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SMALL SCALE WASTE MANAGEMENT PROJECT

Mixed Waste Studies with Viruses and Giardia

by

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MIXED WASTE STUDIES WITH VIRUSES AND *GIARDIA*

by

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Summary: When the septic tank soil field fails on a farm that has a slurry system for animal manure, septic tank effluent may be mixed with manure slurry, for disposal to land surface. Viruses and parasite cysts, the principal public health concerns, undergo relatively rapid degradation in mixed waste.

Keywords: animal manure, *Giardia* cysts, mixed waste, septic tank effluent, viruses

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ABSTRACT

When the soil field of a farm on-site wastewater treatment system fails, it may be impossible to install another soil field. If the farm is handling manure in liquid suspension, an alternative is to run the septic tank effluent (STE) into the stored animal manure slurry, for eventual surface disposal to land as if only manure were present. Public health concerns with this approach are mainly about human enteric viruses--pathogenic bacteria and parasites present are as likely to be of animal as human origin. We found previously that poliovirus and cysts of *Giardia lamblia* are degraded relatively rapidly in mixed waste and that specific bacteria from manure would attack poliovirus. We have now determined that hepatitis A virus (HAV) is also subject to such degradation, both in mixtures of dairy cattle manure slurry with STE and of swine manure slurry with STE. Mixtures comprised 80% animal manure slurry and 20% STE. They were sampled weekly during storage at 5° and 22°C (ca. 41° and 72°F), roughly representing a seasonal range of mixed-waste temperatures. Some of the bacteria isolated from the waste suspensions have been shown to exert direct antiviral effects, and cell-free filtrates of the bacterial cultures appear to degrade HAV. The first point of attack is evidently the coat protein of the virus. Along with several other considerations, the observed viral degradation affords significant reason to consider mixed waste systems in particular farm situations.

Keywords: animal manure, *Giardia* cysts, mixed waste, septic tank

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effluent, viruses

MIXED WASTE SYSTEMS

Land that is good for farming may not be good for subsurface disposal of septic tank effluent. When the soil field of an existing septic tank system fails in Wisconsin, a farmer may be denied a permit to install a new soil field because the available land does not meet Wisconsin's more stringent, current criteria. The present legal alternative is a holding tank, which must be emptied frequently (often every 1-2 weeks) throughout the year.

A proposed alternative--for farms that have existing slurry systems for animal manure--has been to mix the effluent from the septic tank with the manure slurry, for eventual surface disposal to land **as animal manure** (Snowdon et al., 1989a). The four Wisconsin state agencies that have oversight in such situations met with University of Wisconsin-Madison faculty and agreed that the mixed waste alternative should be tested on an experimental basis. The agreement applies only to existing farms with failed septic tank soil fields, denial of permit to replace the soil field, and an existing animal manure slurry system of such a scale that the septic tank effluent does not exceed 25% of the total waste volume. It has been determined that the most recent EPA regulations do not preclude this practice, where the human waste has been treated in a septic tank and does not include the solids that accumulate in the bottom of the tank. Yet to be resolved is the disagreement with the Grade A Pasteurized Milk Ordinance of the United States, which forbids mixture of human and animal wastes on Grade A dairy farms.

Most disease agents in body wastes are shed in feces, rather than urine. These pathogens are categorized as viruses, bacteria, and parasites. Many of the most significant bacteria (e.g., *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* sp.) are more likely to be shed in animal manure than in human feces. The same is apparently true of the more commonly waterborne parasites, *Cryptosporidium parvum* and *Giardia lamblia*. Therefore, the most significant public health concern in connection with mixtures of human and animal wastes should be the viruses, which are quite host-specific (Snowdon et al., 1989a). We had shown earlier that poliovirus is inactivated relatively rapidly in mixtures of septic tank effluent and animal manure slurry (Deng and Cliver, 1992a; Snowdon et al., 1989b). However, the virus of hepatitis A is apparently more durable in environmental settings, so it seemed important to determine the fate of this agent in mixed wastes. We review here the findings regarding degradation of poliovirus 1 and *Giardia lamblia* in mixed wastes, and summarize the latest findings regarding hepatitis A virus in this context.

