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Project Summary

Regrowth of Salmonellae in Composted Sewage Sludge

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Research was conducted to investigate the regrowth of salmonellae in composted sewage sludge. Though composting effectively stabilizes and disinfects sewage sludges, the decrease in salmonellae may be only temporary, because this pathogen can survive and grow without a human or other animal host.

Modification of an agar medium improved our ability to detect salmonellae in composts. Salmonellae were detected in four composts from 30 composting sites across the United States. However, all composts supported salmonella growth when sterilized by radiation. These results and those by others suggest that the microflora in composts suppress salmonella growth.

To determine the nature of salmonella suppression in composts, we investigated the effects of groups of the compost microflora and the characteristics of the substrates used by salmonellae in composts. Results indicated that suppression of salmonella regrowth is mainly a result of bacterial competition for a limited number of substrates that these organisms use in common with salmonellae.

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

Composting is a very effective process for stabilizing and disinfecting sewage sludge. The high temperatures achieved in the composting process inactivate pathogenic organisms and result in population densities that approach or are below analytical detection limits. For viruses, certain bacteria, and parasites requiring specific hosts for survival, inactivation results in a permanent decrease in their densities. For salmonellae, which can propagate in the absence of specific hosts, the reduction in numbers may be only temporary. Repopulation of compost by salmonellae may occur through regrowth of the organisms existing in the compost at an undetectable concentration or through the growth of organisms introduced from an outside source. A likely source may be feces from salmonella-infected birds, reptiles, or other animals. Salmonellae infecting these animals are also infectious to humans. Thus even though the composting process achieves treatment conditions that meet the further pathogen reduction criteria set forth in 40 CFR Part 257, "Criteria for Classification of Solid Wastes Disposal Facilities and Practices: Interim, Final, and Proposed Regulations" (as corrected in the Federal Register of September 21, 1979); there may still be a potential for repopulation of composted sewage sludge by salmonellae.

Anecdotal and a few scientific reports of salmonellae in composted sewage sludge have been made. Studies using a few composts have indicated that salmonellae can grow extensively only if the compost has been sterilized. This finding indicates that the microflora present in composts prevent salmonella regrowth through antagonistic effects that are not understood.

To evaluate the potential for salmonellae to grow in sewage-sludge com-

post, we modified an agar medium used in the most-probable-number (MPN) method to improve our ability to detect salmonellae and used it to assay the salmonella content and salmonella growth potential of sewage-sludge composts collected from 30 compost sites across the United States.

The factors involved in preventing growth were studied by methods designed to segregate the microbial populations of the compost on the basis of temperature growth range and other physiological and biochemical properties, so that individual and groups of organisms could be tested for their antagonistic capabilities.

To gain information on the number of soluble, usable substrates involved in salmonella regrowth, kinetic studies of salmonella growth in composts were conducted and analyzed according to Monod's growth equations.

Modified Agar Method for Detecting Environmental Salmonellae by the MPN Method

Methods of detecting and enumerating low numbers of salmonellae from environmental samples have used MPN methods, which require careful selection of colonies from a plated agar medium. Xylose lysine brilliant green (XLBG) agar was modified to control the loss of selectivity caused by heating the brilliant green component. The agar content was increased to reduce colony spreading. Brilliant green (BG) dye and reagents to form the H_2S indicator were added after cooling the medium to 50°C and just before pouring. H_2S-positive salmonellae were easily distinguished from most other gram-negative bacteria present in sewage sludge compost.

Salmonella recovery from compost increased strikingly as a result of the suppression of competing organisms when BG dye was added after autoclaving. In previous analyses of composts and sewage sludges using brilliant green (BG) and bismuth sulfite (BS) agars, only 7% of the salmonella-like colonies picked were confirmed biochemically and serologically as salmonellae. In analyses using commercial XLBG agar, 27% of the colonies picked were confirmed as salmonellae. However, salmonellae were detected using BG and BS agars in two of the samples that had been negative using XLBG agar. In recent surveys of 15 composts using the XLBG agar in which the BG dye was added after autoclaving, 21 of 26 (81%) of salmonella-like colonies picked were confirmed biochemically and serologically as salmonellae.

The use of modified XLBG agar has resulted in fewer nonsalmonellae being picked for further MPN analysis and has greatly reduced the work load associated with the MPN method. Direct plating was possible for enumerating salmonellae in laboratory composts containing about 10^3 or more salmonellae.

