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Occurrence of enteroviruses and *Giardia* cysts in sewage sludge before and after anaerobic digestion

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OCCURRENCE OF ENTEROVIRUSES AND GIARDIA CYSTS IN SEWAGE SLUDGE BEFORE AND AFTER ANAEROBIC DIGESTION

By

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STATEMENT BY AUTHOR

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To my parents Horacio and Bia Soares whose constant love and encouragment have given me the strength to pursue my goals.

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ABSTRACT

The disposal of anaerobically digested sewage sludge onto farmland in Pima county has created the need to evaluate the potential public health impact of pathogens which are indigenous to sewage and may be present in sludge. The occurrence of enteroviruses and Giardia cysts in sewage sludge before and after anaerobic digestion was monitored for a period of 14 months. This study showed that significant concentrations of enteroviruses and Giardia cysts are present in anaerobically digested sewage sludge being applied to farmland in Pima County. The concentration of Giardia cysts ranged from 1.33×10^{3} to 8.6×10^{4} per liter of raw sludge and 2.0×10^{3} to 2.8×10^{4} per liter of treated sludge. The concentration of enteroviruses in sludge ranged from 1.74×10^{2} to 1.28×10^{4} per liter before anaerobic digestion and from < 2 to 5.63×10^{2} per liter after treatment. The percentage of virus removal after anaerobic sludge digestion varied from 73% to > 99.95%. Methods to study the fate of enteroviruses in the sludge-soil matrix were also evaluated. An increase in the ratio of eluant to solids seemed to enhance virus recoveries from sludge:soil mixtures.

INTRODUCTION

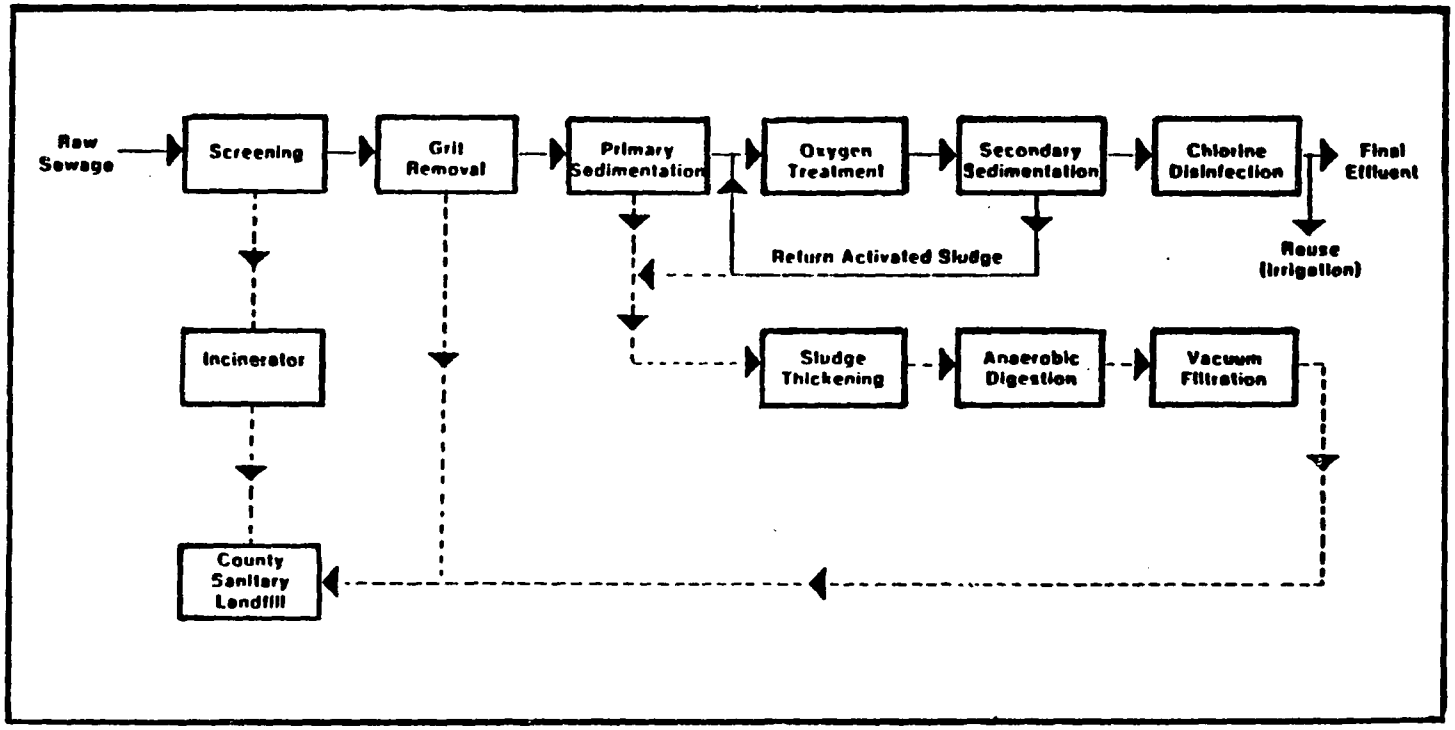
Disposal of domestic sewage sludge is a major problem faced by most communities in the United States. Land disposal avoids pollution of surface waters, in particular lakes, rivers, and streams and it is an economical alternative of disposal, since the soil enrichment and fertilizing qualities of sludge are utilized as a resource. Sewage sludge contains organic matter, nitrogen, phosphorous, calcium, magnesium, and certain trace elements which are of value to agriculture (Coker 1983). Sludge may also improve soil structure and the water-retention capacity of the soil due to its rich organic matter content (De Hann 1976; cited by Coker 1983, Bitton 1980). Population increases in the metropolitan areas and the federal and state regulations for higher treatment levels of sewage, makes land disposal of sewage sludge a promising method (Bitton 1980). Dorn et al. (1985) stated that the amount of sewage sludge requiring disposal has increased since the Federal Government required sewage plants to include secondary treatment. In addition, the Water Pollution Control Act Amendments of 1972 required communities to consider alternative treatment techniques (including sludge recycling to land) before applying for wastewater construction grants. Incineration of sludge requires large amounts of energy and must be done in compliance with the Air Quality Act of 1967 (Dorn et al. 1985).

The application of human and animal wastes to farmland is in fact, an ancient practice (Engelbrecht 1978; Hurst 1989 ; Rose 1986). In the United States, land application of sewage sludge to farmland dates back to at least 1922 (Wolman 1977 ; cited by Hurst 1989). According to Scanlan et al. (1989) an average of 16% of domestic sewage sludge in the U.S. is applied to land for agricultural utilization, land reclamation, forest utilization, and land disposal.

LAND APPLICATION OF DOMESTIC SEWAGE SLUDGE IN PIMA COUNTY

In Pima County, sludge application to farmland has been the major mechanism of disposal since 1984. Anaerobically digested sludge is applied to cotton fields at a rate suitable to meet the nitrogen requirements of cotton which is approximately thirty thousand gallons per acre. At the Ina Road wastewater treatment plant, located near Tucson, sewage sludge results from settling of raw sewage solids during primary treatment of wastewater and the activated sludge treatment processes. Figure 1 represents a flow diagram of the treatment process at the Ina Road plant. This plant also receives sewage sludge from the Roger Road wastewater treatment plant for treatment. The sludge generated during primary and secondary sedimentation is combined and treated by anaerobic digestion with a mean retention time of 16 days before disposal. This process reduces the bulk of organic matter and prevents putrefaction by decreasing biological oxygen

FIGURE 1
THE INA ROAD WATER POLLUTION CONTROL FACILITY



demand levels (Hurst 1989). However, most of the viral, bacterial, and parasitic human pathogens are concentrated in sludge during settling of primary and secondary sludges. The four major groups of enteric pathogens which are excreted in feces and therefore present in sewage are viruses, bacteria, protozoan cysts, and helminthic ova (round worms, tape worms) (Fradkin et al. 1989). Viruses are too small to settle, but are also concentrated in sludge due their association with particulate matter. The application of sewage sludge to farmland poses some questions about the human health and animal health consequences of this practice (Dorn et al. 1985). Bitton (1980), stated that viruses associated with sludge flocs made of microbial biomass, organic, and inorganic debris, are not totally inactivated, which is an important issue to consider when considering safe measures of sludge disposal.

In Pima county, sludge is applied to cotton fields only, which eliminates the risk of contamination of food crops. However, it still creates a concern due to the potential for groundwater contamination, Pima county currently relies solely on groundwater for drinking water and domestic use. Viruses which are shed in feces or urine and capable of producing infection when ingested have the potential to be transmitted by inadequately treated water (Grabow 1968; Melnick et al. 1978; Mosley 1967; WHO Scientific Group, 1979). Quoting from the IAWPRC Study Group in Water Virology: " Water is one of the most universal and common

potential sources of infection, therefore, the virological quality of water needs serious attention". Reports on the minimum infectious dose of animal viruses have been reviewed by Ward and Akin (1984). Gerba and Haas (1988) have also reviewed literature on minimum infectious dose of enteric viruses, incidence of clinical illness, and mortality in order to assess the risks which may be associated with exposure to human enteric viruses. Gerba and Haas (1988) reinforced that such studies are necessary to assess the public health impact of various methods of sludge disposal and suggested that significant risks of illness (>1 : 10 000) and mortality (>1 : 1 000 000) may arise from the exposure to low levels of enteric viruses. Groundwater accounts for almost half of the waterborne disease outbreaks in the United States and enteric pathogens are the most common cause of water and foodborne illness (Craun, 1984). Therefore, any disposal mechanism of domestic sewage which presents the risk of microbial contamination of water supplies should be carefully monitored.

Other detrimental aspects of sludge are the presence of heavy metals which can be incorporated into the food chain. Accumulation of high levels of cadmium has been observed in the livers and kidneys of cattle exposed to a sewage recycling site and caws grazing on sludge-treated pastures (Kienholz et al. 1977; Fitzerald 1980). Nitrate pollution of groundwater is another concern since high levels of nitrate have been associated with a condition known as methemoglobinemia (blue-

babies) which affects infants under four months (Bitton 1980).

This study was focused on the occurrence of enteroviruses and Giardia cysts in domestic sewage sludge and the level of reduction of these enteric pathogens after treatment. In addition, sensitive techniques for the recovery of low numbers of viruses from sludge treated soils were investigated. Such techniques are necessary for the proper assessment of virus survival and movement in sludge-treated soils.

U.S. ENVIRONMENTAL PROTECTION AGENCY SEWAGE SLUDGE REGULATIONS

The U.S.EPA is in the process of preparing new regulations for the utilization and disposal of domestic sewage sludge. The final regulation promulgation is schedule for completion in October of 1991. A review prepared by the members of the Water Pollution Control Federation (WPCF) Residuals Management Committee, summarized the EPA proposed rules in which three pathogen reduction classes were established. These being class A, B, and C, which stipulate the detection levels of pathogenic organisms which are not to be exceeded in sludge. Class A requires that pathogenic organisms be equal to or less than 3 Salmonella sp., 1 plaque forming virus unit, 1 protozoan organism, and 1 helminth ova per gram of volatile suspended solids (VSS), or that the sludge temperature be raised to 53°C for 5 days, or an equivalent treatment. It further

requires that the concentration of indicator organisms be equal to or less than 2 $\log_{10}$ fecal coliforms, and 2 $\log_{10}$ fecal streptococci per gram of dry volatile suspended solids. This must be accomplished with required vector (flies, rodents, mosquitoes, and other organisms) attraction reduction. If vector attraction reduction is by injection, the concentration of fecal coliforms and fecal streptococci cannot exceed 3 $\log_{10}$ per gram of dry weight volatile suspended solids before injection. Class B requires a 2 $\log_{10}$ decrease in Salmonella sp. and viruses, and an average fecal coliform and fecal streptococci concentration equal to or less than 6 $\log_{10}$ per gram of VSS. Class C requires a 1.5 $\log_{10}$ reduction in Salmonella sp. and viruses, and average densities (expressed in $\log_{10}$) equal to or less than 6.3 for fecal coliforms and 6.7 for fecal streptococcus. The reduction is measured by the difference of treatment plant influent and final sludge product concentrations. Class B and C sludges result from the following treatments: biological, lagoon, air drying, or chemical addition methods, or storage for at least one day. Vector reduction for class A, B, and C may be accomplished by any of these means: (1) 38% reduction in VSS; (2) 15% or less reduction in volatile solids by anaerobic digestion, plus additional processing for 40 days at 30°C by anaerobic digestion; (3) a specific oxygen uptake that is less than 1 mg O_2 / g solids . h for anaerobically digested sludge; (4) a raise in sludge pH to 12 or above without further alkali adjustment, pH remaining at 12 for two hours and then at 11.5 for

22 hours; (5) percent solids is 75% or greater; (6) sludge is injected below soil surfaces with no evidence of liquid on the surface within 1 hour of injection. The regulations also include specific monitoring (determining the concentration of chemical pollutants and determining compliance with pathogen and vector attraction reduction requirements), record keeping, and reporting requirements for each method of disposal. Other general requirements for land application of sludge state that land application must no cause harm to endangered species. In addition, it cannot restrict flood flow or the temporary water storage capacity of the floodplain, it cannot cause hazard to human and animal health or water sources through runoff, and it cannot be applied to frozen, snow covered, or flooded land unless it can be applied without posing a treat to human health or the environment. Sludge cannot be applied within ten meters of surface water, or to land at rates in excess of the nitrogen requirements of the vegetation grown, and it cannot cause leaching of nitrogen to groundwater. When class A sludge is applied, public access is not permitted until a vegetative cover is established, and for class B and C, human access is not permitted for 12 months and a vegetative cover is also required. Sites for land application are referred to as agricultural land if crops or animals are raised for human consumption and non-agricultural land if neither food nor animal feed crops are grown. For agricultural land food crops, where harvest parts are totally above ground but touch either the sewage sludge

or the mixture of soil, must not be grown for a period of 18 months after sludge application. When the harvested parts are bellow ground, food crops cannot be grown for 5 years after sludge application. The waiting period may be reduced to 18 months if it can be shown that no viable helminth ova are present in the soil. Feed crops cannot be harvested for 30 days after application of class A and B sludges and 60 days after application of class C sludges.

