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# Effects of Moisture Content on Long-Term Survival and Regrowth of Bacteria in Wastewater Sludge

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The effects of moisture content on the survival and regrowth of seeded and indigenous enteric bacteria in raw sludge were determined. Cultures of six strains of fecally associated bacteria grown in sterilized, liquid sludge (5% solids) were all quite stable at this moisture level for over 90 days at 21°C. When the moisture content of the sludge containing these organisms was reduced by evaporation and the samples were stored at 21°C for extended periods, bacterial inactivation rates were generally proportional to the moisture losses of the samples. A dramatic reversal in this effect was observed in samples containing more than 90% solids. In this dried sludge, every bacterial species studied except *Proteus mirabilis* was found to be extremely stable. Bacteria indigenous to sludge were also found to survive for long periods in dried sludge. Regrowth of bacterial isolates in sterilized raw sludge was found to occur readily at 37°C in samples containing ≤75% solids, but no growth was observed in samples with ≥85% solids. Some growth, but to less than saturation densities, occurred with 80% solids. Growth of seeded *Salmonella typhimurium* was also found to occur in the presence of indigenous organisms in both liquid and dewatered raw sludges. However, the population density attained was well below that found in sterilized samples of the same sludges. In addition, the number of salmonellae dropped below detectable limits within a few days in sludges containing viable indigenous organisms, whereas little decrease occurred during this time with salmonellae grown in previously sterilized sludges.

Use of human wastes on land as a fertilizer or soil conditioner is a common method of resource conservation practiced worldwide. In developed countries, wastes applied to land in the form of sludge are normally given some type of pretreatment. Sand-bed drying and extended storage of wastewater sludges are representative types of such treatments.

In addition to beneficial ingredients, sludges may also contain indigenous populations of human enteric pathogens. Some of the enteric bacteria comprise one group of such pathogens. In the accompanying paper, it was shown that removal of water from sludge by evaporation to near dryness decreased the population of most bacterial species about one order of magnitude or less (10). Although other factors such as heat and ultraviolet radiation may help reduce the bacterial population during sand-bed drying, these results imply that, in itself, this process may be an insufficient means of disinfection. However, it is possible that in combination with long-term storage, sand-bed drying may be quite effective as a disinfection process.

Little is known about the effects of long-term storage on the numbers of bacterial pathogens in sludge. One important reason for this scarcity of knowledge is that slight variations in moisture contents of stored sludges could have large effects on the persistence of these pathogens. Factors such as the types of sludge treatments used before storage and variations in climatic conditions could greatly modify the moisture content of a stored sludge.

Bacterial pathogens also have the potential to regrow to large numbers in sludge. Such regrowth could follow partial disinfection of sludge or recontamination of highly disinfected sludge. Because of an absolute requirement of water for bacterial growth, the regrowth potential of bacteria in sludge will be related to the moisture content of the sludge.

The study reported here was performed to determine how the moisture content of sludge affects both the inactivation rates of bacteria

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during long-term storage and bacterial regrowth potential.

## MATERIALS AND METHODS

The materials and methods used in this report, except those described below, are presented in the companion paper (10).

**Regrowth of bacteria in dewatered sludge.** Studies on the effects of moisture content on the growth potential of bacteria in sterilized raw sludge were performed as follows. Samples of raw sludge dried by natural evaporation at 21°C were further dehydrated by heat (80°C). This material was pulverized and then treated with 6 Mrad of ionizing radiation, using $^{60}Co$. The sterilized, powdered sludge was readjusted to different moisture levels by the addition of distilled water and a constant amount of a bacterial suspension (*Streptococcus faecalis*, *Proteus mirabilis*, or *Salmonella typhimurium*). Each sample was thoroughly mixed, blended for 2 min at high speed, and assayed immediately (in duplicate) for recoverable colony-forming units (CFU). *S. faecalis* was assayed by pour plating (1 ml) in KF *Streptococcus* agar, and the two *Enterobacteriaceae* were assayed by spreading (0.1 ml) on Hektoen enteric (HE) agar. The plates were incubated at 37°C, and CFU were enumerated after either 48 (*S. faecalis*) or 24 (*Enterobacteriaceae*) h. The remainder of each sample (containing between 10 and 95% solids) was then incubated at 37°C in tightly stoppered bottles and assayed periodically for CFU. The moisture contents of the samples were monitored with each biological assay. A temperature of 37°C was chosen to help provide optimal conditions for regrowth in sludge. This study was repeated three times with essentially identical results. The data shown represent a typical experiment.

