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DRAFT

# Method 1682: *Salmonella* spp. in Biosolids by Enrichment, Selection and Biochemical Characterization

## August 1998

# Acknowledgments

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## Disclaimer

This method is in draft form. It has not been approved by the U.S. Environmental Protection Agency and should not be construed as an Agency-endorsed method. It is being circulated for comments on its technical merit. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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***Caution:*** *This method is a preliminary draft written from three procedures that have not been rigorously tested. Because the method has not been validated, problems may occur if it is applied as written. EPA encourages constructive suggestions for improvement of the method but cautions reviewers that the method should not be reviewed critically until the method is validated and revised based on that validation.*

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# Introduction

Application of treated biosolids to land is helpful as a crop nutrient and soil conditioner but may pose the risk of release of disease-causing microorganisms into the environment if proper disinfection and use criteria are not met. Among these organisms are *Salmonella* spp., pathogenic members of enteric bacteria that can cause salmonellosis in animals and humans if concentrations able to give rise to infections are present. Density of *Salmonella* spp. in Class A biosolids for unrestricted use is to be less than three most probable number (MPN) per four grams of total solids (dry weight basis) at the time the biosolids are used or disposed.

Method 1682 is a performance-based method for detecting *Salmonella* spp. in biosolids. Method 1682 requires calculation of the MPN via enrichment, with selection and biochemical confirmation for determination of *Salmonella*. All calculations are done on a dry weight basis.

Questions concerning this method or its application should be addressed to:

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Note: This method is performance-based. The laboratory is permitted to modify or omit any step or procedure, provided that all performance requirements set forth in this method are met. The laboratory may not omit any quality control analyses. The terms “shall” “must,” and “may not” indicate steps and procedures required for producing reliable results. The terms “should” and “may” indicate optional steps that may be modified or omitted if the laboratory can demonstrate that the modified method produces results equivalent or superior to results produced by this method.

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# Method 1682: *Salmonella* spp. in Biosolids by Enrichment, Selection, and Biochemical Characterization August 1998 Draft

## 1.0 Scope and Application

**1.1** This method is for the detection and enumeration of *Salmonella* spp. (CAS registry number 68583-35-7) in treated sewage sludge (biosolids) by enrichment, selection, and characterization. It is intended to enumerate *Salmonella* spp. to help determine the suitability of biosolids for land application in compliance with 40 Code of Federal Regulations (CFR) Part 503. Although Method 1682 is similar to existing recognized procedures in using separate media for enrichment, selection, and confirmation of the organism, it is intended to be more specific with greater recovery. This method is for use in the Environmental Protection Agency's (EPA's) data gathering and monitoring programs under the Clean Water Act, the Resource Conservation and Recovery Act, the Comprehensive Environmental Response, Compensation and Liability Act, and the Safe Drinking Water Act.

**1.2** This method is designed to meet EPA's survey and monitoring requirements of the U.S. Environmental Protection Agency (EPA) in regulating the use and disposal of biosolids under 40 CFR Part 503 Subpart D and can be specifically applied to determining the density of *Salmonella* spp. Subpart D of the Part 503 regulation protects public health and the environment through requirements designed to reduce the potential for contact with disease-bearing microorganisms (pathogens) in biosolids applied to land or placed on a surface disposal site. A biosolid is Class A if the density of *Salmonella* spp. are less than 3 MPN per 4 grams total solids. This method is based on existing procedures and techniques for the determination of *Salmonella* spp. in biosolids for Class A materials.

**1.3** Although the Part 503 regulation does not specify the total number of samples for Class A biosolids, it suggests that a sampling event extend over two weeks, and that at least seven samples be tested to confirm that the mean bacterial density of the samples is below 3 MPN/4g of total biosolid solids on a dry weight basis. The analysis of seven samples increases the method precision by reducing the standard error caused by inherent variations in biosolid quality.

**1.4** Method 1682 can be used for detection of *Salmonella* spp. bacteria. However, this method cannot differentiate between serovars of *Salmonella* spp.

**1.5** EPA plans to validate this method for use on digested, dewatered, composted, and heat-dried, pelletized biosolids.

**1.6** The MPN is a statistical estimating technique that states the range of the bacterial population with 95% confidence. MPN is used for samples which require selection from a heterogeneous population of organisms such as is found in biosolids. The MPN technique will quantify microorganisms to below the compliance limit of < 3 MPN/4 grams dry weight.

**1.7** This method has not been evaluated for use in water samples and is not intended as a test for organisms other than *Salmonella* spp.

**1.8** Each laboratory that uses this method must demonstrate the ability to generate acceptable results using the procedure in Section 9.3.1.

**1.9** Any modification of the method beyond those expressly permitted is subject to the application and approval of alternative test procedures under 40 CFR Part 136.4 and 136.5.

## 2.0 Summary of Method

**2.1** The selenite brilliant green sulfa enrichment broth (SBG), Rappaport-Vasilliadis agar medium-semisolid modification (MSRV), and Rappaport-Vasilliadis (RV) broth protocols presented in Method 1682 provide enumeration of *Salmonella* spp. in biosolids based on the most probable number (MPN) technique. The determination of *Salmonella* spp. involves initial seeding into either the selective medium SBG (for the SBG protocol), the enrichment medium tryptic soy broth (TSB)

(for the MSRV protocol) or the pre-enrichment medium phosphate buffered peptone water (BPW) plus enrichment in RV broth (for the RV protocol). Positives from SBG are streaked onto the selective and differential plating medium xylose-lysine desoxycholate agar (XLD) and modified lysine iron agar (MLIA). Those organisms that exhibit growth in TSB are spotted onto the selective MSRV medium. Positive RV tubes are isolated onto lysine-mannitol-glycerol (LMG) agar and purified onto MacConkey agar. Confirmatory tests for all three protocols are performed with lysine-iron agar (LIA), triple sugar iron agar (TSI), and urease test medium, followed by positive serological typing using polyvalent antisera. Percent total solids determination is performed on a representative biosolids sample for MPN/g dry weight calculations.

## 3.0 Definitions

**3.1** *Salmonella* spp. are gram-negative, predominately motile, facultatively anaerobic rod-shaped bacteria that comprise about 2,000 serovars. In this method, *Salmonella* spp. are those bacteria that prove positive in the biochemical and serological confirmation methods described.

**3.2** Class A biosolids contain *Salmonella* sp. densities below 3 MPN/4g of total solids on a dry weight basis.

## 4.0 Interferences

**4.1** Since the most probable number (MPN) tables are based on a Poisson distribution, the MPN value will be an underestimate of the bacterial density if the sample is not adequately shaken to ensure equal bacterial cell distribution before portions are removed.

**4.2** Low estimates of *Salmonella* spp. may be caused by the presence of high numbers of competing or inhibitory organisms, or toxic substances such as metals or organic compounds.

**4.3** Bacteria that compete with *Salmonella* spp., such as *Proteus*, may overgrow the medium and mask or prevent detection.

**4.4** Percent total solids interferences: See Appendix A.

## 5.0 Safety

**5.1** The biohazard associated with, and the risk of infection from handling *Salmonella* spp. is high in this method. Method 1682 does not purport to address all of the safety problems associated with its use. It is the responsibility of the laboratory to establish appropriate safety and health practices prior to use of this method. In particular, the analyst/technician must know and observe the safety procedures required in a microbiology laboratory that handles frank pathogens while collecting, preparing, using, and disposing of sample reagents and materials. Equipment and supplies that have come into contact with biohazardous material or suspected of containing biohazardous material must be sterilized prior to disposal or re-use. Field and laboratory staff collecting and analyzing environmental samples are under some risk of exposure to pathogenic microorganisms. Staff should apply safety procedures used for handling pathogens to all samples.

**5.2** The laboratory is responsible for maintaining a current awareness file of Occupational Safety and Health Administration regulations regarding the safe handling of the chemicals specified in this method. A reference file of material safety data sheets should be made available to all personnel involved in these analyses. Additional information on laboratory safety can be found in Reference 16.1.

**5.3** Samples may contain high concentrations of biohazardous compounds and must be handled with gloves. If there is evidence of pressure build-up in the sample container from gasses, these sample containers must be opened in a biological safety cabinet. Any positive reference materials must also be handled with gloves and the analyst/technician must never place gloves in or near the face after exposure to media known or suspected to contain salmonella organisms. Laboratory

personnel must change gloves after handling sewage sludge (biosolids), media tubes or any other items which carry pathogenic organisms. Mouth pipetting is prohibited.

