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Survival of Parasite Eggs Upon Storage in Sludge

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Destruction rates of parasite eggs in stored sludge were examined to help understand the fate of these agents of enteric diseases in sludge lagoons. Eggs from the roundworms, *Ascaris* spp., *Toxocara* spp., *Trichuris* spp., and the tapeworm, *Hymenolepis* spp., were treated with domestic sludges by aerobic or anaerobic processes. Sludge samples seeded with eggs were stored at 4 or 25°C or in a container inserted into the ground to simulate lagoon conditions. The number of eggs recovered from the samples decreased with storage time. The viability and infectivity of eggs recovered were related to the storage temperature; i.e., the eggs stored at 4°C remained viable longer than those stored at 25°C. After 25 months at 4°C, the *Toxocara* eggs and some *Ascaris* eggs remained both viable and infective, whereas most of these eggs stored at 25°C were rendered nonviable after 10 to 16 months of storage in sludge. Although storage temperature was found to be the most important factor affecting the destruction and viability of these eggs, other factors, such as the type of sludge digestion, whether or not the eggs were digested along with the sludge or added later, storage in the soil versus sludge, pH, and egg species also exhibited some minor effects. These controlled laboratory studies suggest that lagooning of sludge can be an effective method for the elimination of parasite eggs, particularly in warmer geographical locations.

Parasite eggs exist and become concentrated in domestic wastewater sludge (11, 12, 14). The usual treatment of raw sewage produces sludges that are usually treated by mesothermic anaerobic or aerobic digestion. These processes eliminate some parasites (8); however, others are resistant to these processes. Eggs of the enteric parasites of the genera *Ascaris*, *Toxocara*, *Toxascaris*, and *Trichuris* pass through digestion processes and remain viable and capable of infection (1–3, 6, 7, 10, 12, 15). The use of digested sludge as a fertilizer to replenish agricultural soil has been proposed as an alternative to discharging, dumping, burying, or incineration (9). Land disposal of digested sludge is relatively common. For example, ca. 60% of municipal sludges in Ohio is disposed of by land application (5). However, once sludge material is placed on the land, viable parasite eggs could become infectious agents, thus posing a potential threat to the health of humans and domestic animals (1, 3, 9, 20). Sludge lagoons are commonly used to store digested sludge before further treatment or land application or as a means of permanent disposal (5). Typically, sludge is stored in permanent lagoons of ca. 5.5 to 10.7 m in depth for periods ranging from several months to years, during which time the solids settle to the bottom. Anaerobic decomposition continues at the bottom of lagoons, and the supernatant is periodically drawn off and returned to the sewage treatment plant for further processing. Lagooning has the advantage of being simple to perform and economical in areas where land is available (4).

The purpose of this study was to investigate the destruction rate of parasite eggs stored in sludge under controlled laboratory conditions to gain insight on their destruction rates in lagoons. Eggs of roundworms commonly found in sludge (9, 12), as well as the eggs of tapeworms, were seeded into sludge before and after its anaerobic or aerobic digestion. We have previously reported on the survival rates of these eggs during the digestion processes (2). In the present study, survival rates under long-term storage conditions were examined.

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MATERIALS AND METHODS

Organisms and digestion of sludge. The sources and methods for obtaining eggs of *Toxocara canis*, *Trichuris vulpis*, *Trichuris suis*, *Ascaris suum*, and *Hymenolepis diminuta* have been reported previously (2). Eggs were collected over a 5-month period beginning in October 1979. The design and operation of anaerobic and aerobic digestors have been previously described (2). Addition of parasite eggs into aerobic and anaerobic bench-top digestors was by methods simulating authentic high-rate (15-day retention time) municipal digestors with constant removals and additions (not digestion of batch samples) (2). These samples in which eggs were added daily and digested along with the sludges were designated the experimental samples. As controls, batch samples of eggs were added to soil (see below), aerobically digested sludge, and anaerobically digested sludge. Thus, there were five different sample types.

Soil controls. Top soil was spiked with eggs and used as a nonsludge control. In an effort to duplicate naturally occurring top soil, the control sample contained one part loam, one part manure, and one part clay (19). It has been previously determined that the aerobically digested sludge used in this study contained 2.0 to 2.9% solids and that the anaerobically digested sludge contained 2.9 to 3.4% solids (2). Therefore, the nonsludge top soil control was made 3% in water.

Storage conditions. Each batch of sludge and soil spiked with parasite eggs, each containing all species of helminth, was divided into 100-g (wet weight) portions and placed into presterilized, 250-ml polypropylene centrifuge bottles with screw caps (Nalge Co., Rochester, N.Y.). Samples were stored at 4 and 25°C and "in ground". The samples placed in ground represented an attempt to simulate conditions in a lagoon. These sample bottles were placed in ca. 208-liter galvanized drums buried outdoors in the ground where they

were subjected to normal temperature fluctuations. The samples were prepared for storage in March 1980. Eight samples subjected to each of 15 different experimental conditions (five different sample types stored at three different temperature conditions) were analyzed at approximately 3-month intervals. The study was terminated in 1983 after 33 months of storage.

