Aspects of Microbiology 13

Environmental Virology

Rao & Melnick

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Preface

We have attempted to provide a concise and up to date account of human viruses in the environment with attention to basic tools now available for monitoring viruses. We have aimed this introductory volume at young students, teachers and investigators in microbiology, virology, environmental engineering and health sciences. As the routes for virus dissemination become more varied, there is an enhanced potential for causing infection in humans. Improvement in the development of control strategies will alleviate the magnitude of viral pollution of the environment. This volume will introduce the reader to an area of science which of necessity must utilize a variety of disciplines.

V. CHALAPATI RAO
JOSEPH L. MELNICK

We humbly dedicate this volume to the cherished memory of our parents

Contents

1 Introduction

Many viruses, belonging to several families and over 100 species, can be found in polluted water and all of them are infectious to man. Not every strain of each virus is virulent, so the fraction of the total number of virus particles present which are capable of causing disease must be very small. The basic properties of pathogens and non-pathogens are similar, so it follows that pathogens must possess highly specialized attributes which enable them to cause disease.

A pathogen must be able to enter the body, lodge in some primary site, multiply, spread to or invade other tissues. While this is happening, the host is mounting non-specific (phagocytic cells and interferon-like materials) and specific (antibodies and cells of the immune system) host defense mechanisms, both of which must be overcome or evaded for the pathogen to be successful.

Some of the earliest studies on virus transmission involved poliovirus. Flexner believed that poliovirus entered the human body through the nasal route and proceeded along nerve fibers directly to the central nervous system. He demonstrated that when zinc sulphate was administered to rhesus monkeys to coat the lining of their noses, the animals were protected against development of paralytic polio when challenged by intranasal inoculation. Flexner entirely overlooked Kling's work in Sweden. As early as 1912, Kling and his associates had reported isolation of poliovirus not only from the throat but also from the feces of both paralytic and nonparalytic patients. In 1936, Sabin and Olitsky reported that olfactory bulb lesions could be detected in monkeys only when infected by intranasal instillation of virus, and not when virus had been administered by other routes. Five years later, Sabin and Ward showed that olfactory bulbs from six fatal human cases were all negative for virus, proof against a nasal route of infection. American investigators turned toward the possibility of an oral-alimentary route of infection. Chimpanzees were especially susceptible to virus administered by the oral route and the infection closely resembled that of human beings both clinically and pathologically (Howe and Bodian, 1941). At that time it was not known how poliomyelitis was spread; it was not understood under what circumstances the paralytic disease occurred, or where the virus was in the infected or diseased individual.

At Yale University the search for poliovirus included specimens other than the spinal cord. In 1938 John Paul made a report to the American Epidemiological Society of some of the findings (Trask *et al.*, 1938). Not only was virus found in large quantities in feces, but it was also recovered repeatedly over a period of weeks from both patients and healthy carriers. This report was met with skepticism by many, but it was soon confirmed and extended. The shift to the concept of poliomyelitis as an enteric infection was under way. It was found that poliovirus could be spread by healthy persons who had inapparent infections. The new concept had direct implications for measures to control fecal contamination in hospitals and households.

Another series of questions was also brought into focus. If polio was an enteric

infection, was it waterborne? Could it be transmitted by direct or indirect contamination from sewage? Pursuit of answers to such questions became one of the first tasks of Melnick when he joined the Yale Poliomyelitis Study Unit. During the period 1940–45, he was engaged in detecting polio viruses in the East River of New York City—almost the only samples of water in the world studied for viruses during that period (Melnick, 1947). The samples were concentrated, purified and injected into test animals, one monkey for each sample. The monkey was examined daily for 4 to 5 weeks for muscle weakness, and every day its rectal temperature was taken. At the end of the observation period, the brain and spinal cord were removed and examined in thin sections under the microscope for lesions of poliomyelitis. What did we learn? Waterborne transmission of polio could not be proved, neither was it disproved. But, during periods of polio prevalence in the community, polioviruses were present in large numbers in sewage and remained infective in flowing sewage for many weeks. We learned the amount of virus excreted by a carrier each day and we determined the ratio of inapparent infections to paralytic cases.

An intriguing problem had been puzzling the investigators in certain poliomyelitis outbreaks: paralytic and nonparalytic polio (meningitis) cases usually had the same seasonal occurrence, but sometimes their prevalence curves did not coincide. This did not make sense in the light of what had been discovered about the relationship between meningitis and paralysis as differing manifestations of infection with the same polio virus. An answer was provided by the introduction of a new test animal—the newborn mouse. Dalldorf and Sickles of the New York State Health Department Laboratories, in their attempts to find an animal more readily available than monkeys for poliomyelitis studies, inoculated newborn (suckling) mice with fecal suspensions from two polio patients from Coxsackie, New York. The mice became paralyzed, but not from poliovirus infection. A new virus had been discovered. It induced fatal disease with paralysis in the baby mice, but not in adult mice or rhesus monkeys. The lesions were located in skeletal muscles rather than in the central nervous system and these isolates subsequently became the first prototypes of the group A coxsackieviruses. In 1949, Melnick, Shaw and Curnen reported a number of mouse-pathogenic viruses.

These viruses clearly differed from the New York isolates of coxsackie A virus in that they caused only limited lesions in muscles but produced degeneration of the brain, pancreas, heart muscle, etc. These new isolates recovered from patients in atypical polio epidemics became the prototypes of the group B coxsackieviruses. Melnick and coworkers studied the prevalence of these newly discovered enteroviruses from 1949 through 1952 in six areas of the United States. In every urban area studied, coxsackieviruses appeared in the specimens collected in the summer and fall, and then virtually disappeared during winter and spring. In general, the isolated coxsackieviruses produced only myositis in test mice. However, a number of strains were neurotropic and myotropic, and others produced lesions only in the brains of mice. If enterovirus tests were conducted only in tissue cultures, as we do today, most such viruses would be missed.

Despite the increasing knowledge of polio and coxsackieviruses gained through investigations that had to depend on monkeys, chimpanzees and suckling mice, investigators were plagued with the problems of irregular supplies of monkeys from Asia and Africa and in dealing with the monkeys' special nutritional needs

and susceptibility to diseases acquired in nature, such as tuberculosis, dysentery and parasitic infections. This led to a search for susceptible cell cultures. Following the original efforts of Levaditi in 1913, there had been two decades of repeated attempts to grow polioviruses in culture. Cell cultures of nerve tissue had been successfully prepared in the United States by Harrison in 1907, and there followed efforts to cultivate polioviruses in such cultures. Sabin and Olisky (1936) reported the successful growth of the MV strain of poliovirus in nerve tissue. This virus, however, had gone through 20 years of intercerebral passage from monkey to monkey at the Rockefeller Institute, which had apparently made it highly neurotropic. Nerve tissue is difficult to obtain and, furthermore, its use as a substrate for growing antigenic material for a possible vaccine was precluded because of the potential of such tissue extracts to produce encephalitis. (More illness has probably been produced by the administration of rabies vaccine prepared from virus grown in nerve tissue of animals than from exposure to supposedly rabid animals.) Another 12 years elapsed before the landmark report by Enders, Weller and Robbins (1949), who demonstrated that poliovirus could be grown in cultures of human cells derived from tissues other than those of the nervous system, and the process could be easily followed by examining the cultures for visible cell damage. The characteristic cytopathic effect in these cultures could be assigned scores and, thus, could serve as a basis for quantification of the virus. The important work of these investigators was recognized by their receipt of the Nobel Prize in 1954. Using tissue cultures, virologists detected a whole new assortment of previously unknown viruses which, at the time of their discovery, could not be associated with specific human diseases. By 1953 these agents were beginning to be called 'orphan' viruses, i.e. viruses in search of a parent disease. A more explicit name was soon agreed upon, enteric cytopathogenic human orphan, or echoviruses. As studies of polioviruses, coxsackieviruses and echoviruses continued, it became clear that these viruses shared not only a human enteric habitat but other properties as well, and they were grouped as the enteroviruses. Four decades of developmental work on these agents has been reviewed in *Portraits of Viruses: Picornaviruses* (Melnick, 1983).

In spite of the fact that polioviruses are detected in largest numbers in sewage, poliomyelitis outbreaks attributed to polluted water have been very few; a total of eight outbreaks in Europe and North America had been reported, but Mosley (1967) believed that only one of them was adequately documented. This occurred in Huskerville, Nebraska in 1952 at which time at least 45 people became ill after contamination of the municipal water system.

Several waterborne outbreaks of viral hepatitis have been reported from different countries, but none have been of the scale of the epidemic which occurred during December 1955 and January 1956 in New Delhi. During the epidemic period one of us was invited to Delhi to participate in virological studies, results of which have been documented elsewhere (Melnick, 1957). During the epidemic there were about 30,000 cases, giving a case rate of 2000 per 100,000 (2%) in the single month of the epidemic. The contamination of the Jumna River, the source of water for the treatment plant, by sewage had taken place for 1 week 6 weeks prior to the epidemic. During the period of contamination, water was coagulated with high doses of alum and disinfected with high doses of chlorine, which prevented the outbreak of bacterial and other enteric viral diseases but failed to prevent the hepatitis epidemic. Following this massive outbreak of

waterborne hepatitis, the Indian government set up the National Environmental Engineering Research Institute at Nagpur to initiate studies on viruses in water and other aspects of environmental pollution. At Baylor College of Medicine in Houston an environmental virology group was organized by Melnick and Wallis, and at the Robert Taft Sanitary Engineering Center of the US Public Health Service in Cincinnati, Kabler, Clarke, Chang and Berg became involved in bacterial and viral pollution of water and wastewaters. Thus, the field of environmental virology came into being in the late 1950s.

Virological research was greatly expanded and made technically more feasible by the development and availability of cell cultures. Research findings showed that enteroviruses are more resistant to chlorine than the coliform bacteria group used to indicate the sanitary quality of water. Enteroviruses have high survival capabilities under various environmental conditions including wastewater and water treatment processes. The minimum human infectious dose for enteroviruses is very low (of the order of one virus particle), which is four to six orders of magnitude lower than the number of enteric bacteria needed to initiate human infection. These findings, coupled with epidemiological studies incriminating water as a vehicle for transmission of infectious hepatitis, led to increased concern about possible spread of other viral diseases by drinking water.

By 1965, interest in the subject of environmental virology had broadened. Gerald Berg and colleagues in Cincinnati convened the first international conference on the subject entitled 'Transmission of Viruses by the Water Route' (Berg, 1967). The deliberations highlighted the problem and urged concerted efforts to develop adequate quantitative methods for detecting low level virus transmission through water, to verify the concern whether viruses can survive undetected under conditions which eliminate coliform organisms, to develop sound judgements of what constitutes an infective dose of virus to humans, to study virus persistance in the deliberate reuse of wastewater, and to intensify efforts to detect the agent of infectious hepatitis.

During the 10 years following the symposium the handful of active pioneer researchers in the field of viruses in water grew considerably, and much progress was made in answering some of the questions that had been raised. Virus monitoring techniques, although still not perfected, were developed during this period to assay as much as 400-liter samples of water, which was then but a far-off goal. Surveys of virus concentrations in water and wastewater have been conducted in many countries. We have a clearer picture of the rate of enterovirus shed by communities into the water environment, their load in raw sewage (100 to 1000 PFU/100 ml) and in treated effluents (1 to 10 PFU/100 ml). In other studies, varying concentrations of viruses were detected in lakes and seawater used for recreation. Here it must be added that so far enteroviruses have been detected only rarely in drinking water supplies which have undergone proper conventional treatment. Many studies advanced our understanding of the virus removal efficiency of conventional as well as low-cost water and wastewater treatment processes.

During this decade several important contributions were made for improving our understanding of the role played by oysters in the transmission of viral diseases. Methods for detection of enteroviruses from shellfish were developed; clams, oysters and mussels were implicated in outbreaks of hepatitis A virus. In

water polluted with human feces, shellfish were found to accumulate enteroviruses and humans became infected by eating shellfish that was improperly cooked. Shellfish themselves did not get infected and, most importantly, the shellfish eventually freed themselves of virus if they were removed to unpolluted water.

In 1974, another international conference on 'Viruses in Water' in Mexico City (Berg *et al.*, 1976) reviewed the advances made during the past decade. Even though water transmission of enteric viruses was often difficult to trace and despite the fact that a single virus monitoring technique had not yet been selected, the time seemed to have arrived for setting standards for the permissible virus content of waters. A number of proposals were discussed including those of (1) the World Health Organization's Committee that drinking water should not have any viruses in a 10-liter sample, (2) Melnick that no more than 1 PFU per 40 liters of recreational water and 1 PFU per 400 liters of drinking water be allowed, and (3) Berg that permissible level of viruses in drinking water should be *none*. Shuval felt that a tentative standard of *no viruses* in 400 liters of water would be a good first step in upgrading water treatment facilities throughout the world. It was urged that standards should also be considered for viruses in water used for cultivating shellfish, for agricultural irrigation, and for recycling. This Conference expressed a consensus that an international body, such as the World Health Organization, in cooperation with interested national authorities and scientific organizations, should set up a committee to select a method for virus monitoring and evaluate the possibility of establishing tentative virus standards for water.

The World Health Organization recognized the growing importance of the problem, invited experts from around the world (Melnick and Rao served as chairman and vice-chairman, respectively, of the group), organized an in-depth discussion on human viruses in water, wastewater and soils, and published a report in 1979 (WHO Report, 1979) aimed at those responsible for public health and economic planning both in developing and developed countries. This report includes 'an assessment of the public health importance of viruses in water, wastewater, and soils, and of the nature of risks for exposed persons. It relates to the methods available for monitoring viruses in different situations and identifies areas for further research.'

In 1982, scientists at another international symposium on 'Enteric Viruses in Water' in Israel raised the question of whether the additional effort and cost of rendering water totally virus free is a justifiable expenditure for public health. A related concern revolved around the difficulties of trying to set virus standards for drinking water with present imprecise methods (Melnick, 1984). No decision was made on the setting up of viral standards at the conference, and none exists yet.

Recent significant developments having potential for opening new frontiers in environmental virology include the successful cultivation of hepatitis A virus (HAV) in cell cultures by Provost and Hilleman in 1979 and the development of a radioimmunoassay for quantification of hepatitis A antigen, development of methods for the concentration of HAV from water, recognition of the involvement of two types of non-A, non-B hepatitis in waterborne epidemics, seroepidemiological surveys of outbreaks of nonbacterial gastroenteritis caused by Norwalk and Norwalk-like agents, involvement of human rotaviruses in infantile diarrhea throughout the world, and recovery of recently discovered

types of enteric adenoviruses from children suffering from acute gastroenteritis.

The routes for the transmission of enteric viruses in the environment (Fig. 1) are so widespread that today there is an increasing awareness that human populations around the world are exposed to enteric viruses through a variety of routes: shellfish that grow in contaminated ocean or estuary water; food crops grown in land irrigated with wastewater or fertilized and conditioned with sludge; recreational waters; and even drinking water that has been polluted with viruses. Waterborne outbreaks of hepatitis caused by type A virus and gastroenteritis caused by rotaviruses and Norwalk viruses are well recognized. Even though there is little direct evidence to connect the presence of enteric viruses in water with incidence of viral disease, they are isolated regularly from sewage and environmental waters, and serve as indicators of pollution by human viruses.

Let us consider the circumstances which have led to clinical recognition of water-associated outbreaks of this disease. The overwhelming majority of reported waterborne outbreaks of hepatitis A, Norwalk and rotaviruses have been attributable to untreated or inadequately treated water supplies. In the case of semipublic and private water supplies, the fault lay usually with sewage contamination of the water source coupled with inadequate or no treatment. With large municipal supplies, the problem usually was one of cross-connections or back-siphonage during distribution. The solutions to these obvious deficiencies are straightforward and attainable.

On the other hand, some viral infections may be spread insiduously by way of continual low level transmission through water. It is the nature of most enteroviral diseases that they elude the epidemiologist in his quest for definitive

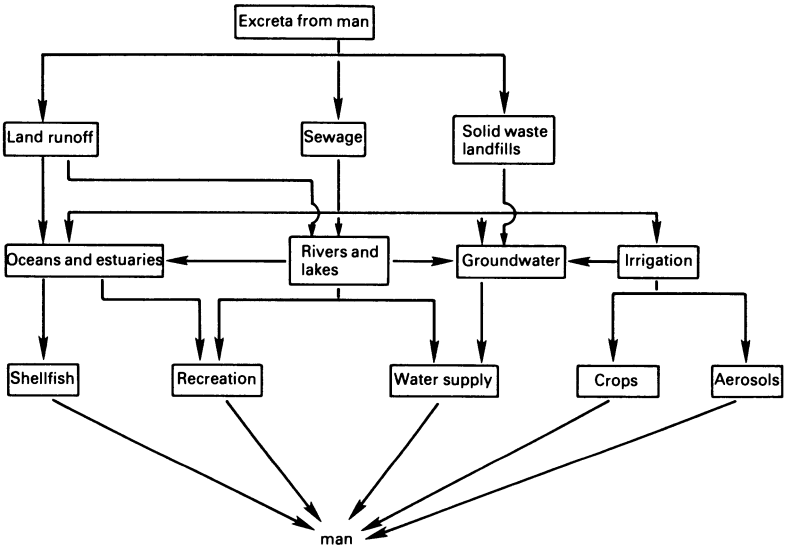

Fig. 1 Routes of enteric virus transmission.

data. Many enteroviruses can cause inapparent or silent infections which go unrecognized until secondary person-to-person spread finally leads to overt disease in scattered, virtually untraceable pockets of the population.

Inadequacies in current water treatment techniques have been demonstrated on several occasions when viruses were isolated from conventionally treated potable supplies which had received treatment adequate to remove bacterial pathogens. The degree to which small detectable numbers of viruses that manage to get through into drinking water become foci of infection in a community remains a question that needs attention.

Virus interactions in cell cultures, commonly called tissue cultures, and in the intact host suggest that in some cases only a few viral particles are present in one tissue culture dose (TCD)—that quantity of virus required to produce in a tissue culture an infection that is easily seen on the basis of cytopathic effect or plaque formation. Tissue cultures vary in their susceptibility to any given virus. However, the number of viral particles that equals one TCD varies with the cell line as well as with the virus. The laboratory procedures used to make these determinations impose further uncertainties. Finally, host cells in culture may not be strictly representative of oropharyngeal and intestinal cells *in vivo;* hence, experiments which try to relate TCDs manifested in cell lines to human infectious doses may produce quite disparate results.

It is important to bear in mind that infection and disease are not synonymous. Viral infections may be highly prevalent while the incidence of viral diseases is low. The prevalence of disease among infected persons is strongly dependent upon the individual hosts' susceptibilities. A detailed summary of this topic is presented by the Safe Drinking Water Committee (1977).

For all of the reasons outlined above, reported levels of viral contamination in water cannot be used directly to assess the risk to human health. Expensive surveillance programs among populations coupled with large laboratory outlays would be necessary to detect virus infections, in order to trace sources of viral disease. Studies to determine virus levels in drinking water, in conjuction with epidemiological surveys of viral disease in the community, remain for the future. At the least, epidemiologists and public health laboratories should have available to them the expertise needed for conducting proper virological studies when an outbreak of disease is suspected of being waterborne and the etiologic agent is unknown. Until information gathered from such investigations is forthcoming, the true public health significance of small numbers of viruses in drinking water will remain unanswered.

Discharge of treated and untreated sewage as well as polluted surface waters into coastal estuarine waters result in viral pollution of recreational and shellfish growing areas. Routinely cultivable enteroviruses have been recovered from water, sediments and shellfish. The greatest portion of virus in polluted water may be associated with suspended solids; virus has been detected in 72% of samples of suspended solids and 51% of sediment samples and in only 14% of clear water samples tested from Galveston Bay, Texas. Viruses adsorbed to sediments remain infectious and survive longer in the marine environment than freely suspended viruses. Sediments can be resuspended in response to storms, movement of boats and swimmers, dredging and changes in water quality. Viruses resuspended in the water column can be transported from polluted to nonpolluted waters used for swimming and shellfish harvesting.

Information on virus types and numbers released into estuarine waters has been obtained mainly from studies on effluents discharged from municipal waste treatment plants. Data is needed on the types and numbers of viruses discharged from small community treatment systems such as package plants with design and operational defects, from septic tanks and nonpoint source run-off from rural areas, and from pleasure boats emptying their fecal wastes into bay waters.

Several outbreaks of viral hepatitis and nonbacterial gastroenteritis have been associated with consumption of raw or inadequately cooked shellfish harvested from waters which met bacteriological standards.

Marine recreational water may also play a significant role in the transmission of viral disease, especially gastroenteritis in young bathers. Recent studies in our laboratory have shown that rotaviruses are present in 63% of sewage polluted coastal water with numbers ranging from 100 to 5000 rotaviruses per 400-liter samples.

The full extent of the health hazard posed by the water and shellfish virus transmission potential is unknown, but an appreciation of the hazard represented can be gained from US Public Health Service and Australian reports of outbreaks involving about 2000 cases of shellfish-associated hepatitis and nonbacterial gastroenteritis. In Australia alone, a country-wide total of at least 2000 cases of shellfish-borne Norwalk virus gastroenteritis occurred in a single month. The known relationship between eating shellfish and clinical cases of type A hepatitis suggests that actual incidence of shellfish-associated hepatitis has been grossly underestimated.

Bacterial monitoring of water and shellfish is not reliable to indicate viral pollution, yet closure of 26% of the total shellfish-growing areas in 13 states in the USA during 1977–1979 was based on bacterially indicated pollution.

Assessements of health hazards in virus-polluted coastal waters have been hampered by an inability to detect and enumerate hepatitis A virus and gastroenteritis virus numbers. Information on their incidence, survival and transport in estuarine water and bioaccumulation and elimination by shellfish should be made available. Information is also needed on the viral sanitary quality of water and shellfish, indicated by hepatitis virus and gastroenteritis viruses themselves, as compared to enterovirus and bacterial indicators.

A great deal of progress has been made in the development of methods for detecting viruses in wastewaters, effluents and drinking waters. Yet the efficiency of these methods probably averages well under 20% and the precision of the numbers obtained with these methods is poor. Moreover, the sensitivity, or lack of it, of the cell culture systems used to demonstrate and quantify the viruses recovered further reduces the accuracy of the total recovery system. Relatively few entero virus types have been used in the development of current methodology and only a few environments have been sampled with any given test procedure.

The available methods should be standardized by comparative tests by workers in different laboratories to insure adequate control over monitoring efforts, and a few efforts are being made in this direction by round-robin testing. Detection of viruses under adverse environmental conditions in which high concentrations of humic acids and suspended solids occur provides challenges quite different from those encountered with finished waters.

The importance for enumerative purposes of the interaction between viruses and suspended solids appears to be highly significant in quantitative estimates of

viruses in sediments, sludges and soils. A quantitative enumeration method should reflect rapid and maximum recovery of as many different virus types as possible including hepatitis A, rota and Norwalk viruses. The numbers of viruses recovered from the environment at present constitutes only the tip of the iceberg.

Current developments in immunological procedures including the use of monoclonal antibodies offer possibilities for detecting enteric viruses that replicate poorly in cell cultures, but it seems unlikely that the sensitivity of such methods can be improved to match the amplification of antigen that tissue culture provides. However, after replication of such viruses in tissue culture, virus is often present in high enough concentration to be detected directly by immunoassay.

As methodology improves, and as data on the numbers of viruses in the environment continue to be generated, our evaluation of the magnitude of viral contamination of the environment also improves. Then, as we understand their true significance, appropriate control measures can be introduced to reduce the hazards from viruses.

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2 Human enteric viruses in polluted water

Over 120 different viruses are excreted in human feces and urine, find their way into sewage, and become water pollutants. They are listed in Table 1 together with diseases that they cause. The term 'enteric virus' is applied to any viruses disseminated by the fecal route. They are divided into several groups, based on morphological, physical, chemical and antigenic differences.

Enteroviruses

The most commonly studied group in natural waters has been the picornaviridae family, which includes poliovirus, coxsackie virus A and B, echovirus, and the high numbered enteroviruses (types 68 to 72). These viruses multiply primarily in the alimentary tract and are excreted in substantial amounts (as many as 10^{10} per gram of feces) for varying periods of times, with a mean shedding period of up to 50 days. The best studied of these enteroviruses are the polioviruses and the least studied are the many types of group A coxsackie viruses, which are usually isolated in suckling mice. Numerous studies have readily demonstrated the presence of enteroviruses in sewage, in effluents from sewage treatment plants, in contaminated rivers and lakes and in treated drinking waters from developing as well as developed countries (refer to Chapter 3).

Polioviruses can cause serious nervous system disease. Clinical disease occurs only in between 1 in 100 and 1 in over 1000 cases of infection, depending chiefly on the virulence of the virus and the age of the host. At present in countries in which live poliovirus vaccine is widely used, the excreted polioviruses are usually vaccine-derived and their pathogenicity is low, although reversion to varying degrees of neurovirulence during several human passages may occur. The other enteroviruses can also cause nervous system disease, usually of a transient nature (aseptic meningitis) but on occasion clinically similar to typical paralytic poliomyelitis. Some enteroviruses, such as enterovirus type 71, have given rise to large outbreaks of central nervous system disease characterized by encephalitis, or paralysis, or both, with many fatalities. Group B coxsackie viruses cause epidemic pleurodynia (Bornholm disease), pericarditis (chiefly in older persons), serious and often fatal myocarditis in infants and congenital cardiac defects in infants born of mothers experiencing infection during pregnancy. In addition, mild respiratory disease may result from certain enterovirus infections. In summary, enteroviruses can cause serious diseases, but fortunately, under most circumstances, do so in only a very small proportion of infections. A comprehensive review updating our knowledge of enteroviruses and their epidemiology has appeared recently (Melnick, 1985).

This infrequent association with severe disease may well help to explain why reports of the waterborne spread of enteroviruses have been so few. Furthermore, severity of the outcome of infection may be directly related to host

age. In developing countries, in which wild polioviruses are still prevalent, infections are typically acquired very early in life, when the risk of serious disease is lowest. Most older children and adults are thus immune. In these areas waterborne spread undoubtedly occurs and may be a significant factor in the process of natural immunization. As sanitation has improved in some of these areas, paralytic poliomyelitis has increased, presumably because infections are delayed to an older age. Data on the prevalence of immunity to the enteroviruses other than poliomyelitis are fragmentary, but several studies suggest that urban populations in particular are frequently exposed to these agents.