MATERIALS AND METHODS

Viruses and cell cultures

Poliovirus 1 (strain CHAT), from the American Type Culture Collection, was propagated and assayed in BS-C-1 established monkey kidney cell cultures by methods described earlier (Deng and Cliver, 1992a). Hepatitis A virus (strain HM175/18f) a rapidly growing and cytolytic variant that is highly adapted to growth in cell cultures (Cromeans et al., 1987), was obtained from Dr. S. M. Lemon, University of North Carolina, Chapel Hill, and used as a model virus in our studies. The strain of FRhK-4 monkey kidney cells used in these studies was also obtained from Dr. Lemon. In the plaque assay of hepatitis A virus, monolayers of FRhK-4 cells were inoculated with serial dilutions of the virus, and a semisolid overlay was added to restrict the spread of virus. After 14-16 days at 37°C, the cells were fixed with formaldehyde at room temperature for 2 hr and stained with crystal violet.

Giardia methods

Methods for extracting cysts of *G. lamblia* from environmental samples and counting them have been described (Deng and Cliver, 1992b). Most important, cysts were scored as viable or nonviable on the basis of the fluorogenic stains fluorescein diacetate ("live" cysts fluoresce yellow-green, or remain unstained) and propidium iodide ("dead" cysts fluoresce red-orange). Viability classifications based on these fluorogenic stains have been validated by high-resolution scanning electron microscopy.

Mixed waste

Effluents (20% by volume) from septic tanks serving Wisconsin farm families were mixed with slurries of swine manure or dairy cattle manure (80% by volume). In many instances, coarse suspended solids were removed by filtration of the mixture through a series of sieves with 2-, 0.850-, and 0.300-mm apertures, to facilitate sampling.

Field experiments

Mixtures of septic tank effluent and swine manure slurry were inoculated with poliovirus or *Giardia* cysts. Forty ml of the inoculated mixed waste was loaded into each of three dialysis-type chambers with polycarbonate filter membranes, which had been developed at the Food Research Institute especially for this study. As controls, poliovirus or *Giardia* was added to Dulbecco's phosphate buffered saline (D-PBS), pH 7.4, containing 2% GG-free calf serum, to the same concentration as in the mixed waste. Forty ml of the seeded D-PBS was loaded into a 100-ml prescription bottle, which was then put in a metal container. The three chambers and the bottle of control suspension were immersed in a mixed waste pit on a swine farm located in northern Dane County, WI.

Laboratory experiments

Bench-scale experiments were done with virus or *Giardia* inoculated into mixtures of septic tank effluent (20%) and slurry of swine manure or dairy cattle manure (80%). Storage temperatures were usually 5° or 22°C, chosen on the basis of expected extremes for mixed waste in Wisconsin. In most instances, entry of oxygen from air was limited by covering the mixed waste in each flask with a layer of mineral oil.

Virus degradation

Virus that had lost its infectivity was assumed to have been affected by physical, chemical, or microbial action. Physical and chemical effects were evaluated as much as possible by inclusion of appropriate controls: this evaluation suggested that microbes were playing a significant part in virus inactivation. Bacterial strains isolated from both the poliovirus and hepatitis A virus experiments were grown in pure culture in thioglycollate medium and tested for inactivation of the respective virus. Bacterial strains selected for further study were those that caused 90% inactivation of virus within 10 days at 30°C. Cell-free filtrates of these pure cultures (heart infusion broth, 24 hr at 37°C) were tested for antiviral effect, with and without added protease inhibitors (α_2-macroglobulin, "E-64," pepstatin, phosphoramidon, and phenylmethylsulfonyl fluoride), singly or all combined.

RESULTS

Virus inactivation

Field experiments. The poliovirus in the mixed waste was inactivated much more rapidly than that in the control buffer suspension. After 16 weeks at the variable temperatures in the mixed waste pit, the poliovirus titer in the mixed waste was 0.51 $\log_{10}$ PFU/mL (mean of six replicate samples), whereas the titer in the control bottle was 4.89 $\log_{10}$ PFU/mL, from an initial level of ca. 10^7 PFU/mL (Deng and Cliver, 1992a). The mean temperature during the period was 20.8°C, and *D* values (days to 90% virus inactivation) were 18.7 for the mixed waste and 56.5 for the buffer control. A second trial yielded similar results (*D* values 29.9 and 51.8, respectively), though total inactivation was somewhat less because temperatures were lower (mean 14.4°C).