VIRUSES IN WASTEWATER AND SLUDGE

Today, more than 120 different types of pathogenic viruses are known to be excreted in feces and several others can be excreted in urine (Hurst 1989), therefore can be expected to be present in domestic sewage. Fradkin et al. (1989) pointed out that the number of human pathogenic enteric viruses present in sewage has gone up by 14 in the past decade and that there may be many more unrecognized viruses present in sewage. Enteric viruses are those which multiply in the intestines, are excreted in large numbers in feces of infected individuals and can be transmitted by the fecal-oral route. Table 1 lists the enteric viruses and the associated health problems.

Enteroviruses are ssRNA spherical, nonenveloped viruses with icosahedral capsid, ranging in size from 25 to 10 nm. The genera enterovirus belongs to the family Picornaviridae and consists of different strains of Poliovirus, Echovirus,

TABLE 1. Human viruses which are shed in feces and may be present in sewage and sludge.

VIRUS GROUP	NUMBER OF SEROTYPES	ILLNESS CAUSED
Adenovirus	41	Pharyngitis,conjunctivitis, respiratory illness, vomiting, diarrhea
Astrovirus	5	Vomiting, diarrhea
Calicivirus	2	Vomiting, diarrhea
Coronavirus	1	Vomiting, diarrhea
Enterically transmitted Non-A non-B hepatitis virus	1	Hepatitis
Enterovirus		
Poliovirus	3	Paralysis, meningitis, fever,
Coxsackievirus A	24	Herpangina,respiratory illness, meningitis, fever
Coxsackievirus B	6	Myocarditis, congenital heart anomalies, rash, fever meningitis, respiratory illness, pleurodynia
Echovirus	34	Meningitis, encephalitis, respiratory disease, rash, diarrhea, fever
Enteroviruses 68-72	4	Meningitis, encephalitis, respiratory illness, acute hemorrhagic conjunctivitis, fever
Hepatitis A virus	1	Hepatitis
Norwalk virus	1	Epidemic vomiting and diarrhea
Parvovirus	2	One type possibly associated with enteric infection
Reovirus	3	Not clearly established
Rotavirus	4	Vomiting, diarrhea
"Samll Round Viruses"	2	Vomiting, diarrhea

Adapted from Hurst (1989). Fate of viruses during wastewater sludge treatment processes. Critical Reviews in Environmental Control. 18: 317 - 343.

Coxsackievirus A and B and HAV (Hepatitis A virus). These are the most commonly studied group of enteric viruses in sewage, (Fradkin et al. 1989), because techniques for the isolation of enteroviruses have been available for several decades and with the exception of HAV they can easily be grown in cell culture lines.

The methodologies for the isolation and identification of viruses from environmental samples are very limited. It is a very expensive and time consuming process which requires working with cell lines, a well equiped laboratory, and well trained technicians. Furthermore, different viruses require different cell culture lines and different conditions of growth.

According to Bitton (1980), concentrations of enteroviruses in raw sewage have been estimated to vary between 5,000 to 28,000 per liter. Melnick and Gerba, (1981) reported that enteric viruses can be excreted in concentrations as high as 10^{10}/g of feces of infected individuals and concentrations as high as 460,000 infectious virus per liter have been found in raw sewage. Fradkin et al.(1989), reported concentrations of enteric viruses to be between 10^{2}-10^{4}/g dry weight of primary sludges, and 3 x 10^{2}/g dry weight of secondary sludges. In anaerobically digested sludge, concentrations of 800 to 4,500 plaque forming units (PFU) per liter have been detected (Cliver 1975). Schwartzbrod and Mathieu, (1986) reported concentrations of enteric viruses in anaerobically digested sludge

to vary from 0 to 130 PFU/l (44% of the samples being positive). Concentrations of 4 to 210 PFU/l and 300 to 3370 PFU/l have been reported by Goyal et al. (1984) and Berman et al. (1981) respectively, as cited by Schwartzbrod and Mathieu, (1986). Other studies have reported an average concentration of 5.1 x 10^3 PFU/l of anaerobically digested sludge (Lewis and Nath, 1983). The amount of viruses present in sewage is, however, highly variable and depends on factors such as the hygienic level of the population, prevalence of infection in the community, the season, climate, and population density (fradkin et al. 1989). In the U.S., peak levels of viruses in sewage occur in the late Summer and early Fall (Melnick and Gerba 1981).

Engelbrecht (1978), pointed out that density of viruses in wastewater do not include all viruses since the methodologies involved are highly selective. Morris and Wait (1980) showed that there are large differences in the recovery efficiency of the method of concentration for different enterovirus. The efficiency of reconcentration using beef extract organic flocculation method, which depends on virus adsorption-elution from an organic floc, was 9% for coxsackievirus B4, 7% for Echovirus 1, 98% for coxsackievirus B3, and 40% for poliovirus 1. Bitton et al. (1979) observed that the efficiency of recovery of poliovirus 1 and coxsackievirus B3 was almost 10-fold greater than that of echovirus 1. This may be due to major differences in their adsorptive behavior.

In a study performed by Williams and Hurst (1988), the concentration of Adenoviruses in raw sewage sludge was estimated to average 10,800 per liter, but when more sensitive methods were used, the virus concentration corresponded to 54,000 viruses per liter. The concentration of enteroviruses was, however, only 1320 per liter. Hurst et al. (in press; cited by Hurst 1989) reported that the concentration of Adenoviruses in raw primary sludge has been estimated to exceed that of the enteroviruses by a factor of 94-fold. Most Adenovirus serotypes cause upper respiratory illness and conjunctivitis, but 80% of the serotypes isolated in raw primary sludge were of the serotypes 40 and 41 which caused gastroenteritis. These examples demonstrate how limitations in the methodologies and the difficulties involved with the isolation and identification of viruses lead to underestimation of their actual numbers in environmental samples, such as sewage. Fradkin et al. (1989) also pointed out that in regard to viruses in environmental samples, only a small percentage is normally detected, even by the best procedures and that the concentrations of these pathogens are only as precise as the assays themselves.

VIRUS INACTIVATION DURING ANAEROBIC SLUDGE DIGESTION

Farrah (1983), reported viral inactivation of 90 to 95% after anaerobic digestion. Eisenhardt et al. (1977) also reported a 95% inactivation during

anaerobic sludge digestion. Berg and Berman (1980) observed 80 to 90% inactivation of viruses during anaerobic mesophilic digestion (35°C, 20 day mean residence time). Hurst (1989) reported virus inactivation ranging from 50 to 99% per day, during anaerobic mesophilic digestion. Because of the initial high concentration of viruses in fresh undigested sludge, significant amounts of viruses remain after anaerobic digestion. The presence of even low numbers of enteric viruses in sludge is a major concern because: (1) not all enteric viruses can be isolated due to lack of appropriate techniques, (2) currently available techniques give less than 50% recovery rates, (3) viruses have a very low infectious dose, the presence of one infectious unit (detectable in tissue culture) can create a potential for disease, (4) studies have suggested that enteric viruses survive longer in the environment than enteric indicator bacteria and have a high resistance to disinfectants such as chlorine (Rose 1986).

Sagik et al. (1980) demonstrated with a hypothetical example how significant amounts of human enteric viruses can be transported to land disposal sites. The authors assumed a concentration of 1×10^{3} enteric viruses per liter mixed liquor suspended solids and a solid level of 0.2 to 0.4%. They demonstrated (theoretically) that even with a 99% reduction in virus level in the anaerobic digestion process, the application of 10 dry T/ acre/yr (22.4 t/ha) to soil, would imply the addition of more than 2×10^{7} plaque forming units (PFU) per acre or

1×10^{3} PFU / ft^{3} assuming injection or plowing to a 6 inch depth. They pointed out that the public health significance of these and other organisms in the soil would depend on factors such as their survival in the soil, their potential for movement to surface or groundwater and the uses to which the site is to be put.

Viruses are removed during wastewater treatment primarily through their association with sewage sludge solids and their partitioning into the various sludge fractions during collection of the suspended solids (Akin et al. 1978). Their association with solids creates an additional problem for the disposal of sludge, since it tends to increase virus survival in the environment (Ward et al. 1976). The mechanism for viral inactivation during anaerobic digestion is not well understood. Ward and Ashley (1977a) and Bitton (1980) believe that the virucidal agent in digested sludge is ammonia in its uncharged form at alkaline pH levels. Ward and Ashley (1977b) observed that two proteins and the RNA of poliovirus are cleaved when the virus is incubated in digested sludge. They believe that ribonucleases and proteases present in sludge do not account solely for viral inactivation since these enzymes in the absence of sludge do not have any detectable effect on viral infectivity (Ward and Ashley 1977b). Enteroviruses are inactivated by ammonia while reovirus is insensitive to ammonia. On the other hand, reovirus inactivation by heat is accelerated by detergents present in sludge while poliovirus and other virus of the Picornaviridae family seem to be protected

against heat inactivation by detergents such as sodium dodecyl sulfate (SDS) (Ward et al. 1976). Therefore, as was suggested by Hurst (1989), viral inactivation during anaerobic digestion of sewage sludge may depend upon virus type. It may be of value to note that the process of settling of sewage sludge and removal of virus is dependent upon the nature of the solids and the virus type and strain (Hurst 1989). How different viruses adsorb onto solids may be related to their protein structure and isoelectric point (Mouillot and Netter 1977; Gerba et al. 1980)

FATE OF VIRUSES IN SOIL

Land disposal of wastewater has several benefits including water recycling and groundwater recharge (Hurst et al. 1979; Gerba and Lance 1978). However, several studies have shown that viruses can survive in the environment for long periods and have the potential to travel great distances both vertically and laterally through the soil profile, (Goyal et al. 1984; Yeager and O'Brien 1979; Vaughn et al. 1983). Enteroviruses have been found in groundwater after land application of wastewater (Goyal 1979). Melnick and Gerba (1981) have reviewed several reports of outbreaks of gastroenteritis due to sewage contamination of groundwater. According to Bitton (1980), sieving may remove bacteria, but not small colloidal size particles such as viruses. However, viral adsorption to solids

plays an important role in their removal by soils (Bitton 1980; Gerba et al. 1975; Nell and Engelbrecht 1982; Goyal and Gerba 1979). Viruses in sludge tend to be solid-associated which along with their further association with soil particles after land disposal, helps in preventing their movement through the soil profile to reach groundwater. Viruses are indeed retained effectively by the sludge-soil matrix, therefore, minimizing their transport to groundwater (Bitton 1980; Damgaard-Larsen et al. 1977). An advantage of sludge application to land is that solid-associated viruses are immobilized at the upper layer of the soil profile (Bitton et al. 1984).

However, the adsorption of viruses to soil particles, as well as sludge, has been shown to be both type and strain dependent, Bitton at al. 1984; Gerba et al. 1980; Gerba and Goyal 1981). Poliovirus has been shown by Gerba et al. (1980) to readily adsorb to activated sludge and a sandy loam soil (99.99%) while adsorption of echovirus and coxsackievirus strains ranged from 0 to 99.99% for sandy loam soil and 87 to 99.99% for activated sludge. Lewis and Nath (1983) demonstrated that 99% of the poliovirus added adsorbed to anaerobically digested sludge. In a study performed by Goyal and Gerba (1979), with 27 different enteroviruses, adsorption of echovirus 1, 12, 29, SA-11 (simian rotavirus) to a Flushing Meadows soil was 55, 78, 14, and 51% respectively. Coxsackievirus 3 varied between 0 and 30% and adsorption of different strains of poliovirus varied

between 98 and 99.9%. The authors suggested that viruses that adsorb poorly to soils may show a greater propensity to travel through the soil profile and not be as readily removed. In fact, Gerba et al. (1980) has shown that enteroviruses which adsorb poorly to soil, tend to migrate further in soil columns. On the other hand, several studies have shown that even viruses that adsorb poorly to soil are retained in the sludge-soil matrix. In Denmark, soils ranging from 5 to 21% clay were able to completely retain sludge-associated coxsackievirus B3, (Bitton et al. 1980). Damgaard-Larsen et al. (1977) also showed that echovirus 1, inspite of its poor capacity for adsorption, was not found in any of the sludge-treated soil leacheates.

