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Factors Affecting Salmonellae Repopulation in Composted Sludges

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The repopulation potential and recovery of *Salmonella* sp. and their close relatives *Arizona* spp. and *Citrobacter* spp. in sewage sludge which had been composted was examined. Salmonellae growth in previously composted sludge was found to occur in the mesophilic temperature range (20 to 40°C), require a moisture content of ≥20%, and require a carbon/nitrogen ratio in excess of 15:1.

Composting of sewage solids is routinely used as an effective means of reducing organisms to very low levels. The review of Kowal and Pahren (8) reported that disease transmission by parasites, bacteria, and viruses in land application of digested and stored sludges was not a significant problem. Others (2, 3, 5), however, have indicated that salmonellae repopulation may be of significant concern under certain conditions.

Studies by Epstein and Wilson (5) have described inoculating *Salmonella enteritidis* serotype Montevideo into sterile composted sewage sludge (60% solids) material. Initial counts were measured at 10^3 bacteria per g of solids. After 2 days of incubation, salmonellae counts were found to be greater than 10^9 bacteria per g.

Brandon et al. (2) observed that *S. enteritidis* serotype Montevideo can grow rapidly in composted sludge having a moisture content of approximately 40% (60% solids). In a subsequent paper, Brandon and Neuhauser (3) determined that a 20% moisture content represented a crucial threshold. Their results indicated that repopulation by salmonellae after disinfection should not occur in composted sludges at or above the 80% total solids (TS) level. These results also indicated that some enteric bacteria, upon desiccation, became dormant and in this state were highly resistant to both heat and radiation.

This report describes selected physical factors affecting salmonellae growth in a commercial soil amendment produced from previously composted sludges. The investigation was conducted to determine whether appropriate control measures could eliminate the potential for salmonellae repopulation in the bagged soil amendment produced with the compost. The variables studied included moisture, temperature, and nutrient levels as measured by volatile solids (VS) and carbon-nitrogen (C/N) ratio.

MATERIALS AND METHODS

Compost description. The Los Angeles County sanitation districts operate a 350-million-gallon/day advanced primary treatment Joint Water Pollution Control Plant located in Carson, Calif. Raw sludge is stabilized by mesophilic anaerobic digestion and mechanically dewatered to >20% TS. An aerobic windrow composting process is used to achieve further stabilization of organics, pathogen inactivation, and volume reduction. The composting process is described in detail elsewhere (6). In general, the compost cycles last 3 weeks, produce temperatures of 50 to 60°C, and reduce moisture to the 60% TS range.

All of the material used in these experiments had been thoroughly composted before use by a fertilizer company.

Temperature-moisture regrowth experiments. A bag of finished compost was opened and allowed to dry in the laboratory at room temperature for approximately 1 year. When used in the experiment, TS was 92.8%, VS was 31.8%, and salmonellae were not detectable (<0.15 most probable number [MPN]/g of TS). A set of 72 250-g samples were weighed into Waring blender jars. These samples were designated "low bacteria"; the indigenous bacterial population in them was not modified. This set was divided into three groups, and the moisture content was adjusted to 9, 16, and 21%, respectively, by adding sterile phosphate-buffered saline (PBS) to achieve the desired moisture level. Each sample was blended at high speed for 30 s and then dispensed into presterilized, cotton-stoppered, wide-mouth 1-liter Nalgene bottles. Subsamples were collected for TS and VS determination. Twenty-four bottles were set up for each moisture level. Ten-gram portions were removed from six of the 9% moisture bottles for determinations of initial salmonellae concentration, and results were averaged for the starting count.

Six bottles from each moisture level were then incubated at one of four temperatures, 4, 28, 36, and 44°C. Three bottles were removed from each incubation temperature after 5 days and again after 21 days for salmonellae assay.