## 6.0 Equipment and Supplies

**Note:** *Brand names, suppliers, and part numbers are for illustrative purposes only. No endorsement is implied. Equivalent performance may be achieved using apparatus and materials other than those specified here, but demonstration of equivalent performance that meets the requirements of this method is the responsibility of the laboratory.*

**6.1** Equipment for collection and transport of samples

- **6.1.1** Sterile plastic bags—1-gal
- **6.1.2** Plastic or glass jars—1-L
- **6.1.3** Sterile auger
- **6.1.4** Sterile scoops— Scienceware, Bel Art No. 436904, or equivalent
- **6.1.5** Ice chest—Igloo, Coleman, or equivalent
- **6.1.6** Ice
  - **6.1.6.1** Wet ice—purchased locally, or
  - **6.1.6.2** Ice packs—Blue Ice, UTek cat. no. 429, or equivalent, frozen for use

**Note:** *Blue Ice and similar products may be used, provided the sample is protected from freezing. Do not allow sample to be in direct contact with ice or blue ice because of freezing potential.*

- **6.1.7** Bubble wrap

**6.2** Equipment for mixing biosolids

- **6.2.1** Sterile trowels
- **6.2.2** Sterile plastic sheet

**6.3** Equipment for growth of microorganisms

- **6.3.1** Sterile culture tubes—18 × 150 mm and 16 × 150 mm, autoclavable slip caps and culture tube racks
- **6.3.2** 5- and 10-mL sterile serological pipets and 10-mL plastic wide-mouthed sterile serological pipets—Falcon, Kimble, or equivalent
- **6.3.3** Pipet bulbs, or automatic pipettor—Pipet-Aid, or equivalent
- **6.3.4** Nichrome wire inoculating loop and needle
- **6.3.5** Bunsen burner or alcohol burner
- **6.3.6** Cornwall syringe—sterile, to deliver at least 5 mL
- **6.3.7** Media dispensing pump—Unispense II, or equivalent
- **6.3.8** Incubator, water-jacketed, humidity-controlled, microbiological type to hold temperatures at either 37°C and 42°C ± 0.5°C—Precision, VWR, or equivalent
- **6.3.9** Plastic sterile petri dishes—microbiological grade, 25 mm × 100 mm
- **6.3.10** Glass templates or slides for agglutination test
- **6.3.11** Erlenmeyer flasks—1-L and 2-L Corning, Nalgene, Kimble, or equivalent
- **6.3.12** Stir bar—Fisher cat. no. 14-511-93, or equivalent
- **6.3.13** Stir plate—Fisher cat. no. 14-493-120S, or equivalent

**6.3.14** Homogenization equipment

**6.3.14.1** Sterile blender jars and motor base—Oster, Corning or equivalent

**6.3.14.2** Stomacher and stomacher bags—Oster or equivalent

**6.3.15** Water bath to temper agar media with a range from room temperature to 100°C—Precision, VWR Scientific, or equivalent

**6.3.16** Media filtration equipment—Sterile, 0.22-$\mu$m pore size Millex syringe filters or larger diameter Millipore 0.22-$\mu$m pore size

**6.3.17** Sterile cotton-tipped applicators

**6.3.18** Magnifying glass or dissection scope—Zeiss, or equivalent

**6.3.19** Latex gloves for handling samples and extraction equipment—Microflex, San Francisco, CA, stock no. UL-315-L, or equivalent

**6.3.20** pH meter—Beckman, Corning, or equivalent

**6.3.21** Vortex mixer—Vortex Genie, or equivalent

**6.4** Equipment for percent total solids determination—see Appendix A

**6.5** Miscellaneous labware and supplies

**6.5.1** Flasks—Erlenmeyer, various sizes

**6.5.2** Beakers—glass or plastic, assorted sizes

**6.5.3** Lint-free tissues—KimWipes or equivalent

**6.5.4** Steel pan of water—30" x 26" x 10"

## 7.0 Reagents and Standards

**Note:** *Reagent-grade chemicals must be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society. The agar used in preparation of culture media must be of microbiological grade.*

**7.1** Deionized water—conforming to Specification D 1193, Annual Book of ASTM Standards.

**7.2** Phosphate buffered water—Prepare phosphate buffer stock by adding 34.0 g potassium dihydrogen phosphate ($KH_2PO_4$) into 500 mL of deionized water. Dissolve completely , then using a pH meter, adjust pH to 7.2 ± 0.5 with approximately 175 mL 1 N NaOH. Dilute to 1 L with deionized water. Prepare magnesium chloride stock by adding 81.1 g $MgCl_2$.•$6H_2O$ into 1 L of deionized water and mixing thoroughly to dissolve. Add 1.25 mL stock phosphate buffer solution and 5.0 mL stock magnesium chloride solution to 1 L of deionized water. Autoclave for 15 min at 121°C. If buffer is to be used for making sample suspensions, add 0.1% Tween-80, mix well, dispense into erlenmeyer flasks, and autoclave at 121°C for 15 min. Store at room temperature.

**7.3** Buffered peptone water (BPW)—(Oxoid cat. No. CM509, or equivalent) Add 20 g of BPW to 1 L of deionized water and mix well. Dispense 10 mL volumes per 16 mm × 150 mm culture tube; sterilize in autoclave at 121°C for 15 min. Store at 4°C. To make 2X BPW, add 40 g of BPW medium to 1 L of deionized water and mix well. Dispense 10 mL volumes per 18 × 150 mm culture tubes and sterilize in autoclave at 121°C for 15 min. Store at 4°C.

**Note:** *Tween lowers surface tension of water. Buffer with 0.1% Tween-80 may "bump" or flash boil when removed from the autoclave. Do not fill flask more than half-full for autoclaving. Remove from autoclave carefully, pointing flask top away from face.*

**7.4** Tween-80—Sigma Chemical Co. cat. no. P1754, or equivalent.

**7.5** Tryptic soy broth (TSB)—(DIFCO cat. no. 0370-15, or equivalent) Add 30 g of TSB in 1 L of deionized water and mix well. Adjust pH with enough 1.0 N NaOH or HCl, if necessary, to achieve a pH of 7.3 ± 0.1 at 25°C. Warm to approximately 80°C and mix to dissolve completely. Dispense 10 mL volumes per 16 mm × 150 mm culture tube; sterilize in autoclave for 15 min at 121°C. 2X TSB is prepared by adding 30 g TSB to 500 mL deionized water, adjusting pH as above. Dispense 10 mL volumes into 18 mm × 150 mm culture tubes and autoclave. Store at room temperature in the dark.

**7.6** Lysine-mannitol-glycerol agar (LMG) (Commercially unavailable)—Prepare LMG agar by adding 3.0 g proteose peptone, 5.0 g yeast extract, 5.0 g lysine, 5.0 g mannitol, 5.0 g glycerol, 5.0 g NaCl, 1.0 g sodium desoxycholate, 4.0 g sodium thiosulfate, 1.0 g ferric ammonium citrate, 0.1 g phenol red and 15.0 g agar into 1 L of deionized water. Boil for 2 -3 min while mixing to dissolve thoroughly and cool to 45 - 50°C. Dispense immediately into 25 × 100 mm sterile petri plates at 15 - 20 mL per plate.

**7.7** Rappaport-Vasilliadis agar medium-semisolid modification (MSRV) (DIFCO cat. no. 1868-17)—Add 31.6 g of MSRV to 1 L deionized water and mix well. Boil the medium, but do not autoclave. Cool to 50°C. Add 1.0 mL of a 2% stock solution of Novobiocin (Section 7.11) per liter of medium. Swirl the medium to mix. Immediately pour thick plates with approximately 25 mL each. Since the medium will not be firm, do not invert plates to store. Store at room temperature and use within 48 hours.

**7.8** Rappaport-Vassilliadis broth (RV) (Oxoid cat. No. CM669)—Add 30.0 g of RV to 1 L deionized water and mix well. Heat gently until dissolved completely. Dispense 10 mL per 16 × 150 mm screw cap or slip cap tube. Autoclave for 15 min at 115°C. Store at 4°C. These tubes will be used for the most probable number analysis in the RV broth method. Prepare at least 15 tubes per sample, plus enough to carry the positive controls through the test.

**7.9** Selenite brilliant green sulfa enrichment broth (SBG) (DIFCO cat. no. 0715-17)—Dissolve 24.2 g SBG in 1 L deionized water for single-strength medium. Use 24.2 g in 500 mL for double strength medium. Prepare fresh on the day of use by heating in a 70°C water bath for 1 hr instead of boiling for 10 min as indicated on the manufacturer's instructions. Cool to room temperature prior to use.

**7.10** Xylose-lysine desoxycholate agar (XLD) (DIFCO cat. no. 0788)—Add 57.0 g of XLD agar to 1 L deionized water. Heat to boiling to dissolve completely but do not autoclave. Cool to 47°C and pour into 25 × 100 mm sterile petri plates. Plated medium may be stored for up to 2 weeks at 2 - 8°C.

**7.11** Novobiocin (2%) stock solution (DIFCO cat. no. 3197)—Dissolve 50 mg into 25 mL of deionized water and filter sterilize using a 0.22 $\mu$m pore-size filter into a sterile container. Aliquot 1.1 mL of the stock solution into 2.0 mL cryovials and freeze at -20°C. Can be stored for up to 1 year at -20°C.