Hepatitis A virus

Hepatitis type A virus (HAV) has been proven beyond doubt to be responsible for waterborne epidemics traced to sewage contamination. The virus is a 27 nm RNA virus whose physico-chemical characteristics place it in the enterovirus group, where it has been classified as type 72. This virus is excreted in feces over a relatively extended period and has been responsible for waterborne epidemics traced to sewage contamination. The largest waterborne epidemic of hepatitis recorded is the New Delhi outbreak of 1955–56, in which about 30,000 cases were reported, although recent evidence indicates that this outbreak may not have been associated with HAV (Wong *et al.*, 1980). Extensive surveillance in the United States revealed fifteen outbreaks of water associated hepatitis A between 1971 and 1978, which accounted for only 6% of 224 outbreaks of waterborne disease in the USA during that period. Mosley reviewed some 50 documented water-related epidemics of hepatitis A virus between 1958 and 1964.

Viruses in faecally polluted water accumulate in shellfish in concentrations several times higher than in surrounding waters. Many outbreaks of viral hepatitis have been traced to the ingestion of raw or inadequately cooked shellfish.

During the past four decades unsuccessful attempts to identify and to characterize the hepatitis A virus have frustrated virologists. The reason for this failure was mainly the lack of a laboratory technique for detection and quantification of the agent. Up to the end of the 1960s, when HAV finally could be transmitted to marmoset monkeys, infection of human volunteers was the only means to identify samples containing the virus. Analysis of the physico-chemical properties of the virus became feasible with the detection by immune electron microscopy of virus-like particles with the antigenic properties of HAV in human feces by Feinstone and coworkers in 1973. This key achievement initiated the development of a method for the quantification of hepatitis A antigen by solid-phase radio immunoassay and finally culminated in the cultivation of HAV *in vitro* in a variety of cell cultures, such as human hepatocellular carcinoma cells, human diploid fibroblast, human amnion, fetal rhesus kidney cells and African green monkey kidney (AGMK) cultures. Virus infection *in vitro* results in no cytopathic effects but establishes persistent infection. Such persistently infected cultures of AGMK cells are a good source of HAV (Simmonds, 1985).

Non-A, Non-B hepatitis

The name 'non-A, non-B hepatitis' includes infections of the liver caused by at least two viruses. These viruses have been characterized as 'blood transmitted', 'coagulation-factor transmitted', and 'epidemic waterborne'. A universally recognized specific serological test has not yet been developed for any of the viruses of non-A, non-B hepatitis. The diagnosis of non-A, non-B hepatitis remains based on the exclusion by serological tests for hepatitis A virus, hepatitis B virus, hepatitis delta virus, cytomegalovirus, and Epstein-Barr virus. A recent review highlights the important aspects of non-A, non-B hepatitis virus in relation to the other viruses that may cause hepatitis (Hollinger *et al.*, 1985).

Waterborne non-A, non-B hepatitis virus has been the cause of five epidemics in India, an epidemic in Nepal, and sporadic cases in India and the Soviet Union. Its epidemiology resembles that of the hepatitis A virus (HAV), but it is serologically unrelated to it and it can infect patients who have recovered from hepatitis A. Infection has been transmitted by water supplies contaminated by infected feces; cases among household contacts suggest that person-to-person spread may also occur. This virus does not seem to cause chronic infections. The viral etiology was shown and the stool-borne transmission was confirmed by filtered feces obtained from nine patients between the first and the eighth days of jaundice. Immune electron microscopy studies in which convalescent serum from the volunteer was used as a source of antibody demonstrated spherical virus particles with a diameter of 27 to 30 nm in stools obtained from the volunteer between 28 and 45 days after ingestion. Antibodies were detected in patients with an illness clinically like hepatitis A, in which no IgM anti-HAV was detected. A 27 nm virus particle in stools and the development of antibodies to it were confirmed in the Nepal epidemic and in the course of infection in a small percentage of marmosets inoculated intravenously with a suspension of stools collected during the epidemic.

The fatality rate may be as high as 12%, although in some outbreaks it may be less than 1%. Epidemic waterborne non-A, non-B hepatitis is most virulent among pregnant women, in whose case fatality rates average 20% but have reached 39%. Although cases of infection with this virus in western countries have not been reported, there is evidence of an association between shellfish consumption and some cases of non-A, non-B hepatitis in two studies from the USA and the UK. It is possible that these were sporadic cases of epidemic waterborne non-A, non-B hepatitis. Cases of non-A, non-B hepatitis associated with shellfish consumption in western countries have not been analyzed separately to determine whether they are clinically similar to epidemic waterborne non-A, non-B hepatitis.

Norwalk and Norwalk-like viruses

Since the first identification of the Norwalk virus during an outbreak of gastroenteritis in an elementary school in Norwalk, Ohio, eight new viruses, capable of producing the same type of human viral diarrhea as the Norwalk virus, have been detected in the stools of patients. They include coronaviruses, caliciviruses, astroviruses and parvovirus-like agents. These viruses are 25 to 30

nm in diameter, could not be cultivated in cell cultures and did not produce disease when inoculated into laboratory animals, including primates. The development of a radioimmunoassay blocking technique for Norwalk antibody in 1978 by Greenberg and colleagues provided a sensitive and specific test for studying Norwalk virus infections.

Kaplan *et al.* (1982) reviewed 74 outbreaks of non-bacterial gastroenteritis investigated by the Centers for Disease Control, Atlanta and attributed 42% of them to Norwalk virus. Of the Norwalk-related outbreaks, water was responsible in 13 and food in 4. The sources of water included municipal as well as semi-public water supplies and recreational swimming water. In Australia, an outbreak of gastroenteritis from oyster-associated Norwalk virus involved 2000 cases during 1979. Seroepidemiological surveys have shown that acquisition of antibodies to Norwalk virus in developed countries begins slowly in childhood and accelerates during adult life. In contrast, infection with this virus occurs early in life in developing countries.

Rotaviruses

Rotaviruses have been found to be the major pathogen in non-bacterial infantile diarrhea throughout the world (Flewett and Woode, 1978). At least 50% of hospitalized cases of gastroenteritis in infants and young children are due to rotavirus infections. Infection has also been reported among adults. Contaminated water has been incriminated in rotavirus diarrhea in the USA, USSR, and Brazil.

The virus particles are approximately 70 nm in diameter, contain double-stranded RNA and represent a separate genus in the family Reoviridae. The virus particle has a double capsid layer, a smooth rim (outer capsid) surrounding spoke-like subunits (inner capsid) radiating outwards from a hub-like core. Because of the wheel-like appearance, the term rotavirus is used for this virus. Enzyme immunoassay suggests that there are two major antigenic types of rotavirus, whereas by neutralization tests at least three and perhaps four subtypes have been identified. Infection caused by one subtype does not appear to protect against infection by the other.

Highest levels of rotavirus are excreted between the second and fourth day of illness (up to 10^{10} particles per gram feces), but a week or so after the onset of symptoms very little virus is detected in stools. The incidence of rotavirus infection in temperate climates peaks during winter months whereas in some tropical developing countries considerable variation in the seasonal distribution of cases has been noticed. Evidence to date indicates that rotavirus infection spreads by the fecal-oral route.

Adenoviruses

Adenoviruses are also found in the feces and have been isolated from domestic sewage. These are large double-stranded DNA viruses that cause respiratory and eye infections among bathers in nonchlorinated swimming pools. In 1975 , previously unrecognized adenoviruses were detected by electron microscopy in

stool samples from infants with diarrhea by Flewett and colleagues. These adenovirus species have been designated enteric adenoviruses (EAds). They are fastidious and cannot be cultivated in cell cultures by conventional means. In several studies EAds have been found in 3.9 to 12% of the stool specimens from infants and young children with acute gastroenteritis. Hospital outbreaks of diarrhea caused by EAds have also been described. Antibodies against EAds have recently been shown to be common and widespread throughout the world.

Recently, EAds have been propagated in selected cell cultures, namely 293 cells, Chang conjunctival cells, and African green monkey kidney cells. Two distinct types of EAds, AD40 and AD41, have now been identified by ELISA and electron microscopy. Uhnoo and coworkers (1984) conducted a one-year study of acute infantile gastroenteritis in children up to fifteen years of age at the University Hospital at Uppsala, Sweden, and detected adenoviruses in the stools or by seroconversion, or both, in 56 of 416 (13.5%) of ill children. Of the adenoviruses detected, 59% of the specimens were identified as adenoviruses 40 and 41. Evidence for established adenoviruses (Ad1 through Ad39, in gastroenteritis was found in 15 of 416 (3.6%) ill children. The predominant symptom of infections with enteric adenoviruses was diarrhea, with a mean duration of 8.5 days (Ad40) and 12.2 days (Ad41). One-third of the children with AD41 infections had prolonged symptoms (>14 days). The established adenoviruses (1 to 39) presented a different clinical picture, characterized by diarrhea of shorter duration, higher fever, and significantly increased occurrence of respiratory symptoms (79%). In this study, adenoviruses were observed throughout the year and displayed no marked seasonal pattern, in contrast to the winter prevalence of rotaviruses. AD40 and AD41 showed similar seasonal distribution with a small peak in July (warmer month), where one-third of all gastroenteritis was due to EAds. The role of EAds in infantile gastroenteritis in developing countries is not known at this time.

Parvoviruses

The adeno-associated viruses (AAV) were the first parvoviruses of human origin to be discovered. AAV, together with adenoviruses, have been recovered from feces, and hence may be present in polluted water. However, data on the frequency of excretion of these viruses are lacking. Seroepidemiological studies indicate that antibodies to AAV, especially types 2 and 3, are widely prevalent in young children.

Enteric viruses in the etiology of waterborne disease

Acute gastrointestinal and diarrheal illnesses continue to be the major waterborne diseases in the more developed countries, usually with no etiological agent being identified. Recently, seroepidemiological studies have identified Norwalk virus as the probable cause in several waterborne oubreaks of gastroenteritis. Rotaviruses have also been suspected. Hepatitis A virus (enterovirus type 72) is the only other viral agent clearly recognized as having caused waterborne disease outbreaks. Several very large outbreaks of drinking-water-transmitted hepatitis have been recognized in India. Outbreaks of swimming pool

adenovirus pharyngoconjunctivitis also have been documented, in which improperly chlorinated and maintained swimming pools were involved. While waterborne transmission of poliovirus and other enteroviruses has been suspected, conclusive evidence has been lacking.

Waterborne outbreaks due to enteroviruses and other enteric viruses are not easily recognized and are difficult to document for several reasons: (i) Many of these viruses cause inapparent infections that are difficult to recognize as being waterborne. A person may contract a viral infection by ingesting contaminated water, and the virus may actively multiply in the alimentary tract without the person developing overt symptoms of the disease. The person may have only mild malaise for a few days or no symptoms at all, yet may act as an effective carrier and transmit the virus to others, who may then develop acute symptoms of the disease. (ii) Epidemiological techniques are not sufficiently sensitive to detect low-level transmission of virus diseases through water. As shown in Table 1, most enteric viruses cause such a broad spectrum of disease syndromes that scattered cases of acute illness would probably have symptoms too varied to be attributed to a single etiological agent.

Table 1 Human viruses that may be present in polluted water

Virus group	Number of serotypes	Disease caused
Enteroviruses:		
Poliovirus	3	Paralysis, meningitis, fever
Echovirus	34	Meningitis, respiratory disease, rash, fever, gastroenteritis
Coxsackievirus A	24	Herpangina, respiratory disease, meningitis, fever, hand, foot and mouth disease
Coxsackievirus B	6	Myocarditis, congenital heart anomalies, rash, fever, meningitis, respiratory disease, pleurodynia
New enterovirus types 68 to 71	4	Meningitis, encephalitis, respiratory disease, rash, acute hemorrhagic conjunctivitis, fever
Hepatitis A (enterovirus 72)	1	Infectious hepatitis
Norwalk virus	2	Epidemic vomiting and diarrhea, fever
Rotavirus	4	Gastroenteritis, diarrhea
Reovirus	3	Unknown
Parvovirus:		
adeno-associated virus	3	Unknown
Adenovirus (feces and urine)	>30	Respiratory disease, conjunctivitis, gastroenteritis
Cytomegalovirus (urine only)	1	Infectious mononucleosis, hepatitis, pneumonitis, immunological deficiency syndrome
Papovavirus, SV40-like (urine only)	2	Associated with progressive multifocal leukoencephalopathy and immunosuppression

Because of the inadequacy of surveillance systems and methods, only a small percentage of enteric viral infections would be expected to be reported or investigated. The importance of low-level transmission is difficult to document because of the occurrence of large numbers of asymptomatic or subclinical cases characteristic of the enteric viruses. However, its importance in maintaining a disease in a community should not be overlooked.

Low level transmission of agents of viral gastroenteritis by water or food may play its most significant role in the spread of these agents between communities distant from each other. For example, viruses may be discharged by one community into a river which is used farther downstream as a water source by another community. Only a few individuals in the downstream community need contract the disease by bathing or water consumption to establish new foci of infection from which viruses can be spread by other routes (i.e. respiratory or fecal-oral). In most cases it is very difficult to identify the point source of viruses that are detected at any distance downstream from their discharge, but sometimes it has been done and the results show that viruses can travel miles from the source of contamination and still be infectious.

Summary

Over 120 different viruses are excreted in human feces and urine, find their way into sewage, and become common water pollutants. A wide spectrum of diseases including paralysis, meningitis, respiratory disease, epidemic vomiting and diarrhea, myocarditis, congenital heart anomalies, infectious hepatitis and eye infections can be caused by these viruses. Although poliovirus, coxsackievirus A and B, and echoviruses have been the most studied of the enteric viruses, they have not been shown to be involved in waterborne outbreaks.

Hepatitis A virus, rotavirus and Norwalk-like agents have been implicated in several waterborne disease outbreaks. Two types of non-A and non-B hepatitis viruses have been the cause of waterborne hepatitis. Two distinct types of enteric adenoviruses, AD40 and AD41, have been recognized as a cause of acute gastroenteritis in children.

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3 Monitoring for viruses in wastewater and water

This chapter explains why viral monitoring of raw domestic wastewater, treated effluents, surface and drinking water is necessary; describes the methods used to concentrate small numbers of viruses from large volumes of water and wastewater; presents data for the actual numbers of viruses isolated in different geographical areas, and discusses the usefulness and limitations of immunological methods for the detection of enteric viruses that are difficult to cultivate in cell cultures.

Why water should be monitored for viral contamination

Monitoring sewage effluents and raw water sources for the presence of human enteric viruses will provide baseline data against which exceptional circumstances, such as may occur during an epidemic, can be compared. In addition, an established system of monitoring in any country will avoid the delay that would be experienced if facilities are provided under emergency in response to sudden outbreaks. The monitoring of wastewater may provide an early indication of viral infection in the community, although the presence of a virus in sewage alone does not necessarily indicate a public health risk. Nevertheless, the detection of virus types not previously observed, or a marked increase in the numbers of a specific virus, may indicate the spread of a virus infection in the community not yet apparent from clinical cases.

During an outbreak caused by an enteric virus, sampling should be aimed at determining whether or not the water supply is responsible for the spread of disease. Samples of raw water, fully treated water and tap water should be examined. It is especially important to test drinking water at the consumer's end when the integrity of the distribution system is suspect, as is often the situation in developing countries with intermittent water supply.

The detection of any enteric virus, even of low pathogenicity, in drinking water is a warning that the water might contain viruses of higher pathogenicity.

Although virological standards for potable supplies are not yet in general application, routine monitoring of drinking water for the presence of viruses can provide an additional degree of public health protection. Such routine monitoring would be particularly justified in cases in which large urban centers use as their source of raw water heavily polluted rivers that carry a significant flow of sewage. Regular virus monitoring should be mandatory in every case of direct reuse of wastewater for potable supplies.

Methods for monitoring viral contamination

The choice of a suitable method for detecting viruses in water and wastewater is dependent on the volume of the sample that must be tested. This volume is dictated by the quantity of virus expected, which is governed by factors such as the degree of treatment of water and wastewater, the density of input viruses in sewage, the prevalence of viral diseases in the community, the season of the year, and the *per capita* consumption of water. Samples may range from a liter of raw sewage to about 20 liters of sewage effluents and polluted surface water, and to about 400 liters or more of treated water.

A number of techniques can be used to detect the presence of viruses. No one method can be applied to all types of virus, and the types of virus which are isolated will therefore depend to some extent on the technique used. Most current investigations are limited to those viruses which are easily grown in tissue cultures. Other viruses require more specialized techniques (e.g. Norwalk viruses are studied by means of immune electron microscopy and radioimmunoassay). Some viruses, such as that of hepatitis A, take at least 4 to 6 weeks to grow in tissue culture. The results obtained from the usual virological examination can therefore represent only a small fraction of the total virus content. The method most generally used consists essentially of three parts: concentration, culture and identification. The culture and identification techniques are similar to those used in other fields of virology, i.e. the virus obtained after concentration of samples is inoculated into cultures of living cells (sometimes test mice are used) and the isolated viruses are identified by means of specific antisera.

Cell cultures sensitive to enteric virus replication and most widely used are primary monkey kidney cells (PMK), two continuous lines, Buffalo green monkey (BGM) cells and human rhabdomyosarcoma (RD) cells. Environmental sample concentrates are inoculated into tube cultures which are fed with maintenance medium and incubated at 37 °C up to two weeks, during which they are examined microscopically for viral cytopathic effects (CPE). If replicate tubes are inoculated (four tubes per dilution) with falling dilutions of virus, it is possible to estimate semiquantitatively the number of infectious virus particles in the sample. This number is based on the sample dilution that causes 50% of the cultures to show viral CPE. This is termed 'tissue culture infective dose 50' ($TCID_{50}$).

Another approach for measuring virus infectivity in monolayer cultures is the plaque assay. This is a simple and rapid method to determine the quantity of virus in a sample. The basic plaque assay for entero- and reoviruses utilizes a monolayer of cells grown in a glass or plastic culture vessel. The growth medium is decanted from the monolayer and a small volume of virus sample (0.2 ml) is adsorbed to a monolayer of $25cm^2$ at 37 °C for 1 h. An overlay containing maintenance medium, serum, neutral red and agar is added to restrict virus dispersion, causing localized CPE and a plaque to form (Hsiung and Melnick, 1955). Plaque assays are more quantitative than tube cultures since one infectious unit produces a single plaque. Another advantage is that each plaque represents one virus type so that different viruses in a single sample can be recovered and identified.

Viruses isolated in cell cultures are identified by a virus neutralization test. The test consists of mixing the virus isolate with the specific antibody for 1 h at 37 °C.

The virus is then assayed for infectivity in cell culture. Neutralization can be determined by a reduction in the titer of the virus causing CPE or by a large (90% or more) reduction in number of plaques. Alternatively, Lim and Benyesh-Melnick (1960) have used pools of antisera to the most common enteroviruses for identification by neutralization. Comparison of the neutralization results of a virus isolate tested against seven pools of antisera containing different combinations will identify any one of 42 different enteroviruses (Melnick *et al.*, 1973).

It is in the field of virus concentration that special techniques have been developed for water sampling. Several excellent reviews are available that deal with virus concentration and methodology (Hill *et al.*, 1971; Sobsey, 1976; Wallis *et al.*, 1979; Goyal and Gerba, 1982). Early development of concentration methods included adsorption and/or entrapment on gauze pads, adsorption to inorganic salts, clay minerals, polyelectrolytes, protamine sulphate, soluble alginate filters, iron oxide, hydroextraction and two-phase polymer separation. In general, these methods have a limited application because they can only be used with small volumes of water, cannot be used with highly turbid water and cannot easily be used in the field. The technique most generally used for a variety of waters is the membrane filter methodology.

Membrane filters of different chemical compositions bearing different trade names have been used in the adsorption and recovery of enteric viruses from water and wastewater. Flat-disk membrane filters of 13 to 293 mm in diameter with pore sizes ranging from 8 μm to 0.22 μm have been evaluated for virus retention in earlier studies. Membranes of larger pore sizes were generally used as prefilters to remove suspended particulates from the sample, while filters with average pore diameters of 0.45 μm were used for virus retention. Metcalf, in the early 1960s, discovered that influenza virus could be retained on Millipore membranes despite the fact that the membrane porosities greatly exceeded the diameter of the virus. Viruses were recovered by grinding the membranes in a mortar with a pestle and resuspending the pulp. Cliver reported that enteroviruses (polio, coxsackie, echo) suspended in tap water, phosphate-buffered saline or deionized water could be adsorbed to Millipore membranes and the virus recovered from the filter surface by treatment with phosphate-buffered saline supplemented with 30% chicken serum. The first application of cellulose nitrate membranes for concentrating viruses from sewage was introduced by Wallis and Melnick. Interfering organic matter was removed by treatment of sewage with anionic resins prior to filtration. Salts (50 mM $MgCl_2$) were then added to the resin-treated sewage to enhance virus adsorption to the filter surface. The viruses were eluted by homogenization of the filter in a medium containing 10% fetal bovine serum. Using this technique, a total of 2795 enterovirus isolates were obtained from a relatively few 1-gallon (3.78-l) samples of sewage during a 7-month period in 1966. Rao and colleagues first demonstrated that adjustment of sample pH to 3.0 was necessary to obtain maximum adsorption of enteroviruses to membrane filters from raw and treated sewage. At this low pH, interfering organic components in sewage passed through the filter leaving the sites on the filter surface available for virus adsorption. Blending of the sewage sample facilitated release of viruses associated with solids. A 30 min retention of the eluent (3% beef extract, pH 8) on the membrane filter enhanced virus recovery. Recovery of poliovirus seeded into raw sewage ranged from 88 to 97%. Using this method, a 1-year monitoring (1970–1) of 80 ml volumes of raw sewage at Nagpur,

India, yielded 1050 to 11,575 PFU of virus/liter.

An important contribution by Wallis, Henderson and Melnick in 1972 paved the way to process large volumes of water for the determination of enterovirus contamination. They observed that trivalent salts (0.5mM $AlCl_3$) could effectively enhance viral adsorption when used at only 1% the concentration required for divalent salts (50mM $MgCl_2$). Thus, only 0.05 kg of aluminum chloride was needed for a 100-gallon sample of water, while the amount of magnesium chloride required was 4 kg. In that report, they also described a second-step concentration procedure (reconcentration) whereby viruses recovered from filters with 0.05 M glycine, pH 11.5, were readsorbed at acid pH to smaller diameter membranes and eluted with smaller volumes of high pH glycine, thus greatly reducing the amount of eluate to be assayed.

Because of the limited surface area of the flat nitrocellulose adsorbent filters (a 142 mm diameter filter has a surface area of 155 cm^2), maximum flow rates of only 11.3 l per min could be achieved with tap water. When turbid seawater was used, average flow rates dropped to 1.89 to 3.78 l per min when 189-l samples had to be processed. To overcome this limitation, in 1977 Farrah and colleagues working in the Wallis/Melnick laboratory tested a variety of filters and found that Filterite fiberglass filters (duofine series) were far less easily clogged and adsorbed greater than 90% of poliovirus added to tap water at pH 3.5. The Filterite filters are manufactured as 10 inch (25.4 cm) long, pleated cartridges (Fig. 2) and have a surface area of 2800 cm^2. A maximum flow rate of 37.8 liters of tap water per minute has been obtained. Using pleated filters in combination with organic

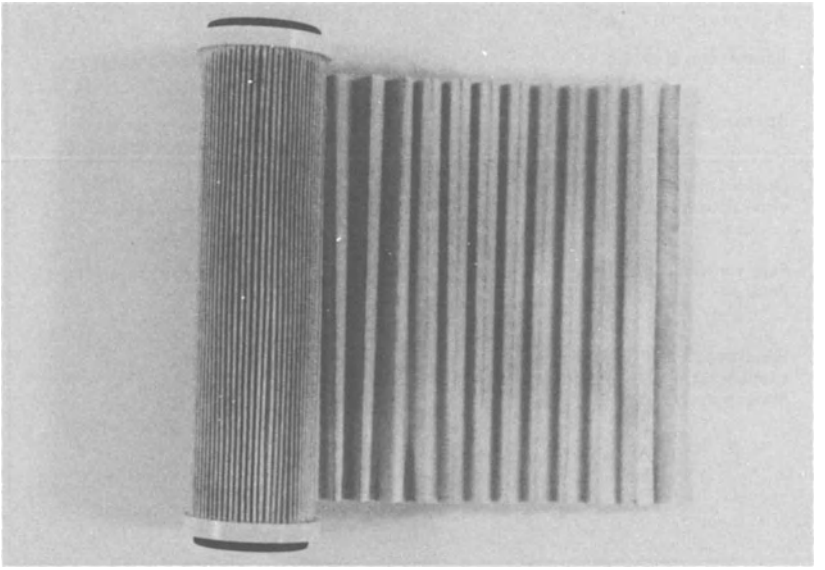

Fig. 2 Filterite filter cartridge (partially unfolded to show the pleated nature).

flocculation, Hill and coworkers detected poliovirus at input levels of ≥3 PFU per 380 liters with an overall reliability of 95% when 1900 liters of tap water were examined. The procedure is briefly outlined in Fig. 3(A).

