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A Modified Scheme for the Isolation and Enumeration of Bacteria in Municipal Sewage Sludge

Kelly Ball

A MODIFIED SCHEME FOR THE ISOLATION AND ENUMERATION OF BACTERIA IN MUNICIPAL SEWAGE SLUDGE

A Thesis

Presented to

the Faculty of the Department of Biology

Western Kentucky University

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Kelly Kozette Ball

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A MODIFIED SCHEME FOR THE ISOLATION AND ENUMERATION OF BACTERIA IN MUNICIPAL SEWAGE SLUDGE

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A MODIFIED SCHEME FOR THE ISOLATION AND ENUMERATION OF BACTERIA IN MUNICIPAL SEWAGE SLUDGE

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Directed by: L. P. Elliott, S. Ford, and M. R. Houston
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Because of the potential health hazards associated with the use of sludge for agricultural purposes, Dudley et al. (1980) published a scheme for the routine analysis of bacteria in municipal sewage sludge. In this study, the Dudley et al. scheme (1980) was modified by updating some of the procedures. Aerobically digested sludge generated by the Bowling Green Wastewater Treatment Plant, Bowling Green, Kentucky, was analyzed using the modified scheme. Sludge samples were collected once every two months over a one-year period from October 1989 to August 1990.

Egg yolk-free cycloserine sulfite agar in conjunction with the reverse CAMP test was used to assay for Clostridium perfringens. This procedure improved the one proposed by Dudley et al. (1980) by achieving a higher confirmation rate, reducing testing time, allowing for easier interpretation of results, and increasing accuracy.

Selective and differential media by Rippey and Cabelli (1979) were added to the scheme to isolate Aeromonas. Aeromonas hydrophila and Aeromonas caviae were successfully isolated and were identified using the system by Cunliffe and Adcock (1989) for speciating aeromonads.

Baird-Parker medium was compared to mannitol salt agar for effectiveness in isolating Staphylococcus from sludge. Statistical analysis showed Baird-Parker medium to be significantly more effective than mannitol salt agar. However, neither agar reduced background flora to acceptable levels. Staphylococcus isolates were subject to species identification by the API Staph Ident system (Analytab Products, Plainview, New York). Staphylococcus xylosus, Staphylococcus haemolyticus, and Staphylococcus epidermidis were found to be present in the sludge.

A procedure by Ottolenghi and Hamparian (1987) was employed to isolate Salmonella in sludge. No salmonellae were isolated over the one year period.

Over the year-long study, bacterial numbers, with the exception of Clostridium perfringens and the total aerobic count, fluctuated with variations in the aerobic digester temperature. Numbers decreased as temperature increased. Clostridium perfringens counts were the most consistent throughout the year and exceeded fecal coliform and fecal streptococci counts in five of the six samplings.

INTRODUCTION

In 1972, the Federal Water Pollution Control Act, now known as the Clean Water Act, was enacted to eliminate the discharge of pollutants into surface waters. One requirement of the Act stated that all wastewater must be treated before it can be reintroduced into waterways to reduce detrimental effects on the environment (Federal Register, Vol. 54, no. 23, February 6, 1989, p. 5746).

As a result, wastewater treatment plants in the United States have been upgraded to "secondary treatment" plants (Jakubowski, 1986). Wastewater is treated in three separate phases at a secondary wastewater treatment plant (Vesiland, 1979). In phase one, incoming wastewater is pretreated by allowing it to pass through large screens or bar racks to remove substantial objects such as rags, paper, and wood. Phase two, the primary treatment phase, routes the pretreated influent into tanks called "primary clarifiers" or "settling tanks." Here the heavier solids settle to the bottom for collection and the lighter solids float to the top forming a removable scum. Phase three, the secondary treatment phase, consists of biochemical oxidation of the wastewater usually involving some type of biological treatment such as activated sludge systems, trickling filters or other attached growth systems (Vimal and Talashilkar, 1985). These processes use microbes to decompose up to 90% of the remaining complex organic substances into lower molecular weight organic molecules, water, and carbon dioxide (Vesiland, 1979). After secondary treatment, the water is usually chlorinated and released back into the waterways.

During the treatment phases, a substance referred to as sludge is generated. According to the Environmental Protection Agency (EPA), sludge is any solid, semisolid, or liquid waste generated from wastewater treatment (Federal Register, Vol 54, no. 23, February 6, 1989, p. 5878). Most wastewater treatment plants pump the sludge into large digester tanks to stabilize it. Stabilization involves reducing the water content, volatile solids, and numbers of microorganisms in the sludge. Some treatment plants operate anaerobic digesters which use anaerobic microbes to break down organic matter in the sludge by means of fermentation. Aerobic organisms are killed by the lack of oxygen and thus decompose. In the aerobic treatment systems, pumps continually circulate and aerate the sludge. The increased aeration kills anaerobic non-spore forming microorganisms and accelerates the degradation rate of organic matter by aerobic microbes. After stabilization, some but not all wastewater treatment plants choose to process the sludge further by a variety of means such as composting, pasteurization, and irradiation (Vesiland, 1979; Vimal and Talashilkar, 1985; Hasit, 1986).

Sludge is disposed of in several ways in this country: (1) by dumping it into landfills and monofills, (2) by surface disposal such as in lagoons, (3) by incineration, (4) by distribution and marketing for garden use, (5) by ocean dumping which will be banned by 1992, and (6) by application to agricultural and non-agricultural land (Converse, et al., 1984; Federal Register, Vol. 54, no. 23, February 6, 1989, p. 5754).

Stabilized sludge contains high amounts of nitrogen, phosphorus, and organic compounds (Converse, et al., 1984). These constituents make sewage sludge very valuable as fertilizer. The EPA estimates that 15.6% of the nation's sewage sludge is being applied to land to improve soil quality (Federal Register, Vol. 54, no. 23, February 6, 1989, p. 5754). Greater crop yields have been reported with the use of

sludge fertilizer over the use of chemical fertilizers (Vimal and Talashilkar, 1985). In 1990, approximately 40 farming operations throughout Kentucky utilized sludge as fertilizer (Telephone conversation, April 20, 1990). Using sludge in land farming is an effective and beneficial means of recycling this waste product.

Despite the beneficial aspects of sludge usage on land, there are some drawbacks. Sewage sludge is known to contain potentially pathogenic microorganisms, parasites, and significant amounts of some heavy metals (Farrah, et al., 1981; Fitzgerald, 1986). Some of the predominant pathogens that have been found to exist in sludge are listed in Table 1. The number and types of pathogens vary with the health of the community that contributes to the sewage sludge (Vimal and Talashilkar, 1985; Fitzgerald, 1986). The numbers of these pathogens are usually reduced but not totally eliminated in the digestion process (Converse, et al., 1984). Some bacteria, viruses, and helminth ova can be quite resistant to secondary treatment (Dudley et al., 1980; Fitzgerald, 1986). These organisms were found to exist for long periods of time in the soil after sludge was applied to farmland (Vimal and Talashilkar, 1985).

Because of the potential health hazards associated with the use of sludge for agricultural purposes, Dudley et al. (1980) designed and published a scheme for the routine analysis of bacteria in municipal sewage sludge. Prior to that time, no such system existed to screen for various pathogenic bacteria in sludge (Dudley et al., 1980). Their scheme was comprised of methods to isolate and enumerate fluorescent Pseudomonas species, Staphylococcus, Mycobacterium, Clostridium, members of the family Enterobacteriaceae with specific tests for Klebsiella, Shigella, Salmonella, and oxidase-positive enteric organisms. Also included were procedures to determine routine indicator organisms such as fecal coliforms, fecal streptococci, total coliforms,

TABLE 1. Pathogenic microorganisms found in sewage sludge[a]

Organisms
Bacteria
Shigella sp.
Salmonella sp.
Vibrio cholerae
Escherichia coli pathogenic strains
Mycobacterium tuberculosis
Erysipelothrix sp.
Leptospira sp.
Clostridium perfringens
Klebsiella pneumoniae
Staphylococcus sp.
Pseudomonas sp.
Aeromonas sp.
Viruses
Enteroviruses
Adenoviruses
Polio viruses
Norwalk viruses
Hepatitus A virus
Parasites
Toxoplasma gondii
Entamoeba histolytica
Giardia lamblia
Helminthic parasites
Ascaris lumbericoides
Fungi
Aspergillus fumigatus

[a]Sources: Rippey and Cabelli, 1979; Dudley et al., 1980; Vimal and Talashilkar, 1985; Fitzgerald, 1986; Federal Register, Vol. 54, no. 23, February 26, 1989, p. 5829.

and total aerobic colony counts (Figure 1). Table 2 summarizes the procedures employed by Dudley et al. (1980).