The entire experiment was repeated with another set of samples designated "high bacteria." Salmonellae

were dosed into these samples to obtain a high initial concentration. The *Salmonella* suspension used to inoculate the high-bacteria experiment was prepared by inoculating three bottles of nutrient broth (Difco Laboratories) plus 0.6% NaCl with three randomly selected *Salmonella* species previously isolated from compost samples. The cultures were incubated overnight at 37°C, centrifuged at 9,000 × *g* for 10 min, washed two times with 50 ml of sterile PBS, combined, and suspended in 400 ml of sterile PBS. Five milliliters of salmonellae suspension was inoculated into each 250-g sample. Moisture additions were adjusted to include the bacterial dose so that the final moisture levels were the same as in the low-bacteria experiment. Incubation and sampling were described above. Figure 1 illustrates these experiments.

Nutrient-oxygen experiments. Unopened bags of the soil amendment produced from the compost were obtained on separate dates and prepared for aerobic and anaerobic incubation as follows. The compost was aseptically transferred from freshly opened bags to presterilized 1-liter Nalgene screw-capped jars in 250-g portions. The jars were stoppered either with sterile cotton plugs (aerobic incubation) or with Nalgene screw caps (anaerobic incubation). The term "anaerobic" as used here includes the possibility of microaerophilic environments existing in the containers. Compost to be assayed was aseptically removed from each bottle in 50-g portions and prepared for culture. The remaining compost was placed in a Whirl-Pak bag and analyzed for TS and VS. For the first experiment, material containing high VS and low bacteria was assayed periodically until salmonellae were below detection levels. Representative salmonelloid isolates from each sample were retained for identification. In the second experiment, material containing naturally occurring high salmonellae levels (≥100 MPN/g) and low VS (26% VS) was incubated at 28°C. Samples were removed periodically for 15 days until salmonellae MPNs dropped below detection levels and VS content was <20% of TS content. This material was then mixed in a Waring blender for 30 s with sterilized uncomposted sludge cake (approximately 30% TS and 50% VS) from the Joint Water Pollution Central Plant and reincubated aerobically and anaerobically as described above for the third experiment. Samples were removed periodically over a period of 15 days until salmonellae were again below the detection level.

Sample preparation. All samples were held in a moist incubator to prevent dryness and assayed for *Salmonella* MPN, TS, VS, and C/N. Chemical oxygen demand and Kjeldahl nitrogen were used to form the C/N ratio. Compost samples were prepared for *Salmonella* MPN assay by blending a 50-g portion of compost aseptically in a presterilized Waring blender jar at high speed for 30 s with 500 ml of PBS plus 0.1% Tween 80.

Bacterial quantitation. Enrichment for salmonellae followed the procedures outlined in *Standard*