Membranes made of cellulose nitrate, cellulose acetate and fiberglass are negatively charged. Efficient virus adsorption to such filters requires the addition of acid and salts to water and wastewater, which is rather inconvenient in field

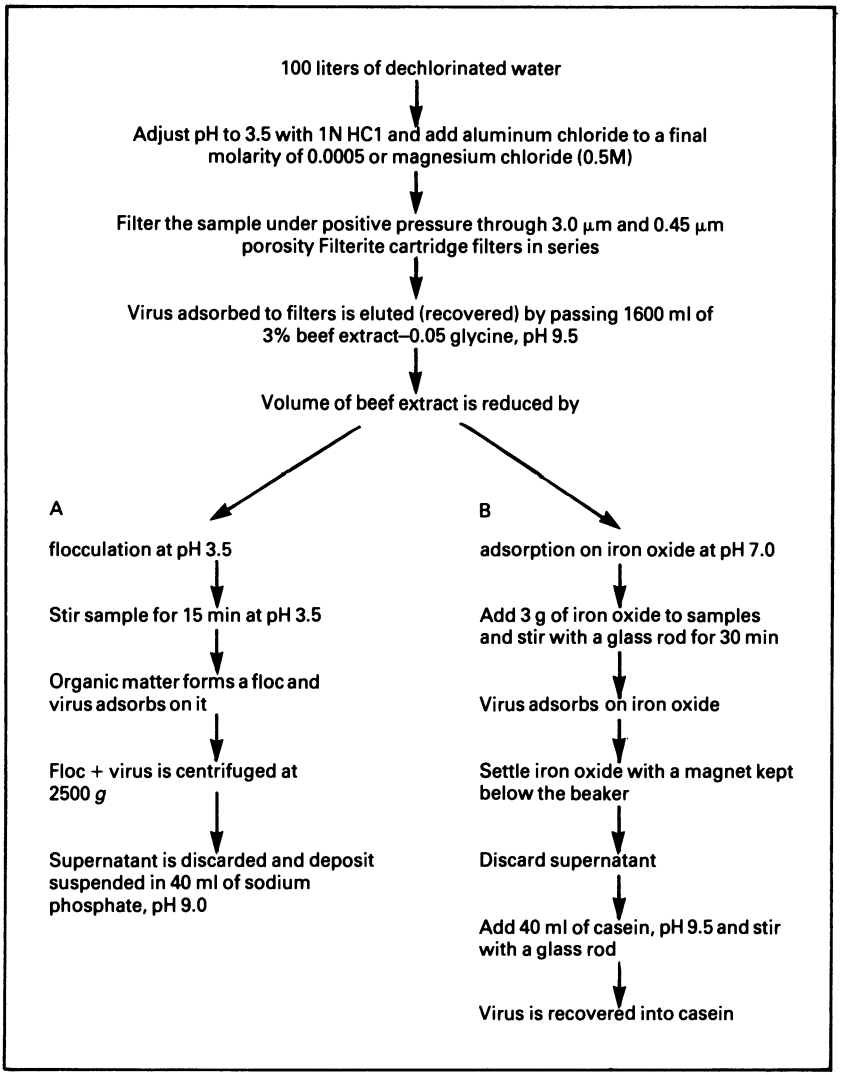

Fig. 3 Virus recovery from water using Filterite filters.

studies. This stimulated a quest for alternative filters that did not require lowering the pH and addition of salts to the water sample. Sobsey and Jones (1979) identified a new type of filter composed of cellulose–diatomaceous earth–'charge modified' resin mixtures subsequently referred to as Zeta plus filters that are positively charged. It was originally anticipated that such filters could be used to process water at ambient pH to obtain virus adsorption. Preliminary studies indicated that poliovirus seeded into tap water was adsorbed on a 47 mm dia. Zetaplus filter with an efficiency of 98 to 99% in the pH range of 5.5 to 7.5. Recovery of virus was 63 to 69%. However, at a pH of 8.5, only 26% of virus was adsorbed on the filter and only 1% of the total input virus was recovered. There is a wide variation in the pH of tap water in different cities. For example, the median value of ambient pH of tap water in Cincinnati during a survey was 8.4 (range 8.0 to 9.3) and in another study, the pH range of Houston tap water was 8.2 to 8.8. In such instances, a pH adjustment to near neutrality was essential to process water by zetaplus filters for efficient virus adsorption.

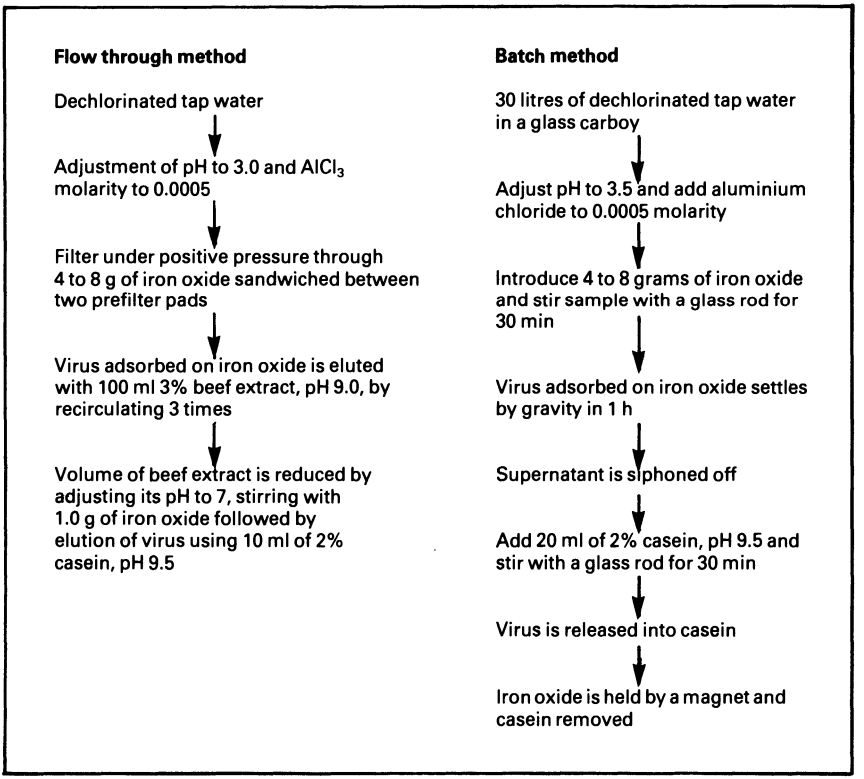

Fig. 4 Scheme for concentration of viruses from water with iron oxide. (Modified from Rao, V.C., Waghmare, S.V. and Lakhe, S.B. (1981). *Applied and Environmental Microbiology* 42: 421–6.)

Membrane filter technology, with all the associated filtration equipment and pressure pumps, is costly for virus monitoring of environmental waters. Developing countries can ill afford such expensive techniques. In countries where drinking water samples of up to 50 l are adequate for virus detection due to occurrence of higher numbers of viruses, the iron oxide method is appropriate and useful. The method is simple, does not require any special apparatus, and is efficient. Iron oxide can be prepared in any laboratory or commercially available material, which is inexpensive, can be used. (It costs $0.015 per gram of iron oxide as compared to $15 for a Filterite cartridge and $50 for a Zetaplus cartridge) . Recovery of poliovirus seeded in tap water ranged from 60 to 80%. Two approaches for the iron oxide method used in field studies by Rao and colleagues are outlined in Fig. 4. The flow-through method can be used in laboratories with facilities for pressure filtration. Otherwise the batch method is equally useful. Using the iron oxide adsorption-elution method, Rao and colleagues reported 60 to 80% recovery of poliovirus seeded into tap water of 1 to 30 l. In addition, a survey of drinking water in Nagpur, India resulted in the isolation of virus in 7 out of 50 samples collected from the distribution system.

Second-step concentration (reconcentration) Primary eluates obtained from membrane filters or iron oxide after processing a large volume of water must be reduced to smaller test volumes so they can be handled economically when viruses are present in the water sample. The volume of such eluates is usually 1 to 2 l and must be reduced to 20 to 40 ml before it can be assayed. Some of the virus concentration methods developed for small volumes of water were adapted for second-step concentration, also referred to as reconcentration. Techniques used for this purpose include two-phase separation, hydroextraction, precipitation with inorganic salts, ultracentrifugation, readsorption to and elution from smaller diameter membrane filters, organic flocculation, and iron oxide adsorption–elution. Reviews by Wallis *et. al.* (1979) and Goyal and Gerba (1982) contain a description of these methods, the associated problems, and their relative efficiencies.

Methods evaluation During 1981–82, six laboratories actively involved in water virology research in the USA participated in a methods evaluation study conducted under the auspices of the American Society of Testing and Materials (ASTM) Committee on Viruses in the Aquatic Environment—Task Force on Drinking Water. Each participant was asked to examine the viradel (virus adsorption–elution) method with cartridge-type negatively charged Filterite filters for virus adsorption and either organic flocculation or aluminum hydroxide-hydroextraction for virus reconcentration. A modification of this procedure, which was originally developed in 1972 by Wallis and Melnick, was introduced as a tentative method in the 14th and 15th editions of *Standard Methods for the Examination of Water and Wastewater.* In addition, the participants were encouraged to examine other methods that had been reported to be useful and reliable. The other methods tested for reconcentration included use of iron oxide and membrane disks. Details of the procedures and test results have been published (Melnick *et al.*, 1984). Considerable variation was noted in the quantity of virus recovered from four 100 l samples of dechlorinated tap water seeded with low (350 to 860 PFU) and high (1837 to 4689 PFU) doses of poliovirus type 1.

Test results of the viradel-organic flocculation method indicated that the average percentage of virus recovered in low input experiments was 66%, with a range of 8 to 20% in two laboratories, 49 to 63% in three laboratories and 98% in one laboratory. For the high input experiments, two laboratories reported recoveries of 6 to 12% whereas four obtained 26 to 46%. Virus recoveries using the viradel-aluminum hydroxide-hydroextraction procedure were in the range of 9 to 34% for low input and 3 to 18% for the high input experiments. The average virus recoveries for the iron oxide and membrane disk procedures were 36% (range 21 to 64%) and 5% (range 3 to 9%), respectively. Possible differences in the quality of water in which the virus was seeded and in the quality of the eluents are some of the variables suspected. The Task Group decided that both the organic flocculation and iron oxide methods are viable approaches for reconcentration and suggested a further evaluation of these two procedures in a number of laboratories.

Virus isolation from sewage, and surface and drinking water

Raw sewage Quantitative estimates of different types of viruses in sewage and treated effluents are essential to evaluate the efficiency of different sewage treatment systems and to estimate the load of virus in treated effluents so that the magnitude of virus hazard associated with receiving waters, irrigated fields and fish culture ponds fertilized by sewage can be determined.

The frequency of virus isolation and quantity of virus recovered from sewage depends on the relative prevalence of infection, number of cases, carriers and the intensity of live poliovirus vaccine use in a community, as well as the efficiency of the method used for virus isolation. Additional factors have also been considered important. These include the time of the day and season of the year that the sample was collected and the water use within the community.

Enteric viral infections are common in children, especially those below five years of age. According to several studies, about 10% of the children under age 15 in the USA were found shedding viruses through the feces at any given time. In developing countries like India, it was found that on an average 45% of children in the age group 1 to 15 years were excreting viruses. Early studies indicated that the number of enteroviruses in raw sewage was proportional to the number of recognized cases in the community. In temperate climates, maximum recovery of viruses from sewage occurs in late summer and early fall. In recent years, with the advent of oral polio vaccination, the predominant enteric viruses isolated from sewage have been the vaccine-derived polioviruses, and these viruses may be found year-round without a dominant summer–fall peak. Other enteroviruses continue their usual seasonal variation. However, our studies in Houston show that rotaviruses are an exception with peak levels in sewage during winter and low numbers during summer; again the seasonal occurrence of rotaviral disease is followed. Development of quantitative methods for concentrating viruses from raw sewage provided data on the numbers of enteroviruses in sewage from different countries. Melnick and Gerba (1980) summarized some of the data. Concentration methods, of which there are a large variety, were used and the recovery efficiency varied substantially. A few investigators attempted to quantitate virus in sewage by direct inoculation of the unconcentrated sample into

the cell culture assay system. In Israel, Buras used such a technique on sewage samples from Haifa and reported the surprisingly high number of 10^6 viral units/liter during mid-summer. The weekly average for eleven consecutive months was about 174,000 units/liter. In the USA, Fannin and colleagues reported up to 440 viral units/liter in Chicago sewage and EPA virologists found in 1450 viral units/liter in a sample of Cincinnati sewage by direct inoculation. The same technique was used by Irving in Australia who reported up to 6850 infectious units of virus/liter in a one-year survey of raw sewage. The rest of the data were generated by a variety of concentration methods and the recovery efficiency of these methods vary. The average virus concentration in raw sewage in the USA would appear to be about 100 PFU per liter. Viral recoveries from sewage in other countries (e.g. Israel, India and South Africa) have generally been higher; average numbers reported were 122201, 3000, 56500 PFU/l, respectively. This finding may reflect (1) a less dilute sewage as a result of water conservation practices, and (2) a higher prevalence of enteric viral infections in tropical and developing countries. It is evident that a variety of virus concentration methods with different recovery efficiencies were used. Since no standardized methodology was used, a true comparison of the quantitative data on viruses in sewage in one area with another is not possible at present.

Since the introduction of the oral vaccine, polioviruses have become the most frequent viruses isolated. They are considered to be derived from the vaccine, and usually have vaccine-like markers. Even though they are considered to be non-pathogenic, they may have a higher capacity for growth in the spinal cord than the original, licensed vaccine virus. There are no estimates of the occurrence of wild polioviruses in raw sewage in urban areas in developed nations. In developing countries like India, it has been reported that poliovirus was the predominant virus present in Nagpur sewage, constituting nearly 80% of the isolates. Between 60% and 80% of these polioviruses appeared to be wild strains characterized by the temperature marker test. Peak numbers of enteroviruses in sewage are encountered in the rainy season (Rao *et al.*, 1978). This may be due to occurrence of 60% of paralytic poliomyelitis cases during the Indian rainy season and also to the fact that, when children defecate in open fields, virus dissemination by rain is faster, resulting in rapid infection of children with subsequent excretion of virus.

There are certain factors which are more important to evaluating the virus hazard in drinking water than the amount of virus present in raw sewage. These include (a) the efficiency of sewage treatment in virus removal, (2) the portion of wastewaters that receive treatment and (3) the degree to which wastewater constitutes the drinking water sources. It is anticipated that, in the near future, surface water entering treatment plants in the United States will not contain more than 25% of wastewater which has been subjected to at least secondary treatment. Therefore, the efficiency of secondary treatment in the removal of viruses from sewage becomes of primary importance in determining the waterborne transmission of viruses.

The term 'secondary treatment' is generally applied to the treatment of primary settled sewage by one of three biologically active processes: (1) trickling filtration, (2) activated sludge digestion, or (3) oxidation pond stabilization. The viral removal/inactivation potential of each treatment has been studied at the laboratory and pilot-scale level, as well as under full-scale field conditions and

these details are discussed in Chapter 4. The contribution of viruses to surface water by effluents from sewage treatment plants can possibly best be determined by focusing on the number of natural viruses found in these effluents rather than on a percent removal by the treatment process and theoretical calculations. In a study conducted by the sanitation districts of Los Angeles, viruses were recovered from 27 out of 60–80 liter samples of activated sludge effluent. The positive samples contained from 0.4 to 136 viral units/liter. Sagik and Sorber have studied the viral content of secondary effluents from three systems in Texas communities. Means of 5 and 179 PFU/l were determined for two trickling filter systems and a mean of 3 PFU/l for the oxidation pond system. Observations from these recent studies have yielded virus levels consistent with previously reported findings from unchlorinated secondary effluents. A year-long period of rotavirus monitoring in the chlorinated effluent of an activated sludge plant in Houston, Texas indicated a range of 0 to 25 fluorescent foci/liter (Rao *et al.*, 1985). In India, 5 to 60 PFU/l of enteroviruses were discharged in the unchlorinated effluent of an activated sludge plant in Bombay and full-scale stabilization ponds near Nagpur (Rao *et al.*, 1978).

Rivers and lakes In view of the increasing demand for water, some countries are seriously considering the intentional recycling of wastewater containing a heavy load of viruses. Also, when treated or partially treated sewage is discharged into surface water which flows downstream for further use, there may not be sufficient time for virus elimination by various natural purification processes such as dilution, adsorption, sedimentation and biological inactivation.

Such unintentional recycling of polluted water occurs in many areas. In some places the load of sewage in surface waters has been so considerable that viruses could be detected even in small volumes of water.

Earlier studies on virus detection in fresh water rivers and lakes were only qualitative and involved the use of a gauze pad suspended in flowing water for varying periods of time (usually 72 h or more), hopefully allowing virus particles to be trapped on the gauze. The pad and its fluid content were then treated with various eluents to release the viruses. Results of these studies indicate that the percentage of river water samples found to contain viruses varied from 0 to 100%, with an overall average isolation rate of 31%. It should be remembered that the success of isolating a virus depends on the numbers of viruses discharged in sewage effluents, the distance of the sampling point from effluent discharge, the degree of dilution in the receiving water, the settling characteristics of the suspended solids on which viruses are adsorbed, and the biological antagonisms. Several of these qualitative studies have been extended over a period of several years and were indicative of the widespread occurrence of enteric viruses in polluted fresh water. In one study, viruses were isolated from river water at least 10 km from the nearest source of pollution.

The development of methods for the concentration of viruses from large volumes of water in the late 1960s and early 1970s has advanced our ability to quantitate and detect enteric viruses in rivers and lakes. Virus has been isolated from polluted lakes and rivers in Canada, England, France, Germany, Mexico and the United States. Virus recovery methods used in generating the data were different and their effectiveness is highly variable. Under these circumstances, comparison of numbers of viruses is difficult. Virus numbers detected should be considered as minimum values. A wide variety of strains of polioviruses,

coxsackie viruses, echoviruses, reoviruses and adenoviruses were recovered. Virus numbers reported from surface waters varied from 0.06 to 620 PFU/l. Occurrence of high concentrations of enteric viruses in surface water and their utilization for recreation or as a source of drinking water should be of potential significance fo users of these waters.

Drinking water The quality of water consumed in different parts of the world varies considerably. While conventionally treated and disinfected water is supplied in major cities of the western world, untreated water from canals, streams and rivers is often the source of drinking water in small towns and villages in many developing countries. The virological quality of these waters is unknown. Thus, data concerning the presence of viruses in drinking water are available only for conventionally purified and chlorinated water, and are summarized in Table 2.

In developing countries, the discharge of raw domestic wastes containing high numbers of human enteric viruses into water courses, consumption of untreated water from canals, streams and wells in villages, and cross-contamination of water in the distribution system because of intermittent water supply in urban areas may expose people to viruses to a greater degree. These conditions increase the need for drinking water to be monitored for viral contamination.

However, data from such countries is meager. Recent work in India has been designed to examine whether or not viruses were present in water treated by conventional procedures and also to evaluate the virological quality of water as delivered to the consumer (Rao *et al.*, 1978). Isolation of human enteroviruses in some samples collected from the distribution system indicated the possibility of the introduction of contaminated ground water through leaks in the distribution pipes at a location not far from the sampling point. In spite of the presence of 0.2 to 0.8 mg/l of total residual chlorine, the occurrence of viruses in certain samples indicated that chlorination had been inadequate. Studies carried out during outbreaks of viral hepatitis A in Yeotmal, Kamptee (small towns near Nagpur) and Bombay resulted in the isolation of other viruses at concentrations of 1 to 7 in 12 to 40 l samples of drinking water collected from the distribution system. Routine monitoring of drinking water from taps during interepidemic periods in Nagpur yielded 1 to 7 PFU from 30 to 60 l of water (of 50 samples examined, 7 contained viruses). No virus was detected in 60 l of finished water at the treatment plant, which is a conventional one.

Gaudalajara, Mexico provided an example of virus occurrence in finished water at the treatment plant and also in tap waters in the distribution system (Deetz *et al.*, 1984). In an initial examination during the rainy season in August 1978, rotavirus was detected in 10 out of 10 twenty-liter drinking water samples and coxsackie B4 or B6 virus in 5 out of 10 samples. In a larger survey during the dry season in December 1979, rotavirus was recovered from 3 and enteric viruses from 8 out of 21 samples. Significantly, enteric viruses were detected in drinking water meeting standards considered adequate to ensure safety from infectious agents, i.e. 0 coliforms/100 ml, turbidity less than 1.0 NTU and a free chlorine level greater than 0.5 mg/l. A recent investigation of finished water from the Villalba plant in Puerto Rico by Gerba and coworkers yielded enteroviruses (0.4 MPN/100 l) and rotaviruses (26 foci/100 l) although the turbidity of water was less than 1 NTU and free chlorine residual was 1.5 mg/l. Coxsackie viruses B1, B2, B3, B4, B6 and adenovirus have been recovered from 1 liter samples of tap water in

Wuhan, China (Zhang *et al.* 1984). All the samples taken were positive for virus, but the authors did not provide any data on the type of water treatment.

Among developed countries, in a study during the 1960s in Paris, Coin and colleagues used gauze pads for virus sampling and isolated polioviruses, coxsackieviruses and echoviruses from 9% of drinking water samples. In north-east France, coxsackievirus B5 and reoviruses were found in urban drinking water treated by conventional methods and disinfected with chlorine or

Table 2 Reported recoveries of human enteric viruses from treated drinking water*

Country	Date	No. samples positive/ No. samples negative	Virus type isolated
France	1960–62	48/553†	Polio and other enteroviruses
	1961–63	2/25	Reovirus, enterovirus
India	1978	19/74	Not identified
Israel	1978	12/18†	Polio, echo
Mexico	1978	8/11	Coxsackie B
	1978	11/11	rotavirus
Romania	1962–71	2/65	Coxsackie A
	1972–77	8/220	polio, coxsackie A
South	1971	2/NR	Reo, entero
Africa	NR	1/464	Reo?
USA:			
Mass.	1969–71	7/64	Reo, echo, polio
Fla.	1975	1/10†	Echo
Virg.	1975	4/12	Polio
Virg.	1976	1/42	Polio
Tx.	1980	3/6†	Coxsackie B, hepatitis A
USSR	1968–71	9/64	Coxsackie A and B, echo, polio
Canada	1985	11/155	Polio, coxsackie B, echo

* Treatment in general was coagulation, rapid sand filtration and disinfection.
† Water only disinfected.
NR= Not reported.
Adapted from Melnick, J. L. and Gerba, C. P. (1982). Viruses in surface and drinking waters. *Environment International* 7: 3–7; Payment, P., Trudel, M. and Plante, R. (1985). Elimination of viruses and indicator bacteria at each step of treatment during preparation of drinking water at seven water treatment plants. *Applied and Environmental Microbiology* 49: 1418–28

ozone. Later, Foliguet *et al.* in 1981 reported the presence of enteroviruses in 11% of drinking water samples containing residual chlorine. More recently, Festy monitored Paris drinking water disinfected by either chlorine or ozone and reported isolation of poliovirus 1, 2, 3, coxsackie virus A, coxsackievirus B4 and echovirus 11. Six of 819 samples tested with the gauze pad procedure were positive to virus.

Virus presence in drinking water in the USA was first reported in 1970 in 10% of samples of conventionally treated water in a New England community. Again, in 1977 poliovirus 1 was isolated from 5 out of 129 samples of treated drinking water containing 1.3 to 1.7 mg/l total chlorine. In the summer of 1980 a large outbreak of suspected viral gastroenteritis involved approximately 79% of 10,000 persons living in Georgetown, Texas, and virus studies were carried out at Baylor College of Medicine (Hejkal *et al.*, 1982). During the outbreak coxsackie viruses B2 and B3 were isolated from wells serving the city water utility. Free total chlorine content of the well water was 0.8 mg/l. Coxsackie virus B3 was also isolated from one sample of chlorinated tap water. An outbreak of 36 cases of hepatitis type A followed 4 weeks after the gastroenteritis outbreak. Hepatitis A antigen was detected both in the community's sewage and in one of the wells serving the city's water supply. This is the first instance in which hepatitis virus was actually detected in a water supply 1 month prior to the outbreak.

In Canada, Payment, Trudel and Plante (1985) monitored the virological quality of finished water from seven conventional treatment plants in Montreal for 12 months and reported the recovery of poliovirus 3 and coxsackie virus B5 from five of the plants. Treated water at these plants was disinfected with ozone and chlorine. Viruses were detected in 7% (11 out of 155) of the finished water samples (1000 liters) at an average density of 0.0006 viral units/l, the highest virus density measured being 0.02 viral units/l. It is significant that the finished water was essentially free of indicator bacteria measured in 1-l volumes.

In South Africa, Nupen recovered viruses from five out of one hundred 10 l samples of conventionally treated drinking water. Recently Tyler examined 553 samples (20 l per sample) of drinking water in England during 1979 and 1982. 16% of samples were positive to virus and contained 1 PFU/20 l. Virus types detected were poliovirus 1, 2, 3, coxsackie virus B3 and B5, and echovirus 7, 11 and 22. 60% of the isolates were polioviruses. Viruses were found in drinking water with a residual chlorine concentration in excess of 0.3 mg/l. In the USSR, Kazantseva and Drozdov reported the isolation of poliovirus 1 and 2 and echovirus 1 and 7 in 17% of drinking water samples. In Romania, Nestor and collaborators isolated coxsackie viruses in 2 out of 65 drinking water samples treated by coagulation, sand filtration and disinfection.

Indicators for viruses

Isolation of the viruses themselves is the most appropriate means of virus detection. However, at present, widespread direct testing for virus is hampered by such factors as the long time required to obtain test results, variations in the precision and accuracy of detecting various virus types, the shortage of competent personnel, and the high cost of viral analysis.

Consequently, it is desirable to identify, if possible, reliable indicator organisms and analytical methods to serve as surrogates for the presence of

viruses. Biological and chemical indicators are often used in public health. For example, public health and environmental health practitioners currently use fecal coliform bacteria as an indicator organism for fecal bacterial pathogens.

No universal indicator presently exists that is suitable for detection of all viruses. Virus occurrence in the environment is sporadic and there are differences between viruses and proposed indicators in survival capabilities, ease of detection, responses to environmental stress, and susceptibility to disinfection.

Indicators may be drawn from bacterial, yeast and viral groups. Candidate bacterial indicators include the coliform group (total and fecal), fecal streptococci, anaerobic spore formers (clostridia species) and non-spore formers (bifido-bacteria), acid fast forms, and standard total plate counts. Candidate viral indicators include either bacteriophages or selected enteric viruses.

Conceptually, any of the above may serve as surrogate indicators for viruses. Certain candidate indicators may prove more useful than others in a given situation, such as in the examination of sludges, soils, leachates or water. The choice of the surrogate virus indicator should be determined by the nature of the environmental sample to be tested.

Historically, coliforms have gained wide acceptance as indicators of fecal pollution. The acceptability of members of the coliform group as indicators of the potential presence of viruses has been based upon the argument that there are many more coliform bacteria present in sewage than viruses. While some general relationship may exist between numbers of indicator bacteria and viruses in grossly polluted waters, discrepancies sometimes occur in high quality waters, perhaps due to differences in sample sizes used in test for viruses and coliform bacteria (e.g. 400 l vs 50 to 100 ml, respectively for drinking water) or perhaps due to the lack of nutrients to sustain bacterial life.

There are other limitations on the use of bacterial indicators. Viruses are generally more resistant to disinfection than coliform bacteria. Moreover, the greater persistence of viruses in polluted water and sediment may lead to a further change in the ratio of surviving bacteria and viruses.

In addition, some members of the total coliform group such as *Klebsiella* may manifest growth outside the excreting host, which is impossible for viruses in an extracellular environment.

Fecal streptococci biotypes are also ubiquitous in aquatic environments. Since fecal streptococci are more resistant to disinfection than coliform bacteria, some strains of fecal streptococci persist for days in irrigation waters, sludges and landfill leachates. Since fecal streptococci do not multiply in the environment, they would appear in some circumstances *a priori* to be more suitable indicators of enteric viruses than fecal coliforms (e.g. in sludges).