During the past decade, new techniques have been developed for assaying some of these pathogenic bacteria. Also, some species of bacteria, which occur in sludge and which were thought to be harmless, have been shown to produce adverse effects in humans. For these reasons, the purpose of this thesis was to up-date the Dudley et al. scheme (1980) as shown in Figure 2.

Specific procedures for the isolation of mycobacteria, Shigella, and Klebsiella included in the Dudley et al. scheme (1980) were not performed in this study. Since Klebsiella were isolated on MacConkey and XLD agars and identified with the API 20E system (Analytab Products, Inc., Plainview, New York) along with other gram-negative bacteria, it was deemed unnecessary to use an additional procedure for isolating Klebsiella. Dudley et al. (1980) and Ottolenghi and Hamparian (1987) did not detect Shigella in most sludge samples. They stated that Shigella does not survive well in sludge and is rapidly inactivated. Since Shigella is not an important sludge bacteria, an isolation procedure for Shigella was not included in the modified scheme. The Dudley et al. (1980) procedure for mycobacteria was not employed in this study due to expense and time.

Figure 1. Schematic diagram of sludge analysis by Dudley et al. (1980).

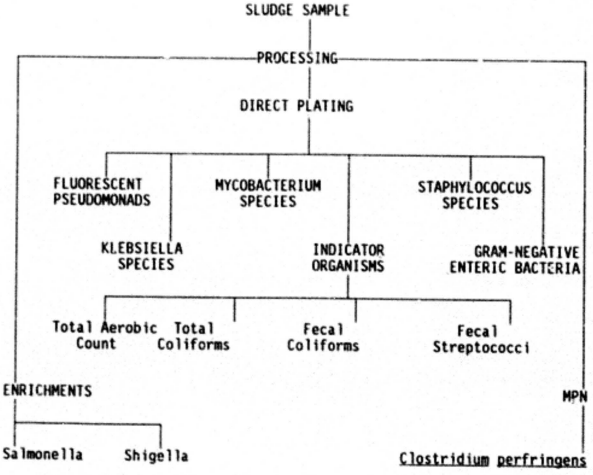

SLUDGE SAMPLE
PROCESSING
DIRECT PLATING
FLUORESCENT PSEUDOMONADS
MYCOBACTERIUM SPECIES
STAPHYLOCOCCUS SPECIES
KLEBSIELLA SPECIES
INDICATOR ORGANISMS
GRAM-NEGATIVE ENTERIC BACTERIA
Total Aerobic Count
Total Coliforms
Fecal Coliforms
Fecal Streptococci
ENRICHMENTS
MPN
Salmonella
Shigella
Clostridium perfringens

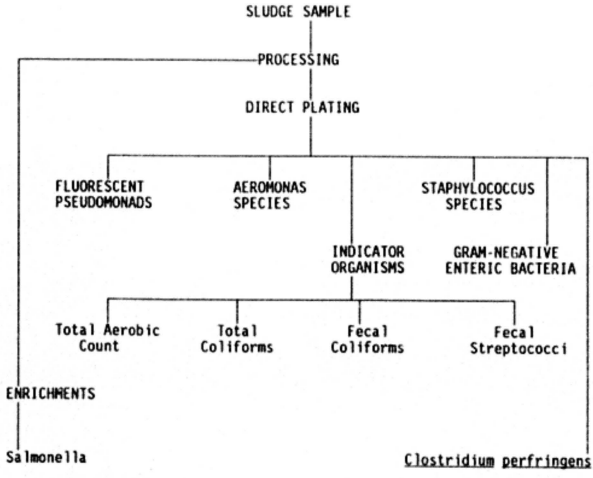
TABLE 2. Procedures used in Dudley et al. sludge processing scheme (1980)

Bacteria	Enrichment	Isolation medium	Additional procedures
Total aerobic count		Plate count agar	
Total coliforms		m-ENDO agar LES	
Fecal coliforms		m-FC agar	
Fecal streptococci		m-Enterococcus agar	
Salmonella sp.	Selenite broth	Brilliant green agar	Oxidase test, triple sugar iron and lysine iron media, serological test
Shigella sp.	GN broth	Xylose lysine	Oxidase test, triple sugar iron and motility indole ornithine media
Mycobacterium sp.	Treated with benzalkonium chloride (500 µg/ml)	Middlebrooks 711 agar with oleic-albumin-dextrose-catalase and 3 µg/ml amphotericin B	Subculture on Lowenstein-Jensen medium

TABLE 2. Continued

Bacteria	Enrichment	Isolation medium	Additional procedures
Fluorescent pseudomonads		Cetrimide agar	Exposure to fluorescent light for 18 to 24 hours followed by observation under long-wave ultraviolet light
Klebsiella sp.		Eosin-methylene blue agar	Oxidase test, triple sugar iron and motility indole ornithine media
Staphylococcus sp.		Mannitol salt agar	Gram stain
Clostridium perfringens		Modified differential reinforced Clostridium medium in MPN tubes	Litmus milk and Gram stain
Gram-negative enterics		MacConkey agar and xylose lysine deoxycholate agar	Oxidase test and API 20E identification system

Figure 2. Schematic diagram of sludge analysis by Ball and Elliott.

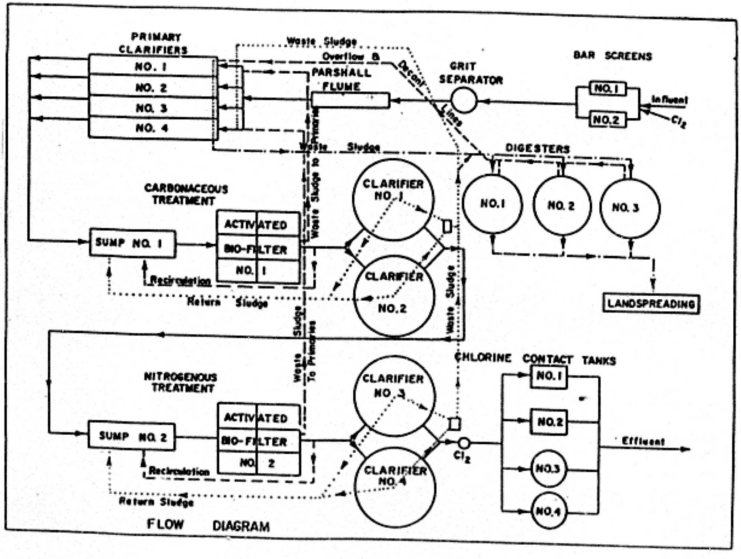

SLUDGE SAMPLE
PROCESSING
DIRECT PLATING
FLUORESCENT PSEUDOMONADS
AEROMONAS SPECIES
STAPHYLOCOCCUS SPECIES
INDICATOR ORGANISMS
GRAM-NEGATIVE ENTERIC BACTERIA
Total Aerobic Count
Total Coliforms
Fecal Coliforms
Fecal Streptococci
ENRICHMENTS
Salmonella
Clostridium perfringens

MATERIALS AND METHODS

Description of sampling site

Sludge samples used in this study were obtained from the Bowling Green Waste Water Treatment Plant located in Bowling Green, Kentucky (Figure 3). The facility receives an average of 5.5 million gallons of sewage per day (Personal interview, April 1989). The plant employs an activated biological-filter system to treat the raw sewage and then discharges the treated effluent into the Barren River. During the wastewater treatment, approximately 35,000 gallons of sludge are generated each day. The sludge is directed to one of three aerobic digesters. At the time this study was conducted, the sludge was disposed of by two means: (1) application to farm land in Simpson county in Kentucky and (2) dumping into a nearby landfill. The sludge which was transported to farm land was always procured from digester #2 at the treatment plant. Samples of sludge for this study were likewise obtained from digester #2. They were collected at approximately the same time of day (10:00 - 10:30 am). The samples were obtained once every two months from October 1989 to August 1990 (Table 3). Data pertaining to various physical factors influencing the sludge on the day of sampling were obtained from the laboratory technicians on staff at the treatment plant (Table 4). The sludge samples were collected in sterile quart mason jars and were placed on ice to transport to the laboratory. All samples were processed within three to four hours after collection.