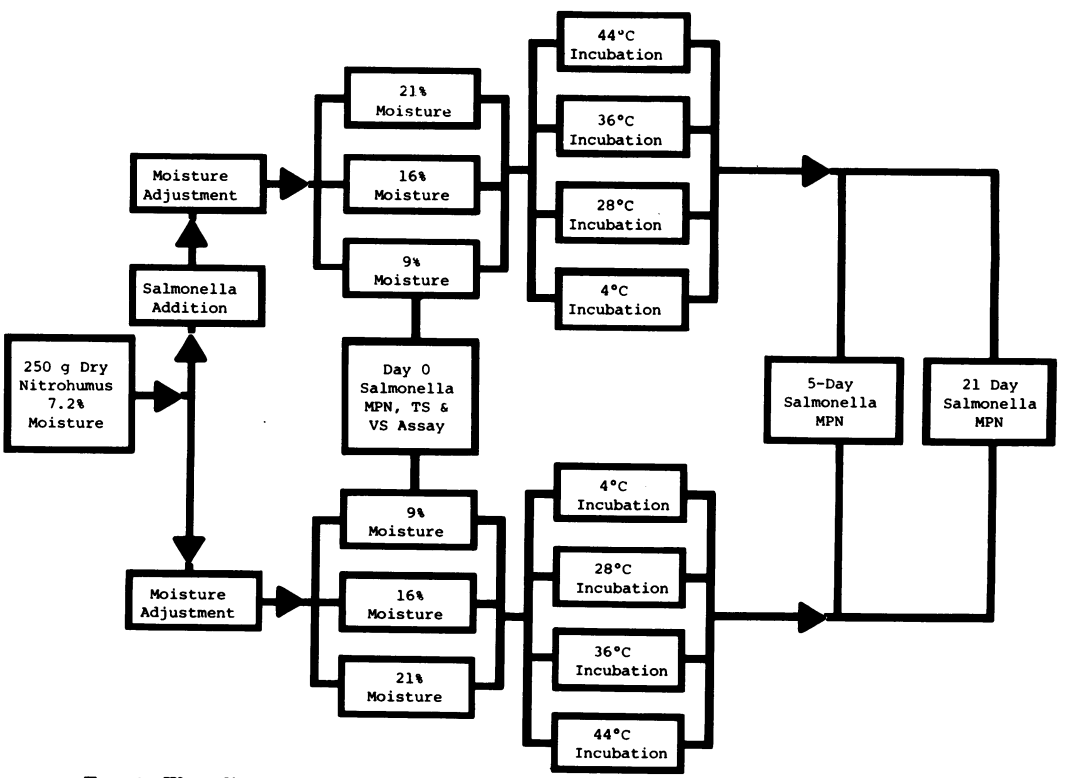

FIG. 1. *Flow diagram of the bagged compost temperature-moisture repopulation experiment.*

Methods (1). The medium chosen for initial enrichment was tetrathionate broth (Difco), based on work done in this laboratory and the efforts of others (7, 9, 10). The three-tube MPN test was used for initial selective enrichment. Tetrathionate tubes were streaked to bismuth sulfite agar, xylose lysine desoxycholate agar, and brilliant green sulfa agar (all Difco products) at 24 and 48 h. Representative isolates were picked from all plates showing positive colonies and transferred to triple sugar iron agar (Difco), lysine iron agar (BBL Microbiology Systems), and urease test medium (BBL). Tubes showing a salmonellae-positive fermentation pattern were serotyped with *Salmonella* O antiserum, Poly A-I, and Vi (Difco). Positive cultures from the first nutrient-oxygen regrowth experiment were reserved for biochemical identification by the procedures of Edwards and Ewing (4).

The procedures for TS, VS, chemical oxygen demand, and Kjeldahl nitrogen were those described in *Standard Methods* (1).

RESULTS

Temperature-moisture experiments. Table 1 shows the effects of moisture and incubation temperature on salmonellae growth in composted sludge with a VS content of ~30% TS. The reported analyses represent the average of three replicates. Optimal recoveries in the low-bacteria sample occurred at the 21% moisture level at 28 to 36°C after a 5-day incubation. The population increased more than four orders of magnitude under these conditions. The indigenous salmonellae initiating this growth had survived in a desiccated state for over 1 year prior to providing the proper moisture-temperature combination for the repopulation to occur. *Salmonella* spp. were still detectable at this moisture level after 21 days of incubation at 28°C. Optimal salmonellae recovery in the high-bacteria samples occurred after 5 days at both 28 and 36°C. All other samples showed negative or declining values for *Salmonella* populations.