The spore-forming clostridia have the disadvantage of much greater persistence than other bacterial indicators and viruses in the environment. Consequently, at best, certain clostridia may be used as tracers of remote pollution rather than as indicators of viruses.

Although some species of bifidobacteria are specifically associated with human fecal pollution and although they are less likely to regrow in the environment, they may be subject to environmental stresses, especially the presence of oxygen. Bifidobacteria are unsuitable as indicators because they are difficult to detect and because their relationship to enteric viruses is unknown.

Standard bacterial total plate counts have been suggested as an indicator of the

quality of reclaimed water. However, only limited information exists about the suitability of this heterogeneous group of bacteria as an indicator of viruses.

Coliphages such as f2, MS2 and members of the T series may have a restricted, yet useful, indicator function. These phages may serve as laboratory or field models to assess the rate or extent of virus removal in water and wastewater treatment plants, and may possibly be used as tracers. Two aspects of this work need further study. One concerns the ecological relationship that exists between phages and bacterial host cell, (i.e. multiplication of phages in the test samples). The second concerns the relationship between the rate of survival of phages and enteric viruses. Comparative studies of the survival of phages and enteric viruses have yielded conflicting results that cast further doubt on the usefulness of phages as indicators of enteric viruses.

A vaccine strain of poliovirus (type 1) has been suggested as a potential indicator for other enteric viruses. This vaccine strain is shed in large numbers by vaccinated individuals, is relatively safe to handle (in seeding studies), and may be more readily detected in environmental samples than wild enteric viruses. One of the most serious objections to the use of a single virus indicator, such as poliovirus type 1, is that neither poliovirus type 1 nor any other enteric virus is always found in fecal samples.

Standards for viruses in drinking water

Before legal standards for acceptable virus numbers in drinking water are set, it would be desirable to know the concentration of each enteric virus required to produce infection when present in drinking water. However, it is unreasonable to expect that we shall ever have a direct answer to this question. At our present state of knowledge, the alternative is to monitor for viruses under different circumstances to obtain the information base on which to set criteria for safe water and then to establish standards to meet these criteria.

For setting standards we need uniform methods; otherwise, the standards cannot be monitored. To establish such standard methods, certain criteria have to be met: determination of the limits of accuracy, and reproducibility of tests in the different laboratories and indeed in a single laboratory.

Can the detection and measurement of other agents be used for setting virus standards for safe water? We already knew that the absence of bacteria cannot serve as an indication of the absence of viruses. The use of bacteriophage as an indicator of the presence of human viruses has been suggested, but the evidence is too meager to say whether their measurement may serve as a criterion on which a standard can be based.

At present, emphasis should be placed on seeking uniformity in methods of concentrating, testing, plaquing and indentifying viruses that are present in water. To establish the criteria that need to be met, a round-robin testing program has been undertaken in the USA and final results are awaited. It would be most desirable if a comparative testing of methods could be initiated in different countries under the direction of the World Health Organization. Out of such a program would come a proper evaluation of current methods that should lead to the promulgation of a standard. The reader should consult the recent recommendation made in this regard by a Committee of Experts convened by the US Environmental Protection Agency (1983).

Future developments in virus detection

Viruses recovered from water and wastewater by detection techniques that depend upon demonstration of cytopathic effects (CPE) in cell culture have been restricted to viruses able to replicate in the cultures used. The majority of isolates have been enteroviruses: polioviruses, group A or B coxsackie viruses, or echoviruses. Reoviruses and adenoviruses have been recovered also.

Enteric viruses such as rotaviruses, Norwalk virus and hepatitis A virus present in environmental samples are difficult to cultivate by conventional cell culture procedures. The advent of solid phase radioimmunoassay (RIA) and enzyme immunoassay (EIA) tests, which use only 50 to 100 μl of test sample, has revolutionized diagnostic medical virology procedures in clinical laboratories. Direct extrapolation of tests from clinical to environmental samples cannot be made due to important differences between virus quantities that exist in the two types of samples. The quantity of virus in clinical samples usually exceeds the 10^5 to 10^6 physical particles of virus needed for recognition of a positive test. Environmental samples contain very low numbers of viruses. Nevertheless, current developments in immunological procedures including the use of monoclonal antibodies may improve the sensitivity of some of these tests for rapid detection of rota, Norwalk and hepatitis A viruses in environmental samples.

Brief descriptions of some of the immunoassays including immunofluorescence, immunoperoxidase technique, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), radioimmunofocusassay (RIFA) or nucleic acid hybridization technique are given here.

Identification of viruses by immunofluorescence (IF) and immunoperoxidase techniques The basic procedure consists of staining viral-specific antigens present in infected cells with a specific antibody conjugated with a fluorescent dye, fluorescein isothiocyanate. Stained cells fluoresce when observed under ultraviolet light. IF tests are rapid and virus-infected cells can be stained as early as 6 to 9 h post infection for polio and rotaviruses while HAV needs at least 7 days for detection. The test detects infectious virus particles and is sensitive enough to detect one such infectious unit as a single focus of fluorescing cells. General procedure for detecting rotavirus or hepatitis A virus is outlined in Fig. 5.

Immunoperoxidase (IP) procedure This immunocytochemical method relies on an enzyme–substrate reaction, the product of which stains the infected cells. In this procedure, the enzyme (horseradish peroxidase) is conjugated to the virus specific antibody molecules with glutaraldehyde. The enzyme is then bound to the virus-infected cells by the antigen–antibody reaction. The substrate (diaminobenzidine-hydrogen peroxide) is added, and the substrate reacts with the enzyme producing a dark reaction product in the virus infected cell. Virus infected cells, stained by IP, appear significantly darker than surrounding cells when observed through a light microscope. This test is 100 to 1000 times more sensitive than immunofluorescence.

Enzyme immunoassay (EIA) The two most commonly used assay systems for the detection of antigens in clinical specimens are so-called direct and indirect assays.

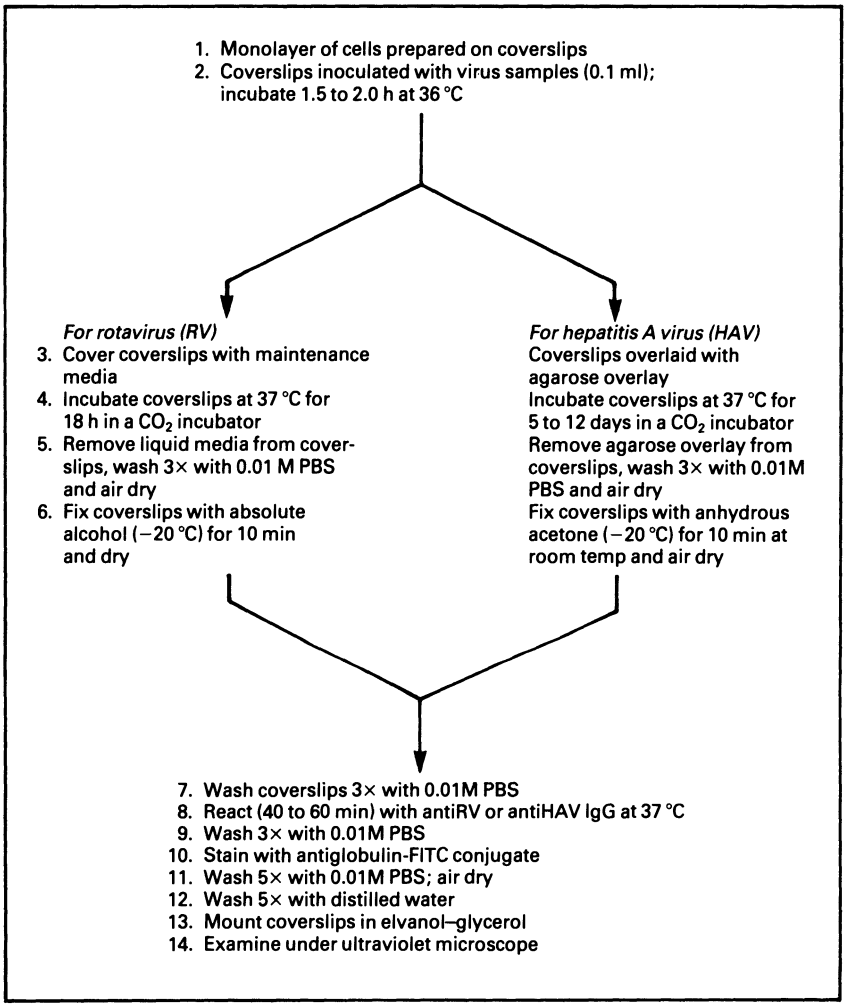

Fig. 5 General procedure for immunofluorescence test for detecting rotavirus and hepatitis A virus.

Direct EIA, which is analogous to direct immunofluorescence assays, utilizes an enzyme directly linked with the antiviral antibody (Fig. 6). Indirect EIA, like indirect immunofluorescence assays, utilizes unlabeled antibody. The unlabeled antibody is measured by the use of an enzyme-labeled antiglobulin which reacts with it. Direct EIA offers the advantage that it requires one less incubation step. In addition, it can be performed with a single antiviral reagent. However, it suffers from the disadvantages that each antiviral reagent must be linked with an enzyme. This is inconvenient if the laboratory wishes to perform a number of different enzyme immunoassays. Indirect assays, on the other hand, do not

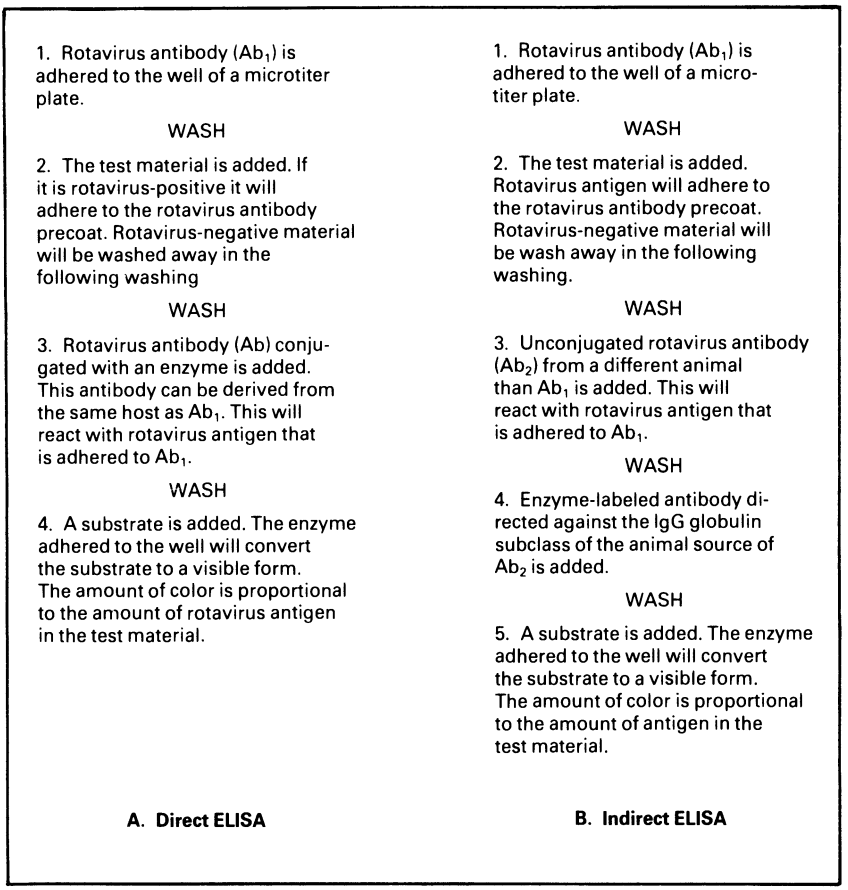

Fig. 6 Direct and indirect ELISA for rotavirus antigen measurement.

require the direct enzyme labeling of the antiviral reagent but rather use enzyme-labeled antiglobulins directed against a species of immunoglobulins, which are available commercially. As in immunofluorescence systems, indirect assays are somewhat more sensitive than direct assays, presumably because a single molecule of antiviral antibody can react with a number of molecules of labeled antiglobulin. However, indirect assays have one disadvantage in that antibody from two different animal species must be utilized to prevent the nonspecific binding of the antiglobulin to the solid phase. Thus the solid-phase antibody must be made in an animal species which will not react with the enzyme-labeled antiglobulin. Fortunately, immunoglobulins demonstrate a large degree of specificity.

Radioimmunoassay (RIA) This highly sensitive technique is based on the binding of an antigen by a specific antibody. The antigen is quantified by labeling

the antibody with the radioisotope ^{125}I and measuring the radioactivity bound to the antigen–antibody complex. The bound labeled virus is then separated from the mixture by centrifugation or filtration (in liquid-phase RIA) or by washing (in solid-phase RIA). Steps involved in solid-phase RIA are given in Fig. 7. The sensitivity and specificity of the RIA and ELISA tests are comparable and both methods require 10^5 to 10^6 virus particles per ml.

The main disadvantage of the RIA is that it depends on radioactive materials which must be handled and disposed of carefully. With the ELISA test, stocks of enzyme-conjugated antibody can be prepared in advance and stored for a long time unlike the loss of activity of radiosotopes used in RIA. While costly equipment is needed to measure radioactivity, the ELISA reaction can be read by the naked eye or by a spectrophotometer. Both RIA and ELISA detect viral antigens and cannot determine whether the antigens are associated with viral infectivity.

The application of both RIA and ELISA for the detection of viruses in environmental samples is expanding in spite of limitations on their sensitivity. RIA was used to assay for HAV in samples of sewage and well water during an outbreak of gastroenteritis and hepatitis in Georgetown, Texas (Hejkal *et al.*, 1982). Using ELISA, Steinman (1981) detected rotavirus in 6 out of 24 samples of raw sewage in West Germany. In England, Goddard and Sellwood (1982) detected rotavirus in sewage by ELISA, but less frequently than enteroviruses. In another West German study, rotavirus was detected by ELISA in 2 out of 8 samples of polluted river water.

Biotin–avidin immunoassay (BAI) or amplified ELISA Biotin–avidin immunoassays have been developed for rapid detection of hepatitis A virus and Norwalk viral antigen and antibody. The biotin-avidin system has been selected for amplification of the basic ELISA protocol based on the following reasons. (i) Biotin binds to avidin receptors with an affinity constant of $10^{15}M^{-1}$, whereas the reported antigen–antibody interactions have an affinitiy value of 10^6 to

1. Antibody specific to the desired antigen is adsorbed onto a solid phase of a well in a microtiter plate.
2. Test specimen containing antigen is added.
3. Antigen combines with the antibody.
4. Unattached antigen is washed out.
5. Antibody labeled with radioactive isotope ^{125}I is added to the well.
6. Labeled antibody binds to the antigen.
7. Well is washed leaving only the antibody–antigen–antibody sandwiches.
8. Radioactivity of the well is measured in a radiation counter and is composed with the counts obtained on a negative sample to quantify the antigen.

Fig. 7 Virus detection by solid phase radioimmunoassay.

$10^9 M^{-1}$. (ii) Each avidin molecule has four receptors for biotin, while an IgG antibody molecule has only two combining sites for antigen. In brief, this system increases antibody reactivity with the enzyme indicator systems through amplifications based on an increase in the number of combining sites available for interaction. Gary *et al.* (1985) detected Norwalk antigen in all stool samples that were positive by the RIA and in two stools negative by the RIA. Of 106 serum samples tested, titers by the BAI generally exceeded those by the RIA by two or four fold. Recent studies in our laboratory indicate that BAI tests are 100 times more sensitive than RIA tests for detection of hepatitis A virus in cell lysates.

Radioimmunofocusassay A plaque assay would be especially useful for studies involving hepatitis A virus, given the exceptionally slow rate of growth of this virus in cell culture. Thus far, this has not been possible, given the noncytopathic nature of HAV. A new method, in which foci of infected cells are detected by staining with radiolabeled anti-HAV followed by autoradiography—described as radioimmunofocusassay (RIFA)—has been developed to quantify HAV in cell cultures (Lemon *et al.*, 1983) (Fig 8). The method, in brief, consists of the following steps: African green monkey cells are grown to confluence in small petri dishes. Monolayers are inoculated with the test sample containing HAV and after incubation for 2 h at 37 °C under 5% CO_2 tension for 14 days. After removing the gel overlay, monolayers are fixed in acetone and ^{125}I–labeled anti-HAV antibody is added. Antibody is removed after 4 h incubation at 37 °C, monolayers dried, and the bottoms of petri dishes cut out for autoradiography. Exposure for 5 days to a nuclear emulsion film at −70 °C followed by processing of the film gives autoradiograms which are examined for the number of radioimmunofocus-forming units per sample unit volume. This new assay method retains many of the advantages of conventional plaque assays for virus.

Nucleic acid hybridization Recent cloning of the RNA genome sequences of

1. Monolayers of primary African Green Monkey Kidney Cells (AGMK) prepared in 35 or 60 mm petridishes
2. Cultures inoculated with virus sample (0.25 ml), inocula adsorbed for 2 h at 37 °C and monolayers overlaid with agarose
3. Plates incubated at 37 °C in a CO_2 incubator for 7 to 14 days
4. Agarose overlay removed at time of test; monolayers washed 5× with tris-saline or PBS and air dried; fixed with anhydrous ethyl alcohol (−20 °C) for 10 min at −20 °C
6. ^{125}I-labeled anti-HAV IgG (500,000 cpm/ml) added (2.0 ml) to monolayers; incubated for 3 to 4 h at 37 °C
7. Plates washed 3× with distilled water
8. Autoradiogram prepared by exposing the monolayer in petridish for 5 days at −70 °C; film developed and read for radiolabeled plaques

Fig. 8 Procedure for quantitating hepatitis A virus by radio immunofocusassay (RIFA).

hepatitis A virus facilitated practical application of the HAV clones in hybridization tests as sensitive probes for the detection of HAV RNA in clinical and environmental specimens. Viral RNA is extracted from specimens by treatment with protease, detergent and organic solvents and applied directly to a nitrocellulose membrane. The bound RNA is hybridized with cDNA and detected by autoradiography. This technique is more sensitive than RIA in detecting HAV in stools (Purcell *et al.*, 1984) and also in HAV water concentrates of beef extract or tryptose phosphate broth.

Dot-blot hybridization assay to detect rotaviral RNA sequences in tissue culture or clinical samples have also been examined (Dimitrov *et al.*, 1985). A full length probe of SA11 gene 6 detected 2.5×10^5 SA11 particles. Combined probes from genes 6 and 9 detected 50% fewer SA11 particles. The low virus concentrations in water and wastewater would probably not allow the direct detection of viral sequences, but enrichment of virus could be attained by even limited growth in cell culture. Only partial growth would be necessary, to the stage where replication of the viral genome or accumulation of viral RNA would reach detectable concentrations. This assay would thus measure viable virus and might prove to be highly sensitive.

Summary

Enteric viruses enter water through feces of infected individuals. These viruses abound in urban sewage due to the continual occurrence of viral infections, usually in children who are often without symptons. The health concern over their waterborne transmission stems primarily from three factors: (1) much virus-laden waste ultimately contaminates surface waters, the source for most drinking water; (2) only a few viral particles may be required to produce an infectious dose, and (3) the enteric viruses can produce a variety of diseases not readily causally identified. Since water is consumed by all, even a low level of viral contamination may significantly contribute to the disease burden of a population.

In comparison to the traditional coliform bacterial indicators of water quality, enteric virus levels are extremely low. Waste and water treatment processes further reduce these levels. Methods are available today for concentrating low numbers of viruses including rotavirus and hepatitis A virus from large volumes of water. Viruses continue to be isolated from many surface waters used as drinking water sources as well as treated drinking waters. Emphasis is being placed on the evaluation of current methods for waters of different qualities. By means of round-robin programs, uniformity will hopefully be achieved in assaying and identifying viruses recovered from water so that standards can be set. The current isolation rate of viruses in drinking water appears to be low, and the numbers reported are believed to constitute a small portion of the total number of viruses actually present.

Bacterial indicators of water quality cannot serve as surrogates for revealing the presence of viruses, for bacteria may be low or absent in virus-containing water. Enteric viruses themselves are the most meaningful, reliable and effective virus index for environmental monitoring.

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4 Virus removal by treatment processes

The most common form of pollution control consists of a system of sewers and waste treatment plants. The sewers collect the wastewater from homes, businesses and industries and deliver it to the plants for treatment to make it fit for discharge into streams, estuaries, or for reuse. During dry weather, when the sewers are handling only the normal amount of wastewater, all of it should be carried to the waste treatment plant. During a storm, when the amount of water in the sewer system is much greater, it may be necessary to allow part of the water—including varying amounts of raw sewage—to bypass directly into the receiving streams. The rest of the wastes are sent to the treatment plant. If part of the increased load of storm water was not diverted, the waste treatment plant would be overloaded and the purifying process would not function properly.

A waste treatment plant's basic function is to speed up the natural processes by which water purifies itself. In many cases, nature's treatment process in streams and lakes was adequate before our population and industry grew to their present size. However, these natural processes, though accelerated in a waste treatment plant, are not adequate for a complete removal of pathogens including viruses, high amounts of nutrients such as phosphates and nitrates, and trace elements.

Introduction of water and wastewater treatment practices has greatly reduced the incidence of waterborne diseases, especially those caused by bacterial pathogens. The importance of these practices is illustrated by comparing the morbidity and mortality statistics for waterborne diseases in countries with and without treatment facilities. Five to ten million people are reported to die each year from waterborne diseases in developing countries.

Treatment of wastewater is designed to reduce or remove suspended solids, dissolved and particulate organic matter, nutrients and heavy metals. The degree of treatment given to wastewater is often indicated by the effluent standards prescribed by regulatory agencies, and by the ultimate use of the effluent.

At present there are two basic stages in the treatment of wastes. They are called primary and secondary. 20% of the US population is served by primary sedimentation, 60% by primary plus secondary treatment, 15% by septic tanks, cess pools or stabilization ponds. 5% of the population has no sewage treatment, and raw sewage is discharged into surface waters. In Canada, wastewater from 40% of the population served by sewers in urban areas is never treated, and in West Germany the Rhine river receives more than 70% of the total sewage. Because of the minimal cost, discharge of sewage into fresh or seawater is commonplace in several developing countries.

Much of the information about the efficacy of treatment processes for virus removal has been developed with laboratory-adapted strains of human enteroviruses in pilot treament units. These systems are not subjected to normal variations in hydraulic and organic loading and other variables found under field conditions. The percentage of the total viral load of domestic sewage which enteroviruses represent is also unknown. This information, when available, may

provide a better evaluation of sewage treatment processes in the removal of a wide spectrum of viruses encountered in domestic sewage. Reviews on the effectiveness of various treatment processes on virus removal have been published (Berg, 1973; Malina, 1976; Gerba, 1981; and Rao *et al.*, 1985).

Primary settling As sewage enters a plant for treatment, it flows through a screen made of parallel steel or iron bars placed in a chamber or channel in an inclined position to remove rags and sticks that might clog pumps and small pipes. The sewage then passes into a grit chamber, which is particularly important for cities with combined sewer systems because the chamber removes sand, grit and gravel that wash off streets or land during a storm and end up at treatment plants. The unwanted grit is usually disposed of as landfill. The sewage still contains dissolved organic and inorganic matter along with suspended solids. When the speed of the flow of sewage through the sedimentation tank (Fig. 9) is reduced, the suspended solids will gradually sink to the bottom. This mass of solids is called raw sludge. Primary treatment of sewage is relatively inexpensive and is the only method of treatment practiced by many municipalities around the world. In this process, sewage is detained for 1 to 3 h and, during this time, 35% of BOD (biochemical oxygen demand—a measure for the oxygen-demanding organic matter of sewage) and 40 to 60% of suspended solids are removed from sewage. In areas where raw sewage is directly discharged into surface water or onto land, primary settling is an inexpensive method of partial treatment of sewage.

A true indication of the extent of virus removal by primary sedimentation is difficult to obtain since there is no accurate method to assess the number of viruses embedded in and absorbed on fecal solids. It is believed that primary settling of solids probably accounts for the removal of a great many of the viruses in sewage because they are largely associated with fecal solids. This seems to be borne out in a recent two-year study (Rao *et al.*, 1977) on virus removal by full-scale primary settling. Using matched raw sewage and effluent samples, the average percent reduction of naturally occurring enteroviruses during monsoon, autumn, winter and summer months was 29, 65, 52 and 64 respectively. The samples were processed by blending in an attempt to release viruses associated with solids. The lower virus removal during the monsoon season was attributed to the continuous disturbance of the settling process by heavy rains. These and other pilot plant studies indicated that an average 50% removal of virus could be obtained during primary settling of raw sewage. It is important to note that the virus removed from

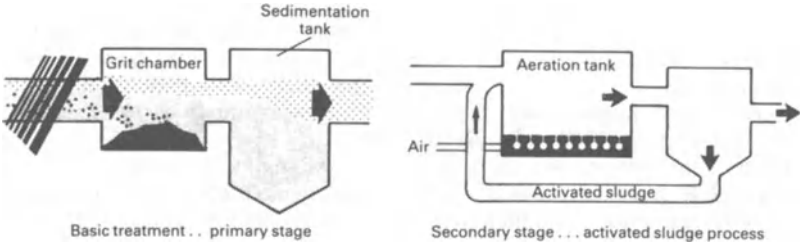

Fig. 9 Treatment of sewage by the activated sludge process.

wastewater by the primary treatment is gathered in raw sludge and is not destroyed, thereby making raw sludge highly infectious.

Secondary treatment After the effluent leaves the sedimentation tank in the primary stage of treatment, it flows or is pumped to a trickling filter or an activated sludge plant. A trickling filter is simply a bed of stones from three to six feet deep through which sewage passes. Bacteria, algae and protozoa grow as mats on these stones and consume organic matter in the sewage. The cleaner effluent trickles out through pipes in the bottom of the filter into a sedimentation tank. The sewage is applied to the bed of stones in two principal ways. One method consists of distributing the wastewater intermittently through a network of pipes laid on or beneath the surface of the stones. Attached to these pipes are smaller vertical pipes which spray the sewage continuously upon the stones. Another much used method consists of a vertical pipe in the center of the filter connected to rotating horizontal pipes which spray the sewage over the stones.

Removal of viruses in trickling filters is generally low and inconsistent. Results of early field studies suggested virus removals of about 40%. Sherman and colleagues conducted quantitative studies of viral removal on a trickling filter of 2.5 million gallon capacity in Maryland. They seeded a bacterial virus at a concentration of 10^6 pfu/ml into the wastewater stream and processed temporally matched composite influent and effluent samples. In three trials, an average 49% removal of virus was noticed. Moore and coworkers monitored a hydraulically underloaded trickling filter plant at Kerrville, Texas and reported a 77% reduction of naturally occurring enteroviruses from raw sewage.