Figure 3. Map of the Bowling Green Waste Water Treatment Plant, Bowling Green, Kentucky, at time of study.



FLOW DIAGRAM

TABLE 3. Samplings and corresponding dates and times

Sampling	Date	Time
1	10/24/89	10:10 am
2	12/13/89	10:00 am
3	02/16/90	10:55 am
4	04/19/90	10:30 am
5	06/06/90	10:30 am
6	08/10/90	9:10 am

TABLE 4. Physical-Chemical Parameters for the Six Samplings

Parameter	Unit	Sampling					
		1	2	3	4	5	6
Air temperature for preceeding 24 hours							
High	°C	24.4	4.4	21.7	15.5	27.7	32.2
Low		10.0	-10.0	15.5	4.4	13.3	17.7
Precipitation for preceeding 24 hours	inches	0.04	0.00	1.29	trace	0.00	trace
Digestor temperature	°C	19.0	7.8	18.0	15.0	21.5	25.0
pH	-log H^+	nd[a]	6.5	nd[a]	6.8	6.3	6.7
Sludge density	% solids by weight	nd[a]	7.0	4.5	5.6	4.5	5.5

[a]no data available

Sample preparation and pH determination

Sludge samples were processed according to Dudley et al. (1980). Twenty ml of a sludge sample and 1 g of sterile 3-mm glass beads were poured into a sterile 50-ml graduated plastic centrifuge tube (Falcon, Oxnard, California 93030) and were mixed at high speed for 2 min using a vortex mixer (Lab-line Instruments, Incorporated, Melrose Park, Illinois 60160) to evenly disperse organisms in the sludge sample. The contents of the centrifuge tube were added to a flask containing 180 ml of sterile phosphate buffer. The solution was shaken thoroughly to mix. Serial dilutions were made in 9-ml sterile phosphate buffer blanks. The pH of the remaining undiluted sludge was determined using a Fisher Accumet pH Meter Model 600 (Fisher Scientific, Pittsburgh, Pennsylvania 15219).

Indicator organisms

Total aerobic colony counts were determined using the standard plate count method described in "Standard Methods for the Examination of Water and Wastewater," Am. Public Health Assoc. (1985). Total coliforms, fecal coliforms, and fecal streptococci were assayed on m-Endo-LES agar (Difco, Detroit, Michigan 48232), m-FC agar (Difco, Detroit, Michigan 48232), and m-Enterococcus agar (Difco, Detroit, Michigan 48232) respectively. Triplicate plates of each medium were inoculated with 0.1 ml of the appropriate sludge dilutions. The samples were evenly spread over the media with sterile bent glass rods. The plate count agar (Difco, Detroit, Michigan 48232) plates and the m-Endo-LES plates were then incubated at 37°C for 24 hours. The m-FC plates were incubated at 37°C for 4 hours and transferred to 45°C for 20 hours. The m-Enterococcus plates were incubated at 37°C for 48 hours.

The resultant colonies were counted. Dark red colonies with golden to greenish metallic sheen were counted as coliforms on the m-Endo-LES medium. On m-FC

agar, only the colonies which were blue in color were counted as fecal coliforms. Pink to dark red colonies on m-Enterococcus were counted as fecal streptococci.

Clostridium perfringens

A selective medium for Clostridium perfringens known as egg yolk-free tryptose-sulfite-cycloserine agar (EYF-TSC) was prepared in accordance to the formula published by Hauschild and Hilsheimer (1974). The medium was prepared exclusive of the D-cycloserine, autoclaved, and maintained at 50°C until needed. Just before pouring plates, filter-sterilized D-cycloserine (Sigma Chemical Co., St. Louis, Missouri 63178) was added to the molten medium so as to achieve a final concentration of 400 μg of D-cycloserine per ml.

One ml of the appropriate 10-fold dilutions of the sludge sample was placed in each of three sterile petri dishes. Approximately 20 ml of the EYF-TSC medium was then poured into the dishes and gently shaken to allow adequate dispersal of the sample in the agar. The plates were then placed in Gas Pak jars (BBL Microbiology Systems, Cockeysville, Maryland 21030) and incubated anaerobically at 37°C for 18 to 20 hours. Typical black colonies were counted. At each sampling time, five typical colonies were randomly picked (Hauschild and Hilsheimer, 1974) and restreaked for purification on sulfite, polymyxin, sulfadiazine (SPS) agar (Difco, Detroit, Michigan 48232) plates with SPS agar overlay. Again, these plates were incubated anaerobically for 18 to 20 hours at 37°C. The isolates were then maintained at 4°C in thioglycollate medium (Difco, Detroit, Michigan 48232) supplemented with 3% agar until needed for identification.

To identify the isolates as Clostridium perfringens, the reverse CAMP test (RCT) as described by Hansen and Elliott (1980) was employed. A stock culture of Streptococcus agalactiae was obtained from T. J. Samson Community Hospital, Glasgow, Kentucky. To perform the reverse CAMP test, S. agalactiae was streaked

down the center of a petri plate containing tryptic soy agar (Difco, Detroit, Michigan 48232) and 5% sterile, defibrinated sheep blood (Cleveland Scientific, Bath, Ohio 44210) (BAP). The presumed C. perfringens isolates were then streaked at right angles to the S. agalactiae making certain that the isolate streak lines began at approximately 2 mm away from the center streak line. The plates were incubated anaerobically at 37°C for 24 hours. Those isolates which produced arrowhead zones of hemolysis were then identified as C. perfringens.

Aeromonas sp.

The media employed to isolate Aeromonas were those developed by Rippey and Cabelli (1979) for use in a multi-step membrane filtration procedure to enumerate Aeromonas in water samples. Since this study was concerned with sludge samples instead of water samples, the membrane filtration step was omitted and the diluted sludge samples were plated directly onto the primary medium. The primary medium (referred to as mA agar) contained trehalose as a carbon source and ampicillin and ethanol as selective inhibitors. The secondary medium (referred to simply as mannitol agar) contained mannitol as a fermentable carbohydrate.

Triplicate plates of the mA agar were inoculated with 0.1 ml of the appropriate sludge dilution and incubated at 37°C for 20 to 24 hours. Colonies which were trehalose-positive and therefore yellow in color were restreaked onto mannitol agar for 24 hours at 37°C. At each sampling time, a minimum of five typical mannitol positive colonies appearing as yellow colonies on the mannitol agar were picked and restreaked for purification on mannitol agar. Each isolate was tested for oxidase production, catalase production, and Gram reaction. Gram-negative organisms which were rod shaped, oxidase-positive, and catalase-positive were then inoculated into glucose fermentation tubes containing durham tubes, esculin iron agar slants (Difco, Detroit, Michigan 48232), and motility-indole-ornithine (MIO) tubes (Difco, Detroit,

Michigan 48232). The media were incubated at 37°C and results were noted after 24 to 48 hours.

Staphylococcus sp.

