APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1980, p. 118-126
0099-2240/80/01-0118/09$02.00/0
Vol. 39, No. 1

Enumeration of Potentially Pathogenic Bacteria from Sewage Sludges

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To ascertain the health risks that may be posed by the land application of sewage sludges, a scheme was devised to determine the types and numbers of pathogenic and potentially pathogenic bacteria present in sludges. A processing treatment was adapted to sludge to give a homogenate which yielded the greatest numbers of viable bacteria. Conventional methods were successful in enumerating *Klebsiella*, *Staphylococcus*, gram-negative enteric bacteria, and commonly used indicator organisms. Modifications of conventional methods improved the enumeration of *Salmonella*, *Mycobacterium* sp., fluorescent *Pseudomonas* sp., and *Clostridium perfringens*. However, *Shigella* methodology yielded only one isolate. Utilizing the proposed scheme, the population densities of these organisms were estimated in three domestic wastewater sludges. In light of these results, the potential impact of land application of sewage sludges is discussed.

The paucity of quantitative data concerning the content of pathogenic and potentially pathogenic bacteria in sewage sludge has been cited by Carrington (4) as being a major obstacle in determining the impact pathogens from sludges may have on the environment. Such sludges could pose a significant health risk through the contamination of vegetables, surface waters, and groundwaters.

Hess and Breer (18) found *Salmonella* species in 90% of the sludges they examined and observed that the organisms could survive for up to 72 weeks in sludges that had been applied to land. Furthermore, they reported that neither aerobic nor anaerobic digestion significantly reduced the contamination of sludge with *Salmonella*. McKinney and co-workers (27) reported that the survival of *Salmonella* in sludge in seeded bench-scale digesters was dependent on the density of the initial population, available nutrients, and detention time. Foliguet and Doncoeur (11) have urged the routine disinfection of digested sludges because retention times and temperatures in field digesters varied so that consistent killing of *Salmonella* was not obtained.

Mycobacterium tuberculosis was found by Jensen (21) in the sewage and digested sludge of towns with tuberculosis sanitoria. The tubercle bacilli were found to survive for as long as 11 to 15 months in sludge on drying beds, but were killed readily by chlorination. Pramer et al. (30) reported finding organisms resembling *M. tuberculosis* at levels approaching 10^5/ml in the raw sludge and 10^4/ml in digested sludge from a sanitorium. Heukelekian and Albanese (19) later made similar observations.

Greer (15) observed densities of up to 10^7 *Clostridium perfringens* organisms per g in sludge, but virtually none in the wastewater effluent. He concluded that *C. perfringens* was concentrated into the sludge. Bonde (3) suggested that *C. perfringens* be used as a routine indicator organism in the examination of both wastewater and sludge.

Other bacteria that may play a role in human disease have been detected in sewage and treated effluents. Drake (7), Lanyi et al. (24), Dutka and Kwan (9), and Guentzel (16) found *Pseudomonas* in sewage or moderately polluted waters. However, none of those investigators attempted to isolate *Pseudomonas* from sludge. Difficulties have been reported in isolating *Shigella* from sewage (35) and stabilization ponds (6). Some researchers (35), however, have had success in recovering *Shigella* from wastewaters. Other organisms that have been isolated from wastewaters and treated effluents include large numbers of *Klebsiella pneumoniae* and low levels of *Staphylococcus aureus* (16). Since these organisms occur in sewage, they also may be found in sludge. Their overt and opportunistic pathogenicity make it important to quantify them to assess any health risk that sludge handling may pose.

Comprehensive bacteriological screens have been developed to enumerate potential pathogens from sewage and sewage effluents by Gra-

bow (13) and Guentzel (16). A project to develop methodology for bacteriological screening of sewage sludges for pathogenic and potentially pathogenic microorganisms was initiated, using the scheme used by Guentzel (16) as a basis. This paper presents methodologies that can provide quantitative data on the population densities in sludge of fluorescent *Pseudomonas* species, *Staphylococcus*, *Mycobacterium*, *Clostridium*, and *Klebsiella*, and semiquantitative data on *Shigella* and *Salmonella*. *Enterobacteriaceae* and oxidase-positive enteric organisms were quantitated using a multi-media rapid identification system. Determinations of routine indicator organisms (total coliforms, fecal coliforms, fecal streptococci, and total aerobic colony counts) also were performed on the sludges for the purpose of comparison with published studies on wastewater.