Activated-sludge treatment The current trend is toward the use of the activated sludge process instead of trickling filters. Like most other techniques, it has advantages and limitations. The size of the units necessary for this treatment is small, thereby requiring less land space, and the process is relatively free of flies and odors. It is more costly to operate than the trickling filter. After the sewage leaves the settling tank in the primary stage, it is pumped to an aeration chamber (Fig. 9) where it is mixed with air for 4 to 5 h. During this time sewage bacteria break down the organic matter and a mass of new bacterial cells are produced. From the aeration tank, the sewage flows to another (secondary) sedimentation tank where it remains for 2 to 3 h and the biological solids settle down as sludge. The sludge, now activated with additional millions of bacteria and protozoa (especially free-living ciliates) can be used again by returning a portion of it into the aeration tank for mixing with new sewage and ample amounts of air. (The term 'activated' stems from the sorptive properties of the biological solids; the newly formed cells have a high sorption capacity for suspended and dissolved organic matter in the incoming sewage.) The rest of the new sludge (called the wasted activated sludge) along with the primary sludge is disposed of after stablization by aerobic or anaerobic digestion).

Activated sludge treatment appears to be best for removal of viruses. Removal of 90 and >99% of poliovirus and coxsackievirus A9, respectively, was obtained with bench model continuous flow activated sludge units in studies of Clarke and colleagues in Cincinnati, Ohio. Plant scale studies substantiated laboratory derived data. A 90 to 99% enterovirus removal was obtained with matched raw sewage and effluent samples collected over 24 h in a Bombay plant treating 5

million gallons of sewage per day. Irving and Smith obtained average removals of 93% for enteroviruses, 85% adenoviruses and 28% reoviruses in a Melbourne, Australia plant over a 1 year period. Moore and coworkers reported a >90% enterovirus removal in an Austin, Texas contact stabilization plant with one reactor for sorption of organic materials and a second reactor for bioxidation of sorbed organic matter. Solids-associated virus in sludge was 5 to 10 fold greater than freely dispersed virus in wastewater liquid fractions.

Removal of viruses during activated sludge treatment is due primarily to adsorption to solids which end up as components of sludge. In laboratory experiments, Glass and O'Brien in New Mexico observed that in the first 20 to 50 min of aeration 75% of virus in sewage was adsorbed to settleable solids, and inactivation of the virus was minimal. As aeration time increased, the percent of inactivated viruses increased and the percent of infective viruses adsorbed to solids decreased. After 4 to 5 h of aeration, equal portions of virus removed are attributable to inactivation and adsorption. After 10 h of aeration, 99% of initial virus was removed, of which 25% virus remained alive associated with settleable solids and 75% became inactivated.

The role of exocellular polymers of floc-forming bacteria and ciliated protozoa in the adsorption and survival of enteroviruses in the activated sludge process have been examined by Smith (1983).

Zooglea ramigera, a Gram-negative, urealytic floc-forming bacterium commonly found in sewage sludge, was used as a model system. By varying carbohydrate levels, large amounts of extracellular polysaccharides or extracellular polyhydroxybutyrate granules were obtained; both materials avidly adsorbed ^{125}I-labeled poliovirus and either precipitated the virions or neutralized them. Ciliated protozoa have been identified as a major factor in the control and removal of viruses from wastewater. They are the dominant protozoa present in activated sludge. As the principal grazing population, they consume much of the absorbed materials in sludge, including viruses. It is unclear whether the majority of ingested virions are digested by the ciliates.

In spite of an apparently impressive removal of enteroviruses in the activated sludge treatment of sewage at first glance, virus numbers discharged in 1 million gallons of effluent at a Bombay, India plant and at two Houston, Texas plants still totaled 2×10^8 and 9.7×10^6 infectious viral units, respectively.

Removal of human rotaviruses in the activated sludge process indicated 93 to 99% reduction of virus. Calculation of virus quantities remaining and discharged in a 1.5 MGD Houston, Texas plant amounts to $4.8 \times 10.^7$ viruses per day. Release of large numbers of both entero and rotaviruses may be a potential public health problem.

Stabilization ponds Among the low cost waste treatment methods, stabilization ponds or oxidation ponds (sometimes called lagoons) are shown to be least expensive and simple to construct and operate. They can be used to treat sewage to the secondary stage of treatment or to supplement other processes. They are used in increasing numbers, especially in developing countries in tropical areas. The number of ponds in use in the USA increased from 45 in 1965 to 4476 in 1971. A good example of the application of these ponds is the Santee, California, water reclamation project. After conventional basic treatment by activated sludge, the town's wastewater is kept in a lagoon for 30 days. After chlorination the effluent is

pumped to land immediately above a series of lakes and allowed to trickle down through sandy soil into the lakes. The resulting water is of such good quality that the residents of the area swim, boat and fish in the lake water.

A stabilization pond is a scientifically constructed pond usually 3 to 6 feet deep in which sunlight, algae and oxygen interact to restore wastewater to a quality that is often equal to effluent from a secondary treatment stage. The ponds usually consist of a number of cells arranged in parallel or in series. Wastewater is retained from a week (in tropics) to about a month (in temperate climate) in these ponds. Due to microbial action, the organic materials are oxidized giving C_2, N_3 and inorganic ions such as sulphates and phosphates, and new microbial cells as end products. The algal population uses the C_2, inorganic nutrients and sunlight to produce dissolved oxygen and new algal cells. Thus, the microbial and algal populations have a synergistic relationship in which both groups benefit from each other. In most ponds used in domestic wastewater treatment, they have dissolved oxygen available in the upper zone of water but the lower zone is anaerobic. Sludge accumulates in these ponds very slowly at a rate which might be only a fraction of an inch per year. Although most stabilization ponds have effluents discharging directly into the receiving body of water, the future trend is the use of intermittent sand beds to remove algae from effluents.

The extent of virus removal or inactivation in waste stabilization ponds is highly variable although a number of factors detrimental to virus survival exist in this treatment process. The effect of sunlight over a long period of time, adsorption to solids, the presence of ammonia in the anaerobic zone and antiviral activity of algae-bacterial populations are some of the factors. In repeated tests, Shuval found between 12 and 4000 PFU of virus per l of influent entering a pond in Israel and 28 to 572 PFU per l in the effluent after a 20-day retention time. Virus removals ranged from 0 to 96%. In a pond covering 16 acres and having a detention time of 30 days in the Santee project, England found viruses in 19% of 87 effluent samples. In South Africa, Nupen released biologically treated and settled effluent containing 3600 PFU of virus into a series of 9 stabilization ponds at the Windhoek wastewater reclamation plant. After 14 days retention, a 95% reduction of virus was noticed in the effluent. In India, Rao and colleagues investigated the performance of both pilot and full-scale stabilization ponds operated in parallel as well as in series with detention times ranging from 2.7 to 17.2 days. Based on assays of 24-h composite samples of matched raw sewage and effluent, indigenous virus removals in a range of 86 to 96% in different seasons were observed.

Septic tanks A septic tank is simply a tank (Fig. 10) buried in the ground to treat the sewage from an individual home. About 50 million people are served by about 15 million private waste disposal systems in the USA. Wastewater from the home flows into the tank where bacteria in the sewage break down the organic matter and the partially treated effluent flows out of the tank into the ground through subsurface drains for final purification in the soil. Because septic tank effluent is consistently of poor quality, the effluent must be purified by the soil before mixing with other subsurface water or it may create health hazards. Virus removal in septic tank treatment of wastewater has not been widely investigated. Limited data are, however, available from the studies of Cliver and associates in Madison. Using 60 cm of sand and a daily dose of 5 cm fluid depth of septic tank effluent

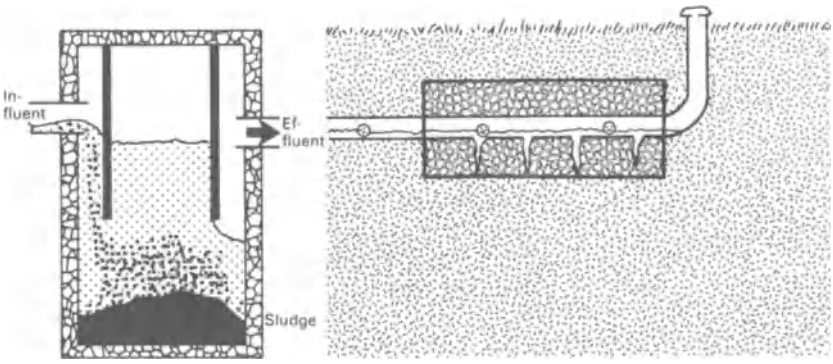

Fig. 10 Treatment of sewage by septic tanks in individual homes.

seeded with poliovirus, virus removal has been complete during more than a year's trial. Virus penetrated only a little more than half the depth of the sand. In laboratory experiments, about 200 gallons of effluent were treated; this is equivalent to the volume produced by a family of four in the USA. Rapid dosing at 50 cm of septic tank effluent per day allowed viruses to penetrate the 60 cm of sand column. At high rates of dosing, the spaces between sand grains are saturated with fluid and virus retention in the soil is poor. The investigators concluded that careful dosing and distribution of the effluent under pressure through narrow pipes with small holes can equalize loading of the effluent in different areas of the seepage bed, avoid saturation of spaces with water between sand grains and thus prevent virus penetration into ground water.

Advanced waste treatment (tertiary treatment)

The increasing need to reuse water now calls for advanced waste treatment. New processes have been developed to achieve any degree of pollution control desired. As waste effluents are purified to higher degrees, the point is reached where effluents become 'too good to throw away'. Such water can be deliberately and directly reused for agricultural, industrial, recreational or even drinking water supplies. An example of reclamation of sewage to potable waters is illustrated by the Stander Reclamation Plant in Pretoria, South Africa. This plant is designed to treat a million gallons a day and a vigilance testing of 100-l samples of finished water never indicated the presence of viruses. On the other hand, viruses have often been recovered from 10 l samples taken during similar testing of treated drinking water from rivers.

There are three processes common to advanced treatment of wastewater and drinking water treatment. They include coagulation-sedimentation, filtration and disinfection.

Coagulation-sedimentation Coagulation and flocculation consist of adding a floc-forming chemical (alum, $A1_2\ (SO_4)_3$ or lime, $Ca(OH)_2$ or ferric chloride,

$FeCl_3$) to water or wastewater to enmesh or combine with nonsettleable colloidal solids and slow settling suspended solids to produce a rapid settling floc. The floc is subsequently removed by sedimentation. Coagulation is the addition and rapid mixing of a coagulant, the resulting destabilization of the colloidal and fine suspended solids (when a solid colloid stays in suspension and does not settle, the system is in a stable condition) and the initial aggregation of the destabilized particles. Flocculation is the slow stirring to aggregate the destabilized particles and form a rapid settling floc.

When a coagulant salt (e.g. aluminum sulphate or alum) is added to water or wastewater, it dissociates and the metallic ion undergoes hydrolysis and creates positively charged hydroxo-metallic ion complexes (aluminum hydroxide) which are adsorbed to the surface of the negatively charged colloids, including viruses. The colloids aggregate by interparticulate attraction (Van der Waal's forces) aided by gentle agitation of water. The floc along with the adsorbed virus settles down. The optimum pH coagulation with alum lies between 6 and 7.5 whereas with iron the optimum pH range is broader.

Coagulation is probably the single most effective chemical process, short of disinfection, for removing viruses from water and wastewater. In the laboratory, Chang and colleagues in Cincinnati found that 25 mg/l of alum removed up to 99% of coxsackievirus A2 seeded into river water. Good removal of virus was dependent on good floc formation which, in turn, depended on coagulant concentration. Ferric chloride and ferric sulphate in a concentration of 25 to 60mg/l removed virus with an efficiency equal to that of alum.

In a pilot scale study in Cincinnati of tertiary treatment (alum) of activated sludge effluent, Wolf, Safferman and Dahling reported poliovirus 1 removal of >99.7% when aluminum to phosphate ratio of 7 : 1 was maintained. Lime flocculation, which is often applied to renovated waters, is very effective provided highly alkaline conditions are maintained. Berg and colleagues, also in Cincinnati, seeded poliovirus 1 into primary effluent and precipitated it with 400 to 500 mg/l of lime. At a pH of 10, little virus destruction occurred, but between pH 10.8 and 11.1 a rapid increase in the rate of virus destruction took place, and at the end of 90 min contact of the coagulation process, >99% of virus was inactivated. Nupen and associates observed a 3.7 to 4.5 log reduction of virus following lime treatment with a settling time of 60 min.

Since the chemical basis for removal of virus in coagulation is a non-specific metal–protein reaction, the efficiency of the process in removing one enteric virus should not differ significantly from that in removing another. In recent studies Rao *et al.* (1985) showed almost identical removal of poliovirus 1, rotavirus SA11 and hepatitis A virus during coagulation of raw water with ferric chloride and alum. It may be noted that viruses in alum sludges are not inactivated and care must be taken in the disposal of infectious sludge. An advantage of lime flocculation is that viruses are destroyed because of high pH maintained in this process.

Filtration In water treatment chemically coagulated and settled waters are filtered to produce high quality drinking water. In wastewater treatment this process is used to filter (1) untreated secondary effluents, (2) chemically treated secondary effluents and (3) chemically treated raw wastewaters. In all three of the uses, the objective is to produce a high quality of effluent.

Filters may be classified according to the types of media used as follows:

(1) *Single medium filters.* These have only one type of medium: usually sand, crushed anthracite or coal.
(2) *Dual medium filters.* These have two types of media: crushed anthracite and sand.
(3) *Multimedia filters.* These have three types of media: crushed anthracite, sand and *garnet.* In both water and tertiary wastewater treatment, dual or multimedia types are used.

The slow sand filter, developed during the mid 1800s, was the first type of filter used for water treatment. Plain sedimentation of the water prior to filtration was usually provided. These filters were single medium filters having an effective sand size of about 0.2 to 0.4 mm and were operated at filtration rates 0.05 to 0.15 gal/m/square foot (0.12 and 1.36 m/h). The filters were cleaned manually, usually every four to six weeks, by scraping off the top layers of clogged sand and clearing the sand with a scouring device. For small and rural communities with limited financial and technical resources, slow sand filtration may be an appropriate technology. Its use is generally limited to waters with low turbidities (less than 50 nephalometric turbidity units). This process is recognized as a biological as well as a physical treatment process. High treatment efficiency is associated with the maturation of 'schmutzdecke' or filtering mat on the surface of the filter sand. The schmutzdecke is a biologically active layer composed of particulates and micro- and macro-organisms including algae, bacteria, protozoa and rotifers. The World Health Organization, through its community water supply schemes, has encouraged a number of pilot projects on slow sand filters in several developing countries since they can be maintained by relatively unskilled personnel.

Forty-seven slow sand filtration plants were identified in the USA and a survey of 27 of them indicated that most of these plants are currently serving communities of fewer than 10,000 persons and are more than 50 years old. All facilities reported excellent turbidity removal and coliform levels of 1 per 100 ml or less in the treated water. Prechlorination is practiced at 22% of plants and postchlorination at 93% of plants. Most plants currently treat water at a cost of less than ten cents per thousand gallons. Assuming water use of 100 gallons/day per capita, a cost to the individual user of about $0.01/day is indicated. Data on virus removal were not available for any of these full scale filters. However, laboratory column studies were recently conducted by McConnel, Sims and Barnett (1984) at Salt Lake City to evaluate reovirus (chosen for study because of its consistent presence and stability in sewage treatment processes) removal from slow-rate sand filtration (SSF) using locally available unsieved construction sand. Infectious virus was not detected in the effluent samples. Reovirus removed from influent water was distributed throughout the entire length of the filter beds (122 cm), the maximum numbers (>70%) occurring in the top 42cm. No infectious virus could be detected in sand samples collected after the experiments at weeks 13 and 21, indicating that reovirus may have been inactivated. In another study by Poynter and Slade (1977) conducted with reservoir water from the Thames River, England, poliovirus removal of 3 to 5 logs in slow sand filters was reported. Because of the large land requirements and the manual labor involved, the slow sand filter has been replaced by the rapid sand filter in towns and cities.

Sand filtration is often practiced in some advanced waste treatment processes.

The adsorption was examined of four different enteroviruses suspended in secondary and tertiary effluents (Fig. 11) to sand columns with low clay content, as found in sandy river beds in West Germany. Viruses in secondary effluent exhibited poorer adsorption at percolation velocities of 1.0 to 1.5 m/day, while all four viruses in tertiary effluent showed excellent adsorption. Sand penetration by virus increased at higher flow rates. Retention of flocculated materials in sand filter beds seems to improve their virus removing efficiency. Removal of lime flocculated secondary effluent by rapid sand filtration (2.25 gal.min/square foot) was reported to be in the range of 82% to 99.8%. Removal of poliovirus by rapid sand filtration with and without coagulation of water has been studied by Robeck and colleagues in Cincinnati. Their results indicate that direct filtration of water could remove 1 to 50% of virus while coagulation with alum without settling if the floc improved virus removal to 90 to 99%. Filtration followed by settling of floc resulted in >99.7% removal of virus.

Sometimes treatment processes remove large amounts of virus, sometimes they do not. Usually better removals are obtained in the laboratory than in the field, perhaps because the control that can be exercised in the laboratory is missing in the field. In any event, treatment processes are designed primarily for purposes other than virus removal, and complete removal of viruses does not occur. However, most treatment procedures reduce virus numbers and also remove many substances that interfere with terminal disinfection. The disinfection process is complex, however, and most effluents and many water supplies are therefore inadequately disinfected.

Disinfection

Methods available for inactivation of viruses in wastewater effluents and water range from the purely physical (ionizing radiation by gamma rays, non-ionizing radiation by ultraviolet light, photodynamic oxidation and heat) to purely chemical (chlorine, chlorine dioxide, ozone, iodine, bromine and bromine

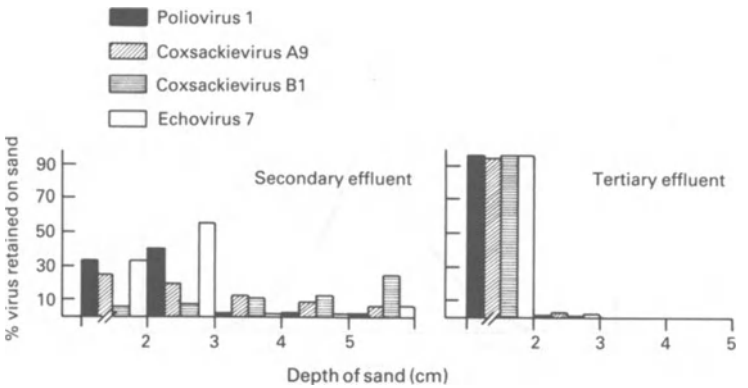

Fig. 11 Sand filtration of sewage effluents seeded with different enteroviruses. (From Nasser, A. *et al.* (1984). In *Enteric Viruses in Water* (edited by Melnick, J.L.). Monographs in Virology 15, pp. 163–170).

chloride). Two treatments such as addition of chlorine to provide a residual following ozonation may be used to produce finished water free from toxic residuals.

Chemical disinfection 1. *Chlorine:* Chlorine is the most widely used disinfectant because it is effective at low concentration, relatively cheap and forms a residual if applied in sufficient dose. It may be applied as a gas or as hypochlorite, the gas form being most common. Chlorine gas reacts readily with water to form hypochlorous acid and hydrochloric acid; the hypochlorous acid produced then dissociates to yield hypochlorite ion:

$$Cl_2 + H_20 \rightarrow HOC1 + HC1$$

$$HOC1 \leftrightarrow H^+ + OC1^-$$

The relative distribution of HOC1 and $OC1^-$ (fig. 12) is a function of pH of water, as shown in Fig. 12. Hypochlorous acid predominates at pH <6 whereas at pH >9.5, hypochlorite is the prevalent form. Chlorine present in the water as HOC1 or $OC1^-$ is described as free available chlorine. Both HOC1 and $OC1^-$ are excellent disinfecting agents. A portion of the available chlorine combines with ammonia in water and forms combined available chlorine that is represented by mono-(NH_2C1, pH>6), di-($NHC1_2$, at about pH 6) and tri-chloramines ($NC1_3$, pH <4.5). The free chlorine residuals have greater disinfecting capability than

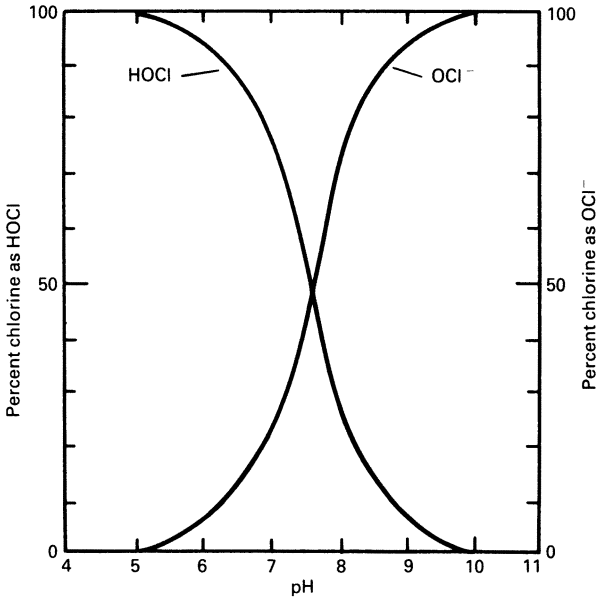

Fig. 12 Relative amounts of chlorine as HOCl and OCl^- at 20 °C versus pH. (Adapted from Fair, G.M. *et al.* (1948). *Journal American Water Works Association* 40: 1051.)

combined residuals. The optimum pH for efficient disinfection of water using chlorine is around 6. Dissolved chlorine also reacts with organic compounds of which two are particularly important. First chlorophenols, formed from the reaction of chlorine with phenols, impart undesirable tastes and odors to water. Secondly, reaction of chlorine with humic substances result in the formation of trihalomethanes: these compounds are limited by drinking water regulation to 0.1 mg/l because of their carcinogenic properties.

The effectiveness of chlorine as a viral disinfectant is highly dependent on a number of factors including temperature, pH, presence of organic matter, and the physical state of the virus (i.e. association with solids, aggregation etc.). Because of the presence of large amounts of organic matter in sewage effluents and because chloramines are not effective virucides, large reductions of virus are not possible. In laboratory studies Shuval examined the effects of different levels and contact times of chlorine on the rate of inactivation of poliovirus 1 and echovirus 9 seeded into an effluent from a biological filter. For a 99% inactivation, 8 mg/l of chlorine and a contact time of 1 h was needed for echovirus 9 and 20 mg/l for poliovirus 1. In respect to viruses naturally occurring in wastewater, a dose of 11 mg/l of chlorine for 30 min could destroy only 16.5% of virus. It required 4 h to bring about more than 99.5% inactivation of the virus. Additional laboratory studies also indicate that high levels and long contact times are required for virus inactivation in effluents. However, high doses of chlorine are not only expensive, but also entail the risk of chloramine toxicity to fish and other aquatic life when such effluents are discharged. Also, carcinogenic chlorinated hydrocarbons may be produced. A further complication is the wide variability in the resistance of solid-associated and aggregated virus. In a study of the chlorine resistance of 20 human enteric viruses suspended in Potomac River water and using 0.5 mg/l of residual chlorine, Liu and colleagues from Narragansett, Rhode Island reported that the the time required for 99.99% inactivation of virus varied from 2.7 min for reovirus 1 to more than 60 min for echovirus 12. A recent report on the development of a progressively more chlorine-resistant poliovirus strain, after a series of sublethal exposures to chlorine, has created additional concern that chlorine-resistant viruses could arise from among the naturally occurring enteroviruses in environmental waters.

Effectiveness of chlorine on rotavirus and hepatitis A virus has recently been studied. Simian rotavirus SA11 is relatively sensitive to free residual chlorine of 0.5 mg/l with 99% inactivation occurring after less than 15 s exposure at pH 6 and 1.4 min exposure at pH 10 (Engelbrecht and colleagues, 1980; Berman and Hoff, 1984). Treatment of HAV with 0.5, 1.0 and 1.5 mg chlorine/l markedly decreased the development of overt hepatitis and seroconversion in marmoset monkeys when compared with controls receiving untreated virus (Peterson *et al.*, 1983). HAV treated with 2.0 and 2.5 mg chlorine/l failed to induce hepatitis or seroconversion in inoculated marmosets. These results suggest that chlorine treatment levels greater than or equal to 1.5 mg/l for 50 min (5 °C, pH 7.0) will effectively decrease the infectivity of HAV by more than four orders of magnitude. This degree of reduction was not always attained after exposure of HAV to 0.5 to 1.5 mg/l of free residual chlorine for 30 min. These results suggest that HAV may be somewhat more resistant to chlorine than other viruses.

2. *Chlorine dioxide* Due to certain potentially toxic byproducts associated with the use of chlorine, alternative disinfectants for wastewater and water have been

sought. One of these methods involves the use of chlorine dioxide (ClO_2). It does not react with ammonia and is only slightly reactive with organic matter. It is a more powerful oxidant than chlorine and persists to maintain a longer lasting residual. The process of preparing ClO_2 for use in water treatment is by excess chlorine method in which chlorine gas and sodium chlorite are used and the reaction is:

$$Cl_2 + H_2O \rightarrow HOCl + HCl$$

$$HOCl + HCl + 2NaClO_2 \rightarrow 2ClO_2 + 2NaCl + H_2O$$

The excess chlorine ensures that all of the chlorite ions are converted to chlorine dioxide.

Poliovirus suspended in municipal effluents required a dose of 2 mg/l of ClO_2 for a 99.9% inactivation. The role of inactivation was not affected by pH. However, its virucidal efficiency depended to a great extent on the concentration of suspended solids. In field studies at ambient temperature, ClO_2 inactivated indigenous coliphages and fecal coliforms in contact stabilization plant effluents 10 to 100 times faster than chlorine. Comparative evaluation of different forms of chlorine and chlorine dioxide in the inactivation of poliovirus 1 are given in Fig. 13. Chlorine dioxide at pH 7 is as efficient as hypochlorous acid at pH 6. At an elevated pH (pH 9.0), ClO_2 is as fast as OCl^-.

3. *Ozone* Ozone is an allotrope of oxygen. It is a powerful oxidant and is more powerful than hypochlorous acid. In aqueous solution, it is relatively unstable, having a half life of 20 to 30 min in distilled water at 20 °C and an even shorter half-life in the presence of oxidant-demanding material in water. Ozone is widely used in drinking water treatment plants in Europe. Today more than 1000 plants throughout the world use ozone. Ozone must be produced on site because, unlike chlorine, it cannot be stored.