The differences of the adsorptive behavior of viruses are based on the configuration of their outer protein coat, which determines the net charge of the virion. Viruses consist of a nucleic acid surrounded by a protein coat, known as capsid, which gives them the colloidal characteristics of proteins (Gerba et al. 1975). Amino acids such as glutamic acid, aspartic acid, histidine, and tyrosine, contain ionizable groups which gives the virion an electrical charge (Gerba 1984). Since viruses behave like proteins, they also have an isoelectric point and the net charges on their surface groups is controlled by the pH of the medium. As a result, environmental factors such as pH of the medium, presence and concentration of cations, soil type (percentage of clay and sand), presence of organic matter, cation-

exchange capacity, all play a role in viral adsorption to soils (Bitton 1975; Gerba and Goyal 1981). According to Gerba and Goyal (1979), pH is the most important factor influencing viral adsorption to soils. Viruses are negatively charged at pH values above their isoelectric point and positively charged at pH values bellow their isoelectric point. Since the isoelectric point for enteric viruses is usually bellow pH 5.0, enteroviruses retain an electronegative charge at most soil pHs, (Schaub et al. 1974). Clays and other particulate matter also retain an electronegative charge, therefore adsorption of viruses to these particles is mediated through the formation of cationic bridges (clay-cation-virus), linking the two electronegative charges (Gerba et al. 1978). This explains how the concentration and species of cations in the medium influence viral adsorption to soils. Gerba et al. (1975) reinforced that any process which disrupts this viral association with solids will enhance their movement through the soil profile. A decrease in the concentration of cations (ionic strenght) of the medium is one example of such disruption which can occur with rainfall or irrigation (Yeager and O'Brien 1979) and lead to migration of viruses to underlying groundwaters. Rainwater has a low ionic strenghten and its percolation through the soil profile will lead to a decrease in the ionic strenght of the soil solution. Some investigators have observed a burst of viruses after a heavy rainfall on land disposal sites due to this effect (Bitton 1980; Schaub and Sorber 1977).

Survival of viruses in the sludge-soil matrix is another important issue that one must face when considering land application of sewage-sludge. Soil temperature and moisture and degree of virus adsorption to soil particles seem to be the main factores in virus persistence (Hurst et al. 1980). Viruses survive better at low temperatures and inactivation is accelerated by a decrease in soil moisture. According to Bitton (1980), evaporation of soil water is the main factor responsible for virus inactivation in drying soils, by causing dissociation of virus components and degradation of the nucleic acid core. Ward and Ashley (1977b) and Yeager and O'Brien (1979) also concluded in their studies that temperature is a very important factor affecting virus survival due to its influence on evaporation (loss of moisture) and subsequent drying of the soil.

In Denmark, at temperatures ranging from 0 to 10°C, it took 161 days to reach a 5 $\log_{10}$ reduction of coxsackievirus B3 in sludge amended soils, and in Florida at temperatures ranging from 21 to 33°C, it took 21 days to reduce poliovirus 1 by more than 5 orders of magnitude. Virus were detected after 21 days under the hot and wet conditions of the summer in Florida (Polio 1 and Echo 1), but were not detected under the hot and dry conditions of the Fall (Bitton 1980). Several studies have shown that viruses can survive in sludge treated soils during the winter months (Bitton 1980 and Eisenhardt et al. 1977). In a study performed by Bitton et al. (1984), enteroviruses (polio and echo) were not

detected in the top 2.5 cm of sludge-treated soil, in the dry fall season, but in the warm and wet summer season, viruses survived for up to 35 days.

Hurst et al. (1978) observed a decrease of 2 $\log_{10}$ per week (PFU/dry weight) of naturally occurring enteroviruses in sewage sludge after land disposal, and no viruses were detected after 3 months of sludge disposal.

METHODS TO STUDY VIRUSES IN SOIL

To determine the potential for groundwater contamination due to land disposal of sewage sludge, one must evaluate the survival and transport of viruses in the soil. As described above, viruses tend to be associated with solids and remain infective. Therefore, any system used to monitor viruses must account for their adsorbance properties. Literature on methods to recover viruses from soils and their efficiencies, is limited. Hurst et al. (1980) used Tris (hydroxymethyl) aminoethane buffer containing 2% fetal bovine serum to dilute the samples of soil containing virus and assayed by direct inoculation. This method may be appropriate when working with large numbers of viruses in small quantities of soil which is usually the case with seeded viruses. Working with environmental samples (sludge and soil) under natural conditions, usually requires techniques that are sensitive enough to recover low numbers of viruses from large volumes of soil and/or sludge. Widely used techniques usually involve the elution of viruses from

particulate matter, followed by a reconcentration method such as organic flocculation. Hurst et al. (1978) evaluated the recovery of enteroviruses using 0.05 M glycine (pH 10.8) and 3% beef extract, but they referred to recoveries from activated sludge only. Goyal et al. (1984) used the method described by Hurst and Gerba (1979) when studying the occurrence of viruses beneath sewage irrigated cropland. Hurst and Gerba (1979) described a method for detection of enteroviruses in soil, using a high pH glycine buffer (pH 11.5) containing EDTA. This method has an average efficiency of 69% for the recovery of four enteroviruses from 25g quantities of loamy sand (FM) soil, and a 10% less efficiency when using 500g of soil. However, it presents a problem since many viruses can be inactivated at such high pHs.

The increasing trend on land disposal of sewage sludge creates the need for developing better methods to study the fate of viruses in soil.

GIARDIA AND GIARDIASIS

Giardia lamblia is a pathogenic flagellated protozoan which infects the upper intestinal tract of humans and many animals. Worldwide, Giardia lamblia is the most commonly isolated intestinal protozoan. In the United States, it is the most common gastrointestinal parasitic infection of humans (Feachem et al. 1983) and it has become the most commonly identified pathogen in waterborne

outbreaks in the United States (Akin and Jakubowski 1986). Kent et al. (1988) stated that giardiasis is becoming an important health problem in the United States. From 1965 to 1984, 90 outbreaks and 23,776 cases of Giardiasis were reported, the majority related to contamination of public water supplies (Craun 1986). According to Craun (1988), 71% of the outbreaks of giardiasis during the period of 1971 to 1985, were caused by surface waters, while 20% were caused by groundwater. The increase in outbreaks and individual cases of Giardiasis has generated an increasing awareness and interest in this parasite.

Giardia exists in the trophozoid and cyst form. The cyst is the environmentally stable form which is excreted in large amounts in feces of infected individuals, typically at concentrations of 10^5 to 10^7 per gram of feces (Feachem et al. 1983). Transmission is by the fecal-oral route, by ingestion of the cyst stage. It can be waterborne when water contaminated with cysts (usually fecal contaminated) is consumed, foodborne transmission occurs when fecally contaminated food is consumed, and person to person transmission when cysts from feces are directly or indirectly transmitted to another person. Population density, hygienic conditions, and proportion of susceptible individuals in the community are important factors in transmission of this parasite. Day care nurseries highlight the susceptible population as children who are in close contact and not yet educated in personal hygiene (Keystone 1982; cited by Harley 1988).

The reservoir of infection is man, although it has been suggested by several investigators that wild animals such as beavers, muskrats, voles can serve as reservoirs of infection. Human isolates have been shown to infect beavers and gerbils but only when large numbers of cysts (order of 10^5) were used. Studies have shown that only 5 cysts of Giardia muris are requires to cause infection in 50% of the time in mice and 10 cysts of Giardia lamblia to cause infection in man (Hoff et al. 1986; Rendtorff 1954). According to Pacho et al. (1987) it appears that only humans serve as a major reservoir of infection for waterborne transmission of giardiasis. Jakubowski and Ericksen (1979) estimated concentrations of 9×10^3 to 2×10^5 Giardia cysts per liter of sewage. Fox and Fitzgerald (1979), reported concentrations of 530 Giardia cysts per liter of raw sewage. Feachem et al. (1983) believe that the methods for isolation of Giardia from environmental samples are very inadequate and cysts may be missed in concentrations less than 4000/l of water and the true count be underestimated by as much as 99%. Erlandsen and Meyer (1984) stated that the continuing occurrence of waterborne outbreaks of giardiasis and the resistance of the cysts to disinfection indicate that they deserve serious consideration. Several outbreaks of Giardiasis have been attributed to sewage contaminated surface waters (Rose et al. 1988; Moore et al. 1969).

Giardia cysts (3 to 140/l) were detected in waters (Arizona, Colorado,

Texas, and Utah) receiving wastewater discharges (Rose et al. 1990). Concentrations of 51 Giardia cysts per liter of raw sewage and 1.3 cysts per liter of treated effluent have been detected by Rose et al. (1988). Sykora et al. (1990) studied the occurrence of Giardia cysts in wastewater and sludge (type of sludge not specified) in different parts of the United States and found numbers ranging from 3087 to 642 cysts per liter of raw sewage. The concentration of cysts detected in sludge ranged from 50 to 30,000 per liter and all samples of sewage and sludge were positive for Giardia cysts. Similar studies performed by Jakubouski et al. (1990) also found levels of Giardia cysts ranging from 3750 to 683 per liter of raw sewage. These authors suggested that the levels of cysts in sewage may reflect disease occurrence in the community.

Rose et al. (1990) reported higher percentages of samples containing cysts in the Fall, while Daly et al. (1988); cited by Rose et al. (1990) reported an increase in cases of giardiasis in the midsummer and peak in September. Sykora et al. (1990) stated that the highest concentration of Giardia cysts were found in late summer, fall and early winter.

According to Bitton (1980), protozoan cysts and helminth ova survive sludge treatment, particularly anaerobic digestion. Although cysts are generally sensitive to drying, helminth ova particularly those of Ascaris lumbricoides, may survive for extended periods of time in sludge and soil. Hays (1976) stated that

the majority of cysts and eggs remain in the sludge and that sufficiently high temperatures are necessary to eliminate infective forms. The author pointed out that many investigators believe cysts are destroyed during anaerobic digestion (temperature and retention time were not specified). Although cysts were detected in sewage in Chicago, none were found in anaerobically digested sludge (Fox and Fitzgerald 1979). It is thought that Giardia cysts may behave in the same manner as E. histolytica cysts, and that survival is a function of time and temperature. Parasites which may be found in sewage are those which have stages in their lifecycle (eggs of helminths and cysts of protozoans) that are adapted to survival in the outside environment. Several protozoans and helminths can be found in sewage and sludges (Fradkin et al. 1989; Arther et al. 1981), however, the focus of this study is on Giardia lamblia only.

To our knowledge, no previous work has been done on the removal or destruction of Giardia cysts during anaerobic sludge digestion. The recent development of immunofluorescence techniques for the detection of Giardia cysts in environmental samples (Rose et al. 1988), which allow quantification of cysts from large sample volumes, has made it possible to evaluate their persistence in sewage and sludge treatment processes. One disadvantage of this method, however, is that it does not differentiate between viable and non-viable cysts.

MATERIALS AND METHODS

DETECTION OF ENTEROVIRUSES IN SLUDGE

Raw and treated sludge was collected monthly from the Ina Road wastewater treatment plant in sterile polypropylene bottles (Nalgene) and kept at 4°C until time of processing. The pH was determined using a digital pH meter model Beckman I70 (Beckman Instruments Inc., Irvine, CA). The solids content was determined by drying a measured volume of sludge in an oven at 100°C for 24 hours and was expressed as a percentage on a weight (grams) to volume (milliliters) basis. Five hundred milliliters of each sample was processed for viruses using the method described by Goyal et al. (1984), which is the method recommended by the Environmental Protection Agency (EPA).

A 500 ml sample of sludge was mixed well in a plastic beaker (VWR Scientific, Inc., San Francisco, CA) using a magnetic stirrer. It was adjusted to pH 3.50 with 5 N HCl, and 0.05 M $AlCl_3$ was added to give a final concentration of 0.0005M $AlCl_3$. The sample was stirred for 30 minutes and centrifuged at 4000 rpm (Beckman, model J2-21 centrifuge, rotor JA-14, Beckman Instruments, Inc., Irvine, CA) for 15 minutes. The supernatant was discarded and the pellet resuspended in 500 ml of 10% buffered beef extract and stirred for 30 minutes. The beef extract was prepared by dissolving 50g of beef extract (Gibco,), 3.15g

of Na_2HPO_4 (J.T. Baker, Inc., Phillipsburg, NJ) and 0.15g of citric acid (Mallinckrodt Chemical Works, St. Louis, NY) in 500 ml of distilled water. The sludge-eluate mixture was centrifuged at 10,000 rpm for 10 minutes and the pellet was discarded. The supernatant was filtered through a #1 Whatmann filter, the pH was lowered to 3.5 and it was stirred for 30 minutes on a magnetic stirrer. It was then centrifuged at 10,000 rpm for 15 minutes and the supernatant was discarded. The pellet was resuspended in 30 to 40 ml of 0.15 M $Na_2HPO_47H_2O$ (pH 9.0), centrifuged again at 10,000 rpm (to remove microbial contaminants) and the pH was adjusted between 7.2 and 7.25. To remove toxicity, the final concentrate was mixed with an equal volume of Freon (1,1,2 Trichlorotrifluoro ethane, Aldrich Chemical Company, Milwaukee, WI) in 50 ml conical tubes, vortexed for 2 to 3 minutes, and centrifuged at 2500 rpm (centrifuge model IEC Centra-7, International Equipment Company, a division of Damon, USA). It was filtered through a stack of a prefilter (Gelman Science Inc., Ann Harbor MI) a 0.65um and a 0.45 um pore size filter (Deltaware menbrane systems, Kimble Division of Owens, Illinois, Toledo, OH). The filtrate was passed through a sterile 0.2 um pore size disposable filter (Nalgene Company, Rochester, NY) and kept at -70°C until time of assay. The filters were pretreated with 3% glycine buffered beef extract at pH 7.2 in order to prevent viral adsorption to the filters.

