Assessment of False Positive and False Negative Confirmation Rates for Fecal Coliforms by EPA Methods 1680 and 1681 in Sewage Sludge Matrices

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- King County Environmental Laboratory: Kate Leone, Despina Strong, Colin Elliot, Joe Calk, Tami Alley, Bobbie Anderson, Karl Bruun, Eyob Mazengia, Robin Revelle, Debbie Turner, and Jodeen Wieser
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Disclaimer

Mention of company names, trade names, or commercial products does not constitute endorsement or recommendation for use.

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Executive Summary

In 2004, EPA completed the report for the interlaboratory validation of EPA Methods 1680 (LTB/EC) and 1681 (A–1) for fecal coliforms in sewage sludge (biosolids) (EPA 821-B-04-007). In 2005 (FR 70:48256) EPA proposed these methods for use in sewage sludge. As a part of the proposal, EPA also published the results of a three laboratory interlaboratory holding time study using these methods to determine whether sewage sludge samples could be held for 24 hours without significant change in bacterial densities for these analytes (Reference 8.1, EPA-821-R-04-029). Between the proposal and the final rule, EPA conducted a more extensive (23 laboratory) holding time study to confirm the results from the preliminary study in a wider variety of sewage sludge matrices (2006, Reference 8.2, EPA-821-R-07-003). The methods and the 24 hour holding time for certain media and sewage sludge treatment type combinations were promulgated in 2007 (FR 72:14220).

In addition to the holding time study, culture data for the false positive and false negative confirmation rates for these two media were collected during the 2006 study but not statistically analyzed at that time. This report presents the statistical assessment of false positive and false negative confirmation rates for Methods 1680 and 1681 in sewage sludge samples analyzed during the U.S. Environmental Protection Agency's (EPA's) second sewage sludge holding time study (2006, Reference 8.2). The purpose of this statistical assessment is to characterize EPA Methods 1680 and 1681 false positive and false negative confirmation rates across multiple laboratories and sewage sludge matrices analyzed during the sewage sludge holding time study.

EPA qualified 23 participating laboratories to study several matrix/method combinations. The laboratories streaked growth from tubes for isolation onto mEndo plates, then shipped the plates to the verification laboratory for confirmation between February 2006 and August 2006. Nine hundred ninety six (767 typical and 229 atypical) colonies were verified as target or non-target colonies. Usable data was obtained from 21 of the 23 laboratories.

Overall false positive rates for Method 1680 ranged from 0% - 17% for all six matrices (aerobically digested, alkaline–stabilized, anaerobically digested, composted, heat–dried, and thermophilically digested). Overall false positive rates for Method 1681 were relatively low for three of the matrices (aerobically digested, alkaline–stabilized, and anaerobically digested) ranging from 3% - 9%. The false positive rate for composted matrices was considerably higher, at 37%, likely due to tubes that appeared turbid (not due to growth) that also produced air bubbles (not gas) when agitated, which thus appeared positive.

Overall false negative rates were very low for Method 1680, ranging from 0% - 5% for all six matrices. In addition, overall false negative rates for Method 1681 were also very low, ranging from 0% - 3% for aerobically digested, alkaline–stabilized, anaerobically digested, and composted matrices. Although the false negative rates were adjusted to include the number of "true" negatives [clear tubes (not submitted for confirmation)], a significant number of negative tubes (turbidity and no gas) submitted to verification verified as false negatives (82/152). This indicates that laboratories may be incorrectly reading the tubes. In some instances gas production may be very weak (especially as sample volume analyzed is decreased) and may be recorded incorrectly as a negative.

Based on the results of this statistical assessment, overall the false positive and negative rates were acceptably low. The high false positive and negative rates observed for some method/matrix combinations may be due to lack of familiarity with the matrices rather than a limitation of the analytical methods. To ensure that analysts are familiar with the different matrices evaluated for fecal coliforms

using Method 1680 and/or Method 1681, we recommend that analysts verify a minimum of ten percent of all positive and negative tubes for each matrix evaluated to increase proficiency.

