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Salmonella Regrowth in Compost as Influenced by Substrate (Salmonella Regrowth in Compost)

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Abstract. Composting can eliminate pathogenic organisms, including salmonellae, from sewage sludge. However, if salmonellae are present in the compost at undetectable levels or are inoculated into the compost by infected animals or from other sources, they may regrow presenting a health hazard for certain uses of compost. In this study, we examined dilute mineral-salt extracts of three composts from widely separate composting sites in the United States and found that they supported growth of *Salmonella typhimurium.* From kinetic studies of the growth of the organism on these extracts, we concluded that each compost produced on extraction a single water-soluble substrate and that the substrates from the different composts were very similar, if not identical.

Introduction

Composting is capable of destroying the primary pathogenic organisms, including salmonellae, that may be present in sewage sludge [2]. However, there have been anecdotal reports of salmonellae in marketed composts. If these reports are valid, possible explanations include growth of organisms (1) that may have survived composting because of failure to obtain a lethal time-by-temperature regime; or (2) that may have been inoculated into the compost by infected birds or other animals, or by equipment contaminated with salmonella-containing sewage sludge.

Available data indicate that salmonellae do not grow extensively when inoculated into compost [1] and sewage sludge unless these materials have been sterilized. Further, repopulation of the sterilized sludge with coliforms will inhibit the growth of salmonellae [10]. A study of compost from a single site indicated that a water content of 20% or greater and a carbon/nitrogen ratio of greater than 15:1 were necessary to support growth. Also, as with sludge, the native flora inhibited growth [9]. But data from studies with sewage sludge and a compost from a single site cannot be considered definitive. To understand

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better the regrowth potential of salmonellae, knowledge of the substrates involved and the nature of the inhibiting microflora is needed.

In this study, the kinetics of salmonella growth in suspensions and extracts of radiation-sterilized compost were studied to determine the number and relative amounts of substrates utilized.

Materials and Methods

Compost

The compost collection, processing, and storage methods have been previously described [5]. Briefly, sewage sludges composted by the Beltsville Aerated-Pile Method [3] were collected from storage piles containing finished composts (composts that had been through the complete process and were ready for utilization), and shipped to our laboratory in sealed 5-gallon containers. Composts were identified by composting site numbers that we assigned. Woodchips were removed by sieving with a 0.6 cm pore-sized screen. The remaining compost was sieved through a 0.147 mm pore-sized screen and stored in plastic bags for periods of less than a week at 4°C. Storage beyond a week was at −20°C. For utilization as substrate, the compost was sterilized by irradiation (3 megarads, ^{60}Co).

Compost Extract

Compost extracts were prepared by shaking overnight weighed amounts of irradiated compost in 100 ml of a minimal medium [4] modified by reducing the NH_4Cl concentration from 1.0 to 0.5 g and omitting the glucose. The mixtures were then centrifuged (19,600 × *g*, 20 min) and decanted to obtain a particulate-free extract. Initially, filtration through a Gelman 0.45 μm pore-sized, 45 mm diameter membrane filter was used to remove the particulate material, but results from the control (membrane-filtered medium without compost) showed that the filter was contributing significant amounts of substrate.

Experimental Inoculum

Salmonella typhimurium ATCC 14028 was used for the experiments. The inoculum was prepared by introducing a loopful of the organism, as grown overnight on a nutrient agar slant, into the minimal medium described above. At first, a filter-sterilized glucose solution was used as the carbon source (final concentration in the medium, 2.0 g/liter) to grow the inoculum. However, use of the glucose-adapted cells for the inoculum resulted in an appreciable lag phase upon inoculation into compost extract. Therefore, an extract of compost #6175 was substituted for the glucose as a carbon source for growth of the inoculum to reduce this lag phase. After overnight growth, the culture was diluted to produce a zero time concentration in the experimental flasks of between 100 and 1,000 colony-forming units (CFU)/ml.