**Growth of *S. typhimurium* in unsterilized sludges.** The growth and survival of *S. typhimurium* was measured in seeded raw sludge containing a natural population of indigenous organisms. The strain of *S. typhimurium* used in these studies was selected for its resistance to streptomycin. To measure its ability to grow and survive in sludges containing indigenous organisms, samples of liquid (5% solids) and dried (reconstituted with distilled water to contain 50% solids) raw sludge were seeded with a constant concentration of the organism. Samples were periodically assayed over 18 days on HE agar containing 0.01% streptomycin. This concentration of the antibiotic reduced the numbers of indigenous CFU on the agar by about two orders of magnitude but had little effect on the recoverable number of the seeded organism. Therefore, the black-centered colonies of *S. typhimurium* that appeared on HE agar were readily detectable, even when present with a much larger population of indigenous organisms. Unseeded control samples were analyzed in parallel throughout this experiment to ensure that the black-centered colonies observed were due to the seeded organism and not indigenous $H_2S$-producing species. No black-centered colonies were ever found on plates with streptomycin unless they contained the seeded *Salmonella* species. In addition, colonies were periodically tested in Enterotubes (Roche Diagnostics) to confirm their identity.

## RESULTS

**Effect of moisture content on the inactivation rates of bacteria in sludge.** The relationship between moisture content and the survival of bacteria in sludge was measured during extended storage at 21°C. The methods used to obtain sludges colonized by a single bacterial genus at different moisture levels are described in the accompanying paper (10). As shown there, partial dewatering of sludge by natural evaporation generally resulted in an increase in the bacterial numbers. However, further dewatering to near dryness caused reductions in the populations of viable bacteria to levels about one-half to one order of magnitude less than originally present in the liquid sludges. The only exception was found with *P. mirabilis*, where the decrease was nearly four orders of magnitude.

To determine the effects of long-term storage at 21°C on bacteria as a function of moisture content, these dewatered samples were kept in tightly sealed containers and periodically assayed for recoverable CFU. Populations of individual bacterial species were all quite stable in sludge containing about 5% solids (Fig. 1). However, this stability decreased in proportion to decreases in moisture content for every bacterial strain studied up to the point where the samples were almost completely dry (about 90% solids). The destabilizing effect of reduced moisture content was totally reversed in these dry samples for every bacterial isolate studied except *P. mirabilis* (Fig. 1D). Indigenous organisms also remained viable for long periods of time at 21°C in dried raw sludge (Fig. 2).

**Regrowth of bacteria in sludge as a function of moisture content.** The moisture requirements for growth of three model strains of bacteria in raw sludge were essentially identical (Fig. 3). Growth in samples containing $\leq$75% solids was rapid for every organism studied, whereas no growth was observed in samples containing $\geq$85% solids. In fact, there was a substantial decrease in CFU in the drier samples, even within minutes of seeding. A similar observation was made during studies on the inactivation of salmonellae in meat and bone meal, where it was postulated that rapid die-off was due to the fact that wet cells were inoculated into dry feed (7). At 80% solids, some growth occurred in sludge, but at a much slower rate than found in samples with higher moisture contents. These results suggest that moisture levels in excess of 15% are required for growth of some if not all strains of enteric bacteria in sludge.