ASSAY FOR ENUMERATION OF ENTEROVIRUSES

CELL CULTURE

The buffalo green monkey kidney cell line (BGM) used to assay sludge samples for enteroviruses. Assay was limited to passage numbers between 50 and 100. The cells were grown in 75 cm^2 and 25 cm^2 plastic tissue culture flasks (Corning Glass Works, Corning, NY) with growth media containing 5% fetal bovine serum. The growth media was prepared by mixing 400 ml of Eagle's Minimum Essencial Media (MEM) with 4 ml of 7.5% sodium bicarbonate (Mallinckrodt Inc., Paris, KY), 12 ml of 1M Hepes buffer (Research Organics, Cleaveland, OH), 4 ml of 200 mM glutamine (Fisher Scientific Company, Fair Lawn, NJ), 1 ml of Penicillin/Streptomycin (final concentration of 100 IU and 100 ug respectively per milliliter of media), Kanamycin, and 1 ml of Mycostatin (final concentration of 10^4 units per 400 ml of media). Antibiotics were obtained from United States Biochemicals Corporation, Cleveland, OH. Twenty five milliliters of fetal bovine serum (Gibco laboratories, Grand Island, NY) was added to the media. The cells were incubated at 37°C until a confluent monolayer was formed, which usually took five days.

ENTEROVIRUS ASSAY

Before exposure to the sample, the growth medium was poured off and the cell monolayer was washed twice with Tris (Sigma Chemical Co., St. Louis, MO) buffered saline solution. Tris (Hydroxymethyl) aminoethane saline buffer was prepared from a stock containing 63.2 g of Trisma base, (hydroxymethyl) aminoethane, (Sigma Chemical Co., St.Louis, MO) 163.6 g of NaCl, 7.46 g of KCl, 1.13 g of Na_2HPO_4 (anhydrous), dissolved in 1600 ml of distilled water and adjusted to pH 7.2 by adding 12 N HCl. This solution was then diluted by adding 320 ml of the stock to 3680 ml of distilled water. To prevent toxicity, growth medium with 8% fetal bovine serum (same amount as the sample volume) was added to the flasks prior to inoculation. Three replicates of a 2 ml volume (final concentrate) were inoculated using 75 cm sq tissue culture plastic flasks (Corning Glass Works, Corning, NY), five replicates of 0.3 ml and three replicates of a 1:10 dilution (0.03 ml) were inoculated using 25 cm sq flasks. In cases where all flasks were positive for enteroviruses, a 1:100 dilution (0.003 ml) was also inoculated into 25 cm^2 flasks. The fasks were incubated at 37°C for 60 minutes and rotated every 15 minutes to allow virus adsorption to the cells. Maintenance medium was the same as growth medium except that it contained 2% fetal bovine serum and 1 ml of Gentamycin (final concentration of 50 ug per milliliter of media) was added. Twenty milliliters of the maintenance medium were added to the 75 cm sq

flasks and eight milliliters to the 25 cm sq flasks. The flasks were incubated at 37^{0}C and examined daily for 14 days for the presence of cytopathogenic effects (CPE). After the cells displayed a cytopathic effect, the flasks were frozen at -70°C and thawed three times. An aliquot from each flask was filter sterilized using 0.2 um porosity filters (Nalgen Company, Rochester, NY.) and inoculated onto a monolayer of BGM cells in 25 cm sq flasks for confirmation of CPE. The concentration of viruses was determined by a most probable number (MPN) authomated statistical analysis (Hurley and Roscoe, 1983).

DETECTION OF GIARDIA CYSTS IN SLUDGE

A twenty ml aliquot of each sample was processed for Giardia cysts. The method used was a modification of that used to detect Giardia and Cryptosporidium from environmental waters as described by Rose et al, (1988). Twenty ml of sludge was mixed in a blender for 3 to 5 minutes. The sample was centrifuged (IEC Centra-7, International Equipment Company, A Division of Damon, USA) at 2500 rpm for 10 minutes in a 50 ml conical tube (Corning glass works, Corning, NY) and the supernatant discarded. The pellet was resuspended with a detergent solution (deionized water containing 1% Tween 80 (J.T. Baker Inc., Phillipsburg, NJ) and 1% sodium dodecyl sulfate, SDS (Sigma Chemical, St Louis, MO)) and homogenized (Vitris "45" Gardiner, NY) at low speed for 3

minutes, with the addition of one drop of antifoam A emulsion (Sigma Chemical, St. Louis, MO). The sample was washed twice with deionized water and centrifugation (2500 rpm), the pellet resuspended in deionized water (1:3 ratio), sonicated (water bath sonicator, 25KHz, Ultrasonic Cleaning System E-module Branson Cleaning Equipment Co., Shelton, CT) and layered onto a gradient of potassium citrate (52% wt/vol, J.T. Baker Inc., Phillipsburg, NJ) in a 1:3 ratio (10ml of sample to 30 ml of the gradient). Tubes were centrifuged at 1600 rpm for 8 to 10 minutes and pellets discarded. The supernatant was diluted with deionized water in a 1:5 ratio and centrifuged at 2500 rpm. Supernatants were removed and sediments combined and resuspended to a final volume of 5ml.

IDENTIFICATION AND ENUMERATION OF GIARDIA CYSTS BY IMMUNOFLUORESCENCE

Cysts were detected by immunofluorescence (described by Rose et al. 1988) by filtering 0.5 ml aliquots of the final concentrate through 5.0 um pore size cellulose nitrate membrane filters (Nuclepore Corporation, Pleasanton, CA.) The filters were cut with a #7 cork pore to a final diammeter of 13 mm, and were placed in filter housings (Nucleopore Corporation, Pleasanton, CA). Volumes of 0.5 ml were passed through the filters using a 6 cc syringe (Monoject, Division of Sherwood Medical, St. Louis, MO) and were washed twice with 10 ml of

phosphate buffer saline (pH 7.2, PBS buffer packets from Meridian Diagnostics Inc., Cincinnati, Ohio). The filter housing was opened and the bottom was plugged with parafilm (American Can Company, Greenwich, CT). Three drops (approximatly 0.1 ml) of the primary antibody were added to the filter and it was incubated at room temperature for 30 minutes. The filters were again washed twice with 10 ml of PBS, the bottom was plugged with parafilm and 3 drops of the secondary antibody were added to the filter. For the secondary antibody the filters were kept closed and protected from light during the 30 minute incubation at room temperature, due to the presence of the flourescent dye (fluorescein isothiocyanate) which is sensitive to light. The primary antibody (murine monoclonal) was obtained from Meridian Diagnostics Inc., Cincinnati, Ohio and the secondary antibody (affinity purified antibody to mouse IgA + IgG + IgM (H + L) (goat), labelled with fluorescein isothiocyanate, FITC) was obtained from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). The filters were washed twice with 10 ml of PBS, removed from the filter housings and mounted on a glass slide (VWR Scientific Inc., San Francisco, CA) with a drop of 95% glycerin/PBS solution and a cover slip (VWR Scientific Inc., San Franscisco, CA) on the top. Giardia cysts were counted using an Olympus BH-2 epiflourescent microscope (Scientific Instruments CO., Tempe, AZ). Giardia cysts are oval shaped, 8 to 12 um long and the cyst wall stains bright apple green. Samples were

counted in triplicates and the cysts were enumerated based on the numbers counted on the entire filter and the volume filtered.

SEEDED STUDIES

GIARDIA

Giardia lamblia stocks were obtained from Diane Swabby (Swabby Gerbco Inc., Phoenix, AZ), and stored at 4°C. For efficiency studies, 20 ml of sludge were mixed with 1 ml of undiluted Giardia stock, and mixed well using a vortex. A 1 ml aliquot was pipetted out and used as the control and the 20 ml left were processed as described above. The control was diluted with PBS and was counted using the method described above.

VIRUSES

VIRUS STOCK PREPARATION

Poliovirus type 1 (strain LSc) was propagated in BGM cells grown in 32 oz glass bottles. After the formation of a complete monolayer, the growth medium was poured off, the cells were washed twice with Tris buffer saline (pH 7.2), and then inoculated at a multiplicity of infection of 0.1 (0.1 ml of the virus suspension). The inoculated cells were incubated at 37°C for one hour for viral adsorption to take place. Fifty milliliters of 2% serum maintenance medium

(described above) was added to the cells which were incubated at 37°C and observed daily until approximately 90% of the cell monolayer showed cytopathogenic effects. The bottles were then frozen at -70°C and thawed three times. The viral suspension was poured into a sterile plastic beaker (VWR Scientific, Inc., San Franscisco, CA), and mixed with Freon (1,1,2 Trichlorotrifluoro ethane, Aldrich Chemical Company, Milwaukee, WI) for 30 minutes using a magnetic stirrer (Thermolyne Corporation, subsidiary of Sybron Corporation, Dubuque, Iowa). The virus-freon mixture was centrifuged at 10,000 rpm in a JA-14 rotor (Beckman model J2-21 centrifuge, Beckman Instruments, Inc., Irvine, CA) for 10 minutes. The Freon and pellet containing cell debris were discarded and the virus stock was dispensed in small volumes and stored at -70°C.

VIRAL RECOVERIES FROM ANAEROBICALLY DIGESTED SLUDGE

Five hundred milliliters of sludge (1.30% solids) was seeded with one ml of poliovirus suspension (8.44×10^7 PFU/ml) and stirred with a magnetic stirrer (Thermolyne Corporation, subsidiary of Sybron Corporation, Dubuque, Iowa). While stirring, one ml of the seeded sludge was pipetted out to serve as the control, and the remaining was processed as previously described. Figure 2 illustrates the procedure and the points at which aliquots were taken to determine the concentration of viruses.

The control (one ml of seeded sludge) was diluted with Tris buffered saline and the 1:100 dilution was filter sterilized (0.2um, Nalgene Company, Rochester, NY), after the filter had been treated with 3% glycine buffered beef extract and it was frozen at -70°C until assayed. Samples from each step were also diluted in Tris buffered saline, filter sterilized (0.2um, Nalgene Company, Rochester, NY) and frozen at -70°C.

VIRAL RECOVERIES FROM SLUDGE AMENDED SOIL

In laboratory studies, sludge was applied to soil based on the field application rate of 14,000 gallons of sludge per acre with an average of 2.25 % solids. Application is to the surface eight inches of soil (average root depths), therefore, an average of 2 million pounds of soil per acre receives sludge (Pepper, personal communication). The sludge sample used in this study had a 1.50% (wt./vol.) solids content which equals to 56.70 g / gallon of sludge and implies that 21,000 gallons of sludge would be applied (the amount applied depends on the solid content of the sludge). Twenty one thousand gallons of sludge per two million pounds of soil equals to a ratio of 8.73 ml of sludge per 100 g of soil.

Figure 3 shows the procedure used to evaluate the percentage of virus recovery from the sludge-soil mixtures. Throughout the study, the same procedure was used varying only the ratios of sludge-soil mixture to the beef extract eluent.

Eight ml of sludge was mixed with 1 ml of poliovirus stock. One ml was removed for assay and the remaining was mixed with 100 g of soil. The seeded sludge-soil mixture was mixed with 100 ml , 250 ml, or 500 ml of beef extract, depending on the ratio being tested. The beef extract (BE-V, BBL Microbiological Systems, Becton Dickinson and CO, Cockeysville, MD) was prepared by mixing 30 g of BE powder with , 0.15 g of citric acid (Mallinckrodt Chemical Works, St. louis, NY) and 3.15 g of $Na_2HPO_47H_2O$ (sodium phosphate buffer, dibasic, anhydrous powder, J.T. Baker Inc., Phillipsburg, NJ) with 500 ml of distilled water and the pH was adjusted to 9.5 using 5 N HCl. The sample was mixed with the beef extract for 15 minutes and centrifuged (Beckman model J2-21, rotor JA-14) in 250 ml centrifuged bottles (VWR Scientific,Inc., San Franscisco, CA) for 30 minutes. The pellet (sludge-soil mixture) was discarded, the volume of the supernatant (beef extract containing the viruses) was measured using a sterile graduated plastic cylinder, and a one ml aliquot was taken at this point. The pH of the supernatant was lowered to 3.5 for organic flocculation (using 5 N HCl), stirred for 15 minutes in a magnetic stirrer, and centrifuged for 10 minutes at 10,000 rpm. The supernatant was discarded and the pellet containing the viruses was resuspended with 30 ml of 0.15 M $Na_2HPO47H_2O$ at pH 9.5 (sodium phosphate dibasic Anhydrous powder, J. T. Baker, Inc., Phillipsburg, NJ). The concentrate was centrifuged at 10,000 rpm for 10 minutes to remove microbial

contaminants. The pH of the final concentrate was adjusted between 7.2 and 7.25, its volume was measured with a 10 ml plastic pipette, and filter sterilized with a 3% glycine buffered beef extract pretreated 0.2 um porosity filter and stored at -70°C until it was assayed.