Experimental Procedure

In the first study, weighed amounts of irradiated compost were added to 100 ml of the minimal medium in 250 ml screw-capped flasks. After adding the salmonellae inoculum, the flasks were placed in a water bath (36 ± 1°C with shaking) and sampled with time. In all other studies, compost

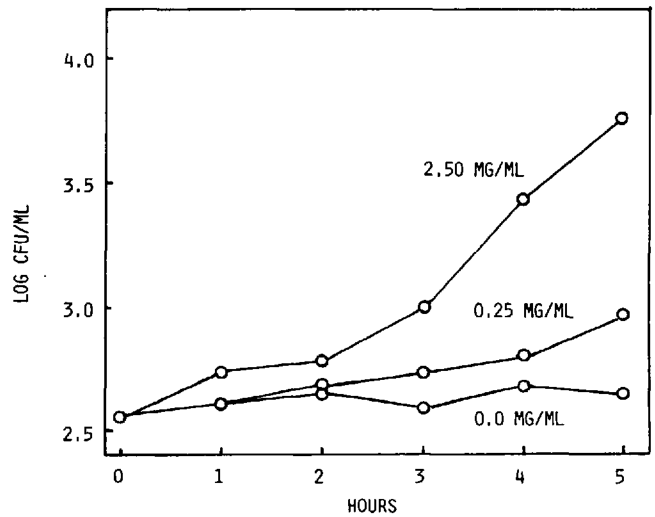

Fig. 1. Growth of salmonellae in a mineral-salts medium as influenced by the amount of compost #6175 added.

extracts were inoculated, incubated, and sampled as above. The amounts used ranged from 0.25–200.0 mg/ml. The specific amounts of compost used in each study will be given in the results section. The sample volume taken was either 0.1 or 1.1 ml depending upon the dilution needed for counting. For counting, tenfold serial dilutions were made and plated by spreading on xylose lysine brilliant green (XLBG) agar. For the first study, flasks were sampled hourly over a 5-hour period. Since 5 hours did not allow achievement of total growth, the sampling time was extended. The sampling interval became 2 hours for an 8-hour period with a final sampling after 24 hours of incubation to insure measurement of the total growth potential of the substrate.

Results

Influence of Quantity of Compost Added on Growth Rate

If growth proceeds by first-order kinetics indicating a readily available single substrate, total growth can be expected to be related to substrate concentration. The rate of growth, however, should not be related, except at very low concentrations [7]. In the first study, 0, 25, and 250 mg of irradiated compost were added to flasks containing 100 ml of the minimal medium lacking the addition of a carbon source. Each treatment was replicated. After inoculation with salmonellae grown overnight in the minimal medium containing glucose, the flasks were incubated with shaking at 37 ± 1°C and sampled at hourly intervals for 5 hours. As shown in Fig. 1, growth occurred with the addition of compost, and the rate of growth increased with the increase in the amount of compost added to the flasks. Five hours was not enough time to achieve maximum growth, and the growth was not indisputably first order.

The variations in growth rate that occurred with different amounts of compost suggested that substrate concentration was controlling the growth rate. However, it was possible that the rate of growth was controlled not only by the quantity of substrate furnished in proportion with the addition of compost but