Bench experiments. Poliovirus was consistently inactivated more rapidly in the mixed waste than in the control buffer. Virus persistence in mixed waste was inverse to temperature. A comparison of poliovirus inactivation in raw mixed waste, autoclaved mixed waste, and bacteria-free filtrate at the same pH and temperatures provided an initial demonstration that the virus inactivation in the mixed waste is related, at least in part, to microbial activity in the waste. At the selected temperatures (25° and 37°C), the virus was consistently inactivated more rapidly in the raw mixed waste than in the autoclaved mixed waste, in which all microbes had presumably been killed. At the same time, the virus inactivation in the bacteria-free filtrate of raw mixed waste was somewhat faster than in the autoclaved mixed waste at both temperatures. It seems likely that there were some metabolites in the filtrate of the raw mixed waste, produced previously by microbes and not removed by the filtration process, which acted on the virus.

As indicated by differences in regression coefficients and *D* values, at both 5° and 22°C, hepatitis A virus was degraded most rapidly in mixed wastes, and the septic tank effluent (D = 58.5 at 5°C; D = 35.1 at 22°C) alone was more effective than the buffer (D = 217.4 at 5°C; D = 90.1 at 22°C). The rates of viral degradation in the two types of mixed wastes were somewhat different at the two temperatures. The mixture with dairy cattle manure (D = 34.6) seemed to be more efficient than that with swine manure (D = 48.5) at 5°C, but it was less efficient (D = 23.0) than the counterpart (D = 17.1) at 22°C. It was also obvious that viral degradation in all the four suspending media at the higher temperature was faster than at the lower. This suggested that the degradation process was temperature-dependent, possibly because degradation was partially biological, and the microorganisms involved were more active at the higher temperature.

Giardia degradation

G. lamblia cysts were also consistently degraded more rapidly in the mixed waste than in the buffer controls under both laboratory and field conditions (Deng and Cliver, 1992b). The rates of cyst degradation in septic tank effluent and buffer were similar. Increasing the proportion of swine manure slurry in the mixed waste favored the cyst degradation. These results suggest that the mixed waste treatment was the predominant factor in the degradation of the *Giardia* cysts in these experiments and that it was swine manure that played the role of degrading the parasite. The strong temperature dependence of the cyst degradation (D = 4.1 at 25°C; D = 129.9 at 5°C) suggests that the process may have at least a partially biological basis. Examination of viable and nonviable *G. lamblia* cysts with high resolution low-voltage scanning electron microscopy revealed an excellent correlation between the viability of the cysts determined by fluorogenic dye staining and their electron microscopic morphology. The staining method appears to be reliable for assessing the viability of *G. lamblia* cysts in experiments such as these.

Virus degradation

The confirmatory experiment with poliovirus and three bacterial cultures isolated from swine manure slurry that had shown antiviral effects in the screening test indicated conclusively that these microorganisms did cause inactivation of poliovirus in the autoclaved mixed waste, in which the effect of other microorganisms was excluded (Deng and Cliver, 1992a). Inhibition (of poliovirus inactivation) by protease inhibitors indicated that the virus inactivation in the mixed waste was in part due to proteolytic enzymes produced by bacteria in the waste.

Of 31 bacterial strains isolated from dairy cattle or swine manure and tested against hepatitis A virus, 10 met the criterion of producing 90% inactivation of the virus within 10 days at 30°C in thioglycollate medium. Beginning with 1.7×10^7 PFU/mL of hepatitis A virus, infectivity tests were negative ($<0.6 \log_{10}$ PFU/mL), and no virus could be detected in the samples by an antigen capture-polymerase chain reaction test (Deng et al., 1994), after 5 (1 strain), 6 (4 strains), and 9 (5 strains) weeks. Most, but not all, of the 10 strains raised the pH of heart infusion broth during growth; comparisons suggested that alkalinity was not a principal antiviral property of these cultures. Cell-free filtrates of nine of these strains, produced as described in Materials and Methods, caused at least 90% inactivation of the hepatitis A virus within 6 days at 37°C; the other did not. Three of the nine active culture filtrates were significantly inhibited by incubation with protease inhibitors before the virus was added, whereas the other six were not.