**Regrowth and survival of *S. typhimurium* in sludge containing a natural popu-**

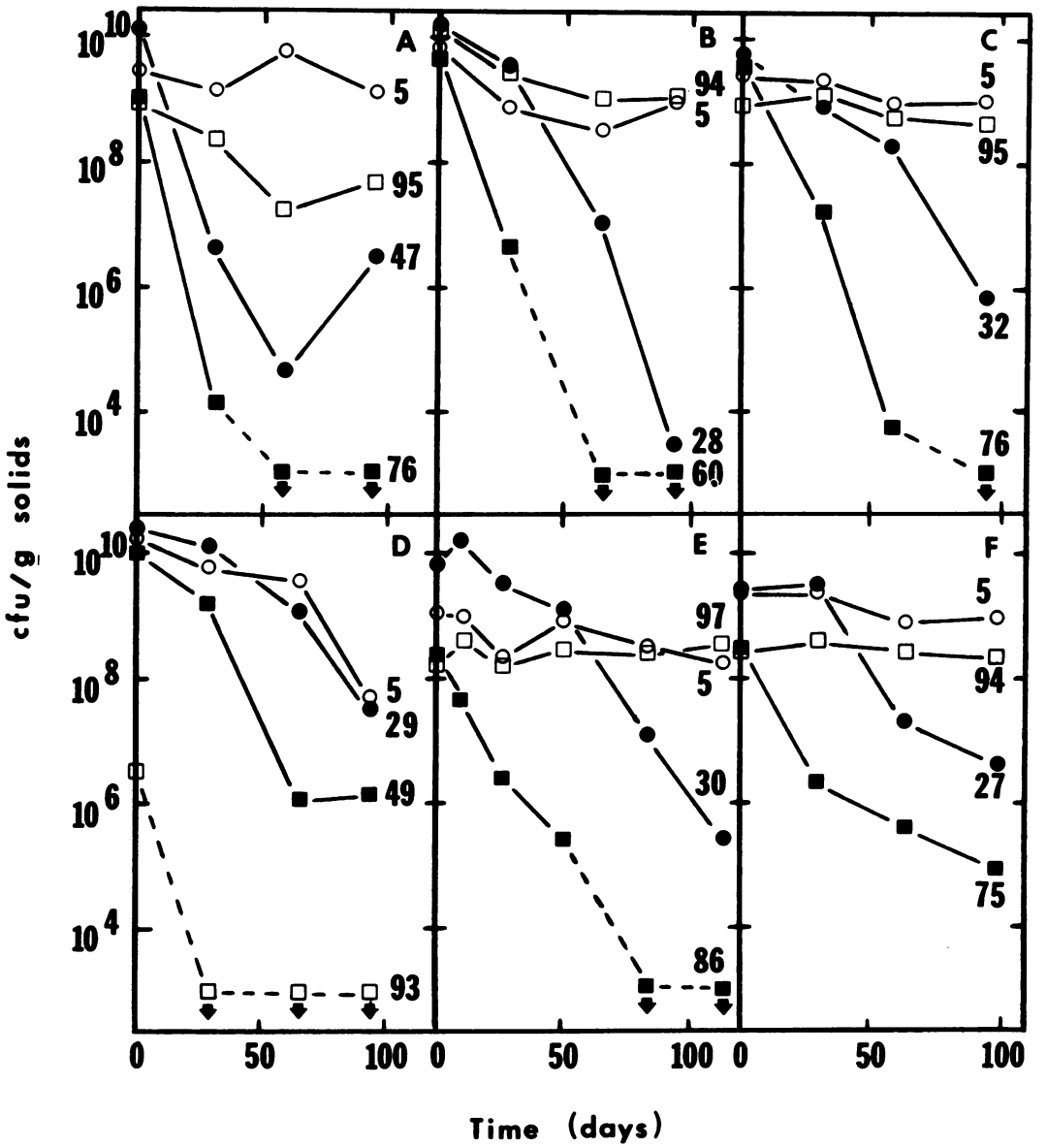


FIG. 1. *Long-term survival of bacterial isolates in seeded raw sludge at different moisture levels. Six isolates of enteric bacteria were grown in sterilized liquid sludge, which was subsequently dewatered by evaporation at 21°C as described elsewhere (10). The dewatered samples were maintained in tightly capped containers at 21°C and periodically assayed for recoverable CFU. The isolates of Escherichia coli (A), Klebsiella sp., (B) and Enterobacter sp. (C) were assayed on M-coliform agar. P. mirabilis (D) and Salmonella typhimurium (E) were assayed on HE agar, and Streptococcus faecalis (F) was measured with KF Streptococcus agar. Numbers are the percentages of solids in the different samples. These values were found to remain constant throughout the experiment. The plotted results represent the average from two samples plated in duplicate. In no case was the difference in the two samples greater than one order of magnitude.*

**lation of indigenous organisms.** The results already presented indicate that bacteria can regrow to high levels in sterilized sludge if the moisture content is greater than about 20%. It has not been shown, however, that these bacteria will grow in sludge that already contains a natural population of indigenous organisms.