Triplicate plates of mannitol salt agar (Difco, Detroit, Michigan 48232) and Baird-Parker agar (Difco, Detroit, Michigan 48232) were inoculated with 0.1 ml of the appropriate sludge dilutions. The plates were incubated at 37°C for 48 hours. On the mannitol salt medium, colonies which were yellow or white in color were counted. Only black smooth colonies on Baird-Parker with or without halos were counted. At each sampling time, five isolates from each type of media were randomly picked and restreaked onto mannitol salt agar for purity. Then, Gram stains and catalase tests were performed. Organisms which were gram-positive cocci and catalase-positive were inoculated into coagulase tubes containing reconstituted coagulase plasma EDTA (Difco, Detroit, Michigan 48232) and were incubated in a water bath held at 37°C. The tubes were examined for clotting at four hours and at 24 hours. These isolates were also inoculated into Staph Ident test strips (Analytab Products, Incorporated, Plainview, New York 11803). The test strips were inoculated, incubated, and read in strict accordance with the manufacturer's instructions.

Salmonella sp.

Salmonella organisms were detected by using a method described by Ottolenghi and Hamparian (1987). Processed, undiluted sludge was swabbed directly onto triplicate plates of Hektoen Enteric agar (HE) (Difco, Detroit, Michigan 48232) using sterile cotton swabs. Also, sterile cotton swabs were dipped into the same sludge sample and were placed in duplicate tubes containing 10 ml of selenite enrichment broth (Difco, Detroit, Michigan 48232). The media were incubated at 37°C for 24 hours and examined. Colonies which appeared blue-green in color with

or without hydrogen sulfide production were counted. Samples from the selenite enrichment broth were streaked onto fresh HE agar plates and incubated at 37°C for another 24 hours.

A minimum of five colonies suspected of being Salmonella were picked for identification and restreaked for purity at each sampling time. The isolates were further examined by performing Gram stains, oxidase tests using spot oxidase reagent (Difco, Detroit, Michigan 48232), catalase tests using 3% hydrogen peroxide, and O-nitrophenyl-B-D-galactopyranoside (ONPG) disk tests (BBL Microbiology Systems, Cockeysville, Maryland 21030). Also, triple sugar iron agar (TSI) (Difco, Detroit, Michigan 48232) slants, lysine iron agar (LIA) (Difco, Detroit, Michigan 48232) slants, and urea broth (Difco, Detroit, Michigan 48232) were inoculated with the isolates. The media were incubated at 37°C and observed at 24 hours and at 48 hours.

Fluorescent Pseudomonas sp.

Triplicate plates of Pseudosel agar (BBL Microbiology Systems, Cockeysville, Maryland 21030) were inoculated by spreading 0.1 ml from the appropriate sludge dilutions onto the plates. The plates were incubated at 37°C for 24 to 48 hours. To enhance pigmentation, the plates were then exposed to fluorescent light for an additional 24 hours. Colonies were counted under ultra violet (UV) light using a Cenco Model 1300 UV lamp (Burton Medic-Quipment Company, El Segundo, California 40245).

At each sampling time, five typical fluorescent colonies were picked and restreaked either on pseudosel agar or pseudomonas A agar (Difco, Detroit, Michigan 48232) for purity. The isolates were then tested for oxidase production, catalase production, Gram reaction, growth at 42 C, growth in 6.5% NaCl, gelatinase

production, motility, indole production, ornithine decarboxylase and growth on MacConkey agar (Difco, Detroit, Michigan 48232).

Gram-negative enteric bacteria

MacConkey agar (Difco, Detroit, Michigan 48232) and xylose lysine deoxycholate (XLD) agar (Difco, Detroit, Michigan 48232) were used to assay oxidase-positive and oxidase-negative, gram-negative bacteria. Triplicate plates of each medium were inoculated with 0.1 ml from the appropriate sludge dilutions and were incubated at 37°C for 24 to 48 hours. Colonies with different colony morphologies were selected from both media from the dilution with countable plates (30 -300 colonies) and were streaked onto MacConkey agar for purity.

Isolates were later gram stained and tested for the presence of oxidase and catalase. API 20E identification strips (Analytabs Products, Incorporated, Plainview, New York 11803) were then inoculated with the isolates according to the accompanying instructions. The test strips were incubated at 37°C for 24 to 48 hours and were then read according to the manufacturer's instructions.

Statistical analysis

The Student's t test for unequal samples (Steel and Torrie, 1980) was employed to determine if there was a significant difference between the number of staphylococci isolated from sewage sludge on mannitol salt agar and Baird-Parker medium.

RESULTS

Bacterial numbers over one year period

The numbers of indicator organisms over the six samplings are recorded in Table 5. Highest colony counts were recorded during the following months: (1) total aerobic count - October and December; (2) total coliforms - December and February; (3) fecal coliforms - December; (4) fecal streptococci - December and April. Bacterial counts for all of the indicator organisms were lowest during the month of August.

The numbers of pathogenic bacteria over the six samplings are recorded in Table 6. The log densities of C. perfringens varied the least, remaining between 5 and 6 throughout the year. Highest colony counts for the other pathogenic bacteria were recorded during the month of December. Lowest bacterial numbers were recorded during the month of August.

The fluctuations in bacterial numbers over the year corresponded with changes in the temperature in the aerobic digester. Due to the fact that the digester tank was not enclosed and that sludge is not heated during mesophilic aerobic digestion, the sludge in the digester was subject to temperature variations according to changes in air temperature. Figure 4 and Figure 5 compare the changes in bacterial numbers with changes in digester temperature over the period of October, 1989 to August, 1990.

TABLE 5. Bacterial counts for indicator organisms over the six samplings

Sampling	Indicator Organisms (CFU/ml)			
	Total aerobic count	Total coliforms	Fecal coliforms	Fecal streptococci
1	3.2×10^8	8.7×10^5	3.0×10^4	3.1×10^3
2	1.3×10^8	3.5×10^6	6.9×10^5	6.3×10^3
3	5.7×10^7	3.4×10^6	3.5×10^4	3.3×10^3
4	5.8×10^7	5.0×10^5	2.1×10^4	1.1×10^4
5	4.5×10^7	5.4×10^5	4.9×10^4	1.5×10^3
6	2.5×10^7	1.2×10^5	4.6×10^3	1.0×10^2

TABLE 6. Bacterial counts for potentially pathogenic organisms over the six samplings

Samplings	Pathogenic organisms (CFU/ml)				
	Clostridium perfringens	Aeromonads	Fluorescent pseudomonads	Gram-negative enteric bacteria	Staphylococci (isolated on BP agar)
1	1.8×10^5	4.3×10^5	2.3×10^4	2.8×10^4	3.5×10^3
2	1.4×10^5	1.5×10^6	1.7×10^5	4.2×10^6	1.6×10^4
3	1.3×10^5	1.3×10^5	1.7×10^4	1.8×10^4	1.4×10^4
4	2.2×10^5	6.8×10^5	1.3×10^4	5.3×10^5	3.5×10^3
5	1.4×10^5	2.4×10^5	1.9×10^3	5.0×10^5	3.5×10^3
6	2.5×10^5	3.6×10^4	8.0×10^2	1.0×10^5	8.7×10^2

Figure 4. Comparison of bacterial counts for indicator organisms with digester temperature over the sampling period. Symbols: ⊞, total aerobic count; ○, total coliforms; ◫, fecal coliforms; ●, fecal streptococci.