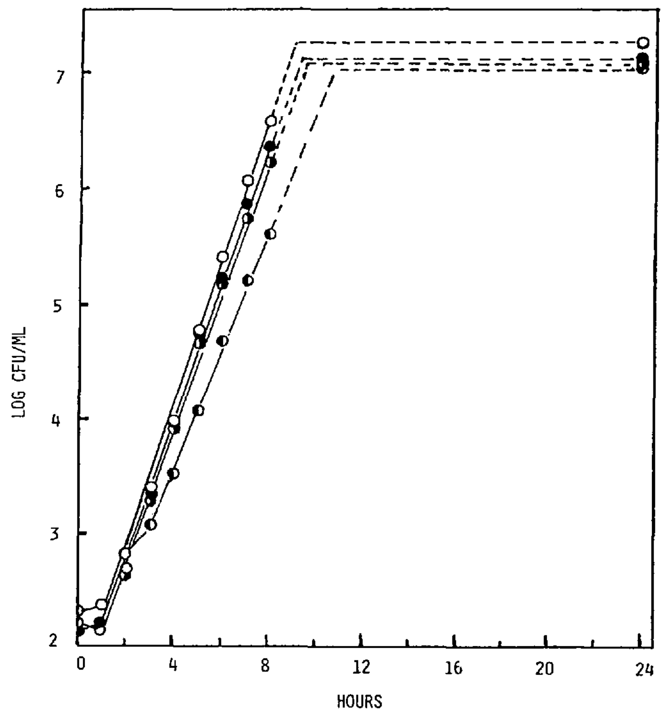

Fig. 2. Growth of salmonellae on extracts of compost #6175 using a mineral salts medium. Length of extraction times were 4 (◑), 8 (●), 16 (○), and 24 hours (◐).

also by the rate of diffusion of the substrate from the matrix of the compost particles, and/or the rate of solubilization of the substrate. To test for these two possibilities, a series of time extracts of the compost was prepared by shaking 500 mg of compost in 100 ml of the carbon-source-free minimal medium and removing the particulate material by centrifugation. Shaking times for extraction were 4, 8, 16, and 24 hours. The inoculum was grown in compost extract to reduce the lag phase.

The plots of the data (Fig. 2) show first-order growth kinetics, because the effect of change in substrate concentration that should produce second-order growth was too small to be evident. The plots also showed a reduction in the lag phase from use of the compost-conditioned inoculum (Fig. 2). Despite the use of the extract from a single compost (#6175), no differences in the shortened lag phase were evident among the three composts indicating that the organisms were encountering similar if not identical substrates in all three composts (compare Figs. 2 and 5). Kinetic data for compost #6266 is not shown. The lag phases must have resulted from some factor such as adjustment of the organisms to the dilutions made of the inoculum broth.

In addition to the shortened lag phase, there was a slight trend of increased growth rate and total population size with increase in extraction time from 4 through 16 hours. Against this trend, the 24-hour extract supported slower growth than the other extracts and a slightly lower total population. These results were difficult to rationalize solely on the basis of release of substrate

Table 1. Regression analyses comparing growth rate constants (k_g) for growth of salmonellae in extracts of compost #6175 as influenced by extraction time

Extraction time (h)	k_g (h^{-1})	ln y intercept	t for H_0: parameter = 0	PR > [t]
4	1.381	3.545	47.23	0.6228
8	1.372	3.752	47.02	0.8342
16	1.493	3.393	49.93	0.0129[a]
24	1.211	3.395	44.06	0.0054[b]

[a] Significant at 0.05%
[b] Significant at 0.01%

with time through diffusion and/or slow solubilization. A regression analysis of the data (Table 1) showed that the 16- and 24-hour compost extract slopes were not the same as the 4-hour extract slope ($P = 0.01$ and 0.05 respectively). The slope of the 8-hour extract was not significantly different from the 4 hour slope ($P > 0.05$). We concluded that these differences, although significant, could be tolerated in kinetic studies for determining the effect of amount of compost added if the extraction period was held within 1 hour's deviation from 16 hours.

Although the data cannot be used to determine the mode of release of substrate, it does appear that the lack of a precise fit of the data to a first-order kinetic plot in the first study, if not merely data scatter, could have been the result of some slow release process or the result of multiple substrates. Therefore, we decided to use extracts instead of compost for the rest of the studies.