The temperature in the drums was constantly monitored (model 4120 thermograph; Weathertronics, Sacramento, Calif.) (Fig. 1). Air temperatures were obtained from the National Weather Service of the National Oceanic and Atmospheric Administration (Greater Cincinnati Airport, Boone County, Ky.). As the seasons progressed, the average monthly temperatures in the drums reflected those of the air with a slight lag time (Fig. 1A), but they never got as cold as the air during the winter months. These records indicated that the in-ground condition was a valid simulation of a lagoon, since lakes, ponds, and other buffered environments typically exhibit this lag (23). Furthermore, the in-ground high and low temperature extremes (Fig. 1B) indicate that the samples were insulated from the broad range of air temperature fluctuations, again properly simulating lagoons (23).

Recovery of eggs from sludge and soil. The pH of each sample was determined before the eggs were isolated. The entire 100-g content of the sample in the plastic bottles was filtered through a U.S.A. standard testing sieve (no. 35 mesh) and washed with water. The filtrate was returned to the same bottle and centrifuged at 400 × *g* for 5 min at 4°C. The supernatant was decanted, and 100 ml of 3% (wt/vol) lactalbumin hydrolysate (Sigma Chemical Co., St. Louis, Mo.) was added to decrease the adherence of sludge material to eggs. The mixture was centrifuged as described above, the supernatant was discarded, and the pellet was washed free of lactalbumin hydrolysate with distilled water. After centrifugation, the pellet was suspended in 50 ml (final volume) of water and layered onto a 150-ml continuous sucrose density gradient (specific gravity, 1.26 to 1.00) then centrifuged at 800 × *g* for 15 min. The pellet that 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 × *g* for 5 min. The pelletable material was washed three times with distilled water.

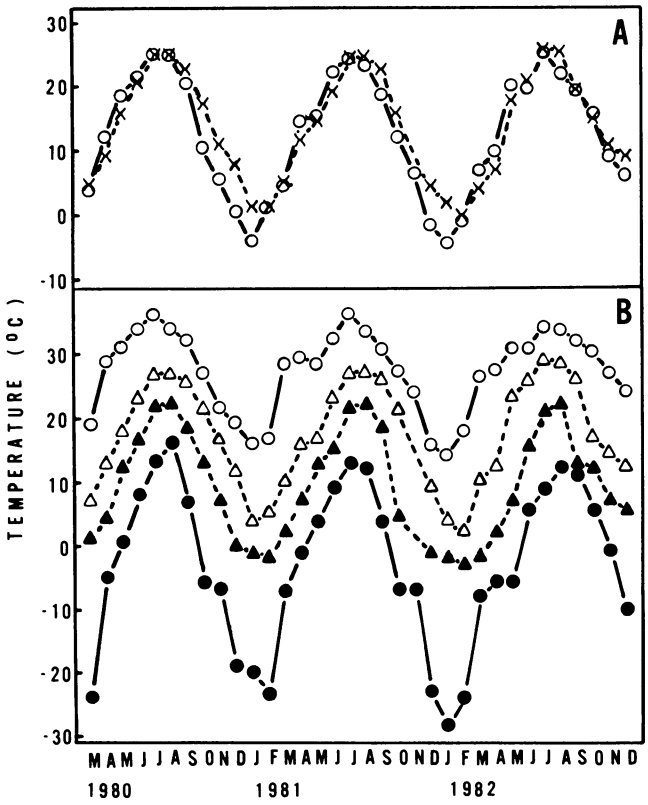

FIG. 1. Temperature fluctuations in the in-ground sample containers as compared with air temperatures (the *x*-axis is the months of the year abbreviated by first letter, beginning with March [M] 1980). (A) Average temperatures of the in-ground containers (×) and average air temperatures (○) recorded over the period of the study. (B) Maximum (△) and minimum (▲) in-ground temperatures and maximum (○) and minimum (●) air temperatures recorded over the period of the study.

Recoveries of eggs from sludge determined after the digestion and at the onset of storage were (number of eggs per 100 g [wet weight] of sludge ± standard error; $n = 3$) 5,300 ± 475 for *Ascaris* spp., 7,533 ± 535 for *Hymenolepis* spp., 1,486 ± 168 for *Toxocara* spp., and 913 ± 63 for *Trichuris* spp. Similar recoveries were not determined for eggs added to soil control samples. The average number of eggs in each 100 g (wet weight) of soil samples was estimated by the amount initially added and determined as 10,353 for *Ascaris* spp., 12,503 for *Hymenolepis* spp., 2,073 for *Toxocara* spp., and 931 for *Trichuris* spp. Procedures for counting the total number of eggs and the criteria for determining the percentage of viable eggs were carried out as described previously (2). An exception was that in the present study, no antibiotic-antimycotic agents were added to the incubation solution used for embryonation of eggs.