When the 1:10 ratio of sludge-soil to beef extract was tested, 50 g of soil were used instead of 100g. The control and the aliquots taken at the elution and reconcentration steps were filter sterilized (0.2 um filter) and diluted with Tris bufferred saline.

PLAQUE ASSAY FOR VIRUS ENUMERATION

Titers of the poliovirus stock for the seeded work were obtained by the plaque forming unit (PFU) overlay method (Smith and Gerba, 1982).

After the formation of a complete monolayer of BGM cells in 25 cm sq tissue culture flasks (grown as described above), the cells were washed twice with Tris buffered saline and inoculated with 0.3 ml of the virus stock or the sample. They were incubated at 37°C for 1 hour and rotated every 15 minutes to allow for viral adsorption. The agar overlay was prepared by mixing an equal volume of maintenance medium (pH 7.2, containing 2 x MEM, 2% serum, 6 ml of 7.5% sodium bicarbonate, 200 mM glutamine, Kanamycin, Streptomycin/Penicillin and Mycostatin) with 1.5% bacto agar (Difco Laboratories, Detroit, MI), maintained

in a liquid state in a 45°C water bath. Eight milliliters of the agar overlay medium was added to each flasks and they were incubated at 37°C for 24 hours. The cells were stained with a solution of 0.5 % crystal violet (Matheson Coleman and Bell, Los Angeles, CA). Two dilutions levels of each sample were assayed in duplicate and the plaques were counted.

500 ml sludge + 1 ml virus stock (7.00×10^7 PFU)
(remove 1 ml for virus titer)

↓

adjust pH to 3.5 and AlCl3 concentration to 0.0005 M
stir for 30 min and centrifuge at 4,000 rpm
(J2-21 centrifuge, JA-14 rotor)
(save 1 ml supernatant for virus titer)

↓

discard supernatant and resuspend pellet in 10% beef extract
stir for 30 min and centrifuge at 10,000 rpm for 30 min
collect supernatant and measure its volume
(remove 1 ml for virus titer)

↓

filter through a Whatmann #1 filter
adjust pH to 3.5 and stir for 30 min
centrifuge at 10,000 rpm for 15 min and discard supernatant

↓

resuspend pellet in 30 to 40 ml of 0.15 M $Na_2HPO_47H_2O$
adjust pH between 7.2 and 7.25
centrifuge at 10,000 for 10 min
measure the final volume
(remove 1 ml for virus titer)

FIGURE 2. Recovery of enteroviruses from sewage sludge (seeded study).

8 ml sludge + 1 ml virus stock (7.00×10^7)
(remove 1 ml for virus titer)

↓

mix (uniformely) with 100g soil
add 3% buffered beef extract and stir for 30 min
(pH 9.5, volume depending on the ratio)

↓

centrifuge at 10,000 rpm for 30 min
(Beckman model J2-21 rotor JA-14)
discard pellet (soil-sludge mixture)
(remove 1 ml of supernatant for virus titer)

↓

adjust pH of supernatant to 3.5 and stir for 30 min
centrifuge at 10,000 rpm for 30 min
discard supernatant

↓

resuspend pellet in 20 to 30 ml of 0.15 M Na_2HPO_47H2O
adjust pH between 7.2 and 7.25
centrifuge at 10,000 for 10 min
discard pellet
measure the final volume
(remove 1 ml for virus titer)

FIGURE 3.Method for the recovery of enteroviruses from sludge amended soil (seeded study).

RESULTS

GIARDIA STUDIES

The levels of Giardia cysts present in raw (combined primary and secondary sludges) and anaerobically digested sewage sludge from the Ina Road Wastewater Treatment Plant were monitored every month for a period of 15 months (from May of 1988 to July of 1989). Tables 2 and 3 show the concentration of Giardia cysts in raw and treated sludge respectively. Data were expressed per liter of liquid sludge and per kilogram of solids. Figures 4 and 5 illustrate the levels of Giardia cysts in both the raw and treated (anaerobically digested) sludges. Because sludges vary in their solid content and microorganisms tend to be associated with solid matter, expression of the results on a dry weight basis (wt./vol.) gives a common frame of reference by standardizing the samples and allows better comparison.

Table 4 gives an overview of the levels of Giardia cysts found in both the raw and treated sludges. For the raw sludges, the highest concentration was 8.60 x 10^{4}/l or 3.30 x 10^{6}/Kg with an average of 1.44 x 10^{4}/l or 5.42 x 10^{5}/kg and the lowest was 1.33 x 10^{3}/l or 7.73 x 10^{4}/Kg. The treated sludge had a high concentration of 2.80 x 10^{4}/l or 4.14 x 10^{6}/Kg with an average of 8.10 x 10^{3}/l or 6.44 x 10^{5}/Kg and a low concentration of 2.00 x 10^{3}/l or 1.03 x 10^{5}/Kg.

TABLE 2. CONCENTRATION OF GIARDIA CYSTS IN RAW SEWAGE SLUDGE.*

DATE COLLECTED	% SOLIDS	GIARDIA/L	GIARDIA/Kg
05/20/88	2.05	1.55×10^4	7.56×10^5
06/20/88	2.00	1.35×10^4	6.70×10^5
07/05/88	4.00	5.00×10^3	1.25×10^5
07/25/88	5.00	8.82×10^3	1.77×10^5
08/12/88	3.50	5.33×10^3	1.52×10^5
09/12/88	3.64	5.88×10^3	1.61×10^5
10/10/88	2.27	6.75×10^3	2.97×10^5
11/17/88	4.77	1.17×10^4	2.47×10^5
12/12/88	2.34	1.07×10^4	4.56×10^5
01/17/89	2.50	2.17×10^3	8.67×10^4
04/24/89	2.61	8.60×10^4	3.30×10^6
05/17/89	1.72	1.33×10^3	7.73×10^4
MEAN	3.03	1.44×10^4	5.42×10^5

* Raw sludge (primary and secondary sludges combined).

TABLE 3. CONCENTRATION OF GIARDIA CYSTS IN ANAEROBICALLY DIGESTED SEWAGE SLUDGE.

DATE COLLECTED	% SOLIDS	GIARDIA/L	GIARDIA/Kg
05/20/88	1.01	1.05×10^{4}	1.03×10^{5}
06/20/88	1.33	2.80×10^{4}	2.10×10^{5}
07/05/88	1.25	5.17×10^{3}	4.14×10^{6}
07/25/88	2.03	1.03×10^{4}	5.09×10^{5}
08/12/88	1.50	6.00×10^{3}	4.00×10^{5}
09/12/88	2.00	1.03×10^{4}	5.15×10^{5}
10/10/88	1.50	2.50×10^{3}	1.67×10^{5}
11/17/88	1.28	3.50×10^{3}	2.73×10^{5}
12/12/88	1.50	8.58×10^{3}	5.72×10^{5}
01/17/89	1.25	6.67×10^{3}	5.33×10^{5}
02/20/89	1.37	2.00×10^{3}	1.46×10^{5}
04/24/89	1.33	1.38×10^{4}	1.04×10^{6}
05/17/89	1.36	3.80×10^{3}	2.79×10^{5}
07/02/89	1.74	2.33×10^{3}	1.34×10^{5}
MEAN	1.46	8.10×10^{3}	6.44×10^{5}

TABLE 4. OVERVIEW OF GIARDIA CYSTS CONCENTRATION IN SEWAGE SLUDGE BEFORE AND AFTER ANAEROBIC DIGESTION.

	GIARDIA/L		GIARDIA/KG	
	RAW	TREATED	RAW	TREATED
MAXIMUM	8.60×10^{4}	2.80×10^{4}	3.30×10^{6}	4.14×10^{6}
MINIMUM	1.33×10^{3}	2.00×10^{3}	7.73×10^{4}	1.03×10^{5}
AVERAGE	1.44×10^{4}	8.10×10^{3}	5.42×10^{5}	6.44×10^{5}

Figure 4
GIARDIA CYSTS PER LITER OF PIMA COUNTY SLUDGE

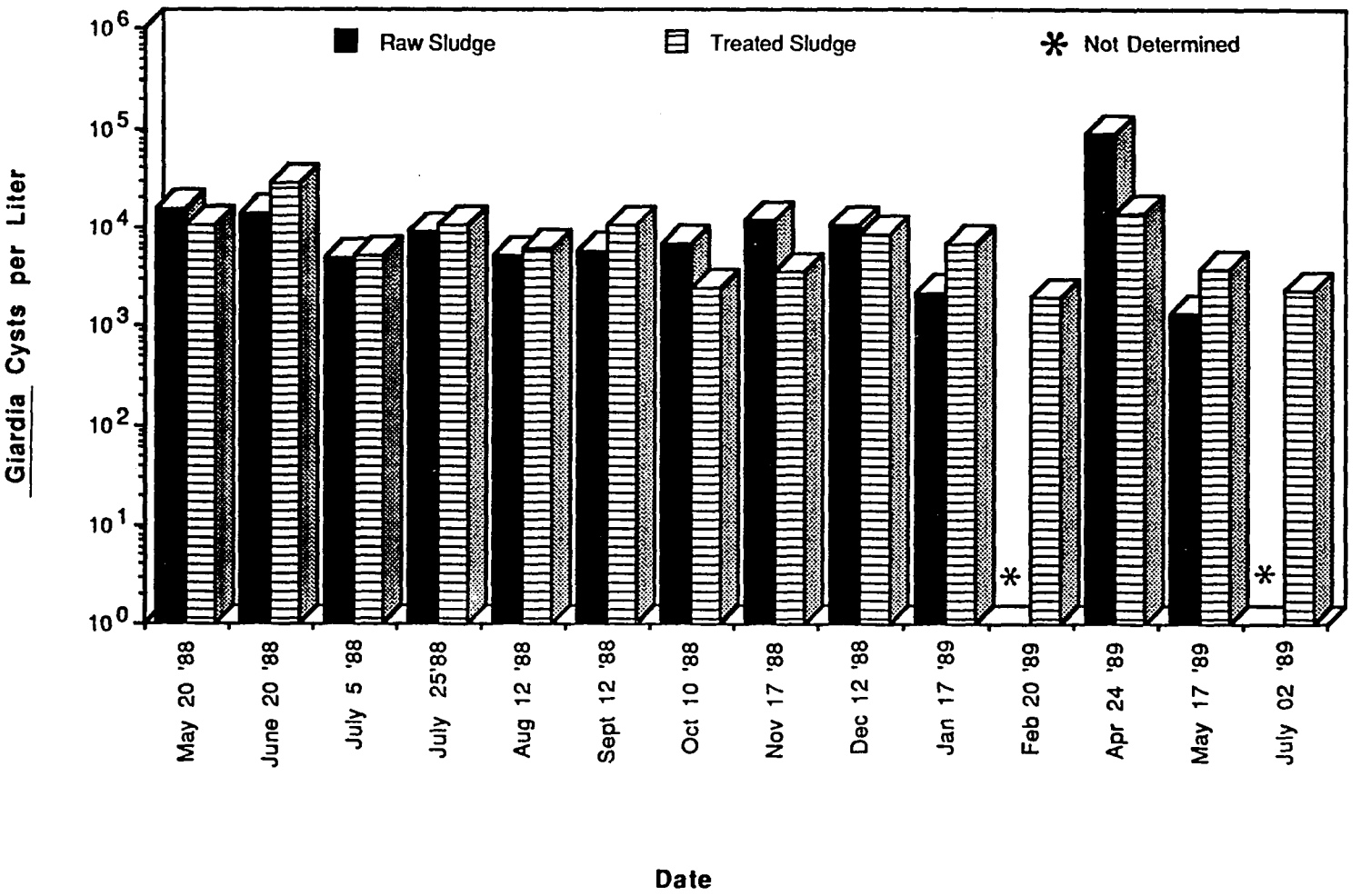
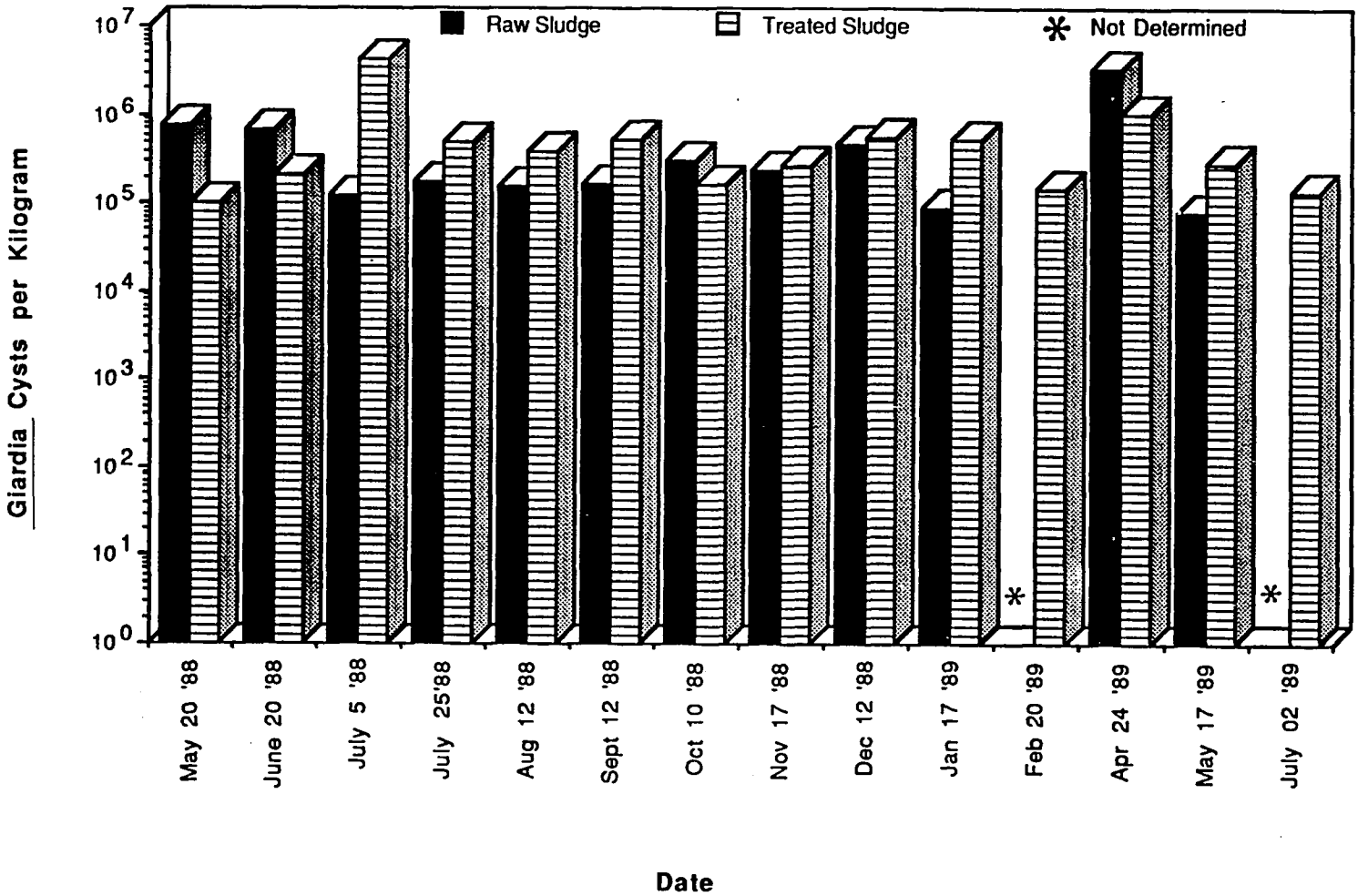


