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Project Summary

Survival of Parasite Eggs in Stored Sludge

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The inactivation rates of digestion-resistant parasite eggs in laboratory-stored sludge were measured to determine their potential fate in sludge lagoons. Eggs from roundworms *(Ascaris, Toxocara,* and *Trichuris)* and a rat tapeworm *(Hymenolepis)* were added to domestic sludges either at the beginning and during, or after aerobic or anaerobic digestion. Digested sludge samples seeded with the parasite eggs were stored in the laboratory at 4°C, at 25°C, and in a container that was inserted in the ground to simulate the conditions of a sludge storage lagoon. Nonsludge soil samples (controls) were seeded with the same parasites as the digested sludges and stored under similar conditions.

The total number of eggs recovered from the samples decreased as storage time and temperature increased. The number of viable eggs and the potential infectivity of recovered *Toxocara* and *Ascaris* eggs were related primarily to storage temperature and time. After 25 months of storage at 4°C, the *Toxocara* eggs and some *Ascaris* eggs remained both viable and infective, whereas most of the eggs stored at 25°C were nonviable after 10 to 16 months of storage in sludge. Though storage temperature and time were the most important factors in the inactivation of these eggs, minor effects were also associated with other factors — type of sludge digestion, timing of egg addition (during or after sludge digestion), type of storage (in sludges versus in soil), pH, and species of egg. These controlled laboratory studies suggest that sludge lagooning can be an effective method for inactivating parasite eggs, particularly in warm geographic locations.

This Project Summary was developed by EPA's Water Engineering Research Laboratory, Cincinnati, OH, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

Enteric parasite eggs become concentrated in domestic wastewater sludge after treatment of the raw sewage. Sludge treatment by mesophilic anaerobic or aerobic digestion inactivates some but not all of the parasites. Digestion-resistant eggs of the enteric parasites *Ascaris, Toxocara, Toxascaris,* and *Trichuris* can remain viable and capable of infection. They must therefore be considered when sludge is disposed of, particularly on land.

Digested sludge is commonly used as a fertilizer to replenish agricultural soil and as an alternative to dumping, burying, or incineration. For example, approximately 60% of all municipal sludge in Ohio is applied to land. Once sludge material is placed on land, however, viable parasite eggs could become infectious agents and pose a potential health hazard to humans and domestic animals.

A promising procedure for minimizing the hazard from these parasite eggs is additional treatment of the sludge in storage lagoons. Sludge lagoons are commonly used to store digested sludge before further treatment or land application, or to provide permanent disposal. Typically, sludge is stored in lagoons for periods ranging from several months to years. During this time, the solids settle

to the bottom. Anaerobic decomposition continues at the bottom of lagoons, and the supernatant is periodically drawn off and recycled to the sewage treatment plant for further processing. Lagooning has the advantage of being a simple method that is economical in areas where land is available. Long periods of storage are known to cause substantial inactivation of digestion-resistant parasite eggs, but quantitative data are sparse.

The purpose of this study was to investigate the inactivation rates of parasite eggs stored in sludge under controlled laboratory conditions to determine their potential fate in storage lagoons. Eggs of roundworms (*Ascaris, Toxocara,* and *Trichuris*) and those of a rat tapeworm (*Hymenolepis*) were seeded into the sludge before and during, or after anaerobic or aerobic digestion. An earlier study reported on the survival rates of these eggs during the digestion processes (M.I. Black et al., "Survival Rates of Parasite Eggs in Sludge During Aerobic and Anaerobic Digestion," Journal of Applied and Environmental Microbiology, 1982, *44*:5,1138-1143). The present study examines the effects of temperature, storage time, and type of sludge digestion on the survival of eggs under long-term storage conditions.

Procedures

Organisms and Digestion of Sludge

Toxocara canis, Trichuris vulpis, Ascaris suum, Trichuris suis, and *Hymenolepis diminuta* eggs were collected over a 5-month period beginning in October 1979. Parasite eggs were added to sludge that was processed by methods simulating authentic high-rate municipal digesters with constant removals and additions (not digestion of batch samples).

Soil Controls

Topsoil was also spiked with eggs and used as a nonsludge control. To duplicate naturally occurring topsoil, the control was made up of one part each of loam, manure, and clay. The aerobically digested sludge contained 2.0% to 2.9% solids, and the anaerobically digested sludge contained 2.9% to 3.4% solids. Thus the topsoil control was made 3% solids in water.