Nutrient oxygen experiments. Figure 2 shows the repopulation of salmonellae from freshly bagged high-VS compost (62% TS and approximately 23% VS) incubated at 28°C under aerobic and anaerobic conditions. After an initial increase, salmonelloid MPNs were found to decrease to detection levels or below in both systems; the VS concentration dropped below 18 g per 100 g of TS, and the C/N ratio responded in a similar manner, decreasing to below 15:1. The anaerobic system required twice the time of the

TABLE 1. *Effect of moisture and temperature on salmonellae repopulation in bagged compost*

Initial Bacterial Count per g TS	Initial Compost Moisture Content, %	Sample Time (Days)	Most Probable Number Counts per Gram Compost as a Function of Temperature			
			4°C	28°C	36°C	44°C
<0.11 Salmonella/g	9.0	5	<0.11	<0.11	<0.11	<0.11
		21	<0.11	<0.11	<0.11	<0.11
	16.0	5	<0.12	<0.12	<0.12	<0.12
		21	<0.12	<0.12	<0.12	<0.12
	21.0	5	<0.14	≥3300	210	<0.14
		21	<0.14	16	<0.14	<0.14
3,300,000 Salmonella/g	9.0	5	<0.33	<0.33	2500	<0.33
		21	<0.33	<0.33	23	<0.33
	16.0	5	3900	290	130	1.1
		21	2.7	4.8	51	0.31
	21.0	5	21,000	≥33,000,000	≥33,000,000	<0.14
		21	330	330	330	<0.14