Coin and coworkers (1964) observed complete destruction of poliovirus in distilled water at a residual of 0.3 mg/l at the end of 3 min of exposure. They also observed the same effectiveness when the virus was suspended in Seine River water only after the ozone demand of the water was completely satisfied before disinfection.

In relatively clean water, ozone at a concentration of <1 mg/l achieves a 3 $\log_{10}$ reduction of viruses in seconds. However, in wastewater ozone reacts with organic components and doses as high as 4 to 15 mg/l are needed for 99.99 to 100% reduction of bacteriophage f2 and poliovirus 2 with a contact time of 1 to 5 min. Plant studies at Laconia, New Hampshire showed that ozone inactivated 99.99% of coxsackievirus B3 and poliovirus 1. These data indicate the high level of efficiency and consistency of ozone treatment of wastewater disinfection. It has been proposed to combine ozone treatment with post-chlorination.

Physical methods for water disinfection Ultraviolet irradiation can effectively disinfect both water and wastewater. The lack of a residual is a major disadvantage in water treatment. Because this process is technically more exacting to apply than chlorination, it is not used routinely on a large scale.

Satisfactory conventional treatment of a water supply (coagulation, sedimentation, filtration and disinfection) to produce finished water should result

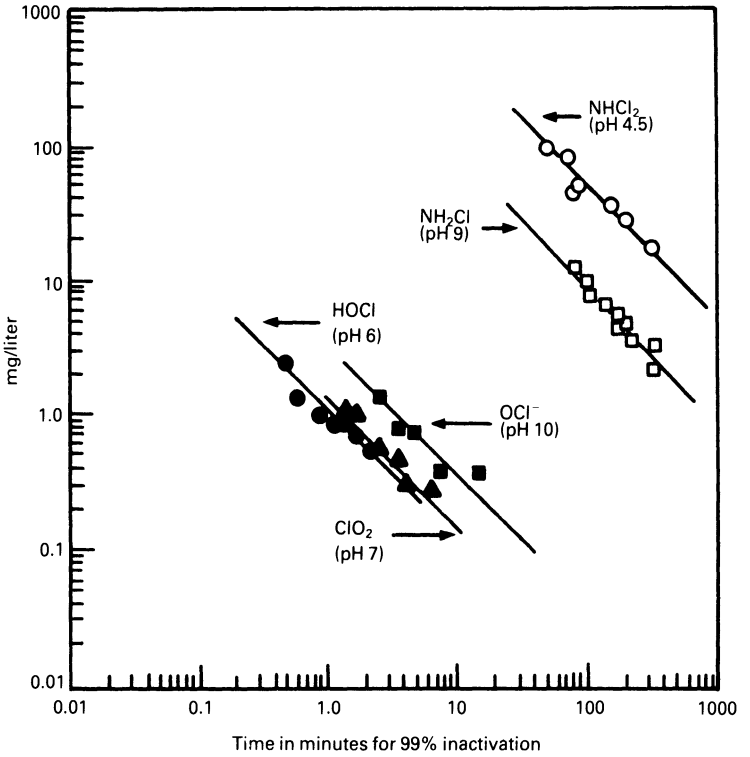

Fig. 13 Comparison of the relative inactivation of poliovirus type 1 by hypochlorous acid, hypochlorite ion, monochloramine, dichloramine and chlorine dioxide at 15 °C. (Adapted from Brigano, F. *et al.* (1979). In *Progress in Wastewater Disinfection Technology* edited by Venosa, A. D.). Mun. Environ. Res. Lab. U.S.EPA, Cincinnati, Ohio.

in removal or inactivation of a minimum of 6 $\log_{10}$ of virus. Nevertheless, viruses have been isolated from treated drinking water in France, India, Israel, Italy, Mexico, Romania, South Africa, USA and USSR. This does not necessarily mean that these processes, when properly carried out, are inadequate for removal of enteric viruses. In many studies the actual water quality and water treatment conditions at the time when viruses were isolated were either not known or not stated. Therefore, any indictment of the procedure for failure to provide adequate treatment for the prevention of waterborne disease cannot be made. Additional field studies are necessary to establish clearly the adequacy of each step.

Summary

Conventional wastewater treatment procedures followed by chlorination were originally developed to reduce biochemical oxygen demand (BOD) and bacteria to acceptable levels. Viruses can survive this treatment level and appear in the effluents. Although an activated sludge process might remove viruses from wastewater, it results in their concentration in primary and secondary sludges. Stabilization ponds also remove viruses and offer a low cost option for treatment of wastewater which is attractive for tropical or subtropical areas. Association of enteroviruses with solids in wastewater suggests the use of coagulation as a process for removing viruses. Among the advanced waste treatment procedures, lime coagulation at high pH levels shows great promise for virus inactivation.

Although HOCl is an excellent disinfectant, ozone and ClO_2 may be more effective in the presence of ammonia because ammonia reacts with HOCl to form chloramines which are less effective as disinfectants but are toxic to aquatic life.

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5 Human viruses in sludges, soils and ground water

In this section we shall consider: how human viruses in sewage are transferred into sludge during sewage treatment; dissemination of viruses in soils upon disposal of sludge onto land; and the ability of viruses to penetrate soil and contaminate underlying ground water.

Human viruses in sludges

The source of viruses in raw sewage is fecal material, and viruses are deeply embedded and adsorbed on fecal solids. Any settling procedure of sewage or effluent results in the removal of large numbers of viruses carried down with solids. Thus, many enteric viruses sediment from wastewater by physical (primary sedimentation), biological (activated sludge, trickling filter humus) and physico-chemical (chemical precipitation with lime, ferric chloride or alum) treatment. The term 'sludge' refers to the solids that settle and are removed when a liquid with suspended solids is passed through a settling tank. Raw primary sludge consists of readily settleable organic matter and fine silt. It is highly putrescible and cannot be stored even for a few hours in warm weather without some type of stabilization to prevent odors from decomposition.

Waste activated sludge is the product of biological multiplication of microorganisms feeding on soluble and suspended organic matter in the presence of dissolved oxygen. Most of the bacteria in waste activated sludge are floc-forming zoogleа. Up to 90% of the zoogleal mass is extra cellular jelly secreted as bacterial capsules. Activated sludge flocs are aggregates of bacterial cells along with protozoa, algae and fungi. This gelatinous mass entraps and adsorbs small particles including solids-associated viruses and settles down leaving clarified wastewater.

The Federal Water Pollution Control Act passed in the USA in 1972 emphasized the need to employ environmentally sound sludge management techniques and mandated at least secondary treatment of municipal sewage. This resulted in huge increases in the amount of sewage sludge. Management of this sludge has become a major problem for many municipalities. Since sewage sludge is an important resource which is sold for agriculture and horticulture for its organic slowly released nitrogen and essential micronutrient content (zinc and copper), a new emphasis has been placed on understanding the fate of enteric viruses.

Virus association with solids in wastewater

The extent of virus adsorption to sewage sludges and their ultimate fate have been

studied using tritium-labeled poliovirus in a laboratory model of the activated sludge system (Malina *et al.*, 1975). 95% of the virus was associated with sludge after 10 min of contact. During a 15 h aeration period, infectivity of poliovirus (measured as plaque forming units—PFU) decreased slowly, and after the 15 h aeration, only 20% of the initial virus content could be detected. These data indicate that poliovirus associated with sludge is inactivated during sewage treatment.

Other reports also showed that viruses added to raw sewage under laboratory conditions, or viruses naturally present in raw sewage, were associated with sludge particles. The reduction of virus numbers during settling of sludge particles in the sedimentation tanks of the activated sludge sewage treatment plants indirectly demonstrated that 76 to 99% of virus could be solids-associated and thus sedimented.

Recovery of viruses from sludges

Since almost all of the enteric viruses contained in sewage sludge are believed to be solids-associated, methods for virus recovery were aimed at elution of viruses from sludge particles using beef extract, serum, detergent or glycine followed by mechanical shaking or sonication and a reduction in volume of the eluate. Recently a number of procedures for recovering enteroviruses from sludges were evaluated in five laboratories in the USA under the auspices of the American Society for Testing materials (ASTM). Based on the results, the ASTM Task Force voted to recommend two procedures as tentative standard methods. A brief outline of these methods is given in Figs 14 and 15. Methods have yet to be developed for recovering rota- and hepatitis viruses.

1. Blend 20 g dry sludge solids with 400 ml chilled sterile distilled water
2. Add 6 g of beef extract; blend 2 min at low speed and 1 min at high speed to release virus from solids and sonicate sample (100 W, 2 min) to maximize virus separation; centrifuge at 10,000 *g* for 30 min and discard sediment
3. Virus in supernatant is concentrated by organic flocculation in which pH is adjusted to 3.5, mixed for 30 min, centrifuged at 10,000 *g* for 30 min and the floc deposit containing virus is dissolved in 5 to 10 ml of sodium phosphate, pH 9.0

Fig. 14 A method for recovering enteroviruses from sludge by sonication-extraction. Glass, J. S., Van Sluis, R. J. and Yanko, W. A. (1978). Practical method for detecting poliovirus in anaerobic digestor sludge. *Applied and Environmental Microbiology* 35: 983–85.

1. 100 ml liquid sludge or 100 g of digested dewatered sludge is mixed with aluminum chloride (final molarity 0.0005), pH adjusted to 3.5 and centrifuged at 2500 *g* for 15 min
2. Virus in the sediment is released by stirring with 100 ml of 10% beef extract
3. Sludge eluate mixture is clarified by centrifugation at 10,000 *g* for 30 min
4. Supernate beef extract containing virus is concentrated by organic flocculation

Fig. 15 A method for recovering viruses from sludges by low pH-$AlCl_3$ Berman, D., Berg, G. and Safferman, R. S. (1981). A method for recovering viruses from sludges. *Journal of Virological Methods* 5: 283–91.

Quantity of viruses in sludges

The studies of Berg (Table 3) provided data on the quantity of naturally occurring viruses in raw and digested sludge. In raw sludge, numbers recovered ranged from 380 to 11,600 PFU/100 ml, while mesophilic sludge (digested at 35 °C for about 20 days) contained 30 to 410 PFU of virus/100 ml and thermophilic sludge (digested at 50 °C) between <1.7 and 16 PFU of virus/100 ml. The ASTM Task Group on

Table 3 Recovery of viruses from raw, mesophilically digested, and thermophilically digested sludges.*

Test		Viruses in sludge, PFU/100ml	
No.	Raw	mesophilically digested	Thermophilically digested
1	380	30	<4
2	550	50	<3.1
3	1530	—	<2.8
4	940	40	<2.7
5	2780	280	3.3
6	1550	250	<1.4
7	1190	240	<1.7
8	1810	100	1.7
9	11600	410	5.0
10	2470	200	5.7
11	1500	360	16.7

*Sludges consisted of one-third activated sludge and two-thirds raw primary sludge digested for about 20 days at 35 °C (mesophilic sludge) or at 50 °C (thermophilic sludge).
Adapted from Berg, G. (1984). *Monographs in Virology* 15: 17–19.

Table 4 Comparative recovery of naturally occurring viruses from sludges *‡

Sludge type	Method	Range of virus recovery (PFU/l)	Average † (PFU/l)
Primary sludge	EPA	132–9303	2567
	Glass	375–3379	1507
Anaerobic digestion	EPA	10–210	93
	Glass	4–635	57

* Based on assays by EPA reference laboratory of sample concentrates submitted by eight participants.
† Average calculated from three trials.
‡ Abstracted from Goyal *et al.* (1984) *Applied and Environmental Microbiology* 48: 531–8.

Viruses in Water conducted a round-robin investigation of methods for detection of viruses from five different sludge types in eight participating laboratories. The results of virus recovery from primary and anaerobically digested sludge varied from 1507–2567 to 57–93 PFU/l respectively (Table 4).

Fate of viruses during sewage sludge treatment and disposal

Approximately 5 million tons of dry sludge were generated in 1976 by more than 20,000 municipal sewage treatment plants in the USA. Although sludge contains solids, the problem of disposal is not only a solid waste problem but also the problem of disposing of water that is in close association with waste solids. A thin, waste-activated sludge from biological treatment may contain well over 100 tons of water per ton of solids. Before disposal, sludge should be reduced in volume by removing water.

Dewatering and drying Drying beds which are usually made of layers of sand and gravel can be used to remove water from sludge. The sludge is spread over the bed and allowed to dry. After a week or two of drying, the residue will be reduced in volume and, consequently, will be more readily disposable. During drying, the solids content of sludge increases from 5% to approximately 40% which, in turn, protects viruses from rapid inactivation. The survival of enteric viruses in sludge dried for 11 days is given in Table 5.

Inactivation of viruses was slow when sludge solids concentration increased from 5% to 30 to 40%. Virus numbers were reduced by >4 to 5 $\log_{10}$ when sludge solids concentration was higher than 90%. Loss of naturally occurring enteroviruses during sludge drying in the field substantiates the laboratory experiments. It was reported that after 7 days of drying the virus content decreased from 33 PFU to 1 PFU/10g of solids (Hurst *et al.*, 1980).

Heat treatment The US Environmental Protection Agency recently stipulated that the minimum temperature for pasteurization of sludge for

Table 5 Persistence of enteroviruses after dewatering sludge by evaporation*

Virus type	Concentration of sludge solids (%)	Virus recovered (PFU/ml)
Poliovirus 1	5	6.5×10^6
	30	3.8×10^6
	91	$<2.5 \times 10^2$
Coxsackie virus B1	5	1.3×10^7
	33	3.0×10^6
	93	$<2.0 \times 10^2$
Reovirus	5	1.2×10^6
	40	7.0×10^5
	94	$<2.0 \times 10^2$

* From Ward, R. L. and Ashley, C. S. (1977). *Applied and Environmental Microbiology* 33: 860–4.

reducing pathogens is 70 °C for a duration of 30 min. At these high temperatures, enteric viruses are inactivated rapidly in sludge. This treatment is nonetheless costly and energy-intensive. The possibility of achieving enterovirus inactivation in sludge at lower temperatures of 43 °C and 51 °C was demonstrated by Ward and colleagues at Sandia Laboratories, Albuquerque, New Mexico (Fig. 16). These investigators detected an organic compound (an ionic detergent) in raw sludge which protected poliovirus. The protective agent was associated with sludge solids and was concentrated during sludge drying and, as a result, enteroviruses were inactivated at 50 °C more slowly in dry sludge (80% solids) than in liquid sludge (5% solids). It was further shown that the protective effect was decreased by ammonia, the virucidal agent present in the liquid portion of the digested sludge. By increasing the pH of the sludge to 8.4 to 9.0, concentration of ammonia was increased and, as a result, the protective effect of the agent was reduced. Thus, a moderate heat-treatment of sludge under alkaline conditions was considered effective in inactivation of enteroviruses. It should be cautioned that hepatitis A virus (HAV) is more heat resistant than poliovirus. The temperature at which 50% of poliovirus particles became disintegrated during heating at pH 7 for 10 min was 43 °C. For HAV the temperature required for the same level of inactivation was 61 °C. In the presence of 1 M MgCl these temperatures for polio- and HAV virus shifted to 61 °C and 81 °C, respectively. During low temperature inactivation of viruses, viral nucleic acid breaks down within unaltered viral capsids. RNA recovered is non-infectious and has been found in a degraded state.

Anaerobic digestion of sludge Anaerobic digestion is the most common sludge treatment in which microbes oxidize organic materials under anaerobic conditions and during the process generate methane, which can be used as a

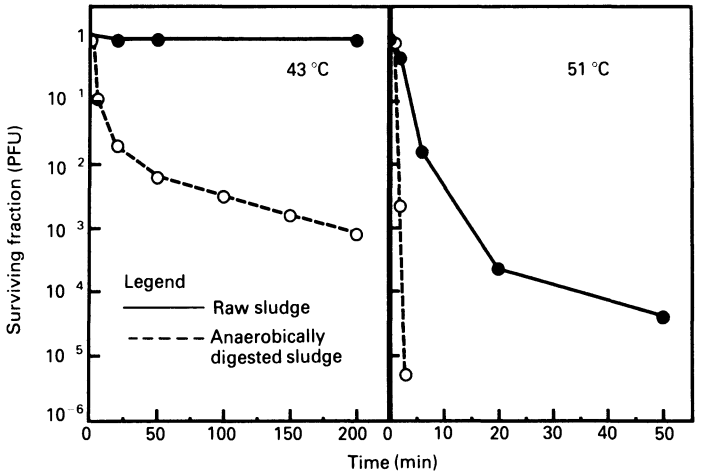

Fig. 16 Rate of inactivation of poliovirus during low temperature heat treatment of sludge. From Ward, R. L. and Ashley, C. S. (1976). *Applied and Environmental Microbiology* 32: 339–46.

fuel. Digester gas consists of methane (55 to 75%) and carbon dioxide (25 to 45%). The gas is used for heating purposes and for power generation. The amount of sludge gas produced may meet all of the energy requirements for a municipal wastewater treatment plant. Heat dried sludges are used as fertilizers on crops and serve as excellent soil conditioners.

Approximately 99.8% of coliform bacteria are destroyed during sludge digestion, but this treatment does not completely destroy viruses. In laboratory experiments poliovirus was shown to survive for more than 30 days when the virus was embedded inside sludge flocs prior to anaerobic digestion (Fig. 17). Mesophilic digestion (35 °C) of raw sludge is not a reliable process for the inactivation of virus, although levels were, on average, reduced by about 80%, and following 45 days storage the virus content was reduced by a further 50%. Thermophilic digestion (50 °C) had a greater virucidal effect than mesophilic digestion. In one study, however, 3 out of 5 samples tested still contained virus although the recovery of infectious virus was decreased by 93%.

An understanding of the mechanisms of virus inactivation during sludge digestion has been provided by investigations also conducted by Ward and colleagues. It was first learned that poliovirus was inactivated to a greater degree in digested sludge than in raw sludge. It was further noticed that the virucidal agent in digested sludge is the uncharged form of ammonia (NH_3) and that, as pH increases from 7 to 10, the concentration of the charged form of ammonia (NH^+_4) decreases from >99% to 13%. Thus, an increase in the pH of digested sludge into the alkaline range would ensure increased concentration of the virucidal agent, NH_3. It was postulated that other virucidal agents may also be involved in virus inactivation during sludge

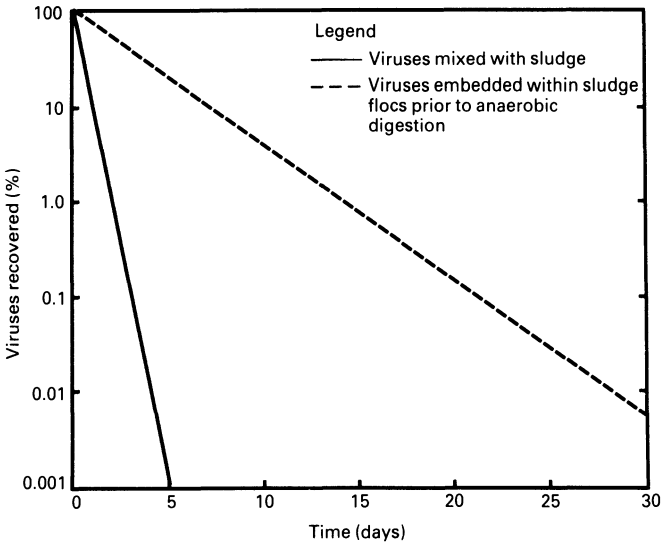

Fig. 17 Poliovirus inactivation during anaerobic digestion of sludge. From Moore, B. E. *et al.* (1976). *3rd National Conference in Sludge management, Disposal and Utilization.* Miami. Fl.

digestion since it was found that ammonia affects only the RNA core without altering significantly the viral capsid.

Sludge composting Another approach to the treatment of sludge is composting. In this process dewatered sludge is mixed with wood chips or leaves and allowed to decompose for about three weeks in summer and for more than one month in winter. Aerobic conditions are maintained either by pumping air into the compost pile or by regularly turning the pile. Temperatures of between 50 °C and 70 °C are developed inside the compost pile and the result is a stabilized odor-free product which is used as a fertilizer and a soil conditioner to improve soil structure, aeration, and water-holding capacity. Compost is also used in landscaping of public buildings, reclamation of marginal lands, and for golf courses.

Inactivation of poliovirus and reovirus in raw and composted sludge was studied by Ward and Ashley (1978). Figure 18 shows that poliovirus is more heat stable in raw than in composted sludge due to degradation of ionic detergents which otherwise protect enteroviruses from thermal inactivation.

Factors such as pH, chemical composition, and moisture content of sludge affect virus inactivation rates during composting. Treatment of sludge with lime prior to composting results in elevated pH levels inside the compost. The presence of ammonia, coupled with a high pH, causes rapid inactivation of enteroviruses. In essence, the degree of virus inactivation is directly proportional to the temperature maintained which, in turn, is related to the efficiency of composting.

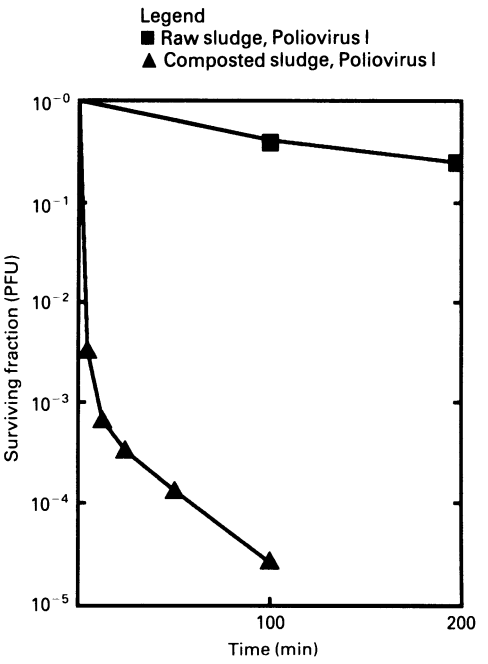

Fig. 18 Comparative rates of heat inactivation (45 °C) of poliovirus 1 in raw and composted sludge. From Ward, R. L. and Ashley, C. S. (1978). *Applied and Environmental Microbiology* 36: 898–905.

Historically, sewage sludges generated by densely populated coastal towns are disposed of into marine waters by ocean falls or by barges. This practice is a potential public health problem because settled sludge becomes a part of bottom sediments, which play an important role in the distribution, survival and transport of human viruses in the marine environment, details of which are presented in Chapter 6. Impact of sewage disposal on the distribution of human enteric viruses into the Atlantic Ocean at two dumpsites near Philadelphia and New York was studied. An estimated 30.5×10^6 kg of sludge was dumped between 1973 and 1980 at the Philadelphia site while the New York site received 3×10^6 tons every year. A variety of viruses including coxsackie B_3, B_5, echo 1 and polio 2 were isolated from sediments and water. A very significant finding was that viruses were isolated from sludge-sediments 17 months after the cessation of sludge dumping in these areas. In the USA, the Marine Protection, Research and Sanctuaries Act of 1972, as amended in 1977, prohibited dumping of any sewage sludge after 1981. However, ocean dumping of sewage sludge still occurs and outbreaks of clam-associated hepatitis and gastroenteritis were reported in the New York area in 1982.

Human viruses in soils

Urbanization and the resulting populaton centers are exerting an increasing demand for technology to manage municipal wastes safely, effectively, economically and within the acceptable limits of environmental tolerances. The need to reuse water, as well as other resources contained in wastes, has led to intense interest in land spreading. At the present time, more than 700 cities in the USA are disposing of a portion, or all, of their wastewater effluents and sludges in some land based system such as forests, golf courses, pastures and agricultural soils for animal crop production. Important considerations favoring land disposal of wastes include: (1) an increased awareness of the hazards associated with sewage pollution of surface waters, (2) the increasing demand on present sources of potable water, (3) the realization that valuable nutrients must be recycled, and (4) the need to recondition depleted soils in order to meet ever increasing demands for human and animal crop production.

Disposal of wastewater and sludge by direct application to land should not be carried out without first considering the following dangers:

Direct virus infections of farm workers and their contacts;
virus contamination of crops used for human consumption;
virus contamination of drinking water sources as a result of surface runoff or infiltration into ground water; and
virus dissemination by aerosols during wastewater sprinkler irrigation with consequent risks of infection through the respiratory tracts of farm workers, residents of adjacent areas or travelers in the vicinity.

Techniques for the extraction, concentration and enumeration of naturally occurring human viruses from soils are only marginally efficient for a few enteroviruses and, at present, are totally inadequate for detecting hepatitis A virus, Norwalk agent and human rotaviruses. This dictates that virus recoveries reported in field studies are only partially quantitative. The detection of any enteric virus is an indication that many other types may be present.

Occurrence and distribution of viruses in soils

Viruses have been isolated from soils receiving domestic wastewaters and sludges. Three different soil types containing 81, 41 and 7.6% of sand were examined for their ability to adsorb a bacterial virus, f_2, suspended in septic tank effluent. The greatest precentage of virus was detected within the first 15 cm of soil below the septic application lines. Several virus isolates were found at 85 cm, with single isolations observed at 100 and 120 cm depths. Hurst and coworkers (1980) seeded poliovirus in soils collected from Flushing Meadows wastewater treatment site near Phoenix, Arizona and detected 91% of virus in the top 2.5 cm of soil. The remaining virus was distributed at depths between 2.5 and 25 cm. Studies by other investigators indicated that 77% of seeded poliovirus in 75 cm-deep soil cores was detected in the top 5 cm of soil. All of these studies indicate that most of the viruses are retained initially in the upper soil layers. Under certain situations, viruses do migrate into deeper layers of soil and ultimately contaminate ground water.