Data analysis. To determine whether each of the various conditions tested had an influence on the recovery or viability of eggs, the data obtained were subjected to statistical analysis. A factorial analysis of variance with repeated measures was employed (BMDP Statistical Software, Inc., University of California). In these analyses, an abridged set of data was used. Data eliminated from these analyses were those from months 0, 19, 30, and 33, which were time points at which complete data sets were not available. Data for soil controls were also not included. The percent recovery data were calculated by using an eggs recovered per eggs added transformation. Similarly, percent viable data were calculated by an eggs viable per eggs recovered transformation. The statistical design used sludge type (aerobically or anaerobically digested sludge), sludge control (eggs added after sludge digestion or eggs digested with sludge), storage temperature, pH, and storage time as the independent variables (grouping factors). The dependent variable was either percent recovered or percent viable.

Infectivity of recovered eggs. The infective potential of the *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 Laboratories, Inc., Wilmington, Mass.). After 8 days, the infected rats were sacrificed by anesthesis with diethyl ether, and the lungs, liver, kidneys, and brain were removed. Each organ was placed into 50 to 100 ml of physiological saline and cut with surgical scissors into small pieces no larger than 3 to 5 mm in the longest dimension. One Baermann apparatus was set up for each organ (13). The funnel was filled with saline (37°C), and two layers of

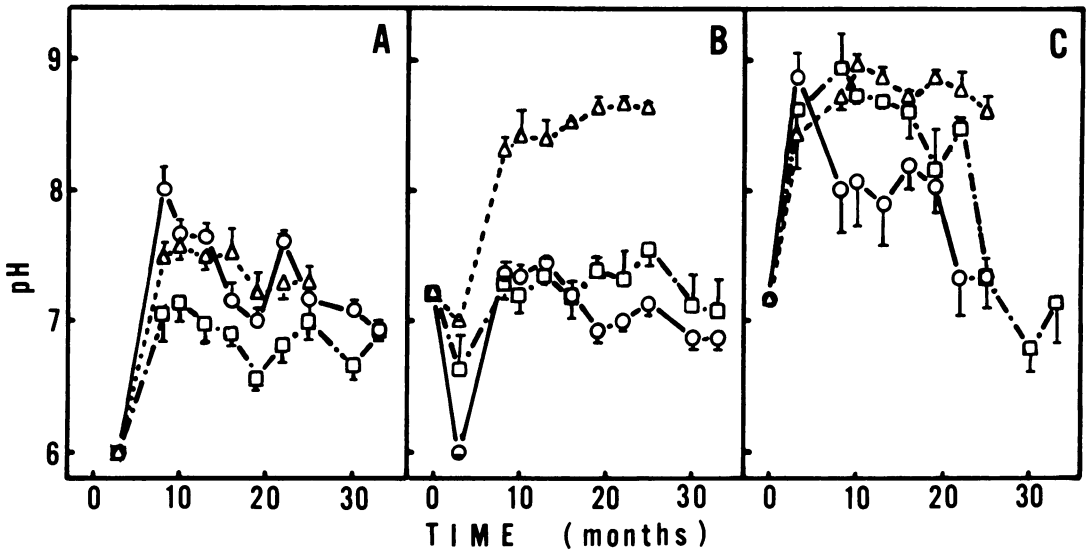

FIG. 2. Changes in pH upon storage of samples at 25°C (○), in ground (□), and 4°C (△). (A) Soil controls. (B) Aerobically digested sludges (experimental and control). (C) Anaerobically digested sludges (experimental and control).

cheesecloth (Curtin Matheson 055-053) were used to suspend the minced organ within the saline in each funnel. Lamps with 60-W tungsten bulbs were placed ca. 5 cm from the junction of head and stem of each funnel 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 a 50-ml glass conical graduated centrifuge tube, and 10 ml of 0.2% (wt/vol) saponin was added to each tube to lyse the erythrocytes (18). The tubes were centrifuged for 2 min at 500 × *g*, and the supernatant was discarded. The total volume of the pellet remaining in each tube was examined. A small pellet (<0.5 ml) required no further treatment and was suspended 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% [vol/vol] 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 [Sigma P-7000; 1:10,000]) in 500 ml of 0.85% (wt/vol) physiological saline plus 3.5 ml of concentrated HCl [22]) and incubated at 30°C for 24 h on a rotating platform. The digested tissue was rinsed free of the pepsin solution and fixed and stored as described above. Each sample was examined under ×125 magnification with a phase-contrast microscope, and the number of *Ascaris* and *Toxocara* larvae was counted.

RESULTS

pH. The pH of soil and sludge samples changed with storage time (Fig. 2). There were large initial increases in the pH values of all soil and anaerobically digested sludge samples, whereas aerobically digested sludge showed an initial decrease in pH. Upon long-term storage, the largest changes in pH were seen in soil at 25°C, aerobically digested sludge at 4°C, and anaerobically digested sludge at 25°C and in ground. During most of the storage time, the pHs of the soil samples and aerobically digested sludges kept at 25°C and in ground were close to neutral. Both aerobically and anaerobically digested sludge kept at 4°C maintained steady pHs after 3 months. The pH of anaerobically digested sludge stored at 25°C or in ground went alkaline after 3 months and then gradually decreased toward neutrality with time. In most cases, the changes in pH of the samples had little effect on the destruction or viability of parasite eggs; however, some exceptions, e.g., *Toxocara* viability, were noted (Table 1).

