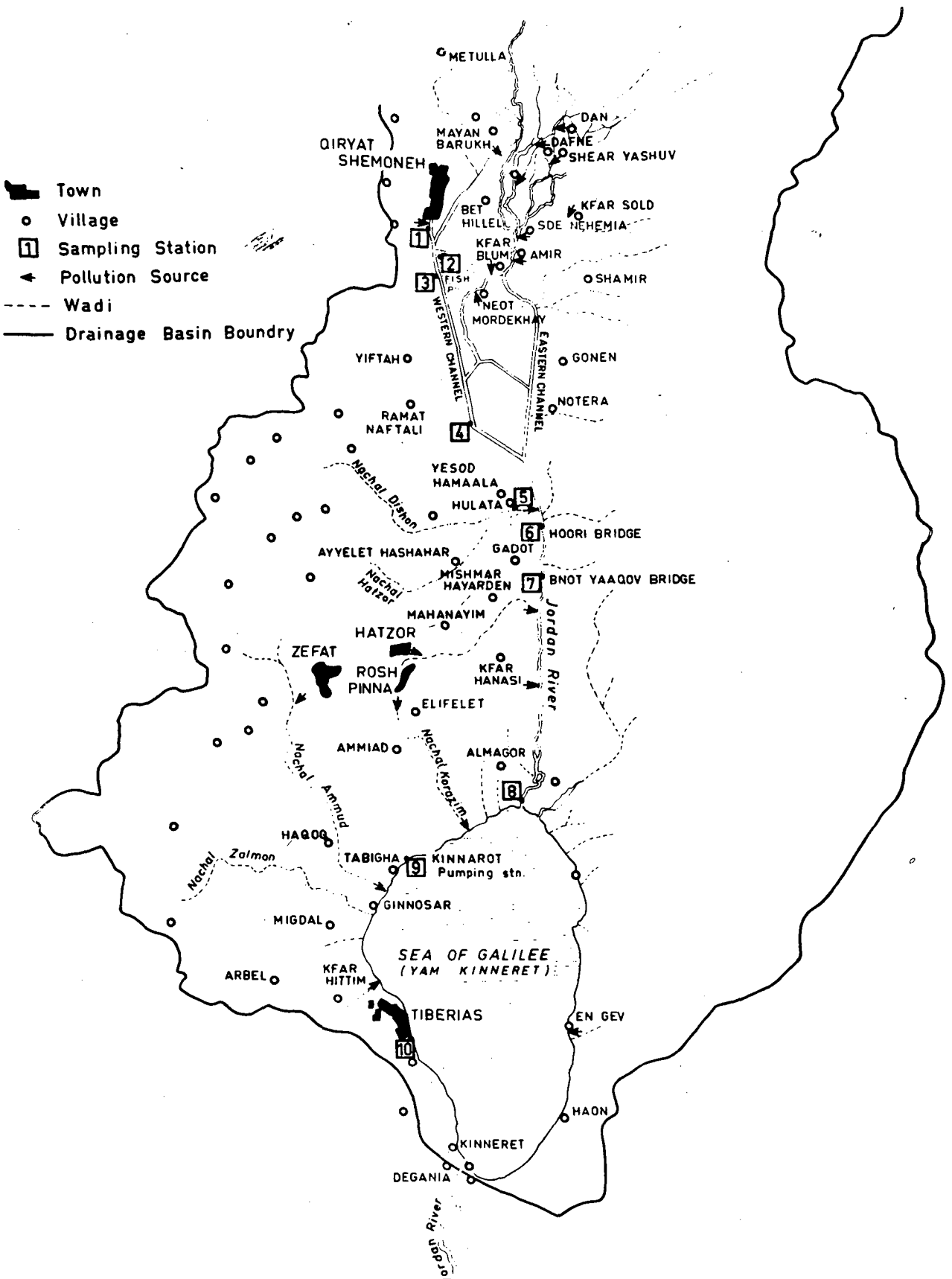
INTRODUCTION

The possibility that diseases caused by enteric viruses, particular infectious hepatitus, can be transmitted by polluted water (10) calls for a better understanding of the patterns of dispersion and disappearance of viruses in natural river systems. Such studies have posed certain difficulties in the past since the natural levels of enteric viruses in polluted water are usually too low to allow for detection in unconcentrated water samples. The recent development of the Phase Separation (PS) method for quantitative assaying of the virus concentration in large volumes of water by our group at the Hebrew University of Jerusalem (11, 12) has helped to make possible studies of viruses in the natural water environment. In this study the dispersion and disappearance of enteroviruses and various bacterial indicator organisms in a river system are compared and related to certain environmental factors.

The Study Area:

The Jordan River-Lake Kinneret watershed in the north of Israel served a model of a watershed system receiving virus laden sewage imputs at a number of points while also acting as a main source of urban water supply. The Israel National Water System, serving a population of about one million persons, draws water from Lake Kinneret (Sea of Galilee) at the Kinnerot Pumping Station in the north-western corner of the Lake (Fig. 1).

FIG. 1 THE JORDAN RIVER - LAKE KINNERET WATERSHED



The area of the Jordan River-Lake Kinneret watershed is approximately 2,730 sq. km. The Jordan River and its tributaries, the Dan, Snir, Hermon and Ayun Rivers as well as several springs are the major water sources of lake Kinneret other than direct surface run-off and rains. The total population of the watershed is about 100,000 persons employed mainly in agriculture and some light industry. It is estimated that the total flow of domestic sewage discharged by the drainage basin directly or indirectly into the lake is about 4.5 million m^3 per year, while some 54 million m^3 of effluent from fish ponds are also discharged into the Jordan and the lake annually. The flow of the Jordan River into Lake Kinneret averages 550 million m^3/year. The summer flow of the river as measured at the Hoori Bridge ranges between 10-20 m^3/sec. while the winter flow ranges between 40-100 m^3/sec. Some of the chemical and physical characteristics of the Jordan River are presented in Table 1. The temperature of the river water varies between 11°C in the winter to 23°C in the summer (Fig. 2).

Lake Kinneret is a sweet-water lake situated 209 meters below sea level with a surface area of 169 sq. km., a volume of about 4,400 million m^3, a maximum depth of 45 m and mean depth of 24 m. The total inflow into the lake averages 770 million m^3 per year (3), 72% of which is derived from the Jordan River. The lake's chemical composition is materially effected by inflow of salt water from saline springs on the bottom and along the shores, which increase the amount of total dissolved solids, in particular chlorides and sodium. The lake water has a chloride ion concentration 15 times greater than that of incoming Jordan River water (3). The lake is showing signs of increasing eutrophication and its pollution by domestic sewage, industrial wastes as well as by agricultural chemicals is of growing concern.

The main source of sewage contamination is from the town of Qiryat Shemoneh having a population of some 15,000 which discharges about 2,500 m^3 of untreated sewage daily into a drainage ditch which leads directly into the western channel of the Jordan River system. Information on the chemical quality of the sewage of Qiryat Shemoneh is presented in Table 2. Minor sources of sewage contamination are

TABLE NO. 1

SOME CHEMICAL AND PHYSICAL CHARACTERISTICS OF THE JORDAN RIVER*

Characteristic	Range	
Total Dissolved Solids	170 - 773	mg/l
Suspended Solids	8 - 559	mg/l
Dissolved Oxygen	8.2 - 10.5	mg/l
Nitrogen (kjeldahl)	0.1 - 2.1	mg/l
NO_2	0.001 - 0.18	mg/l
NO_3	0.2 - 7.9	mg/l
Cl^-	7 - 24	mg/l
Hardness (as $CaCO_3$)	125 - 330	mg/l
P - (total)	0.1 - 0.78	mg/l
pH	7.5 - 8.6	
Temp.	11 - 23	^oC

* Results from reports of the Mekorot Water Co. Laboratory at Tabcha. 1968 - 1969.

TABLE NO. 2

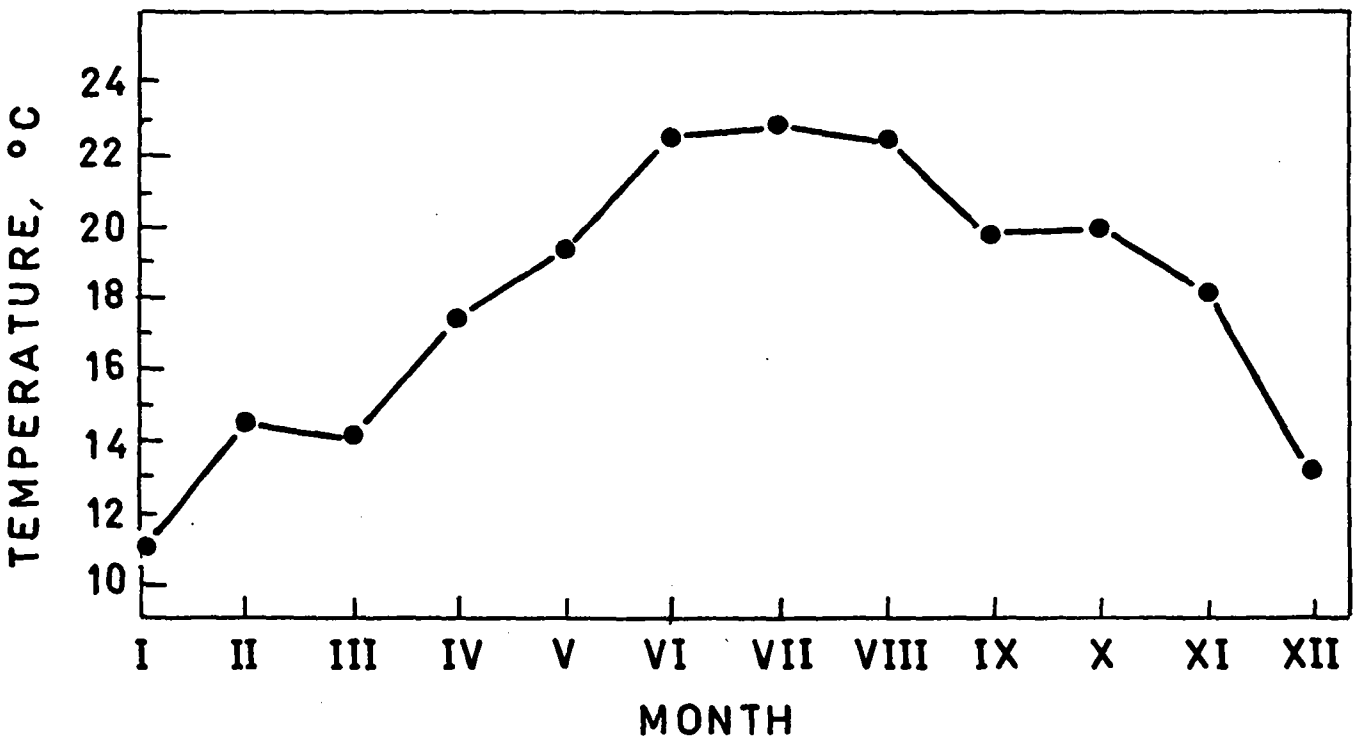
CHEMICAL QUALITY OF QIRYAT SHEMONEH RAW SEWAGE *

Characteristic		
Total Solids	752	mg/l
B.O.D.	520	"
D.O.	0	"
Nitrogen (kjeldahl)	58	"
NO_2	.001	"
NO_3	0.35	"
pH	7.7	"
Temp.	$18^{o}C$	"

* Results from report of the Mekorot Water Co. Laboratory at Tabcha January 1966.

FIG. 2

Mean Monthly Temperature of the Jordan River at the Hoori Bridge
1967



intermittant flows from a number of small kibbutzim (agricultural cooperatives) having populations of 200-500 each, which discharge partially treated sewage indirectly or directly to the Jordan River system when not using it for irrigation or fertilizing fish ponds. Generally speaking most of these sources of contamination (noted by heavy arrows in Fig. 1) do not pollute the river during the dry season from May through October. The same is true for sewage pollution from the towns of Hatzor and Rosh Pina, which actually reaches the river only on occasions. Sewage from the the city of Zefat (Safad) (Pop. - 13,100) can also pollute lake Kinneret directly since it is discharged untreated by the city into the dry river bed of Nachal Ammud, which enters the lake about 3 km south of the Kinnerot Pumping Station. Apparently no sewage reaches the lake during the dry season from this source since it appears to percolate completely into the ground along the route. During the winter rainy season some sewage is undoubtedly washed along with the flood waters into the lake. The sewage of the city of Tiberias (Pop. - 23,600) does not reach the lake since it is completely diverted around the lake to the southern end in a special open channel designed to divert the flow from numerous salt water springs.

From this brief account it can be seen that the sewage of Qiryat Shemoneh serves as the dominant source of fecal contamination entering directly into the river system. This is particularly true for the summer months. It was decided that for the purposes of this study, it would be attempted to determine the natural contribution of enteroviruses and bacterial pollution indicator organisms made by the sewage of Qiryat Shemoneh and to trace their dispersion and disappearance along the river and in the lake up to the water supply system intake pipes. The effect of intervening environmental variables such as dilution in the river, temperature and season, would be considered. In addition to the natural rate of enterovirus contamination a special study was made in an attempt to increase the rate of virus shedding from Qiryat Shemoneh as might occur during an epidemic period. The experiment involved inoculating some 600 school children aged 9 and 14 with polyvalent Sabin polio vaccine.

METHODS AND MATERIALS:

a. Sampling Routine:- Samples of water, sewage and river water were taken from the following sampling stations at various time intervals during the period from December 1968 through September 1970 (see Fig. 1).

Station No.

1 Qiryat Shemoneh
   a. drinking water.
   b. raw sewage.

2 Fish pond-fed partially by raw sewage of Qiryat Shemoneh.

3 Western channel of Jordan River, 3 km. from Qiryat Shemoneh.

4 Western channel of Jordan River, 11 km. from Qiryat Shemoneh.

5 Hulata
   a. drinking water.
   b. sewage.
   c. Jordan River.

6 Jordan River at Hoori Bridge, 19.0 km. from Qiryat Shemoneh.

7 Jordan River at Bnot Yaakov Bridge, 22 km. from Qiryat Shemoneh.

8 Jordan River at Almagor, 34.0 km. from Qiryat Shemoneh.

9 Lake Kinneret at Kinnarot Pumping Station - National Water System intake.

10 Tiberias
   a. drinking water.
   b. raw sewage.
   c. primary effluent.
   d. secondary effluent.

During the period from December 1968 to June 1969, a sample was taken from each main point about every other week. During the period from July 1969 through November 1969, samples were taken every week from stations 3, 4, 6, 8 and 9. The final phase of field testing was carried out during the period from June 1970 through September 1970 in conjunction with the program of polio vaccination of school children aimed at simulating the effects of polio epidemic. During June and July, daily grab and pad samples were taken of Qiryat Shemoneh raw sewage (Station 1) and two to three times weekly similar parallel samples were taken from Stations 3, 4, 6, 8 and 9. From July 13th to September 29th, weekly parallel samples were taken of at Station 1 only.