**7.12** Modified lysine iron agar (MLIA) (DIFCO cat. no. 0849)—Dissolve 34.5 g LIA into 1 L of deionized water. Add 10 g sucrose, 10 g lactose and 1.5 g bile salts No. 3 (DIFCO # 0130). Heat to boiling and cool to 47°C. Add 3.7 mL stock Novobiocin (Section 7.11) and stir before pouring into 25 × 100 mm sterile petri dishes. Store at 4°C.

**7.13** Triple sugar iron agar (TSI) (DIFCO cat. no. 0265)—Weigh out 65 g TSI into 1 L of deionized water. Heat until boiling to dissolve completely. Dispense 5 mL amounts into 16 × 100 mm test tubes in slant racks, cap and sterilize for 15 min at 121°C and 15 psi. Allow the autoclaved medium to solidify in the slant rack such that half the surface area is on a slant and half is in the butt of the tube. Store at 4°C.

**7.14** Lysine iron agar (LIA) (DIFCO cat. no. 0849)—Add 34.5 g into 1 L of reagent-grade water and mix. Heat to boiling to dissolve completely. Dispense 5 mL amounts into 16 × 100 mm test tubes and sterilize at 121°C for 12 min. Allow the autoclaved medium to solidify in the slant rack such that half the surface area is on a slant and half is in the butt of the tube. Store at 4°C.

**7.15** Urease test broth (DIFCO# 0283-15)—Dissolve 38.7 g urease test broth in 1 L deionized water. Filter sterilize using a 0.22 $\mu$m filter into a sterile flask. Aseptically dispense 3 mL into sterile 16 × 100 mm test tubes using a sterile pipet or sterile Cornwall syringe. Store at 4°C.

**7.16** Sterile physiological saline (0.85% w/v)— Dissolve 8.5 g NaCl in 1 L deionized water. Autoclave for 20 minutes at 121°C. Store at room temperature.

**7.17** MacConkey agar without salt (DIFCO cat.no.0331-17)— Dissolve 47 g MacConkey agar in 1 L deionized water. Sterilize by autoclaving for 15 min at 121°C but avoid prolonged heating. Cool to 45 - 50°C and dispense 25 mL per 25 × 100 mm sterile petri plates. Plated medium may be stored for up to 2 weeks at 2 - 8°C in the dark.

## 8.0 Sample Collection, Preservation, and Storage

**8.1** The most appropriate location of biosolids sample collection is the point prior to leaving the wastewater treatment plant. Samples may be taken from pipes, conveyor belts, bins, compost heaps, drying beds and stockpiles.

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**Note:** *If samples are not to be tested within 1 hour after collection, they should be cooled to below 10°C during a maximum transport time of 6 hours. If samples are brought to 4°C by* ***prompt*** *chilling, 24 hours between sampling and analysis should not adversely affect the results. Samples should not be frozen.*

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**8.2** All sampling containers and equipment must be clean and sterile to prevent contamination or overgrowth by other bacteria or mold.

**8.3** Equipment and container cleaning procedure

- **8.3.1** Wash apparatus with laboratory grade detergent and water.
- **8.3.2** Rinse with tap water.
- **8.3.3** Rinse with 10% HCl acid wash.
- **8.3.4** Rinse with distilled water.
- **8.3.5** Allow to air dry or rinse with an organic solvent such as hexane to promote drying process.
- **8.3.6.** Cover with foil and autoclave for 15 minutes at 121°C.

**8.4** Procedure for sampling digester biosolids

- **8.4.1.** Collect the digester biosolids sample from the outlet pipe used to fill the truck.
- **8.4.2** Purge the pipe of old biosolids and warm to the digester temperature by allowing biosolid to flow through the pipe into a bucket.
- **8.4.3** Position a 1-gal. sterile bag under the flow so that nothing but sample touches the inside of the bag. Fill the bag, but leave 0.5 in. of head space in the bag for gas production. Leaving head room is extremely important when taking samples of biosolids that have been anaerobically digested.

**8.5** Procedure for sampling conveyor belt biosolid

- **8.5.1** Collect the biosolids sample directly into the sample container without mixing or transferring to another area.
- **8.5.2** Using a sterile scoop, transfer the pressed biosolids directly off of the conveyer and into a sterile container.
- **8.5.3** Pack the sample into the container. Head space is not as important as with a liquid sample because there is less gas formation.

**8.6** Procedure for sampling from a bin, drying bed, truck bed or similar container

**8.6.1** Divide the material to be sampled into four quadrants.

**8.6.2** Use a scoop or core the sample if material is deep.

**8.6.3** Take a sample from each of the quadrants and combine in a stainless steel or plastic bucket.

**8.6.4** After all the samples have been taken, pour the bucket contents out onto a plastic sheet and mix by folding the sample back onto itself several times.

**8.6.5** Reduce the sample size by "coning and quartering." Divide the bucket contents into four even piles. If sample size is still too large, divide each quarter into quarters and discard half of each quarter. Put into glass or plastic sampling container.

**8.6.6** An alternate method to "coning and quartering" is to randomly take flat shovel-fulls of biosolid from the bucket that has been dumped onto a plastic sheet and put those samples into a sampling container. (Curved scoops have been shown to favor a certain size particle and therefore should not be used.)

**8.7** Record the following into your log book:

**8.7.1** Facility name and location

**8.7.2** Date

**8.7.3** Arrival time

**8.7.4** Name of facility and contact

**8.8** Record the following onto sample container when known:

**8.8.1** Sample number

**8.8.2** Date and time

**8.8.3** Sample name

**8.8.4** Sample location

**8.8.5** Parameters

**8.8.6** Volume

**8.8.7** Observations

**8.9** Ensure that the chain of custody form is filled out

**8.10** Sample preservation and handling—Ice or refrigerate bacteriological samples at a temperature of 1°C to 4°C during transit to the laboratory. Do not freeze sample. Use insulated containers to assure proper maintenance of storage temperature. Sample bottles should be placed inside sealable ziplock-type bags, excess air purged, and bags sealed to ensure that bottles remain dry during transit or storage. Refrigerate samples upon arrival in the laboratory and analyze as soon as possible after collection. Bring samples to room temperature before analysis.

**8.11** Chlorinated samples—Add a reducing agent to containers intended for the collection of biosolids containing residual chlorine or other halogen. Sodium thiosulfate ($Na_2S_2O_3$) is a satisfactory dechlorinating agent that neutralizes any residual halogen and prevents continuation of bactericidal action during sample transport. If $Na_2S_2O_3$ is used, add a sufficient volume of $Na_2S_2O_3$ to a clean sample bottle, to give a concentration of 100 mg/L in the sample. In a 120-mL sample bottle, a volume of 0.1 mL of a 10% solution of $Na_2S_2O_3$ will neutralize a sample containing about 15 mg/L residual chlorine.

**8.12** Holding time limitations—Analyses should begin as soon as possible, preferably, within 8 hours of collection. If it is impossible to examine samples within 8 hours, refrigerate samples at 4°C and analyze within 24 hours.

**Note:** *Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Sample results will be considered invalid if those conditions are not met.*

## 9.0 Quality Control

**9.1** Each laboratory that uses this method is required to operate a formal quality assurance (QA) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability through analysis of positive and negative control samples (Section 9.4) and blanks (Section 9.5), and analysis of positive and negative control samples and blanks as tests of continued performance. Laboratory performance is compared to the performance criteria specified in Sections 9.4 and 9.5 to determine if the results of analyses meet the performance characteristics of the method. Specific quality control (QC) requirements for Method 1682 are provided below. General recommendations on QA and QC for facilities, personnel, and laboratory equipment, instrumentation, and supplies used in microbiological analyses are provided in the USEPA microbiology methods manual, Part IV, C (4) (Reference 16.2).

**9.2** If an analytical technique other than the techniques specified in this method is used, that technique must have a specificity and recovery equal to or better than the specificity and recovery of the techniques in this method for *Salmonella* in the sample of interest. Specificity is defined as producing results equivalent to the results produced by this method for *Salmonella*, and that meet all of the QC acceptance criteria stated in this method. Recovery is defined as the ability of the test to enumerate viable *Salmonella* spp. without losses as a consequence of environmental parameters or the test method.

**9.2.1** Each time a modification is made to this method, the analyst is required to repeat the positive and negative control samples (Section 9.4) to demonstrate that the modification produces results equivalent or superior to results produced by this method.

**9.2.2** The laboratory is required to maintain records of modifications made to this method. These records include the following, at a minimum:

**9.2.2.1** The names, titles, addresses, and telephone numbers of the analyst(s) who performed the analyses and modification, and of the quality control officer who witnessed and will verify the analyses and modification.

**9.2.2.2** A listing of the analyte measured (*Salmonella* spp.).

**9.2.2.3** A narrative stating reason(s) for the modification.

**9.2.2.4** Results from all QC tests comparing the modified method to this method, including initial precision and recovery (analysis of positive and negative control samples (Section 9.4), and analysis of blanks (Section 9.6).