MATERIALS AND METHODS

Source of samples. Sludges utilized during the methods development phase of this study were obtained from the Rilling Road Regional Wastewater Treatment Plant in San Antonio, Texas, as either primary or anaerobically digested sludges. Additionally, wasted secondary sludge samples generated by an activated sludge treatment process were collected at the Kerrville, Texas, Wastewater Treatment Plant. Samples were held for at most 24 h at 4°C before being processed.

Bacterial surveys were performed on sludges undergoing land application at sites in the continental United States. Representative samples were collected, cooled to approximately 4°C, and shipped cold in insulated containers by air to the laboratory, where testing was initiated within 24 h of sample collection.

Bacteriological media. Commercially available dehydrated media used in this study were manufactured by Difco Laboratories, except where noted. Bonde medium was made according to the published formulation (3). Differential reinforced clostridia medium (DRCM) used during this study was a modification of that suggested by Gibbs and Freame (12) (Table 1), and the formulation for sulfite-polymyxin B-sulfadiazine medium suggested by Angelotti et al. (2) was used.

Indicator organisms. Total aerobic colony counts as defined by the standard plate count were determined as described in *Standard Methods for Examination of Water and Wastewater* (1), using processed sludge samples diluted in phosphate-buffered saline (pH 7.3 ± 0.1).

Coliforms in sludge were enumerated by direct plating on appropriate selective media. Samples were diluted serially in sterile phosphate-buffered saline, and 0.1-ml samples were spread with sterile glass L-rods over each of three replicate plates. Total coliforms were assayed on m-Endo agar LES, fecal coliforms were assayed on m-FC agar, and fecal streptococci were determined on m-Enterococcus agar. Incubation was at 35°C for 24 h for total coliforms and 35°C for 48 h for fecal streptococci. Fecal coliform plates were incubated at 35°C for 4 to 5 h and then transferred to 44.5°C for an additional 20 h.

Staphylococcus. Appropriate dilutions were spread onto mannitol salt agar plates, which were incubated at 37°C for 24 to 48 h. Colonies showing a typical yellow zone of fermentation were isolated for Gram staining. Those colonies identified as being gram-positive cocci were counted as staphylococci.

Klebsiella. Appropriate dilutions were spread-plated in triplicate on eosin-methylene blue agar. After incubation at 37°C for 24 to 48 h, mucoid colonies were transferred to heart infusion agar slants. After 24 h of incubation, oxidase-negative isolates were inoculated to triple sugar iron agar and motility indole ornithine medium. Determinations of *Klebsiella* were based on typical reactions in these biochemical tests (25).

Pseudomonas. To determine fluorescent *Pseudomonas* levels in sludge, appropriate dilutions were spread-plated in triplicate on cetrimide agar. Plates were incubated at 37°C for 24 h, then exposed to fluorescent light for 18 to 24 h to enhance pigmentation. Colony counts were made under long-wave ultraviolet light in an Ultra-Violet Products, Inc. (San Gabriel, Calif.) Chromato Vue Box (model CC-20).

Shigella. Enrichment procedures were deemed necessary for detection of *Shigella* species, so only semiquantitative enumeration by range was accomplished. Samples (25, 10, 1, and 0.1 ml) of processed sludge were inoculated into separate volumes of GN enrichment broth to yield a final volume of 100 ml and were incubated at 37°C for 18 to 24 h. Serial dilutions were prepared from each enrichment and spread-plated on xylose lysine deoxycholate agar (BBL Microbiology Systems, Cockeysville, Md.). The plates were incubated at 37°C for 24 h. Isolated suspect colonies (red or colorless) were transferred to heart

TABLE 1. *Modified DRCM medium*[a]

Basal medium	Amt for 1 liter
Peptone	10 g
Beef extract	12 g
Sodium acetate	5 g
Yeast extract	1.5 g
Soluble starch	1 g
Glucose	1 g
5% solution of L-cysteine	10 ml
Distilled water	990 ml

[a] Preparation:

1. Add peptone, beef extract, sodium acetate, yeast extract, and starch to water. Boil to dissolve.
2. Add glucose and adjust pH to 7.1 to 7.2 with 10 N NaOH.
3. Filter while hot through Whatman filter paper, then autoclave at 121°C for 15 min.
4. Add the L-cysteine and dispense aseptically in 10-ml volumes to sterile screw-cap test tubes.
5. On the day the medium is to be used, steam the tubes in the autoclave for not more than 3 min. Add 0.1 ml of a 4% solution of sodium sulfite and a 7% solution of ferric citrate to each tube.

infusion agar slants; after 24 h of growth, oxidase-negative isolates were transferred to triple sugar iron and motility indole ornithine media. Isolates displaying biochemical reactions characteristic of *Shigella* (25) were confirmed using commercially available antisera (Difco).