Various sampling techniques to be described later were used. In all cases samples were placed in insulated ice chests and were shipped by air freight from Rosh Pina to Jerusalem the same day or were refrigerated at 4^{o}C till the next morning for air shipment to Jerusalem. On a few occasions samples for virus assay were frozen and held at -20^{o}C for a few days before shipment to the laboratory. Sample temperatures were checked on arrival at the laboratory in Jerusalem and varied between 4-10^{o}C. When samples arrived at the laboratory in Jerusalem in the evening they were refrigerated at 4^{o}C until the next morning. Comparative bacteriological tests indicated that except for raw sewage the bacterial counts remained stable with proper refrigeration over a 24 hour period. In sewage, coliforms and fecal coli tended to increase by up to one log when held refrigerated for 24 hours, while fecal streptoccoci counts remained stable. To overcome this problem raw sewage samples in the latter stages of the study were diluted 1:100 on arrival at the laboratory and held over-night at 4^{o}C. No change was noted in bacterial count if samples were so treated.

b. Sampling Methods for Virus Assay:- Two sampling methods were used. When samples for quantitative assay by the Phase Separation (PS) method were required, a 3-5 liter grab sample was taken in chemically disinfected plastic jerry-

cans with the aid of a specially designed weighted holder. For samples by the absorbant pad method, 3,200 cm^2 of guaze were used per pad. Usually two pads were tied to a string and suspended in the sewage, river, or lake from 1-7 day period. On collection they were carefully lifted from the water and placed undisturbed into plastic bags for shipment to the laboratory for assay.

c. Samples for Bacteriological Tests:- Samples were taken in sterile 200 ml. glass bottles with ground glass stoppers or from the large plastic jerry-cans.

d. Virus Assay:- Samples of 3-5 liters were concentrated by the PS method described previously (11), and assayed for enteroviruses on primary Rhesus Kidney Cells (RKC) monolayers by the plaque forming method used routinely in this laboratory. The results are reported as plaque forming units (PFU) per 100 or 1,000 ml. In the case of pads, the liquid was expressed from the pads by sterile methods and assayed for enteroviruses as mentioned above. In the case of samples of river or lake water the expressed fluid was concentrated further by the PS method. There were somewhat different procedures for the assay of samples taken in the summer of 1970, which will be reported on in full elsewhere.

e. Virus Typing:- Field samples of sewage or water were assayed for viruses, a number of plaques from each positive plate were transferred to tubes, which were frozen and kept at $-20^{\circ}C$ for identification. Virus typing procedures were similar to those used in the routine clinical virus typing work of this laboratory (8). For the purpose of this study, viruses were classified in gross categories only. They were either identified as one of the three types of poliovirus (P_1, P_2, P_3) or, when found negative in neutralization tests against poliovirus antisera but produced paralysis when injected in suckling mice, are classified as coxsackievirus B types or echovirus type 9. Strains found negative with polio antisera as well as in suckling mice, but exhibiting CPE, were considered as possible strains of echovirus (ECHO), or other unidentified enteroviruses.

f. Coliform Bacteria:- The membrane filtration (MF) method was used with Gelman Metrical 0.45 Filters and m Endo Media (Difco) as described in Standard Methods for the Examination Water and Wastewater (13).

g. Fecal Coliform:- The MF method with FC broth (Difco) and incubation at 44^{o} was followed(13).

h. Fecal Streptococcus:- The MF method with Enterococcus Agar (Difco) and incubation at 37^{o}C for 48 hours was followed (13).

i. Chemical Tests:- Procedures recommended by Standard Methods for the Examination of Water and Wastewater (13) were followed for all chemical tests.

j. Simulated Polio Epidemic:- It became apparent towards the end of the study that it was difficult to trace the kinetics of enterovirus disappearance in the Jordan River system in a manner similar to what was obtained with bacterial pollution indicator organisms due to the low initial concentration of enteroviruses in the Qiryat Shemoneh sewage and the high dilution obtained in the Jordan River. An attempt was made to overcome this by vaccinating 600 school children aged 9 and 14 in Qiryat Shemoneh with polyvalent Sabin vaccine. The vaccination was carried out on June 14, 1970. Sewage samples from Qiryat Shemoneh were taken daily from June 11th through Jyly 12th and then weekly till September 27th. Composit samples of sewage for concentration by the PS method were taken between 7-8 o'clock while absorbant pads were held for 1-3 day periods. Blood specimens were taken from a sample of about 75 children at the time of vaccination and 14 days afterwards. Stools were taken from about 50 of the same children 3, 7 and 14 days after vaccination. Routine samples for virus assays were taken from the critical sampling stations in the river and lake at 2-3 day intervals.

TABLE 3

Enteroviruses and Bacterial Pollution Indicators
in the Qiryat Shemoneh Raw Sewage
1968 - 1970

Date	viruses PFU/100 ml	coli-MF per 100 ml	F. coli-MF per 100 ml	F. strep.-MF per 100 ml
20.12.68	61.4	7.2×10^7	5.5×10^7	6.4×10^5
12. 1.69	117.5	5.8×10^8	7.0×10^6	2.6×10^6
16. 2.69	13.5	7.3×10^9	5.4×10^7	2.4×10^7
2. 3.69	18.3	8.8×10^8	-	2.2×10^6
16. 3.69	129.2	1.3×10^9	1.0×10^7	1.2×10^6
30. 3.69	65.2	1.5×10^{10}	3.0×10^9	5.9×10^6
20. 4.69	56.5	4.5×10^{10}	6.2×10^8	1.1×10^7
4. 5.69	552.0	3.3×10^9	2.5×10^8	1.3×10^7
18. 5.69	1.1	1.7×10^8	4.9×10^7	1.7×10^6
1. 6.69	11.7	5.4×10^8	2.7×10^8	8.2×10^5
15. 6.69	3.5	7.0×10^8	6.5×10^8	1.7×10^7
29. 6.69	5.3	6.8×10^9	-	2.7×10^7
11. 6.70	91.0	1.2×10^8	5.3×10^7	3.2×10^6
14. 6.70	155.8	6.0×10^9	7.3×10^8	5.3×10^7
17. 6.70	233.8	4.1×10^8	4.3×10^7	1.4×10^7
21. 6.70	204.3	9.0×10^7	1.4×10^7	5.4×10^6
24. 6.70	110.5	4.9×10^7	6.0×10^6	2.0×10^6
28. 6.70	129.6	6.8×10^8	4.0×10^7	8.8×10^6
1. 7.70	38.7	2.3×10^7	7.2×10^6	4.2×10^5
8. 7.70	60.6	1.7×10^8	3.6×10^7	2.9×10^6
12. 7.70	130.9	1.2×10^8	2.4×10^7	1.0×10^6
2. 8.70	169.0	8.5×10^7	3.0×10^7	7.9×10^6
9. 8.70	24.2	9.4×10^7	7.0×10^7	-
16. 8.70	43.3	4.8×10^8	6.6×10^7	6.2×10^5
23. 8.70	33.2	6.2×10^7	4.0×10^7	4.0×10^6
30. 8.70	11.9	3.8×10^7	5.4×10^7	1.2×10^7
6. 9.70	36.4	4.4×10^7	1.8×10^7	1.1×10^6
13. 9.70	4.5	1.2×10^7	6.1×10^6	5.0×10^5
27. 9.70	56.7	1.2×10^8	4.1×10^7	4.0×10^6
	85.7	$3.5 \times 10^{8*}$	$5.4 \times 10^{7*}$	$3.7 \times 10^{7*}$

* Log mean

RESULTS:

Isolation of Enteroviruses in the Watershed:- During the course of the study 452 assays of water supply, sewage, river and lake water from 16 different sampling stations were made. In most cases the samples were concentrated by the PS method with 110 samples taken by the absorbant pad method. The mean enterovirus concentration in Qiryat Shemoneh sewage based on 58 PS assays is 120 PFU/100 ml. Table 3 shows the results of 29 quantitative virus assays of the Qiryat Shemoneh sewage for which parallel bacteriological results are available. The mean virus concentration for this series is 85.7 per 100 ml. with a maximum of 552.0 and a minimum of 1.1. The log mean coliform count of the sewage is 3.5×10^8/100 ml. The log mean of fecal coli is 5.4×10^7/100 ml. and that for fecal streptococcus is 3.7×10^6/100 ml.

Table 4. presents a summary of all 452 enterovirus assays in the Jordan River-Lake Kinneret watershed during the period of the study (1968-1970). While viruses were detected in essentially all samples of raw and treated sewage from Qiryat Shemoneh, Hulata and Tiberias the number of virus isolations from the Jordan River and Lake Tiberias were few. 6 out of 45 or 13.3% of the samples taken from Station 3 in the northern part of the western channel of the Jordan were positive. This Station is 3 km. south of the inlet of raw sewage from Qiryat Shemoneh. At Station 6, the Jordan River at the Hoori Bridge, 2 out of 45 samples or 4.4% were positive. This point is 19 km. from the inlet of Qiryat Shemoneh sewage. It is interesting to note that on one occasion out of 43 assays viruses were detected at the inlet of the Kinnerot Pumping Station in Lake Kinneret. Out of 9 positive virus findings in the Jordan River and Lake Kinneret, one was detected by the pad method. No viruses were detected in the 41 tests of drinking water samples. The virus concentration found in these positive river water samples varied from 1.2-73 PFU/liter.

Between the dates of June 12 and July 12, 1970 a series of 30 daily comparisons were made of the viruses detected in Qiryat Shemoneh sewage in grab samples, concentrated by the PS method and swabs. These tests were part of a study of the

TABLE 4

Isolations of Enteroviruses in the Jordan River - Lake Kinneret Watershed
1968 - 1970

Sampling Station	No. Samples assayed	No. positive	% positive
1. Qirat Shemoneh			
a. Drinking water	13	0	0
b. Raw Sewage	160 (51)*	159 (51)*	99.4
2. Sasa Fish Pond	13	0	0
3. Western Channel North	45 (13)	6 (1)	13.3
4. Western Channel South	14 (6)	0	0
5. Hulata			
a. Drinking water	13	0	0
b. Jordan River	8	0	0
c. Sewage Effluent	3	1	33.3
6. Jordan River at Hoori Bridge	45 (12)	2	4.4
7. Jordan River at Bnot Yaakov Bridge	1	0	0
8. Jordan River at Almagor	33 (14)	0	0
9. Lake Kinneret at Kinnerot Pumping Station	43 (14)	1	1.75
10. Tiberias			
a. Drinking water	15	0	0
b. Raw Sewage	16	16	100
c. Primary effluent	15	15	100
d. Secondary Effluent	15	15	100
Total	452 (110)	215 (52)	

* absorbent pad method

TABLE 5

Virus Types Isolated from Sewage
at Qiryat Shemoneh and Tiberias
1968 - 1970

Virus Type	Q. Shemoneh Raw Sewage		Tiberias Raw Sewage		Tiberias Effluent		Total	
	No.	%	No.	%	No.	%	No.	%
Polio I	20	19.0	15	16.7	53	32.3	88	24.5
Polio II	7	6.7	19	21.1	18	11.0	44	12.3
Polio III	36	34.3	34	37.8	68	41.5	138	38.4
Cox. B.	13	12.4	11	12.2	10	6.1	34	9.5
Echo or other	29	27.6	11	12.2	15	9.1	55	15.3
Total	105	100%	90	100%	164	100%	359	100%

effectiveness of the pad method which is to be reported on elsewhere. A preliminary analysis of these data indicate that while the average virus concentration of the sewage as shown by the test of the series of grab samples was 12.8 PFU/100 ml. The expressed fluid from the series of pads removed from this sewage stream over the same period had a mean virus concentration of 792.0 PFU/100 ml. or 62 times more than that of the sewage in which they were immersed.

The results of typing virus strains picked from assays of sewage from Qiryat Shemoneh and Tiberias is shown in Table 5. 75% of all strains proved to be poliovirus. 13% of the polio strains tested showed strong C.P.E. when incubated at 40°C. The remaining 87% grew at 37°C only. The latter are attenuated polio vaccine strains. About 10% of the strains were coxsackie B types and 15% were echovirus. From samples of water from the river and lake strains of poliovirus and echovirus were also isolated.

Bacterial Pollution Indicator Organisms:-

Parallel with the assays for enteroviruses, tests were made for coliforms, fecal coli and fecal streptoccocus in the same samples of water and sewage. Fig.3 summarizes the results of 195 bacterial tests during the period December 1968 to June 1969, from Stations 1, 4, 6, 8 and 9. While the log mean coliform count in the Qiryat Shemoneh sewage was about 10^9/100 ml. the fecal coli count was about 10^8/100 ml. with the fecal streptoccocus still another log lower. In the last station in the Jordan River (no. 8) at Almagor near the outlet into the lake, the coliform count has dropped to about 10^4/100 ml. with the fecal coli and fecal streptoccocus counts each about one half a log lower, respectively. However, in the lake at the inlet to the Kinnerot Pumping Station the coliform count averages about 300/100 ml. while the fecal streptoccocus count is next in order with the fecal coli practically disappearing.