Glucose Equivalent

Tests were conducted to determine the amount of glucose used by the organisms. The results were used to calculate the glucose equivalent of the substrate in the compost so that a relative measure of the substrate concentration in the compost could be obtained. Glucose was added to flasks of minimal medium to form a glucose concentration series as follows: 0.004, 0.40, 4.00, 40.0, and 400.0 μg/ml. The growth rates for all concentrations were not first order (not shown), but after 48 hours, the total growth was correlated with the amount of glucose used (Fig. 3). Each CFU required 4.82×10^{-8} mg of glucose. The mean glucose equivalents for the substrate, as based upon total CFUs formed, were 0.26, 0.083, and 0.050 mg/g of compost respectively, for composts #6175, #6252, and #6266. The standard deviations were respectively, 0.14, 0.055, and 0.24 reflecting the large amount of variability among samples of each compost.

Lack of first-order growth on glucose could be explained by the fact that bacteria require appreciable CO_2 tension for first-order growth on simple carbohydrates, but the CO_2 requirement can be ameliorated by succinate, purines, and pyrimidines [6].

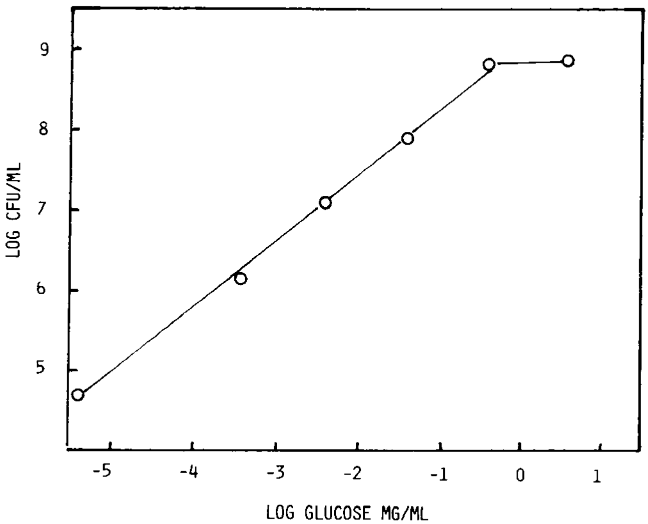

Fig. 3. Amount of salmonellae growth as influenced by amount of glucose added to a mineral salts medium.

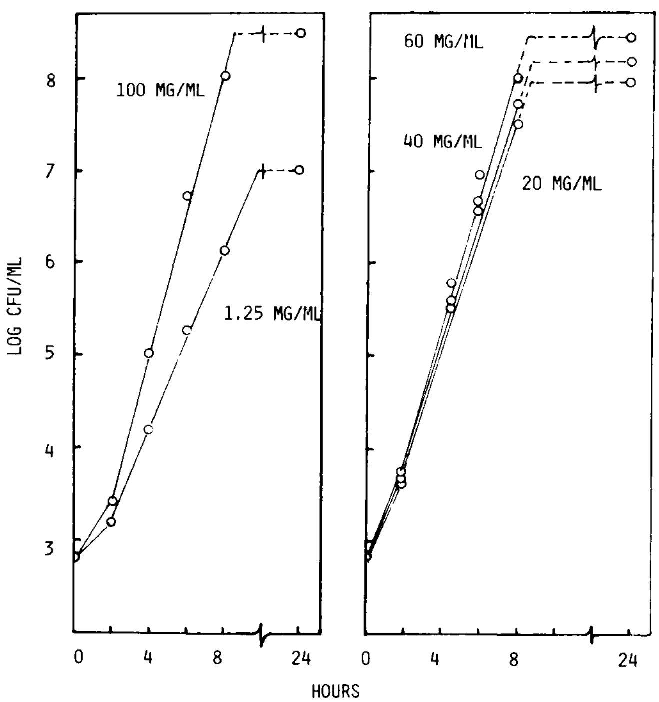

Fig. 4. Growth of salmonellae in a mineral-salts extract of compost #6175 as influenced by the amount of compost extracted.