Salmonella. Because of the high solids content of sludge and the anticipated low densities of *Salmonella*, a semiquantitative enrichment procedure was selected for use. As suggested by Dutka and Bell (8), a variety of enrichment broth-plating agar combinations were examined to determine the combination that yielded the highest level of *Salmonella*. Tetrathionate–salmonella-shigella agar, selenite–salmonella-shigella agar, tetrathionate-brilliant green agar, and selenite-brilliant green agar combinations were compared, and the selenite-brilliant green agar combination was selected. Spino (32), Harvey and Price (17), Cheng et al. (5), and Yoshpe-Purer et al. (37), among others, have suggested an elevated temperature for the isolation and enumeration of *Salmonella* from polluted waters. These investigators preferred to incubate inoculated enrichment and plating media at 42°C. However, the low levels of *Salmonella* indigenous to sludge, possible environmental damage to stressed organisms, and the inhibitory nature of the enrichment medium suggested that a 37°C incubation temperature would facilitate the detection of these organisms. For these reasons, 37°C was used to enhance growth of *Salmonella* in enrichment, followed by 42°C incubation upon transfer to brilliant green agar.

Sludge samples of 10, 1, 0.1, and 0.01 ml were inoculated into separate volumes of selenite broth to yield a final volume at 100 ml, then were incubated at 37°C for 24 h. Subsequently, serial dilutions were prepared from each enrichment culture and plated onto brilliant green agar plates. After incubation at 42°C for 24 h, suspect colonies (pink-colored) were subcultured to heart infusion agar slants. After one day of growth on heart infusion agar, oxidase-negative isolates were inoculated into triple sugar iron and lysine iron agar media. Those isolates showing biochemical reactions characteristic of *Salmonella* (25) were confirmed serologically using commercially available antisera (Difco). Using this procedure, semiquantitative estimations of *Salmonella* levels could be determined in a minimal amount of time.

Mycobacterium. Guentzel (16), using benzalkonium chloride at a concentration of 500 μg/ml, treated sewage for 30 min before plating it onto Middlebrooks 7H11 agar supplemented with oleic-albumin-dextrose-catalase enrichment. Plates were examined over a 4-week period of incubation at 37°C in a 5% CO_2-in-air atmosphere. Suspect colonies were tested for acid-fastness using the Ziehl-Neelsen stain and were then subcultured onto Löwenstein-Jensen tubed media and biochemically tested. This procedure gave excellent recovery of many *Mycobacterium* species, while suppressing most sewage saprophytes.

A modification of this procedure was found to be more suitable for sludges. Nine milliliters of processed sludge was mixed in a Vortex blender with 1 ml of diluted Zephiran chloride concentrate to give a final concentration of 1,600 μg of benzalkonium chloride per ml. After a treatment period of 35 to 40 min at room temperature (24 to 25°C), the mixture was diluted, and samples were spread-plated onto Middlebrooks 7H11 agar with oleic-albumin-dextrose-catalase enrichment, modified by the addition of 3 μg of amphotericin B (Fungizone) per ml. Plates were incubated at 37°C in a 5% CO_2-in-air atmosphere and observed over a period of 4 weeks for growth. Isolated colonies were stained and observed for acid-fast bacilli. Additionally, all nonchromogenic colonies were subcultured to tubes of Löwenstein-Jensen medium to be tested for niacin production, a distinguishing characteristic of *M. tuberculosis*.

Gram-negative enteric bacteria. To quantitate oxidase-positive and oxidase-negative gram-negative enteric bacteria, processed sludge samples were diluted serially in phosphate-buffered saline and plated in triplicate on both MacConkey and xylose lysine deoxycholate agars. After incubation at 37°C for 24 h, colonies were isolated from both media at that dilution which had approximately 200 discrete colonies over the six plates. These colonies were streaked to quadrants of MacConkey agar for growth and confirmation of purity.

Subsequent identification involved oxidase testing by the Kovacs procedure (25) and the use of a commercial identification system, the API 20E system marketed by Analytab Products Inc. (Plainview, N.Y.). Each strip contains a preset battery of 20 microtubes which gives the results of 22 biochemical tests for the identification of 49 species of *Enterobacteriaceae* and 38 groups or species of gram-negative bacteria.