Essentially this same pattern of dilution and dieaway appears for the 126 bacterial determinations during the period June-July 1970 as shown in Fig.4. However,

FIG. 3

HE CONCENTRATION OF BACTERIAL POLLUTION INDICATOR RGANISMS AT VARIOUS POINTS IN THE JORDAN RIVER AND LAKE KINNERET

(DEC. 1968 - JUNE 1969)

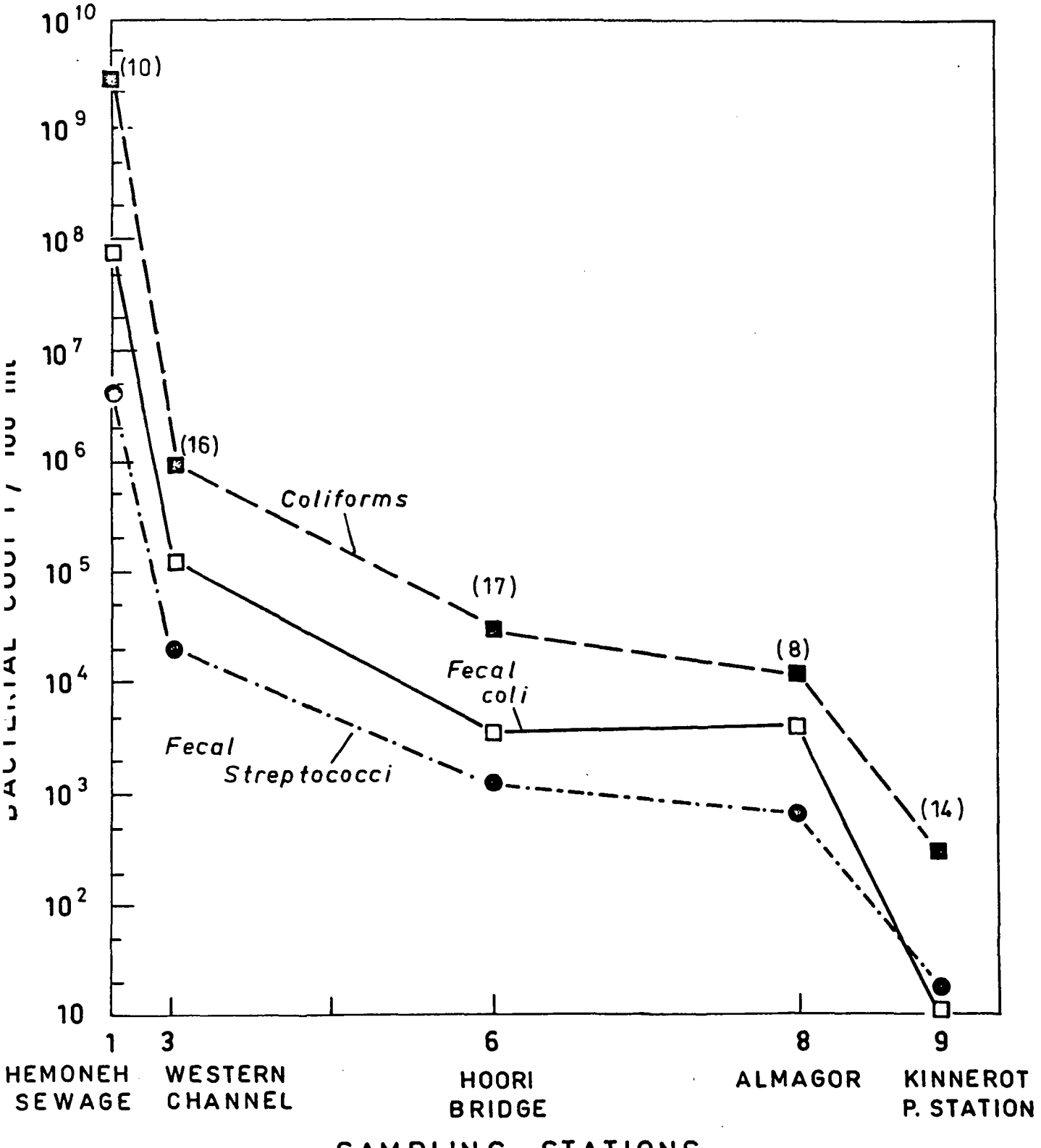
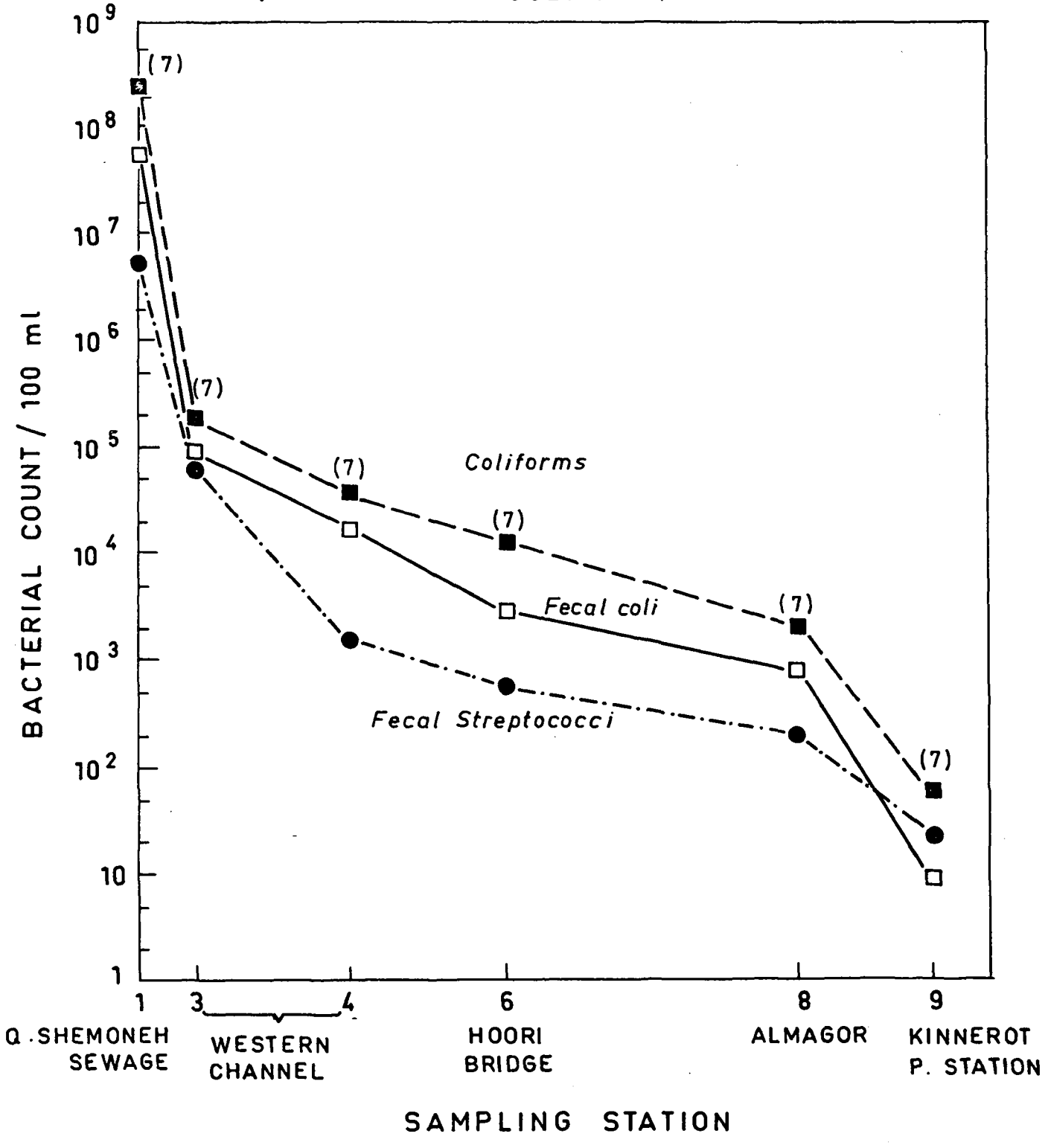


FIG. 4.

THE CONCENTRATION OF BACTERIAL POLLUTION INDICATOR ORGANISMS AT VARIOUS POINTS IN THE JORDAN RIVER AND LAKE KINNERET
(JUNE 1970 - JULY 1970)



the average initial coliform count in the raw sewage of Qiryat Shemoneh is about 10^8/ 100 ml., a full log lower than the results obtained in the previous year (Fig. 3), before improvements had been made in the bacteriological methods. Fig. 5 shows the seasonal variation as log mean monthly coliform counts at two points along the Jordan River. In "A" the variation at station 3 - western channel - 3 km. from Qiryat Shemoneh is shown. The log mean count is 6.9×10^5. There appears to be a slight drop in the average coliform count during the months of April - June. In "B" at the Hoori Bridge, 19 km. from Qiryat Shemoneh, the log mean is 2.5×10^4 but no clear cut seasonal pattern of variation can be detected.

Sewage Treatment Plant Tiberias:- Although the treated sewage of the city of Tiberias does not empty directly into the lake an evaluation of the virological and bacteriological aspects of the treatment plant were included in the study as an example of what improvements might be obtained from treatment in similar conventional biological filtration plants.

From Table 6 it can be seen that the virus concentration in the raw sewage varied from 1.5 - 754.6 PFU/100 ml. with a mean of 95. The virus concentration of the treatment plant effluent varied from 3.5 - 120.7 PFU/100 ml. with a mean of 30.4. The mean virus removal efficiency based on each individual day's removal efficiency and considering negative removals as zero is 40.1%. The log mean coliform count for the raw sewage is 1.9×10^9/100 ml. with a log mean of the effluent of 2.5×10^8/100 ml. The mean coliform removal efficiency again based on individual series is 76%.

Table 7 shows the relationship between the concentration of enteroviruses, coliforms, fecal coli, and fecal streptoccocus in the sewage of Qiryat Shemoneh and Tiberias. The calculated ratio of virus:coli varies between 1:8,200,000 to 1:78,000,000. The concentration of fecal coli is about 1/10 of that of coliforms, while the fecal streptoccocus is about 1/100 of the concentrationof the coliforms.

TABLE 1

Enterovirus and Coliform Removal in the Tiberias
Biological Filtration Treatment Plant
1969

Date	Raw Sewage		Treated Sewage		virus removal %	coliform removal %
	virus conc. PFU/100 ml	coliforms-MF /100 ml	virus conc. PFU/100 ml	coliforms-MF /100 ml		
1. 1.69	754.6	5.4×10^8	120.7	2.8×10^7	84	95
12. 1.69	16.1	7.4×10^8	40.0	2.9×10^8	negative	61
2. 2.69	36.2	8.2×10^8	12.7	4.1×10^7	65	95
16. 2.69	14.3	-	25.9	4.4×10^8	negative	-
2. 3.69	18.4	3.2×10^9	-	-	-	-
16. 3.69	42.6	2.1×10^{10}	23.0	2.1×10^9	46	90
30. 3.69	1.5	8.0×10^8	20.1	7.1×10^8	negative	11
20. 4.69	126.9	-	58.8	2.3×10^8	54	-
4. 5.69	20.3	5.6×10^8	38.0	4.5×10^8	negative	20
18. 5.69	44.2	3.6×10^9	6.0	1.7×10^8	86	95
1. 6.69	69.6	5.4×10^9	5.2	4.1×10^8	93	92
15. 6.69	7.0	2.3×10^{10}	10.9	4.7×10^8	negative	98
29. 6.69	7.5	3.0×10^8	3.5	2.5×10^6	53	99
mean	95	1.9×10^{9}*	30	2.5×10^{8}*	40.1	75.6

* Log mean.

TABLE 7

Relationships Between the Mean Concentration of Enteroviruses, Coliforms, Fecal Coliforms and Fecal Streptococci: in Sewage Samples Qiryat Shemoneh and Tiberias

1968 - 1970

	Qiryat Shemoneh Raw Sewage	Tiberias Raw Sewage	Tiberias Effluent
No. of samples	29	13	12
Viruses - PFU/100 ml	86	95	30
Coliforms - MF/100 ml*	3.5×10^8	1.9×10^9	2.5×10^8
F. Coli MF/100 ml*	5.4×10^7	1.3×10^8	4.1×10^7
F. Strep MF/100 ml*	3.7×10^6	7.0×10^6	3.5×10^6
Virus: Coli	$1:8.2 \times 10^6$	$1:7.8 \times 10^7$	$1:1.3 \times 10^7$
Virus: F.C.	$1:1.1 \times 10^6$	$1:4.7 \times 10^6$	$1:1.9 \times 10^6$
F.C.: Coli	1:6.5	1:17	1:6
F. Strep: Coli	1:95	1:210	1:70
F. Strep: F. Coli	1:15	1:15	1:12

* Log. mean

Simulated Polio Epidemic - June 1970:- Fig. 5 presents the virus concentrations found in the Qiryat Shemoneh sewage before and after vaccinating 600 school children on June 14, 1970. What appears as a peak of enteroviruses in the raw sewage was detected five days later reaching 3,200 PFU/1000 ml. and subsided 4 days afterwards. However, similar, although slightly lower, peaks appeared both one and two months after the initial vaccination experiment, as well as on a number of occasions previously. The mean virus concentration in Qiryat Shemoneh sewage based on 58 samples during 1968 - 1970 was 1,200/1000 ml. The mean for June - July 1970 alone was the same.

DISCUSSION

The main enterovirus input into the Jordan River system comes from the 2,500 m^3 per day of sewage from the town of Qiryat Shemoneh. As shown in Table 3, the mean enterovirus concentration of the sewage as determined by the Phase-Separation method is 85.7/100 ml. for the 29 samples shown. The mean for all 58 tests during the study is 120 PFU/100 ml. It can be calculated that the mean total daily contribution of enteroviruses to the river from Qiryat Shemoneh is about 3.0×10^9 PFU. Despite this heavy virus input, viruses were detected in only 13% of the samples at Station 3 in the western channel of the Jordan, 3 km. from Qiryat Shemoneh and in only some 5% of the samples at Station 6, 19 km. from the source of the sewage.

The flow in the western channel of the Jordan has been estimated to average some 1,200,000 m^3/day which means that on entering the western channel the sewage is diluted on the average by a factor of about 1:500. This may vary from a minimum as low as 1:100 to a maximum of 1:5,000. By dilution alone the mean virus concentration would be reduced to about 2 PFU/1000 ml. at Sampling Station 3 in the western channel, 3 km. from Qiryat Shemoneh.