Growth of Salmonellae in 30 Composted Sewage Sludges

Sewage sludge composts from 30 municipalities were sampled, and 4 samples (12%) contained salmonellae. Salmonellae inoculated into the composts died out unless the compost had been sterilized. In radiation-sterilized composts, the salmonellae grew. Growth and death rates were found to be moisture and flora associated. The growth and death rates for antibiotic-resistant salmonellae were the same as those of nonresistant strains. In nonsterile air-dry composts, salmonellae persisted longer than in nonsterile moist composts. It was concluded that the active, indigenous flora of composts establish a barrier to colonization by salmonellae, and that in the absence of competing flora, reinoculated salmonellae may grow to potentially hazardous densities.

Microbial Suppression of Salmonella Regrowth

Recent studies of a few composts and the studies of this report have indicated that the microflora of composts suppress the regrowth of salmonellae. In this work, compost microflora were examined for the antagonistic effect of individual microorganisms and groups of microorganisms on salmonella growth in compost.

Compost microflora from different temperature zones in compost piles were compared for their abilities to inhibit salmonella growth. Pure culture isolates of compost microbes were tested individually in agar plates and in groups in sterile and experimental composts to determine their contribution to suppression. The microflora were removed from the compost in extracts, fractionated by centrifugation and filtration, and reintroduced into sterile compost to compare the activities of the different fractions on salmonella growth.

Of several hundred isolates from compost, 23 bacteria, 61 actinomycetes, and 42 fungi were chosen to represent a range of morphologically and taxonomically different compost microorganisms. None of the bacteria or actinomycetes inhibited salmonella growth in agar-plate inhibition assays. In contrast, six fungal isolates did, but no growth inhibition was evident when three of the fungi, chosen because they expressed the greatest antagonism, were inoculated with or before salmonella into sterile compost.

The capability of microorganisms from different compost temperature regimes to inhibit salmonella growth was determined. Compost from the 70°C zone of a compost pile did not suppress salmonella growth. Compost from a 55°C adiabatic incubator was more suppressive, and compost from a curing pile from a surface area that was near ambient temperature was completely suppressive.

Studies involving the size fractionation of the flora obtained in compost extracts again showed the lack of the ability of fungi to suppress salmonella growth and indicated that although the actinomycetes suppressed growth to some extent, gram-negative bacteria played a larger role. Of the gram-negative bacteria, the coliforms were much more effective than the noncoliform organisms.

Given the diversity of the microbial population of cured compost at ambient temperature, it was concluded that salmonella regrowth would be negligible. Because total inhibition is not related to the activities of any single group of microorganisms, no microbial assay can be recommended to determine the capability of a compost to suppress salmonella regrowth.

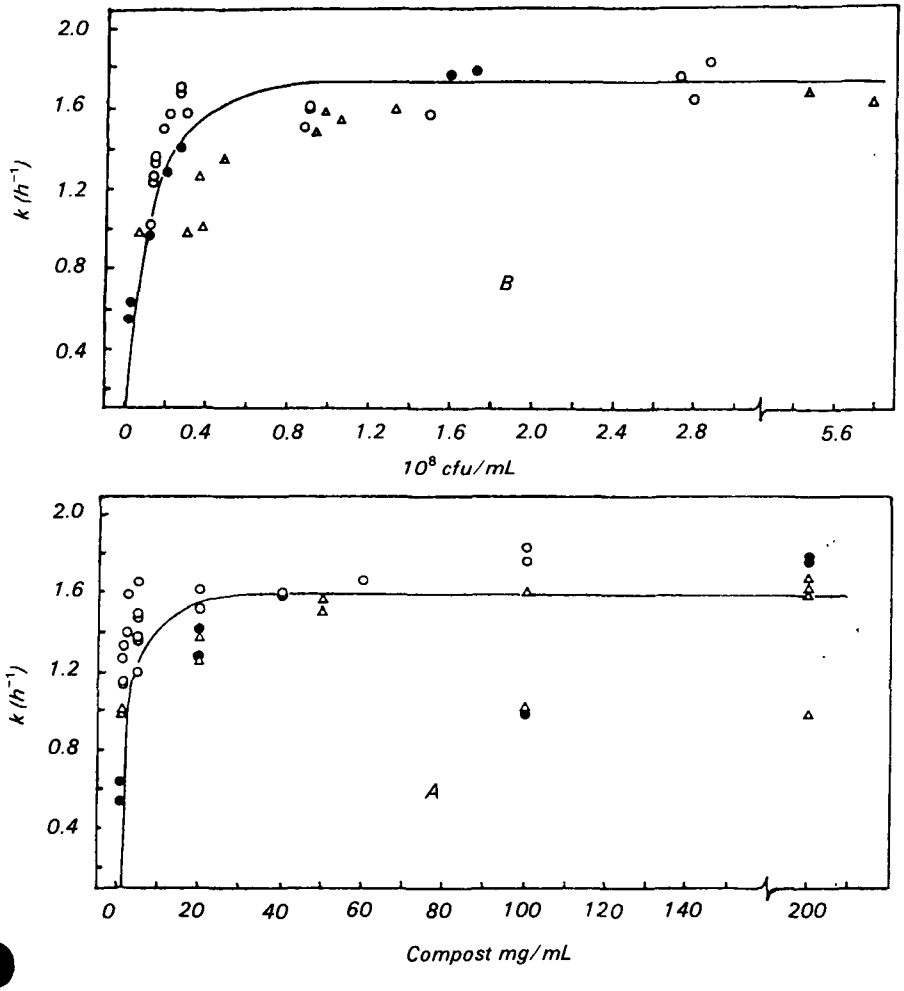
Influence of Substrate on Salmonella Regrowth