TABLE 1. Factorial analysis of variance

Source of variability[a]	% Recovery of the following genera (*f* value, tail probability):				% Viability of recovered eggs of the following genera (*f* value, tail probability):		
	Ascaris	*Toxocara*	*Trichuris*	*Hymenolepis*	*Ascaris*	*Toxocara*	*Trichuris*
Aerobically versus anaerobically digested sludge	8.22, 0.0043	3.06, 0.0809	17.53, 0.0000	0.64, 0.4228	33.77, 0.0000	41.85, 0.0000	4.35, 0.0376
Control versus experimental	46.48, 0.0000	16.15, 0.0001	31.09, 0.0000	15.23, 0.0001	1.73, 0.1883	2.14, 0.1436	7.32, 0.0071
Storage temperature	2.34, 0.0967	11.73, 0.0000	3.30, 0.0376	125.13, 0.0000	186.95, 0.0000	717.72, 0.0000	8.72, 0.0002
Storage time	8.40, 0.0000	15.21, 0.0000	6.08, 0.0000	18.93, 0.0000	44.20, 0.0000	144.11, 0.0000	22.82, 0.0000
pH	0.87, 0.3500	0.21, 0.6477	0.02, 0.8969	7.36, 0.0069	1.64, 0.2005	26.97, 0.0000	0.60, 0.4382
No. of determinations	641	636	641	637	627	615	385

[a] Degrees of freedom: type of digestion, 1; control versus experimental, 1; storage temperature, 2; storage time, 6; pH, 1.

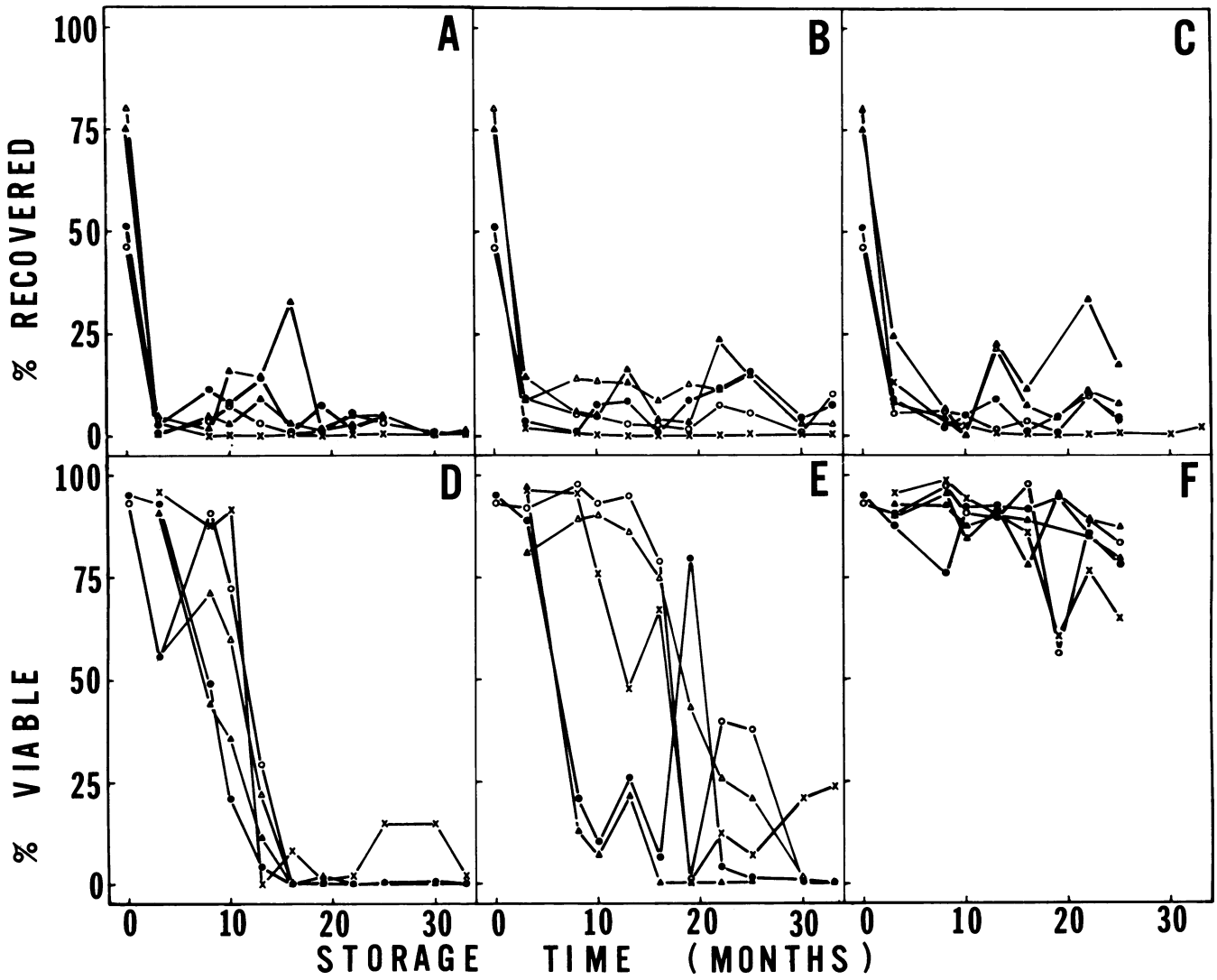

FIG. 3. Destruction (A, B, C) and viabilities (D, E, F) of *Ascaris* eggs stored at 25°C (A, D), in ground (B, E), and 4°C (C, F). The eggs were added to soil (×), aerobically digested sludge (△), or anaerobically digested sludge (▲) or treated with sludge in aerobic (○) or anaerobic digestors (●).

