MEMBRANE CONCENTRATION PROCEDURES TO ASSESS QUANTITATIVELY THE EFFECTIVENESS OF THE TUCSON, ARIZONA PILOT FILTER IN REMOVING ENTEROVIRUSES FROM WASTEWATER

by

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A Thesis Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY AND MEDICAL TECHNOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

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ACKNOWLEDGMENTS

The author wishes to express her sincere appreciation to Dr. P. P. Ludovici for direction, advice, and guidance throughout this study.

Grateful acknowledgment is made to Richard G. Coleman for technical as well as for academic assistance. Gratitude is also expressed to Mrs. Alex Hodge and Mrs. Dorothy McKenrick for their thoughtful cooperation in the preparation of reagents, tissue cultures, and supplies.

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ABSTRACT

A membrane virus concentration procedure was used to demonstrate the effectiveness of a tertiary treatment Pilot Filter in reducing the virus concentration of activated sludge effluent from the Tucson sewage treatment plant. This technique, which allows the quantitative determination of virus infective units per sample volume, showed that the Pilot Filter reduced the virus concentration by an average of 96%. No significant difference in removal of virus content was observed when the Pilot Filter was loaded from two different basins with horizontal travel distances of approximately 50 and 100 feet.

For identification of virus types, a rapid and economical plastic panel micro culture technique showed an excellent correlation to the standard tube culture method in virus neutralization studies. Virus types detected during the eight month period of the study included poliovirus types 1, 2, and 3, one adenovirus, and several echovirus types.

Preliminary studies were carried out to determine whether the membrane virus concentration procedure could be improved. These studies included pre-treatment of membranes with serum, use of a Dowex anion exchange column,

and the investigation of an aluminum hydroxide virus-adsorption method. Each of these procedures significantly improved the efficiency of virus recovery.

The aluminum hydroxide method was superior to the other procedures and gave 100% recovery of virus added to wastewater samples.

INTRODUCTION

The population "explosion" coupled with the intensified interest in water for recreational purposes have stimulated investigations to provide information on the advisability of reclaiming wastewater. In many areas, especially the Southwest, political problems arise over derivation of future supplies of water to meet an increasing demand. Desalination plants are being constructed for processing sea water to render it suitable for human consumption. The Glen Canyon Dam has been constructed and other dams will be built in the future with the regrettable result that some of the country's most scenic and irreplaceable areas will be destroyed.

One of the most obvious sources of water is wastewater. At the present time, many studies are under way to determine the feasibility of reclaiming wastewater. These include studies conducted in Nassau County, New York, Lebanon, Ohio, Pomona, California, the Sanitation Districts of Los Angeles County, and the Orange County Sanitation District (1). Another similar study initiated in 1966, is the Santee Water Reclamation Project at Santee, California. The primary purpose of the study was to

determine the suitability of reclaimed water for unrestricted recreational use (2).

Ubiquity of enteroviruses. Since viruses as disease causing entities are a threat to successful reclamation of wastewater, their detection and elimination are necessary. Virus populations present in sewage are included in three major groups, the echoviruses, the polioviruses, and the coxsackieviruses. Over 70 individual types are recognized. Adenoviruses and reoviruses have also been isolated. These virus types are ubiquitous and are excreted in large numbers especially during the late summer and early fall and may subsequently be found in sewage. Epidemiological studies show that infections caused by these agents are widespread in the population with the highest morbidity rate in infants and young children (3).

Outbreaks of disease attributed to water supplies. Published information on outbreaks of disease attributed to water supplies is limited. There have been only two well documented outbreaks of poliomyelitis which are considered to be water-borne. Bancroft, Englehard and Evans in 1957 (4), reported an outbreak occurring in Huskerville, Nebraska in which contamination of the water distribution system due to faulty plumbing was cited as being responsible. Epidemiological evidence

that the cause had a water-borne source was indicated by a high incidence of the disease in people drinking water near the source of contamination, whereas a decrease in number of cases occurred at further distances from the source. The second well documented outbreak occurred in Edmonton, Alberta, Canada in 1953 (3). Failure of chlorination facilities of a sewage treatment plant at a town 20 miles upstream on the Saskatchewan River was responsible for the outbreak. There were 322 cases and 16 deaths.

Up to 1966, no association of other enteroviruses with water-borne epidemics had been found. However, one must appreciate the difficulties in assigning the etiology of epidemics to a water source. Person to person spread in outbreaks is the most common disseminating factor rather than water. It is doubtful that serious attempts to incriminate water-borne sources in epidemics due to enteroviruses have been made.

For future investigations, the paucity of information on hand at present should stimulate further epidemiological studies especially when one considers that in the years 1946 to 1960, 142 outbreaks of gastroenteritis and diarrheal disease occurred in the United States. During this period, 18,790 cases were reported (5). Weibel et al. (5) also report that gastroenteritis and diarrheal diseases are the most commonly water transmitted diseases.

Ironically, the only clearly documented cases of water-borne spread of disease due to viruses have involved the infectious hepatitis virus for which no laboratory assay is available at the present time. Presence of the virus in sewage has been shown only by use of human volunteers. Probably the most common single water-borne disease in the United States at the present time is infectious hepatitis. The number of well-documented cases in this country is relatively small when compared to world-wide episodes (3, 6, 7, 8). Water supplies incriminated in these outbreaks had as sources of contamination, sewage polluted wells, springs, lakes, and rivers. The 1955-56 epidemic in New Delhi (8) is the largest attributed to a water-borne source.

Density of enteroviruses in sewage. Kelly and Sanderson (9) report in testing 308 raw sewage samples that poliovirus was found by grab sampling to be present in sewage at concentrations of less than two and up to 44 plaque forming units (PFU) per 100 ml. A more accurate estimate would probably be in the range of 500 PFU per 100 ml according to Clark and Kabler (6). Wallis and Melnick (10) report as many as 701 PFU per gallon in raw sewage. Conflicting results are obtained by different researchers and may be attributed to variations in host systems and techniques. Isolations of all agents

present in a single sample would require use of several host systems.

Survival time of viruses in sewage. In considering the effect of organic material in sewage, one might expect that this material would exert a protective action on the virus and thus prolong survival. However, some reports indicate that virus survival is longer in clean or treated water. Clark and Kabler (6) have shown that poliovirus may survive in sewage up to 110 days at 4 C, 23 days at 20 C, and 17 days at 28 C. Echovirus type 7 survived 130 days at 4 C and echovirus type 12 may be present at 60 days. These viruses exceed the survival time of Escherichia coli, Aerobacter aerogenes, and Streptococcus fecalis in sewage at 4 C.

Effectiveness of sewage treatment in removal of viruses. The effectiveness of sewage treatment methods in removing viruses that might cause water-borne diseases has been studied by Clarke and Kabler (6). Primary sewage treatment had little or no effect on the enterovirus population, secondary sewage treatment with trickling filters removed only about 40%, and secondary treatment by activated sludge effectively removed 90 to 98% of the enteroviruses. Chlorination of treated sewage effluent further reduced the number of viruses present but did not completely eliminate them.

Disinfection of water has long been accomplished by chlorination. Chlorine residuals in water which are sufficient to kill bacteria, however, will not completely inactivate viruses. Factors which influence virus reduction in presence of chlorine are time of exposure, temperature, pH, and the concentration of the disinfectant. Kelly and Sanderson (11) reported that in water at pH 6 at 25 C, exposure to residual chlorine at concentrations of at least 0.3 ppm for at least 30 minutes was necessary to inactivate poliovirus. When the pH was raised or the temperature lowered, higher concentrations of chlorine were required. Infectious hepatitis virus can withstand 40 minutes of contact with 1.0 ppm free residual chlorine (12, 13). Even at superchlorination residual levels of 15 to 23 ppm for 30 to 60 minutes, the virus was somewhat attenuated but disease was still produced in human volunteers. Mosely (14) reports, however, that after treatment with a concentration of 1.1 ppm total residual chlorine for 30 minutes, hepatitis virus was inactivated.

Qualitative methods for detecting viruses in sewage. Early attempts to isolate virus from sewage were studied by injection of material into monkeys (15). Reliable laboratory assay systems were not available until the advent of the use of tissue cultures in the 1950's. More

sophisticated techniques became available in 1953 with the establishment of stable cell lines.

The gauze pad method of sampling is a modification of the method originally used in 1916 for detection of the tuberculosis bacillus. This sampling method has been widely used in conjunction with various concentration procedures to detect presence of virus in sewage (16, 17, 18, 19, 20, 21). Gravelle and Chin (21) in 1961, reported use of gauze pad sampling in testing two concentration techniques. They showed that ultracentrifugation of the sample was superior to an ion exchange resin technique in concentrating virus. The ion exchange resin technique, previously developed by Kelly (16), consisted of adsorption of viruses onto Dowex 1 resin. This method yielded isolations of virus in 21% of the sewage samples tested over a two year period.