**9.2.2.5** Data that will allow an independent reviewer to validate each determination by tracing the processing and analysis steps leading to the final result.

**9.3** Analytical QC procedures required per batch of field samples (batch is defined as 20 samples or one 12-hour laboratory shift, whichever comes sooner).

**9.3.1** Perform one positive control sample and one negative control sample (Section 9.4) per batch of field samples

**9.3.2** Perform one blank sample (Section 9.5) per batch of field samples

**9.3.3** Perform duplicate analyses on at least one sample per batch. In laboratories with more than one analyst, have each make parallel analyses on at least one positive sample monthly.

**9.3.4** Check dilution water for sterility by adding 20 mL water to 100 mL of a nonselective broth such as tryptic soy broth. Incubate at 35 ± 0.5° C for 24 hours and observe growth. If any contamination is indicated, reject analytical data from samples tested with these materials.

**9.3.5** Test medium sterility by subjecting a representative portion of each batch to incubation at 37 ±0.5°C for 24 to 48 hours and observe for indications of growth.

**9.4** Positive and negative control samples. Obtain reference cultures from qualified outside sources and use these to establish pure stock cultures that are maintained for the laboratory. Analyze positive and negative control samples with each batch of field samples and with each new lot of media. For positive control, inoculate the medium with a known positive *Salmonella* sp.(e.g. S. typhimurium); for negative control, inoculate the medium with a known negative *Salmonella* sp. (e.g. *Escherichia. coli*). Test each batch of each individual medium, reagent, or antisera with both positive and negative controls to verify correct response.

**9.5** Process a positive sample through the entire analytical procedure and examine for appropriate responses. Process a negative control sample until it is verified that culture is negative.

**Table 1. Positive and Negative Control Results**

| Medium | Reaction for *Salmonella* spp. | Negative Reaction for *Salmonella* spp. |
|---|---|---|
| Selenite brilliant green | "Brick" red color | Medium remains clear green color |
| Rappaport-Vasilliadis broth | Turbidity indicates growth of multiple types of microorganisms | |
| Tryptic soy broth | General purpose growth medium. Growth of any organism results in turbid solution. | |
| Lysine mannitol glycerol agar | Colonies have black centers surrounded by a yellow-orange to pink zone. *S. typhi* is yellow with a yellow zone and a black center after 48 h. *Salmonella* spp. which are $H_2S$ negative are pink or colorless with pink zones. | Yellow colonies with yellow zone. *Proteus* spp. are small, with dark greenish-grey or black centers surrounded by a pink zone. |
| Rappaport-Vasilliadis agar medium-semisolid modification | Migrated cells visible as a gray-white turbid zone extending out from the inoculated drop | Medium remains blue-green around the drop with no gray-white turbid zone |
| Xylose lysine desoxycholate agar | Pink to red colonies with black centers. (*Shigella* spp.are red) | *Enterococcus* spp. and *E. coli* are yellow with bile ppt |
| MacConkey Agar | Transparent or slightly opaque colonies | Other color indicators are organisms but not *Salmonella* spp. |
| Modified lysine iron agar | Purple medium with colonies having "fried egg" appearance and smooth edges. Centers are black and nucleated. | Atypical growth and color change to yellow if *Enterobacteriaceae* are present |
| Lysine iron agar | Alkaline slant (purple) with alkaline butt (purple) with or without $H_2S$ | Other color indicators are organisms but not *Salmonella* spp. |
| Triple sugar iron agar | Good growth with alkaline slant (red) with acid butt (yellow) and $H_2S$ production (Black butt) | Other color indicators are organisms but not *Salmonella* spp. |
| Urease test broth | No color change (negative urease means it's positive for *Salmonella* spp.) | Pink (positive urease, but negative for *Salmonella* spp.) |

## 10.0 Calibration and Standardization

**10.1** Check temperatures in incubators daily to ensure operation is within stated limits of the method and record daily measurements in incubator log book.

**10.2** Check/calibrate thermometers twice per year against a NIST certified thermometer or one that meets the requirements of NIST Monograph SP 250-23. Check mercury columns for breaks.

**10.3** Calibrate pH meter prior to each use using standards of pH 4.0 and 7.0.

**10.4** Calibrate top-loading balances once per month with reference weights of ASTM Class 2.

**10.5** All equipment must be calibrated according to manufacturer's instructions and maintained on a schedule as dictated in their maintenance manual.

## 11.0 Procedure

**11.1** Three types of procedures are given in the following sections. All procedures entail identical homogenization, biochemical and serological confirmation steps, however, the beginning steps of each method are different. The first method is the selenite brilliant green sulfa enrichment broth (SBG) procedure which involves enrichment in SBG followed with selection in xylose lysine agar (XLD) and modified lysine iron agar (MLIA). The second procedure is the Rappaport-Vasilliadis agar medium-semisolid modification (MSRV) procedure that enriches with tryptic soy broth (TSB) then selects in MSRV. The third procedure is the Rappaport-Vasilliadis broth (RV) method which pre-enriches with buffered peptone water, enriches with RV broth, isolates onto LMG agar with purification of isolates onto MacConkey's agar. All procedures are followed by identical biochemical and serological confirmation steps.

**11.2** Homogenization

**11.2.1** Prepare sample suspensions of biosolids by weighing out a sufficient amount of biosolids so that at least 4 g dry weight will be present. If the sample contains more than 10% solids in the sample then use 50 g wet weight. If less solids are present, then use a sample known to contain at least 4 g dry weight. Add Tween-80 directly to liquid samples to a 0.1% v/v concentration.

**11.2.2** Place the material in a stomacher bag or blender and add 100 mL of phosphate buffered water plus 0.1% Tween-80 if 4g dry weight is used or 500 mL phosphate buffered water plus 0.1% Tween-80 if 50 g is weighed out.

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**Note:** *This procedure may be altered as a result of testing to standardize biosolids preparation.*

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**11.2.2.1** Homogenization using a using a stomacher

**11.2.2.1.1** Seal the stomacher bag and stomach for approximately 5 min. at normal speed.

**11.2.2.1.2** Cut almost through the top corner seal of the stomacher bag with scissors. This will avoid contamination of the scissors and the contents of the bag.

**11.2.2.1.3** Tear off the bag corner and pour the contents into a 250 mL sterile disposable centrifuge tube.

**11.2.2.2** Homogenization using a mechanical mixer:

**11.2.2.2.1** Place a cover on the blender and mix at medium speed for 2 to not more than 5 min.

**11.3** Enrichment and selection

**11.3.1** Selenite brilliant green sulfa enrichment broth (SBG) procedure

**11.3.1.1** Preparation

**11.3.1.1.1** Prepare phosphate buffered water (Section 7.2) and Novobiocin stock solution (Section 7.11) according to their recipes. Follow instructions for storage.

**11.3.1.1.2** Prepare selenite brilliant green sulfa enrichment broth (SBG) (1X and 2X) (Section 7.9), and cool to room temperature. Prepare most probable number (MPN) tubes by aseptically dispensing 10 mL of 2X SBG into five sterile 18 mm × 150 mm tubes per each biosolids sample. Aseptically dispense 10 mL of 1X SBG into twelve sterile 16 mm × 150 mm tubes per each biosolid sample.

**Note:** *SBG must be used on the same day it is made.*

**11.3.1.1.3** Prepare 400 mL of each of the primary isolation media: XLD (Section 7.10) and MLIA (Section 7.12 ) per biosolids sample. Pour 15 plates (25 mL per plate) of each media per sample. Allow plates to solidify and excess moisture to evaporate prior to use. Follow instructions for storage.

**11.3.1.1.4** Prepare 30 tubes of the biochemical medium TSI (Section 7.13), LIA (Section 7.14) and urease test broth (Section 7.15) for each sample.

**11.3.1.1.5** Assemble five 2X SBG tubes and 12, 1X SBG tubes per biosolid sample for MPN determination. (One 1X SBG tube will be used for positive control and one 1X SBG tube will be used for negative control.)

**11.3.1.2** Enrichment procedure required for calculation of most probable number (MPN)

**11.3.1.2.1** Pipet 10 mL of the homogenized biosolids from Section 11.2 into each of the 5 tubes containing 10 mL 2X SBG.

**11.3.1.2.2** Pipet 1.0 mL of the homogenized biosolids into each of the 5 tubes of 1X SBG, and 0.1 mL into each of the remaining 5 tubes of 1X SBG.

**11.3.1.2.3** Inoculate one tube 1X SBG with a *Salmonella* culture and one tube SBG with an *E. coli* culture for positive and negative controls, respectively.

**11.3.1.2.4** The controls will be carried through to the plating step as a QC measure to detect media or incubator failure.