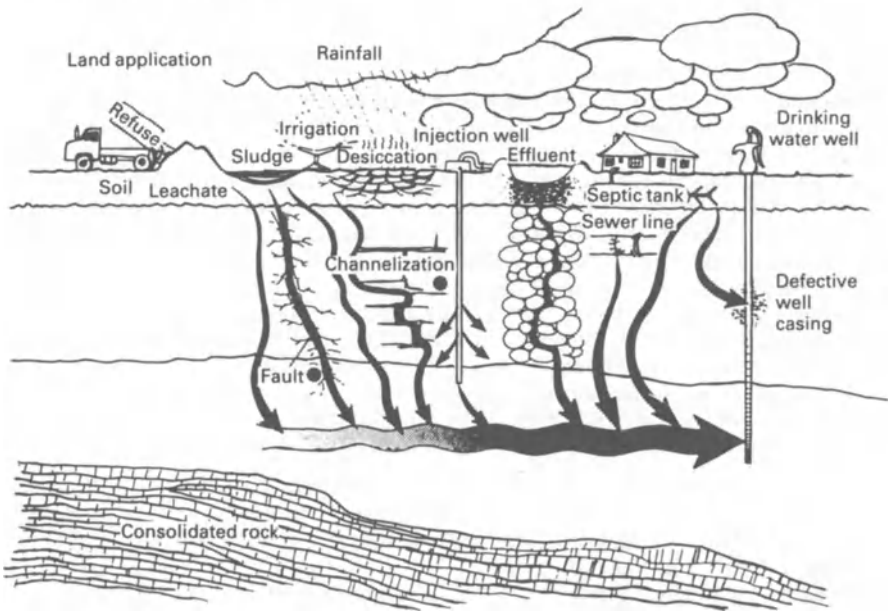

Fig. 19 Factors that influence the entry, survival and migration of viruses in ground water. Soils rich in clay minerals and percolating fluids with high ionic composition enhance virus retention; high levels of moisture and low water temperatures in the soil promote virus survival while increased microbial activity inactivates viruses faster. Higher percentage of soil saturation with water and low ionic composition of soil fluids (like rain water) promote virus desorption from soil and help movement of virus in ground water. From Melnick, J. L. and Gerba, C. P. (1980). *Public Health Reviews* 9: 185–213.

Adsorption, persistence and desorption of viruses in soils

The various factors associated with human enterovirus entry, survival and migration in soils are depicted in Fig. 19, and are discussed below.

Adsorption of viruses to soils Virus retention by soil is largely caused by adsorption, a process that is controlled by the composition of soil and by the ionic strength, pH, and flow rate of water. Among the soil components, clay minerals are actively involved in the adsorption of both biological and chemical pollutants due to their large surface area and ion-exchange properties. Large amounts of viruses are retained on clays, especially on kaolinite. The sorptive ability of organic matter, a colloidal fraction of soils in retaining viruses, is not known, but recent evidence suggests that organic soils are not as effective as mineral soils for virus retention. The degree of saturation of soil pores with water affects virus retention on soils. Saturated pores reduce the chances of contact between viruses and soil particles and

thus facilitate their migration. One simple approach to prevent virus desorption from soil is to allow it to dry between sewage effluent applications. Virus adsorption to soil is enhanced when the hydrogen ion concentration of virus containing effluents is below 7. This is due to the fact that both enteroviruses and clay particles are negatively charged at alkaline pHs and, naturally, they repel each other. Minimal virus adsorption to soil occurs at pH 8 and above. Virus adsorption to soil is also influenced by the concentration and species of cations. An increase in the cation concentration results in enhanced virus adsorption, monovalent cations (Na^+, K^+) facilitating better adsorption of virus than divalent (Mg^+, Ca^+) or trivalent (Al^{3+}, Fe^{3+}) cations. Certain types of soluble organic matter in water and soil will decrease virus adsorption to soil. Of 34 different soil materials tested for adsorption of poliovirus and reovirus, a muck soil and a silt loam, both of which had high organic matter content, exhibited poorest virus adsorption (Moore *et al.*, 1981). Humic and fulvic acids are highly colored organic compounds in water and soils and interfere with virus adsorption to soils. Studies at the University of Florida indicate that 80% of poliovirus suspended in highly colored cypress dome water (containing high concentrations of humic and fulvic acids) passed through the soil columns and was detected in the leachates. Treatment of these leachates with activated carbon to remove organic components restored the adsorption capacity of the soil.

Persistence of viruses in soils An assessment of the degree of public health hazard associated with land disposal of wastewater depends on the survival of human viruses in soil. During the past two decades many reports have addressed the persistence of enteroviruses in soil. Reviews by Duboise *et al.* (1979) and Gerba *et al.* (1975) identified several factors which influence virus survival in soils. They include temperature, moisture, microbial activity, pH, salt concentration, virus association with soil and other particulate matter, virus aggregation, soil texture and virus type. For many of these factors the precise mechanisms by which they cause virus inactivation or protection have not been determined. Natural soils are dynamic ecosystems and interactions between some of the factors add to the complexity of elucidating their precise roles in virus survival or inactivation. However, soil temperature and moisture content appear to be the controlling factor in virus persistence.

Temperature Temperature has a considerable influence on virus inactivation rates in soils. Viruses persist longer at lower temperatures and the survival times are shorter at higher temperatures. Coxsackievirus B1 inactivation rates in sandy and sandy loamy soils suspended in river water, ground water and septic tank wastewater increased as temperatures were increased from 4 °C to 37 °C. In pilot scale outdoor studies on poliovirus persistence in soils irrigated with sewage effluent in Cincinnati, Ohio, Tierney *et al.* (1977) found that 99% inactivation in soils required about two months during winter and only 2 to 3 days in warm summer months. The soil temperature in warmer months rarely exceeds 44 °C and viral inactivation around these temperatures is believed to be due to inactivation of viral RNA.

Soil moisture Soil moisture affects virus survival in soils. Bagdasaryan in the

USSR reported that enteroviruses survived 3 to 6 times longer in soils with 10% moisture content than in air-dried soils. Sagik and coworkers (Austin, Texas) found that poliovirus was inactivated more rapidly in drying soil as the moisture content decreased from 13 to 0.6% than in the same soil type maintained at 15 or 25% moisture content. Inactivation of 99% of the initial viruses occurred within one week in drying soil but took 7 to 8 and 10 to 11 weeks in soils with 25 and 15% moisture content, respectively. Yeager and O'Brien (New Mexico) compared the degree of poliovirus inactivation in eight different soils saturated with river water, ground water or septic wastewater and in the same soils that were allowed to dry out during the course of the experiment. Upon drying, none of the initial viruses was detectable in any of the dried soils (>99.99% inactivation), but considerable quantities of virus were still present in the same types of saturated soils. In a field study on virus survival in a rapid infiltration system for wastewater, Hurst and colleagues (Houston, Texas) reported that virus inactivation rates were greater in more rapidly drying soils. Virus inactivation was enhanced by allowing soils in rapid infiltration systems periodically to dry and become aerated between waste-water applications. Yeager and O'Brien (1979) studied the mechanism of virus inactivation in soils and found that the loss of poliovirus infectivity in moist and dried soils results from irreversible damage to the viral particles, including (i) dissociation of viral genomes and capsids, and (ii) degradation of viral RNA. In both moist and dried, non-sterile soils, viral RNA was released from capsids and found in a degraded form. In dried, sterile soils, viral RNA was released but remained largely as intact molecules. Viral capsid components were not readily recoverable from drying soils due to irreversible binding, but they could be recovered as empty capsids from moist soils. These results suggest that polioviruses are inactivated by different mechanisms in moist and dry soils.

Microbial and chemical activity Soil microbial activity and chemical activity due to microbial enzymes appear to decrease virus survival in soils. Sterile and non-sterile suspensions of different soils were seeded with poliovirus and their survival studied in the laboratory. The time required for 99% inactivation of virus was always shorter in non-sterile soils than in sterile suspensions in which microbes were destroyed. The mechanism of microbially mediated virus inactivation in soils appears to be the result of degradation of virion structural proteins and their use as substrate by soil bacteria.

Association of viruses with soil particles may either enhance or decrease their survival, depending on the chemical properties of the soil particle. Poliovirus adsorption to oxide particles of manganese, aluminium and copper result in significant and rapid inactivation while adsorption to silica and iron oxide did not inactivate the virus.

Virus adsorption to soil is not an irreversible process and, under appropriate conditions, soil particles desorb virus and then become entrained with the percolating fluids. Virus desorption from soils is attributed to concentration of ions in the percolating fluids, virus type, pH, soil depth, soil continuity and degree of saturation.

Desorption The ion concentration or conductivity of percolating fluids

appears to play a major role in virus desorption from soils. Duboise *et al.* (1979) reported that when soil columns containing adsorbed coliphage T_7 were rinsed with deionized water, a number of phages desorbed and were recovered in column effluents. Based on these studies, the authors suggested that natural rainfall, with its low ionic composition, may act in the same manner, thus triggering virus migration at land application sites. In fact, a number of enteric viruses were detected in ground water beneath a spray irrigation system following a heavy (71 cm) rainfall in Florida. To prevent virus migration through soils into ground water as a result of heavy rains, a practical and effective method was to flood the areas with sewage effluents, thereby restoring the ionic concentration in the soil.

Desorption of viruses from soil appears to depend upon the virus type. Laboratory study results showed that <2% of a vaccine strain of poliovirus were eluted from soil cores while large numbers of mutants of this strain were extensively eluted with rain water. A survey of human virus occurrence in Long Island ground water that had been recharged with wastewater indicated the least numbers were among the polioviruses, thereby suggesting that polioviruses may elute poorly from soil particles.

A number of other factors may also influence desorption of viruses from soils. High pH fluids appear to create strong electrostatic repulsive forces between negatively charged soils and virus particles carrying similar charge, causing their desorption. Once desorbed, several soil characteristics may contribute to virus movement towards ground water. Prominent among them are soils riddled with cracks or fissures, depth to the ground water aquifier and degree of saturation of pores in the soil. There were reports to indicate that a bacterial virus under saturated soil conditions moved laterally over a distance of 900 m in a relatively short period of time, covering about 350 m/day.

Viruses in aerosols

Treatment of sewage and disposal of effluents on land generate aerosols containing human enteric viruses. Dispersion of aerosolized viruses may cause health risk among people.

During sprinkler irrigation, which is commonly used for wastewater application to the land, between 0.1 and 1% of the liquid is aerosolized, depending on the type of spray device, the pressure, and wind speed. Pathogenic bacteria and viruses can become entrapped in airborne water droplets ranging in size from 1 to 50 μm. Aerosols containing enteric microorganisms formed by wastewater treatment processes have been detected 1200 m downwind, while microorganisms from sprinkler irrigation of food processing wastes might be spread as far as 25 km. Enteroviruses have been detected 40 to 100 m downwind of sewage spray irrigation fields. It was found that the detectable concentration of viable airborne microorganisms increases with an increase of relative humidity and a decrease of solar radiation. Darkness facilitates survival of aerosolized enteric microorganisms up to 10 h more than during daytime. Of viable particles detected in one study, 30% were in the respirable size range of under 5 μm.

Humans may be infected by aerosols containing pathogenic bacteria or viruses primarily by inhalation of particles of 0.2 to 2 μm size which penetrate the alveoli, but larger droplets in the 2 to 5 μm range or greater, which are trapped in the upper respiratory tract, are removed by ciliary action and may find their way into the digestive tract.

Studies have shown that in droplet formation of the surface of aerated liquids, the droplet formed scavenges organic material and microorganisms; the result is that the aerosolized droplet may contain a bacterial concentration 100 times or greater than that of ambient water. This suggests that bubbles formed during the aeration of sewage treatment such as activated sludge may lead to the aerosolization of liquid droplets containing very much higher concentrations of pathogens than the wastewater itself.

An epidemological study of possible health risks associated with sprinkler irrigation with wastewater was carried out in Israel (Katzenelson *et al.*, 1976). In 77 agricultural settlements practicing sprinkler irrigation with oxidation pond effluent after 3 to 7 days of retention time, the incidence of typhoid fever, salmonellosis, shigellosis, and infectious hepatitis was from 2 to 4 times higher than in 130 control settlements not practicing sewage irrigation.

Human viruses in ground water

About 19% of the water supply in the USA is at present taken from ground water sources and as the demand for clean water increases and supply from surface sources decreases, the use of ground water is expected to increase. Ground water with no disinfection continues to be the first choice as a drinking water source in West Germany.

Application of wastewater and sludge, whether for agricultural irrigation or as a method of treatment and disposal, poses the risk of microbial contamination of ground water. Since ground water is often used for human consumption with little or no treatment, transmission of infectious disease becomes a matter of primary concern. Of the 550 documented waterborne disease outbreaks reported during the period 1946 to 1977, about 65% have a probable viral etiology (hepatitis A, poliomyelitis, and gastroenteritis).

Virus isolation from ground water associated with waterborne disease outbreaks

During 1976 and 1980, eight waterborne gastroenteritis outbreaks associated with Norwalk-like agent(s) were reported in the USA. Although the Norwalk agent cannot be isolated from water at present, its presence was confirmed from stools of infected individuals. Of these outbreaks, seven originated from contaminated well water while a lone outbreak was attributed to ground water. The number of cases ranged from 10 to 467 (Kaplan *et al.*, 1982). The entry of enteroviruses into aquifiers, their survival, migration, and reported viral outbreaks attributed to ground water have been reviewed (Keswick and Gerba, 1980). A variety of enteroviruses were isolated from drinking water wells associated with waterborne disease outbreaks. Wellings and colleagues

isolated an echovirus 22/23 complex in 378 l samples from a 12.2 m deep well during an outbreak of gastrointestinal illness at a migrant labor camp in Florida. The well was located 30.5 m from a solid waste field, and was in the middle of an area bordered by septic tanks. The echovirus was isolated from sewage, from potable well water containing 0.4 to 0.6 mg/l residual chlorine and from stools collected from individuals living in the camp. It appears that well water must have been contaminated by leachates from septic tanks and chlorination of the water reduced bacterial counts to undetectable levels, and yet the virus survived. This raises concern about ground water disinfection based on current standards. Shuval reported the isolation of poliovirus type 1 (4 to 7 PFU/1) from a well under suspicion during an outbreak of hepatitis A in Israel. In June 1980, Hejkal, Gerba, Melnick and colleagues studied an outbreak of gastrointestinal illness and infectious hepatitis associated with drinking water wells in Georgetown, Texas. Prior to chlorination (the only treatment used), well water samples were found to be heavily contaminated with coliforms. Potable water samples taken for virological analysis contained 0.8 mg/l of residual chlorine. The attack rate of gasterenteritis among the 10,000 population served by the contaminated wells was 80%. Coxsackie virus B2, B3 and hepatitis A virus antigen were detected in the well water. Also, in 1980, enteric viruses (polio 1, echo 27 and 29) were isolated from ground water at a summer camp in western Maryland during an outbreak of gasteroenteritis. The water supply for the camp was a 95-foot well which during the outbreak contained 0.7 to 1.0 mg/l disinfectant (iodine), a concentration considered adequate to destroy bacteria. Coliform counts in the drinking water were well within allowable standards. Mack and colleagues demonstrated the presence of poliovirus 2 in a well associated with an outbreak of gastroenteritis in Michigan. The source of contamination was traced to a close waste drain field from which the virus penetrated 20.5 m through several soil profiles and travelled 91.5 m laterally.

Virus isolation from land application sites

Even though there have been no reports of disease outbreaks associated with land treatment of wastewater, viruses have been detected in ground water after waste-water application to land or direct ground water recharge. At St Petersburg, Florida, when secondary effluents are discharged into a cypress dome containing a layer of black organic soil (4 to 12% clay) and sandy clay, a variety of viruses including polio 1, coxsackie B4, and echovirus 7, 11 and 14 were recovered from 3 m-deep wells in the vicinity. Viruses migrated 7 to 38 m laterally from the wastewater application point and survived for 28 days. At a spray irrigation site containing sandy soil with little or no silt or clay in Gainesville, Florida, Wellings and coworkers isolated coxsackie virus B4 and poliovirus 1 and 2. These viruses are reported to survive chlorination and sunlight, and migrated through 3 to 6 m of soil.

Virus survival in ground water

Since sunlight (normally known to inactivate viruses through its ultraviolet

component) is not available in ground water and the temperature is lower, viruses should survive longer there than in surface waters. Laboratory strains of coxsackie virus A9, poliovirus 1, coxsackie virus B1 and echovirus 7 suspended in ground water (10 °C) showed only 1 to 2 log loss of titer after 300 days according to a study in Berlin, West Germany.

Since the factors influencing the movement of viruses in soil are still not fully elucidated and since effluent and soil conditions vary so extensively, caution should be exercised with regard to wells supplying drinking water in the vicinity of wastewater irrigation or land disposal sites. Reasonable safety measures should include the siting of such wells at a suitable distance from such sites and the routine virological monitoring of water quality.

Summary

During wastewater treatment and settling, enteroviruses are transferred into sludge and accumulate in large numbers. The most common method of sludge treatment—anaerobic digestion—does not completely destroy viruses but they are partially inactivated by a virucidal agent identified as the uncharged form of ammonia. Sludge composting, a thermophilic and aerobic process, destroys large numbers of viruses.

Land application of wastewater and sludge is an economical and effective way to turn wastewater into a valuable water resource while minimizing the pollution of surface water. Relatively thin layers of soils remove viruses by adsorption. Exceptions are very course sands and gravels that have high infiltration rates and may allow direct channeling to the ground water. The most important soil characteristic affecting virus removal is the soil permeability.

Aerosols containing human enteric viruses are generated during wastewater application to soil. Dispersion of aerosolized viruses may be a health risk.

Water-borne viral outbreaks related to contaminated ground water have occurred and different virus types have been isolted from such waters and also from drinking water wells, from beneath wastewater application sites, and in the vicinity of septic tank systems.

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6 Viruses in seawater, sediments and shellfish

This chapter examines the fate of human enteric viruses in the marine environment. We shall consider the factors that prolong their survival when they are associated with solids, the conditions facilitating virus uptake by shellfish, their retention in and release from shellfish, and transmission to humans resulting in outbreaks of hepatitis and gastroenteritis.

Many communities discharge their wastes into estuaries, bays, harbors and other coastal waters. The oceans thus receive billions of gallons of both treated and untreated domestic and industrial wastes, sewage sludges as well as polluted surface waters. The coastal waters of the United States receive more than eight billion gallons of municipal sewage in a single day. A wide variety of human enteric viruses in amounts up to 50 PFU and more per liter are present even in treated effluents, and most sewage is hardly treated adequately. A high percentage of viruses is associated with solids in sewage effluents and these viruses are thereby protected from chlorine inactivation. The distribution of viruses in coastal waters is shown in Fig. 20.

Viruses associated with large sized particulates (> 6μm) soon leave the water column and settle down in the bottom sediments while viruses adsorbed on fine solids including colloids (> 3μm) tend to stay afloat in the water for longer. Viruses associated with suspended solids initially accumulate in a loose, fluffy layer over the compact bottom sediments. Sediments in coastal waters serve as a reservoir of human enteric viruses from which virus can be released into the water

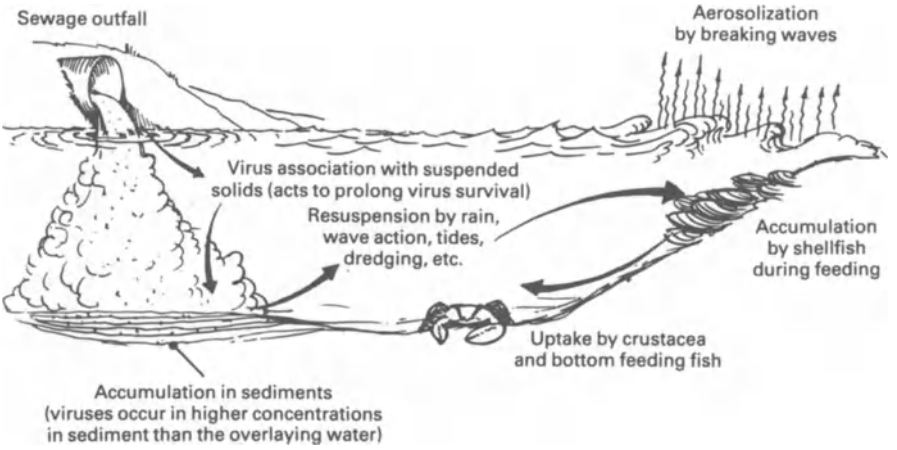

Fig. 20 Scheme depicting the fate of viruses in coastal systems. From Melnick, J. L. and Gerba, C. P. (1980). *Public Health Reviews* 9: 185–213.

column by storm action, dredging, boating, etc. Viruses from fluffy sediments can be more easily resuspended by mild turbulence or water movements. These resuspended viruses from polluted waters can be transported to remote non-polluted areas used for shellfish production and bathing, depending on the current velocity, water circulation pattern and prevailing winds. Several studies in laboratory aquaria have shown that oysters, mussels and clams, which are filter feeding animals (i.e. they sieve out suspended food particles from a current of water passing through the shell cavity) could concentrate enteric viruses from the water in their tissues. Since the entire shellfish is often consumed raw or is inadequately cooked, it can serve as a passive carrier of human pathogenic enteric viruses. Another source of seafood, crabs, can derive human viruses from polluted waters and by feeding on contaminated shellfish.

Viruses transported to bathing beaches can cause a health hazard during recreational activities, primarily swimming (especially if the head is immersed), but infection is also possible as a result of wading and boating.

Monitoring of enteroviruses in marine and estuarine water and sediments

The magnitude of the health hazard associated with the discharge of virusladen sewage wastes into the oceans depends upon the quantity of viruses and their survival in water, sediments, and on suspended solids.

Recent advances in methods of virus concentration from marine waters and sediments have made field studies on virus occurrence and distribution possible. Enteroviruses and rotaviruses can now be concentrated from the required sample size, 400 l of turbid seawater or 1000 g of sediments, with an efficiency of about 50%. An outline of these methods is given in Fig 21 and 22.

1. 400 liters of estuarine water is adjusted to pH 3.5, additional salts (like aluminium chloride) are added and filtered through microporous filters having a large surface area
2. Virus adsorbed to filter material are recovered by passing a liter of proteinaceous solution (like beef extract) at an alkaline pH, 10.5
3. Volume of beef extract is reduced down to 10 ml by a simple and inexpensive process. pH of beef extract is adjusted to 7 using dilute HC1. One gram of magnetic iron oxide is added and the sample stirred with a glass rod for 30 min. Virus adsorbed on iron oxide is settled with a magnet. Supernate is discarded. Virus is released from iron oxide by stirring with 10 ml of isoelectric casein, pH 8.5. Iron oxide is again settled by a magnet and the supernate containing virus is recovered
4. Sample is made sterile by treating with antibiotics
5. Sample is then inoculated into appropriate cell cultures and the virus plaques counted

Fig. 21 Concentration of enteroviruses from estuarine water. Adapted from Rao, V. C. and Melnick, J. L. (1983). Annual Meeting, Amer. Soc. for Microbiol., New Orleans, Q8.

1. 300 g of sediment is mixed with 900 ml beef extract (1 : 3 w/v) containing a chaotropic agent, 2M sodium nitrate, pH 5.5 and shaken for 15 min
2. Virus from sediment is eluted into beef extract. Sediment is deposited by low speed centrifugation and discarded
3. Large volume of supernatant containing virus is reduced to a small volume of 10 to 20 ml. pH of beef extract supernate is adjusted to 3.5 to generate a floc on which virus is adsorbed. Interference from the chaotrope in floc formation is reduced by adding ammonium sulphate. Additional flocculating material (cat floc) is added to the solution and mixed for 20 min
4. Sample is centrifuged to sediment the floc + virus. Pellet is resuspended in sodium phosphate to separate the virus
5. The concentrate is made sterile by treating with antibiotics and inoculated into cell cultures

Fig. 22 A practical method for recovery of enteroviruses from estuarine sediments. Adapted from Waite, D. A. and Sobsey, M. D. (1983). *Applied and Environmental Microbiology* 46: 379–85.

Table 6 Occurrence of enteroviruses in marine and estuarine waters

Country	Sampling site	% of samples positive to virus	Quantity of virus (PFU/10 l)
Israel	Mediterranean (near sewer outfall)	27	600
	Near Bathing beach	13	320
USA	Houston Ship Channel; Houston, Texas	67	0.5–8
USA	Coastal canal community Galveston, Texas	65	0–5
USA	Atlantic Ocean Miami, Florida	100	0–1
West Germany	Baltic Sea	36	5–125
Italy	Mediterranean Sea	100	0.5–40
Romania	Black Sea	22	

Adapted from Melnick, J. L. and Gerba C. P. (1980). CRC critical reviews in *Environmental Control* 10: 65–93.

Enteric viruses have been detected in marine and estuarine waters in various parts of the world (Table 6). In Israel, enteroviruses have been recovered from 20 of the 72 samples taken near a marine outfall with virus concentrations as high as 600 PFU/10 l. In addition, 2 out of 16 samples taken at a bathing beach a mile away

contained enterovirus at about half that concentration. Enteroviruses have been demonstrated in 67% of seawater samples from Houston ship channel. Baltic Sea samples taken near a sewer outfall were positive for virus in four of eleven samples, with concentrations of 5 to 125 PFU/10 l. Italian workers have isolated viruses in seawater near sewage outfalls in 100% of samples, with virus concentrations ranging from 0.5 to 40 PFU/10 l. Investigations in the vicinity of deep-sea sewer outfalls near Miami, Florida yielded virus concentrations from 0 to 1 PFU/10 l.

Reports on the quantity of virus in estuarine sediments have been few. Data given in Table 7 were based on a volume–volume comparison of virus concentration in water and in sediment and probably do not give a true comparison of concentration differences. The data show an approximately 10 to 10,000 fold higher concentration of virus in the sediment than in the overlying water at sites in both Texas and Florida.

Our own recent studies in the Galveston Bay were designed to determine and compare the quantitative distribution of naturally occurring enteroviruses and rotaviruses in water, suspended solids, fluffy sediments, and compact sediment. Data presented in Table 8 indicate that the virus was most often found attached to suspended solids (< 3μm size). 72% of these samples were positive for enteroviruses compared to only 14% of the water samples. Fluffy sediments yielded enteroviruses in 47% of samples, while only 6% of compact bottom sediment samples tested were positive for virus. Rotaviruses were associated with 50% of suspended solids, more than in any of the other fractions. While a greater number of enteroviruses were recovered from fluffy sediments than from suspended solids (39 to 398 PFU per 250 l), the reverse was true of rotaviruses

Table 7 Recovery of enteroviruses from estuarine and marine sediments

Type of sediment	Site location	Virus concentration (PFU/100 l)	
		Water	Sediment
Marine sediment*	Hollywood	0.25	3160
	Miami	0.50	2160
	Miami beach	7.30	9830
Estuarine sediment	1. Coastal canal community along the Texas coast (1 m from sewage outfall)	160	2000
	2. 300 m from sewage outfall	90	2080

* Adapted from Gerba, C. P. Smith, E. M. Schaiberger, G. E. and Edmond T. D. (1979). *Methodolgy for Biomass Determinations and Microbial Activities in Sediments* (edited by C. D. Litchfield and P. L. Seyfried). American Society for Testing and Materials, pp. 64–74.