***Ascaris* spp.** Destruction of *Ascaris* eggs occurred, especially within the first 3 months after the onset of storage (Fig. 3). Recovery of these eggs from sludges was greater than that from soil samples. The temperature condition at which *Ascaris* eggs were stored had little effect on the recovery rates.

The temperature at which the eggs were stored had the most dramatic effect on their viabilities. This was evident by the large *f*-values obtained for the influence of storage temperature on percent viability, as determined by statistical analytical consideration of the different parameters (Table 1). Egg viability decreased very slowly when stored at 4°C. After 33 months, >50% of the *Ascaris* eggs recovered from samples kept at 4°C were still viable; however, 10 to 16 months of storage at 25°C was sufficient to render most of the recovered eggs nonviable. This decrease in viability was first exhibited by eggs stored in anaerobically digested sludge (control and experimental) and later by those stored in aerobically digested sludges. The decrease in viability of eggs from anaerobically digested as compared with aerobically digested sludges was more pronounced in samples stored in ground than in those kept at 25°C. That is, the eggs stored in ground in anaerobically digested sludge lost viability much earlier than those stored in aerobically digested sludge. In general, the viability of *Ascaris* eggs recovered from the soil controls was similar to that of eggs from the aerobically digested sludges.

The infectivity of recovered *Ascaris* eggs that were incubated under conditions supporting embryonation reflected their viability (Table 2). Eggs stored at higher temperatures decreased in infectivity more rapidly than those stored at lower temperatures. After 33 months, some eggs from samples stored in ground were still infective. Therefore, even if most of the population of eggs appeared to be nonviable, a few were still capable of causing infections in test animals.

***Toxocara* spp.** The type of sludge (i.e., digested under aerobic or anaerobic conditions) had no effect on the survival rates of *Toxocara* eggs; however, fewer eggs were recovered from the soil samples stored at 25°C and in ground (Fig. 4). The data on *Toxocara* spp. were more scattered than

TABLE 2. Recovered ova infectivity

Time in storage (mo) and storage condition	Recovery of the indicated genera of ova under the following conditions:									
	Aerobically digested sludge (control)		Aerobically digested sludge (experimental)		Anaerobically digested sludge (control)		Anaerobically digested sludge (experimental)		Soil (control)	
	Ascaris	*Toxocara*	*Ascaris*	*Toxocara*	*Ascaris*	*Toxocara*	*Ascaris*	*Toxocara*	*Ascaris*	*Toxocara*
3										
25°C	QNS[a]	QNS	−	+	−	+	QNS	QNS	+	+
In ground	+	+	+	+	−	+	−	+	−	+
4°C	+	+	QNS	QNS	QNS	QNS	+	+	+	+
8										
25°C	+	−	+	−	−	−	−	+	QNS	QNS
In ground	+	+	+	−	−	−	+	−	+	+
4°C	+	+	+	+	+	+	−	+	+	+
10										
25°C	+	−	+	−	−	−	+	−	−	+
In ground	+	+	+	+	−	−	+	−	−	+
4°C	QNS	QNS	−	+	QNS	−	−	+	−	+
13										
25°C	+	−	+	−	−	−	+	−	−	+
In ground	+	−	+	−	+	−	+	−	−	−
4°C	+	+	+	+	+	+	+	+	−	+
16										
25°C	−	−	+	−	−	−	−	−	−	−
In ground	+	−	+	+	−	−	−	−	+	+
4°C	+	+	+	+	+	+	−	+	+	+
19										
25°C	−	−	−	−	−	−	−	−	−	−
In ground	+	−	−	−	−	−	+	−	−	−
4°C	+	+	−	+	+	+	+	+	−	−
22										
25°C	−	−	+	−	−	−	−	−	−	−
In ground	+	−	+	−	−	−	+	−	−	−
4°C	+	+	+	+	+	+	+	+	+	+
25										
25°C	−	−	−	−	−	−	−	−	−	−
In ground	−	−	−	−	−	+	−	−	−	+
4°C	−	+	−	+	−	+	+	+	+	+
30										
25°C	−	−	+	−	ND[b]	ND	ND	−	+	−
In ground	+	−	−	−	ND	ND	+	−	+	−
4°C	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
33										
25°C	−	−	−	−	ND	ND	−	−	−	−
In ground	+	−	−	−	ND	ND	+	−	+	+
4°C	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] QNS, Quantity not sufficient for analysis.
[b] ND, Not determined.

those obtained for *Ascaris* spp., which was probably due to the smaller numbers of *Toxocara* eggs initially seeded in the samples. Storage temperature also had an effect on the recovery of *Toxocara* eggs as it did with *Ascaris* eggs. The recoverability of eggs decreased inversely with the average temperature at which the samples were stored.