**11.3.1.2.5** Incubate SBG tubes for 20 to 24 hours at 37°C.

**11.3.1.2.6** Record all “brick red” tubes as presumptive positive along with sample number in lab notebook.

**11.3.1.3** Selection

**11.3.1.3.1** Streak the positive SBG tubes, including positive control tubes, to plates containing the selective medium xylose-lysine desoxycholate agar (XLD) and modified lysine iron agar (MLIA)

for primary isolation of *Salmonella* spp. Incubate plates for 18 to 24 hr at 37°C. Inoculate plates with fresh negative control.

**11.3.1.3.2** Do not discard the SBG broth tubes. Instead, retain the SBG broth tubes for an additional 24 hours at 37°C until after the isolation plates have been incubated and examined.

**11.3.1.3.3** Examine the primary isolation plates (XLD and MLIA) and determine whether the pattern of *Salmonella* spp. isolation follows a logical dilution distribution pattern. The pattern should exhibit more total positives in the least dilute and fewer positives in the most dilute. If this pattern is not established then inoculate 1 mL from the least dilute/negative 48 hour SBG tubes into fresh SBG tubes, and incubate 18 to 24 hours at 37°C to find the *Salmonella*.

**11.3.1.3.4** Proceed to Section 11.4 for biochemical confirmation of *Salmonella* and biochemical characterization using TSI, LIA , urease test broth and polyvalent O antisera reaction.

**11.3.2** Rappaport-Vasilliadis agar medium-semisolid modification (MSRV) procedure

**11.3.2.1** Preparation

**11.3.2.1.1** Prepare phosphate buffered water (Section 7.2) and Novobiocin stock solution (Section 7.11) according to their recipes. Follow instructions for storage.

**11.3.2.1.2** Prepare tryptic soy broth (TSB) (1X and 2X) (Section 7.5) according to directions. Prepare MPN tubes by aseptically dispensing 10 mL of the 2X TSB into 5 sterile 18 mm × 150 mm tubes for each biosolid sample. Aseptically dispense 10 mL of the 1X TSB into 10 sterile 16 mm × 150 mm tubes per each biosolid sample. Dispense 2 more tubes of 1X TSB for controls per set of samples.

**11.3.2.1.3** Prepare 400 mL of the MSRV primary isolation medium (Section 7.6) per biosolids sample. Pour 15 plates (25 mL per plate) of the medium per biosolid sample. Allow to cool and solidify before use. Store up right as media will not be firm.

**11.3.2.1.4** Prepare 400 mL of the isolation medium xylose-lysine desoxycholate agar (XLD) (Section 7.10) per biosolids sample. Pour 15 plates (25 mL per plate) of the media per biosolid sample. Allow plates to solidify and dry slightly prior to use. Follow instructions for storage.

**11.3.2.1.5** Prepare 30 slant tubes of each of the biochemical confirmation media: TSI (Section 7.13), LIA (Section 7.14) and urease test broth (Section 7.15).

**11.3.2.1.6** Assemble five 2X TSB tubes and twelve 1X TSB tubes per biosolid sample for MPN determination. (One 1X TSB tube will be used for positive control and one 1X TSB tube will be used for negative control.)

**11.3.2.2** Enrichment procedure required for calculation of most probable number (MPN)

**11.3.2.2.1** Add 10 mL aliquots of homogenized biosolid sample from Section 11.2 to five tubes containing 10 mL of 2X TSB.

**11.3.2.2.2** Pipet 1.0 mL of homogenized sample into each of five tubes of 1X TSB, and 0.1 mL to the remaining 5 tubes of 1X TSB. The MPN enrichment is carried out using 10 mL of inoculum per 10 mL 2X broth. The 1X broth is inoculated with 1.0 and 0.1 mL quantities of sample.

**11.3.2..2.3** For positive and negative controls, inoculate one tube of 1X TSB with a loopful of *Salmonella* culture and one tube TSB with a loopful of an *E. coli* culture, respectively. The controls will be carried through to the plating step as a QC measure to detect media or incubator failure.

**11.3.2.2.4** Incubate the TSB MPN tubes and controls for 24 hours at 35°C.

**11.3.2.2.5** Record all turbid tubes as positive along with sample number in lab notebook. Because of the non-inhibitory nature of the enrichment medium, all tubes will be positive in most instances. If the tubes do not appear positive, then this may indicate the presence of a toxic substance.

**11.3.2.3** Selection

**11.3.2.3.1** Apply six, 30-$\mu$L drops from each positive tube onto a corresponding MSRV plate. Allow the drops to absorb into the agar for 1 hour at room temperature, and incubate each plate, inoculated side up, at 42 ±0.5°C for 16 to 18 hours in a humidity-controlled hot air incubator. An open pan of water placed in the bottom of the incubator will serve the same purpose.

**11.3.2.3.2** Examine plates for the appearance of motility surrounding the spots, as evidenced by a "whitish halo" of growth approximately 2 cm from the center of the spot.

**11.3.2.3.3** Material from the outer edge of the halo is taken up with a sterile inoculating needle and streaked onto XLD agar for isolation.

**11.3.2.3.4** Incubate XLD plates for 18 to 24 hours at 35°C.

**11.3.2.3.5** Proceed to Section 11.4 for confirmation of *Salmonella* spp. and biochemical characterization using TSI, LIA , urease test broth and polyvalent O antisera reaction.

**11.3.3** Rappaport-Vasilliadis broth (RV) method procedure

**11.3.3.1** Preparation

**11.3.3.1.1** Prepare buffered peptone water (BPW) (Section 7.3). Follow instructions for storage.

**11.3.3.1.2** Prepare Rappaport-Vasilliadis (RV) broth (Section 7.8), and cool to room temperature prior to use.

**11.3.3.1.3** Prepare lysine-mannitol-glycerol (LMG) agar according to its recipe. (Section 7.6). Pour 15 plates (25 mL per plate) of the medium per biosolid sample. Allow plates to solidify and dry slightly prior to use. Follow instructions for storage.

**11.3.3.1.4** Prepare MacConkey agar (Section 7.17) and follow instructions for storage. Pour 15 plates (25 mL per plate) of the media per biosolid sample. Allow plates to solidify and dry slightly prior to use. Follow instructions for storage.

**11.3.3.2** Pre-enrichment procedure for calculation of most probable number (MPN)

**11.3.3.2.1** Inoculate 10 mL of the homogenized biosolid sample from Section 11.2 into each of the 5 tubes containing 10 mL of 2X BPW.

**11.3.3.2.2** Pipet 1.0 mL of the homogenized biosolids into each of 5 tubes of 1X BPW, and 0.1 mL into each of the remaining 5 tubes of 1X BPW.

**11.3.3.2.3** Inoculate one tube 1X BPW with a lab stock *Salmonella* culture and one tube BPW with an *E. coli* culture for positive and negative controls, respectively.

**11.3.3.2.4** The controls will be carried through to the plating step as a QC measure to detect media or incubator failure.

**11.3.3.2.5** Incubate the pre-enrichment PBW MPN tubes and controls at 37°C for 24 hours.

**11.3.3.2.6** Record all turbid tubes as positive along with sample number in lab notebook. Because of the non-inhibitory nature of the enrichment medium, all tubes will be positive in most instances. If the tubes do not appear positive, then this may indicate the presence of a toxic substance.

**11.3.3.3** Enrichment step for *Salmonella* spp. in RV broth

**11.3.3.3.1** Transfer 0.1 mL from each turbid buffered peptone water pre-enrichment tube to a corresponding tube of RV broth.

**11.3.3.3.2** Incubate the enrichment RV tubes and controls at 43°C for 48 hours.

**11.3.3.4** Isolation

**11.3.3.4.1** After 48 hours, subculture each of the positive RV broth tubes onto an individual plate of lysine-mannitol-glycerol (LMG) agar. Subculturing may be performed by transferring the organisms using a sterile cotton swab or flamed loop. The plate should be streaked for single cell colony formation.

**11.3.3.4.2** Incubate the LMG plates at 37°C for 24 hours.

**11.3.3.5** Purification

**11.3.3.5.1** After the 24 hour incubation, colonies suspected of being *Salmonella* spp. are purified onto MacConkey agar. The suspect colony is picked up via sterile loop from the LMG plate and is streaked for single cell colony formation onto MacConkey agar.

**11.3.3.5.2** Incubate MacConkey agar plates at 37°C for 24 hours.

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**Note:** *Experienced analysts in the isolation of single cell colonies may be able to culture isolated Salmonella. colonies on the LMG plate such that purification is redundant. If this is the case, it is allowable that biochemical confirmation (Section 11.4) follows the isolation step (Section 11.3.3.4).*

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**11.3.3.5.3** Proceed to Section 11.4 for biochemical confirmation of *Salmonella* and biochemical characterization using TSI, LIA , urease test broth and polyvalent O antisera reaction.