RESULTS

Sludge processing. The procedures compared included: (i) Vortex mixing at high speed for 2 min of 20 ml of sludge containing approximately 1 g of sterile 3-mm glass beads in a 50-ml centrifuge tube; (ii) homogenization at high speed in a Waring blender for 10 s or 3 min; (iii) sonication at various wattage outputs (58, 66, and 76.5 W) using a Branson Sonifier model 200; and (iv) direct plating without treatment. As a part of each processing test, serial dilutions were prepared in sterile phosphate-buffered saline and plated to compare the maximal yield of total aerobic colonies, fecal coliforms, and fecal streptococci.

The results are presented in Table 2. Sonication at all wattage levels was found to be bactericidal. Sludge sonicated at an output of 66 W yielded the highest recoveries from sludges that were sonicated. The best recovery of viable organisms was obtained in samples dispersed by Vortex mixing with glass beads. Therefore, this method was selected as the sludge handling procedure before any bacterial analyses on subsequent sludge samples.

Routine indicator organisms. Total coliforms, fecal coliforms, and fecal streptococci

were enumerated in Vortex-blended sludge samples by using three different methodologies: (i) completed multiple-tube fermentation (1); (ii) membrane filtration (1); and (iii) spread-plating directly onto appropriate selective media.

The results of representative studies are shown in Table 3. For total coliforms and fecal streptococci, direct plating was the superior method. However, the most-probable-number (MPN) method yielded higher fecal coliform recoveries. It was considered likely that the recovery of fecal coliforms by direct plating would have been enhanced had the plates been incubated at 35°C for 4 to 5 h before incubation at 44.5°C (14); therefore, subsequent fecal coliform assays were done in this manner.

Decreased analysis time was a significant advantage of spread-plating over the multiple-tube fermentation technique. Because of the handling advantage and better average recoveries, direct plating was chosen as the method to be used when enumerating the routine indicator organisms.

C. perfringens. Four media were evaluated for the recovery of *C. perfringens*. Litmus milk, used by Greer (15) (modified by addition of 0.1% reduced iron), and DRCM (modified) were employed in MPN procedures. Sulfite-polymyxin B-sulfadiazine agar and Bonde medium (3) were compared in an evaluation of available plating media. After processing, the sludge sample was divided into two aliquots. One portion was used for the enumeration of vegetative *C. perfringens* by direct inoculation to the medium from appropriate dilutions, and the other aliquot was heat-shocked at 80°C for 30 min and then inoculated to enumerate spores of *C. perfringens*. For litmus milk and DRCM, a diluted sludge sample

TABLE 2. *Effect of sludge treatment on the recovery of indigenous bacteria*[a]

Sample source	Treatment	Fraction surviving (% of direct plating)			
		Total aerobic colonies	Fecal coliforms	Fecal streptococci	Aerobic count on EMB[b]
Secondary sludge	Sonication (66 W):				
	1 min	12	42		
	5 min	6.2	4.7	NT	NT
	10 min	2.8	1.4		
	Blender homogenization (3 min)	54	43	NT	NT
Secondary sludge	Vortex mixing (2 min)	163	200	150	151
	Blender homogenization (10 s)	66	109	85	100
	Blender homogenization (3 min)	146	91	120	119
Digested sludge	Vortex mixing (2 min)	242	154	87	88
	Blender homogenization (3 min)	158	100	103	95

[a] After each processing trial, sample dilutions were plated in triplicate onto the different media. The resulting density of bacteria, determined by direct plating with no processing treatment, was designated arbitrarily as 100%. NT, Not tested.

[b] Plate count on eosin-methylene blue (EMB) agar, 37°C for 24 h.

TABLE 3. *Enumeration of indicator organisms—methodology comparison*

Methodology	Total coliforms in sample:			Fecal coliforms in sample:			Fecal streptococci in sample:		
	1	2	3	1	2	3	1	2	3
Multiple-tube fermentation (MPN/100 ml)									
Confirmed	7.9×10^7	2.4×10^8	1.3×10^8	5.4×10^7	1.3×10^8	3.4×10^7	9.4×10^4	1.4×10^7	2.8×10^5
Completed	1.3×10^8	3.5×10^8	2.4×10^8	9.2×10^7	2.4×10^8	1.3×10^8	1.4×10^5	1.4×10^7	2.8×10^5
Membrane filtration (CFU/100 ml)[a]	1.8×10^8	1.5×10^8	2.6×10^8	4.6×10^7	3.8×10^7	5.3×10^7	4.5×10^6	3.0×10^6	5.2×10^6
Direct plating (CFU/100 ml)[b]	2.9×10^8	3.5×10^8	4.2×10^8	5.0×10^7	4.2×10^7	7.7×10^7	4.3×10^8	3.0×10^6	8.8×10^6

[a] CFU/100 ml, Colony-forming units per 100 ml of sludge. Utilizing Gelman GN-6 0.45-μm membranes.