As with *Ascaris* spp., storage temperature was a very important factor influencing the viability of recovered eggs (Table 1). The *f*-value for the effect of storage temperature on percent viability was the largest for all variables and helminth species. After 2 years in storage at 4°C, the recovered *Toxocara* eggs were still viable. The viability of eggs stored in aerobically digested sludges (control and experimental) decreased more slowly than those in the anaerobically digested sludges. The eggs recovered from samples stored at 25°C and in ground were virtually nonviable after 10 months of storage in sludge. The viability of *Toxocara* eggs in the soil controls was not affected by storage at any temperature. The effect on viability of *Toxocara* eggs stored in sludges as compared with soil was the most dramatic observed among all the parasite species examined. Both aerobically and anaerobically digested sludges, control and experimental, caused a reduction in the viability of these eggs, whereas soil did not. Although fewer

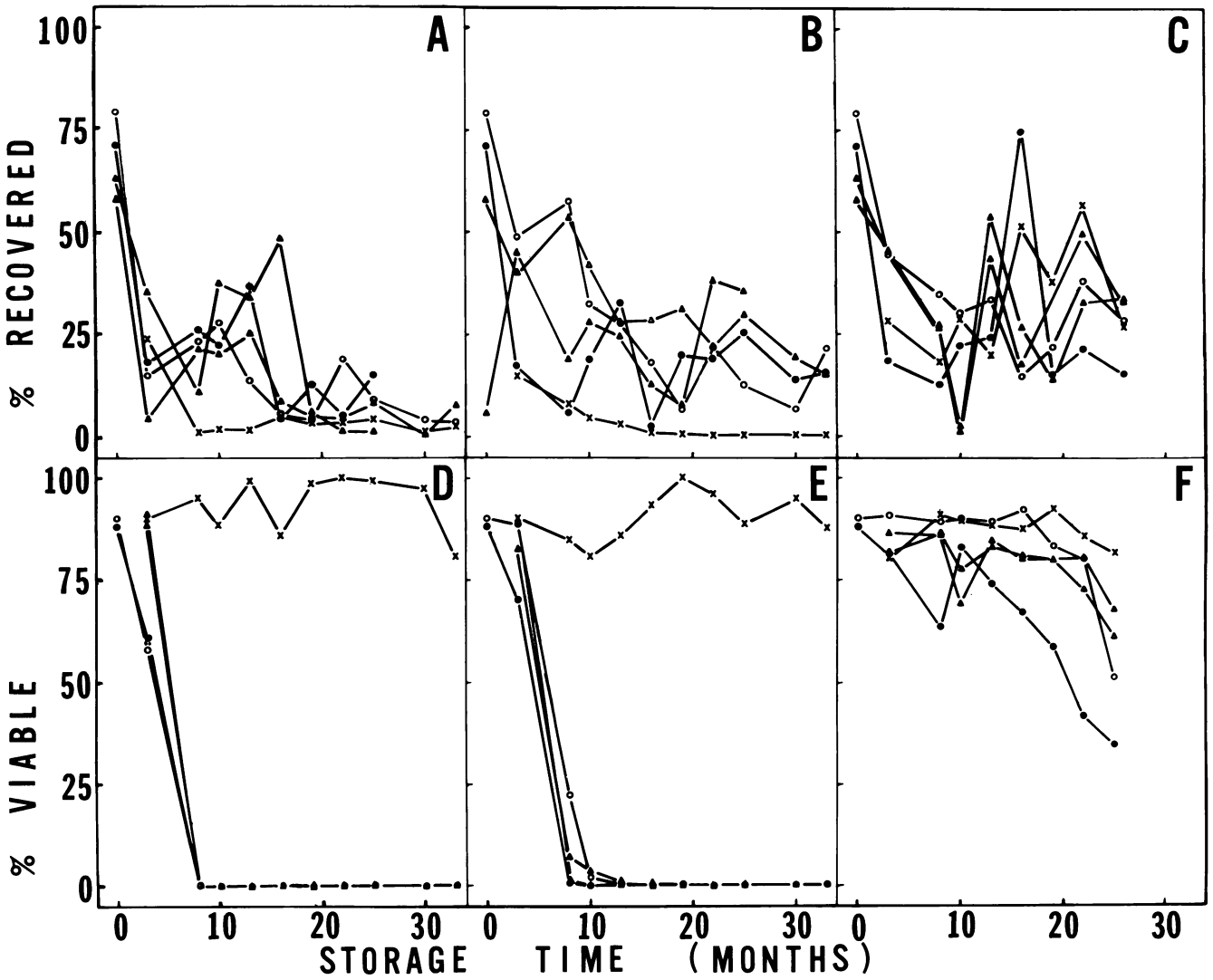

FIG. 4. Destruction (A, B, C) and viabilities (D, E, F) of *Toxocara* eggs stored at 25°C (A, D), in ground (B, E), and 4°C (C, F). The eggs were added to soil (×), aerobically digested sludge (△), or anaerobically digested sludge (▲) or treated with sludge in aerobic (○) or anaerobic digestors (●).

