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by

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HUMAN AND ANIMAL WASTES MIXED FOR DISPOSAL TO LAND: INACTIVATION OF VIRUSES AND PARASITES IN A LABORATORY MODEL

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Water is costly. It often serves as a suspending medium both for human and animal wastes. Some areas that are well suited to animal agriculture have soils that are poorly suited to on-site disposal of human waste. Disposal of human waste supensions to land surfaces is likely to be prohibited or much more restricted than the surface disposal of animal waste slurries, partly out of concern for health. On the other hand, animal waste slurries are commonly stored and can thus be discharged at selected, favorable times, whereas human waste suspensions may require frequent disposal--regardless of unfavorable weather, etc.--for lack of detention facilities. These considerations led us to examine the potential health effects of mixing a human waste suspension (septic tank effluent) with a dairy manure slurry for eventual disposal to land as animal waste.

A 43-state survey on mixed waste disposal (J.C. Converse, 1983, unpublished) revealed that 67% of the responding states prohibit mixing household waste with manure from cattle, swine, or poultry. Grade A milk regulations also prohibit mixing domestic wastes with animal wastes. However, Wisconsin (and perhaps other states) permits spreading septage and holding tank wastes on forage crops if done at least 8 weeks before consumption of the forage by animals. Because these regulations appear to be generally arbitrary, we wished to develop at least some data concerning the possible public health effects of mixing waste for surface disposal to land.

"Mixed waste" (MW), for purposes of this study, comprised 15% septic tank effluent (STE) and 85% dairy manure slurry. Selected pathogenic agents were added to this mixture and compared, for rate of loss of infectivity, with the same pathogens in STE alone or in phosphate-buffered saline or distilled water. Clearly, the focus was on events during detention of the liquid waste--before land spreading.

Pathogens (disease agents) in wastes comprise viruses, bacteria, and parasites (cysts of protozoa and eggs of worms; Ward and Morrison 1983). Viruses are highly host-adapted, or species-specific. Viruses present in animal feces are not a threat to human health; these agents might infect animals after surface disposal of a manure slurry, but this situation occurs without mixing wastes. Human viruses present in septic tank effluent would represent a risk to human health, but not animal health, if mixed waste were inappropriately applied to land. Bacteria vary in

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species-specificity. Those that are most strongly adapted to humans tend not to persist for long periods in the environment, whereas fecal bacteria of animal origin, notably Salmonella, are hardy. This suggests that mixing human waste with an animal manure slurry would not add to whatever hazard might result from surface disposal of the latter. Protozoa (particularly Giardia lamblia) are frequent causes of waterborne disease in humans and are apparently capable of some transmission among species (Benenson 1985). Some parasitic worms have a life cycle in which two host species (e.g., humans and cattle) must be infected alternately; others may infect only a single species, but may present an environmental hazard because they persist for long periods in the soil to which waste has been applied.

Model agents selected for this study comprised a vaccine strain of poliovirus 1 as a representative human enteric virus, cysts of Giardia lamblia as a representative protozoon, and eggs of Ascaris suum to represent a relatively species-specific but environmentally stable parasitic worm (Ascaris lumbricoides, which might have been even more appropriate, was unavailable). Tapeworms, which must infect two alternate species during their life cycle, would also have been of interest; however, the tapeworm species that we were able to obtain were not those that infect food animals and gave equivocal results, which will not be reported here. Bacteria were omitted because none seemed of specific concern in the context of mixed waste.

EXPERIMENTAL

Infectious Agents

Poliovirus 1, strain CHAT, had originally been obtained from the American Type Culture Collection and had been used experimentally for many years in our laboratory (Cliver and Herrmann 1972, Stramer and Cliver 1984). The virus was replicated in monolayer cultures of established monkey kidney cell lines, usually the BGM line. Virus infectivity was assayed by the plaque technique in monolayer monkey cell cultures maintained with agar medium. Results were recorded as plaque-forming units (PFU) per milliliter.