SECTION 1.0 INTRODUCTION

Land application of sewage sludge is a critical component of solid waste management. Under Subpart D of Title 40 Code of Federal Regulations (CFR) Part 503, sewage sludges (biosolids) are required to be processed prior to land application in order to minimize pathogen levels and the potential public health risks associated with contact or exposure. Subpart D further defines and classifies sewage sludge (biosolids) for land application purposes based on pathogen concentrations. In 2004, EPA validated Methods 1680 (Reference 8.3) and 1681 (Reference 8.4) for fecal coliforms in Class A and B sewage sludge. As a part of the proposal to approve these methods for use in sewage sludge, EPA also published the results of a three laboratory interlaboratory holding time study using these methods to determine whether sewage sludge samples could be held for 24 hours without significant change in bacterial densities for these analytes (Reference 8.1, EPA-821-R-04-029). False positive or false negative rates were not collected for these media in this initial study. Between the proposal and the final ruling, EPA conducted a more extensive (23 laboratory) holding time study to confirm the results from the preliminary study in a wider variety of sewage sludge matrices and to collect data to determine false positive and false negative rates for fecal coliforms (2006, Reference 8.2, EPA-821-R-07-003). The methods and the extended holding time for certain media and sewage sludge treatment type combinations were promulgated in 2007 (FR 72:14220).. The objective of this statistical assessment report was to assess false positive and false negative confirmation rates for Methods 1680 and 1681 as they were not characterized via biochemical verification during the validation study.

1.1 Summary of EPA Method 1680 for Fecal Coliforms

Fecal coliforms were evaluated in Class A and Class B sewage sludge using Method 1680 (Reference 8.3). Sewage sludge samples were homogenized and inoculated into lauryl tryptose broth (LTB) a presumptive medium. Following incubation at 35.0°C ± 0.5°C for 24 ± 2 and 48 ± 3 hours, growth from positive tubes was transferred to EC broth (confirmatory medium) and incubated at 44.5°C ± 0.2°C for 24 ± 2 hours. All tubes exhibiting turbidity and gas production were considered positive for fecal coliforms. All tubes that did not exhibit both turbidity and gas production were considered negative for fecal coliforms.

1.2 Summary of EPA Method 1681 for Fecal Coliforms

Fecal coliforms were evaluated in Class A and Class B sewage sludge using Method 1681 (Reference 8.4). Sewage sludge samples were homogenized and inoculated into A-1 medium and incubated at 35.0°C ± 0.5°C for 3 hours and 44.5°C ± 0.2°C for 21 ± 2 hours. All tubes exhibiting turbidity and gas production were considered positive for fecal coliforms. All tubes that did not exhibit both turbidity and gas production were considered negative for fecal coliforms.

SECTION 2.0 STUDY OBJECTIVES AND STUDY DESIGN

2.1 Study Objectives

The following study objective was established for the biochemical verification study:

- Characterize EPA Methods 1680 and 1681 false positive and false negative confirmation rates across multiple laboratories and sewage sludge matrices.

To accomplish this objective, qualified volunteer laboratories submitted mEndo plates to verification that were streaked from positive and negative EC and/or A-1 tubes that were analyzed during the holding time study.

The following data quality objective was established for this study:

- Data produced under this study must be generated according to the analytical and QA/QC procedures as described in each of the analytical methods or approved changes to these procedures in order to ensure that data will be of known and reliable quality.

2.2 Technical Approach: Identification of Laboratories

The study required two types of laboratories: participant laboratories and a verification laboratory. The role of these laboratories is described below.

2.2.1 Participant Laboratories

Participant laboratories were representative of the general user community, with experience analyzing sewage sludge samples for fecal coliforms and had access to representative sewage sludge matrices within driving distance (2 hours).

To reduce cost, volunteer laboratories were recruited from a pool of 400 laboratories contacted for potential participation in the study. To reduce the burden on the participant laboratories and encourage volunteer participants, EPA provided the media, reagents, and disposable supplies required for the study. EPA also provided a contractor [Computer Services Corp (CSC)] to manage the study.