Quantitative methods for detecting viruses in sewage. Even though poliovirus was recovered from sewage as early as 1940 and has been repeatedly isolated since that time, no accurate quantitative methods were available as late as 1965. The inherent lack of detection sensitivity requires collecting large volumes of sewage and concentrating the viruses in some manner prior to attempting detection.

One of the most definitive attempts to quantitate echoviruses in sewage was that of Kelly and Sanderson in 1960 (9). They employed a plaque assay on monkey kidney cell monolayers. A gauze pad immersion sampling procedure was used. Concentration was accomplished on ion exchange resins. Calculations were based on the numbers of plaques counted per sample volume expressed from the pad. This did not solve the problem of determining viruses per unit volume of sewage, however, since no accurate estimate of the amount of water flowing through the gauze pad was possible.

With the report of Metcalf in 1961 (22) of the use of membrane filters for recovering influenza viruses from aqueous suspensions, application of this method for virus detection in sewage was considered. According to Cliver's report in 1965 (23), use of a 0.45-u porosity Millipore membrane would result in at least 50% probability of detecting virus at a level of two PFU per liter. The concentration of virus on a membrane with a porosity greater than the diameter of the virus particles filtered was explained on the basis of virus adsorption to the matrices of the membranes. In 1967, Melnick, Ver, and Wallis (24) reported the use of Millipore membranes for the effective concentration of enteroviruses. In an effort to obtain quantitative recovery of viruses from sewage, the use of

Millipore membrane procedures for virus concentration was further developed and Wallis and Melnick (10) report that sewage in Houston, Texas yielded 2,795 isolations over a seven month period.

STATEMENT OF THE PROBLEM

The need for increasing the water supply in areas where a shortage exists or is imminent in future years has prompted city, county, and federal support of water reclamation studies. The Tucson Water Reclamation Project is a cooperative effort of the United States Public Health Service, The Department of Water and Sewers of the City of Tucson, The Public Health Department of Pima County, and the Departments of Sanitary Engineering, Fisheries, and Microbiology of the University of Arizona. The purpose of the project is to determine the suitability of using reclaimed water for recreational purposes.

Since viruses as contaminating organisms present a threat to successful reclamation of such water, their detection and elimination are necessary. This study as originally outlined was concerned with assessing the effectiveness of a natural sand and gravel tertiary treatment pilot filter in removing viruses and thus rendering the water safe for reuse.

Previous viral detection studies on wastewater have supplied only qualitative data and no quantitation of the removal of virus infective units has been reported. Using a Millipore membrane virus concentration procedure

as suggested by Wallis (25), the virus population in terms of virus infective units per volume of water may be assessed. In addition, this technique permits concentration and detection of small numbers of virus infective units in large volumes of water.

Using this method, it was possible to determine the effect of travel distance within the Pilot Filter on virus removal from wastewater. A knowledge of the average number of enterovirus infective units in Tucson's sewage was obtained as well as an indication of the seasonal distribution of enteric viruses in the Tucson area during the study period.

Other aspects of the study included adapting a plastic panel micro culture technique for rapid and economical identification of viral isolates, evaluating the effectiveness of the Millipore membrane concentration and detection technique, modifying and improving the technique, and comparing it with other methods for virus concentration and detection.

MATERIALS AND METHODS

Wastewater sampling sites. A schematic representation of the Tucson Sewage Treatment Plant is shown in Figure 1. The treatment plant proper is composed of an activated sludge plant operating in parallel with a trickling filter. Activated sludge flows directly into an irrigation ditch and is used to fill four basins which in turn load the Pilot Filter. Water added to these basins, Pilot Filter influent (PFI), travels a horizontal distance of up to 250 feet and a vertical distance of 15 to 18 feet within the filter before it emerges as Pilot Filter effluent (PFE). The filter is composed of natural sand and gravel from river beds in the Tucson area. The Pilot Filter effluent is channeled to fill four experimental fish ponds represented by "T" in the diagram.

Our study was concerned only with Pilot Filter loading from Basins 1 and 3. Basin 1 leading occurred from October through December 1966. Wastewater added exclusively to Basin 1 travelled approximately 100 feet horizontally and 15 feet vertically before emerging as PFE. From January to May 1967, Basin 3 was loaded in the same manner and in this case the horizontal travel distance was reduced to approximately 50 feet. Pontacyl Brilliant

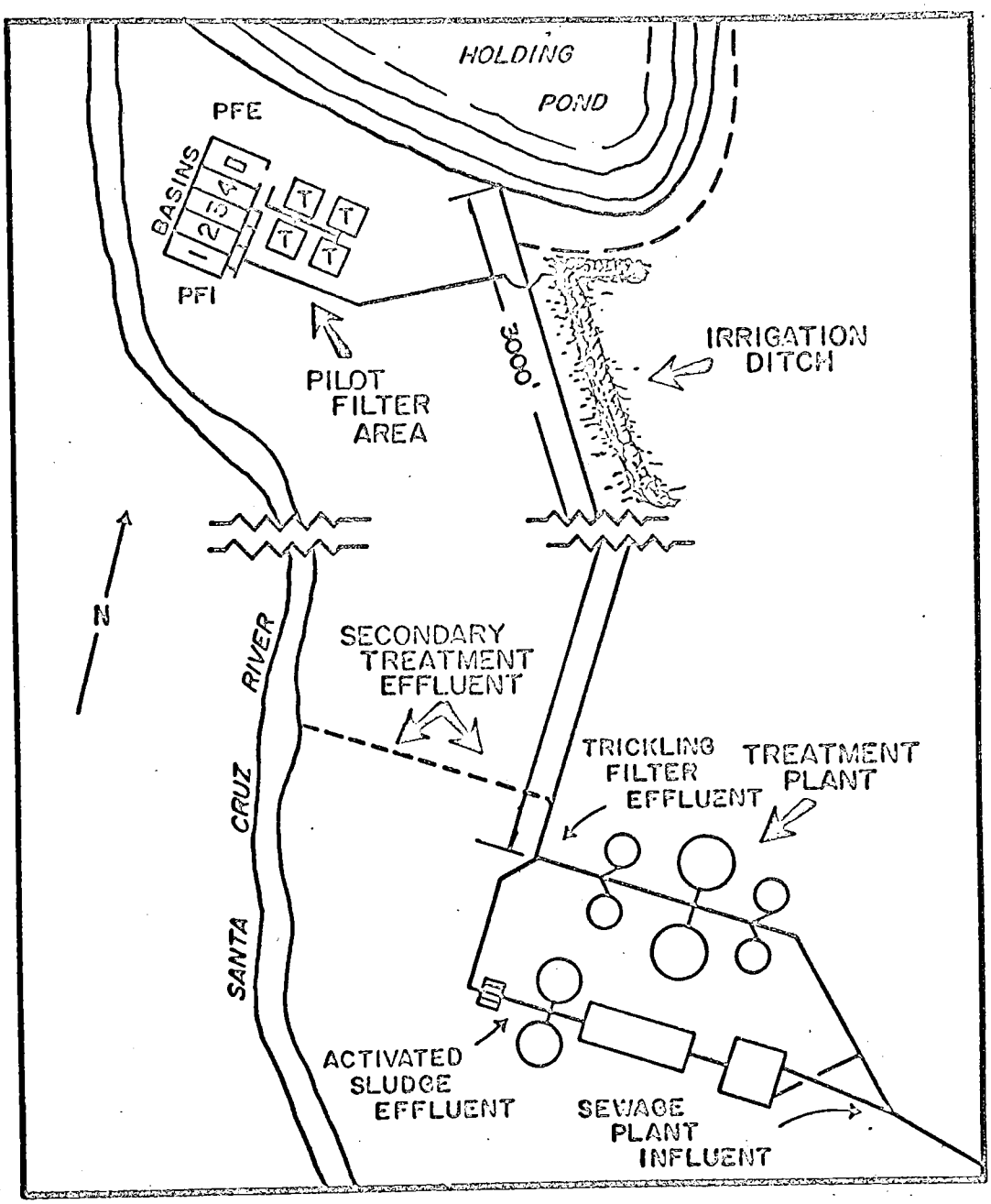

Fig. 1. Schematic Representation of Tucson Sewage Treatment Plant Facilities.

Source: Shriver, Patricia, 1967. (44)

Pink B(PBPB) dye studies carried out by the Sanitary Engineering Department indicated a travel time of approximately 21 days for Basin 1 loading compared to five days for Basin 3 loading.

Grab samples were collected in one gallon volumes in Millipore pressure containers (Millipore Corporation, Bedford, Massachusetts) from Basin 1 or 3 for the PFI. Pilot Filter effluent samples were collected in five gallon volumes. These samples were transported directly to the laboratory where they were processed immediately.