[b] Processed sample was spread with sterile glass L-rods on plates (100 by 15 mm) containing appropriate selective media.

was inoculated according to usual MPN procedures over a range of five decimal dilutions, using five tubes per dilution, and incubated for 3 days at 37°C. For the agar media, a standard pour plate was employed, with triplicate plating for each dilution followed by incubation at 37°C for 3 days. Anaerobiosis of solid media was obtained by covering the agar surface with an ultraviolet-sterilized square of cellophane.

Confirmatory tests were performed to assess the efficiency of test media in recovering clostridia. Wet mounts and Gram stains were prepared from each positive tube of litmus milk and DRCM and were observed for the presence of nonmotile, encapsulated, gram-negative rods. In addition, from each tube of DRCM, one loopful of growth was transferred to fresh litmus milk and examined after 1 and 3 days of incubation at 37°C for stormy fermentation. Selected colonies from the agar media were examined in the same manner. Table 4 shows representative results of these studies. No recoveries of *C. perfringens* were made from Bonde medium, whereas all other media yielded positive isolations of the organism.

Selection of a routine procedure involved considerations of handling ease and selectivity, as well as reproducibility of results. The use of pour-plating media necessitated special handling to achieve anaerobiosis, and confirmation from the agar media was more difficult because colonies were embedded in the agar. In addition, fungal overgrowth proved to be a problem with the solid media. Litmus milk had several disadvantages, including extensive handling time in confirmation and equivocal results based on relatively subjective judgment in determining stormy fermentation. DRCM was judged to be the best medium of those tested in the quantitative recovery of *C. perfringens* from sludge. The high confirmation rates (92% in this study), rapid confirmation, unequivocal results (positive or negative), and the use of aerobic incubators were among the advantages. This experience is consistent with the report of Gibbs and Freame (12), who noted that the DRCM medium was sensitive for *C. perfringens* while maintaining selectivity against most other sulfite-reducing organisms.

The final procedure was similar to that recommended by the World Health Organization (36) for drinking water. Samples of untreated and heat-shocked sludge were inoculated into two different sets of a five-decimal dilution series, three tubes per dilution. After 3 days of incubation at 37°C, a loopful of medium from each tube was transferred to a corresponding tube of fresh, sterile litmus milk. Growth of *C. perfringens* in DRCM was confirmed when the litmus milk exhibited stormy fermentation after 1 to 3 days of incubation at 37°C. Quantitative determinations of *C. perfringens* levels were made using MPN tables (1).

Application to field samples. Figure 1 is a schematic diagram of the general methodology used to isolate selected bacterial populations from a variety of sewage sludges. Raw primary, wasted activated, digested, and lagooned sludges ranging in solids content from 22,000 to 50,000 mg/liter were examined, and their bacterial populations were quantitated. Table 5 shows the levels of microorganisms isolated from digested sewage sludges obtained from three operating sewage treatment plants in the southern United States.

In the presence of high densities of aerobic and facultatively anaerobic organisms as determined by the total aerobic count, the proposed scheme was successful in enumerating densities of other component populations whose levels may be as high as the aerobic count. For example, the *Mycobacterium* densities from site 2 equaled the total aerobic count for that sludge, and the vegetative *C. perfringens* levels in sludge from site 3 approached the total aerobic counts. The *Mycobacterium* levels at the other two sites