The tests for bacterial pollution indicator organisms shows that their concentration is reduced from 500-5,000 fold at Station 3 on entering the western channel as

FIG. 5

Seasonal Variation in Coliform Counts at Points Along the Upper Jordan River (1968 - 1969)

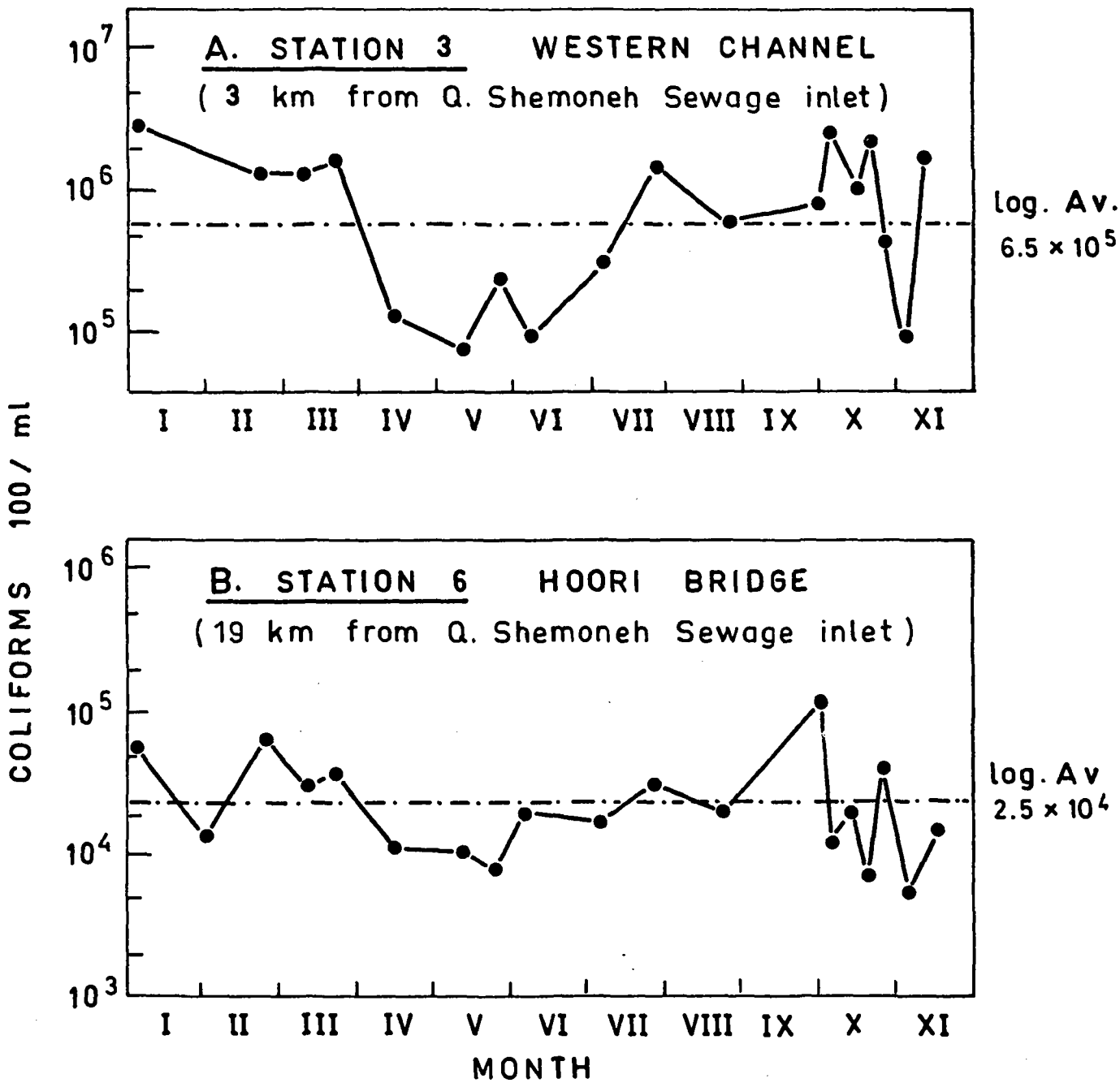
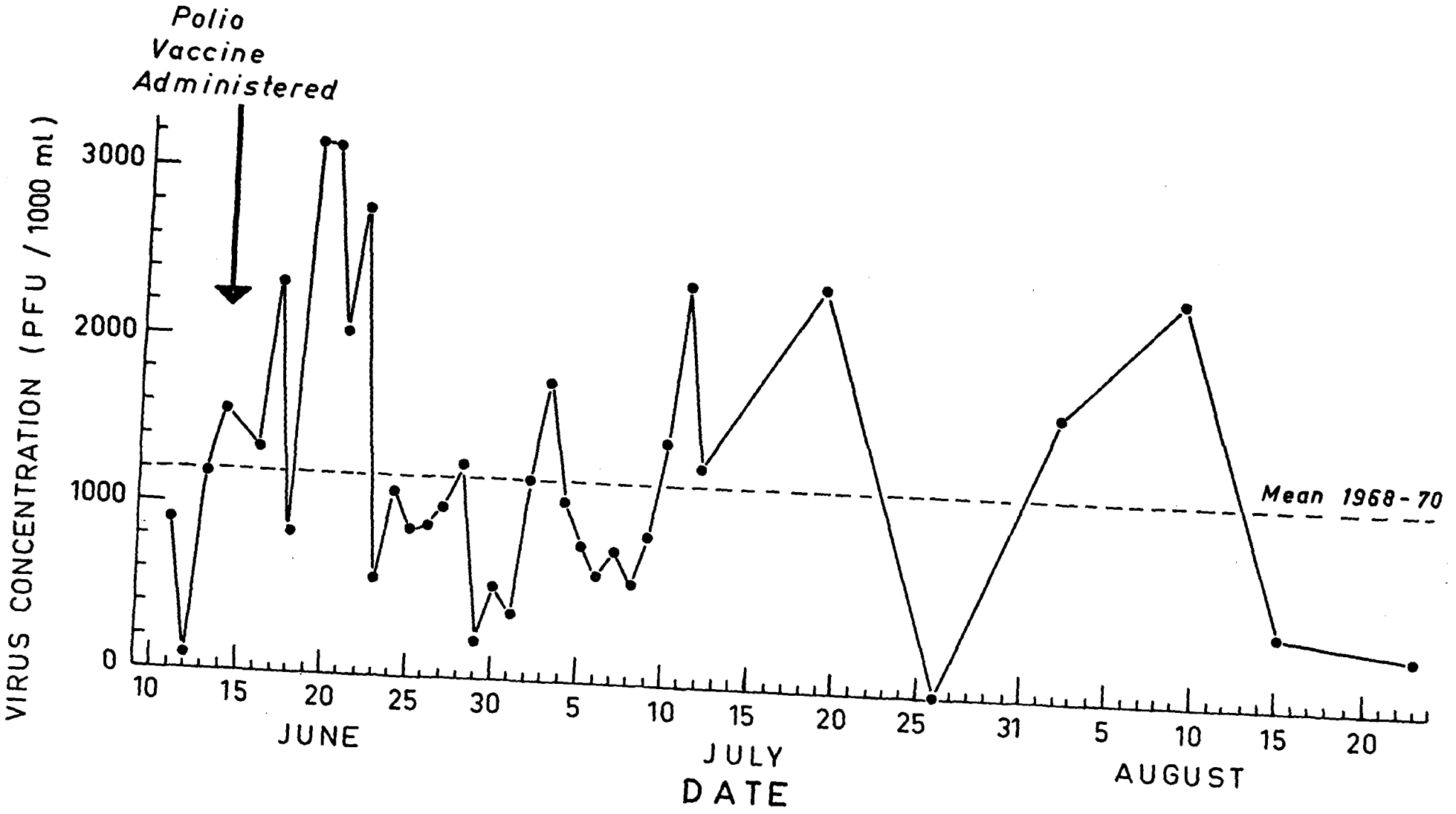


FIG. 6

ENTEROVIRUS CONCENTRATIONS IN THE RAW SEWAGE OF QIRYAT SHEMONEH
(JUNE-AUG 1970)



compared to the original counts in the raw sewage at Qiryat Shemoneh, depending on the period of the year and the flow in the western channel. Some 30-50% of this reduction in bacterial count may result from sedimentation of sewage solids in the slow flowing drainage ditch leading from Qiryat Shemoneh to the western channel. It might be anticipated that there would be a similar reduction in viruses.

The limit of the PS method in detecting viruses in water has been shown to be about 2 PFU/liter (11). Thus the high rate of dilution plus sedimentation in the drainage ditch together with the limits of detection of the method used, may explain the relatively small percentage of viruses detected in the northern station in the western channel (no. 3), which nevertheless is heavily contaminated with sewage flow. Further dilution and sedimentation and perhaps some die-away may explain the even lower rate of virus detection at Station 6, the Hoori Bridge in themain channel of the Jordan, some 19 km. south of Qiryat Shemoneh. The fact that never-the-less viruses were detected on one occasion in lake Tiberias at the intake of the Kinnarot Pumping Station may indicate that some viruses do infact pass down the river and into the lake or come from local lakeside sources of contamination, and could be detected more frequently if methods were available to assay very large samples of water. It is obvious from these data that despite our original expectations, the PS method is not sensitive enough to carry out a quantitative study of the kinetics of enterovirus disappearance in such a river system as the Jordan where a high dilution reduces the initial virus count to levels lower than 2 per 1000 ml. Another possible explanation is that the PS method has been shown to be somewhat selective in its ability to concentrate viruses (5). Wallis et. al. (14) have been studying virus concentration systems aimed at detecting as few as 1 PFU per 400 liters of water. The use of such a sensitive method would be essential if quantitative results are to be obtained in studies of the behavior of enteroviruses in the natural water environment.

The absorbant pad method was used on 59 occasions in this study for tests of river or lake water and it is worth noting that of the 9 cases positive for viruses one was with the aid of the pad method. However, only five of the positives were tested

both by pads and the PS method and the one positive case with the pad was negative in the PS method. Although this method is not a quantitative one and has been shown to have a relatively low efficiency of a few percent in clear water, (6, 9) there is evidence that it can be an effective method of detecting viruses in more polluted water. This higher efficiency could possibly be associated with contaminated suspended particles which are trapped more readily by the pads. Another possible advantage of the pad method is the fact that by remaining immersed in theflowing stream for 24 hours or more they may pick up slugs of viruses, excreted by individual carriers, which enter the sewage system intermittantly. Grab samples may completely miss such peaks in virus concentration. For example, a single child in Qiryat Shemoneh who is infected with enteroviruses may excrete as many as 10^9 PFU into the sewage system at a given moment. Even if this is diluted in 10,000 liters by the general sewage flow of the town and another 1000 times by the flow of the river it might be expected that the concentration of viruses in that particular slug of river water might be as high as 100 PFU/liter. Therefore, a large composite sample taken over a period of many hours might be needed to gain a more complete picture of the true level of viruses in a given flowing river system.

The preliminary information on the virus concentration in sewage in grab samples as compared to the expressed fluid from pads held for 24 hours in the same sewage stream seems to indicate that about 60 times more virus per unit volume is found in the expressed fluid. Gravell and Chin (4) and others, (6, 9) have shown that pads are more effective than unconcentrated grab samples in detecting enterovirus in contaminated water. It is not possible to estimate the actual volume of sewage which has passed through a pad so as to provide a quantitative estimate of the virus recovery efficiency. Hoff et al. (6) and Liu et al. (9) have attempted to evaluate the virus recovery efficiency of pads in a special flow-through device. Their measurements vary from about 0.3% efficiency for clean tap water to as high as 47% for sea water. Pads in turbid water showed a higher degree of virus pick-up (about 1%) than in clear tap water. If we assume that the pad's efficiency is indeed

only 1% and that the pad's water retention volume is about 100 ml., than about 600 liters would have had to flow through the pad in 24 hours to obtain the virus concentration detected. That would only require about 40 ml. of sewage to filter through the pad per minute. Such a filtration rate is not completely unreasonable to expect under the conditions of a rapidly flowing sewage stream having a velocity of about 0.5 - 1.0 meters per second. These are only rough estimates based on numerous assumptions which never-the-less may help explain the apparent effectiveness of pads in detecting viruses in sewage and water. This method is well worthy of further study to try to determine its actual virus pick-up efficiency under varying environmental conditions.

The results of typing the various virus strains detected during the course of this study show that poliovirus (75%) is dominant. The fact that 13% of the poliovirus strains tested proved positive for the 40°C temperature marker indicative of wild polio strains, does not necessarily mean that all such isolates are true wild poliovirus being shed in the sewage of Qiryat Shemoneh and Tiberias. Studies have shown that a high percentage of vaccine strains of poliovirus can easily lose the temperature marker after one human passage. Still these data do never-the-less point to the possibility that some wild poliovirus strains are still in circulation in the communities studied despite the fact that almost universal vaccination of children with Salk vaccine or live Sabin polio vaccine has been practiced in Israel since 1958.

The effort to induce a simulated polio epidemic in Qiryat Shemoneh in June 1970 by vaccinating 600 school children with Sabin polio vaccine seems to have succeeded only to a limited degree. The initial plan had called for postponing the primary vaccination of all new born infants for a one month period and vaccinating them all on a single day. This would have included about 40 infants. However, the Ministry of Health follows a policy of initiating vaccination of each infant routinely at a fixed interval after birth. They opposed any postponement or change in schedule as an unnecessary health risk.

The idea of revaccinating school children was the alternative proposed by the health authorities. Although it could not normally be expected that many revaccinated children would excrete massive doses of poliovirus, it was considered a possibility in this case since most of the 14 year olds had received only Salk vaccine containing killed virus. The Ministry of Health was also interested to learn if there would indeed be a response to such a revaccination and cooperated fully. Stool examination of a sample of vaccinated children indicated that some 90% showed stools positive for polio virus from 3 to 4 days latter, 20% showed virus titers of 10^4 P.F.U./gm. feces, while only two children reached titers as high as 10^5 P.F.U./gm. It can be estimated that the total amount of poliovirus shed by all the children vaccinated reached no more than 10^8 - 10^9 P.F.U./day. This amount could be excreted by a single infant receiving primary vaccination who might excrete as much as 10^6 P.F.U./gm. in his stools, or 10^9 P.F.U. per day.

It can be assumed that the peak virus concentration in the sewage of 3,200 P.F.U./1000 ml. detected from 4 to 9 days after the mass vaccination may indeed be associated with the shedding of viruses by the vaccinated school children. However, this peak was not appreciably higher than other peaks detected throughout the study period. It had been hoped to achieve a peak for several days at least, one order of magnitude higher than any previously detected. In this way it was anticipated that it would have been possible to boost initial virus levels in the river to allow for their detection along the full length to gain the desired information on disappearance and die-away characteristics under natural river conditions. This objective was not achieved and during the course of the experiment in the summer of 1970, viruses were detected in the river only on one occasion.

Little field information is available on rates of virus inactivation in the natural sweet water environment. A number of laboratory studies recently reviewed by Akin et al. (1) indicate that viruses can survive for many days in river water. Their own studies do however indicate that under simulated field conditions poliovirus inactivation rates are faster under natural conditions than under laboratory conditions. The

role of natural fauna and flora and other environmental factors such as U.V. radiation are difficult to replicate under laboratory conditions. All these factors point to the need of carrying out a field evaluation of the kind intended in this study.