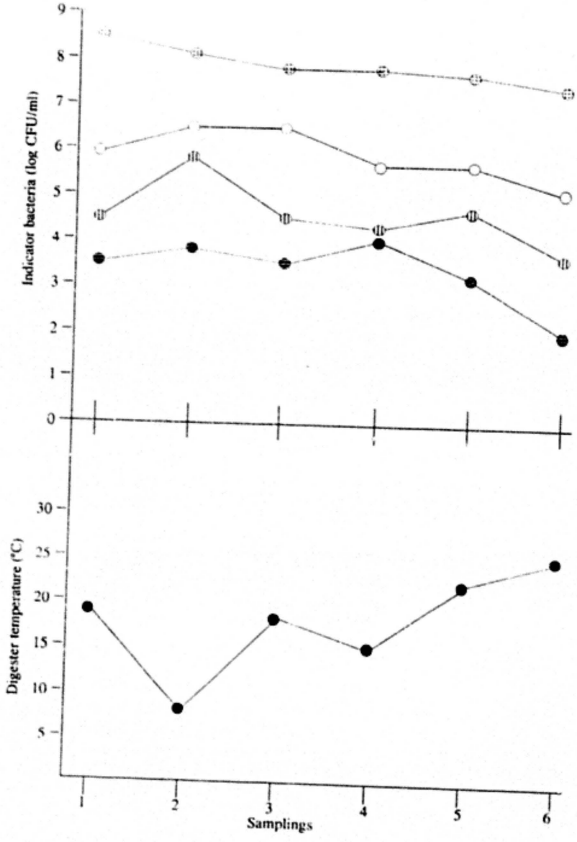
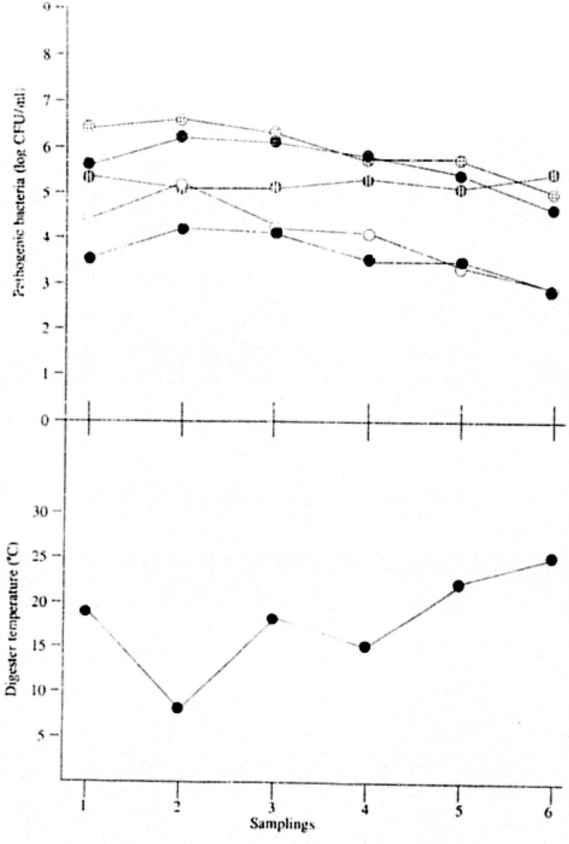

Indicator bacteria (log CFU/ml)
9
8
7
6
5
4
3
2
1
0
Digester temperature (°C)
30
25
20
15
10
5
1
2
3
4
5
6
Samplings

Figure 5. Comparison of bacterial counts for pathogenic organisms with digester temperature for the sampling period. Symbols: ⊕, gram-negative enteric bacteria; ●, aeromonads; (|), Clostridium perfringens; ○, fluorescent pseudomonads; ●, staphylococci (isolated on Baird-Parker agar).


Pathogenic bacteria (log CFU/ml)
9
8
7
6
5
4
3
2
1
0
Digester temperature (°C)
30
25
20
15
10
5
1
2
3
4
5
6
Samplings

Clostridium perfringens

C. perfringens was isolated from sewage sludge over the one year study (Table 6). Six of the 30 colonies chosen for confirmation over the six sampling times were not viable for identification. Three out of the remaining 24 colonies (13%) were found to be gram-variable filamentous bacilli. The rest of the isolates were found to be gram-positive blunt rods and 20 of these isolates (95%) produced arrowhead zones of hemolysis characteristic of C. perfringens in the reverse CAMP test. C. perfringens produces an alpha-toxin which acts synergistically with the CAMP factor in S. agalactiae to produce the distinctive hemolysis pattern (Hansen and Elliott, 1980).

Aeromonas sp.

Aeromonads were successfully isolated from sewage sludge on mA agar in this study (Table 6). Of the total number of colonies selected for identification over the six samplings, 25 remained viable for identification. Nine isolates (36%) were oxidase-negative, gram-negative bacilli and 16 isolates (64%) were oxidase-positive, gram-negative bacilli. Three of the oxidase-positive isolates were indole-negative and were presumed to be members of the genus Pseudomonas (Finegold and Baron, 1986). The other 13 oxidase-positive isolates were presumed to be aeromonads. To speciate the aeromonads, a scheme published by Cunliffe and Adcock (1989) was employed (Table 7). Ten of the Aeromonas isolates were esculin-positive and failed to produce gas in glucose. These were identified as Aeromonas caviae. Three of the Aeromonas isolates were able to produce gas in glucose and were able to hydrolyze esculin. These were identified as Aeromonas hydrophila. Of all of the aeromonads isolated, 77% were A. caviae and 23% were A. hydrophila.

TABLE 7. Cunliffe and Adcock scheme (1989) for speciating aeromonads

Test	Species		
	A. caviae	A. hydrophila	A. sobria
Esculin hydrolysis	+	+	-
Glucose fermentation	A*	A* + gas	A* + gas

*A = acid

Staphylococcus sp.

Staphylococci were isolated from sewage sludge over the year-long study (Table 6). During the six samplings, a total of 34 isolates from the mannitol salt (MS) agar plates were purified for further testing. Gram stains were prepared from the isolates and were observed. Only one of the isolates (3%) proved to be gram-positive cocci. Further testing revealed the identity of this isolate to be Staphylococcus xylosus. All of the other isolates were gram-positive to gram-variable bacilli.

Thirteen of the colonies picked from Baird-Parker (BP) medium for identification over the six samplings remained viable and were purified. Three (23%) of the isolates were gram-positive cocci. Of these three isolates, two were identified as Staphylococcus epidermidis, and one was identified as Staphylococcus haemolyticus. The other isolates which exhibited colony morphology like staphylococci on Baird-Parker agar were gram-negative bacilli identified as members of the genus Klebsiella and gram-positive coccobacilli which produced a salmon-orange colored pigment on tryptic soy agar. Based on the results of further tests, these gram-positive organisms were presumed to be in the genus Rhodococcus.

Statistical analysis was performed on the data from both agars by using the Student's t test (Steel and Torrie, 1980). At the 95% confidence level, there was a significant difference between the number of staphylococci isolated on MS agar and the number of staphylococci isolated on BP agar. BP agar proved to be the most effective for isolating staphylococci from sewage sludge.

Salmonella sp.

Twenty-three out of the total number of isolates picked for confirmation over the six samplings were viable for identification. None of these colonies produced hydrogen sulfide. Results of the biochemical tests performed indicated that 65% of

the isolates were pseudomonads, 22% were Providencia alcalifaciens, and 9% were Proteus. None of the isolates were identified as members of the genus Salmonella.

Fluorescent Pseudomonas sp.

Fluorescent pseudomonads were isolated throughout the six samplings (Table 6). A total of 29 colonies growing on Pseudosel agar were randomly selected for identification; however, five of these did not remain viable. Of the 24 remaining isolates, all were gram-negative bacilli. Sixteen (67%) were identified as Pseudomonas sp., five (21%) were oxidase-negative, glucose fermentors, and three (12%) were oxidase-positive, glucose fermentors. To speciate the fluorescent pseudomonads, the appropriate isolates were tested for their ability to produce gelatinase and their ability to grow at 42°C. P. fluorescens produces gelatinase and does not grow at 42°C (Table 8). P. putida does not produce gelatinase nor does it grow at 42°C. P. aeruginosa does possess the ability to grow at 42°C, may or may not produce gelatinase, and may or may not produce pyocyanin. Using these criteria to speciate fluorescent pseudomonads, four isolates were identified as P. aeruginosa, eight were identified as P. fluorescens, and four were identified as P. putida.