TABLE 4. *Recovery of clostridia from sludge*

Sample	Test	Litmus milk (MPN/100 ml)		DRCM (MPN/100 ml)		SPS agar (CFU/100 ml)[a]	
		25°C	80°C	25°C	80°C	25°C	80°C
Secondary sludge	Presumptive	2.4×10^8	2.4×10^8	2.4×10^8	1.6×10^8	NT	
	Confirmative	2.4×10^8	2.4×10^8	2.4×10^8	1.6×10^8	NT	
Secondary sludge	Presumptive	2.6×10^6	5.4×10^7	3.4×10^6	1.6×10^7	4.8×10^6	9.1×10^6
	Confirmative	1.7×10^6	5.4×10^7	3.4×10^6	1.6×10^7	*	*
Digested sludge	Presumptive	2.4×10^8	2.4×10^8	2.4×10^8	1.6×10^8	NT	
	Confirmative	2.4×10^8	2.4×10^8	2.4×10^8	1.6×10^8	NT	
Digested sludge	Presumptive	1.7×10^8	2.6×10^6	1.1×10^7	7.0×10^5	1.6×10^6	4.3×10^5
	Confirmative	3.4×10^7	2.6×10^6	1.5×10^6	7.0×10^5	*	*

[a] CFU/100 ml, Colony-forming units per 100 ml of sludge. NT, Not tested. *, 12 to 15 colonies confirmed from each sample.

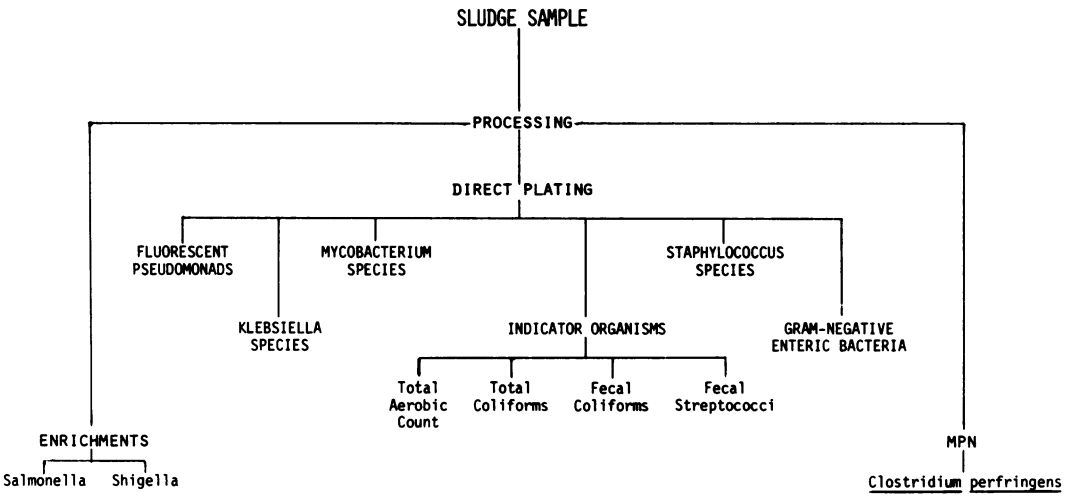

FIG. 1. *Schematic diagram of sludge processing procedures for the enumeration of selected organisms. Procedural details are described in the text.*

were identical, albeit at levels two decimal dilutions lower than that of site 2. The sporulated *C. perfringens* densities were one decimal dilution lower than vegetative *C. perfringens* in two of the three sludges. However, in the other sludge, sporulated counts equaled vegetative counts, demonstrating the unique nature of each sludge. In addition, organisms isolated as *Klebsiella* on eosin-methylene blue agar were quantitated at higher densities than were fecal coliforms in two of the three sludges, perhaps indicating the higher sensitivity of the *Klebsiella* isolation procedure or the better survival of the organism. The sludge from site 2 contained higher densities of virtually every genus of organisms isolated, and yielded a wider variety and higher quantities of gram-negative enteric bacteria.

Salmonella was easily detected and rapidly estimated semi-quantitatively in each sludge, whereas *Staphylococcus* and *Shigella* were isolated from only one of the three sludges reported. The modified methods used to enumerate *Mycobacterium*, *C. perfringens*, fluorescent pseudomonads, and *Salmonella* successfully quantitated their population densities in sludge. The methods utilized to isolate *Klebsiella* and the indicator organisms also worked well.