DISCUSSION

This study was intended to evaluate the potential public health effects of mixing on-farm septic tank effluent with animal manure slurry, for eventual disposal to land surface as manure. The emphasis was on viruses infectious for humans because these are the one category of pathogens that would be unlikely to occur in manure slurry that had not received human waste.

Both poliovirus and hepatitis A virus were shown to be much more rapidly inactivated in mixed waste than in laboratory buffer or in septic tank effluent without manure. Some of the poliovirus inactivation experiments were run in special dialysis-type chambers that could be filled with mixed waste and immersed in a pit of mixed waste for periodic retrieval and sampling. The remainder of the experiments were done in the laboratory, where temperatures could be controlled more closely and more frequent sampling was possible. Containment of hepatitis A virus was, of course, critical. Removal of the bacterial cells by filtration of mixed waste caused a decrease in antiviral activity, whereas autoclaving the mixed waste caused a greater loss of antiviral activity. Several strains of bacteria isolated from manure, and now known to belong to several different species (*Bacillus brevis*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus* sp., *Enterococcus faecium*, and *Pseudomonas alcaligenes*), have been shown to inactivate virus under laboratory conditions. Cell-free filtrates of cultures of most of these bacteria are antiviral, and some are inhibited by protease inhibitors, indicating that they attack the coat protein of the virus. Others are not affected by the protease inhibitors used in these studies and may act on viruses in some other way. Attempts are continuing to characterize the non-protease antiviral substances from these cultures and to determine how the virus is affected by them.

The *Giardia* results were similar, in that microbial action was possibly associated with the loss of viability of the cysts. In electron micrographs of *Giardia* cysts from mixed waste, what appeared to be bacterial cells were sometimes seen adhering to the cysts, but these may or may not have been actively causing degradation (Deng and Cliver, 1992b). In that *Giardia* cysts in wastewater or in the environment are often not of human origin, the enhanced degradation of these in mixed waste is significant but not critical to the safety of mixing septic tank effluent with animal manure slurry.

The public health implications of mixing septic tank effluent with animal manure slurry for eventual disposal to land surface as manure do not depend solely on these microbiological findings. It is noteworthy and encouraging that bacteria in farm animal manure appear to be capable of biodegradation of viruses under practical conditions. Biodegradation of waterborne viruses has been demonstrated previously, but conditions were aerobic and hepatitis A virus was not included (Cliver and Herrmann, 1972; Herrmann et al., 1974). However, it should also be noted that a holding tank is the present legal alternative, at least in Wisconsin, when a septic tank soil field fails and cannot be replaced because the site does not meet current requirements. A farmer with a holding tank will be obliged to empty it every few weeks, on a year-around basis. This wastewater may be spread onto land surface even when the ground is frozen, whereas a sufficiently capacious manure slurry pit could accept the septic tank effluent (which may be a useful diluent) for land aplication once or twice a year, when the soil is being worked. This would favor incorporation of any remaining pathogens into the soil, where they are unlikely to cause harm.

Mixture of human waste with animal manure is prohibited nationwide by the Grade A Pasteurized Milk Ordinance, but is permitted on a trial basis on other farms in Wisconsin under a memorandum of understanding among the four state agencies that have oversight in the matter. The Wisconsin delegation to the 1993 Interstate Milk Shippers' Confernece in Arlington, Texas, proposed that mixing waste be permitted on Grade A dairy farms under the conditions outlined in the Wisconsin plan. The proposal was not adopted, due partly to a misunderstanding of the proposal. A critique by the committee that considered the proposal seemed to center on the hazards of dairy cattle manure, which are real but are not exacerbated by addition of septic tank effluent. The matter is supposed to be studied by an ad hoc committee and reconsidered at the 1995 conference. Meanwhile, very limited trials in Wisconsin seem not to be turning up any unforeseen problems. We believe that the experimental data and the rationale presented here recommend the mixed waste practice as a positive contribution to public health under the circumstances described.

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