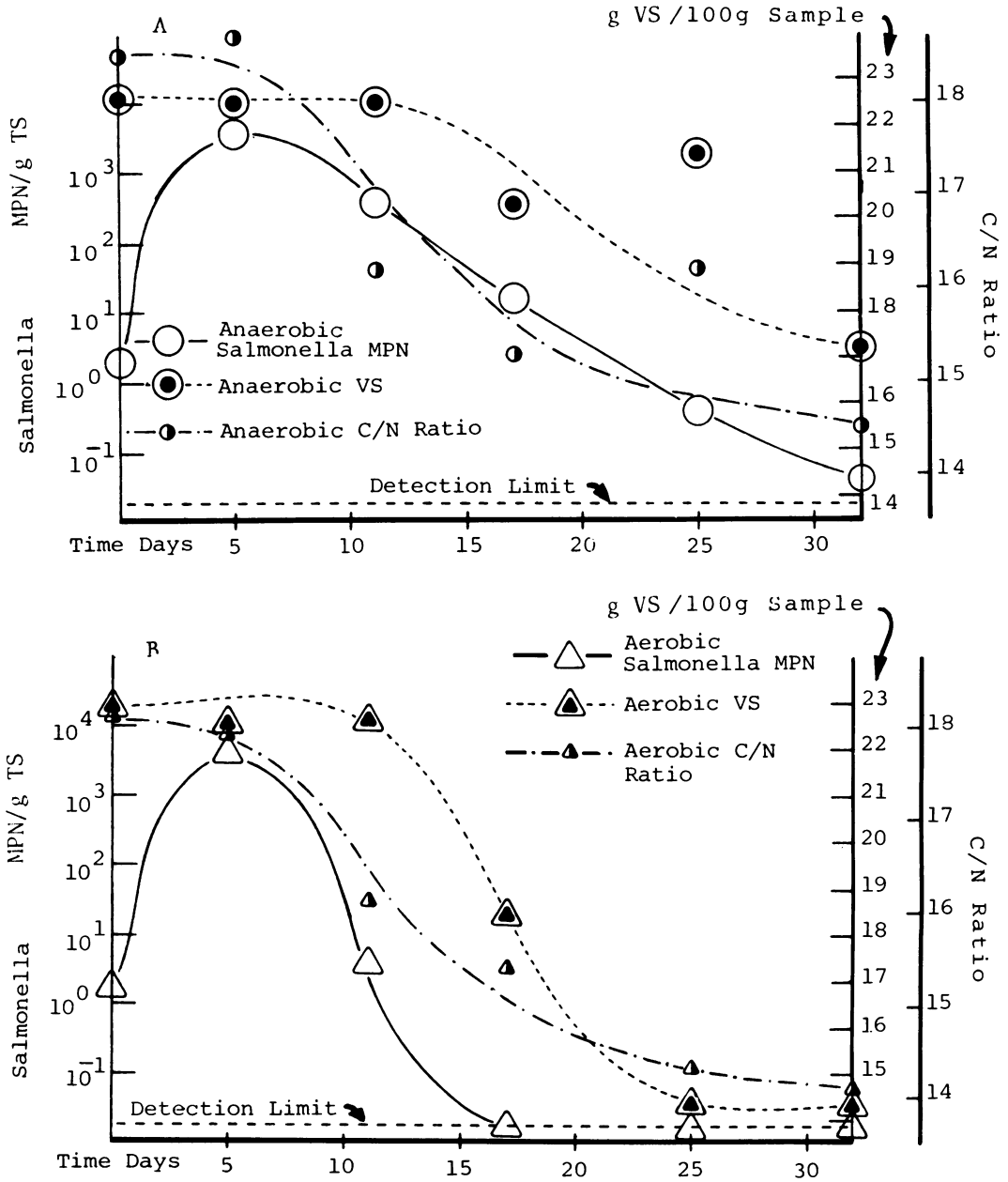

FIG. 2. *(A) Effects of anaerobic incubation on salmonellae populations, VS, and C/N ratio in bagged compost. (B) Effects of aerobic incubation on salmonellae populations, VS, and C/N ratio in bagged compost.*

aerobic system to achieve the same results.

Table 2 summarizes results of the growth potential experiment for salmonellae in freshly bagged low-VS (VS, <18 g per 100 g of TS; C/N ratio, <15.1) compost incubated at 28°C. No growth was observed; however, aerobic conditions showed a faster die-off rate for salmonellae when compared with the anaerobic treatment. The low initial VS (<18 g per 100 g of TS) did not permit growth to occur, and VS did not decline further as salmonellae die-off occurred. The C/N ratio, however, did decrease as die-off occurred.

Table 3 summarizes the repopulation of salmonellae from the same low-VS compost used in the previous experiment and shown in Table

TABLE 2. *Salmonella growth in low-VS compost under aerobic and anaerobic conditions*

Sample Day	Aerobic Incubation				Anaerobic Incubation			
	TS g/100 g Sample	VS g/100 g Sample	C/N Ratio	Salmonella MPN/g TS	TS g/100 g Sample	VS g/100 g Sample	C/N Ratio	Salmonella MPN/g TS
0	67.2	16.8	15.1	37	67.6	16.9	15.4	37
1	67.1	17.0	15.0	21	67.0	16.5	15.3	89
2	68.4	17.7	14.9	a	67.0	16.5	15.0	60
3	67.5	16.4	14.6	15	67.3	16.3	14.6	96
5	68.9	16.1	14.0	9.4	67.3	14.6	13.9	13
11	70.9	14.3	13.1	<0.14	67.4	11.8	12.8	<0.15

a. Laboratory accident, sample lost.

TABLE 3. *Salmonella repopulation in low-VS compost augmented with VS from sterilized sludge cake under aerobic and anaerobic conditions*

Sample Day	Aerobic Incubation[a]				Anaerobic Incubation[a]			
	TS g/100 g Sample	VS g/100 g Sample	C/N Ratio	Salmonella MPN/g TS	TS g/100 g Sample	VS g/100 g Sample	C/N Ratio	Salmonella MPN/g TS
0	65.1	16.7	18.0	<0.15	65.5	17.4	18.3	<0.15
1	65.1	16.7	16.8	7700	65.5	17.4	17.2	2300
2	63.3	16.7	16.2	1400	63.3	16.8	16.9	2200
3	65.3	16.6	14.9	870	63.6	16.8	15.4	630
5	68.9	18.5	16.3	820	64.1	17.9	15.7	300
10	65.1	15.2	15.0	0.69	65.1	16.4	12.4	<0.15
15	70.4	17.7	14.6	<0.15	68.6	15.4	12.9	<0.15

a. Control Nitrohumus in which sterile PBS was added to boost moisture content showed no salmonelloid regrowth.