Sample processing. Samples were processed according to the method described by Wallis (25). Wastewater samples were clarified by filtration through a Millipore AP-20 microfiber glass prefilter pad (142 mm diameter). This step removed a large percentage of suspended particulate matter which would possibly clog filter membranes in later steps. Subsequent filtration through a GS 0.22-u Millipore membrane (diameter 142 or 293 mm) removed concomitant contaminating bacteria, yeasts, and molds. Magnesium chloride was then added to the water to make a 0.1 M solution which stabilized the viruses present and enhanced viral adsorption to the concentration membrane in a later step by making the wastewater isotonic. Addition of protamine sulfate (Salmine, Nutritional Biochemicals Corporation) at a 1:400,000 final concentration

functioned to aggregate non-viral protein material. The sample containing $MgCl_2$ and protamine sulfate was then stirred by means of a magnetic stirring device for 30 to 60 minutes. The protamine sulfate-protein aggregates were removed by filtration of the sample through an AP-20 clarifying pad. This step removed many of the components which would coat the concentration membrane and prevent virus adsorption. Virus concentration was accomplished by filtration of this processed sample through a Millipore HA 0.45-u membrane (diameter 47 or 142 mm). Virus particles are electrostatically attracted and adsorbed to the matrices of this membrane. To free the concentrated virus, the membrane was placed in a mortar with one or two grams of alundum and one ml of fetal bovine serum (FBS) and triturated with a pestle. Three ml of Melnick's B Medium (MEL-B) (Grand Island Biologicals) containing 2% FBS, 200 units of penicillin and 200 ug of streptomycin per ml were added. After uniform mixing, the material was placed in a Kahn tube and centrifuged at 1000 rpm for ten minutes in an IEC International Centrifuge, Universal Model U V. Supernatant fluid was collected and stored in a Revco at -60 C until tissue cultures were ready for inoculation.

Cell cultures. Primary human amnion cultures (HAM) were prepared by standard tissue culture procedures (26). Monodispersed cells were suspended in growth medium

and planted in Wallis-Melnick tubes at a concentration of 3 X 10^5 to 5 X 10^5 cells per tube.

Growth medium for HAM cultures consisted of Melnick's A Medium (MEL-A) (Grand Island Biologicals) containing 10% calf serum, 0.78% $NaHCO_3$ supplemented with 100 units of penicillin, 50 ug of streptomycin, and 2.5 ug of fungizone per ml. After cell monolayers formed, cultures were maintained with MEL-B medium with 2% FBS and antibiotics at concentrations equal to those in growth medium.

The established cell lines of Human Embryonic Lung (HEL) (27) and human epidermoid carcinoma (HEp-2) (28) were carried by serial passage in this laboratory. Growth medium consisted of Eagle's Basal Medium (BME) supplemented with 10% calf serum, 0.78% $NaHCO_3$, and antibiotics at concentrations previously mentioned. Tube cultures were prepared by trypsinization of monolayers grown in 16 ounce prescription bottles. Cells in suspension were dispersed at a concentration of 1 X 10^5 to 2 X 10^5 cells per ml into Wallis-Melnick tubes for tube assays and at a concentration of 4 X 10^4 cells per ml into plastic panel wells for the plastic panel micro culture technique. Maintenance medium for established cell lines was identical to growth medium with the exception that 2% FBS instead of 10% was used.

African Green Monkey kidney (AGMK) tubes were obtained from Flow Laboratories, Inglewood, California, and were used when monolayers became confluent. Maintenance medium MEL-B, was the same as that used for HAM cultures. For use in plaque assays, AGMK cells were received frozen in liquid nitrogen from Microbiological Associates, Bethesda, Maryland. Upon receipt in this laboratory, frozen cells were stored in liquid nitrogen until they were thawed for planting. One ounce prescription bottles were seeded with approximately 1 X 10^6 cells suspended in BME prepared with Hank's balanced salts and supplemented with 2.0 mM of glutamine and nonessential amino acids at a concentration of 0.01 mM. Monolayers usually formed in these bottles within 7 to 12 days.

Quantitative virus detection procedures. Cultures of HAM were used for quantitative determinations of virus infective units (VIU) present in wastewater. Each water sample concentrate was inoculated at dilutions of 1:3, 1:9, and 1:27 for PFI or undiluted, 1:3, and 1:9 for PFE. A 0.1 ml volume of each dilution was inoculated into each of ten tube cultures from which the medium had been decanted. After adsorption for one hour at 37 C, one ml of MEL-B medium was added to each tube. These tubes were incubated at 37 C for 14 days. Readings for cytopathic

effect (CPE) were made at two day intervals. Tube cultures showing CPE were frozen at -60 C for later virus identification studies. When a particular water concentrate was negative after 14 days incubation, the cultures were frozen and thawed, and like sets were pooled. Two tenths ml of this fluid was inoculated into each of six fresh HAM tube cultures according to the procedure for primary passage. Tubes were again observed at two day intervals for 14 days. Positive tube cultures were frozen as before. Those showing no CPE after the second passage were again frozen and thawed, like sets pooled and passed a third time before they were considered negative for enteroviruses. If cultures were again negative after seven to ten days, three tubes from each set of six were challenged with echovirus type 11 (strain Gregory, Baylor University) in order to detect the presence of an interfering type virus (29). Tube cultures showing CPE when challenged with echovirus type 11 were considered negative for an interfering virus of the rubella type. Tubes challenged with echovirus type 11, but showing no CPE would be considered positive for interfering virus.

In order to determine if viruses were present which were not detectable in HAM, all HAM negative sample concentrates were subsequently tested in tube cultures of AGMK.

Estimation of virus concentration. Calculations for VIU per inoculum were made as follows. The number of tube cultures positive at each dilution were translated into percentage values and the probable VIU per inoculum were estimated on the basis of standard statistical tables (30). When two or more dilutions of a sample produced positive tubes, the VIU per inoculum for each dilution was determined and averaged to give a mean VIU number for the sample. All values were expressed on the basis of a one gallon sample volume. For the samples which became positive in the first or second passage, an arbitrary value of one VIU per sample volume was set.

Identification of enterovirus isolates. Because of the convenience and availability of cultures of HEL in the laboratory, most identification procedures were carried out in this established cell line. When a particular isolate did not grow in HEL, primary cultures of HAM were used to identify the virus.

A pool was made of the contents of all HAM positive tubes from each sample. As a first step in identification, these pooled materials were inoculated into HEp-2 monolayers to separate polioviruses or coxsackie B viruses from the echoviruses (31). Appropriate antisera were then used in subsequent testing of the original sample of HEp-2 positives in HEL.

Another means of separating pooled specimens containing more than one virus type was by the end point dilution method. The virus type was subsequently identified by using appropriate antisera. Usually after determination of individual types, the original isolate was tested again against a pool of the individual antiserum types for confirmation.

Prior to neutralization tests, log dilution titrations were made and a dilution calculated to contain approximately 100 TCD_{50} per 0.1 ml was used in neutralization tests using a modification of the intersecting antiserum pool scheme of Schmidt, Gunther, and Lenette (32). Each antiserum in the pool was diluted to contain 20 antibody units per 0.1 ml. Standard tube tests were performed using three tubes for each antiserum-virus combination. Antisera used in these studies were poliovirus types 1, 2, and 3, coxsackievirus type B 1-6, echovirus types 1-25 except 4, 18, and 21, and coxsackievirus type A 9.

Plastic panel micro culture neutralization tests. In order to minimize volumes of reagents and tissue culture cells used for neutralization tests, a plastic panel viral identification procedure, reported by Rosenbaum et al. (33) Lamb et al. (34), and Smith, Canchola, and Channock (35) was adapted for studies in our laboratory. Plastic panels (Linbro Chemical Company, New Haven, Connecticut) were

sterilized by immersion in 70% alcohol over night. Using plastic pipettes (Cook Engineering Company, Alexandria, Virginia), calibrated to deliver 0.025 ml per drop, one drop of each antiserum pool was delivered into four wells. One drop, 0.025 ml, of virus at a dilution giving 100 TCD_{50} per 0.025 ml was added to wells containing antiserum and to four control wells containing medium alone. Panels were then covered with aluminum foil and placed in a 5% carbon dioxide 95% air incubator for one hour. During this incubation period, cultures of HEL cells grown in 16 ounce prescription bottles were trypsinized and counted. After the virus-antiserum incubation period, 1.0×10^4 to 1.2×10^4 HEL cells suspended in 0.025 ml BME containing 10% FBS were added to each well. Wells were overlaid with Drakeol (Drakeol 6 VR, Pennsylvania Refining Company, Butler, Pennsylvania) in order to prevent dessication. Panels were then covered with pressure stick adhesive covers and incubated at 37 C. Monolayers formed in wells in which virus was neutralized, whereas, in wells in which the virus was not neutralized, cells attached to the plastic bottom of the wells, but were soon destroyed by the virus. Tests were readable in 24 to 72 hours. Wells of control cells alone maintained their integrity for seven or eight days. Tests were observed daily both

microscopically for CPE as well as macroscopically for colorimetric pH changes.