Influence of Water-Soluble Compost-Substrate Concentration on Growth

To determine the effect of concentration, 16-hour extracts were made from different quantities of the compost #6175 and inoculated with the test strain grown on extract of compost. The quantities of compost extracted per ml of

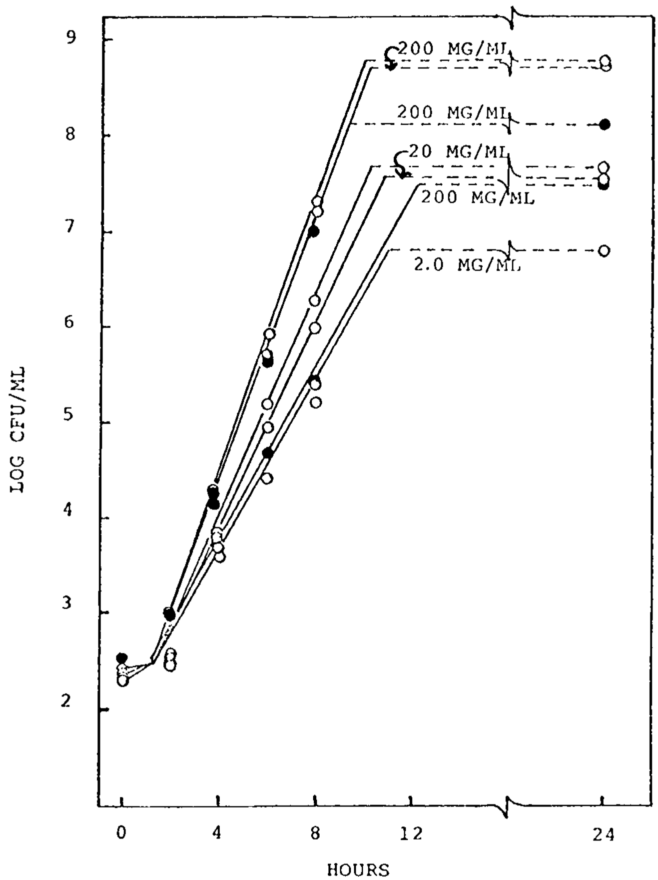

Fig. 5. Growth of salmonellae in a mineral-salts medium as influenced by amount of compost (#6252) extract added. Data for 200 mg/ml extracts that appear to be out of order (●).

basal medium were 1.25, 20, 40, 60, and 100 mg. The results showed only small differences in the rates of growth at concentrations of 20 mg/ml and above. Only the lowest concentration of 1.25 mg/ml produced a large difference in growth rate (Fig. 4). Total growth, however, appeared to be proportional to the amount of compost extracted. Similar studies with composts #6252 and #6266 were conducted. The results for some of the data for compost #6252 are shown in Fig. 5. The data for #6266 are not shown.

Regression analyses of the data from the three studies showed that the growth-rate equations were first order ($P = 0.01$). Also there appeared to be a positive relationship between growth rate, total growth, and amount of compost extracted, but some of the curves were out of order as were those for two of the 200 mg/ml curves (filled circles) in Fig. 5. In these two samples the mass of the sample relative to the other samples shown in Fig. 5 was not proportional to the microbial response to substrate, which prompted us to utilize total salmonella growth as a measure of substrate concentration, as discussed in the next section.