Although it was not possible to study virus disappearance rates in the river system by factors other than dilution, the data for bacterial indicator organisms presented in Figs. 3 and 4 are of interest in this respect. It is noted that after an initial large drop in bacteria count at Station 3 due primarily to initial dilution with river water the rate of bacterial reduction from the raw sewage up to Station 8, some 30 km. away is about 90% - 95%. This may be explained by die-away and partially by sedimentation in addition to the dilution factor.

Between Station 4 on the western channel and Station 6 on the main Jordan River channel, the flow of the river more than doubles. However, the reduction in bacteria count is somewhat less than 50% between these two points. This may partially be due to the contamination in the eastern channel which is also exposed to some sewage flows from small agricultural settlements. The reduction between Station 6 and Station 8 at Almagor, some 13 km. away is slight. The estimated time of flow in the dry season is about 12 hours so that the 90 - 95% reduction in bacterial pollution indicator organisms indicates a definite self-purification process. The final major reduction in bacterial count appears in the lake where dilution alone can account for the improvement in the microbial quality of the water.

The relationships between the concentrations of enteroviruses, coliforms, fecal coli and fecal streptoccocus in the various sewage sources are summarized in Table 7. The virus: coli ratio appears to be $1:10^7$. However, this may be based on overly high counts for coliforms that resulted from holding sewage samples for 24 hours before analysis. A more reasonable estimate of the true ratio is felt to be about $1:10^6$.

The fecal coli: coli and strep: coli ratios found can also only be considered

as approximations. One point of special interest however, is the fact that fecal streptoccocus organisms are found in about 1/100 the concentration of coliforms in raw sewage and about 1/10 the concentration of fecal coli. However, by the time these bacteria reach Lake Kinneret, the fecal streptococci are found in higher numbers than the fecal coli and at only about 1/10 the level of the coliforms. This adds further evidence supporting the usefulness of streptococcus fecalis, as a resistent pollution indicator organism that can show signs of old or distant pollution (27).

SUMMARY AND CONCLUSIONS:

A study of the dispersion and disappearance of enteroviruses and bacterial pollution indicator organisms was carried out in the Jordan River-Lake Kinneret watershed in Israel in 1968-1970. 452 assays of water supply, sewage, river and lake water samples at 16 different sampling stations were made. Detection methods for enteroviruses were based on 3-5 liter grab samples concentrated 500 fold by the Phase-Separation method and on the use of the qauze pad method. In an effort to boost the level of virus shedding from the town of Qiryat Shemoneh, the main source of fecal contamination, 600 school children were revaccinated with Sabin polio vaccine.

The main findings are as follows:

1. The mean concentration of enteroviruses in the sewage of Qiryat Shemoneh is 1,200 P. F. U. per 1000 ml. and the ratio of viruses: coliforms is about 1:1,000,000.

2. Enteroviruses were detected in western channel of the Jordan River at a point 3 km. below the sewage inlet in 13% of the samples and in about 5% of the samples, 19 km. downstream. On one occasion out of 43, enteroviruses were detected in Lake Kinneret at the intake of the Israel National Water System. The low level of virus detection in the river is explained by the high level of dilution of the sewage averaging 1:500 and the limits of detection of the method used of about 2 P.F.U./1000 ml. The revaccination with polio vaccine of 600 school children in Qiryat Shemoneh resulted in a detectible peak of virus concentration in the town's sewage 4-9 days latter, but in

no detectable increase in virus concentration in the river.

3. In a comparative study of pads vs. grab samples, it appears that the expressed fluid from qauze pads held in a sewage stream for 24 hours contain about 60 times more virus per ml. than that found in the sewage stream by grap samples. This phenomenon may be explained by the relative large volumes of sewage which may pass through the pad in 24 hours rather than by their high level of efficiency in picking-up viruses. This finding does not contradict other information indicating that such gauze pads have a virus pick-up efficiency of about 1%.

4. 75% of the virus strains identified were poliovirus. The remainder were echovirus and coxackievirus. 13% of the poliovirus strains showed C. P. E. at 40°C which is indicative of wild strains. Some of the poliovirus strains may indeed be wild types despite the fact that Israel has practiced universal polio vaccination since 1958. Some however, may be vaccine strains which may lose the T. marker on a single human passage.

5. Bacterial pollution indicator organisms studied showed rapid reductions due to dilution of sewage in the river but also showed relatively rapid die-away. In a 30 km. stretch of the river with a flow time of about 12 hours coliforms, fecal coli and fecal streptococcus counts were reduced by 90-95%. This may be indicative of active self-purification processes in the river.

6. Fecal coli counts in sewage and heavily polluted river were 1/10 of the coliform count with fecal streptococcus counts averaging 1/100 of the coliforms. However, in Lake Kinneret, distant from major sources of pollution, fecal coli practically disappeared, while fecal streptococci organisms remained and were found in concentrations 1/10 of the coliforms. These findings add further support to the contention that fecal streptococci are more resistent to environmental factors than either coli or fecal coli and serve as a valuable indicator of old or distant pollution.

7. Despite the limited information obtained in this study as to the ability of enteroviruses to survive in a natural river system such as the Jordan, the fact that it is exposed to heavy loads of fecal contamination and feeds a major source of water supply requires a cautious approach.. The single case of detecting enteroviruses at the intake of the Water Supply System may be indicative of the fact that enteroviruses are indeed more resistent than bacterial pollution indicators which practically disappear by the time they reach the lake. Methods for monitoring large volumes of water are the only way to provide a definitive answer to this question. Eventually water supply systems should be in a position to monitor 100-500 liter samples before ruling the water to be free of viruses.

ACKNOWLEDGEMENTS

Dr. Moshe Nishmi of the Department of Virology invested many weeks of work on the virus typing.

Miss Yael Wiener and Mrs. Nachama Biderman devotedly carried a major share of the burden of the virus assay work, and Mr. Andrew Thompson assisted in the bacteriological work.

Mr. I. Tikvati of the Mekorot Water Co. skillfully coordinated the field sampling program. Without his tireless efforts the study could not have been made. The cooperation offered by the management of the Mekorot Co. and Mr. Shem tov and Mr. E. Netzer in particular is appreciated.

A special note of thanks is extended to Mr. Shalom Levy and to the Public Health nurses of the Ministry of Health in Qiryat Shemoneh who assisted in the polio vaccination campaign. Mr. E. Larzarov, Mr. S. Ben Yehuda and Mr. M. Nevo also played key roles in this campaign. Many others in the Medical School and in the field participated in this study each in his own way. It would be too complicated to list each individually, but their cooperation is appreciated.

REFERENCES

1. Akin, E.W., Benton W.H., Hill, W.H. (1971). Enteric viruses in ground and surface waters: a review of their occurrence and survival. Proc. 13 water quality conf. — viruses and water quality. Ed. V. L. Snoeyink Univ. of Ill. (in press)

2. Geldreich, E.E. and Kenner, B.A. (1969). Concepts of fecal streptococci in stream pollution. Jour W.P.C.F. Vol. 41: R 336 - 352.

3. Goldshmid, Y. (1970). Water Quality Management of the Israel National Water System. Developments in Water Quality Research. Ed. H.I. Shuval, Ann Arbor - Humphrey Science Publishers, Ann Arbor.

4. Gravelle, C.R. and Chin, T.D.Y. (1961). Enterovirus isolations from sewage: a comparison of three methods. J. Infec. Dis. 109: 205-209.

5. Grindrod, J. and Cliver, D.O. (1970). A polymer two-phase system adapted for virus detection. Archiv. fur die gesamte virusforsforschung 31:365 - 372.

6. Hoff, J.C., Lee, R.D. and Becker, R.C. (1971). Evaluation of a method for concentration of micro-organisms in water in 1967, p. 37 - 49. Proc. Northwest Shellfish Sanitation Research Planning Conf. E.F.A. Gig Harbor, Wash.

7. Lee, R.D., Symons, J.M. and Robeck, G.G. (1970). Watershed human-use level and Water Quality. J.A.W.W.A. 62: 412 - 422.

8. Lennette, E.H. and Schmidt, N.J. (1964). Diagnostic Procedures for Viral and Rickettsial Disease. Am. Publ. Hlth. Ass., New York.

9. Liu, O.C., Brashear, D.A., Seraichekas, H.R., Barnick, J.A., and Metcalf, T.G. (1971). Virus in water: I. A preliminary study on a flow-through gauze-pad sampler for recovering viruses from water. Applied Micr. 21: 405 - 410.

10. Mosley, J.W. (1968). Transmission of viral disease by drinking water. p. 5 - 23 in G. Berg (Ed.), Transmission of viruses by the water route. John Wiley and Sons, New York.

11. Shuval, H.I., Fattal, B., Cymbalista, S. and Goldblum, N. (1969). The Phase-Separation method for the concentration and detection of viruses in water. Water Research 3: 225 - 240.

12. Shuval, H.I. (1970). Detection and control of enteroviruses in the water environment. Developments in Water Quality Research. Ed. H.I. Shuval, Ann Arbor - Humphrey Science Pub. Ann Arbor

13. Standard Methods for the Examination of Water and Wastewater (1965) 12th ed. Am. Public Health Assoc. New York, N.Y. 769 pp.

14. Wallis, C., Grinstein, S., Melnick, J.L. and Fields, J.E. (1969). Concentration of viruses from sewage and excreta on insoluable polyelectrolytes. Appl. Microbiol. 18: 1007 - 1014.

MARINE ANTIVIRAL ACTIVITY

Hillel I. Shuval, Andrew Thompson,

Badri Fattal, Samuel Cymbalista and Yael Wiener

INTRODUCTION

The phenomenon of natural processes of virus inactivation in sea water was first reported upon by Plissier and Therre at Nice in 1961 and since that time has been observed by others in the Atlantic Ocean, North Sea and Baltic Sea. Little however is known to date of the nature of this phenomenon. The presence of antibiotic substances in the sea capable of inactivating non-marine bacteria was noted as early as 1889, by Degiaxa, and has been studied by many workers over the years. These studies have been fully reviewed by Greenberg (1956), Orlob (1956) and Mitchell (1968), and point to the existence of biological factors as the primary mode of bacterial inactivation in the sea although some weight is given to purely chemical factors.

This paper reports on further developments of the studies on marine antiviral activity (MAVA) in the eastern Mediterranean and Red Sea reported on previously (Shuval, 1970) and deals in particular with the association of this phenomenon with marine bacteria.

2. METHODS AND MATERIALS

2.1 Virus Strain

The virus strain used in these experiments was a poliovirus type 1 strain originating from an epidemic which occurred in Israel in 1961. This strain was passed a number of times in monkey kidney cultured cells and BSC_1 cells. The virus stock was grown on BSC_1 cells and was concentrated by the phase separation (PS)[1] method (Shuval et al., 1969) so that when diluted its final concentration was $10^5 - 10^6$ PFU/ml.

2.2 Virus Assays

When primary isolations of enteroviruses in sewage or the sea were made, quantitation of viruses was carried out on primary Rhesus kidney cell (RKC) monolayers. BSC_1 cells were used in laboratory experiments.

All titrations were made using the plaque technique, and results reported as plaque forming units (PFU). The methods used for plating virus suspensions were those routinely employed in this laboratory. The cells were grown in diasposable plastic petri dishes 60 mm dia., in a humid atmosphere of 6% CO_2 at 37°C. Complete monolayers were formed in 4 days with BSC_1 and 6 - 8 days with RKC. Following virus adsorption for 30 - 45 min., an agar overlay was applied and the plates re-incubated for an additional period of 3 days and then neutral red vital stain incorporated in 2% agar was added as an overlay to the plates. After an additional period of 24 - 48 hours the plates were counted for plaques.

2.3 Isolation of Viruses From the Sea

Samples of sea water taken from an area north of Tel Aviv contaminated with sewage, were assayed for the presence of enteroviruses. Samples of the raw sewage flowing to the sea were also assayed for enteroviruses. Grab samples of 3 - 5 litres taken at regular intervals, from sampling points fixed by buoys, were assayed for viruses by the phase separation method.

This method (Shuval et al., 1969) has been shown to be capable of detecting as few as 1 - 2 enteroviruses per litre of water sample tested. Similar results have been demonstrated with sea water.

In addition, on a number of occasions polyethylene sponges were suspended at the sampling points for 24 - 48 hours. The expressed fluid from these sponges were assayed for the presence of enteroviruses. Parallel samples were tested in each case for coliforms, faecal coliforms, enterococci and salmonellae (to be reported on elsewhere).

2.4 Virus Typing

When field samples of sewage or sea water were assayed for enteroviruses, a number of plaques from each positive plate were transferred to tubes, which were frozen and kept at -20°C until identification. Virus typing procedures were similar to those used in the routine clinical virus typing work of this laboratory (Lennette and Schmidt, 1964).

2.5 Assay of Sea Samples for Marine Antiviral Activity (MAVA)

Samples of sea water to be assayed for the presence of MAVA were inoculated with a suspension of poliovirus type 1 so that the final concentration was 10^5 - 10^6 PFU/ml. Usually the suspension was diluted 1:100 or 1:1000 in the sea water sample. Samples were incubated at room temperature (22± 3°C) for 7 days unless otherwise noted. Parallel samples of sea water heated to 90°C for one hour, inoculated with an equal amount of poliovirus and incubated under the same conditions

served as a control. Samples taken at regular intervals over the period of the test were assayed for virus concentration. The degree of virus inactivation was expressed as the "MAVA coefficient" which was calculated by subtracting the 7-day virus concentration (expressed in logs to the base 10) of the sample tested from that of the 7-day control. As suggested by Magnusson et al. (1967) a log difference of 2.0 or greater was considered as positive for natural virus inactivation. The heat treated controls usually showed a reduction in virus concentration of less than 1 log in 7 days.

2.6 Assay of Pure Cultures of Marine Bacteria for MAVA

A similar procedure was followed when testing pure cultures of marine bacteria for MAVA. However in those tests, overnight broth cultures of the strain to be tested were diluted in heat-treated sea water samples so that the final density of the bacteria was about 10^3/ml or similar to that found in most natural sea samples tested. The MAVA coefficient was calculated in the same manner as described above. In each case heat-treated sea water and normal sea water inoculated with poliovirus were used as negative and positive controls respectively.

2.7 Enumeration of Marine Bacteria

Sea water samples were membrane filtered — MF (Gelman-Metricel 0.45u) in appropriate dilutions made with sterile sea water. The filters were placed in pre-sterilized plastic petri dishes containing 5 ml of 1 5% agar medium similar to ZoBell's medium 2216. The plates were incubated at room temperature ($22 \pm 3^{o}C$). The formula of the medium used in these studies either as a broth, with an added 1.5% agar for plates, or with double strength peptone and 1.5% agar for stock culture slopes is as follows:

5.0g Bacto Peptone
1.0g Bacto Yeast Extract
0.2g Sodium Thiosulphate made up to 1,000 ml with sea water or distilled water (when distilled water was used 40.0g sodium chloride was added)

pH after autoclaving 7.4.