Poliovirus seeding of wastewater. Standard Millipore membrane viral concentration procedure. Studies were carried out to evaluate the viral concentrating efficiency of the standard Millipore membrane procedure by its ability to recover poliovirus type 1 (Sabin strain, Wyeth Laboratories) added to PFI and PFE water samples. This poliovirus produces plaques in AGMK cells in 48 hours, whereas indigenous viruses were incapable of forming plaques but did show CPE in tube cultures in four to seven days. Thus, it was possible to compare variations in water processing steps on the recoverability of seeded poliovirus. The virus was assayed by standard plaque methods in one ounce bottle cultures of AGMK. Agar overlay medium consisted of Earle's balanced salt solution, 1:60,000 neutral red, 0.1% skim milk, 1.5% Bacto-agar (Difco), 0.78% $NaHCO_3$, 25 mM $MgCl_2$, 2.0 mM glutamine supplemented with 100 units of penicillin and 50 ug of streptomycin per ml.

Millipore membrane Dowex resin procedure. Wallis and Melnick (10) incorporated a Dowex resin to replace protamine sulfate in the membrane viral concentration procedure in an effort to obtain higher virus recoveries by eliminating more membrane coating components (MCC).

Dowex anion exchange resin (50-100), mesh, 1-X8 (Cl^-) was prepared by washing several times in a beaker containing approximately ten volumes of distilled water. For each wash, water and resin were stirred approximately ten minutes by a magnetic stirring device. The water was siphoned off after each wash and finally replaced with 0.15 M NaCl. The resin was similarly washed with saline and the pH was tested after each wash until it equaled that of fresh 0.15 M NaCl. The slurry was then poured into a 30 X 400 mm column and additional saline was run through to ensure a uniformly packed column. In early preparations, the column was autoclaved, however, this step was omitted in later preparations since bacterial contamination from this source was not a problem in the assay.

Aluminum hydroxide viral adsorption procedure. Aluminum hydroxide for adsorption and concentration of viruses was prepared essentially by the method described by Wallis and Melnick (36). One hundred ml of 25 mM $AlCl_3$ in distilled water and three ml of 2 M $NaCO_3$ were mixed for 15 minutes on a magnetic stirrer. The resulting precipitate was removed by centrifugation at 2000 rpm for 15 minutes. This sediment was washed twice with 0.15 M NaCl and autoclaved at 121 C for 15 minutes. After cooling, the solution was again centrifuged and the sediment was

resuspended in 100 ml of sterile 0.15 M saline. This $Al(OH)_3$ preparation was used in PFI and PFE samples at a volume of ten ml per liter of wastewater.

After removal of bacterial contaminants from the water sample, $Al(OH)_3$ was added and stirred magnetically for two hours. The sample was then filtered through a HA 0.45-u Millipore membrane which retained the $Al(OH)_3$-virus complex. This material was removed from the membrane with a spatula and ground in a mortar with alundum and four ml of FBS. After gravity sedimentation for ten minutes, the supernatant fluid containing the eluted virus was removed and used for tissue culture inoculation.

RESULTS

Viral isolations from Pilot Filter influent and Pilot Filter effluent Basin 1 and 3 loading. During a seven month period, October 10, 1966 to May 4, 1967, a total of 28 wastewater samples were tested for the presence of viruses by the membrane virus concentration technique.

The results of these studies for the PFI are shown in Table 1. Of the seven samples tested from Basin 1, four, or 57% were positive. Five of eight samples, or 62.5%, from Basin 3 were positive. It is of interest to note that in run 93, one out of ten tissue culture tubes was positive at both the 1:9 and 1:27 dilutions, but no positives were seen at 1:3. Subsequent passage confirmed presence of virus in these higher dilution tubes. Run 103 was a questionable positive in the first passage of the originally inoculated HAM tubes. Subsequent passage in HAM revealed an isolate mainfesting an adenovirus type CPE.

There were three instances in which one-half gallon samples were tested instead of the usual one gallon volume. In all three instances, however, virus was detected. Values for estimated virus infective units per sample ranged from 0 to 491.4.

Table 1. Detection of enteroviruses in human amnion cell cultures for Pilot Filter influent during loading of Basins 1 and 3.

Sample number	Positive cultures out of 10 Dilution of concentrate 1:3	1:9	1:27	Volume (gallons)	VIU* per sample
Basin 1					
36	0	0	0	1.0	0
42	0	0	0	1.0	0
47	3	2	0	1.0	61.0
52	10	7	4	1.0	491.4
55	4	2	0	1.4	140.4
71	3	2	0	0.5	21.6
75	0	0	0	1.0	0
Basin 3					
84	2	1	0	0.5	33.0
88	5	0	0	0.5	72.2
93	0	1	1	1.0	79.2
97	0	0	0	1.0	0
101	2	0	0	1.0	26.4
103**	0	0	0	1.0	1.0
107	0	0	0	1.0	0
110	0	0	0	1.0	0

*Virus infective units.

**Positive in subsequent passages and given an arbitrary value of 1 virus infective unit per gallon.

Table 2 shows similar data for PFE samples. Three of these samples were initially positive when inoculated into HAM cultures. Runs 70 and 106 became positive in subsequent passages. Of Basin 1 loaded samples tested, two of five were positive representing a 40% isolation rate. Three out of eight samples were positive for Basin 3 loading to give a percentage isolation of 37.5. Estimated VIU per sample ranged from 0 to 264.4 for the PFE.

Effectiveness of the Pilot Filter in removing viruses. An indication of the effectiveness of the Pilot Filter was determined by calculating the estimated VIU per gallon, for each sample. As shown in Table 3, for Basin 1 loading, PFI yielded an average of 110.7 VIU per gallon, whereas PFE yielded an average of only 5.5 VIU per gallon. Thus the Pilot Filter removed 95% of the ingoing virus detectable by our technique. Similar data for Basin 3 indicated virus removal by the Pilot Filter of 97%.

Seasonal distribution of enterovirus isolations. Seasonal distribution of viruses in PFI is shown graphically in Figure 2. In October, only one isolation was obtained while on November 15, the maximum number of VIU was observed in the PFI. Thereafter a decline in number of VIU occurred in December followed by a rise in January with a second though somewhat lower peak than that of November 15

Table 2. Detection of enteroviruses in human amnion cell cultures for Pilot Filter effluent after loading Basins 1 and 3.

Sample number	Positive cultures out of 10 Dilution of concentrate 1:3	1:9	1:27	Volume (gallons)	VIU* per sample
Basin 1					
56	ND**	7	3	5.0	264.6
66	0	0	0	5.0	0
70***	0	0	0	5.0	1
76	0	0	0	4.0	0
77	0	0	0	3.5	0
Basin 3					
78	5	0	0	5.0	27.6
83	0	0	0	4.0	0
87	3	2	0	4.0	11.6
94	0	0	0	4.5	0
98	0	0	0	5.0	0
102	0	0	0	5.0	0
106***	0	0	0	5.0	1
117	0	0	0	5.0	0

*Virus infective units

**Not done

***Positive in subsequent passages and given an arbitrary value of 1 virus infective unit per gallon.

Table 3. Estimated virus infective units per gallon of Pilot Filter influent (PFI) and Pilot Filter effluent (PFE).