Cysts of Giardia lamblia--a strain of human origin that had been passed through gerbils--were obtained from Dr. Hibler (Colorado State University, Ft. Collins). Gerbil pellets containing the cysts were stored under water (distilled, sterilized) at 4°C until the start of the experiment. Cysts in experimental samples were counted with a hemacytometer. Viability was assessed by differential staining with propidium iodide and fluorescein diacetate. Propidium iodide crosses the membranes of nonviable Giardia cysts, intercalates in the nucleic acid, and fluoresces a bright red-orange when seen in a microscope with appropriate ultraviolet excitation. Fluorescein diacetate can only cross a membrane by active metabolism (i.e., into a viable cell) and will fluoresce green in an ultraviolet microscope if the cell's metabolism has cleaved the acetates from the molecule. Each cyst seen with the microscope was first identified (based on size; shape; and the presence of a refractile edge, two nuclei, and a median body) with visible light and then viewed with ultraviolet excitation and scored as nonviable (red) or viable (green).

Eggs of Ascaris suum were obtained from the intestinal contents of infected swine, with the assistance of Dr. Dwight Bowman (School of Veterinary Medicine, University of Wisconsin-Madison). Viability of eggs recovered from experimental samples was assessed by clearing the eggs with a 20% solution of household bleach, holding the eggs (after washing) in a

0.5% formalin solution at 27°C for 4 weeks, and examining the eggs microscopically for the presence of active embryos.

Inactivation Studies

Suspending media in the first virus experiment comprised phosphate-buffered saline (PBS, pH 7.2), STE from a household system serving two adults and two children, and MW made from 15% STE and 85% of a mixture of fresh manure and urine from one dairy cow. Crude poliovirus (i.e., as obtained from the cell cultures) was mixed with each of the suspensions. A magnetic stirbar and 200 ml of each of the suspensions were placed in a sterile Erlenmeyer flask, covered with sterile mineral oil (attempting to maintain anaerobic conditions in the suspension), and closed with a sterile black rubber stopper. Portions (20 ml) of each suspending medium, without virus, were frozen and used to replace a similar amount of the contents of each flask at weekly intervals: this was done in the case of the MW to afford a fresh supply of nutrients to whatever active microflora might be present; the other suspensions were treated the same way to keep the applicable dilution factors constant. Two flasks of each suspension were incubated at 15°C; duplicate samples were processed and analyzed for virus content every third week from the material removed from the flask for replacement. In one follow-up experiment, some of the MW was irradiated with cobalt-60 gamma rays (courtesy of Dr. Donald Thayer, USDA Eastern Regional Laboratories, Philadelphia) to kill the indigenous microflora; in another, the manure portion of the MW comprised urine and feces composited from three cows rather than one.

Giardia experiments were done with smaller volumes of suspension (100 ml/flask), with four flasks per suspension sampled singly, rather than in duplicate. Suspending media were sterile double-distilled water (DW), STE, and MW from an experimental system in northern Wisconsin. An estimated 6.75×10^6 Giardia cysts were inoculated into each flask. Samples were collected in random order from the flasks, at weekly intervals during storage at 15°C.

Ascaris experiments were conducted with 1 L of suspension per flask and two flasks per suspending medium. Suspending media were DW, STE, and MW prepared from 15% STE and 85% mixture of cow feces and urine. Each flask was inoculated with 53 ml of intestinal contents from a pig infected with Ascaris suum, mixed, sampled, and stored at 15°C. The gas space in these larger flasks was filled with nitrogen; the stopper closing the flask was fitted with a gas trap filled with mineral oil to exclude oxygen-containing air. In view of the great stability of Ascaris and the relative difficulty of recovering the eggs from the MW, only four sample sets were collected, one each on days 0, 4, 90, and 180.

Recovery of Agents

Obtaining representative samples and recovery of the model infectious agents from them were especially critical with MW in these experiments. Samples were taken after vigorous mixing, usually with a magnetic stirbar that had been placed in the vessel at the beginning of the experiment. Glassware was coated with silicone preparation to minimize adsorption of the agents to vessel surfaces.

Poliovirus was recovered from 5-ml samples to which 5 ml of a half-and-half mixture of PBS and calf serum had been added. The suspension was adjusted to pH 9 with 1.5 N NaOH, treated in an ultrasonic bath, and clarified by centrifugation at 16,500 x g for 1 h. The supernatant fluid was filtered at 0.45 µm and 0.22 µm porosities to remove bacteria before plaque assay. Most of the virus was recovered from PBS and STE by this method, but only 7 to 14% from the MW.

Giardia was recovered from MW by serial passage through sieves of 2.0, 0.85, 0.30, and 0.149 mm porosity; solids retained on each sieve were rinsed with a 0.01% solution of Tween 20, which passed through the sieve. Sieving was not needed in processing the DW and STE samples. Cysts were collected by centrifugation (5 min, 650 x g), resuspended in Tween solution, collected by centrifugation onto a layer of Percoll-sucrose solution (specific gravity = 1.09), and washed twice more by centrifugation and resuspension in Tween solution. They were counted in a hemacytometer, and viability was determined as described above.