Fresh sea samples were also tested by MF methods for coliforms, faecal coliforms and streptococcus fecalis in order to establish the degree of contamination with sewage organisms.

2.8 Isolation of Pure Cultures of Marine Bacteria

Discrete colonies from membrane filters of fresh sea water samples were picked and sub-cultured on slopes of either stock culture agar (Difco) or the special marine bacteria medium mentioned above. All pure culture isolates were sub-cultured at about 6-week intervals onto fresh slopes incubated at $22 \pm 3^{o}C$ for 48 hours and subsequently stored at $4^{o}C$. The morophological and biochemical characteristics of selected isolates were studied and will be reported upon elsewhere.

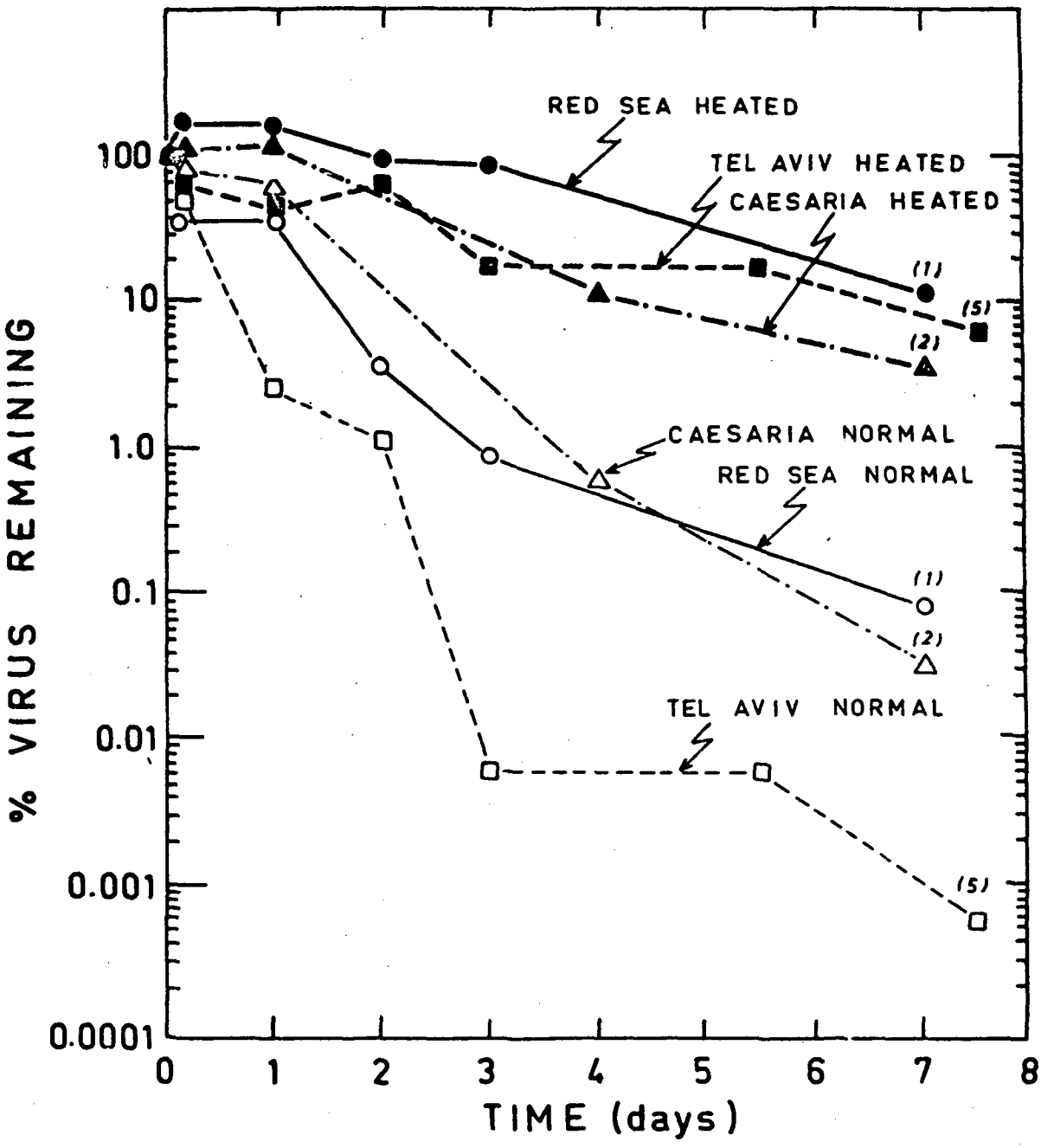
3. RESULTS

3.1 Field Studies

In the summer and fall of 1968, 72 samples of sea water showing various degrees of sewage contamination were assayed for enteroviruses by the PS method or by the sponge method. Enteroviruses were isolated in 20 cases and included polio I, II and III and types of echovirus and coxsackievirus. Most of the isolations were in the highly contaminated area immediately above the end of the outfall sewer discharging some $100,000m^3$ of sewage daily: the sewer extends 880m into the sea. In that area 12 out of 24 samples were positive for enteroviruses ranging in concentration as high as 60 PFU/1. The median coliform MPN in that area was 2.4×10^6/100 ml. The physical dilution factor of the sewage was measured by the use of radioisotopes (Gilat et al., 1970) and found to be from 1:10 to 1:100. At the most distant sampling station 1,500m to the northeast, opposite a popular bathing beach enteroviruses were isolated in 2 out of 16 samples. The mean coliform MPN was 460/100ml. The physical dilution factor at that point was determined to be from 1:100 to 1:1000.

Fig. 1.

INACTIVATION OF POLIOVIRUS IN NORMAL AND HEAT-TREATED SEA WATER SAMPLES FROM THE MEDITERRANIAN AND RED SEA



(n) = number of experiments

During the same period 16 samples of raw sewage from the Tel Aviv main pumping station, before disposal to the sea, were assayed for enteroviruses. All were positive and the mean concentration was 664 PFU/1. The median coliform MPN was 4.6×10^8/100ml.

3.2 Assays for MAVA in Sea Samples

In order to study virus inactivation in sea water under more controlled conditions it was decided to carry out a series of laboratory tests.

Ten samples of sea water taken from various points along the Mediterranean coast from Haifa in the north to Palmachim in the south and two samples from the Red Sea, one from the Eilat beach and the second from a point further south in the open sea were assayed for MAVA. The results of 8 of these tests are shown graphically in fig. 1. The MAVA coefficient of each sample is presented in Table I. It should be pointed out that the mean MAVA coefficient is 4.1 for samples 1 - 6 taken from the sewage polluted sea near Tel Aviv and Haifa while it is 2.7 for the 6 samples taken from the other relatively unpolluted areas.

TABLE I

Marine antiviral activity (MAVA) coefficient for polluted and unpolluted samples of sea water from the Mediterranean and Red Sea

Sample No.	Place	Coefficient of Inactivation
1	Tel Aviv	4.0
2	Tel Aviv	3.5
3	Tel Aviv	5.0
4	Tel Aviv	4.5
5	Tel Aviv	3.2
6	Haifa	4.2 Av. $\overline{4.1}$ (polluted)
7	Caesarea	2.7
8	Red Sea	2.2
9	Palmachim Beach	4.0
10	Eilat	2.1
11	Palmachim	3.2
12	Palmachim	2.2 Av. $\overline{2.7}$ (unpolluted)

FIG. 2

THE STIMULATION OF MARINE BACTERIAL FLORA IN NORMAL SEAWATER BY TISSUE CULTURE FLUID + 16°C

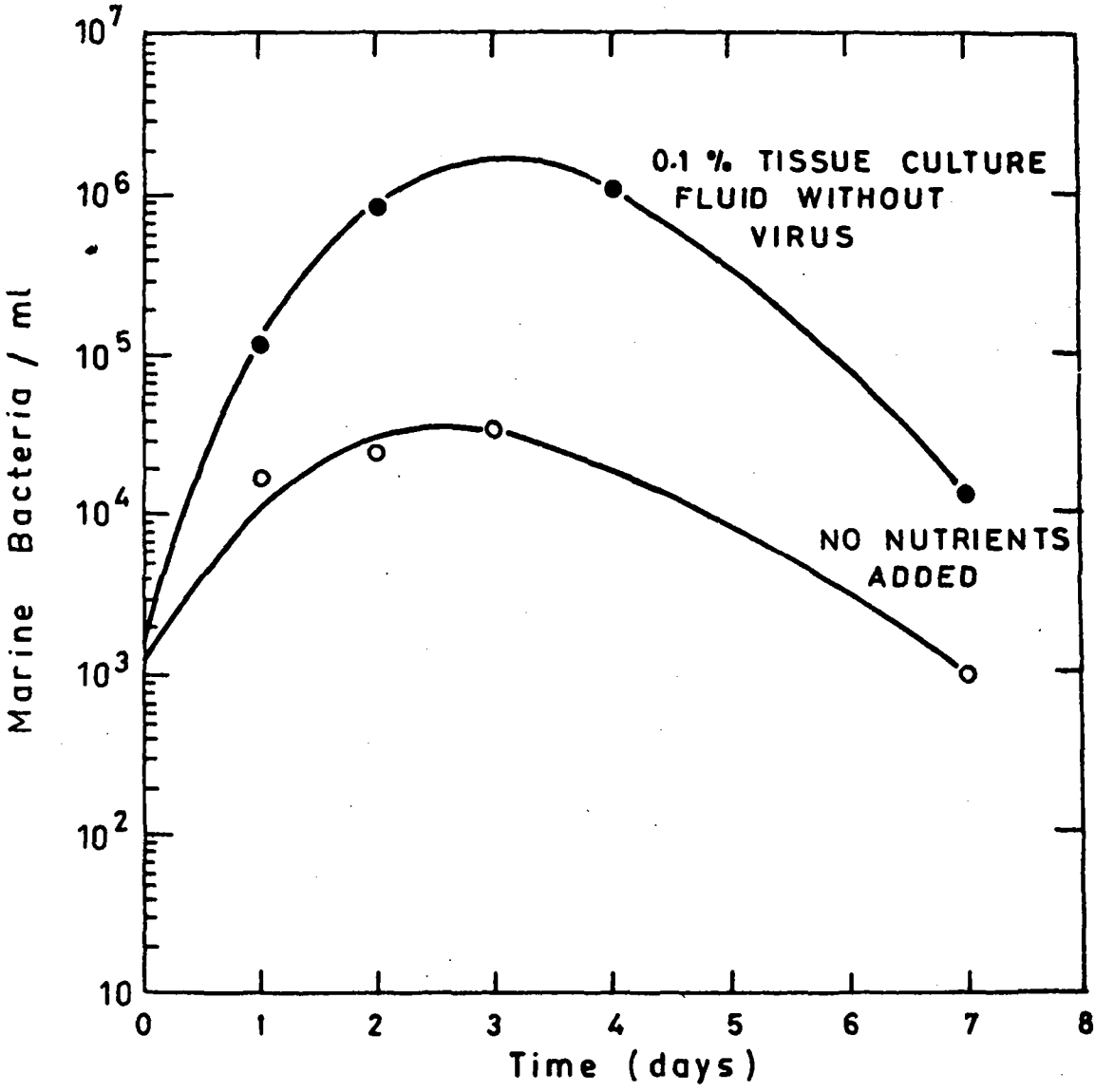


Table II shows the MAVA coefficient (from 1.2-4.0) for repeat assays of a single sample of sea water tested over a 60-day period as compared to the count of marine bacteria in the sample. This sample, as all other sea water samples, held for laboratory studies was stored at 4°C. In a sample of sea water from the Red Sea the initial MAVA coefficient was 2.2 while after 18 months it was still positive. At the same time the marine bacteria count was 10^2/ml.

TABLE II

MAVA coefficient and marine bacteria population in stored sea water (4°C)

Days	3	6	9	28	39	44	60
MAVA coefficient	2.2	1.2	3.2	2.8	4.0	3.3	4.0
Bacteria per ml	$1x10^2$	$6x10^1$	$2x10^3$	$3x10^3$	$1x10^3$	$2x10^3$	$2x10^3$

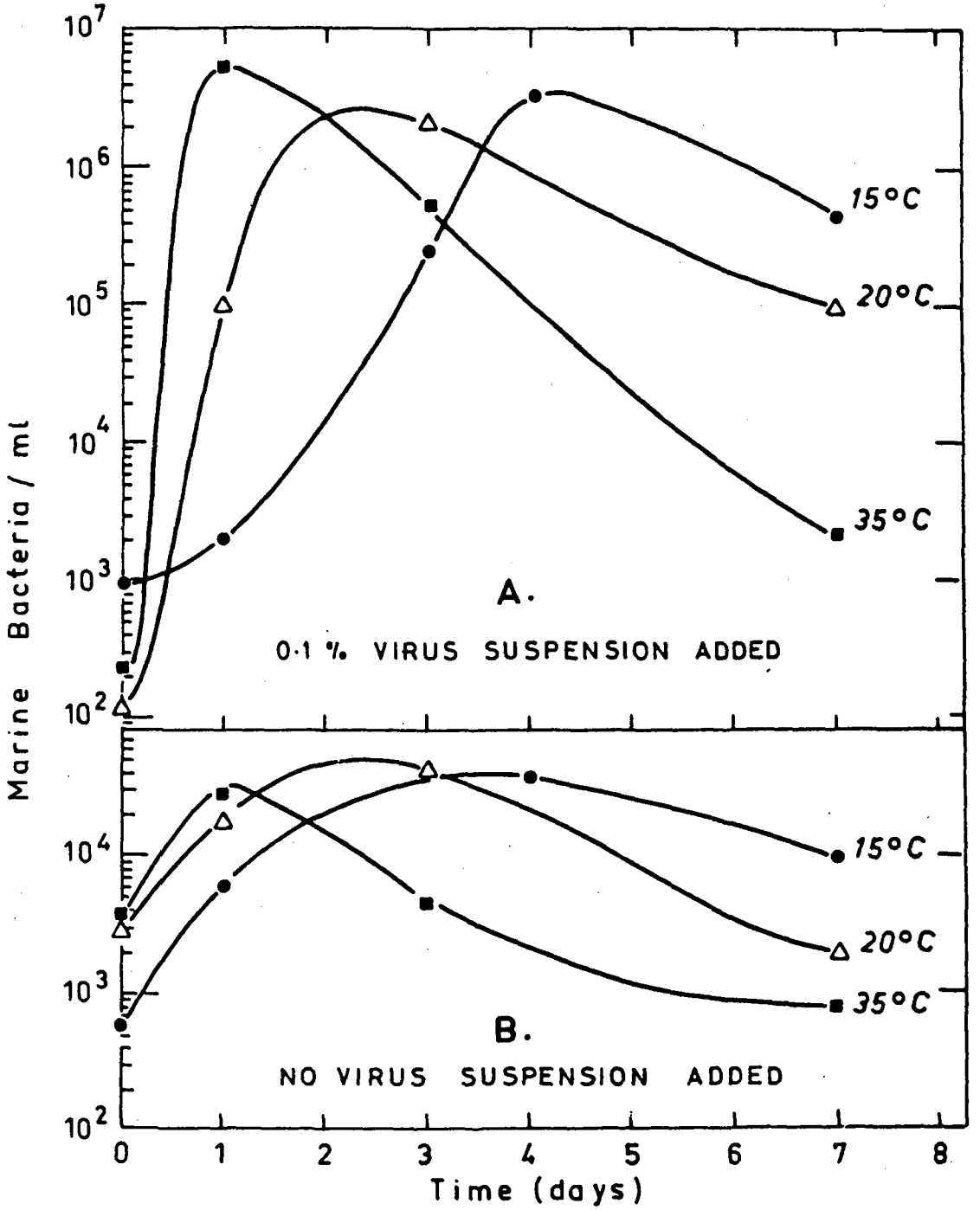
3.3 MAVA Assay Tests and the Growth of Marine Bacterial Flora

Marine bacteria were enumerated before testing samples for MAVA and during the period of the test. It was noted that the bacterial count increased during the test to a maximum of about 10^6 organisms/ml while initial counts were about 10^3/ml.