	BASIN 1			BASIN 3	
SAMPLE	PFI	PFE	SAMPLE	PFI	PFE
1	0	27.3	1	66.0	5.5
2	0	0	2	165.6	0
3	61.2	0.2	3	79.2	5.1
4	491.4	0	4	0	0
5	100.3	0	5	26.4	0
6	122.4	ND*	6	1.0	0
7	0	ND	7	0	0.2
			8	0	0
Mean	110.7	5.5		42.3	1.4
Estimated Reduction by the Pilot Filter	95%			97%	

*Not Done

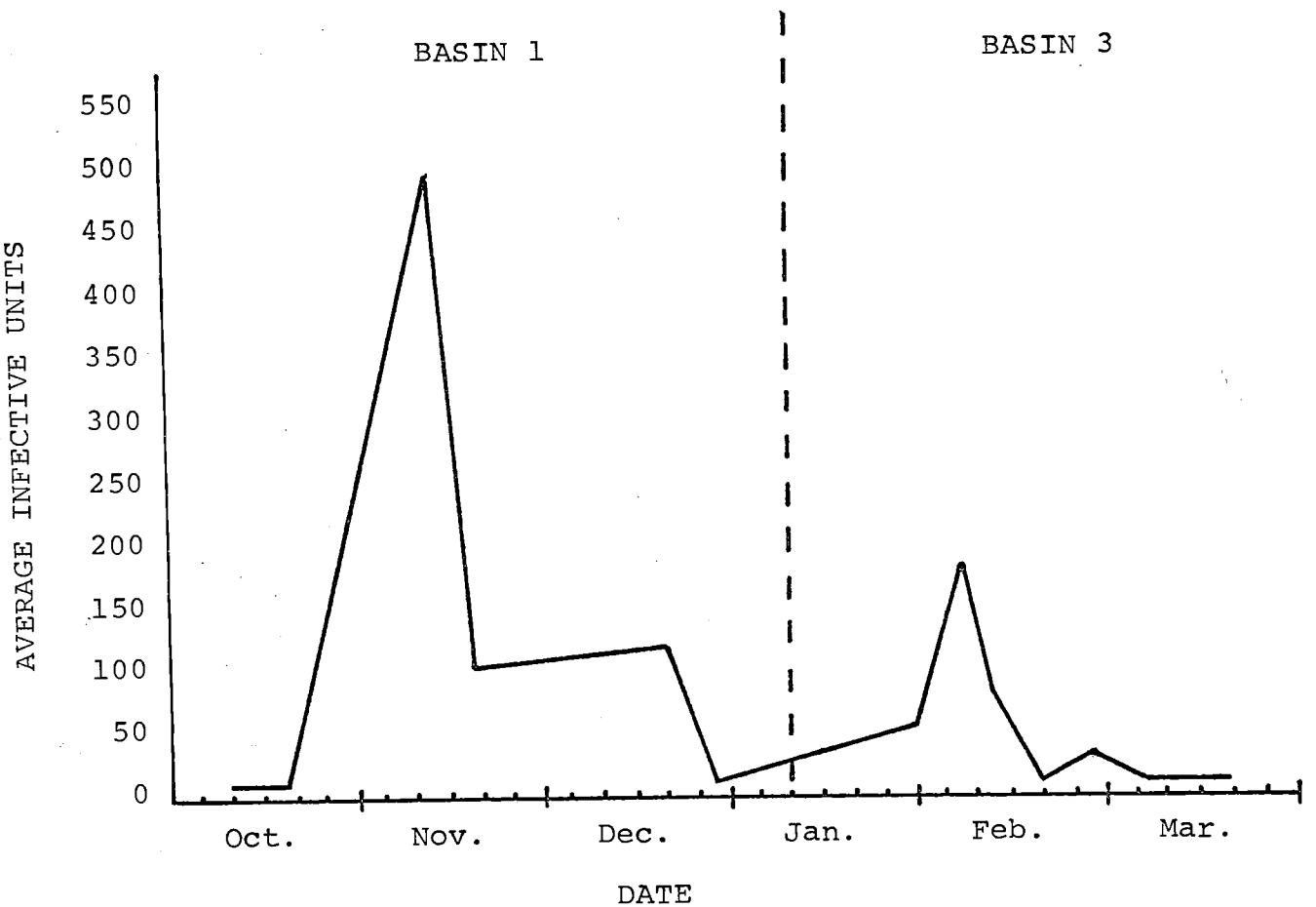

Figure 2. Seasonal distribution of viruses in Pilot Filter influent from Basins 1 and 3.

occurring on February 7. During March, isolations declined and none were obtained from the last samples taken that month.

In general, VIU isolated per sample from PFE followed the same pattern as that for the PFI. However, the number of VIU was considerably less for PFE samples. As shown by Figure 3, the highest isolation occurred in November with a sharp decline in December followed by a small peak in January and another in February. Then a general decline occurred through March and April. As can be seen by comparing Figures 2 and 3, Basin 3 yielded fewer VIU from both PFI and PFE than did Basin 1.

Virus detection studies with AGMK. Not included for quantitation, but of interest in virus detection studies were four of the 15 HAM negative samples. When the original Millipore membrane concentrate from these HAM negative samples was inoculated into AGMK tube cultures, one sample (run 110) became positive within 72 hours. The other three samples were negative in AGMK cultures on first passage but in subsequent passage two samples (runs 83 and 97) became positive. The original membrane concentrate of run 103 from which the adenovirus type was isolated was also inoculated into AGMK. An additional virus type was isolated showing enterovirus

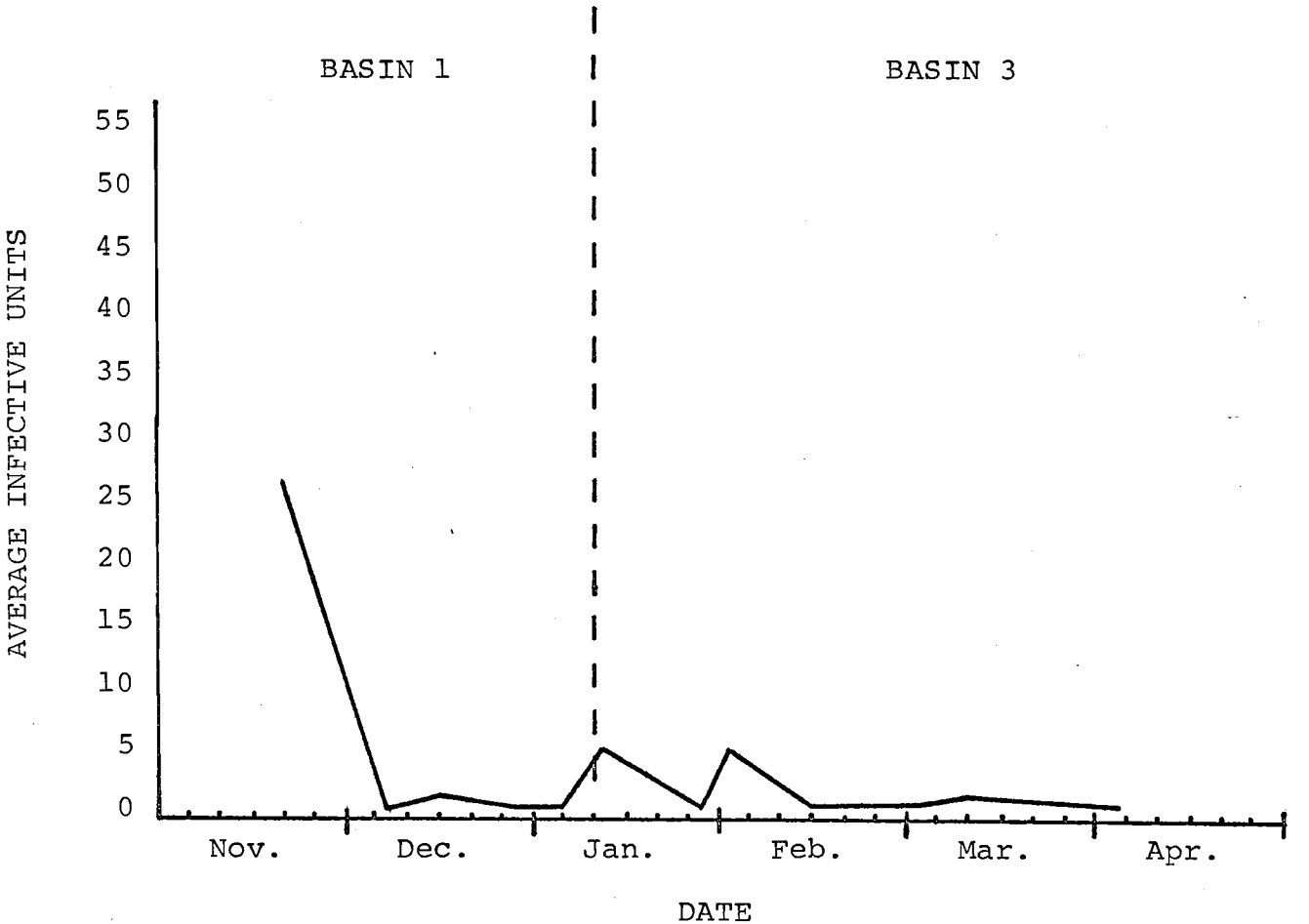

Figure 3. Seasonal distribution of viruses in Pilot Filter effluent after Basins 1 and 3 loading.

type CPE, however, no adenovirus type CPE was observed in AGMK cell cultures.

Attempt to detect the presence of interfering viruses by echovirus type 11 challenge. A representative number of third passage tubes of all HAM negative PFI and PFE samples were challenged with echovirus type 11. In every case, these infected tubes showed CPE thus ruling out the possible presence of an interfering virus. Positive samples were not challenged with type 11 echovirus since it would be laborious and time consuming to block all the virus types present by use of appropriate antisera before challenging with the echovirus. It was felt that the 13 negative samples tested were a fair representation of the wastewater and further testing of positive samples would be in the realm of diminishing returns.