Figure 5

GIARDIA CYSTS PER KILOGRAM OF PIMA COUNTY SLUDGE



The levels of Giardia cysts found in the raw and treated sludges were fairly constant throughout the period of study with no significant difference (Wilcoxon nonparametric test) between the concentration of cysts found in the raw and treated sludges. The Minitab computer program (student version, Addison Wesley 1988) was used for the statistical analysis. In some instances the treated sludge seemed to have higher numbers of cysts than the raw sludge, but the levels of cysts were not significantly different and may to some extent be a reflection of the variability in the sampling and detection method. Peak levels were seen in the month of July of 1988 and April of 1989 in both the raw and treated sludges, which may be due to an increased incidence of giardiasis in the community during those months.

The efficiency of the method used for the detection of Giardia cysts in sewage sludge was also evaluated with sludge samples seeded with high concentrations of Giardia lamblia cysts. The method used in this study was a modification of the method developed by Kayed (1986) for the detection of cryptosporidium in sludge. The results are given in Table 5 for raw sludge and Table 6 for treated sludge. The recovery efficiency averaged 3.3 % with a standard deviation of 2.1 for the raw sludge and 1.4 % with a standard deviation of 0.2 for the treated sludge.

TABLE 5. EFFICIENCY OF GIARDIA CYST DETECTION IN RAW SEWAGE SLUDGE*

TRIALS	SEEDED SLUDGE TOTAL NUMBER OF CYSTS	FINAL CONCENTRATE TOTAL NUMBER OF CYSTS	PERCENT RECOVERED
1	2.22×10^{3}	1.17×10^{2}	5.2
2	1.79×10^{6}	9.13×10^{4}	5.1
3	3.08×10^{5}	4.67×10^{3}	1.5
4	6.93×10^{5}	1.03×10^{4}	1.5
MEAN			3.3
STD DEV.			2.1

* Seeded study with Giardia lamblia cysts.

TABLE 6. EFFICIENCY OF GIARDIA CYST DETECTION IN ANAEROBICALLY DIGESTED SEWAGE SLUDGE.

	SEEDED SLUDGE	FINAL CONCENTRATE	PERCENT RECOVERED
TRIALS	TOTAL NUMBER OF CYSTS		
1	1.17×10^{6}	1.93×10^{4}	1.6
2	4.53×10^{5}	6.00×10^{3}	1.3
3	5.20×10^{5}	6.50×10^{3}	1.2
MEAN			1.4
STD DEV.			0.2

* Seeded study with Giardia lamblia cysts.

ENTEROVIRUS STUDIES

The occurrence of enteroviruses in raw (combined primary and secondary sludges) and anaerobically digested sludge was monitored on a monthly basis from May of 1988 to July of 1989. Tables 7 and 8 give the levels of enteroviruses found in raw and treated sludges respectively on a per liter basis and per kilogram basis. Figure 6 describes the results on a per liter basis and Figure 7 describes the results on a per kilogram basis for both raw and treated sludges. A summary of the concentration of enteroviruses in sewage sludge before and after anaerobic digestion is given in Table 9. For raw sludge, the concentration varied between 1.28×10^{4}/l or 7.00×10^{5}/Kg and 1.74×10^{2}/l or 4.36×10^{3}/Kg with an average of 2.60×10^{3}/l or 1.05×10^{5}/Kg. For the treated sludge the concentration of enteroviruses varied between 5.14×10^{3}/l or 2.52×10^{5}/Kg to less than 2/l or less than 6.25/Kg with an average of 4.80×10^{2}/l or 2.67×10^{4}/Kg.

As expected, peak levels of enteroviruses were observed in midsummer and early fall which is due to a high incidence of infection with enteroviruses during this time of the year. The levels of enteroviruses in raw and treated sludges were highly variable and the levels in raw sludge were significantly higher than the levels in treated sludge. Results from studies on the removal or reduction of

TABLE 7. CONCENTRATION OF ENTEROVIRUSES IN RAW SEWAGE SLUDGE.*

DATE COLLECTED	% SOLIDS	VIRUSES/L	VIRUSES/Kg
05/20/88	2.05	6.76×10^{2}	3.30×10^{4}
06/20/88	2.00	2.34×10^{2}	1.17×10^{4}
07/05/88	4.00	1.74×10^{2}	4.36×10^{3}
07/25/88	5.00	3.00×10^{3}	6.00×10^{4}
08/12/88	3.50	1.22×10^{4}	3.50×10^{5}
09/12/88	3.64	7.08×10^{2}	1.94×10^{4}
10/10/88	2.27	1.24×10^{3}	5.44×10^{4}
11/17/88	4.77	5.63×10^{2}	1.18×10^{4}
12/12/88	2.34	8.14×10^{2}	3.48×10^{4}
01/17/89	2.50	1.74×10^{2}	6.96×10^{3}
03/27/89	2.17	2.59×10^{2}	1.04×10^{4}
04/24/89	2.61	1.79×10^{3}	6.87×10^{4}
05/17/89	1.72	1.20×10^{4}	7.00×10^{5}
MEAN	2.97	2.60×10^{3}	1.05×10^{5}

* Raw sludge (primary and secondary sludges combined).

TABLE 8. CONCENTRATION OF ENTEROVIRUSES IN ANAEROBICALLY DIGESTED SEWAGE SLUDGE.

DATE COLLECTED	% SOLIDS	VIRUSES/L	VIRUSES/Kg
06/20/88	1.33	3.2	2.40×10^{2}
07/05/88	1.25	14.7	1.18×10^{3}
07/25/88	2.03	10.9	5.35×10^{2}
08/12/88	1.50	84.8	5.66×10^{3}
09/12/88	2.00	57.0	2.85×10^{3}
10/10/88	1.50	55.5	3.70×10^{3}
11/17/88	1.28	48.1	3.76×10^{3}
12/12/88	1.50	13.1	8.93×10^{2}
01/17/89	1.25	< 2	< 6.2
02/20/89	1.37	21.1	1.54×10^{3}
03/27/89	1.43	< 2	< 6.9
04/24/89	1.33	48.9	3.68×10^{3}
05/17/89	1.36	1610	1.18×10^{5}
07/02/89	1.74	104	5.97×10^{3}
07/20/89	2.03	5137	2.52×10^{5}
MEAN	1.53	480	2.67×10^{4}

TABLE 9. OVERVIEW OF ENTEROVIRUS CONCENTRATION IN SEWAGE SLUDGE BEFORE AND AFTER ANAEROBIC DIGESTION.

	ENTEROVIRUSES/L		ENTEROVIRUSES/Kg	
	RAW	TREATED	RAW	TREATED
MAXIMUM	1.28×10^{4}	5.14×10^{3}	7.00×10^{5}	2.52×10^{5}
MINIMUM	1.74×10^{-2}	< 2	4.36×10^{3}	< 6.25
AVERAGE	2.60×10^{3}	4.80×10^{2}	1.05×10^{5}	2.67×10^{4}