The kinetics of salmonella growth in suspensions and extracts of irradiation-sterilized composts were studied to determine the number of substrates and the relative amounts of the substrates used. Three composts from widely separated compost sites in the United States were used. Initial studies showed that growth of salmonellae in suspensions of compost did not appear to be first order; but growth in extracts was ($p < 0.01$), indicating a soluble substrate and an insoluble substrate that became solubilized as growth proceeded in the presence of the compost solids.

The magnitudes of the growth-rate constants obtained using the extracts were sensitive to the quantity of compost used up to a maximum amount, and a hyperbolic relationship was found when growth-rate constants were plotted against the amount of compost extracted (Figure 1A). Plotting growth-rate constants against the maximum amount of salmonella growth brought some of the outlying data points in closer to the hypothetical curve (Figure 1B).

The linear forms of the hyperbolic curves generated by the data all appeared to fall on a single curve (Figure 2). The correlation coefficients for the three curves all exceeded 0.997. Models involving multiple versus single parameters were tested for relative fit of the data to the regression line of Figure 2. The correlation coefficient of a model with three separate intercepts and three separate regression coefficients was 0.9988, whereas that for one combined equation (one intercept and one regression coefficient) was 0.9977. Although the multiple parameters improved the fit, the difference was so small that on the basis of parsimony, the simpler model combining the three equations to yield a single intercept and a single regression coefficient was preferred.

Figure 1. *Growth-rate constants (k, h^{-1}) for salmonellae as plotted against: A. amount of compost extract added and B. total amount of salmonellae growth (compost 6175, open circles; compost 6266, closed circles, and compost 6252, triangles).*



The results of this study showed that it is possible to extract a water-soluble substrate from compost that will support first-order growth of salmonellae. The first-order nature of the kinetics and the combined data for the three composts used suggest that there is a single substrate among the composts supporting salmonella growth. The identification of this substrate and the testing for its presence in other composts might possibly furnish valuable information as to the factors involved in the regrowth of salmonellae in composts.

Conclusions

Selecting salmonella colonies when enumerating low numbers of salmonellae in sewage-sludge and compost samples can be difficult because of the growth of organisms that mimic salmonellae. This difficulty can be greatly alleviated by modification of the standard XLBG agar medium. The modification involves using high concentrations of BG dye that has not been heated beyond 50°C.

Studies of composts collected from 30 composting sites throughout the United States show that inhibition of the growth of salmonellae by the indigenous microflora of composts is a general phenomenon.