Identification of enterovirus isolates. Standard monolayer tube neutralization tests. Virus was detected in HAM tube cultures in 14 of the 28 wastewater samples tested during the study. Five of these isolations contained only one virus type in HAM cultures. Nine others were a mixture of two to four types, and one sample, run 78, yielded an unidentifiable type.

Of ten HEp-2 positive isolates, all contained at least one poliovirus type except runs 55 and 84 in which no poliovirus could be identified. Of the four HEp-2

negative samples, run 103 contained poliovirus type 2 as shown by AGMK studies. The remaining HEp-2 negative samples contained no poliovirus. No coxsackie B viruses were detected.

Runs 78, 84, and 103 did not propagate well in HEL beyond the second passage of the virus, therefore identification was attempted in HAM. Run 84 contained echovirus type 7 and run 78 was not identifiable. Since no adenovirus antiserum was available in this laboratory for testing run 103, a stock of this virus was made in bottle cultures of Chimp Liver kidney kindly supplied by Dr. Vern Hartwell at the CDC Phoenix Field Station of the United States Public Health Service, Phoenix, Arizona. This virus stock was tentatively typed by Dr. Hartwell in a micro agar precipitin test (37, 38) as adenovirus type 16.

Of the four AGMK isolates not included in the quantitative study, run 83, 110, and 103 contained polio virus type 2. Type 3 poliovirus was obtained from run 97. Of special interest is run 103 from which adenovirus type 16 was the only type detected in the HAM isolation studies.

Plastic panel micro culture technique identifications. To determine the dependability of this technique, 5 of the 11 HEL identifiable isolates were identified both

by the standard tube technique and the plastic panel micro culture technique. As may be seen in Table 4, with only one exception an excellent correlation was shown. Run 56 was a multiple isolate with echovirus type 20 and poliovirus type 3 identified by the standard tube method and echovirus type 20 and poliovirus type 2 identified by the micro technique. On the basis of these favorable results, this more rapid, less expensive technique was employed for identification of the remaining isolates.

Table 4. Comparison of standard tube and the plastic panel micro culture technique for the identification of isolates.

Run number	Identity standard tube		Identity micro technique	
	Echovirus type	Poliovirus type	Echovirus type	Poliovirus type
52	20	3	20	3
56	20	3	20	2
70		3		3
71	20	3	20	3
101	6		6	

Enterovirus types detected in the Tucson wastewater. Identification of PFI isolates are shown in Table 5. Seventy-three percent of the samples tested were positive including both HAM and AGMK isolations. A total of 24 isolations of virus were obtained. Table 6 shows

Table 5. Isolation and identification studies on Pilot Filter influent during Basin 1 and 3 loading.

Sample number	Virus type
36	none
42	none
47	echo 6, 7, 12, and 19
52	echo 20 and polio 3
55	echo 6, 11, and 19
71	echo 20 and polio 3
75	none
84	echo 7
88	echo 6, 11, 19 and polio 3
93	polio 1, 2, and 3
97	polio 3
101	echo 6
103	adeno 16 and polio 2
107	none
110	polio 2

Table 6. Isolation and identification studies on Pilot Filter effluent after Basin 1 and 3 loading.

Sample number	Virus type
56	echo 20, polio 2 and 3
66	none
70	polio 3
76	none
77	none
78	unidentified
83	polio 2
87	echo 7 and polio 3
94	none
98	none
102	none
106	polio 3 and echo 3
117	none

46% of the samples tested for the PFE were positive with ten isolations and four different types represented.

The frequency of detecting each virus type is shown by Figure 4. This data represents a combination of both PFI and PFE samples and includes HAM and AGMK isolations. Poliovirus type 3 was the most commonly isolated virus with nine isolations obtained. Types 1 and 2 poliovirus were detected one and five times, respectively giving a total of 15 poliovirus isolates. Other predominant virus types isolated included echovirus types 6, 7, 19, and 20. Types 3, 11, and 12 were present though infrequent, and a single adenovirus was detected during the study. One isolate was unidentifiable in tests using antisera available in our laboratory. Thirty-four total isolations were made with 11 different virus types represented.

Seasonal occurrence of virus types. As shown in Table 7, poliovirus type 3 was prevalent from November through March. Echovirus types 6, 7, and 19 were detected in October, November and February and type 20 echovirus existed in sewage during the late fall and early winter months. Other virus types appeared sporadically so that no seasonal pattern could be established for their presence in wastewater.

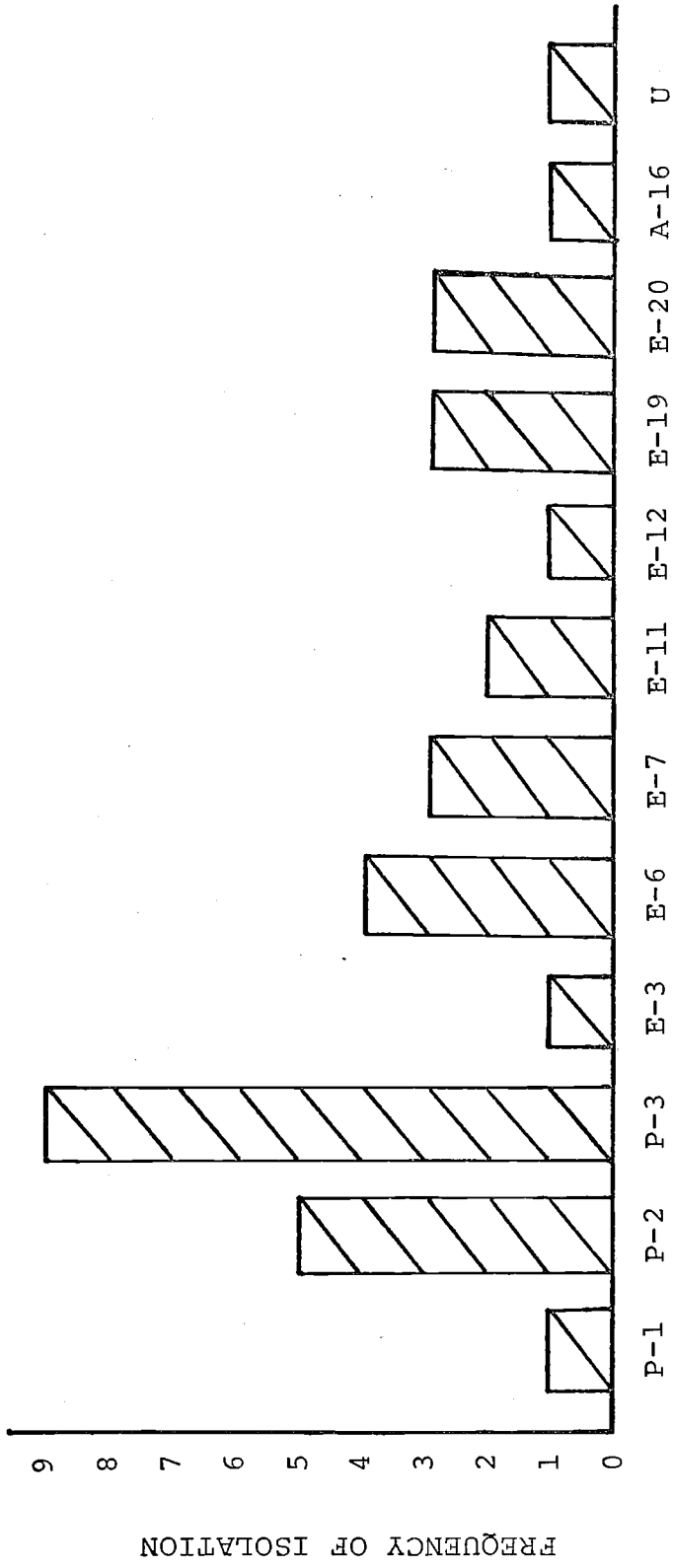

Figure 4. Frequency of virus types detected. (P=poliovirus type, E=echovirus type, A=adenovirus type, U=unidentified)

Table 7. Seasonal occurrence of viruses in Tucson wastewater.

Month	Sample number	Virus type
October	47	echo 6, 7, 12, and 19
November	52	echo 20 and polio 3
	55	echo 6, 11, and 19
	56	echo 20, polio 2 and 3
December	70	polio 3
	71	echo 20 and polio 3
January	78	unidentified
	84	echo 7
	83	polio 2
February	87	echo 7 and polio 3
	88	echo 6, 11, 19 and polio 3
	93	polio 1, 2, and 3
	101	echo 6
	97	polio 3
March	103	adeno 16 and polio 2
	106	echo 3 and polio 3
	110	polio 2

Poliovirus seeding of wastewater. Recoverability of added virus after standard Millipore membrane viral concentration processing. Having satisfied the primary purpose of the study, namely to determine the effectiveness of the Pilot Filter in removing viruses from wastewater, the next area of concern was an evaluation of the viral concentration efficiency of the processing technique. In these studies, a number of variables were investigated including the volume of the samples, the size of the pre-filter membranes, the size of the viral-adsorption membrane and the concentration of virus added to the wastewater. Results are shown in Table 8. The virus recovery rate ranged from 0.6% to 3.8% for the PFI and from 1% to 5% for the PFE. These surprisingly low recoveries were obtained in experiments regardless of the variables in volumes of water tested, differences in quantity of virus added to the sample, and differences in the diameter of filter membranes used.