Ascaris was recovered from smaller numbers of larger volume samples than the other agents because of the laborious process involved (Reimers et al. 1981). A 25-ml sample was suspended in 150 ml of a 1% solution of Linbro "7X" anionic detergent and passed through a series of sieves as described for Giardia. More "7X" solution was used in rinsing the sieves, and again sieving was omitted with DW and STE samples. Solids in the suspension that had passed the finest sieve were allowed to settle for an hour (the supernatant fluid was discarded), suspended in "7X" solution, and collected by centrifugation (3 min, 870 x g). The sediment was resuspended in 50 ml of zinc sulfate solution (specific gravity = 1.2); after another centrifugation--the eggs remained suspended--the supernatant fluid was passed through a 35 µm porosity sieve. The eggs were collected from the sieve mesh with "7X" solution and sedimented by centrifugation. The sediment was resuspended in water, held overnight at 7°C, and collected again by centrifugation. Eggs from this were pelleted, counted in the hemacytometer, and tested for viability as described previously.

RESULTS

Poliovirus 1

Plaque counts obtained by the methods described were transformed to $\log_{10}[(PFU/ml)+1]$. Statistical analyses of variance were based on a General Linear Model, with tests for non-linearity. Comparisons of inactivation rates are based on D values--the number of days required for 90% inactivation of the agent, derived from the slope of the linear-least-squares curve representing the log-transformed data. Where the presence of significant differences was demonstrated, the t-test was used under restricted rules to determine which of several differences was significant.

The first experiment compared the persistence of poliovirus in PBS, STE, and MW for a period of 105 days. D values were 71, 72, and 20 days, respectively; inactivations in PBS and STE were similar to each other, but significantly lower than in MW. The present report is not intended to address the influence of temperature on inactivation, but it might be noted in passing here that D values for MW did not vary significantly with temperature (5° to 25°C), whereas temperature was quite influential in the other two suspending media.

Attempts to associate the higher rate of inactivation of poliovirus in MW with microbial action produced only equivocal results. Gamma irradiation seemed likely to be the bactericide that would least perturb the complex ecosystem in the MW. However, the irradiation did not produce sterility, presumably because bacterial spores, which are known to be relatively resistant to irradiation, withstood the treatment. In another experiment, the MW was simply frozen at -20°C and thawed: this seemed to reduce the bacterial population to some extent and (not necessarily for that reason) reduced the rate of poliovirus inactivation compared with that in mixed waste that had not been frozen.

MW was produced with manure composited from several animals in another experiment. The D value for poliovirus in this suspension was 21 days (versus 20 days for MW in the first experiment). Surprisingly, the D value for STE in this experiment was only 25 days. Comparisons of "total" microbial levels (aerobic plate count) and of pH indicated that these were not significant factors in virus inactivation in these experiments. Identification of specific bacteria capable of inactivating viruses has been undertaken previously (Cliver and Herrmann 1972), but biodegradation of viruses is relatively difficult to demonstrate in anaerobic systems.

MW was obtained from an experimental on-site system on a dairy farm in northern Wisconsin. During a 105-day experiment, the D value for poliovirus was 33 days, which was significantly less than with MW that had been frozen and thawed before the experiment (D = 64 days) or with PBS (D = 90 days). The D values for virus in the frozen and thawed MW and in the PBS did not differ significantly from each other. In all, then, it appears that 90% inactivation of poliovirus in MW might take approximately a month at 15°C.

Giardia lamblia

The percentage of viability was high (generally >80%) in samples of Giardia cysts; this varied little with the suspending medium or the length of time in storage at 15°C (total elapsed time in this experiment was only 35 days). Reductions in numbers of viable Giardia cysts with time, then, were based principally on reductions in the total numbers of cysts that could be recovered. Presumably, the cysts that could no longer be recovered had disintegrated. D values were 47 days in DW, 40 days in STE, and 33 days in MW. Differences among these were not statistically significant.

Ascaris suum

Ascaris eggs persisted quite well in each of the suspending media throughout the 180-day storage period. However, the eggs in STE and MW lost viability, whereas those in DW essentially did not: the calculated D value in DW was 10,000 days, but the hypothesis of zero slope (i.e., no inactivation with time) could not be rejected statistically. D values were 400 days in STE and 280 days in MW; these did not differ significantly from each other, but the MW value was significantly different from that for DW. Statistics aside, it is clear that Ascaris eggs die very slowly under the conditions of this experiment.