**11.4** Biochemical confirmation

**11.4.1** Label all tubes with date, sample, and experiment. Pick those isolated colonies exhibiting *Salmonella* sp. morphology as defined in Table 1 for the medium being used, from the plate to slants of triple sugar iron agar (TSI), lysine iron agar (LIA), and urease test broth. Use the same colony to inoculate all three media. Inoculate positive and negative control organisms into each medium. Inoculate agar medium with a needle into the butt of the tube and streak along the slant. Incubate for 24 hours at 35°C. A positive TSI reaction is an acid butt (yellow in color) and an alkaline slant (purple in color) with or without $H_2S$ gas production. A positive LIA reaction is an alkaline butt, alkaline slant with or without the production of $H_2S$. Urease is an orange medium and will change to pink or cerise if positive. A negative urease test is one that exhibits no color change after inoculation. *Salmonella* spp. are negative for urease.

**11.4.2** To confirm cultures via polyvalent O antiserum: Emulsify isolates on the slant portion of either LIA or TSI using sterile physiological saline (0.85%). Place one drop of suspension into each of 2 slide wells. Add one drop of sterile saline to one well as a control and one drop of polyvalent O antiserum (DIFCO) to the second well. Observe the template under magnification for an agglutination reaction.

**11.4.3** Correlate all positive plates and tubes to original positive tube from which sample was plated. Record all positive samples. Determine the MPN from this information (see Section 12.0). Record all data clearly into a laboratory notebook. When percent total solids has been determined, record the information with results.

**11.5** Percent total solids determination: See Appendix A

## 12.0 Data Analysis and Calculations

**Note:** *See the USEPA microbiology manual, Part II, Section C, 3.5, for general counting rules (2). The Most Probable Number (MPN) procedure is used to estimate bacterial density following the multiple-tube fermentation technique. Conversion is made from MPN/100 mL to MPN/g. Bacterial density is recorded as MPN/g dry weight.*

**12.1** Estimation of *Salmonella* spp. density

**12.1.1** The estimated density of *Salmonella* spp. bacteria, based on the confirmation test using the polyvalent O antiserum reaction, is computed in terms of the most probable number (MPN) procedure. The MPN value may be obtained from the MPN table (Table 3) below using the number of positive tubes in three significant dilutions.

**12.1.2** Only three of the four series of five selection tubes will be used for estimating the MPN or "MPN code." The MPN code represents the total number of positive tubes for *Salmonella* spp. per dilution. The three dilutions are called significant dilutions.

**12.1.3** Scenarios for selection of significant dilutions

**12.1.3.1** When more than three dilutions are positive, use results from only three dilution series to compute MPN. Choose the highest dilution (the most dilute, with least sample volume, e.g., 0.01 is higher than 0.1) giving positive results in all five tubes inoculated and the two succeeding higher dilutions.

**12.1.3.2** If there are positive tubes in higher dilutions than the dilutions selected, positive results are moved up from these dilutions to increase the positive tubes in the next highest dilution selected.

**12.1.3.3** If all tubes are positive, choose the three highest dilution series. If all tubes are negative, choose the three lowest dilution series.

**12.1.3.4** In the significant dilution example table (Table 2), the numerator represents the number of positive tubes per sample dilution and the denominator represents total number of tubes inoculated per sample dilution.

**12.1.4** Use Table 3 to estimate the MPN index/100 mL from the significant dilutions chosen.

**12.1.5** The MPN for combinations not appearing in the Table 3 may be estimated by Thomas' formula (Equation 2). If such unlikely tube combinations occur in more than 1% of the samples, it is an indication that the procedure is faulty or that the statistical assumptions underlying the MPN estimate are not being fulfilled.

**Equation 2 (Thomas' formula)**

$$MPN \ / \ 100\ mL = \frac{number\ of\ positive\ test\ tubes\ X\ 100}{\sqrt{(mL\ sample\ in\ negative\ tubes\ X\ mL\ sample\ in\ all\ tubes)}}$$

**12.1.6** Due to the extreme variability of the solid content of biosolids, results from biosolid samples are converted to MPN/g using the Equation 3.

**Equation 3**

$$MPN \ / \ g = \frac{10\ (MPN\ index\ from\ Table\ 3)}{(largest\ significant\ dilution\ volume)(\%\ total\ solids)}$$

**12.1.7** After the MPN/g has been calculated, the biosolid sample can be evaluated according to Class A biosolid pathogen requirements.

**TABLE 2. SIGNIFICANT DILUTIONS IN DETERMINING MPN INDEX (SIGNIFICANT DILUTIONS ARE UNDERLINED)**

| *Sample* | *10.0 mL* | *1.0 mL* | *0.1 mL* | *0.01 mL* | *Significant Dilution Results* |
|---|---|---|---|---|---|
| *A* | 5/5 | 5/5 | 3/5 | 0/5 | 5-3-0 |
| *B* | 5/5 | 3/5 | 1/5 | 1/5 | 5-3-2 |
| *C* | 0/5 | 1/5 | 0/5 | 0/5 | 0-1-0 |

**Table 3. MPN Table**

| Combination of Positives | MPN Index/ 100 mL | 95% Confidence Limits | | Combination of Positives | MPN Index/ 100 mL | 95% Confidence Limits | |
|---|---|---|---|---|---|---|---|
| | | Lower | Upper | | | Lower | Upper |
| 0-0-0 | < 2 | --- | --- | 4-2-0 | 22 | 9.0 | 56 |
| 0-0-1 | 2 | 1.0 | 10 | 4-2-1 | 26 | 12 | 65 |
| 0-1-0 | 2 | 1.0 | 10 | 4-3-0 | 27 | 12 | 67 |
| 0-2-0 | 4 | 1.0 | 13 | 4-3-1 | 33 | 15 | 77 |
| | | | | 4-4-0 | 34 | 16 | 80 |
| 1-0-0 | 2 | 1.0 | 11 | | | | |
| 1-0-1 | 4 | 1.0 | 15 | 5-0-0 | 23 | 9.0 | 86 |
| 1-1-0 | 4 | 1.0 | 15 | 5-0-1 | 30 | 10 | 110 |
| 1-1-1 | 6 | 2.0 | 18 | 5-0-2 | 40 | 20 | 140 |
| 1-2-0 | 6 | 2.0 | 18 | 5-1-0 | 30 | 10 | 120 |
| | | | | 5-1-1 | 50 | 20 | 150 |
| 2-0-0 | 4 | 1.0 | 17 | 5-1-2 | 60 | 30 | 180 |
| 2-0-1 | 7 | 2.0 | 20 | 5-2-0 | 50 | 20 | 170 |
| 2-1-0 | 7 | 2.0 | 21 | 5-2-1 | 70 | 30 | 210 |
| 2-1-1 | 9 | 3.0 | 24 | 5-2-2 | 90 | 40 | 250 |
| 2-2-0 | 9 | 3.0 | 25 | 5-3-0 | 80 | 30 | 250 |
| 2-3-0 | 12 | 5.0 | 29 | 5-3-1 | 110 | 40 | 300 |
| | | | | 5-3-2 | 140 | 60 | 360 |
| 3-0-0 | 8 | 3.0 | 24 | 5-3-3 | 170 | 80 | 410 |
| 3-0-1 | 11 | 4.0 | 29 | 5-4-0 | 130 | 50 | 390 |
| 3-1-0 | 11 | 4.0 | 29 | 5-4-1 | 170 | 70 | 480 |
| 3-1-1 | 14 | 6.0 | 35 | 5-4-2 | 220 | 100 | 580 |
| 3-2-0 | 14 | 6.0 | 35 | 5-4-3 | 280 | 120 | 690 |
| 3-2-1 | 17 | 7.0 | 40 | 5-4-4 | 350 | 160 | 820 |
| | | | | 5-5-0 | 240 | 100 | 940 |
| 4-0-0 | 13 | 5.0 | 38 | 5-5-1 | 300 | 100 | 1300 |
| 4-0-1 | 17 | 7.0 | 45 | 5-5-2 | 500 | 200 | 2000 |
| 4-1-0 | 17 | 7.0 | 46 | 5-5-3 | 900 | 300 | 2900 |
| 4-1-1 | 21 | 9.0 | 55 | 5-5-4 | 1600 | 600 | 5300 |
| 4-1-2 | 26 | 12 | 63 | 5-5-5 | ≥1600 | --- | — |

## 13.0 Method Performance

**13.1** Expected performance data will be included into this method when the validation studies have been completed.

## 14.0 Pollution Prevention

**14.1** Selenite is a carcinogen and should be disposed of as indicated on the media package. The other solutions and reagents used in this method pose little threat to the environment when recycled and managed properly.

**14.2** Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be disposed.

## 15.0 Waste Management

**15.1** The laboratory is responsible for complying with all Federal, State, and local regulations governing waste management, particularly hazardous waste identification rules and land disposal restrictions, and for protecting the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required. An overview of requirements can be found in *Environmental Management Guide for Small Laboratories* (EPA 233-B-98-001).

**15.2** Samples, reference materials, and equipment known or suspected to have viable *Salmonella* spp. attached or contained must be sterilized prior to disposal.

**15.3** For further information on waste management, consult *The Waste Management Manual for Laboratory Personnel* and *Less Is Better: Laboratory Chemical Management for Waste Reduction*, both available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington D.C. 20036.