Figure 6
ENTEROVIRUSES PER LITER OF PIMA COUNTY SLUDGE

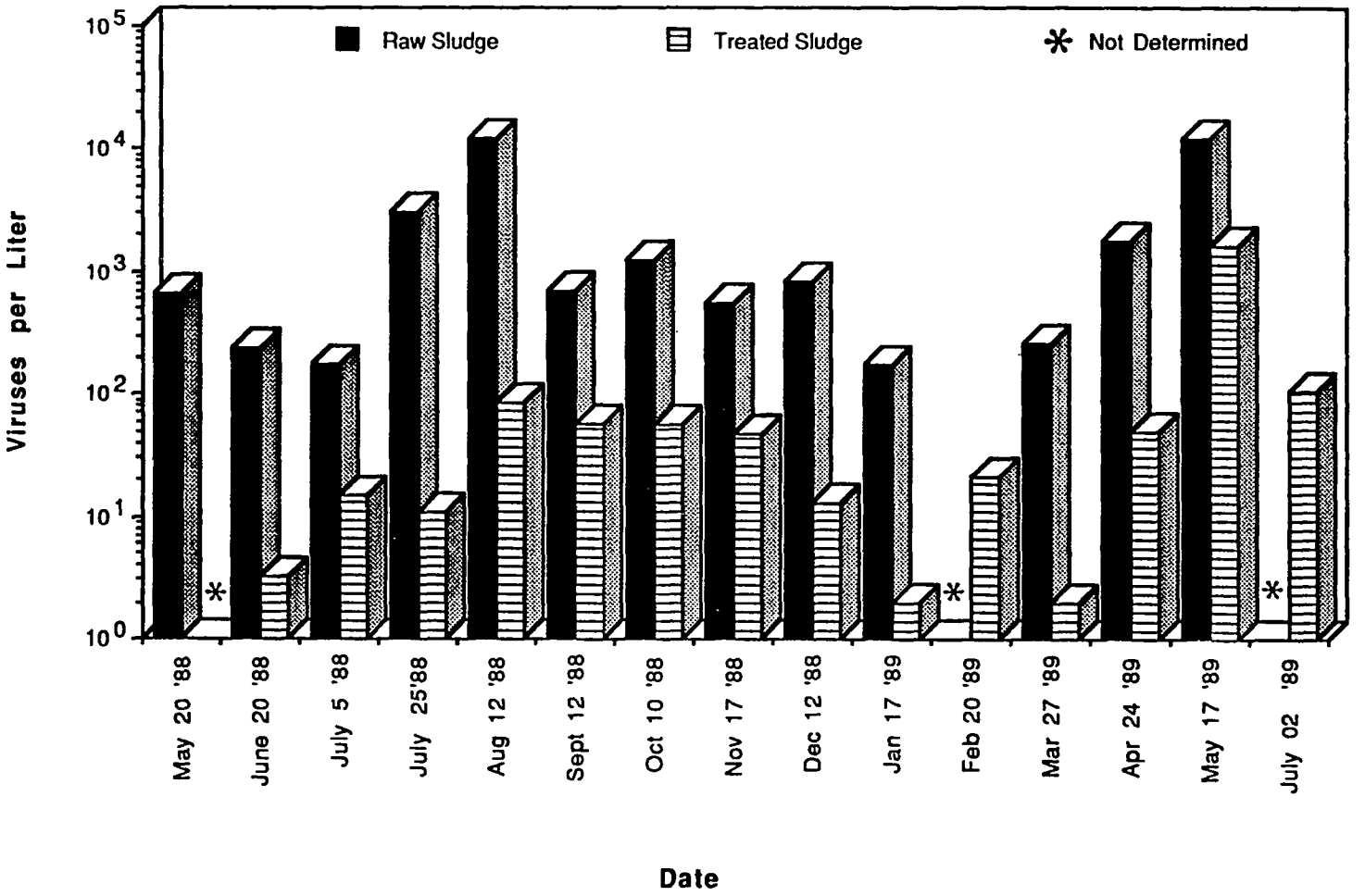
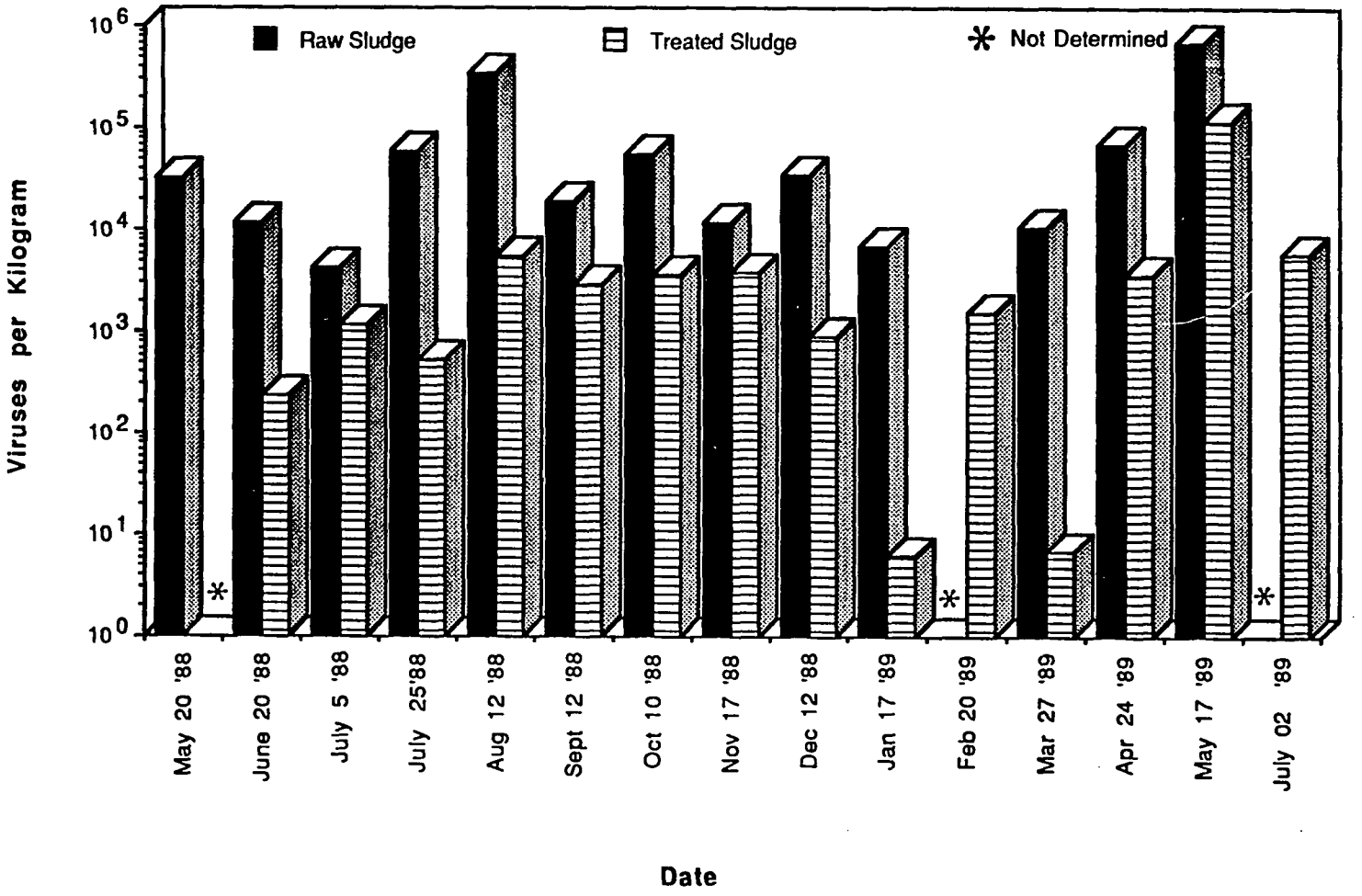


Figure 7
ENTEROVIRUSES PER KILOGRAM OF PIMA COUNTY SLUDGE



enteroviruses after anaerobic digestion of sewage sludge are shown in Table 10 and Figure 8. The percentage removal of enteroviruses after anaerobic digestion of sewage sludge varied from greater than 99.95% to 73% and was highly variable during the period of this study.

STUDIES WITH POLIOVIRUS TYPE 1 (STRAIN LSc)

SLUDGE STUDIES

The efficiency of recovery of enteroviruses from sewage sludge was tested by seeding anaerobically digested sludge with poliovirus type 1 (strain LSc) with approximately 5.00×10^{5} viral particles. Table 11 gives the number of polioviruses added to the sludge and the number recovered at the elution and reconcentration steps. Table 12 gives the percent recovery of poliovirus also at the elution and reconcentration steps. The percent recovery at the reconcentration level was determined with the initial virus input and not with the number of viruses in the eluent, therefore it can be regarded as the overall efficiency of the method since this is the last major step where viral particles may be lost.

Elution of poliovirus from anaerobically digested sludge gave an average percent recovery of 119 %. Reconcentration by biofloculation yielded a 29.6% recovery efficiency.

TABLE 10. VIRUS REMOVAL AFTER ANAEROBIC DIGESTION OF SEWAGE SLUDGE

DATE SAMPLES COLLECTED	% VIRUS REMOVAL
06/20/88	98
07/05/88	73
07/25/88	99.1
08/12/88	98.4
09/12/88	85.3
10/10/88	99.3
11/17/88	96.9
12/12/88	97.4
01/17/89	> 99.95
03/27/89	> 99.94
04/24/89	94.7
05/17/89	83.2

Figure 8
VIRUS REMOVAL AFTER ANAEROBIC SLUDGE DIGESTION

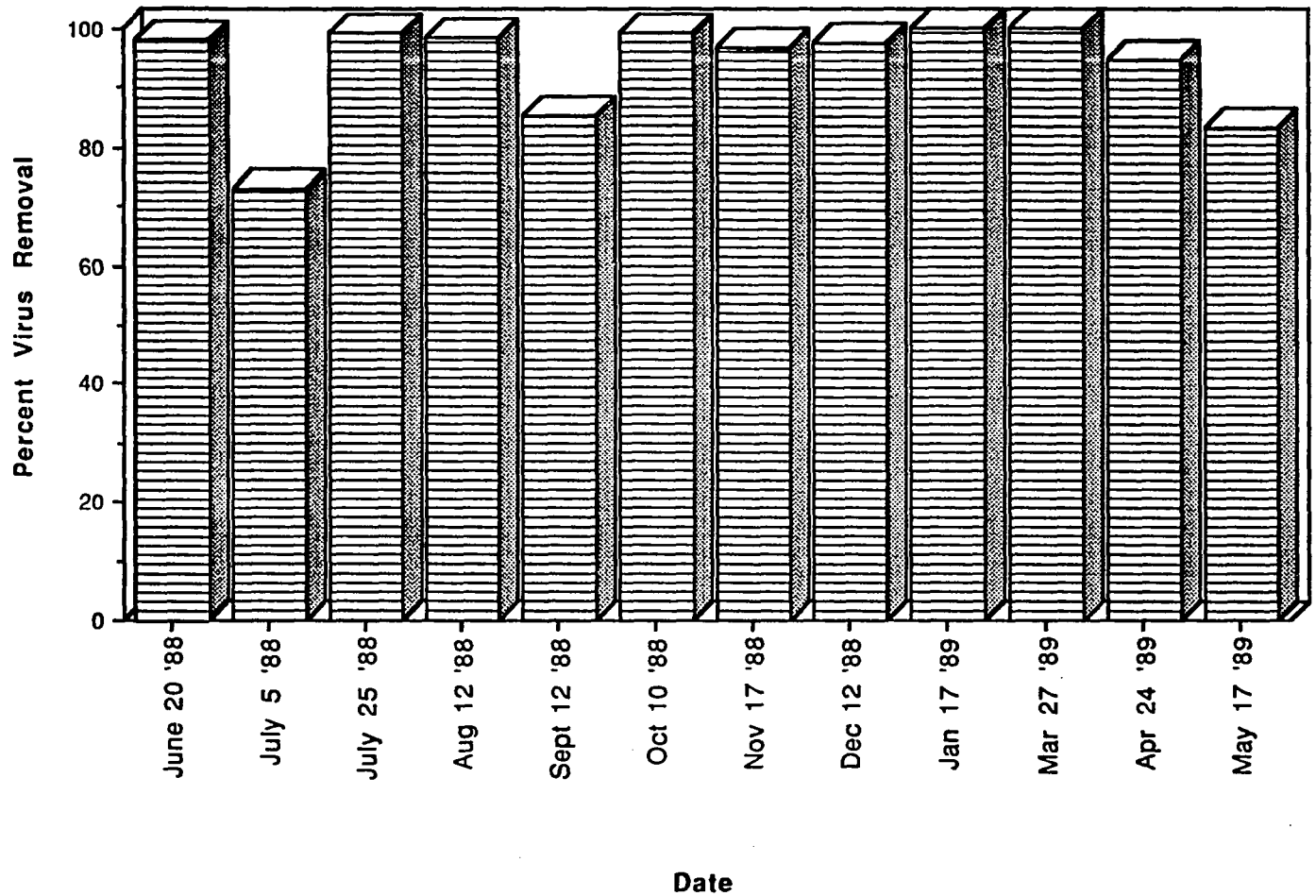


TABLE 11. NUMBER OF POLIOVIRUS RECOVERED FROM SEEDED ANAEROBICALLY DIGESTED SEWAGE SLUDGE.*

VIRUS INPUT TOTAL PFU	TRIAL	NUMBER OF VIRUSES RECOVERED TOTAL PFU	
		ELUTION	RECONCENTRATION
4.08×10^7	1	3.96×10^7	1.66×10^7
5.00×10^7	2	6.05×10^7	8.75×10^6
4.83×10^7	3	6.72×10^7	1.48×10^7

*Sludge seeded with poliovirus type 1, strain LSC. A solution of 10% buffered beef extract (Gibco) was used for elution and reconcentration.

TABLE 12. EFFICIENCY (%) OF POLIOVIRUS ELUTION AND RECONCENTRATION FROM ANAEROBICALLY DIGESTED SEWAGE SLUDGE.*

	PERCENT RECOVERY	
TRIALS	ELUTION	RECONCENTRATION
1	97	40.7
2	121	17.4
3	139	30.6
MEAN	119	29.6
STD DEV.	21	11.7

* Sludge seeded with poliovirus type 1, strain LSC. A solution of 10% buffered beef extract (Gibco) was used for elution and reconcentration

EFFICIENCY STUDIES OF POLIOVIRUS RECOVERY FROM SLUDGE:SOIL MIXTURES

Methods to recover enteroviruses from sludge:soil mixtures were evaluated. Treated sludge seeded with poliovirus type 1 (strain LSc) was mixed with a Sandy Loam soil (appendix A) to simulate field conditions. This mixture was suspended in a solution of 3% buffered beef extract (pH 9.5) in amounts equal to 1, 2.5, 5, and 10 times the volume of solids (sludge:soil mixture) for elution of the viruses. The results of this evaluation are given in Tables 13 and 14. Table 13 shows the number of viruses added to the sludge:soil mixtures and the numbers recovered at the elution and reconcentration steps. Table 14 gives the percent recoveries of poliovirus at the different ratios of eluant to solids. Elution of poliovirus at a 1:1, 1:2.5, 1:5, and 1:10 ratios yielded percent recoveries with an average of 1.7, 14.2, 67.0, and 98.8 respectively. Results of this study showed that the percentage of virus recovered increased significantly with an increase in the eluant volume. A regression analysis of the data (Minitab student version, Addison Wallis 1988) showed that there is a linear correlation (correlation coefficient equal to 0.92) between the volume of the eluant and the percent of viruses recovered. The relationship is described by the equation $Y = - 5.60 + 11.4 X$ (Y represents the

volume of the eluant and X represents the percent recovery of viruses). As shown in Tables 13 and 14, reconcentration of the virus suspension by organic flocculation of the beef extract at pH 3.5 was evaluated for the 1:1 and 1:5 ratios giving average recoveries of 1.1% and 48.6% respectively. The data shows that the reconcentration step gives an average loss of 20% (15% to 26%) of the virus and/or loss of infectivity.