2 day 11, after being amended with sterilized fresh anaerobic sludge cake (50% VS). The additional VS from the sterile fresh sludge cake resulted in the rapid repopulation of salmonellae (Table 3). VS was not increased above the starting level demonstrated in Table 2 and, therefore, did not reflect the population die-off shown above. The C/N ratio in this sludge-augmented system was equivalent to that found in the freshly bagged high-VS fertilizer shown in Fig. 2. The repopulation and decline of the salmonellae populations were reflected by the decline of the C/N ratio in this system as it returned to a preamended state.

The salmonellae and closely related species isolated during the freshly bagged compost repopulation experiment summarized in Fig. 2 were identified by the procedures of Edwards and Ewing (4). The species distribution indicated that aerobic incubation produced a lower variety of species than anaerobic incubation. *S. typhi* was not isolated at any time during the procedure; *S. cholerasuis* was the most common isolate of the type genus. *Citrobacter* was the salmonelloid isolated over the longest period of time, and the genus *Arizona* was isolated only under anaerobic incubation conditions.

DISCUSSION

Monitoring of the sanitation districts' composting operation during the previous 5 years has indicated that dewatered, anaerobically digested sludge contains an average of approximately 10^5 salmonellae per g of TS. Composting the sludge reduces the salmonellae population to a level below the detection limits of the test system used (<0.2 MPN per g of TS). Although it was recognized that composting did not sterilize the sludge, it was felt that adequately composted sludges presented little, if any, salmonellae hazard to the user of the resulting fertilizer product. Recent work (2, 3, 5), however, has indicated that salmonellae may grow in composted sludges, and therefore salmonellae repop-

ulation may occur in previously composted material. Salmonellae repopulation in well-composted sludge was slow to occur during this study and was found to be dependent on moisture level, temperature, and nutrient content of the composted solids.

Salmonellae growth was found to occur, in spite of competing coliforms and other bacteria, optimally in the mesophilic temperature range (20 to 40°C) and required a moisture content of ≥20% as reported by Brandon et al. (2) and Brandon and Neuhauser (4). In addition, the VS content served as indicator of growth potential when the concentration was in excess of 18 g of VS per 100 g of TS. A VS content of less than 18 g per 100 g of TS did not preclude growth potential. It was, however, no longer a dependable indicator of salmonellae repopulation. A more consistent indicator of salmonellae growth potential was found in the C/N ratio. When this ratio, as determined from the chemical oxygen demand and Kjeldahl nitrogen, was approximately 15:1 or less, repopulation did not occur. It is felt that VS represents the immediately available source of nutrient for the cells present, but the C/N ratio serves as the long-term nutritional indicator of salmonellae repopulation potential, provided carbon is the rate-limiting factor.

Both aerobic and anaerobic incubation conditions showed equal growth potential. The aerobic system, however, was clearly more efficient in VS destruction, C/N reduction, and salmonellae die-off than the anaerobic system. The distribution of isolates for each system was different as well. The anaerobic system showed the greatest variety of salmonellae for the longest duration. The organisms present continued the composting process until nutrients were no longer able to maintain cell viability.

Although salmonellae repopulation was demonstrated to occur in thoroughly composted sludges, the effect was transient, with population peaks occurring around 5 days followed by subsequent die-off. It should be noted, however, that the moisture-nutrient variables manipulated during this study were controlled within a fairly tight range which would be typical for a well-composted sludge. Poorly or partially composted sludges, under the proper conditions, may exhibit a much higher salmonellae growth peak and maintain the level for a longer period.

Nevertheless, as long as a demonstrated potential exists for repopulation of salmonellae in a commercial soil amendment product produced from composted sludge, a potential health hazard exists for the user. The data from this project suggest that salmonellae repopulation can be minimized or eliminated by using VS and C/N ratios as the determining factors for assessing the adequacy of composting. Appropriate storage or stockpiling of the material after composting may be required to achieve the necessary levels.

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