Gram-negative enteric bacteria

Gram-negative microorganisms were isolated on MacConkey agar and XLD agar over the six samplings (Table 6) and were easily identified using the API 20E system. The various species isolated and identified are listed in Table 9.

TABLE 8. Scheme for speciating fluorsecent pseudomonads

Test	Species		
	P. aeruginosa	P. fluorescens	P. putida
Growth at 42°C	+	-	-
Gelatinase production	+/-	+	-

TABLE 9. Gram-negative bacteria isolated from sludge over the six samplings on XLD and MacConkey agars.

Organisms
Enterobacteriaceae
Klebsiella pneumoniae
Klebsiella oxytoca
Enterobacter cloacae
Enterobacter agglomerans
Providencia alcalifaciens
Gram-negative, oxidase-positive bacteria
Fluorescent pseudomonads
Aeromonas hydrophila
Acinetobacter calcoaceticus biotype Iwoffi
Vibrio cholerae
Bordetella bronchiseptica

DISCUSSION

Clostridium perfringens

In this study, the species of Clostridium isolated from sewage sludge was C. perfringens. C. perfringens is a gram-positive, endospore-forming, anaerobic bacillus proven to be the causative agent of gas gangrene and gastroenteritis (Buffaloe and Ferguson, 1981; Finegold and Baron, 1986; Jensen and Wright, 1989). C. perfringens is more widely distributed in the environment than any other pathogenic bacterium. It exhibits a very high survival potential in adverse environments compared to many bacteria due to its ability to sporulate. Its principal habitats are the soil and the intestinal tracts of humans and animals. It has been isolated in large numbers from sewage sludge, a fact which has lead some researchers to suggest C. perfringens be used as an indicator organism in the examination of sludge (Dudley et al., 1980). Thus it was not surprising that Hansen and Elliott (1982) isolated this bacterium from the discharge pipe of the Bowling Green, Kentucky, sewage disposal plant. Dudley et al. (1980) isolated this bacterium from sewage sludge but the methods used for isolation and identification are questionable.

In the Dudley et al. scheme (1980), the selective medium chosen for assaying C. perfringens was modified differential reinforced clostridium medium (DRCM). They used DRCM in most probable number (MPN) procedures over a range of five decimal dilutions using five tubes per dilution. They incubated the tubes for three days at 37°C. Gram stains were prepared from each tube and one loop full from

each DRCM tube was inoculated into litmus milk. They incubated the litmus milk tubes for one to three days at 37°C. Isolates which exhibited stormy fermentation in litmus milk and which were gram-positive, spore forming bacilli were identified as C. perfringens. Using this procedure, Dudley et al. (1980) reported a high confirmation rate of 92%.

As described previously, EYF-TSC agar developed by Hauschild and Hilsheimer (1974) was employed to select for C. perfringens in this study. The reverse CAMP test (RCT) developed by Hansen and Elliott (1980) was used as the confirmatory test. A 95% confirmation rate was achieved in this study using these procedures.

Several advantages were attained using the EYF-TSC/RCT procedure over the Dudley et al. (1980) procedure for isolating C. perfringens. First, less time was required. The EYF-TSC/RCT procedure required two to three days to perform as opposed to four to six days using the Dudley et al. (1980) procedure. Second, the EYF-TSC/RCT procedure allows for direct quantitative enumeration of C. perfringens instead of relying on MPN tables to estimate bacterial numbers. Third, the reverse CAMP test is preferable over litmus milk to confirm the presence of C. perfringens because the reverse CAMP test is more accurate and the results are easier to read. In their report, Dudley et al. commented that stormy fermentation was sometimes difficult to observe in the litmus milk tubes. Fourth, a higher confirmation rate was achieved using the EYF-TSC/RCT procedure over the procedure used in the Dudley et al. scheme (1980).

Aeromonas sp.

Members of the genus Aeromonas are motile, gram-negative, oxidase-positive, facultatively anaerobic, glucose-fermenting bacteria that are widely distributed in aquatic environments (Von Graevenitz, 1980; Namdari and Cabelli, 1989). Aeromonads have been isolated from sewage sludge in large numbers according to

Rippey and Cabelli (1979) and Hazen et al. (1978). Neilson (1978) reported detecting between 10^5 to 10^7 aeromonads per ml in sewage sludge comprising ten percent or more of the total number of gram-negative bacteria in his samples.

Aeromonads were considered at one time to be harmless to humans. However, in the past twenty years, researchers have linked Aeromonas sp. such as A. caviae, A. hydrophila, and A. sobria to acute diarrheal disease, wound infections, septicemia, and other infections such as urinary tract infections and otitis (Von Graevenitz, 1980; Finegold and Baron, 1986). Thus methods should be used to detect these bacteria during bacteriological surveys of sewage sludge.

The Dudley et al. scheme (1980) does not include a procedure to isolate Aeromonas sp. in sludge. However, due to the high levels of aeromonads known to exist in sewage sludge and due to their potential pathogenicity, a test for aeromonads was added to our modified scheme. The procedure selected was one developed by Rippey and Cabelli (1979) for isolating and enumerating A. hydrophila in fresh water. This same procedure was used successfully by Namdari and Cabelli (1989) to isolate aeromonads from sewage.

In our study, 81% of the oxidase-positive organisms isolated with the Rippey and Cabelli (1979) procedure were identified as aeromonads. The two species isolated were A. caviae and A. hydrophila with A. caviae being the predominant species. Monfort and Baleux (1990) also found A. caviae to be the dominant Aeromonas species at a pond system in-flow regardless of season. In their literature review, Namdari and Cabelli (1989) found that the majority of aeromonads existing in high nutrient waters such as sewage water are anaerogenic strains of Aeromonas such as A. caviae. Aerogenic strains such as A. hydrophila tend to predominate in low nutrient waters. Despite extensive research performed to elucidate the ecology and taxonomy of these bacteria, the systematics of Aeromonas remain unsettled (Janda,

1991). Since Bergey's Manual of Systematic Bacteriology, Volume 1, was published (1984), new species that exhibit special phenotypic characteristics have been described (Altwegg, et al., 1990; Carnahan, et al., 1991).

Staphylococcus sp.

Staphylococci are catalase-positive, gram-positive, facultatively anaerobic cocci which are found primarily on the skin and mucous membranes of mammals (Finegold and Baron, 1986; Jeasen and Wright, 1989). Staphylococci have also been isolated from water, especially recreational waters, and in low levels from sewage (Dudley, et al., 1980; Klapes, 1983). S. aureus, a pathogenic staphylococci, produces the enzyme coagulase and can be easily distinguished from other staphylococci by performing a coagulase test. Although other species of staphylococci may be coagulase-positive, S. intermedius and S. hyicus, they are not usually clinically important agents of human disease. The coagulase-negative staphylococci are less virulent than S. aureus (Finegold and Baron, 1986). However, coagulase-negative S. epidermidis has been shown to cause infections in immunocompromised patients and in patients with implanted prosthetic devices (Kloos and Smith, 1980). Also, S. epidermidis and S. aureus are among the most drug resistant gram-positive cocci (Rogolsky and Gobert, 1990). S. saprophyticus, another coagulase-negative member of the genus Staphylococcus, has been linked to urinary tract infections especially in young women (Finegold and Baron, 1986). Because various staphylococci are potentially harmful and because they have been isolated from water and sewage, it is important to incorporate a procedure for assaying staphylococci into a scheme for isolating pathogenic bacteria from sewage sludge.

In the Dudley et al. scheme (1980), mannitol salt (MS) agar was employed to isolate staphylococci. The agar, which inhibits the growth of gram-negative microbes, contains mannitol as the carbon source and 7.5% NaCl as an inhibitor (Finegold and

Baron, 1986). Those colonies which fermented mannitol, thus having yellow zones around them, and which were gram-positive cocci were counted as staphylococci. No attempt was made by Dudley et al. to speciate the isolates. The fact that the isolates were not identified to the species level presents a weak point in the Dudley et al. scheme (1980). In order to accurately survey pathogenic bacteria in sewage sludge samples, pathogenic staphylococci need to be distinguished from non-pathogenic staphylococci .