Storage Conditions

Each batch of digested sludge or soil controls was divided into 100-g (wet weight) aliquots and placed into presterilized, 250-mL polypropylene bottles sealed with screw caps (Nalge Co., Rochester, New York). Samples were stored at 4°C, at 25°C, and in the ground. The 4°C and 25°C samples were stored in constant-temperature rooms. The purpose of the sample placed in the ground was to simulate conditions in a sludge storage lagoon. In-ground samples were placed in covered, 55-gal. galvanized drums. These were buried with their rims flush with the ground's surface in an area where they were subjected to the normal temperature fluctuations of the adjoining earth. Digested sludge samples spiked with all of the test species of helminth eggs were prepared for storage in March 1980. Eight aliquots of each sample for each of 15 different experimental conditions were analyzed at approximately 3-month intervals (a total of 120 samples for each sampling period). The study was terminated in 1983 after 33 months of storage.

Temperatures inside the drums were constantly monitored (Weathertronics 4120 thermograph*, Weathertronics, Sacramento, California). Air temperatures were obtained from the National Weather Service of the National Oceanic and Atmospheric Administration (Greater Cincinnati Airport, Boone County, Kentucky). As the seasons progressed, the average monthly temperatures inside the drums reflected those of the air with a slight lag time, but they never got as cold as the winter air. These records indicated that the in-ground condition was a valid simulation of a sludge lagoon, since lakes, ponds, and other buffered environments typically exhibit this lag. Furthermore, the in-ground high and low temperatures indicated that the samples were insulated from the broad range of air temperature fluctuations, again properly simulating sludge lagoons.

Sampling of Spiked Sludges and Spiked Soil Controls

The experimental setup for this study consisted of four spiked, digested test sludges plus one spiked soil control sample for each of three different test storage conditions (or a total of 15 different experimental conditions). To determine the rate of inactivation of the parasite eggs, eight samples for each of the 15 experimental conditions (or a total of 120 samples for each sampling period) were taken for examination approximately every 3 months over a total period of 25 months. A modified sampling schedule was maintained after 25 months of storage.

Recovery of Eggs from Sludge and Soil

The pH of each sample was determined before eggs were isolated. The entire content (100 g) of the sample in the plastic bottles was filtered through a U.S.A. standard testing sieve (No. 35 mesh) and washed with distilled water. The filtrate was returned to the same bottle and centrifuged at 400 X g for 5 min at 4°C. The supernatant was decanted, and 100 mL of 3° (w/v) lactalbumin hydrolysate (Sigma, St. Louis, Missouri) was added to decrease adherence of the eggs to the sludge. The mixture was centrifuged as above, the supernatant was discarded, and the pellet was washed free of lactalbumin hydrolysate with distilled water. After centrifugation, the pellet was resuspended in 50 mL (final volume) distilled water and layered onto a 150-mL continuous sucrose density gradient (specific gravity 1.26 to 1.00); then it was centrifuged at 800 X g for 15 min. The pellet formed at the bottom of the gradient was discarded, and the material within the gradient was collected, diluted with an equal volume of distilled water, and centrifuged at 800 X g for 5 min. The pellet material was washed three times with distilled water.

At the onset of storage, recoveries of eggs from sludge were determined for tests involving eggs added at the beginning and during aerobic or anaerobic digestion. The recoveries are listed in Table 1.

Similar recoveries were not determined for eggs added to soil controls because of lack of manpower at this labor-intensive time when hundreds of samples were being prepared for this study. Estimates of the average number of eggs in each 100-g, wet-weight soil sample were based on the number initially added. These averages were as follow:

Ascaris	10,400
Hymenolepis	12,500
Toxocara	2,100
Trichuris	900

Eggs recovered from all samples were incubated at 25°C in the dark for 30 days to cause embryonation.

*Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Data Analysis

The data were analyzed to determine whether each of the various storage conditions had an influence on the recoverability and viability of the test parasite eggs. Two-factorial analysis of variance (ANOVA) with repeated measures was used (BMDP Statistical Software, Inc., University of California). The first ANOVA used an abridged set of data. The data eliminated from these analyses were those of months 0, 19, 30, and 33 because complete data for the parasite-spiked sludge samples were not available. Also, data for the parasite-spiked soil samples were not included in this ANOVA because they might not be comparable with those of sludge samples. The soil samples were not treated aerobically or anaerobically, and eggs were not added to the soils over a period of 15 consecutive days (as would be required for a strict statistical analysis of variance evaluation).