2.2.2 Verification Laboratory

The Orange County Sanitation Districts, Environmental Sciences Laboratory in Fountain Valley, CA served as the verification laboratory. To assess false positive and negative rates, the verification laboratory speciated typical and atypical colonies on mEndo plates submitted by the participant laboratories. Colonies were identified using the Vitek® automated biochemical identification system with bioMerieux's industrial/environmental database. Verification laboratory instructions are in Appendix A.

The verification laboratory was also recruited as a volunteer in an effort to reduce costs. To reduce the burden on the verification laboratory, EPA provided all necessary verification media, reagents, and supplies.

2.3 Technical Approach: Sample Collection, Storage Conditions, and Holding Times

A single bulk sample of at least 1000 g was collected and transported to the laboratory on ice and maintained at less than 10°C and above freezing. At the laboratory, the bulk sample was split into replicates, spiked (if necessary), and stored in the refrigerator at less than 10°C and above freezing. After 6 and 24 hours from sample collection, unspiked replicates were analyzed by the appropriate procedure (**Table 1**) and submitted for verification.

2.4 Technical Approach: Methods

The false positive and false negative confirmation rates were assessed for both Class A and Class B unspiked matrices analyzed during the sewage sludge holding time study using Methods 1680 and 1681.

2.5 Technical Approach: Study Design and Analyses

For the verification of false positive and false negative confirmation rates, Methods 1680 and 1681 were used to analyze unspiked samples at multiple laboratories. Table 1 summarizes the method/matrix combinations that were evaluated during the holding time study and submitted to verification to meet the objectives listed in Section 2.1.

Table 1. Study Analysis Summary

Matrix	Method	No. of Tubes Submitted to Verification[a]	
		Positive	Negative
Aerobically digested	1680/1681	25	25
Alkaline–stabilized	1680/1681	25	25
Anaerobically digested	1680/1681	25	25
Compost	1680/1681	25	25
Heat–Dried	1680	25	25
Thermophilically digested	1680	25	25

a Laboratories were instructed to submit 25 positive (turbidity and gas) and 25 negative (turbidity and no gas) tubes to the verification laboratory, if available

2.5.1 Assessment of Methods 1680 and 1681 False Positive and False Negative Confirmation Rates

Methods 1680 and 1681 false positive and false negative rates were assessed during the 2006 (Reference 8.2, EPA-821-R-07-003) holding time study by submitting tubes with growth from unspiked samples to biochemical verification according to study instructions. For each matrix and method, the participant laboratories were requested to submit growth from 25 positive (turbidity and gas) tubes and 25 negative (turbidity and no gas) tubes. No attempt was made to verify the contents of those tubes which did not exhibit visual evidence of growth (turbidity). All tubes (positive and negative) with growth were submitted to the following verification procedure:

- For each positive and negative tube that was verified, growth from tubes were streaked for isolation onto mEndo plates and incubated at 35.0°C ± 0.5°C for 24 ± 2 hours.

- Participant laboratories prepared mEndo plates for shipment to the verification laboratory by wrapping the edges of the plates with parafilm and then wrapping the stack of plates associated with each sample with bubble wrap. Wrapped plates were placed into a cooler lined with a trash bag and

surrounded by blue ice. The cooler was sealed with shipping tape and shipped to the verification laboratory using pre-addressed FedEx shipping labels.

- At the verification laboratory, the following number of isolates were verified using the Vitek® automated identification system:

  - Positive tube verification: Up to five isolated "typical" colonies from each mEndo plate (red with characteristic metallic sheen) were submitted to biochemical verification. If any one of the five colonies verified as a positive fecal coliform result, the tube was categorized as a true positive. If none of the five colonies verified as a fecal coliform, the tube was categorized as a false positive, and further colonies were not selected for verification due to cost constraints.
  - Negative tube verification: Five isolated "atypical" colonies from each mEndo plate (colonies not having typical color and morphology) were submitted to biochemical verification. If there were less than five typical colonies on each mEndo plate, "atypical" colonies were also submitted to ensure that five colonies per plate were submitted to verification. If none of the five colonies verified as a fecal coliform, the tube was categorized as a true negative. If any of the five colonies verified as a positive fecal coliform result, the tube was categorized as a false negative.
  - No growth/unidentified: If no growth was observed on mEndo, or blood agar, the tube was not included in the total number submitted to verification. In addition, any isolates that were not identified by the Vitek® were not included in the total number submitted to verification.