## 16.0 References

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**16.6** Dr. Robert C. Cooper: Paper in Progress: *Detection of Salmonella in Biosolids Using Rappaport-Vassiliadis Semisolid Agar*. BioVir Laboratories, Benicia, CA.

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**16.8** Rappold, Helmut, and Robert F. Bolderdijk. *Modified Lysine Iron Agar for Isolation of Salmonella from Food.* Applied and Environmental Microbiology.1979. 38 (1): 162 -163.

**16.9** Hu, Catherine J., Gibbs, R.A., and Goen E. Ho. 1995. *Detection of Salmonella in Composted Wastewater Sludge.* Environmental Science Report No.95/5. Murdoch University. ISBN No. 0-86905-429-5.

## 17.0 Tables, Diagrams, Flowcharts, and Validation Data

To be included in future draft, as required

## 18.0 Glossary of Definitions and Purposes

These definitions and purposes are specific to this method but have been conformed to common usage as much as possible.

**18.1** Units of weight and measure and their abbreviations

**18.1.1** Symbols

| | |
|---|---|
| °C | degrees Celsius |
| μL | microliter |
| < | less than |
| > | greater than |
| % | percent |

**18.1.2** Alphabetical characters

| | |
|---|---|
| cm | centimeter |
| gal | gallon |
| g | gram |
| L | liter |
| M | molar |
| mg | milligram |
| min | minute |
| mL | milliliter |
| mm | millimeter |
| MPN | most probable number |
| OD | outside diameter |
| s | second |
| v/v | volume per unit volume |
| w/v | weight per unit volume |

**18.2** Definitions, acronyms, and abbreviations (in alphabetical order).

Analyte—The microorganism tested for by this method. The analytes in this method are *Salmonella* spp.

Differential medium—A solid culture medium that makes it easier to distinguish colonies of the desired organism.

Deionized water—water that has been through mixed bed resin columns and is demonstrated to be free from the analytes of interest and potentially interfering substances.

Enrichment—Using a culture medium to increase growth of the target organism prior to isolation of that organism.

Field blank—An aliquot of sterile biosolids or other reference matrix that is placed in a sample container in the laboratory or the field, and treated as a sample in all respects, including exposure to sampling site conditions, storage, and all analytical procedures. The purpose of the field blank is to determine if the field or sample transporting procedures and environments have contaminated the sample.

Laboratory blank—See Method blank.

Laboratory reagent blank—See Method blank.

May—This action, activity, or procedural step is neither required nor prohibited.

May not—This action, activity, or procedural step is prohibited.

Method blank—An aliquot of sterile biosolids or designated matrix that is treated exactly as a sample including exposure to all glassware, equipment, media, procedures that are used with samples. The method blank is used to determine if analytes or interferences are present in the laboratory environment.

Most probable number method (MPN)—A statistical determination of the number of bacteria per weight or volume of sample. It is based on the fact that the greater the number of bacteria in a sample, the more dilution is needed to reduce the density to the point at which no bacteria are left to grow in the tubes in a dilution series.

Must—This action, activity, or procedural step is required.

Negative control—A suspension of bacteria that will not give a positive reaction during any portion of this method such as *E. coli*.

Positive control—See Ongoing precision and recovery standard.

Preferred—Optional

Preparation blank—See Method blank.

Selective medium—A culture medium designed to suppress the growth of unwanted microorganisms and encourage the growth of desired ones.

Should—This action, activity, or procedural step is suggested but not required.

Stock suspension—A suspension containing an analyte that is prepared using a reference material traceable to EPA, the National Institute of Science and Technology (NIST), or a source that will attest to the purity and authenticity of the reference material.

# Appendix A: Total Solids in Solid and Semisolid Matrices

## 1.0 Scope and Application

**1.1** This procedure is applicable to the determination of total solids in such solid and semisolid samples as soils, sediments, biosolids separated from water and wastewater treatment processes, and biosolid cakes from vacuum filtration, centrifugation, or other biosolid dewatering processes.

**1.2** This procedure is taken from EPA Method 1684: *Total, Fixed, and Volatile Solids in Solid and Semi-Solid Matrices.*

**1.3** Method detection limits (MDLs) and minimum levels (MLs) have not been formally established for this draft procedure. These values will be determined during the validation of Method 1684.

**1.4** This procedure is performance based. The laboratory is permitted to omit any step or modify any procedure (e.g. to overcome interferences, to lower the cost of measurement), provided that all performance requirements in this procedure are met. Requirements for establishing equivalency are given in Section 9.1.2 of Method 1682.

**1.5** Each laboratory that uses this procedure must demonstrate the ability to generate acceptable results using the procedure in Section 9.2 of this appendix.

## 2.0 Summary of Method

**2.1** Sample aliquots of 25-50 g are dried at 103°C to 105°C to drive off water in the sample.

**2.2** The mass of total solids in the sample is determined by comparing the mass of the sample before and after each drying step.

## 3.0 Definitions

**3.1** Total Solids–The residue left in the vessel after evaporation of liquid from a sample and subsequent drying in an oven at 103°C to 105°C.

**3.2** Additional definitions are given in Sections 3.0 and 18.0 of Method 1682.

## 4.0 Interferences

**4.1** Sampling, subsampling, and pipeting multi-phase samples may introduce serious errors (Reference 13.1). Make and keep such samples homogeneous during transfer. Use special handling to ensure sample integrity when subsampling. Mix small samples with a magnetic stirrer. If visible suspended solids are present, pipet with wide-bore pipets. If part of a sample adheres to the sample container, intensive homogenization is required to ensure accurate results. When dried, some samples form a crust that prevents evaporation; special handling such as extended drying times are required to deal with this. Avoid using a magnetic stirrer with samples containing magnetic particles.

**4.2** The temperature and time of residue drying has an important bearing on results (Reference 1). Problems such as weight losses due to volatilization of organic matter, and evolution of gases from heat-induced chemical decomposition, weight gains due to oxidation, and confounding factors like mechanical occlusion of water and water of crystallization depend on temperature and time of heating. It is therefore essential that samples be dried at a uniform temperature, and for no longer than specified. Each sample requires close attention to desiccation after drying. Minimize the time the desiccator is open because moist air may enter and be absorbed by the samples. Some samples may be stronger desiccants than those used in the desiccator and may take on water.

**4.3** Residues dried at 103°C to 105°C may retain some bound water as water of crystallization or as water occluded in the interstices of crystals. They lose $CO_2$ in the conversion of bicarbonate to carbonate. The residues usually lose only slight amounts of organic matter by volatilization at this temperature. Because removal of occluded water is marginal at this temperature, attainment of constant weight may be very slow.

**4.4** Results for residues high in oil or grease may be questionable because of the difficulty of drying to constant weight in a reasonable time.

**4.5** The determination of total solids is subject to negative error due to loss of ammonium carbonate and volatile organic matter during the drying step at 103°C to 105°C. Carefully observe specified ignition time and temperature to control losses of volatile inorganic salts if these are a problem.

## 5.0 Safety

**5.1** Refer to Section 5.0 of Method 1682 for safety precautions.

## 6.0 Equipment and Supplies

**Note:** *Brand names, suppliers, and part numbers are cited for illustrative purposes only. No endorsement is implied. Equivalent performance may be achieved using equipment and materials other than those specified here, but demonstration of equivalent performance that meets the requirements of this method is the responsibility of the laboratory.*

**6.1** Evaporating Dishes–Dishes of 100-mL capacity. The dishes may be made of porcelain (90-mm diameter), platinum, or high-silica glass.

**6.2** Watch glass–Capable of covering the evaporating dishes (Section 6.1).

**6.3** Steam bath.

**6.4** Desiccator–Moisture concentration in the desiccator should be monitored by an instrumental indicator or with a color-indicator desiccant.

**6.5** Drying oven–Thermostatically-controlled, capable of maintaining a uniform temperature of 103°C to 105°C throughout the drying chamber.

**6.6** Analytical balance–Capable of weighing to 0.1 mg for samples having a mass up to 200 g.

**6.7** Container handling apparatus–Gloves, tongs, or a suitable holder for moving and handling hot containers after drying.

**6.8** Bottles–Glass or plastic bottles of a suitable size for sample collection

**6.9** Rubber gloves (Optional)

**6.10** No. 7 Cork borer (Optional)

## 7.0 Reagents and Standards

**7.1** Reagent water–Deionized, distilled, or otherwise purified water.

**7.2** Sodium chloride-potassium hydrogen phthalate standard (NaCl-KHP).

**7.2.1** Dissolve 0.10 g sodium chloride (NaCl) in 500 mL reagent water. Mix to dissolve.

**7.2.2** Add 0.10 g potassium hydrogen phthalate (KHP) to the NaCl solution (Section 7.2.1) and mix. If the KHP does not dissolve readily, warm the solution while mixing. Dilute to 1 L with reagent water. Store at 4°C. Assuming 100% volatility of the acid phthalate ion, this solution contains 200 mg/L total solids, 81.0 mg/L volatile solids, and 119 mg/L fixed solids.