Nevertheless, in the real world, soils are not normally digested aerobically or anaerobically to stabilize against putrefaction or to reduce vector attraction. Also, the test parasites were added directly to the soil samples in a manner similar to that used with the test parasites added to the sludges after digestion, and they were exposed to the same temperatures and storage time conditions. We therefore decided that useful information would be provided (1) by performing another ANOVA that included the test parasite soil samples and (2) thereby enabling comparisons with the ANOVA that excluded the soil samples. Again, data for months 0, 19, 30, and 33 were not included because complete data sets were not available.

Infectivity of Recovered Eggs

The infective potential of *Ascaris* and *Toxocara* eggs was analyzed. Samples containing viable and nonviable eggs were concentrated to 2 mL and intubated into male Holtzman albino rats (Charles River Breeding Lab., Inc., Wilmington, Massachusetts). After 8 days, the infected rats were sacrified by anaesthesia with diethyl ether, and their lungs, livers, kidneys, and brains were removed. Each organ was placed into 50 to 100 mL of physiological saline solution and cut into small pieces. One Baermann apparatus was set up for each organ. The funnel was filled with saline at 37°C, and two layers of cheesecloth were used to suspend the minced organ within the saline in each funnel. Lamps with 60-W tungsten bulbs were placed approximately 2 in. from the junction of the head and stem of each funnal to create a thermal gradient. After 50 to 60 min, the suspended organ was removed and discarded. The contents of each funnel were placed into 50-mL, glass, conical tubes, and 10 mL of 0.2% (w/v) saponin was added to each tube to lyse red blood cells. The tubes were centrifuged for 2 min at 500 X g, and the supernatant was discarded. The volume of the pellet remaining in each tube was examined. A small pellet (0.5 mL) required no further treatment and was resuspended in 20 mL of a solution containing 0.1% Tween-80 and 10% formalin-saline (5.4 mL of 37% formalin in 14.6 mL of 0.1% (v/v) Tween-80 physiological saline solution) to fix and preserve the larvae. Fixed samples were stored at 4°C until examined for larvae.

Tubes containing 0.5 mL or more of tissue pellet required further reduction before they could be analyzed. These pellets were suspended in 30 mL of a pepsin digestion solution (2.5 g of pepsin in 500 mL of 0.85% (w/v) sodium chloride plus 3.5 mL conc. HCl) and incubated at 30°C for 24 hr on a rotating platform at 200 rpm. The digested tissue was rinsed free of the pepsin solution with distilled water and fixed and storage as above. The number of *Ascaris* and *Toxocara* larvae in each sample were counted under 125X magnification using a phase-contrast microscope.

Table 1. Egg Recoveries from Sludge

	Eggs/100 g of Sludge (wet weight) ± Standard Error of Measurement (n=3)	
Egg Type	*Aerobic Digestion*	*Anaerobic Digestion*
Ascaris	4,800 ± 176	5,300 ± 475
Hymenolepis	4,800 ± 199	7,500 ± 535
Toxocara	1,700 ± 127	1,500 ± 168
Trichuris	400 ± 40	900 ± 63

Results and Discussion

Effects of Sludge Digestion

During the 15-day period of mesophilic anaerobic digestion approximately 23% of the *A. suum* eggs were inactivated. *T. canis* and *Trichuris* spp. eggs were not affected. Aerobic digestion inactivated 38% of the *Ascaris* eggs, 11% of the *Trichuris* eggs, but none of the *Toxocara* eggs. Therefore, it appears that laboratory aerobic digestion inactivated the parasite eggs more effectively than laboratory anaerobic digestion.

pH

The pH of soil and sludge samples changed with storage time. Large initial increases occurred in the pH values of all soil samples (from pH 6 to pH 7 to 8) and anaerobically digested sludge samples (from pH 7.2 to pH 8.5 to 9), whereas aerobically digested sludge samples showed an initial decrease in pH (from pH 7.2 to pH 6 to 7). After long-term storage, the largest changes in pH were seen in soil stored at 25°C, aerobically digested sludge stored at 4°C, and anaerobically digested sludge stored at 25°C, and in the ground. No obvious correlations were noted between the pH of samples and the recovery, viability, or infectivity of parasite eggs.