TABLE 13. NUMBER OF POLIOVIRUS RECOVERED FROM SLUDGE SOIL MIXTURES USING DIFFERENT RATIOS OF SLUDGE:SOIL TO BEEF EXTRACT.*

VIRUS INPUT TOTAL PFU	RATIOS[a]	TRIAL	NUMBER OF VIRUSES RECOVERED (TOTAL PFU) ELUTION	RECONCENTRATION
3.0×10^7	1:1	1	4.1×10^5	3.5×10^5
2.6×10^7		2	5.3×10^5	4.3×10^5
1.1×10^7		3	1.8×10^5	1.1×10^5
2.2×10^7	1:2.5	1	3.5×10^6	NDb
5.0×10^7		2	6.6×10^6	ND
3.0×10^7	1:5	1	2.7×10^7	2.0×10^7
1.0×10^7		2	6.3×10^6	5.4×10^6
2.3×10^7		3	1.2×10^7	9.4×10^6
2.2 x 10 7	1:10	1	2.4 x 10 7	ND
4.9 x 10 7		2	4.2 x 10 7	ND

* Using 3% buffered beef extract (BBL-V) adjusted to pH 9.5.

[a] For the 1:1, 1:2.5, and 1:5 ratios, 100ml, 250ml, and 500ml of the eluant were added to 100g of soil respectively. For the 1:10 ratio, 500ml of the eluant was added to 50g of soil.

[b] Not determined.

TABLE 14. PERCENT RECOVERY OF POLIOVIRUS FROM THE SLUDGE SOIL MIXTURES. *

		PERCENT RECOVERY	
RATIOS[a]	TRIALS	ELUTION	RECONCENTRATION
1:1	1	1.4	1.2
	2	2.0	1.7
	3	1.7	1.1
	MEAN	1.7	1.3
	STD DEV.	0.3	0.3
1:2.5	1	15.2	ND[b]
	2	13.3	ND
	MEAN	14.2	ND
	STD DEV	1.3	ND
1:5	1	90.2	63.6
	2	61.2	52.5
	3	49.6	37.9
	MEAN	67.0	48.6
	STD DEV.	21.0	11.8
1:10	1	112.2	ND
	2	85.4	ND
	MEAN	98.8	ND
	STD DEV.	19.0	ND

* Using 3% buffered beef extract (BBL-V) adjusted to pH 9.5.

a For the 1:1, 1:2.5, and 1:5 ratios, 100ml, 250ml, and 500ml of the eluant were added to 100g of soil respectively. For the 1:10 ratio, 500ml of the eluant was added to 50g of soil.

b Not determined.

TABLE 15. SUMMARY OF EFFICIENCY STUDIES FOR POLIOVIRUS RECOVERY FROM SLUDGE:SOIL MIXTURES.

RATIO OF SLUDGE:SOIL TO BEEF EXTRACT*	VIRUS RECOVERY (%)	
	ELUTION	RECONCENTRATION
1:1	1.7	1.3
1:2.5	14.2	ND
1:5	67.0	48.6
1:10	98.8	ND

* 3% buffered beef extract (BBL-V), adjusted to pH 9.5.

DISCUSSION

This study showed that significant concentrations of Giardia cysts and enteroviruses are present in Pima County's anaerobically digested sewage sludge which is disposed of into farmland.

Very high concentrations of Giardia cysts were present in both raw and treated sludge, and there was no significant difference between their occurrence in the raw and treated sludge. These results suggest that sludge treatment by anaerobic digestion at the Ina Road Wastewater Treatment Plant has had no effect on the removal of Giardia cyst from sewage sludge. It should be noted, however, that the viability of the cysts was not studied and although high numbers were shown to be present in sludge, these may have been inactivated during treatment. Sykora et al. (1990) reported concentrations of Giardia cysts in different parts of the United States ranging from 3375 to 642 per liter of raw sewage and concentrations ranging from 50 to 30,000 per liter of sludge (sludge type not specified). Jakubowski et al. (1990) also found high concentrations of Giardia cysts (ranging from 3750 to 683 per liter) in raw sewage.

Although the concentration of Giardia cysts in Pima County's raw sewage was not investigated, the studies by Sykora et al. (1990) and Jakubouski et al., (1990) showed that high concentrations of Giardia cysts can be found in sewage

and they suggested that their levels in sewage may reflect the level of infection in the community. Based on their data from examination of Giardia cysts from different sewage treatment plants, Jakubowski et al. (1990) estimated cyst levels of 1500 and 2600 per liter of raw sewage as representing one case of giardiasis per month per 100,000 population. Microorganisms tend to concentrate in the sludge fractions during sewage treatment due to their association with particulate matter, particularly fecal matter as is the case with sewage. Therefore, sludge would be expected to have higher concentrations of cysts than raw sewage. The viability of Giardia cysts in raw sewage was studied by the dye exclusion method and these were found to be 100% infective (Hew Smith, personal communication). Similar studies should be performed to assess the viability of cysts after treatment of sewage and sludges. Bitton, (1980) and Fox and Fitzgerald, (1979) suggested that the high temperature and retention time necessary to inactivate or destroy cysts are usually not maintained in wastewater treatment plants and this seems to be the case at the Ina Road Wastewater Treatment Plant. The Class A Microbial Standards proposed by the United States Environmental Protection Agency for land application of sewage sludge has set a limit of one protozoan parasite per gram of volatile solids. This was never met by the Ina Road Wastewater treatment plant during this period of study.

The efficiency studies showed that the method used to recover Giardia

cysts from sludge is still very poor. The low recoveries obtained in this study suggest that the numbers found greatly underestimate the actual number of cysts present in the sludge. Sykora et al. (1990) pointed out that detection of cyst by sucrose flotation gave results ranging from 0.4% to 78% of the direct counts. This may be due to the high solid content of sewage and sludges which hinders the detection of cysts. More work should be done to improve these methods.

The study of enteroviruses in raw and treated sludge demonstrated that there was a significant difference between the raw and the anaerobically digested sludge. Reduction in the number of enteroviruses after anaerobic digestion was highly variable ranging from greater than 99.95% TO 73%. The United States Environmental Protection Agency has proposed microbiological standards for the disposal of sewage sludge onto farmland. Class A standards were met only 42% of the times, while Class B standards were met 8% of the times, and Class C standards were met 17% of the times. Thirty three percent of the times, the treated sludge did not meet even Class C standards. The stringency on the pathogen reduction levels stipulated by Classes A, B , and C depend on the use to which the land is put. In Pima County, only cotton is grown and animal grazing is not allowed. The only restriction implemented is that between successive sludge applications in a field a crop must be grown. Which class applies to Pima County's sludge disposal method has not been established by the Pima County

Wastewater Managment Division (Pepper, personal communication).

The results of this study show that in some occasions the plant was capable of reducing the concentration of viruses in sludge by 2 logs or even more. Careful monitoring of the digestors or improvement of the treatment process should be done in order to maintain a constant reduction of viruses in sludge and to comply with the proposed standards. The high variability in the reduction of the number of virus in treated sludge may in part be due to the fact that raw sludge is fed into the digestors on a continuous basis and the possibility of mixing digested sludge with fresh undigested sludge exists. Also, a 16 day retention time of the sludge in the digestors was not always maintained. A new digester was under construction in 1989 and officials believe they will be able to maintain a longer and more constant retention time which may allow for better removal of pathogens (Pepper, personal communication).

Data on the occurrence of enteroviruses in raw and treated sludge shows that even with viral reduction levels ranging from 73% to greater than 99.95%, significant numbers of viruses are still present in sludge which is being applied to farmland in Pima County. This is mainly due to the high numbers of enteroviruses initially present in raw sludge. As shown in Table 1, more than 120 different types of enteric viruses can be associated with sewage. Enteroviruses represent only a small portion of the viruses which may be present in sewage, but

the cost of virus isolation and assay in cell culture limited this study to the detection of enteroviruses only. In addition, for many enteric viruses, methodologies for their isolation from environmental samples and growth in cell culture have not yet been developed. Also, the efficiency studies demonstrated that even within the enterovirus group, only a small percentage (average of 30%) of the actual numbers present in sludge were recovered. It is clear that our results greatly underestimate the actual number of viruses present in sludge which is being applied to farmland at very large quantities, approximately 30,000 gallons of sludge per acre. Therefore, the detection of even low numbers of viruses in sludge being disposed of onto land generates a public health concern due to the possibility of movement through the soil profile and contamination of the underground aquifers.

In order to assess the fate of enteroviruses in sludge as they contact the soil, methods to recover viruses from the sludge:soil mixture were evaluated. Evaluation of the different ratios of beef extract to soil:sludge mixtures showed that the eluting effectiveness of the buffered beef extract at pH 9.5 increased as its volume increased. A regression analysis showed a linear correlation between the eluant volume and the percent recovery of viruses ($r = 0.92$). Since the pH and the concentration of beef extract were the same, it seems that the improved elution efficiency is a reflection of the volume of the eluant. Hurst and Goyke

(1986) observed an increase in elution efficiency when the volume of eluant increased up to a 1:5 ratio of sludge solids to eluant, greater ratios (1:6) lead to a decrease in elution efficiency. This decrease in elution efficiency at ratios greater than 1:5 was not observed in our study, where ratios up to 1:10 were tested. However, Hurst and Goyke worked with sludge solids only, and our study involved soil particles which may behave differently due to a different soil particle-virus association. The mechanism behind this increased efficiency with increasing volumes of eluant is not clearly understood. It is likely that increasing volumes of beef extract may increase the number of sites available to the viruses in the system. The ratio of viruses to the number of adsorptive sites in the soil matrix may become increasingly low (as compared to the eluant), therefore, causing a shift in the equilibrium towards the eluant side.

The use of large volumes of eluant, however, puts some limitations for the field studies necessary to assess virus survival and movement in the sludge:soil matrix. It becomes prohibitive in terms of time and costs involved. Reconcentration by bioflocculation described by Katzenelson et al. (1976) causes a loss in the recovery efficiency of viruses (average of 20% loss) but it becomes less time consuming and less expensive since it concentrates the eluted virus into smaller volumes which can be assayed in tissue culture. However, this is still not a practical method since only small amounts of soil can be tested due to the large

volumes of eluant necessary for good recoveries.

It is possible that greater elution efficiencies may be achieved with lower volumes by changing other parameters such as the type of eluant, pH, and concentration of the eluant. Beef extract is the most common eluant used due to the advantage that it offers with the reconcentration step. In addition, only one type of virus (poliovirus type 1, strain LSc) and one type of soil and sludge was used in this study. Other viruses and other soils may behave differently due to different isoelectric points and different chemical composition. Soil type strongly influences the behavior of viruses (Gilbert et al. 1976). Pancorbo et al. (1981) also found that sludge type strongly influences the effectiveness of virus recovery. These authors also suggested that studies conducted with artificially contaminated samples may differ from field conditions because laboratory viruses, unlike indigenous viruses, tend to adsorb to solid matter, while indigenous viruses are believed to be mostly embedded within sludge solids. However, such studies are necessary and provide valuable information. Pancorbo et al. (1981) concluded in their studies that with a contact time of 10 to 60 minutes there was no inactivation of poliovirus seeded into anaerobically digested sludge and that the virus stock could be used as a control to determine the number of viruses present in sludge. On the other hand, this study gave a 40 to 50 % reduction of poliovirus added to anaerobically digested sludge for a maximum contact period of 15

minutes. The initial concentration of viruses was determined using small aliquotes of the sludge:virus mixture instead of the original stock.

CONCLUSION

The occurrence of enteroviruses and Giardia cysts in Pima County'sewage sludge before and after anaerobic digestion was studied. The results of this study suggest that significant concentrations of enteroviruses and Giardia cysts are present in anaerobically digested sewage sludge being disposed of onto farmland. High concentrations of Giardia cysts were found in both raw and treated sewage sludge suggesting that anaerobic mesophilic (32° C) digestion of sludge is not effective at removing Giardia cysts. The viability and survival of Giardia cysts after anaerobic digestion and upon application of sludge to farmland should be assessed.

Removal and/or reduction of enteroviruses by anaerobic sludge digestion was significant but highly variable. Treatment of sewage sludge at the Ina Road Wastewater Treatment Plant may have to be upgraded or better controlled in order to obtain consistent removal of viruses and comply with the United States Environmental Protection Agency's proposed standards.

Wastewater treatment processes were designed to meet the demands of physico-chemical standards rather than microbiological standards, therefore, the removal of pathogens ought to be regarded as fortuitous (Morris, 1984). However, modern society demands for the protection of the environment and to minimize the health risk associated with pathogens indigenous to sewage, require that reduction of pathogens be an integral part of sewage treatment. The effect of

operational parameters such as the retention time and temperature of the sludge digestors could be evaluated in order to upgrade the treatment process and control the pathogen levels of sewage.

It is also necessary to assess the survival and movement of enteric viruses in soil after sludge application due to their potential of leaching into the groundwater beneath the site. The use of large volumes of beef extract (increasing the ratio of eluant to solids) for the elution of viruses from soil:sludge mixtures enhanced recoveries of viruses. However, it makes the method less practical since only small amounts of soil can be sampled at a time.

APPENDIX A

SOIL ANALYSIS*

Soil class: Sandy Loam

Soil texture: Sand = 61.6 %
Silt = 24.8 %
Clay = 13.6 %

Exchangeable Na = 70 ppm
K = 1300 ppm
Ca = 2048 ppm
Mg = 162 ppm

pH = 8.2

EC_e = 0.64 mmhos

* Soil analysed on a 1:2 soil to water suspension.

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