DISCUSSION

We are aware of no previous comprehensive bacteriological screens for overt and opportunistic pathogens recoverable from sewage sludge. Smith et al. (31) examined aerobically digested sludge for fecal coliforms, fecal streptococci, total aerobic count, *Salmonella* species, and *Pseudomonas* species. They found that aerobic treatment of sludge at 56°C resulted in significant reduction of these bacterial populations. In a comprehensive examination of piggery-waste digester sludge for the functional organisms involved in sludge digestion, Hobson and Shaw (20) also found *Escherichia*, *Streptococcus*, *Clostridium*, and *Bacteroides*. Matthews et al. (26) examined dairy effluent sludge for mycobacteria, and found that 42% (27 out of 63) of the sludge samples examined contained mycobacteria, from which 32 different strains were isolated. However, none was pathogenic for experimental animals. Others (11, 18, 22, 27) have reported only on the presence or fate of *Salmonella* in digested sludge. Carrington (4), in an extensive review of the literature, lists *Salmonella*, *Shigella*, *Escherichia coli*, *Clostridium*, and *M. tuberculosis* as major pathogens that may be recoverable from sludges. Most of the studies reviewed were concerned with only one specific pathogen or with groups of indicators. The present study is the first attempt to screen sludges for these organisms, as well as for a variety of opportunistically pathogenic organisms and indicator organisms, simultaneously. Whereas the methodology described in this report may not provide for absolute quantification of every organism that may be present in sewage sludge, it does enumerate a wide variety of enteric organisms recoverable from sludge.

The high solids content and high levels of saprophytic bacteria and fungi associated with a sludge sample necessitated that selective media and techniques be used in the enumeration of certain microorganisms. Nevertheless, where

TABLE 5. *Enumeration of bacteria in sludges undergoing land application*[a]

Organism	Enumeration (CFU/g)[b]		
	Site 1 (digested sludge)	Site 2 (lagooned sludge)	Site 3 (digested sludge)
Total aerobic count	4.8×10^8	2.2×10^9	1.2×10^8
Total coliforms	4.5×10^6	6.1×10^7	5.1×10^6
Fecal coliforms	4.8×10^5	4.7×10^6	3.1×10^6
Fecal streptococci	2.4×10^5	4.5×10^5	6.9×10^4
Fluorescent pseudomonads	6.4×10^4	7.1×10^4	2.0×10^4
Staphylococcus sp.	$<7.9 \times 10^4$ (ND)	1.2×10^6	$<6.7 \times 10^4$ (ND)
Clostridium perfringens (MPN/g)			
Vegetative (room temp)	2.6×10^7	2.2×10^7	3.1×10^7
Sporulated (80°C)	2.6×10^7	4.7×10^6	4.9×10^6
Mycobacterium sp.	4.0×10^7	2.4×10^9	4.1×10^7
Salmonella sp.	≥2.4–<24	>2.0	≥2.0
Shigella sp.	ND	ND	≥20
Klebsiella sp.	8.8×10^6	1.2×10^7	4.7×10^5
Enterobacteriaceae[c]			
Citrobacter diversus subsp. Levinae	—	—	1.0×10^6
C. freundii	7.1×10^5	5.9×10^6	6.1×10^5
Enterobacter aerogenes	—	2.0×10^6	—
E. agglomerans	2.4×10^5	4.9×10^6	—
E. cloacae	1.6×10^5	1.6×10^7	1.0×10^6
E. sakazakii	—	9.8×10^5	2.0×10^5
Escherichia coli	1.7×10^6	8.8×10^6	2.0×10^5
Hafnia alvei	—	—	8.0×10^6
Klebsiella oxytoca	6.2×10^5	9.8×10^5	6.1×10^5
K. ozaenae	7.9×10^4	9.8×10^5	2.7×10^6
K. pneumoniae	1.6×10^5	2.0×10^6	6.1×10^5
Proteus morganii	—	—	2.0×10^5
Serratia liquefaciens	7.9×10^4	3.9×10^6	—
S. marcescens	—	9.8×10^5	—
S. rubidaea	—	2.0×10^6	—
Yersinia enterocolitica	—	—	2.0×10^5
Y. ruckeri	—	—	1.0×10^6
Oxidase-positive, gram-negative enteric bacteria[c]			
Achromobacter sp.	—	—	2.0×10^5
A. xylosoxidans	—	—	6.1×10^5
Acinetobacter calcoaceticus var. *lwoffi*	—	2.0×10^6	—
Aeromonas hydrophila	2.9×10^6	5.3×10^7	2.0×10^6
Alcaligenes sp.	7.9×10^4	—	2.0×10^5
Bordetella bronchiseptica	—	—	2.0×10^5
CDC Group V E-1	7.9×10^4	—	2.0×10^5
Flavobacterium odoratum	1.6×10^5	—	—
Pseudomonas aeruginosa	7.9×10^4	—	—
P. cepacia	5.5×10^5	9.8×10^5	—
P. fluorescens	3.1×10^5	9.8×10^6	—
P. maltophilia	—	9.8×10^5	—
P. paucimobilis	—	9.8×10^5	—
P. putida	6.2×10^5	1.3×10^7	4.7×10^6
P. putrefaciens	—	9.8×10^5	—
P. stutzeri	3.1×10^5	9.8×10^5	—
Vibrio alginolyticus	—	9.8×10^5	2.0×10^5
Total suspended solids (mg/liter)	41,900	50,700	49,100

[a] Sludge from site 1 had undergone digestion in a two-stage high-rate anaerobic digestion system. Sludge from site 2 was lagooned after undergoing anaerobic digestion for 11 days. Sludge from site 3 had undergone standard anaerobic digestion for 20 to 30 days with no mixing or heating.