In fig. 2 the growth of the natural marine bacterial flora of a sea water sample incubated at 16°C is shown when no nutrients are added and again when 0.1% tissue culture fluid (without viruses) is added. This tissue culture fluid was prepared in the same way as normal virus stock suspension would have been grown in tissue culture except that no virus was used to infect the cells. The sample with tissue culture fluid added reached a maximum bacterial count of about 10^6/ml in

FIG. 3

THE GROWTH OF MARINE BACTERIAL FLORA IN NORMAL SEAWATER AT VARIOUS TEMPERATURES



3 days while the sample without added nutrients increased in bacterial count to about 10^4/ml in the same period.

Fig. 3B shows the growth of marine bacterial flora in normal sea water at 15°C, 20°C and 35°C with no virus suspension added while fig. 3A shows the same samples with 0.1% virus suspension added for MAVA assay.

The point of maximal bacterial growth is 1 day at 35°C, 2-3 days at 20°C and 4 days at 15°C. The maximal bacterial growth levels of the samples to which virus suspensions have been added are about 2.0 logs higher than those with no virus suspension added.

Fig. 4 presents the virus inactivation curves for each of the three samples shown in Fig. 3A. The maximum of the corresponding bacterial growth curve given in Fig. 3A is shown by an arrow.

Fig. 5 presents a typical virus inactivation curve at 15°C over a 30 day period showing a MAVA coefficient of 2.8 in 7 days and 4.1 at the end of the test.

3.4 Attempts to Demonstrate MAVA After Treatment to Remove Marine Bacteria

A series of experiments were carried out with the aim of testing for MAVA in sea samples from which the marine bacteria had been removed or inactivated. In each experiment a sample of heat treated sea water with virus suspension added was used as a negative control while a sample of normal sea water with virus inoculum added was used as a positive control.

Samples of sea water were centrifuged at 65,000 or 100,000g for 1 hr.; ultrasonicated while cooled in crushed ice for up to 15 minutes at 22Kcs; and shaken in the presence of glass beads of 0.1mm diameter in a liquid CO_2 cooled Braun Disintegrator for 2 minutes. In each of the above cases the treated sample proved positive, with a MAVA coefficient greater than 2.0. In each case the sample after treatment proved positive for viable marine bacteria in concentrations

FIG. 4

INACTIVATION OF POLIOVIRUS IN NORMAL SEAWATER AT DIFFERENT TEMPERATURES

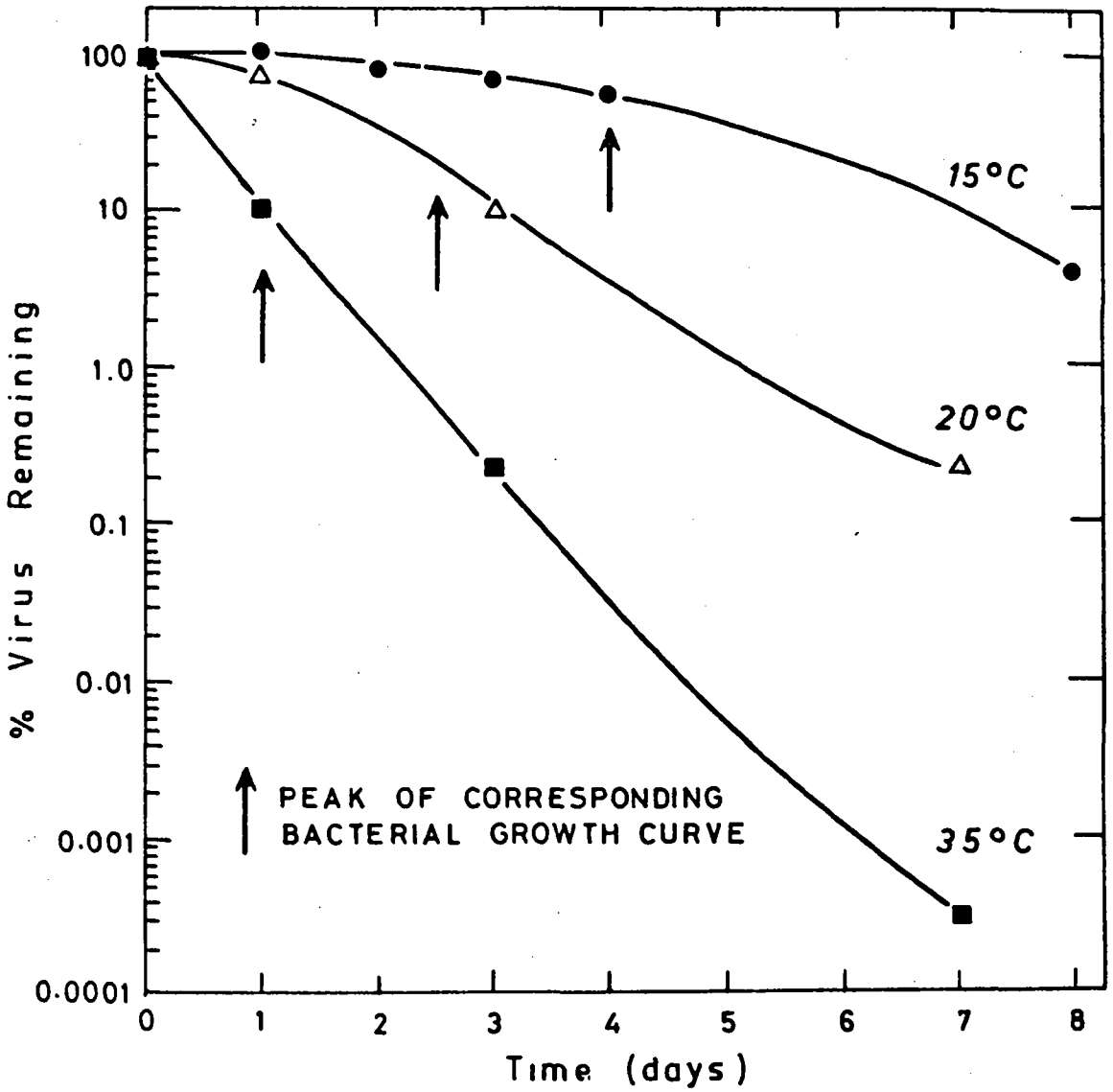
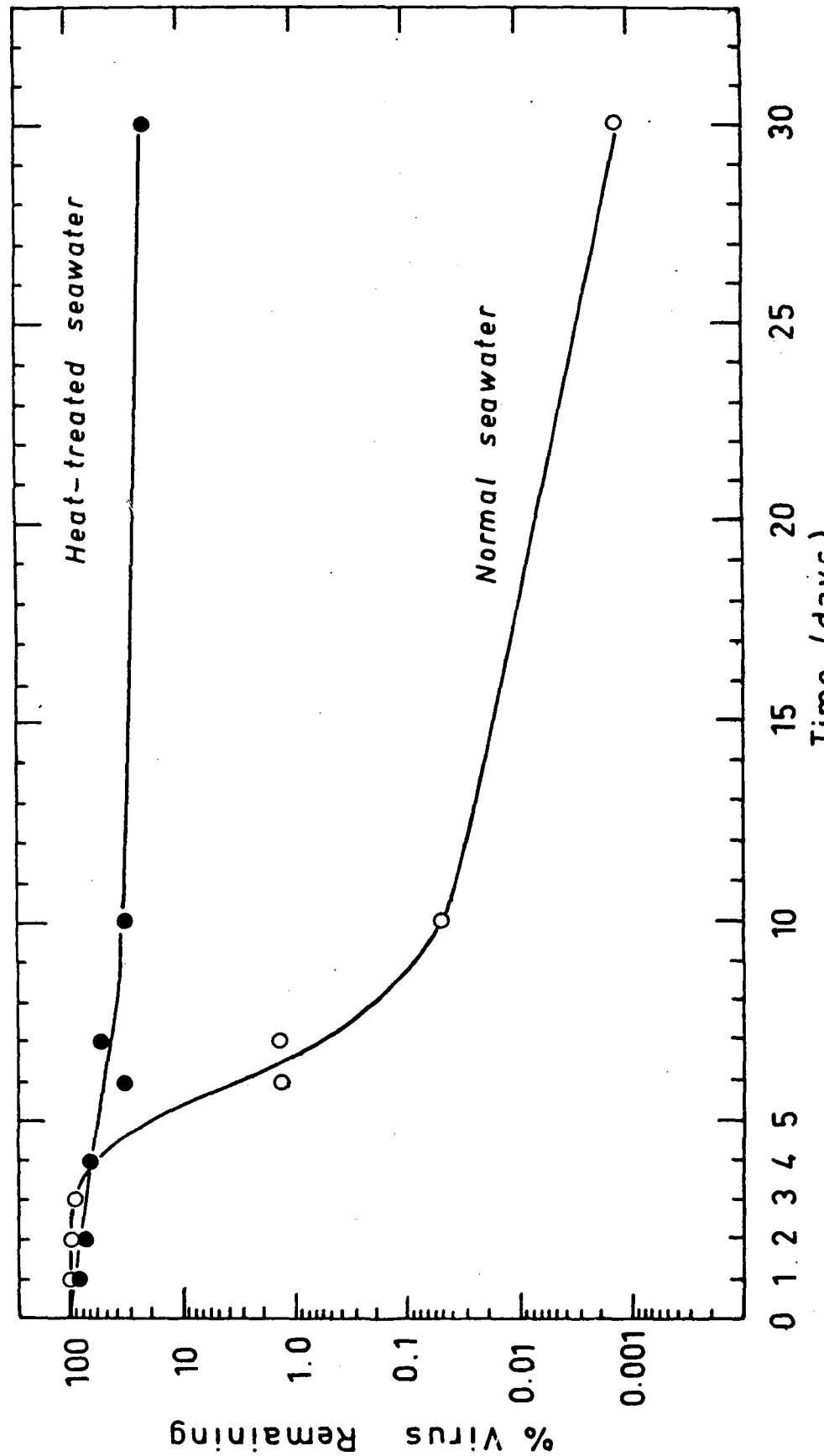


FIG. 5

INACTIVATION OF POLIOVIRUS IN NORMAL AND HEAT-TREATED SEAWATER AT 15°C



ranging from 10-100/ml. The treated sea water was tested for marine bacteria again at the end of the 7 day MAVA test at room temperature. In all cases the bacterial count in the sea water samples being tested was of the order of 10^3-10^4/ml.

Sea water samples filtered through membrane filters (Millipore) of 0.22u and 0.3u pore size were negative for MAVA. No bacteria were found.

Virus suspension was added to heat treated sea water and placed in a sterile cellulose dialysing bag suspended in normal sea water enriched with 0.1% tissue culture fluid. The results were negative for MAVA. No viable marine bacteria were isolated from the bag: the enriched seawater supported an active population.

3.5 Isolation and Testing of Pure Cultures of Marine Bacteria

A number of pure culture isolations were made from samples of sea water from various points along the Mediterranean coast and from the Red Sea. 58 of these cultures were tested for MAVA and 8 showed MAVA coefficients of greater than 2.0 (See Table III), while the others showed practically no virus inactivation activity. Some of these cultures represented bacteria of widely differing morophological and biochemical characteristics. These cultures were retested after an interval of several months and 7 of the 8 now showed MAVA coefficients less than 2.0. Only Strain #13 was positive with a coefficient of 3.5. On further retest strain #13 was also negative. The cultures were all subcultured at 4°C according to a procedure followed by Magnusson et al. (1967) and tested again for MAVA; however their virus inactivating activity was not regained.

TABLE III

MAVA coefficients of marine bacteria pure culture isolates in repeat tests

Culture No.	Place of Isolation	MAVA coefficient 1st test	2nd test
1	Tel Aviv	4.0	0.2
13	Eilat	3.8	3.5
15	Eilat	3.8	0.6
23	Eilat	4.6	1.3
26	Tel Aviv	2.7	1.9
35	Tel Aviv	3.3	1.2
39	Palmachim Beach	3.2	0.5
47	Palmachim Beach	4.0	0.4

4. DISCUSSION

The isolation of pathogenic enteroviruses in contaminated sea water has been reported upon by a number of workers (Metcalf et al., 1967). However, of particular interest here is the detection of enteroviruses at a site 1,500m from the point of sewage discharge where physical dilution, together with natural dieaway has reduced the coliform count by a factor of some 10,000. Enteroviruses were detected in this area, opposite a popular bathing beach, generally showing coliform bacteria counts in the neighborhood of those considered acceptable for bathing (2400/100ml).