In our modified scheme, we also used MS agar to isolate staphylococci. Baird-Parker (BP) medium was used as well for isolating staphylococci and was compared to the MS agar to determine which medium was more effective for isolating staphylococci from sewage sludge. Since some coagulase-positive, as well as coagulase-negative, staphylococci do not produce yellow zones of fermentation on MS agar, those colonies which fermented mannitol and those colonies which did not were counted. Lambe et al. (1990) described many species of coagulase-negative, mannitol-negative staphylococci which can produce diseases in humans. They therefore stressed the importance of assaying for coagulase-negative staphylococci and the importance of speciating the isolates.

Baird-Parker (BP) agar was also used in this study to isolate Staphylococcus. The agar was chosen for this study for two reasons: (1) to determine if BP agar could be used to isolate staphylococci in sewage sludge and (2) to determine if BP agar would be more selective for staphylococci in sewage sludge compared to MS agar. The medium was developed by Baird-Parker (1962) specifically for isolating Staphylococcus species. The medium contains pyruvate, glycine, lithium chloride, egg yolk, and tellurite. Colonies of staphylococci growing on this agar appear as smooth black colonies due to their ability to reduce tellurite. Coagulase-positive staphylococci, which are egg yolk-positive, produce smooth, black colonies

surrounded by clear zones. BP agar is the recommended medium for isolating and enumerating S. aureus in food (Niskanen and Aalto, 1978) and it has been used successfully to isolate S. aureus in hydrotherapy pools and in swimming pools (Klapes, 1983).

To speciate staphylococci in this study, the Staph Ident rapid identification system by Analytab Products, Inc. (Plainview, New York 11803) was used. The system is comprised of 10 microcupules which contain media and various substrates. Isolates to be identified are emulsified in 0.85% saline solution and are inoculated into the test strips. Identification of the isolates can be determined after only five hours of incubation. The API Staph Ident system allows for the identification of 15 different species of staphylococci. The system is recommended throughout the literature for the speciation of staphylococci and proved very useful in this study (Finegold and Baron, 1986; Lambe, et al., 1990; Rogolsky and Gobert, 1990).

As previously discussed, only 3% of the colonies on MS agar were identified as Staphylococcus. The majority of the microorganisms which grew on the MS agar were gram-positive and gram-variable, catalase-positive bacilli. They were almost impossible to distinguish from staphylococci. Of those colonies on BP agar which were smooth and black in appearance, 23% proved to be staphylococci. Many gram-positive bacilli also grew on BP agar but most were easily distinguished because they produced very rough gray colonies. Overall, differentiation of colonies was somewhat easier on BP agar than on MS agar. Statistical analysis indicated that there was a significant difference in the ability of the two media to isolate Staphylococcus from sewage sludge with BP medium being the most effective. However, neither medium proved to be sufficiently selective against background flora to obtain accurate counts of staphylococci.

Salmonella sp.

Salmonella species are ubiquitous. In animals and birds, many of these species comprise part of the normal intestinal flora. Unfortunately, these same species can cause illnesses in humans. They are transmitted via the fecal-oral route. The species Salmonella typhi is known to be the etiologic agent for typhoid fever. Other serotypes of Salmonella have the potential to produce gastroenteritis. Salmonella is responsible for the majority of food-borne gastroenteritis with more than two million cases of salmonellosis reported annually in the United States (Finegold and Baron, 1986; Jensen and Wright, 1989).

Dondero et al. (1977) report that many researchers have isolated Salmonella species frequently in sewage treatment plant effluent, industrial wastes, and in streams receiving these wastes. Others reported isolating Salmonella from treated sewage sludge as well (Dudley et al., 1980; Ottolenghi and Hamparian, 1987). In light of these findings, it is important to include procedures to isolate Salmonella in a scheme for the routine analysis of sewage sludge. The isolation procedure for Salmonella published by Ottolenghi and Hamparian (1987) was employed in this research rather than the method used by Dudley et al. (1980) because the Ottolenghi and Hamparian procedure was shown to be slightly more sensitive for detecting Salmonella (Ottolenghi and Hamparian, 1987). They were able to detect as few as 11 CFU/ml with their method. Despite this degree of sensitivity, no salmonella were isolated during the six samplings in this study. There are several possible explanations for these results.

Salmonellae may have been present in the sludge but at levels lower than 11 CFU/ml thus making it unlikely that they would have been detected using the Ottolenghi and Hamparian (1987) isolation procedure. The bacteria may have been present at low levels because salmonellae, especially the more virulent strains, do not

compete well with other bacteria in sewage (Calabra et al., 1972; Dondero et al., 1977; Hussong, Burge, and Enkiri, 1985). Also, if salmonellae were present in the sludge, they may not have survived the aerobic digestion of the sludge. Farrah and Bitton (1983) discovered that salmonellae exhibit a higher rate of inactivation in aerobically digested sludge than many other bacteria.

Based on their studies, several authors have reported fluctuations in the rate of isolation of Salmonella in sewage sludge from season to season and from treatment plant to treatment plant (Dudley et al., 1980; Jones et al., 1980; Ottolenghi and Hamparian, 1987). Standard Methods for the Examination of Water and Wastewater (1985) states that the occurrence of Salmonella in wastewater is highly variable and this may be attributed to sporadic incidences of infection with Salmonella in the community contributing to the wastewater. Farrell et al. (1990) examined sewage influent and sewage sludge from four sewage treatment plants from November 1980 to February 1983. They frequently did not find salmonellae in the influent solids or the sludge solids.

The isolates chosen from the hektoen enteric (HE) agar for confirmation in this study proved to be members of the following genera: Proteus, Providencia, and Pseudomonas. Members of these three genera are known to grow well in selenite enrichment media and on HE agar and to exhibit colony characteristics similar to salmonellae (The Difco Manual, Difco Laboratories, Detroit, Michigan). Proteus, Providencia, and Pseudomonas are known to be present in sewage sludges in substantial numbers. If Salmonella species were present, it is possible that they were not able to compete well with these other bacteria in the same media and, therefore, were not detected. Gundstrup (1974) and Nemedi and Lanyi (1971) reported that when Pseudomonas species and Salmonella species were inoculated into selenite

media and tetrathionate media the pseudomonads grew faster than and sometimes greatly overgrew the salmonellae making the salmonellae very difficult to isolate.

Pseudomonas sp.

Members of the genus Pseudomonas are gram-negative, non-fermentative, indole-negative bacteria which are ubiquitous in the environment (de Vincente et al., 1986). They are biochemically very versatile, exhibit a high level of resistance to antibacterial agents, and are known to produce inhibitory substances against other microorganisms such as Bacillus (Hoadley, 1968; Gundstrup, 1974; Keeven and deCicco, 1989). Many species in this genus are considered to be important opportunistic pathogens of man and animals. P. aeruginosa is associated with a large percentage of nosocomial infections and it is the most frequently isolated nonfermentative bacilli in clinical microbiology laboratories (Finegold and Baron, 1986; Keeven and deCicco, 1989). De Vincente et al. (1990) studied the serological and pyocinogenic characteristics of P. aeruginosa strains isolated from natural waters. They found serotype 1 was the most frequently isolated. They speculated that this may be due to a higher resistance of the serotype to the aquatic environment and to water treatment processes. P. aeruginosa pyocin type 17B was only detected in the most polluted sites. The major source of pollution at the sampling sites was sewage.

As in the Dudley et al. scheme (1980), a medium containing cetrimide as the selective agent (Pseudosel agar) was used to assay for fluorescent pseudomonads in this study. All three species in the fluorescent pseudomonad group were isolated from the sewage sludge samples with P. fluorescens being most predominant. Lambe and Stewart (1972) reported that fluorescent pseudomonads grew well on Pseudosel agar, and that it was an excellent medium for the enhancement of pigment production in P. aeruginosa. However, they also reported that Pseudosel agar is not

highly selective against various other gram-negative bacilli. Therefore, background flora may impede detection of fluorescent pseudomonads.