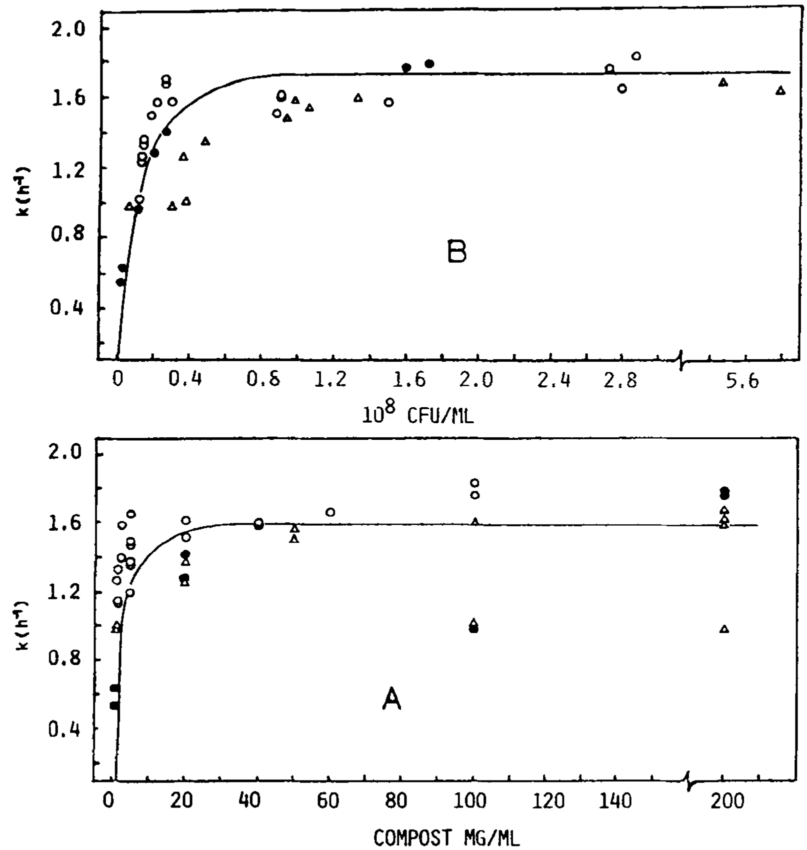

Fig. 6. A comparison of plots of the growth-rate constants for salmonellae vs **A.** amount of compost extract added, and **B.** total amount of salmonellae grown. ○, △, and ● refer to composts #6175, #6252, and #6266 respectively.

Discussion

With the exception of the results shown in Fig. 1, analysis of the growth-rate data for all three composts showed first-order regressions, but the rate coefficients were not constant for the lower concentrations. The dependency of growth rate on concentration was evaluated by plotting the growth-rate constants against the substrate concentrations for the three composts (Fig. 6A). The data appeared to describe a single curve indicating that the substrate could have been the same or very similar in all three composts. However, there were outlying points that did not seem to fit into the general pattern, just as in Fig. 5 two samples gave curves that were out of order respective to the other curves with regard to the masses used.

The quantity of extractable substrate should have been directly related to the amount of compost mass for any single compost unless the compost mass from which the samples were taken was not uniform or some change in the available substrate had occurred between samplings. If either was true, then

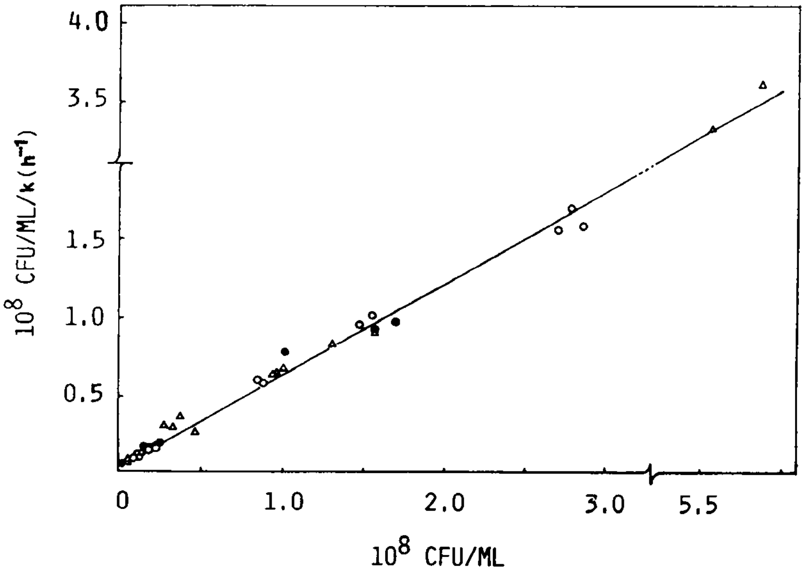

Fig. 7. Plot of the population and rate-constant data of Fig. 6B using the linear form of Monod's equation. For meaning of symbols see caption of Fig. 6.

maximum growth might be a better measure of substrate quantity than compost mass was, and should be better correlated with the growth-rate constant (k) than mass was. It also should compensate for differences in substrate concentrations among the different composts. Plotting k versus total population grown (P) produces fewer outlying data points possibly indicating the instability of the substrate with time despite refrigeration and/or its uneven distribution in the refrigerated compost samples when they were subsampled (Fig. 6B).