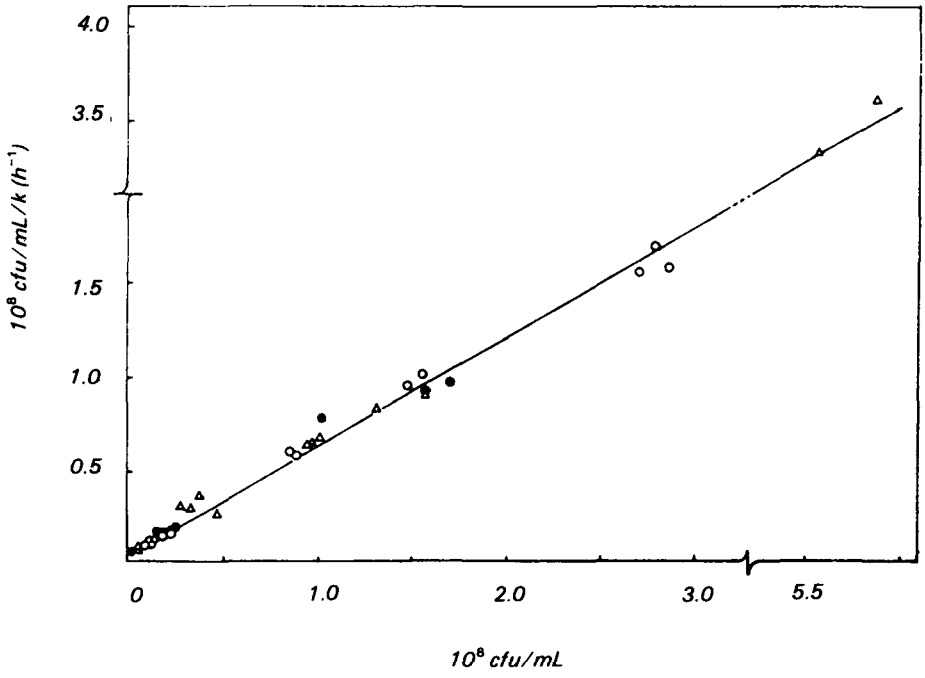
When the complete microflora of compost (bacteria, actinomycetes, fungi, and protozoa) are present or introduced into sterile compost, they fully suppress the regrowth of salmonellae. A major proportion of suppression comes from the coliforms, with complementing activity from other gram-negative bacteria. Thermophilic and mesophilic actinomycetes also supplement the suppressive activity, but the effect of fungi is negligible. The contribution, if any, by protozoa was not defined.

Three composts from widely separated composting sites in the United States contained water-extractable substrates that supported the growth of *Salmonella typhimurium*. Kinetic studies of salmonella growth indicate that these substrates in the different composts are very similar, if not identical, and that total salmonella growth is a sensitive assay for their concentration in composts.

Recommendations

Modification of the xylose lysine (XL) agar base (agar increased to 2% and 6 to 7 ppm BG dye added to the autoclaved

Figure 2. *A plot of the population and rate-constant data of Figure 1B according to the linear form of Monod's equation (see Fig. 1 for meaning of symbols).*



medium after cooling to 50°C) appears to provide a useful alternative to other plating media for salmonella assay of sewage sludges and sewage-sludge composts. Increasing the BG dye content of the modified XLBG to 9 ppm was found to increase the effectiveness in discriminating for salmonella colony growth. We suggest, however, that each user run a study to determine what concentration works best for salmonella measurement. Additional studies are recommended to compare the recovery of salmonellae from similar samples (i.e., sludges, composts) with other procedures. Also, comparison of the modified medium and other media to recover indigenous salmonellae is recommended.

The resident microflora in the composts apparently provide a safety factor preventing the colonization of sewage-sludge composts by salmonellae. It has been suggested that composts be sterilized by irradiation. We suggest that complete sterilization may result in unchecked growth of salmonellae if the composts become inoculated. The possibility that partial sterilization may destroy pathogens and yet inhibit salmonella growth needs evaluation.

The fungi play essentially no role in suppressing the growth of salmonellae introduced into composts. Schemes to prevent or control fungal growth can be used if they do not eliminate gram-negative bacteria, particularly coliforms, from the compost.

The findings that bacteria most closely related to salmonellae play the major role in suppressing salmonella growth and that similar water-soluble substrates in the three composts studied support salmonella growth suggest that a study to determine the identity of these substrates may furnish the key to understanding and perhaps controlling the regrowth of salmonellae in composts.

Studies are recommended to determine the contribution of protozoans and other parasites for suppressing salmonella regrowth in composted sewage sludge.

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The complete report, entitled "Regrowth of Salmonellae in Composted Sewage Sludge," (Order No. PB 87-129 532/AS; Cost: $13.95, subject to change) will be available only from:
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