Ascaris

A substantial reduction occurred in the recovery of *Ascaris* eggs, especially within the first 3 months of storage; but more eggs were recovered from sludges than from the soil samples. The temperature at which *Ascaris* eggs were stored had little effect on the fraction recovered. Viability, however, was dramatically affected by the storage temperature and time. Viability decreased very slowly with storage at 4°C. After 25 months, more than 50% of the *Ascaris* eggs recovered from samples stored at 4°C were still viable; but 10 to 16 months of storage at 25°C rendered most of the recovered eggs nonviable. *Ascaris* eggs stored in the ground in anaerobically digested sludge lost their viability much earlier than those in aerobically digested sludge. However, after 30 months of storage, low levels of viable eggs were still recovered from the digested sludge stored in the ground. Viable *Ascaris* eggs were recovered from the soil controls from each storage condition over the 33-month test period. Overall, anaerobically digested sludge appeared to have a greater effect on reducing *Ascaris* egg viability than did

aerobically digested sludge or the soil control.

The infectivity of recovered *Ascaris* eggs incubated under conditions supporting embryonation reflected their viability. Eggs stored at higher temperatures decreased in infectivity more rapidly than those stored at lower temperatures. *Ascaris* eggs aerobically digested *along* with the sludge (Phase II) were noninfective (did not cause infections in test rats) after 25 months when stored at 25°C, in the ground, and at 4°C. In the case of *Ascaris* eggs added *after* aerobic digestion (Phase I), a small number of eggs from samples stored in the ground for 33 months still appeared capable of causing low levels of infectivity, even though there was no visual evidence of viability. Thus even when the egg population appeared to be nonviable, a few were occasionally capable of causing infections in test animals. Also, some eggs that appeared to be viable did not produce infection in the test animals. Thus tests for egg viability alone may not be sufficient to ensure noninfectivity.

Toxocara

The type of sludge (aerobic or anaerobic) had no affect on the recovery of *Toxocara* eggs; however, fewer eggs were recovered from the soil samples stored at 25°C and in the ground. The data on *Toxocara* were more scattered than those obtained for *Ascaris*, probably because of the smaller numbers of *Toxocara* eggs initially seeded in the samples. Storage temperature and time affected the viability of *Toxocara* eggs in a manner similar to *Ascaris* eggs. The viability of eggs decreased as the average storage temperature increased.

The viability of eggs stored in aerobically digested sludges decreased more slowly than in eggs stored in anaerobically digested sludges. The eggs recovered from samples stored at 25°C and in the ground were essentially nonviable after 13 months of storage in sludge. After 2 years in storage at 4°C, a significant number of the recovered *Toxocara* eggs were still viable. The viability of *Toxocara* eggs in the soil controls was not affected by storage at any temperature. The difference in the viability of *Toxocara* eggs stored in sludge and in soil was the most dramatic among all the parasite species tested. Thus it appears that the sludges also had some detrimental effect on *Toxocara* egg viability.

Though fewer *Toxocara* eggs were recovered than *Ascaris* (and therefore fewer total eggs were intubated into rats), these eggs were just as capable of causing infections. Higher storage temperatures decreased the infectivity of the eggs more effectively than did lower temperatures. Also, sludge storage of eggs decreased their infectivity more effectively than did soil storage.

Trichuris

Recoveries of *Trichuris* eggs from all samples were similar: all were low after 3 months of storage. The total number of *Trichuris* eggs seeded into each sample was smaller than for any other species. Because a low number of *Trichuris* eggs were recovered, the viability data on this species were very scattered. But eggs stored at lower temperatures generally had greater viabilities, and eggs stored in soil maintained their viabilities longer than the eggs stored in the sludges.

Hymenolepis

Though these eggs were not viable at the onset of sludge digestion and sample storage, they could still be recovered from the samples, especially those stored at 4°C. Temperature also affected the recovery of *Hymenolepis* eggs, which decreased more rapidly in the samples stored at higher temperatures. As with *Toxocara*, fewer *Hymenolepis* eggs were recovered from soil stored at 25°C and in the ground than were recovered from sludges.

Conclusions and Recommendations

The destruction of roundworm eggs was minimal during the 15-day treatment period of mesophilic anaerobic or aerobic digestion. After 3 months of storage at 25°C, at 4°C, and in the ground, the roundworm eggs recovered from both types of sludges were viable. *Ascaris* and *Toxocara* eggs were infective in rats.

After 16 months in storage at 25°C, very few of the recovered *Ascaris* eggs in the digested sludges appeared to be viable; nevertheless, a low level of infection in rats was observed after 22 months of storage at 25°C. At least 2 years of storage in sludge at 25°C was needed to inactivate most of the *Ascaris* eggs. When *Ascaris* eggs added after aerobic digestion (Phase I) were stored in the ground and exposed to normal temperature fluctuations, inactivation (both nonviable and noninfective) took at least 33 months. Storage at 4°C had little effect on decreasing *Ascaris* viability and infectivity. Thus the temperature and length of time at which digested sludge was stored appeared to be the most important factors for inactivating *Ascaris*. The higher the storage temperature and the longer the storage time at this high temperature, the more rapidly the roundworm eggs were rendered harmless.