To test the ability of an enteric bacterial path-

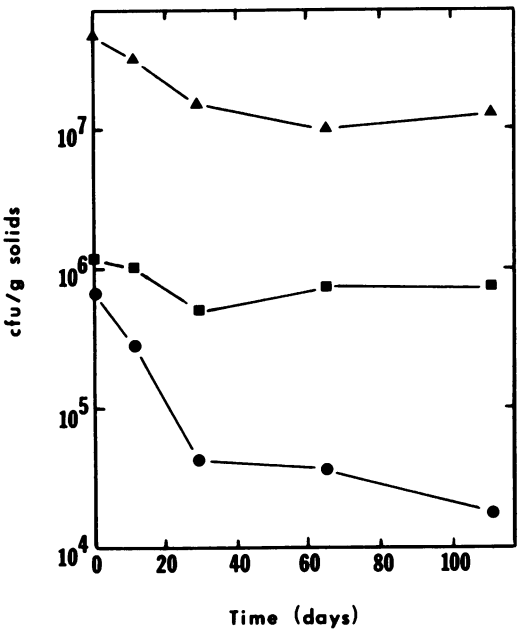


FIG. 2. *Long-term survival of indigenous bacteria in air-dried raw sludge. Liquid raw sludge was dewatered by evaporation at 21°C to contain about 95% solids. This material was stored in a tightly capped container at 21°C and periodically assayed for total recoverable CFU on nutrient agar (▲), in KF Streptococcus agar (■), and on HE agar (●). The results represent the average from two samples which never differed by more than one order of magnitude.*

ogen to grow in sludge in the presence of indigenous species, both liquid raw sludge (5% solids) and dried raw sludge reconstituted to contain 50% solids were seeded with *S. typhimurium* and incubated at 37°C. The seeded *Salmonella* grew in both liquid and dewatered sludges, but to levels that were significantly less than found in radiation-sterilized samples of these same sludges (Fig. 4). However, upon further incubation, the number of seeded *Salmonella* decreased to below detectable limits in both sludges that contained indigenous organisms. *Salmonella* grown in sterilized sludge remained at a high concentration throughout this experiment. These results indicate that a pathogenic enteric bacterium can regrow in sludge containing a natural population of viable organisms, but its ability to survive for long periods may be limited, especially at higher temperatures.

## DISCUSSION

Because it has been suggested that long-term storage of dried wastewater sludges could, in some cases, be used as a low-cost method of disinfection, it is important to understand what effects this process has on enteric pathogens in these sludges. This study has shown that bacterial survival can be dependent on the moisture content of the stored sludge. Although these organisms could be quite stable in liquid sludges, their abilities to survive over long periods at 21°C were found to decrease with a decrease in the moisture contents of the sludge. This pattern was reversed in dried sludges, where all bacteria tested except *P. mirabilis* were extremely resistant to inactivation during storage of sludge.

These results indicate that long-term storage may be an effective means of inactivating bacterial pathogens in sludge, but the moisture content of the sludge should probably be maintained between about 10 and 50%. Because regrowth of these organisms at an optimal temperature (37°C) was prevented at moisture levels of less than 20%, a moisture content of sludge of about 15% should produce rapid inactivation of bacteria during extended storage and, at the same time, prevent bacterial regrowth. A reduction of the moisture content of sludge to this level may also effectively decrease its population of viable parasites and viruses (8, 9). Therefore, it is suggested that long-term storage of sludge at the proper moisture content after natural evaporation may greatly reduce the numbers of all major groups of enteric pathogens.