Toxocara eggs were recovered than *Ascaris* eggs, and therefore a less total number of eggs was intubated into rats, these eggs were just as capable of causing infections in rats (Table 2). Higher storage temperatures decreased infectivity of the eggs to a greater degree than did lower temperatures. Storage in sludges (aerobically and anaerobically digested) decreased infectivity of the eggs more than did storage in soil.

***Trichuris* spp.** The recovery of *Trichuris* eggs from all samples was similar, i.e., all samples had low levels of eggs after 3 months of storage (Fig. 5). The total number of eggs seeded into each sample was the smallest for this species, as compared with the other species. Due to the low numbers of recovered *Trichuris* eggs, the viability data on this genus was very scattered. However, in general, eggs stored at lower temperatures had greater viabilities, and eggs stored in soil maintained their viabilities longer than did the eggs stored in the sludges.

***Hymenolepis* spp.** Although 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 (Fig. 6). Temperature also had an effect on the recovery of *Hymenolepis* eggs. The recovery of *Hymenolepis* eggs decreased more rapidly in the samples stored at higher temperatures. As with *Toxocara* spp., the recovery of *Hymenolepis* eggs from soil stored at 25°C and in ground was less than that recovered from the sludges.

DISCUSSION

There are few reports on die-off and viability rates of parasite eggs upon long-term storage in sludge. Schatzle saw a 10% reduction of *Ascaris* egg viability after 7 months of storage at 0 to 20°C (16). Stern and Farrell found no reduction in the viability of *Ascaris* eggs recovered from sludge stored for 6 months at 4 to 20°C (17). Veerannan reported a 50% reduction in *Ascaris* egg viability after 1 year in storage and practically no survival after 3 years (21). The present study was conducted under controlled laboratory conditions and may not precisely reflect the conditions that occur in actual lagoons currently used for storage of sludge. However, the results obtained from these studies clearly indicate that the eggs of some common enteric helminths