Although the eggs of *Toxocara* were more resistant to digestion than those of *Ascaris*, they were more quickly destroyed during storage. After only 10 months in storage at 25°C, none of the *Toxocara* eggs recovered from the sludge were capable of producing an infection in rats. *Toxocara* eggs stored in the ground for 25 months could not produce an infection in rats even though they appeared to be viable. Storage at 4°C for more than 2 years did not effect their infectivity. As with *Ascaris*, *Toxocara* eggs were more quickly inactivated in sludges at higher storage temperatures with longer storage time.

Comments are difficult to make on the viability of *Trichuris* eggs recovered. As mentioned earlier, the recovery rates for these eggs were low and erratic, and large standard deviations were observed. Therefore only a most general statement can be made about the viability of these eggs. After 13 months of storage in sludges at 4°C, at 25°C, or in the ground, most of the *Trichuris* eggs were no longer viable. Eggs stored in soil, however, appeared to retain their viabilities for at least 25 months.

A summary of specific conclusions follows:

1) During the 15-day period of mesophilic anaerobic digestion, approximately 23% of the *A. suum* eggs were inactivated; *T. canis* and *Trichuris* spp. eggs were not affected.
2) Aerobic digestion inactivated 38% of the *Ascaris* eggs, 11% of the *Trichuris* eggs, but none of the *Toxocara* eggs.
3) The total number of eggs recovered from the stored samples decreased with time.
4) Eggs of all species studied were mostly inactivated faster (16 months in anaerobic digested sludges and about 25 months in aerobic digested sludges) when stored at higher (25°C) rather than at lower temperatures (4°C or in the ground).
5) *Ascaris* eggs aerobically digested along with sludge (Phase II) were noninfective after 25 months when stored at 25°C, in the ground, and at 4°C.
6) *Ascaris* eggs anaerobically digested with sludge (Phase II) were non-

infective by 16 months when stored at 25°C, but were still infective after 22 months when stored at 4°C or in the ground.

7) Either aerobic or anaerobic digestion makes *T. canis* eggs more susceptible to inactivation when compared with eggs stored in soil controls.
8) *Trichuris* spp. eggs in the digested sludges (Phase II) appeared to be inactivated by storage at 25°C and in the ground after 16 months, but could be viable even after 25 months when stored at 4°C. However, a conclusive statement on *Trichuris* viability is difficult to make because of the low initial innoculum levels and subsequent low recovery of these eggs.
9) *H. diminuata* proved very sensitive, since storage of the eggs at 4°C alone before seeding into the sludges rendered them nonviable and noninfective.
10) Viability of the eggs did not always predict infectivity; that is, eggs that did not look viable were sometimes infective and vice versa.

This 3-year investigation into the persistence of certain roundworm eggs during storage after anaerobic or aerobic digestion indicates the need for additional research. The following studies should be done on the survival of parasitic eggs in sludges.

1) An investigation of the types and numbers of microbial agents present in stored sludges and their contributions to the destruction of parasite eggs.
2) An investigation of the factors in the digestion processes that apparently increase the resistance of *A. suum* eggs to inactivation when they are subsequently placed in storage.
3) A thorough measurement of the redox potential of sludges during storage and the relation of the electrochemical potentials to the organic and inorganic chemical reactions that may affect parasite destruction.
4) A larger-scale investigation into the effects of long-term storage on the destruction of eggs from certain parasites (it should more closely duplicate existing lagoon conditions).
5) A study of the inactivation of parasite eggs within 3 months of storage in the absence of retrieving them from either sludge or soil (the object here is to conclusively establish whether they were inactivated or whether they became more difficult to isolate).
6) Studies of die-off rates in sludge-amended soils.
7) Biochemical and physiological analyses of parasite eggs to determine what is altered in eggs exposed to sludge as compared with eggs stored in controls (soil or water).
8) Research to improve the analytical methods for measuring the recovery and viability of parasite eggs in sludge (e.g., improve the standard deviation of the method).

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The complete report, entitled "Survival of Parasite Eggs in Stored Sludge," (Order No. PB 86-137 148/AS; Cost: $16.95, subject to change) will be available only from:

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