If the substrates were qualitatively different, then the maximum rates at which they would be utilized by *S. typhimurium* should not be the same. Linearization of the data for the three composts would make it possible to compare the fit of the data to a common regression line.

Growth-rate data such as plotted in Fig. 6 can be described by Monod's equation [7]. This equation can take the following form for use in linearization of the data:

$$\frac{P}{k} = \frac{P_l}{k_m} + \frac{1}{k_m} P$$

in which P = the maximum population achievable (CFU/g compost extracted), and P_l = the population concentration when the growth-rate constant k = one-half the maximum growth rate achievable (k_m).

Analysis of the results from the transformations of the data in Fig. 6A and 6B showed that using total growth as opposed to compost mass resulted in smaller differences among the parameters (intercepts and slopes) for the transformed equations (Table 2). Also, the values for the correlation coefficients

Table 2. Comparison of intercepts (a), slopes (b), and correlation coefficients (CC) of equations using amount of compost or total growth as the independent variable

	x = amount of compost			x = total growth		
Compost	a	b	CC	a	b	CC
#6175	0.883	0.563	0.996	3.228	0.571	0.995
#6263	1.834	0.714	0.872	6.126	0.594	0.999
#6266	7.877	0.568	0.901	4.796	0.533	0.999

Table 3. Determination of the relative linear fit of multiple vs single parameters in use of the transformation of Monod's equation to describe dependence of the growth-rate coefficient on maximum population as a measure of substrate concentration

	No. of parameters		
Model	a	b	Correlation coefficient
1	3	3	0.9988
2	3	1	0.9983
3	1	3	0.9987
4	1	1	0.9977

were increased for composts #6252 and #6266 showing that using total growth was more representative of the amount of the substrate available than was compost mass. Therefore, we plotted the transformation for the total growth versus k data of Fig. 6B in Fig. 7.

The constant k_m can be used to compare the efficiency with which substrates are utilized [7]. According to Ostle [8], the correlation coefficient can be used to compare regression parameters for goodness of fit. In Table 3, four variations of a model equation are compared for their effect on the magnitude of their resulting correlation coefficients. Model 1 utilized an intercept and a regression coefficient for each of the sets of data for the three composts. Models 2 and 3 eliminated respectively two regression coefficients and two intercepts. Model 4 utilized only one regression coefficient and one intercept. There was a decrease in the value of the correlation coefficient with each decrease in the number of parameters used, but the total decrease from model 1 to model 4 was only 0.0011 (0.11%). This change was small, and dictated on the basis of parsimony that model 4 was preferred. There was no good reason to believe that more than one kind of substrate among the composts was needed to produce these results.

The water-soluble substrate in the compost was decomposed in accord with first-order kinetics, but glucose was not. For minimal media with single simple carbon sources, high concentrations of CO_2 are required to produce first-order growth [6]. Although growth may not be first order, given enough time the substrate will be exhausted producing growth proportional to the amount of substrate added. The CO_2 requirement can be met by providing additional metabolites. These results indicated that there probably was a water-extractable single energy source in each compost extract producing the observed first-order growth. If it was a simple compound such as glucose or some other sugar, there must also have been metabolites available to compensate for the lack of an adequate CO_2 concentration.

The results of this study showed that it was possible to extract a water-soluble substrate from compost that would support first-order growth of *S. typhimurium*. The first-order nature of the kinetics, and the high degree of correlation for the combined data using the linear form of Monod's equation suggested that there was a single substrate among the composts. 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.

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