[b] CFU/g, Colony-forming units per gram of total suspended solids, unless indicated otherwise. ND, None detected. —, Not detected.

[c] Highest numbers were recovered off either MacConkey or XLD agar. Approximately 200 colonies (100 from each agar) were isolated from appropriate dilutions of each sludge and inoculated to API 20E strips.

the level of a selected bacterial species was less than approximately 100 per ml of sludge, the direct-plating technique may have failed to detect the organism sought due to the low probability of recognizing these bacteria against background microbial growth.

The sensitivity of enrichment procedures may have been similarly limited. For example, only one *Shigella* organism was recovered from the attempted *Shigella* isolations. Wang et al. (35) found that temperature was a vital factor in determining the probability of *Shigella* survival in sewage treatment, with higher temperatures decreasing survival. The recovery of only a single isolate from digested sludge is therefore not surprising.

The entire isolation and enumeration scheme was designed to handle sludge samples from field sites on a routine basis. In enumerating *Salmonella*, Phirke (29) and Kenner and Clark (23) suggested using extensive MPN procedures with a series of three or five tubes per dilution, five decimal dilutions per sample. Confirmation required conventional identification of *Salmonella* from each tube. Cheng et al. (5) state that the wide confidence limits inherent to MPN determinations are exacerbated in *Salmonella* MPN determinations. Because of this disadvantage and the time required to carry out such procedures, population determination by range from enrichments was employed in enumerating *Salmonella*.

The use of the API 20E Identification System broadened the scope of the bacteriological examination. The system identified the environmental isolates and provided estimates of a large and diverse number of species of organisms, including the *Enterobacteriaceae* and gram-negative, oxidase-positive enteric bacteria. The use of the API 20E system enlarged the scope of the study to a comprehensive survey capable of identifying and enumerating many pathogenic and potentially pathogenic bacteria likely to be found in domestic wastewater sludges.

Van der Drift et al. (34) reported that the removal of bacteria from wastewater was a biphasic process. Initially, the organisms are sorbed rapidly to the solids biomass, followed by predation on the organisms by ciliated protozoa. Moore et al. (28) have shown that enteric viruses are also transferred into the secondary biomass. Organisms thus removed from raw sewage by secondary wastewater treatment are recoverable from wasted sludges. Subsequent sludge treatment and handling must consider the viability of these microorganisms, because they are introduced into the soil environment by land application.

Edmonds (10) has shown that conventional indicators of fecal pollution can be recovered for several months from sites where dewatered anaerobically digested sludge has been applied. Hess and Breer (18) recovered viable *Salmonella* in sewage sludge 72 weeks after land application. Salmonellae were recovered from 23 of 103 randomly selected vegetable samples by Tamminga et al. (33). Stormwater runoff from application sites and direct contact with sludge also pose potential health hazards (10). The data presented here indicate that organisms with greater survival potential, including mycobacteria, *Klebsiella pneumoniae*, and clostridia, also were present in high densities in sludge.

The growing practice of disposal to land of these pathogen-containing materials should be coupled with land use limitations for such sites. Pathogens removed through wastewater treatment should not be reintroduced into a population via new reservoirs that may be established by irresponsible land management of application sites. Acreage set aside for food crops that undergo heat processing, for fiber crops, or for forest products can be utilized reasonably for land disposal. However, other agricultural and recreational lands should not be utilized as application sites for primary and digested sludge disposal unless the residual has been further treated by composting, irradiation, or pasteurization.

ACKNOWLEDGMENTS

We thank Charles A. Sorber for his guidance and advice, H. Jac Harding and John B. Moring, Jr., for their technical assistance, and Helen Ramirez and Dorothy Baskin for preparation of the manuscript.

This work was funded by the U.S. Environmental Protection Agency through Southwest Research Institute, San Antonio, Tex.

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