Experimental constraints prevented all of these trials from being conducted simultaneously, so conditions in the various suspensions were not identical. Nevertheless, D values at 15°C are compared in Table 1, for whatever additional insights such comparisons may afford.

DISCUSSION

The data in this study are extremely variable, due principally to the heterogeneity of the MW and the great difficulty of recovering these agents quantitatively from MW. It may well be that some real differences (e.g., between treatment effects) were not significant statistically because of this variation. Therefore, it may or may not be only fortuitous that each of the agents studied was least stable (had the smallest D value) in MW.

The assumption was that human infectious agents would be present in STE, which might be spread on land surfaces in that form or after mixing

Table 1. Representative D Values[a] for Three Agents in Three Suspending Media at 15°C

Agent	Suspending medium		
	Phosphate-buffered saline or distilled water (days)	Septic tank effluent (days)	Mixed waste (days)
Poliovirus 1	71	72	20
Giardia lamblia (cysts)	47	40	33
Ascaris suum (eggs)	10,000	400	280

[a]Time for 90% inactivation

with manure slurry and storage for as much as 6 months or perhaps even a year. Obviously, even if the manure storage unit is emptied only once per year, some of the waste it contains will not have been in the mixture for long. It may well be that in northern states such as Wisconsin the greatest value of the storage basin is that it permits application of wastewater at times when the soil is not frozen, and perhaps not saturated with moisture.

Polioviruses have often been used to represent human viruses transmitted through the environment. This is because the vaccine polioviruses are relatively safe to handle and can be quantified with relative ease. Their stability in environmental contexts probably does not differ drastically from those of the hepatitis A virus and of other human intestinal viruses that are more often implicated in waterborne and foodborne disease. Temperature has appeared to be a preeminent factor determining the rate of poliovirus inactivation, even as pH and other environmental influences varied (Salo and Cliver 1976), but neither temperature nor pH seemed to be influential in MW. Biodegradation is another possible basis for virus inactivation in this system (Cliver and Herrmann 1972), but as was stated previously, this tends to be most significant in aerobic, rather than anaerobic systems.

For whatever reasons, Giardia lamblia is presently the leading cause of reported waterborne disease in the US. However, Giardia cysts are obviously less stable in water, and reputedly in soils as well, than Ascaris eggs. It is unfortunate that circumstances did not permit valid studies on tapeworm eggs--perhaps such studies could better be undertaken in an area of the US where cysticercosis is more prevalent in food animal species. Although both cysticercosis and human tapeworms are a relative rarity in Wisconsin, it is important that precautions be taken to keep it that way.

In addition to the inactivation of infectious agents that appears to occur during detention of MW, we believe it is important from a public health standpoint that detention in a storage unit would permit

application of the waste to land surfaces under more favorable conditions. In particular, virus present in wastewater (e.g., STE) applied to frozen soil is likely to be preserved until the spring thaw, at which time it may run off the fleld and contaminate surface waters. Some kinds of parasites are inactivated or killed by freezing, but that would not necessarily be true of those we studied.

SUMMARY

For reasons yet unknown, poliovirus 1 was more rapidly inactivated at 15°C in mixed waste (15% septic tank effluent plus 85% bovine manure slurry) than in phosphate-buffered saline (pH 7.2) or septic tank effluent. Degradation of the virus by bacterial action was suspected, but not proven in this context.

Giardia lamblia cysts disappeared more rapidly at 15°C from mixed waste than from distilled water or septic tank effluent, but the rates of decrease were not significantly different by the statistical test applied. In general, fewer cysts were recoverable with time, but the observed viability rate (roughly 80 to 90%) varied only slightly with time.

Ascaris suum eggs persisted, but lost viability with time at 15°C--especially in the mixed waste. Loss of viability in water was essentially not measurable during 180 days.

These observations do not prove the safety of mixing septic tank effluent with dairy animal manure for eventual disposal to land surfaces, but they do show that some human infectious agents that might be present lose infectivity or viability more rapidly in the mixed waste than in septic tank effluent or in water. This suggests that septic tank effluent would be safer to apply in this way than directly to the soil surface. It should be noted that the human waste in all of the experiments had been processed in a septic tank, rather than being dealt with in raw wastewater.

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