† Adapted from Gerba C. P. Goyal S. M. Smith, E. M. and Melnick, J. L. (1977). *Marine Pollution Bulletin* 8: 279–282.

Table 8 Recovery of entero- and rotaviruses at Kema and Seabrook in Galveston Bay

Virus group	Sample type	No. examined	% positive to virus	No. of PFU*
Enterovirus†	Water	35	14	3–12
	Compact sediment	35	6	7–10
	Fluffy sediment§	15	47	39–398
	suspended solids**	18	72	4–40
Rotavirus‡	Water	31	16	119–1000
	Compact sediment	8	12	1200
	Fluffy sediment§	15	40	800–3800
	suspended solids**	18	50	1800–4980

* Plaque forming units of virus estimated for 250 liters of water and 1000 grams of compact sediment.
† Data adapted from Rao, V. C. Seidel, K. M. Goyal, S. M. Metcalf, T. G. and Melnick, J.L. (1984). *Applied Environmental Microbiology*. 48: 404–9.
‡ Data adapted from Rao, V. C. and Melnick, J. L. (1984). ASM Meeting, St. Louis, Missouri.
§,** Obtained by filtering 40 to 60 liters of water containing fluffy sediment (loose upper layer of bottom sediment disturbed and suspended in water near the bottom) and a similar volume of water containing suspended solids near the surface. Plaque forming units of virus were calculated for 250 liters.

which were 800 to 3800 PFU per 250 l versus 1800 to 4980 PFU from suspended solids.

Persistence of enteroviruses in the marine environment

Human enteroviruses sustain themselves inside living cells, and once they are discharged into environmental waters, they will eventually die. Laboratory studies indicated that different viruses (polio, coxsackie B, echo and reo) survive for different periods of time at different temperatures.

Many of the viruses tested lost 99.9% of infectivity between 2.5 and 28 days at 20 to 26 °C, but survived considerably longer, between 36 and 130 days, at 3 to 5 °C. Coxsackie virus B3 survived for only 3 days in Mediterranean waters, while it persisted for 28 days in Atlantic waters in the same temperature range. This raises an important point, that besides temperature, there may be other factors influencing virus survival in marine water. This led to a variety of investigations of the effects of salinity, solar radiation, bacterial antagonism, chemicals pollution, suspended solids and sediments. We have chosen to highlight some of those studies which yielded definitive information on virus inactivation or persistence.

Light Inactivation of microorganisms by the ultraviolet component of sunlight has long been recognized, but because of its high attenuation in natural waters, it is only effective near the surface of the water. Using a dye that absorbs visible light, photodynamic oxidation of viruses has been demonstrated by Gerba, Wallis

and Melnick (1977). Natural waters may contain substances such as lignins, fulvic acids, humic acids and vitamins, which may act as photosensitizers. These substances absorb radiation and selectively transfer that energy to dissolved oxygen which is excited to its highly oxidized state. Other environmental factors, such as pH, temperature, time in the water and dye concentration (like methylene blue) affect the sensitivity of the virus. The duration and intensity of light exposure can also influence the rate of virus inactivation. The discovery of a photodynamic antiviral substance in algal cells give support to the idea that photochemical reactions may be important to viral inactivation in nature.

Biological factors A number of investigators have reported a virus inactivating factor in seawater and these results have been reviewed (Akin *et al.*, 1971). Shuval's laboratory observed a 3 log reduction of virus in ten days in normal seawater while only 15 to 20% of virus was lost in heated seawater. A marine bacterium, *Vibrio marinus,* may have been responsible. Virucidal activity has been demonstrated in the Mediterranean seawater and in the Gulf Coast estuarine water. In these studies, neither filtration nor autoclaving of the water destroyed the virus inactivation factor. Thus, it appears that the data on the involvement of biological factors in virus inactivation are not conclusive.

Beside water temperature and association with solids, another reason for the long survival time of enteric viruses, compared to many other viruses, is their relative resistance to proteolytic enzymes. Cliver and Herrmann (1972) indicated that a wide range of proteolytic enzymes did not inactivate three types of poliovirus and five types of coxsackie B viruses.

Other possible mechanisms by which certain bacteria and other microorganisms inactivate enteroviruses have been investigated. Cliver and Herrmann (1972) reported that *Pseudomonas aeruginosa* produced substances with a molecular weight below 500, which appeared to dismantle the virus. Substances of low molecular weight could not be enzymes, and they were referred to as virolytic substances. Some bacteria also produce substances that react with a virion to prevent its adsorption to host cells. In addition, microorganisms can produce certain substances that, while they themselves do not inactivate viruses, sensitize them to inactivation by other processes, such as photodynamic inactivation or enzymic degradation. Other products of microorganisms, such as humic acids, phenolic compounds, and tannin may inactivate viruses by oxidation, reduction or other mechanisms yet to be elucidated.

Adsorption to solids Enteric viruses are known readily to attach themselves to solids in water, and several laboratory experiments yielded data indicating prolonged survival of solids-associated viruses. Potential adsorbents found in marine water include sand, pure clays (montmorillonite, kaolinite, bentonite, illite), naturally occurring suspended solids (algae, bacteria), silts and sediments. Addition of as little as 5 mg/l of kaolinite to artificial seawater seeded with poliovirus resulted in survival of 5% of virus after 6 days while in natural seawater 50 mg/l was required to maintain the same level of virus (Gerba and Schaiberger, 1975). In another experiment, virus seeded natural seawater, artificial seawater and suspensions with 500 mg/l kaolinite were monitored for their virus content for 32 days. In natural seawater a loss of 5.3 logs was observed in 10 days whereas in the sample containing kaolinite it took 32 days for the same loss of virus.

Since estuarine sediments are composed of a variety of clays, bacteria, and sand, a question has been raised as to whether enteric virus survival on pure clays like kaolinite would be comparable to virus survival in field samples of sediment. An answer has been provided by the experiments of Smith, Gerba and Melnick (1978). In a comparison of the survival times of four virus types in seawater and in seawater containing sediments collected from the Galveston Bay, all of the four virus types survived longer when sediment was present.

In the presence of sediment, echovirus 1 and coxsackie B3 had the longest survival time, 18 days or greater; poliovirus, 14 days; and coxsackievirus A9, 4 days. In the sample containing seawater alone, coxsackievirus A9 was undetectable after 2 days, and others persisted for 4, 6 and 10 days.

In a recent study, we examined the survival of poliovirus 1 and rotavirus (SA11) seeded in seawater supplemented with fluffy sediments and suspended solids collected from the Galveston Bay. Since these two fractions of sediment have the greatest chances of being transported to distant areas, data on the survival of viruses associated with them is especially significant. Test results indicated that both the viruses survived longer when associated with solids, whether suspended or sedimented. Both virus types could be detected in the fluffy sediment and suspended solids even on the nineteenth day, but could not be detected beyond the ninth day in seawater (Rao *et al.*, 1984).

Studies on the survival of enteroviruses under field conditions were also carried out at two sites near a sewer outfall in the Galveston Bay. Cellulose dialysis tubes filled with seawater or a mixture of sediment and seawater seeded with known numbers of polio- and echoviruses were held in a plastic bait bucket with perforated sides at a depth of 1.5m. The quantity of virus remaining in the samples was determined at one day intervals for seven days. Both polio- and echoviruses were inactivated much faster in seawater alone than in the presence of sediment, and this confirmed the protective effect of sediment on viruses (Fig. 23).

Although the exact mechanism of increased virus survival while adsorbed to solids and sedimentary particulates is unknown, we may speculate. Adsorbed viruses offer less of their surface for interaction with antagonistic chemicals and it is possible that attachment to a solid surface may stabilize the virion. Degradation of the virus particle would involve a re-orientation of the viral capsid, and the energy binding the virus to the particulate matter would have to be overcome. Thus, energy, either as temperature or as a high energy chemical bond, would be required.

Other factors Aggregation of virus and genetic variability among strains of viruses are also considered relevant to the survival of viruses in the aquatic environment.

Enteric viruses are released in a highly aggregated state from infected host cells, and they probably remain so upon entering waste waters and natural waters (Young and Sharp, 1977). Ionic conditions prevailing in natural waters do not lead to dissociation of these aggregates. Cations such as Ca^{2+} and Al^{3+}, at appropriate concentrations and in combination, have been found to induce aggregation. Poliovirus exhibits aggregation at salinities equal to those present in seawater. Although viral aggregation has not been demonstrated to enhance virus survival in marine water, aggregates are known to exhibit an increased resistance to inactivation during disinfection.

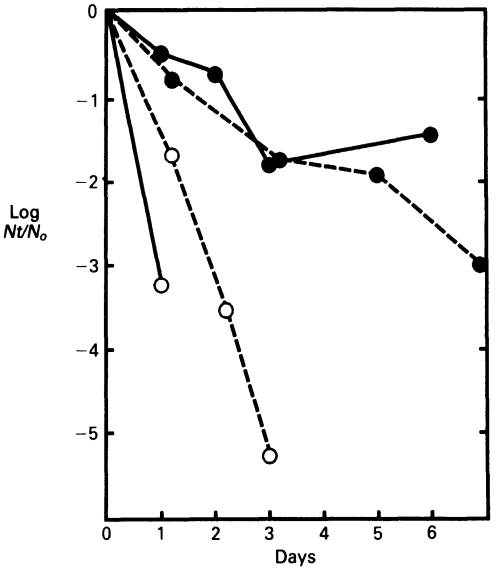

Fig. 23 Survival of poliovirus 1 and echovirus 1 in polluted estuarine water in the Galveston Bay. o — — o: poliovirus suspended in seawater; • — — •: poliovirus suspended in seawater and sediment; o —— o: echovirus suspended in seawater; • —— •; echovirus suspended in seawater and sediment. Log N_t/N_o: the log of virus concentration at time of sampling divided by the virus concentration at time 0 hour. Adapted from Smith *et al.* (1978). *Applied and Environmental Microbiology* 35: 685–9.

The degree of viral aggregation appears to depend not only on the type of virus but also on the specific strain. If aggregation offers a survival advantage in natural waters, we may speculate that some form of genetic selection may be operative for aggregation.

Genetic variability among some strains of viruses may play some role in virus survival in natural waters. For example, it has recently been reported that repeated exposure of poliovirus to sublethal doses of chlorine and passage in cell culture may result in increasing resistance to inactivation by chlorine.

Transmission of enteric viruses by recreational water resources

Recreational use of marine waters may play a significant role in the transmission of enteric viral disease. The US Environmental Protection Agency recently completed a 7-year study on epidemiological–microbiological water quality and gastrointestinal symptoms among bathers. The finding was that a swimming-associated gastroenteritis, primarily in children, could be quantitatively associated with the quality of the bathing water as measured by enterococcus and *Escherichia coli* densities. The gastroenteritis typically had a short incubation period, an acute onset, a short period of relatively benign symptoms and no sequelae, although in some individuals the symptoms were

disabling enough for them to remain at home, remain in bed, or seek medical advice. The association between illness and the presence of as few as 10 *E. coli* per 100 ml suggested that the agent(s) responsible for the illness was highly infectious, was present in sewage in large numbers and/or survived much longer than *E. coli* in the marine environment. These characteristics, along with the nature of illness, suggested a viral etiology.

Role of shellfish in the transmission of enteric viral disease

Shellfish (oysters, mussels and clams) sieve out suspended food particles from a current of water passing through the shell cavity during filter feeding. They can filter from 4 to more than 20 l of water per hour. The feeding rates depend on salinity, temperature, particulate matter and availability of food. Along with food particles, bacteria and viruses are taken in by ciliary action with incurrent water and become entrapped in the mucous secreted on the gills. Then, the mucous is pushed by ciliary action toward the mouth. Since the entire shellfish may be consumed raw or inadequately cooked, they can act as passive carriers of human pathogenic viruses.

Several species of shellfish rapidly accumulate viruses if it is present in polluted waters. Uptake of viruses has been demonstrated in Mediterranean mussels, European oysters, Eastern and Gulf Coast oysters, hard clams, New Zealand marine blue mussels, Pacific oysters and Manila clams. Substantial amounts of virus accumulate within 12 to 24 h, and the level is maintained as long as sufficient virus is present in the surrounding water. The level of virus inside the shellfish is much higher than in the surrounding water. As the concentration of virus decreases in the water, the viral content in shellfish also falls.

Experimental studies in laboratory aquaria revealed that virus uptake varies from one species to another and is enhanced in low turbidity waters having temperatures over 7 °C in a flow-through system over that in a stationary condition (in which water is not replaced continuously). Viruses accumulate mainly in the shellfish digestive tract, and soon diffuse into the haemolymph and mantle cavity fluids. We have noted that food particles are generally entrapped within a mucous mass secreted by the shellfish. Then the mucous is pushed into the mouth by ciliary action. Attachment of virus to mucous is dependent on the salinity and pH of the water.

When placed in clean water in an aquarium, contaminated shellfish have the ability to cleanse themselves from viruses, bacteria and silt. This cleansing process is called 'depuration'. It is the elimination of their fecal contaminants including viruses. In field conditions, when shellfish are shifted from polluted areas on to seabeds in non-polluted waters, they also depurate. Artificial purification or cleansing of shellfish is also accomplished by placing them in large tanks or basins filled with seawater sterilized by filtration, chlorination or ozonation.

A number of factors are known to optimize the process of depuration. They include feeding activity of shellfish on the microorganisms in clean water, a temperature of 20 °C, and a flow-through system. In spite of providing certain optimum conditions based on experimental data, a major problem encountered in commercial purification systems is the lack of a source of clean water close to

oyster harvesting areas. Even if a source of clean water is found, it is susceptible to sporadic contamination by overland runoff.

Crustaceans also accumulate viruses. In aquaria, the Pacific coast shore crabs (*Pachygrapsus* and *Hemigrapsus Sp.*) and the Texas coast blue crab (*Callinectus sapidus*) were experimentally shown to accumulate enteroviruses. When allowed to feed on virus-contaminated mussels, the Pacific shore crabs accumulated 74 to 94% of the virus present in the shellfish. The special problem associated with viral uptake by crabs is that, unlike sedentary bivalve shellfish, they may move back and forth between polluted and non-polluted waters, thus acting as vectors of viral disease.

Viral disease outbreaks attributed to shellfish

Infectious hepatitis and gastroenteritis are the only viral diseases known to be associated with contaminated shellfish consumption. The first documented outbreak of infectious hepatitis traced to consumption of raw oysters occurred in Sweden in 1955 and later such outbreaks were detected in the United States in 1961. These outbreaks have been reviewed in detail (Gerba and Goyal, 1978). Outbreaks that have been documented since then are listed in Table 9 (Melnick and Gerba, 1980).

Table 9 Recent documented outbreaks of viral disease due to shellfish consumption*

Year	Source	Place	No. of cases	Agent
1973	Oysters	Houston, Texas	263	Hepatitis A
1976	Mussels	Australia	7	Hepatitis A
1978	Oysters	Australia	150	Norwalk agent
1978	Oysters	Australia	2000	Norwalk agent
1978	Mussels	England	41	Hepatitis A
1979	Oysters	Alabama; Georgia	8	Hepatitis A
1976–1979	Cockles	England	797	gastroenteritis due to small, round virus-like particles
1980†	Oysters	Florida, USA	6	Gastroenteritis
1980‡	Oysters	Philippines	7	Hepatitis A
1982§	Clams	New York, USA	150	Hepatitis A, gastroenteritis due to Norwalk-like virus
1984**	Cockles	Singapore	322	Hepatitis A

* Adapted from Melnick, J. L. and Gerba, C. P. (1980). *Public Health Reviews* 9: 185–213.
† Gunn, R. A., Janowski, H. T. Lieb, S. Prather, E. C. and Greenberg, H. B. (1982). *American Journal of Epidemiology* 115: 348–51.
‡ Ohara, H., Naruto, H. Watanabe, W. and Ebisawa, I. (1983). *J. Hyg. Camb.* 91:163–5.
§ Morbidity and Mortality Weekly Report, Center for Disease Control, Atlanta, Georgia (1982). 31: 449–51.
** Goh, K. T., Chan, L. Ding, J. L. and Oon, C. J. (1984). *Bulletin of the World Health Organisation* 62: 898–9.

In one of the examples reported from Australia, 7 individuals out of a family of 16 developed infectious hepatitis one month after a family picnic. These individuals consumed incompletely cooked mussels (*Mytilus edulis*). The disease was confirmed serologically (by detection of hepatitis A antigen in stools) and a relationship was demonstrated between shellfish consumption and development of the disease. Recent studies indicate that Norwalk-like agents may be responsible for a significant number of shellfish-associated cases of gastroenteritis. In addition to epidemics, sporadic cases of infectious hepatitis can be attributed to the ingestion of contaminated shellfish. Even so-called sporadic cases may not be truly sporadic because a common source is often not suspected and no investigations are carried out.

A prospective, controlled epidemiologic study was conducted to determine the modes of transmission of nonepidemic infectious hepatitis cases among patients in Boston hospitals. Ingestion of raw shellfish was significantly more frequent (34/185) in infectious hepatitis patients than in controls (10/185). Only 4 of the 34 patients who had eaten raw shellfish had a history of contact with jaundiced persons. Also, ingestion of steamed clams (13/104) was more common in patients than in matched controls (2/104); only 3 of the 13 patients had been exposed to jaundiced persons. When considered together, ingestion of steamed clams or raw shellfish was as frequent a potential exposure to hepatitis as was contact with jaundiced persons.

Studies in Germany indicated that consumption of contaminated mollusks accounted for an estimated 19% of hepatitis A cases of Frankfurt. The cases were chiefly attributed to eating oysters and mussels from the Mediterranean littoral, especially from the shores of southern France and Italy.

In addition to causing infectious hepatitis, polluted shellfish may also be responsible for outbreaks of nonbacterial gastroenteritis. In a series of 33 outbreaks, 797 people suffered from food poisoning during one year in Southampton and Chelmsford, UK. Epidemiologically, all of the outbreaks were traced to consumption of cockles gathered from waters known to be polluted by sewage, and at a time when gastroenteritis was apparently widespread in a nearby area. Small, round, virus-like particles were detected by electron microscopy in a high proportion of fecal specimens from three of the outbreaks. On the basis of morphology, these particles were thought to be the agent of winter vomiting disease but proved to be serologically distinct,

In January 1980, six out of 13 persons (46%) in a small town in Florida, USA, became ill with Norwalk virus gastroenteritis after eating raw oysters. Norwalk virus infection was identified by a sensitive and specific serologic radioimmunoassay.

Viral gastroenteritis in association with HAV is not known to have been reported following shellfish consumption. Such outbreaks have recently been reported from the State of New York. During 1982, fourteen separate outbreaks of gastroenteritis associated with consumption of raw clams have occurred. Of 150 persons affected, seven individuals subsequently developed hepatitis A 21 to 37 days after eating clams. The presence of IgM antibody to hepatitis A has been demonstrated. The clinical findings observed in several of the New York outbreaks are compatible with a viral etiology, such as the Norwalk virus: a short incubation period, abrupt onset of gastrointestinal illness and brief duration (1 to

2 days). Absence of bacterial pathogens and virus-like particles in clams from one outbreak support a viral etiology in several of these outbreaks.

Isolation of viruses from shellfish

Several studies on the occurrence of enteric viruses in shellfish have been summarized (Gerba and Goyal, 1978). Virus recoveries have been made from *Crassostrea virginica, Mercenaria mercenaria, Mytilus edulis, Mya arenaria,* etc. Viruses have been isolated most often from shellfish collected from heavily polluted waters closed to shellfish harvesting, but on occasion shellfish from approved waters have also yielded viruses. The isolation of echo viruses in mussels from an Italian market, coxsackie A viruses from a French market, and poliovirus in oysters imported into the United States from Japan are examples of the occurrence of human enteric viruses in marketed oysters even in developed countries.

Adequacy of bacterial standards

It is time consuming and expensive to monitor seawater and shellfish routinely for viruses. Presence of fecal coliform bacteria is used as evidence of recent fecal pollution of water. Bacterial standards in the United States for shellfish and shellfish-growing waters state that the most probable number of coliforms in water should not exceed 70 per 100 ml, and no more than 10% of samples should exceed 230 coliforms per 100 ml. Shellfish meat itself should contain no more than 230 fecal coliforms per 200 g. Several outbreaks of viral disease have been associated with shellfish harvested from waters which met bacteriological standards and which were certified for oyster harvesting. Effective control of enteric bacterial diseases spread by recreational water use and shellfish has resulted from the establishment of bacteriological standards. No outbreaks of shellfish-associated typhoid fever have occurred since 1954 in the USA. However, controversy has centered around the adequacy of bacteriological standards to reflect a viral disease hazard and several studies have now demonstrated the occurrence of enteroviruses in shellfish, shellfish harvesting waters and marine recreational waters that met currently accepted bacteriological standards. Recently, we conducted a statistical analysis of several major field studies on the occurrence of viruses and indicator bacteria in the marine environment. Enteroviruses were detected 44% of the time in recreational water considered acceptable as judged by fecal coliform standards; they were detected 35% of the time in water which met acceptable standards for shellfish harvesting. Our evaluation is that the bacteriological standards do not reflect the occurrence of enteroviruses, and perhaps other human pathogenic viruses, in marine water.

Virus detection in shellfish

A number of methods for recovering enteric viruses from laboratory-contaminated as well as field samples of shellfish have been described. Details of

1. Shellfish meat is homogenized in distilled water, pH 5 and conductivity <2000 mg/NaCl. Sample is centrifuged to sediment the particulates on which virus is adsorbed. Supernate is discarded
2. Viruses are recovered by resuspending sediment in glycine–saline, pH 7.5 and conductivity ≥8000 mg/l NaCl
3. Virus in a large volume of glycine–saline is concentrated to a small volume by adjusting its pH to 4.5 and adsorbing virus on the floc which is sedimented by centrifugation. Sediment is resuspended in 0.1N Na_2HPO_4, pH 7.4 for releasing the virus
4. Concentrate is treated with antibiotics to eliminate bacteria and then inoculated into appropriate cell cultures for virus detection.

Fig. 24 Scheme for recovering viruses from oysters. Adapted from Sobsey *et al.* (1978). *Applied and Environmental Microbiology* 36: 121–8.

one method are given in Fig. 24. When this procedure was tested on (groups of 3) oysters experimentally contaminated with 4 different enteroviruses in low concentrations (30 PFU per pool), virus recovery averaged 63%.

Summary

A wide variety of human enteric virus pathogens including those causing poliomyelitis, infectious hepatitis and acute gastroenteritis are present in amounts up to 50 infectious particles and more per liter even in treated sewage effluents. Most of them are associated with suspended solids. Solids-associated viruses in effluents discharged into coastal waters settle down into bottom sediments. From 10 to 10,000 times more viruses per unit volume may be found in sediments than in overlaying seawater at some polluted sites. Coastal sediments represent a reservoir of viruses and a source from which viruses can be released into the water column by water turbulence.

Physical, chemical and biological factors control virus persistence in estuarine and marine waters. Temperature is probably the most important factor in virus survival; viruses survive longer at lower temperatures. The ultraviolet component of sunlight and natural photodynamic processes may inactivate viruses. Although marine microorganisms have been implicated in virus inactivation in seawater, the virucidal agent(s) remain(s) yet to be identified. Virus survival is prolonged by adsorption to clays and other suspended solids.

Viruses adsorbed to sediments may be transported to distant non-polluted recreational or shellfish growing waters by water movement patterns. Bioaccumulation of virus by shellfish is the basis for shellfish-transmitted hepatitis and acute gastroenteritis following consumption of uncooked virus-carrying shellfish. Several outbreaks of shellfish transmitted hepatitis and acute gastroenteritis have been reported throughout the world.

Marine recreational water may play a significant role in the transmission of enteric viral disease. Swimming-associated gastroenteritis, probably related to rotavirus or Norwalk virus, has been recently reported.

Not only bivalves, but also crabs have been shown to acquire viruses from polluted water and, in addition, by feeding on contaminated mussels. Disease outbreaks attributed to consumption of contaminated crabs have not been reported, but the potential for a public health hazard exists.

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Glossary

Allotrope: a chemical element that exists in two or more different forms as oxygen and ozone.

Anaerobic digestion: complex organics in the sludge from sewage treatment plants are converted into methane and carbon dioxide by bacteria in the absence of oxygen at 32 to 35 °C.

Antibody: protein produced in the body to neutralize an antigen.

Antigen: a virus, an enzyme or a toxin to which the body reacts by producing antibodies.

Bacterial antagonism: killing or inhibition of viruses by bacteria.

Common source outbreak: a viral disease oubreak traced to a common source like contaminated water or food.

Convalescent serum: serum collected between the end of a disease and the patient's restoration to complete health. It usually contains higher levels of antibody against a pathogen than the original serum sample.

Cytopathic effect: diseased condition of a cell caused by a virus. A sensitive and rapid method for recognition of enterovirus infection in cell culture.

Dialysis: separation of crystalloid from colloidal substances (or smaller molecules from larger ones) in a solution by interposing a semipermeable membrane between the solution and water.

ELISA: abbreviation for Enzyme Linked Immunosorbent Assay. A test for the detection of antigen in clinical samples and for demonstrating viral antibody in serum samples.

Encephalitis: an acute inflammation of the brain and the spinal cord.

Hydroextraction: removal of water through a semipermeable membrane sorrounded by polyethylene glycol which absorbs water. A procedure used in concentrating viruses from small volumes of water.

Immune electron microscopy: concentrating virus particles on antibodies for observation in electron microscope.

Intercerebral passage: passage or transfer of poliovirus from the cerebral tissue of one monkey to that of another monkey.

Myocarditis: inflammation of the muscular walls of the heart.

Myositis: inflammation of a muscle.

Myotropic: having an affinity for the muscular system.

Neurotropic: having an affinity for the nervous system.

Pericarditis: inflammation of the pericardium, the membrane around the heart.

PFU: Plaque forming unit. Plaques are colourless areas of dead cells surrounded by viable cells stained with neutral red, a vital dye. A plaque is produced by a single virion or an aggregate of virions on a monolayer culture of cells.

Photodynamic oxidation: inactivation of viruses in water or wastewater by visible light in the presence of photosensitizing dyes such as methylene blue.

Pleurodynia (Bornholm disease): pain in the pleura (membrane enveloping the lungs and lining the thoracic wall in the chest).

Seroconversion: effectiveness of a viral vaccine in inducing development of antibody in humans.

Sludge: solids from sewage or water that settle to the bottom of sedimentation tanks.

Temperature marker test: A test in tissue culture to differentiate virulent (viruses growing at 40 °C) from attenuated (viruses growing at 36 °C) in regard to poliovirus.

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