One difficulty encountered in this research was distinguishing between weakly fluorescent colonies and non-fluorescent colonies on Pseudosel agar under ultra-violet light. Therefore, to obtain data that are more accurate, it would be advantageous to employ a medium that either is more selective for fluorescent pseudomonads or allows for better differentiation between fluorescent pseudomonads and other bacteria. Thus a parallel study was done assaying for P. aeruginosa from sewage samples using a newly developed, highly selective medium by Keeven and deCicco (1989). The relatively inexpensive selective agent in the medium was 1,10-phenanthroline. Despite four trials over four months, P. aeruginosa was not isolated from sewage sludge using the new medium.

Gram-negative enteric bacteria

The majority of gram-negative bacteria isolated from sewage sludge were identified as the following members of the family Enterobacteriaceae: Klebsiella pneumoniae, Klebsiella oxytoca, Enterobacter cloacae, and Enterobacter agglomerans with K. pneumoniae predominating. This is consistent with the findings of Dudley et al. (1980). They reported that K. pneumoniae, clostridia, and mycobacteria exhibit the highest survival potentials of bacteria in sewage sludge and are therefore present at high densities in sludge. Campbell et al. (1976) also states that K. pneumonia can be isolated in high numbers even when stress in the environment reduces the total number of bacteria.

No Escherichia coli were isolated on XLD and MacConkey agars over the 6 samplings. Farrah and Bitton (1983) found that E. coli does not exhibit a very high degree of stability in aerobically digested sludge. Standard Methods for the Examination of Water and Wastewater (1985) states that other coliform bacteria such

as Enterobacter and Klebsiella may be present in freshly polluted environments in the absence of E. coli. According to Seidler et al. (1977), Klebsiella may be present in the absence of E. coli because Klebsiella is able to obtain nutrition from a wider range of substances allowing it to compete better in the environment than E. coli and some other enteric bacteria.

Pseudomonads and aeromonads were also consistently isolated in high numbers from sewage sludge. Pseudomonads were the most prevalent gram-negative, oxidase-positive bacteria isolated on the MacConkey and XLD agars. Farrah and Bitton (1983) found P. aeruginosa to be very stable in aerobically digested sludge.

Bacterial numbers over one year period

As in the Dudley et al. scheme (1980), standard procedures for isolating and enumerating indicator organisms were employed to determine the relative bacteriological quality of the sewage sludge. A well-operated sewage treatment plant should generate sludge with fecal coliforms and fecal streptococci log densities of 6.0 or less (Federal Register, Vol. 54, no. 23, February 6, 1989, p. 5832). The fecal coliform and fecal streptococci numbers over the year-long study were under a log density of 6.0 indicating that the Bowling Green Wastewater Treatment Plant, according to the EPA, was adequately treating sludge to reduce bacterial numbers to acceptable levels.

Farrah and Bitton (1983) conducted a study to determine the effect of variables such as pH, temperature, and total solids on the survival of bacteria during aerobic sludge digestion. They found that the temperature of sludge digestion was the variable most highly correlated negatively with the change in bacterial numbers. They also found positive significant values of correlation between pH, total solids, and type of bacteria and changes in bacterial numbers. The results of this study concur with those of Farrah and Bitton (1983). Bacterial numbers increased in

December when digester temperature dropped drastically and the amount of sludge solids increased. Sludge solids were at higher than normal levels because sludge had not been removed from the tank for transport to farmland for two months. Decreased bacterial numbers were noted in June and August when digester temperatures rose to 21.5°C and 25°C respectively. Fitzgerald (1986) stated that pathogens (not including spore-formers and bacteria with protected cells such as mycobacteria) tend to survive longer at lower temperatures and tend to survive for shorter lengths of time as temperature increases. Farrah and Bitton (1983) offered some explanations for this phenomenon. They proposed that bacterial numbers increased during lower temperatures because (1) the rate of bacterial metabolism is lowered and (2) the activity of protozoan predators diminishes. They also suggested that higher sludge solids can contribute to higher bacterial numbers by providing protection for the bacteria.

C. perfringens and the total aerobic counts were not affected by fluctuations in temperature to the extent that the other organisms were affected. In their study, Farrah and Bitton (1983) found that total aerobic counts were more stable as temperature approached 28°C compared to those of some other types of bacteria in sludge. The ability of C. perfringens to form spores in adverse conditions can account for its stability. Due to its persistence in environmental extremes, some have proposed, as discussed previously, that C. perfringens would be an appropriate indicator organism in the examination of sewage sludge (Dudley, et al., 1980). Routine procurement of C. perfringens counts would allow for more accurate data on the effectiveness of the digestion process in reducing bacterial numbers to acceptable levels. In five of the six samplings, C. perfringens counts exceeded those of fecal coliforms and fecal streptococci. Therefore, these traditional indicator organisms cannot be relied upon to reflect accurately the bacteriological quality of

the sludge. Certain European countries have been using C. perfringens for many years as an indicator organism in the examination of water quality (Buffaloe and Ferguson, 1981). Hansen and Elliott (1982) suggested that if counts of C. perfringens at river sites such as tributaries and discharge pipes exceed a level of 31 CFU/ml, the count might be indicative of fecal pollution occurring at that site. The U. S. EPA should consider the usefulness of C. perfringens as a sludge indicator organism. The EYF-TSC/RCT procedure used in this study would be an excellent procedure to use on a routine basis. The procedure is relatively inexpensive and could be performed in most water and wastewater treatment plant laboratories.

SUMMARY

A modification of the Dudley et al. scheme (1980) for the routine examination of bacteria in sewage sludge was proposed in this study. The modified scheme was used successfully to study bacteria in sewage sludge over a one-year period. The information gathered in this study may prove helpful to those involved in the bacteriological screening of sewage sludge on a regular basis.

EYF-TSC agar in conjunction with the reverse CAMP test (RCT) proved reliable for isolating and identifying C. perfringens in sewage sludge. This procedure was shown to be an appropriate substitute for the procedure for C. perfringens in the Dudley et al. scheme (1980) because it allowed for a reduction in testing time, direct enumeration of bacteria, greater ease in interpreting results, and increased accuracy.

Aeromonas species were easily isolated and identified using selective and differential media developed by Rippey and Cabelli (1979) and a scheme by Cunliffe and Adcock (1989) for the speciation of aeromonads. The substantial numbers of aeromonads isolated indicate the need for the addition of this procedure for aeromonads to the Dudley et al. scheme (1980).

Low numbers of staphylococci were isolated from sludge using MS agar and BP agar. More staphylococci were isolated on the BP agar. Statistical analysis showed a significant difference between the number of staphylococci isolated on BP agar and those isolated on MS agar, indicating BP medium was more effective in isolating staphylococci from sewage sludge. However, both agars failed to reduce background flora sufficiently. Therefore, their use in the routine analysis of sludge cannot be

advocated. Further studies need to be conducted to discover or develop a highly selective and/or highly differential medium for the isolation and enumeration of staphylococci in sewage sludge. The API Staph Ident system (Analytab Products, Inc., Plainview, New York), however, proved to be a useful and rapid procedure for speciating staphylococci from sludge.

No Salmonella species were isolated from sludge using the procedure proposed by Ottolenghi and Hamparian (1987). This may be due to the presence of undetectable levels of Salmonella or to the lack of Salmonella in the sludge. More research is needed to determine if the Ottolenghi and Hamparian (1987) procedure would be an improvement over the one in the Dudley et al. scheme (1980).

Over the year-long study, variations in the aerobic digester temperature and the percentage of sludge solids affected most bacterial counts. Bacterial numbers increased as temperature decreased and sludge solids increased. The numbers decreased as temperature increased. C. perfringens counts were relatively stable over the one year period and exceeded fecal coliform and fecal streptococci counts in all but one sampling. In light of these facts, C. perfringens would be an excellent choice as an indicator organism in the examination of sewage sludge.

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