## 8.0 Sample Collection, Preservation, and Storage

**8.1** Use resistant-glass or plastic bottles to collect sample for solids analysis, provided that the material in suspension does not adhere to container walls. Sampling should be done in accordance with Reference 13.2. Begin analysis as soon as possible after collection because of the impracticality of preserving the sample. Refrigerate the sample at 4°C up to the time of analysis to minimize microbiological decomposition of solids. Preferably do not hold samples more than 24 hours. Under no circumstances should the sample be held more than seven days. Bring samples to room temperature before analysis.

## 9.0 Quality Control

**9.1** Quality control requirements and requirements for performance-based methods are given in Section 9.0 of Method 1682.

**9.2** Initial demonstration of laboratory capability - The initial demonstration of laboratory capability is used to characterize laboratory performance and method detection limits.

**9.2.1** Method detection limit (MDL) - The method detection limit should be established for total solids using diluted NaCl-KHP standard (Section 7.2). To determine MDL values, take seven replicate aliquots of the diluted NaCl-KHP solution and process each aliquot through each step of the analytical method. Perform all calculations and report the concentration values in the appropriate units. MDLs should be determined every year or whenever a modification to the method or analytical system is made that will affect the method detection limit.

**9.2.2** Initial Precision and Recovery (IPR) - To establish the ability to generate acceptable precision and accuracy, the analyst shall perform the following operations:

**9.2.2.1** Prepare four samples by diluting NaCl-KHP standard (Section 7.2) to 1-5 times the MDL. Using the procedures in Section 11, analyze these samples for total solids.

**9.2.2.2** Using the results of the four analyses, compute the average percent recovery (x) and the standard deviation (s, Equation 1) of the percent recovery for total solids.

Equation 1

$$s = \sqrt{\frac{\sum x^2 - \frac{(\sum x^2)}{n}}{n-1}}$$

*Where:*

*n = number of samples*
*x = % recovery in each sample*
*s = standard deviation*

**9.2.2.3** Compare s and x with the corresponding limits for initial precision and recovery in Table 2 (to be determined in validation study). If s and x meet the acceptance criteria, system performance is acceptable and analysis of samples may begin. If, however, s exceeds the precision limit or x falls outside the range for recovery, system performance is unacceptable. In this event, correct the problem, and repeat the test.

**9.3** Laboratory blanks

**9.3.1** Prepare and analyze a laboratory blank initially (i.e. with the tests in Section 9.2) and with each analytical batch. The blank must be subjected to the same procedural steps as a sample, and will consist of approximately 25 g of reagent water.

**9.3.2** If material is detected in the blank at a concentration greater than the MDL (Section 1.3), analysis of samples must be halted until the source of contamination is eliminated and a new blank shows no evidence of contamination. All samples must be associated with an uncontaminated laboratory blank before the results may be reported for regulatory compliance purposes.

**9.4** Ongoing Precision and Recovery

**9.4.1** Prepare an ongoing precision and recovery (OPR) solution identical to the IPR solution described in Section 9.2.2.1.

**9.4.2** An aliquot of the OPR solution must be analyzed with each sample batch (samples started through the sample preparation process (Section 11) on the same 12-hour shift, to a maximum of 20 samples).

**9.4.3** Compute the percent recovery of total solids in the OPR sample.

**9.4.4** Compare the results to the limits for ongoing recovery in Table 2 (to be determined in validation study). If the results meet the acceptance criteria, system performance is acceptable and analysis of blanks and samples may proceed. If, however, the recovery of total solids falls outside of the range given, the analytical processes are not being performed properly. Correct the problem, reprepare the sample batch, and repeat the OPR test. All samples must be associated with an OPR analysis that passes acceptance criteria before the sample results can be reported for regulatory compliance purposes.

**9.4.5** Add results that pass the specifications in Section 9.4.4 to IPR and previous OPR data. Update QC charts to form a graphic representation of continued laboratory performance. Develop a statement of laboratory accuracy for each analyte by calculating the average percent recovery (R) and the standard deviation of percent recovery (SR). Express the accuracy as a recovery interval from R-2SR to R+2SR. For example, if R=05% and SR=5%, the accuracy is 85-115%.

**9.5** Duplicate analyses

**9.5.1** Ten percent of samples must be analyzed in duplicate. The duplicate analyses must be performed within the same sample batch (samples whose analysis is started within the same 12-hour period, to a maximum of 20 samples).

**9.5.2** The total solids of the duplicate samples must be within 10%.

## 10.0 Calibration and Standardization

**10.1** Calibrate the analytical balance at 2 mg and 1000 mg using class "S" weights.

**10.2** Calibration shall be within ± 10% (i.e. ±0.2 mg) at 2 mg and ± 0.5% (i.e. ±5 mg) at 1000 mg. If values are not within these limits, recalibrate the balance.

## 11.0 Procedure

**11.1** Preparation of evaporating dishes–Heat dishes and watch glasses at 103°C to 105°C for 1 hour in an oven. Cool and store the dried equipment in a desiccator. Weigh each dish and watch glass prior to use (record combined weight as "$W_{dish}$").

**11.2** Preparation of samples

**11.2.1** Fluid samples–If the sample contains enough moisture to flow readily, stir to homogenize, place a 25 to 50 g sample aliquot on the prepared evaporating dish. If the sample is to be analyzed in duplicate, the mass of the two aliquots may not differ by more than 10%. Spread each sample so that it is evenly distributed over the evaporating dish. Evaporate the

samples to dryness on a steam bath. Cover each sample with a watch glass, and weigh (record weight as "$W_{sample}$").

**Note:** *Weigh wet samples quickly because wet samples tend to lose weight by evaporation. Samples should be weighed immediately after aliquots are prepared.*

**11.2.2** Solid samples–If the sample consists of discrete pieces of solid material (dewatered biosolid, for example), take cores from each piece with a No. 7 cork borer or pulverize the entire sample coarsely on a clean surface by hand, using rubber gloves. Place a 25 to 50 g sample aliquot of the pulverized sample on the prepared evaporating dish. If the sample is to be analyzed in duplicate, the mass of the two aliquots may not differ by more than 10%. Spread each sample so that it is evenly distributed over the evaporating dish. Cover each sample with a watch glass, and weigh (record weight as "$W_{sample}$").

**11.3** Dry the samples at 103°C to 105°C for a minimum of 12 hours, cool to balance temperature in an individual desiccator containing fresh desiccant, and weigh. Heat the residue again for 1 hour, cool it to balance temperature in a desiccator, and weigh. Repeat this heating, cooling, desiccating, and weighing procedure until the weight change is less than 5% or 50 mg, whichever is less. Record the final weight as "$W_{total}$."

**Note:** *It is imperative that dried samples be weighed quickly since residues often are very hygroscopic and rapidly absorb moisture from the air. Samples must remain in the dessicator until the analyst is ready to weigh them.*

## 12.0 Data Analysis and Calculations

**12.1** Calculate the % solids or the mg solids/kg biosolid for total solids (Equation 2).

Equation 2

$$\%\ total\ solids = \frac{W_{total} - W_{dish}}{W_{sample} - W_{dish}} * 100$$

*or*

$$\frac{mg\ total\ solids}{kg\ sludge} = \frac{W_{total} - W_{dish}}{W_{sample} - W_{dish}} * 1{,}000{,}000$$

*Where:*

$W_{dish}$ = *Weight of dish (mg)*
$W_{sample}$ = *Weight of wet sample and dish (mg)*
$W_{total}$ = *Weight of dried residue and dish (mg)*

**12.2** Sample results should be reported as % solids or mg/kg to three significant figures. Report results below the ML as < the ML, or as required by the permitting authority or in the permit.

## 13.0 Method Performance

**13.1** Method performance (MDL and quality control acceptance criteria) will be determined during the multi-lab validation of this method.

**13.2** Total solids duplicate determinations must agree within 10% to be reported for permitting purposes. If duplicate samples do not meet this criteria, the problem must be discovered and the sample must be run over.

## 14.0 Pollution Prevention

**14.2** Pollution prevention details are given in Section 14 of Method 1682.

## 15.0 Waste Management

**15.1** Waste management details are given in Section 15 of Method 1682.

## 16.0 References

**16.1** "Standard Methods for the Examination of Water and Wastewater," 18th ed. and later revisions, American Public Health Association, 1015 15th Street NW, Washington, DC 20005. 1-35: Section 1090 (Safety), 1992.

**16.2** U.S. Environmental Protection Agency, 1992. Control of Pathogens and Vector Attraction in Sewage Sludge. Publ 625/R-92/013. Office of Research and Development, Washington, DC.

## 17.0 Tables, Diagrams, Flowcharts, and Validation Data

**17.1** Tables containing method requirements for QA/QC will be added after the validation study has been performed.