Loss of seeded poliovirus during the standard Millipore membrane processing steps. In an attempt to learn where in the water processing procedure loss of virus was occurring, two experiments were done in which the filtrates were tested for virus after each step in the water processing procedure. Results are presented in Table 9. It is obvious that the greatest loss of virus occurs during the

Table 8. Virus recoverability after seeding wastewater with poliovirus and processing by the standard Millipore membrane virus concentration procedure.*

Sample No.	Volume sample (ml)	AP-20 Prefilter size (mm)	HA 0.45 u Membrane size** (mm)	Poliovirus input (PFU/ml)***	Virus Recovered on 0.45 u Membrane (%)
Pilot Filter Influent (PFI)					
1	2000	142	47	3	0.6
2	4000	142	47	3	1.1
3	225	47	47	4200	1.7
4	225	47	47	5900	3.8
5	4000	142	142	4300	3.0
6	2000	142	47	25	1.3
Pilot Filter Effluent (PFE)					
1	4000	142	47	40	5
2	4000	142	47	2.5	1
3	4000	142	47	2	3

*All samples were filtered through 142 mm, GS 0.22 u membranes to remove bacteria after AP-20 prefiltration.

**Size of the viral adsorption membrane.

***PFU/ml - plaque forming units per ml.

Table 9. Loss of seeded poliovirus during the standard Millipore membrane processing steps.*

Processing step	% of poliovirus lost	
	Sample 1	Sample 2
After seeding	0	0
AP-20 prefiltration (gross debris removal)	34	ND**
GS 0.22-u membrane (bacteria removal)	54	ND
Total loss during lst two steps of processing	88	90.8
HA 0.45-u membrane (loss due to failure to adsorb to membrane)	8.2	6.2
Total virus lost in filtrates	96.2	97.0

*Samples 1 and 2 were 225 and 4000 ml of PFI water samples respectively.

**Not done individually.

first two processing steps (88 and 90.8%) in which water is freed of gross debris and bacteria. Since wastewaters are presumably hypotonic, no virus should be lost during these preliminary processing steps according to Wallis and Melnick (10). Of the remaining virus available for recovery (12 and 9.2%), a further loss of 8.2 and 6.2% occurred due to failure of virus to adsorb to the HA 0.45-u membrane thus resulting in a total virus recovery of only 3.8 and 3% respectively for the two experiments.

Prevention of virus loss by coating the pre-concentration membranes with serum. In an effort to prevent virus loss due to adsorption to the AP-20 pad and the GS 0.22-u membrane during the pre-concentration steps, these filters were treated with FBS according to the method of Ver, Melnick, and Wallis (39) prior to filtration of the seeded wastewater. Sampling after passage through the treated AP-20 pad revealed an actual increase in PFU per ml from an input of 4600 PFU to 5500 PFU. However, a 30% loss of original virus input occurred after filtration through the GS 0.22-u membrane.

Comparison of protamine sulfate and Dowex anion resin columns for the removal of membrane coating components from wastewater. The second area in which virus was lost was due to failure to adsorb to the HA 0.45-u membrane. Wallis and Melnick (40) have reported that several types

of MCC are present in wastewater. These include proteins, lipoproteins, and other organic components. As shown by our studies, protamine sulfate was somewhat ineffective in removing these components under our experimental conditions since virus was found in the filtrate after the concentration step (Table 9).

In pilot studies, protamine sulfate was compared to a Dowex anion exchange resin column to determine efficiency in removing MCC. A 500 ml sample of PFI was seeded with poliovirus type 1 and the bacteria were removed by the usual method. The sample was subsequently divided into two 250 ml aliquots. One 250 ml aliquot was processed using protamine sulfate to remove MCC. The other sample was passed through the anion exchange resin column. The efficiency of MCC removal in each case was determined by sampling the HA 0.45-u membrane filtrate. Table 10 shows that using the anion exchange column, no virus was passed through the HA 0.45-u membrane after 50, 100, and 200 ml volumes were filtered. Conversely, after 50 ml passed through the HA 0.45-u membrane, 940 PFU per ml were detected in the filtrate with the protamine sulfate method of removing MCC. Increasing volumes filtered gave increasing PFU of virus escaping concentration by the membrane.

Table 10. Comparison of Dowex resin and protamine sulfate for removal of membrane coating components from wastewater.

Volume filtered (ml)	Virus in membrane filtrate (PFU/ml)			
	Experiment 1		Experiment 2	
	Dowex	P. SO_4*	Dowex	P. SO_4
50	0	940	ND	ND
100	ND**	ND	0	730
200	0	2280	0	2090

*Protamine sulfate

**Not done

Comparison of three quantitative processing techniques for the concentration of viruses in wastewater. In order to find a more economical and efficient method for the quantitative determination of virus concentration in wastewater, an $Al(OH)_3$ viral-adsorption method was investigated. This procedure reported by Wallis and Melnick (36) in 1967, is described under Materials and Methods. A preliminary study was carried out comparing the $Al(OH)_3$ viral-adsorption method with the standard Millipore membrane procedure using protamine sulfate or Dowex resin to remove MCC from wastewater. A PFI or PFE water sample was clarified and the bacteria removed by the usual process of membrane sterilization. These samples were then seeded with poliovirus type 1 and each was divided into three

one gallon aliquots and processed according to the three methods described. The comparative results are shown in Table 11. A 100% recovery was obtained with the $Al(OH)_3$ technique while only 28% and 3.4% for PFI samples, and 32% and 29% for PFE samples were obtained using the Millipore membrane protamine sulfate and Millipore membrane Dowex resin column techniques respectively.

Table 11. Comparison of three quantitative processing procedures for the concentration of viruses in wastewater.*

Method	Percentage poliovirus recovered	
	PFI	PFE
Millipore membrane protamine sulphate	28	3.4
Millipore membrane Dowex resin	32	29
$Al(OH)_3$ viral-adsorption	100	100

*Three gallon samples were filtered through AP-20 prefilters and 0.22-u membranes to remove bacteria prior to seeding with poliovirus. One gallon aliquots were then processed by each of the three methods.

DISCUSSION

This is the first study to report the application of a membrane virus concentration procedure to assess quantitatively the effectiveness of virus removal from wastewater. Using this method, I showed that a tertiary treatment Pilot Filter effectively reduced the viral population in activated sludge effluent (PFI) approximately 95 and 97% for Basin 1 and 3 loading respectively.

One would expect that water placed in Basin 1 would travel a greater distance through the Pilot Filter than water loaded in Basin 3 and, therefore, a correspondingly greater percentage of virus reduction should occur. However, this was not the case and one can only speculate as to the reasons for this finding. It may be possible that a threshhold or saturation point occurred within the filter during the Basin 1 loading period. In agreement with this suggestion is the fact that larger quantities of VIU were present during Basin 1 loading as seen in Table 3 (110.7 and 42.3 mean VIU for Basin 1 and Basin 3 respectively). Since the filter works partially on the basis of removal of viruses by their adsorption to the filter media, a large number of viruses may have attached to sites, and thus subsequent virus adsorption did not occur.

This hypothesis is made untenable, however, by the fact that viruses are present in PFI at fairly low concentrations (maximum infective units 491 per gallon). This number would hardly seem adequate to saturate the Pilot Filter system.

It is not objectionable, however, to postulate that conditions within the filter may change over a period of time. The filter was operational in the early fall of 1966. Sampling of Basin 1 influent began on October 15, therefore a change within the filter media over a period of months may have occurred before Basin 3 loading began. If adaptation occurred, Basin 3 might then be more efficient than Basin 1 in reducing viral populations.

Another factor to take into consideration is the Pilot Filter clogging factor. Pilot Filter effluent from Basin 1 loading decreased from approximately 30 million gallons per day (MGD) to 19 MGD and from approximately 48 MGD to 30 MGD for Basin 3 loading during the period of study. If a clogging effect occurred, virus would be required to travel greater distances in and around matrices of filter media and would, therefore, be exposed to more adsorptive surfaces resulting in a reduction of virus populations. This may account for detection of fewer VIU in the PFE as the study progressed for both basins.