The effects of moisture content on survival of bacteria in wastewater sludge observed here are similar to observations made previously on bacterial survival in foods, on clothing, and in dried animal feces (see reference 6 for review). In these studies, it was observed that much more rapid die-off of bacteria occurred under moist conditions in these materials than in the same materials after they had been dehydrated. It was also noted that death was generally accelerated when the moisture content was near the level needed for bacterial growth. Thus, the more rapid die-off in moist materials may in part be due to an attempt by bacteria to multiply under conditions where a limitation in water eventually results in their death. From this, it follows that when materials such as feed or sludge are dehydrated, even nonsporeforming bacteria may attain some resistant state where their metabolic rates are so low that survival for extended periods of time is possible. The observation made here that regrowth of bacteria in sludge occurs just above a moisture level where the maximum die-off rate was found during long-term storage supports these suggestions.

The results reported here concern experiments performed with raw sludges under laboratory conditions. It is possible that these results

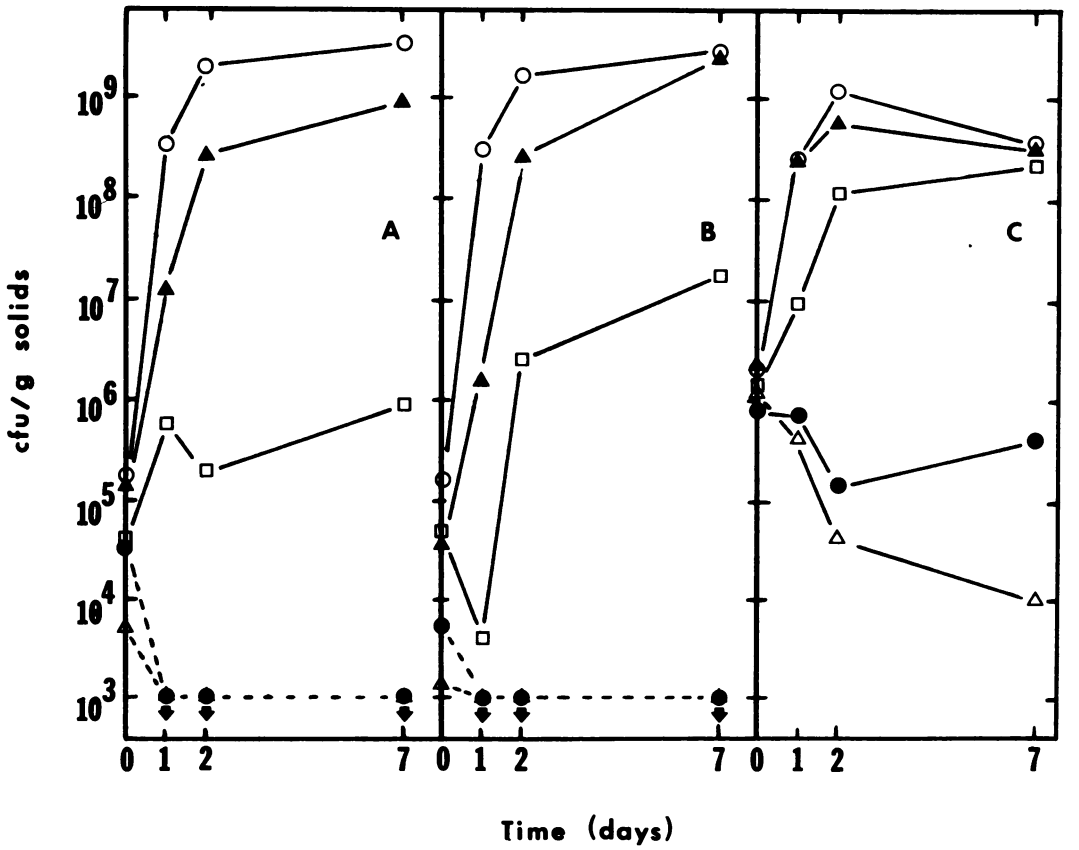


FIG. 3. *Growth of bacterial isolates in seeded raw sludge as a function of moisture content. Samples of dry, sterile raw sludge were adjusted to different moisture levels and seeded with a constant concentration of either P. mirabilis (A), Salmonella typhimurium (B), or Streptococcus faecalis (C). After each sample was thoroughly mixed, it was assayed for recoverable CFU both immediately and after 1, 2, and 7 days of incubation at 37°C. The percentages of solids in the samples shown here were 70* (○), *75* (▲), *80* (□), *85* (●) *and 90* (△).