Parallel studies to determine the degree of physical dilution of the sewage in sea in that same area were made with the aid of radioisotopes (Gilat et al., 1970). In view of the initial virus concentration in the raw sewage, which averaged 664 PFU/1 and a physical dilution of between 100 - 1,000, it can be surmised that the enteroviruses showed little or no dieaway in the sea from the time of discharge until being carried to the bathing beach many hours later, where they were detected. During that same period the coliform count was reduced by 3 logs by dieaway alone. This

indicates that enteroviruses appear more resistant to natural inactivation factors in sea water than coliforms and shows the limitations of depending upon these bacteria as pollution indicator organism, at least as far as viruses are concerned.

Despite its apparent slow acting effect in the marine environment we decided to investigate the processes of virus inactivation in sea water under laboratory conditions.

The presence of virus inactivating capacity in the sea has been noted by a number of workers (Plissier and Therre, 1961 and 1963; Cioglia and Loddo, 1962; Hedstrom and Lycke, 1964; Matossian and Garabedian, 1967; Metcalf and Stiles, 1967; and Mitchell and Jannasch, 1969). Our initial studies of a similar phenomenon in samples from the eastern Mediterranean and Red Sea led us to believe that we are dealing with a biological factor since sea samples lost their antiviral action when membrane filtered, treated with ether or when heated to 90°C (Shuval, 1970). For convenience we have called it MAVA (Marine antiviral activity). However it is not our intention to imply any specific mode of action or beneficial use.

The finding of higher MAVA coefficients in samples taken from the Tel Aviv and Haifa shore area which are polluted with sewage, as compared with samples from cleaner areas (Table I) might suggest that MAVA, which is apparently associated with marine bacteria, is stimulated by the nutrients in the sewage. We shall discuss this later.

Magnusson et al. (1967) have already pointed out the possible relationship between MAVA and marine bacteria and Gundersen (1967) reported on the isolation of a pure culture of Vibrio marinus (later identified as Vibro parahaemolyticus*) which possessed virus inactivating capacity similar to that found in natural sea water samples. All samples of sea water we tested for MAVA showed initial bacteria concentrations ranging from 100-1000/ml. In one MAVA positive sea sample from the Red

---

* Personal communication from K. Gundersen.

Sea stored for 18 months at 4°C we still detected about 100 bacteria/ml. Magnusson (1966) reported finding no difference in the virus inactivating capacity of varying dilutions of sea water. In the light of our finding concerning bacterial regrowth during the test procedure this is understandable. On a number of occasions control samples heat treated to 90°C for 1 hour showed MAVA coefficients higher than 2.0 although on almost all occasions they were nil or less than 1.0. On one occasion, when checked for sterility, it was found that the control did indeed have an active bacterial population. This may be a regrowth of heat resistant strains not destroyed at 90°C. The possibility of contamination cannot be ruled out, however.

We decided to study the growth of the marine bacterial flora in samples of sea water being tested for MAVA. The aim was to determine whether the addition of virus suspension containing tissue culture fluid influenced bacterial growth and the rate of MAVA. Fig. 2 clearly shows that the addition of the tissue culture fluid — host cell mixture in which the viruses are suspended serves as a nutrient stimulus for the growth of the marine bacteria. In fig. 3 it can be seen that the maximal bacterial growth of the order of 10^6 organisms/ml is reached in one day at 35°C, in 2-3 days at 20°C and in 4 days at 15°C. Fig. 4 shows the corresponding virus inactivation patterns at the three different incubation temperatures. It is interesting to note that the more rapid virus inactivation phase is reached in each case on or about the time of maximal bacterial growth. There was no rapid bacterial growth or MAVA at 5°C.

Fig. 5 showing the virus inactivation in a sea water sample at 15°C over a 30 day period indicates that the most rapid phase of virus inactivation has ended by about the 10th day at which time our studies have shown that marine bacterial counts have returned to figures approaching their initial concentrations of about 10^3/ml or slightly above them.

These data give further support to the idea that there appears to be a very close association between the growth rate and/or level of marine bacteria in the sea water sample and the point of onset and rate of virus inactivation.

That the rate of virus inactivation appears to be temperature dependent is to be expected in light of the known kinetics of virus inactivation in relation to temperature with other inactivation agents. It is difficult to say at this time whether the shape of the inactivation curves in fig. 4 are a result of the effect of the bacterial growth rate and the point of the maximal bacterial concentration or the temperature dependence of the inactivating activity, or a combination of both.

The higher MAVA coefficients found in polluted sea water may also be a result of the higher nutrient levels in the sample which stimulates bacterial growth under laboratory test conditions.

One question that comes to mind is whether the rapid rate of virus inactivation shown in these and similar laboratory experiments of others is not some sort of laboratory artifact associated with the rapid growth and high levels of the marine bacteria stimulated artificially by the addition of the nutrient rich virus suspension and incubation at temperatures much higher than naturally found in the marine environment. In any event even the rate of virus inactivation found under artificial laboratory conditions indicates that the time required for a 90% reduction in virus count (T_{90}) is in the order of 48 hours as compared to 1 - 3 hours for coliforms at 15°C (Gilat et al., 1970). This may mean that MAVA is of minor importance in the self-purification processes involved in polluted sea water near bathing beaches, particularly where the time of passage is only a matter of hours.

It has been hypothesized that MAVA is an enzyme produced by certain marine bacteria and we have attempted to investigate this possibility. Our attempts to obtain virus inactivation in sea water without the presence of marine bacteria have failed to date. Attempts at total bacterial removal or kill with ultracentrifugation, ultrasonication and disintegration failed. It is not surprising that all such treated samples showed MAVA when one realizes that despite a considerable initial reduction in concentration, the final bacterial count in the sea samples tested reached the same high level in a matter of a few days of incubation at room temperature.

Membrane filtration of small sea samples removed all bacteria and the filtrate showed no MAVA. This was repeated with the filtration of sea samples of 1 liter with the aim of ensuring the passage of any free enzyme or other antiviral factor which might have been completely adsorbed in the membrane filter matrix when smaller volumes were filtered. The filtrate of these samples also showed negative MAVA coefficients as well as no bacteria.

Our attempts to determine whether the active antiviral effect could be transmitted through a dialysing bag held in a normal sea water sample in the laboratory also proved negative. Metcalf and Stiles (1967) reported on the inactivation of a virus suspension held in the open sea in a sealed dialysing bag. Since they had inadequate controls there is no certainty that the inactivation was in fact a result of the passage of some antiviral factor through the walls of the dialysing bag or was a result of dieaway due to other environmental factors such as light. Plissier and Therre (1963) also showed active dieaway of viruses held in a dialysing bag in the open sea; however their controls held in samples of the same sea water in the laboratory showed a much slower inactivation rate, again raising the doubt that the antiviral factors actually passed through the dialysing bag.

Our efforts to demonstrate the activity of a virus inactivating agent independent of viable marine bacteria with which it is apparently associated have not succeeded so far. There may be several possible explanations for this. One might be that the inactivation of viruses in normal sea water or in pure cultures or certain marine bacteria may be associated directly with the micro-organisms themselves and not some agent produced by them. It could possibly be some form of surface adsorption which becomes effective quantitatively when bacterial densities approach the 10^6/ml.

Another possible explanation might be that some marine bacteria do indeed produce a form of virus inactivating agent which is very short lived in the test environment and works effectively only when bacteria are present in sufficiently high numbers to allow the agent to reach concentrations for effective virucidal action.

Whether this agent is an enzyme or some type of oxidant is yet to be determined. All of the above hypotheses are being actively pursued.

It should also be pointed out that among the 8 organisms found to show MAVA, a number had widely differing morphological and biochemical characteristics indicating that the phenomenon is apparently associated with more than the single organism isolated by the Swedish group (Gundersen, 1967). Magnusson (1966) finds that virus inactivating capacity was effective for poliovirus 1-3 coxsackie B_5, echo 32, adeno 7, vaccinia, and influenza A_2.

The loss of virus inactivating capacity by marine bacteria on multiple passaging at 23°C and its being regained after subculturing at 4°C has been reported by Magnusson (1967). Our positive cultures have completely lost their activity, however. New pure culture isolates are now being evaluated and subcultivation at lower temperatures is being tried to prevent the loss of this characteristic of certain marine bacteria.

The finding of the MAVA in the Atlantic Ocean, Baltic Sea, North Sea, western Mediterranean and in all samples of sea water tested from points along the Mediterranean coast of Israel and in the Red Sea as well as in 8 pure culture isolates of marine bacteria from those samples indicates the ubiquity of this phenomenon.

5. SUMMARY AND CONCLUSIONS

A study of the distribution and dieaway of enteroviruses in polluted coastal waters was made which led to an investigation of virus inactivation in the marine environment. Marine antiviral activity (MAVA) which is felt to be of a biological nature and closely associated with specific marine bacteria is reported upon.

The main findings are as follows:

5.1 Enteroviruses are more resistant to inactivation processes in the marine environment than coliform organisms which have been used as the classical

pollution indicator organism. Enteroviruses were detected in a sewage polluted coastal area on numerous occasions including opposite a bathing beach showing relatively low coliform counts.

5.2 Twelve samples of sea water from the Mediterranean coast of Israel and from the Red Sea showed marine antiviral activity, when inoculated with poliovirus under laboratory conditions. Samples from the areas polluted with sewage showed higher MAVA coefficients.

5.3 Heat and ether treated samples lose their antiviral activity, as do samples passed through membrane filters.

5.4 All MAVA positive sea samples contained viable marine bacteria at an average density of 10^3/ml while a sample stored for 18 months at 4^o still contained as many as 100 organisms/ml.

5.5 The addition of virus suspension during the laboratory test for MAVA with incubation at $22^o \pm 3^oC$ for 7 days led to the stimulation of the bacterial numbers to 10^6/ml while incubated sea water with no nutrients added showed a much lower growth of organisms.

5.6 The time at which the rapid inactivation phase of the viruses starts, appears to be closely associated with the period of the logarithmic growth phase and the maximal bacterial concentration in the sea water samples.

5.7 The rapid rate of virus inactivation detected under laboratory conditions (a 4 - 5 log reduction in 7 days) may be associated, to a great extent, with the artificial stimulation of marine bacterial growth upon the addition of nutrients and the high incubation temperatures of the laboratory test situation.

5.8 The effectiveness of MAVA in the self-purification processes in the open sea may be of minor importance, particularly when sewage pollution is carried to adjacent bathing beaches in a matter of hours, since the T_{90} of poliovirus in sea water appears to be about 48 hours at 15^oC.

5.9 It was not possible in the experiments reported upon to demonstrate active antiviral factors in sea water in the absence of marine bacteria. Whether the antiviral activity requires the presence of the bacteria or is associated with a very short-lived agent produced by the bacteria has not been determined.

5.10 An effect similar to MAVA as seen in normal sea water samples was detected in 8 pure culture isolates of marine bacteria. The morphological and biochemical characteristics of a number of these organisms differed widely. On multiple sub-cultivation their antiviral activity was lost.

5.11 The fact that virus inactivation activity in the sea has been observed in the Atlantic Ocean, North Sea, Baltic Sea, Mediterranean Sea and Red Sea indicates the ubiquity of this phenomenon.

Acknowledgements: The continuing advice and guidance of Prof. Nathan Goldblum is sincerely appreciated. Without the devoted assistance and the skillful organization of the program of field sampling by Mr. Naftali Cohen this study could not have progressed as it did. Dr. Moshe Nishmi's work in identifying the virus types is appreciated. The valuable comments of Dr. Eliyahu Katzenelson in the analysis of the data and in the writing of this manuscript has contributed much to this effort.

REFERENCES

1. Cioglia, L. and B. Loddo, The process of self-purification in marine environment III, Resistance of some enteroviruses. Nuovi Ann. d'Igiene Microbiol., 13: 11-29. 1962

2. Degiaxa, V., Uber das Verhalten E meger Pathogene Microorganismen in Meerwasser. Z. Hyg. Infektionskrank., 16: 162-225. 1889

3. Gilat, Ch., H.I. Shuval, Y. Yoshpe-Purer, N. Cohen, "Developments in Water Quality Research; the use of radioisotope tracers in the study of the dispersion and inactivation of bacteria discharged in coastal waters." Ann Arbor-Humphrey Science Publishers, Ann Arbor-London, 269 pp. 1970

4. Greenberg, A.E., Survival of enteric organisms in sea water. Publ. Health Reports., 71:77. 1956

5. Gundersen, K., A. Brandberg, S. Magnusson, E. Lycke, Characterization of a marine bacterium associated with virus inactivating capacity. Acta. path. et. microbiol. scandinav., 71: 281-286. 1967

6. Hedstrom, C.E. and E. Lycke, An experimental study of oysters as virus carriers. Am. J. Hyg. 79:134. 1964

7. Lennette, E.H. and N.J. Schmidt, Diagnostic procedures for viral and rickettsial disease. Am. Publ. Hlth. Assn., New York. 1964

8. Lycke, E., S. Magnusson, E. Lund, Studies on the nature of the virus inactivating capacity of sea water. Arch. ges. Virusforsch., 17:409. 1965

9. Magnusson, S., K. Gundersen, A. Brandberg, E. Lycke, Marine bacteria and their possible relation to the virus inactivating capacity of sea water. Acta. path. et. microbiol. scandinav., 71: 274-280. 1967

10. Metcalf, T.G. and W.C. Stiles, "Transmission of Viruses by the Water Route: Survival of enteric viruses in estuary waters and shellfish." Interscience Press, New York, 439 pp. 1967

11. Mitchell, R., Factors affecting the decline of non-marine microorganisms in sea water. Water Research, 2: 535. 1968

12. Mitchell, R. and H.W. Jannasch, Processes controlling virus inactivation in sea water. Environ. Sci. & Tech., 3:941. 1969

13. Matossian, A.M. and G.A. Garabedian, Virucidal action in sea water. Am. 1967 J. of Epidem., 85: 1-8.

14. Orlob, G.T., Viability of sewage bacteria in sea water. Sewage and Indus- 1956 trial Wastes., 28: 1147.

15. Plissier, M. and P. Therre, Recherches sur l'inactivation du poliovirus 1961 dans l'eau de mer. Ann. Inst. Pasteur., 101 : 840-844.

16. Plissier, M., L'inactivation dans l'eau de mer et l'eau d'alimentation de 1963 certains enterovirus. Arch. ges. Virusforch., 13: 76-81.

17. Shuval, H.I., B. Fattal, S. Cymbalista, N. Goldblum, The phase-separa- 1969 tion method for the concentration and detection of viruses in water. Water Research., 3: 225-240.

18. Shuval, H.I., "Developments in Water Quality Research;" The detection 1970 and control of enteroviruses in the water environment. Ann Arbor-Humphrey Science Publishers, Ann Arbor-London.