It is unlikely that pH or temperature were significant factors in the efficiency of virus removal by the filter with water loaded in either basin. The pH values of PFI ranged from 7.4 to 7.6 for both basins and the average pH value for PFE was 7.75. Temperatures of water within the filter averaged about 25 C during the study from October to May. At this temperature, poliovirus would be expected to survive 17 to 23 days (6) and would not be inactivated during the time required for water to become effluent.

No definite conclusions can be drawn concerning the effectiveness of the Pilot Filter in removing specific virus types. Since sampling was not done on a daily basis an exact knowledge of all of the virus types in the water would be impossible to assess. Grab sampling may miss certain virus types altogether. Berg (19) has stated that virus release in sewage occurs infrequently and therefore virus populations vary constantly. This is demonstrated by isolations of poliovirus at various times during the year suggesting the presence of the virus in sewage continuously. Failure to detect it at any given time may be due to inadequate sampling techniques. However, no superior sampling techniques have been devised at the present time and until a continuous monitoring system is functional, results of this nature can only be evaluated

over a period of months with a reduction in estimated virus infective units as a criterion of the efficiency of a tertiary type treatment.

The fact that all virus is not removed by the Pilot Filter is a matter of concern since the exact dose of virus which will produce a human infection is still debatable (41). For this reason, the Pilot Filter effluent may have to be treated further by chlorination or by some other method before it can be considered safe for recreational uses.

Severe limitations have been placed on the quantitation of viruses in sewage because of a lack of suitable sampling and concentration methods. The commonly used gauze pad sampling procedure provides no way to measure the actual amount of sewage flowing through a suspended pad nor is there any means of detecting how much of the available viruses have been absorbed. Inconsistencies due to elution are common. It must be considered also, that the nature of the sewage varies over the several days during which the pad is immersed.

In grab sampling these variables are eliminated. At any given time, for any given volume of wastewater, the population of viruses may be assessed per volume tested. It is also possible to sample large volumes of

wastewater by this method, whereas only a few hundred ml at most may be extracted from a gauze pad.

Concentration of viruses from gauze pad samples is usually accomplished by ultracentrifugation (2, 3, 6, 7, 20, 21). This technique is superior to ion exchange resins (21). But, it would not be feasable to ultracentrifuge large volumes of water. The paramount disadvantage of grab sampling is the use of chemical or physical methods to concentrate viruses and it therefore, cannot be considered absolutely quantitative. Virus concentration by Albertsson's two phase system using dextran sulfate and polyethylene glycol (42) has been reported by Shuval et al. (43) to recover 39 and 60% of an input seed of poliovirus. According to Wallis and Melnick (10), concentration of virus from grab samples of raw sewage by the Millipore membrane concentration technique recovers 100% of an input poliovirus seed.

Use of this technique on the Tucson wastewater in our laboratory compared to gauze pad sampling with ultracentrifugation concentration (44) over the same time period to detect indigenous viruses, resulted in a frequency of isolation of 60% compared to 83% respectively for the two methods. However, the true value of the membrane concentration method lies in its ability to assess

virus infective units present per volume of water which is impossible by the other method.

Qualitative studies of isolates show that of all virus types detected, poliovirus comprised the majority. It was not within the scope of this study to determine if these poliovirus isolates were wild types or vaccine strains. This situation warrants further study.

Of special interest was the isolation of adenovirus. The reports of Davis (45) and Hartwell, Love, and Eidenbock (46), and Hatch and Siem (47) on the isolation of adenoviruses from overt cases of infectious hepatitis have produced interest in adenoviruses or associated viruses with respect to possible etiology in the disease.

Since three successive passages were required in HAM to detect the presence of this virus in the water sample concentrate, attention should be given to repeated serial passage of negative specimens in the future. It is obvious that certain viruses may be missed altogether if only two serial passages are done as was the previous acceptable standard. In this study, three water samples showed presence of virus only after three successive passages.

The isolation of poliovirus in AGMK from four water sample concentrates which were HAM negative indicates the necessity of using different cell culture types in qualitative studies. However, since the purpose of our study

was to monitor the Pilot Filter, comparing detectable virus going in with detectable virus in the effluent, use of HAM alone for quantitation was considered to be sufficient.

Use of HEL cell cultures for identification of HAM isolates is advantageous since the HEL stable cell line may be easily maintained in serial passage in the laboratory. Thus it provides a readily accessible source of tissue cultures whereas, HAM processing is time consuming and not always dependable as a source of cells. Studies of Moore et al. (48) and Berquist and Love (49) have shown the HEL cell line to be susceptible to a large majority of the enteroviruses. In a comparable study (44) conducted during the same period as the present investigation in which HAM and AGMK were used for isolation and identification, AGMK was shown to be superior for detection of polioviruses. The frequency of isolation of polio in HAM was only 9% whereas, in AGMK a 90% frequency was obtained. However, in our study using HAM for isolation and HEL for identification, polioviruses were detected with a frequency of 44.5%. It may be suggested that while polioviruses may be isolated in HAM, they tend to be overgrown by echovirus types in subsequent passage and are therefore masked in identification procedures. Using HEL, apparently this suppression of poliovirus occurs to a lesser degree than if HAM were used for

identification. Kono et al. (50) report in studies of mixed infections of poliovirus type 1 and echovirus type 7 in the FL human amnion established cell line, that a pre-emption of susceptible cells by one virus caused a reduced yield of the other virus. The suppressive effect was mutual. It must be stated however, that AGMK is apparently superior to HAM for poliovirus isolation, while the reverse is true for echoviruses.

A second advantage of using the HEL cell line is that it lends itself well to the plastic panel micro culture technique in the identification of isolates. In comparative identification studies of the standard tube and micro panel techniques, results were comparable with the exception of run 56 which yielded poliovirus type 3 by tube and poliovirus type 2 by micro panel. In evaluating these results, we considered that both poliovirus types were present in the original sample and that a sampling error might account for the discrepancy in results between the two tests.

The failure to detect interfering virus types using echovirus type 11 as a challenge virus in negative samples is not surprising. By the nature of the sewage and its treatment, viruses included in the myxovirus group might be inactivated. This and the fact that HAM may not be the ideal cell type with which to detect these viruses

does not absolutely rule out their presence, however it is unlikely that such viruses as rubella would survive in sewage.

That sewage contains substances which inhibit virus isolation in cell cultures is well known (51). This may account for isolation of virus at the 1:9 and 1:27 dilutions and failure to isolate virus at the 1:3 dilution in run 93.

Evaluation studies on the Millipore membrane virus concentration technique indicate that only 0.6% to 5.0% of the added virus may be recovered. These percentages must be viewed with caution, however, when one considers that very small quantities of indigenous virus may be detected in wastewater while heavily seeded test samples may prevent complete recoverability simply because of a membrane clogging effect.

It must be considered also, that wastewater in different locations may vary in chemical, organic, and microbial constituents. Therefore, while the Millipore membrane virus concentration technique resulted in 100% recovery of virus in seeded raw sewage samples in Houston, Texas (25), the constitution of the Tucson wastewater may differ to such an extent that seeded virus in our experiments was poorly recoverable.

It was shown, however, in seeding experiments using the Millipore concentration technique that recovery could be improved by coating pre-concentration membranes with serum and by using Dowex anion exchange resin columns in place of protamine sulfate to remove membrane coating components.

Use of the $Al(OH)_3$ adsorption technique for detection of virus in wastewater has, in preliminary studies, shown superiority to the Millipore methods. This technique is much less complicated, more economical, and apparently more efficient in concentrating virus.

SUMMARY

Over an eight month period, October 1966 to May 1967, the removal of viruses from wastewater by a tertiary treatment Pilot Filter was quantitatively assessed. A Millipore membrane concentration procedure, applied for detection and quantitation, showed that the Pilot Filter effectively removed an average of 96% of the ingoing viruses when the filter was loaded from two different charging basins.

Isolation studies show November, January, and February as peak months for virus isolations. Virus infective units ranged from 0 to 491 per gallon sample. Of 15 Pilot Filter influent samples tested, 73% were positive compared to only 46% positive samples from 13 tested for the Pilot Filter effluent. The predominant virus type isolated was poliovirus type 3. Poliovirus types 1 and 2 as well as several echoviruses and one unidentified type were also detected. One isolation of adenovirus was obtained. The majority of the isolates were identified in neutralization tests by a plastic panel micro culture technique. Thirty-four isolations were obtained of which nine were poliovirus type 3. A total of 15 polioviruses

were detected and seemed to be present sporadically throughout the study. Ten different enterovirus types were identified.

Laboratory studies showed that the efficiency of the Millipore membrane virus concentration procedure could be improved by serum coating pre-concentration membranes with serum and by using a Dowex anion resin column to remove membrane coating components prior to concentration steps. Use of an $Al(OH)_3$ virus adsorption method gave excellent recovery of virus added to wastewater samples. This technique is being investigated for use in future quantitative studies.

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