will not be directly applicable to field conditions, especially when other types of sludges are involved. However, we have recovered nearly $10^5$ $H_2S$ producers on HE agar per g of sand-bed dried, anaerobically digested sludge from the City of Albuquerque said to have been stored for more than 3 months (results not shown). This sludge contained less than 10% moisture. Based on the findings reported here and on reports that salmonellae can survive for years on dry cloth and in dried feces (6), long survival times could be expected in sludge with less than 10% moisture. Furthermore, the moisture requirements for bacteria in raw sludge found here are the same as those observed by others for the growth of salmonellae in composted sludge (1; C. Russ, Los Angeles County Sanitation Districts, personal communication). Therefore, these results may be directly applicable to a variety of other sludges, even under field conditions.

It has been shown by other investigators that detection of bacteria in environmental samples can be difficult, and elaborate methods have been used to increase recoveries (2, 4, 5). A procedure that may increase the natural affinity of bacteria for sludge particulates and, as a result, further inhibit bacterial detectability is reducing the moisture content of the sludge. However, processing of sludge samples by Vortex mixing or blending followed by direct plate counting, as was done here, was shown to yield better bacterial recoveries from sludge than other methods tested (3). Furthermore, in the present study all time-dependent changes in the bacterial populations in sludge at any moisture level were determined relative to a control sample with the same moisture content analyzed at

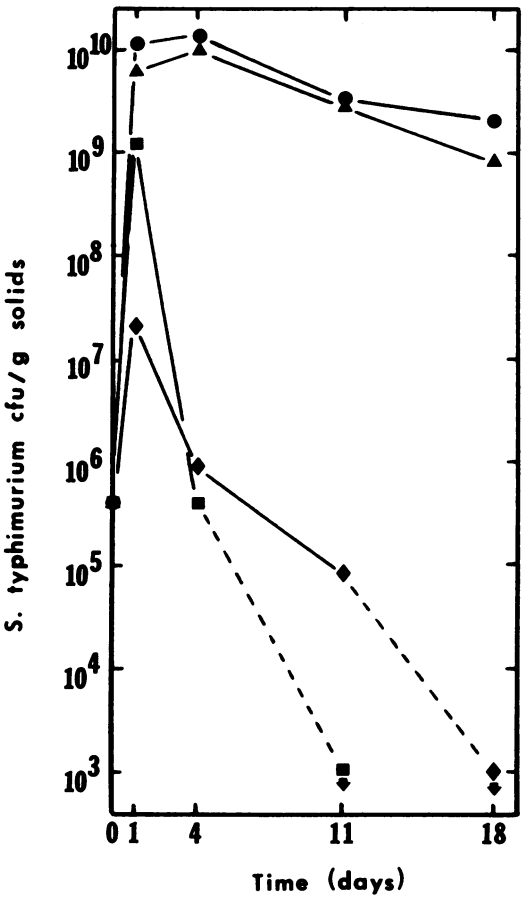


FIG. 4. *Growth and survival of a streptomycin-resistant strain of Salmonella typhimurium in liquid and dewatered sludges containing indigenous organisms. Both irradiated (6 Mrad) and unirradiated samples of liquid (5% solids) and dewatered (50% solids) raw sludges were seeded with* $4 \times 10^5$ *CFU of Salmonella per g of solids and incubated at 37°C. After 1, 4, 11, and 18 days the samples were assayed for recoverable* $H_2S$*-producing CFU on HE agar containing 0.01% streptomycin. Symbols: ●, Irradiated, dewatered sludge; ▲, irradiated, liquid sludge; ■, unirradiated, dewatered sludge; ◆, unirradiated, liquid sludge.*

time zero. Therefore, unless the ability to detect bacteria in sludge samples held at a particular moisture content changes with time, the conclusions drawn in this study should remain valid.

## ACKNOWLEDGMENTS

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