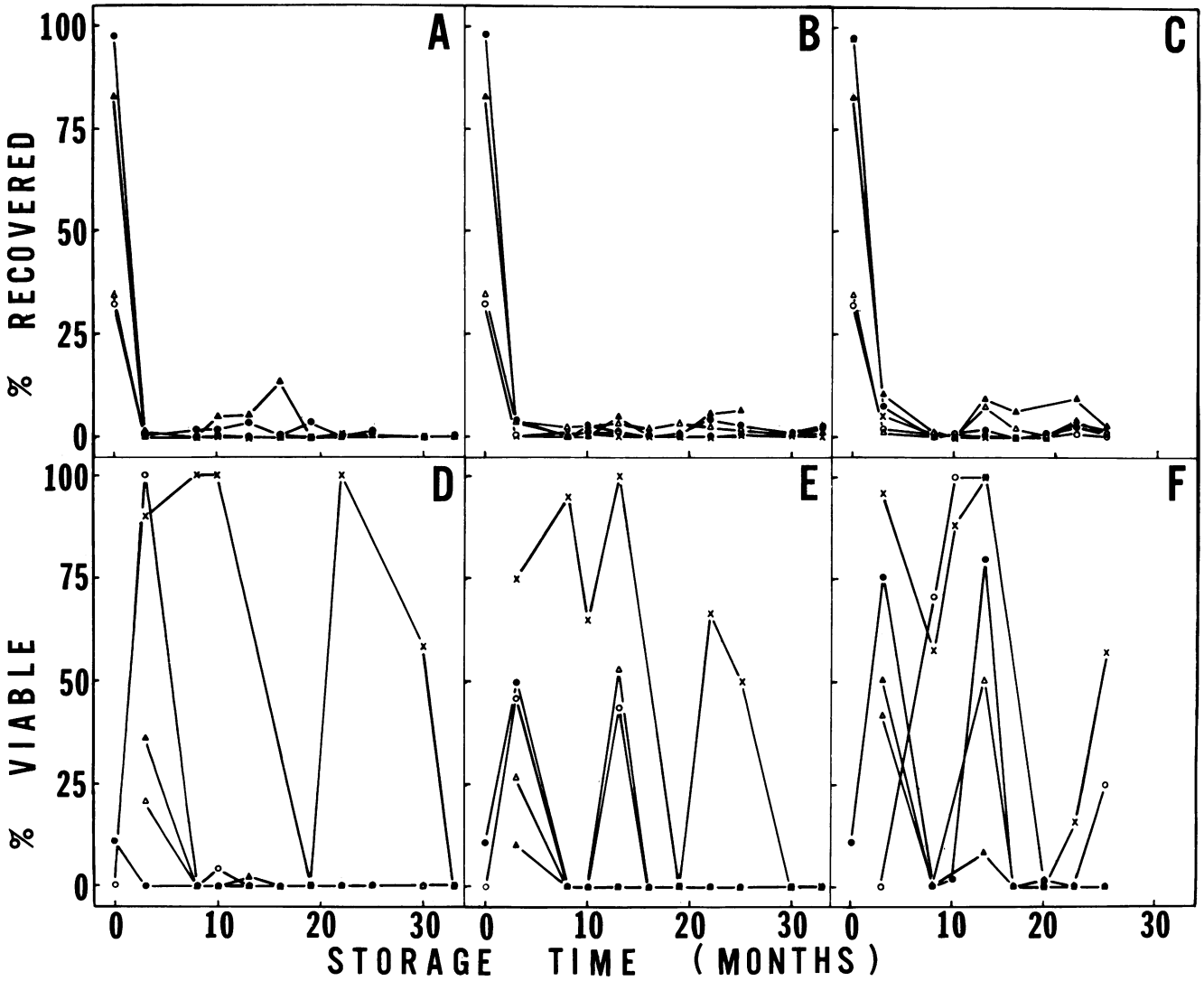

FIG. 5. Destruction (A, B, C) and viabilities (D, E, F) of *Trichuris* eggs stored at 25°C (A, D), in ground (B, E), and 4°C (C, F). The eggs were added to soil (×), aerobically digested sludge (△), or anaerobically digested sludge (▲) or treated with sludge in aerobic (○) or anaerobic digestors (●).

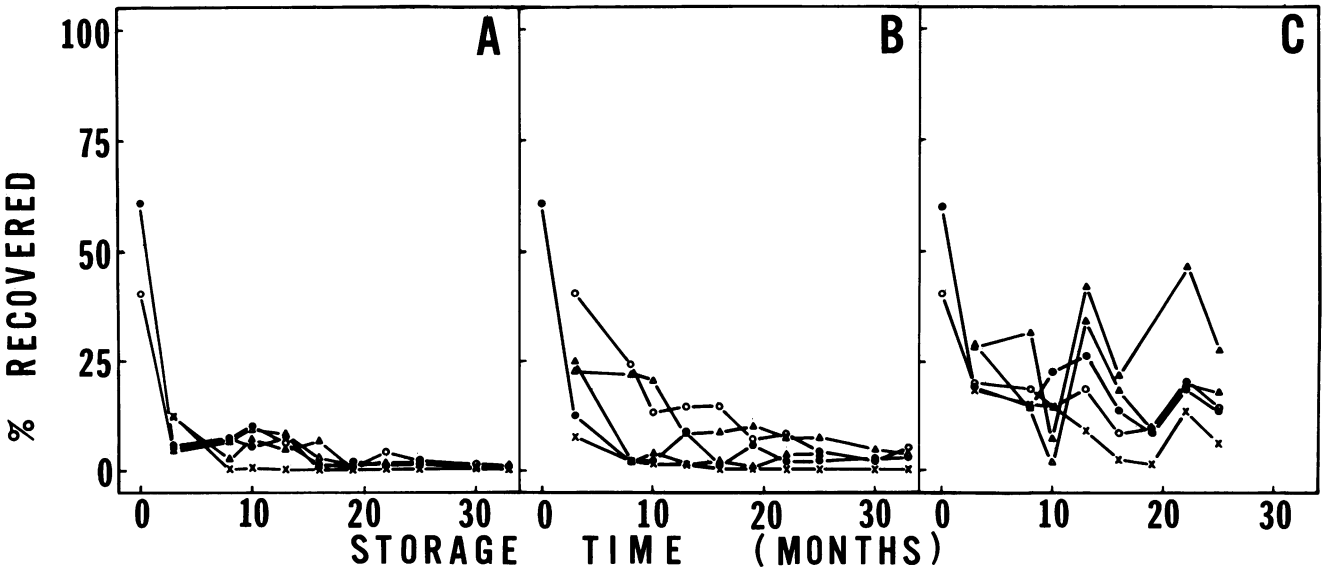

FIG. 6. Destruction of *Hymenolepis* eggs stored at 25°C (A), in ground (B), and 4°C (C). The eggs were added to soil (×), aerobically digested sludge (△), or anaerobically digested sludge (▲) or treated with sludge in aerobic (○) or anaerobic digestors (●).

that are resistant to the usual sludge treatment processes are destroyed faster when stored in sludge at higher rather than lower temperatures. Although the effects of aerobic versus anaerobic digestion, soil versus sludges, exposure to sludge digestion processes versus no exposure to sludge digestion, and, in a few cases, pH were significant on the recovery and viability of various species of helminth eggs, the factor that had the greatest influence was that of storage temperature (Table 1). Thus, it appears that lagooning of sludge can be an effective method for destruction of parasite eggs in warm climates. On the other hand, eggs of these parasites could survive for more than 3 years if kept in lagoons in geographical locations where the bottoms of lagoons remain at low temperatures. The high specific gravity of these eggs causes them to settle and remain at the bottom if the lagoons remain unstirred (12). Even though these parasites are commonly regarded as endemic to tropical regions, the increased mobility of greater numbers of travelers may be accompanied by the appearance of ascarid eggs in sludges from temperate regions. Indeed, *Ascaris* eggs have been isolated from domestic sludge samples taken from widely scattered regions of the United States (1, 13, 15).

In a previous report (2), we reported that some of the roundworm eggs were destroyed during the sludge digestion treatments. Furthermore, immediately after eggs were processed through the digestors, the viabilities of *Ascaris* and *Toxocara* eggs were greater in anaerobically treated than aerobically treated material. It is presently unclear why the converse was true of the viabilities of these eggs upon long-term storage in sludges.

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