  Note: The Vitek® automated identification system is a fully automated system that performs bacterial identification of isolates using fluorescent technology. After primary isolation, an isolated colony is prepared at a known optical density in saline and inoculated into the Vitek® system. The gram negative card contains 41 fluorescent biochemical tests that are read every 15 minutes. Algorithms are used for organism identification.

2.6 Quality Control (QC) Analyses

Participating laboratories completed the following QC requirements: media sterility checks, dilution water sterility checks, blender jar sterility checks, method blanks, positive controls, and negative controls.

Table 2 summarizes the positive and negative control cultures used during the study.

Table 2. Positive and Negative Control Cultures

Method	Medium or Test	Positive Control	Negative Control
1680/1681	EC, A-1	*Escherichia coli*	*Enterobacter aerogenes*
1680	LTB	*Escherichia coli*	*Pseudomonas aeruginosa*
1680/1681	mEndo	*Escherichia coli*	*Enterobacter aerogenes*

SECTION 3.0 STUDY IMPLEMENTATION

3.1 Study Management

This study was designed under the direction of the Office of Science and Technology, Engineering and Analysis Division within the U.S. Environmental Protection Agency's (EPA's) Office of Water (OW). The EPA technical lead was Robin K. Oshiro. Coordination of activities for the study was performed by the CSC Microbiology and Biochemistry Studies Group.

3.2 Study Schedule

Fecal coliform analyses were conducted between February 2006 and August 2006. Prior to analyzing holding time study samples and submitting to verification, each laboratory was required to analyze four initial precision and recovery (IPR) samples, one unspiked reference matrix sample, and one matrix spike (MS) sample to demonstrate method proficiency.

3.3 Participant Laboratories

The 23 laboratories and one verification laboratory that participated in the assessment of false positive and false negative confirmation rates are provided in **Table 3,** below. No endorsement of these laboratories is implied, nor should any be inferred. Participant laboratories were randomly assigned numbers for purposes of presenting data in this report.

Table 3. Laboratories Participating in the Assessment of Methods 1680 and 1681 False Positive and False Negative Confirmation Rates

Albuquerque Water Utility Authority Water Quality Laboratory Steve Glass, Bill Lindberg, Lauren Tapps 4201 2nd Street, S.W., Albuquerque, NM 87105-0511	**The Industrial Laboratories Company, Incorporated** Tania Vogel, Geoff Henderson, Lenka Teodorovic 4046 Youngfield Street, Wheat Ridge, CO 80033
Alexandria Sanitation Authority John Benard, Leulu M. Gebremedhin, Lisa Racey, Paul Carbary, George Floyd 1500 Eisenhower Avenue, Alexandria, VA 22314	**Katahdin Analytical Services, Incorporated** Deborah Nadeau, Shelly Brown, Greg Lull, Amy Broadbent, Jane April, Mandi Greenleaf 340 County Road #5, Westbrook, ME 04092
Amtest Laboratories Kathy Fugiel, Neila Glidden, Melinda Woomer 14603 NE 87th Street, Redmond, WA 98052	**King County Environmental Laboratory** Kate Leone, Despina Strong, Colin Elliot, Joe Calk, Tami Alley, Bobbie Anderson, Karl Bruun, Eyob Mazengia, Robin Revelle, Debbie Turner, Jodeen Wieser 322 West Ewing Street, Seattle, WA 98119-1507
Analytical Laboratories, Incorporated Sandy Koch, Robert Voermans, Lynn Murray 1804 North 33rd Street, Boise, ID 83703-5814	**Madison Metropolitan Sewerage District** Kurt Knuth, Montgomery Baker, Kris Farrar, Carol Mielke 1610 Moorland Road, Madison, WI 53713
Barton Laboratory, Jefferson County Commission J. Lynn King, Henry Word, Ronstead Claughton, Don Lovell, Bob Spigner 1290 Oak Grove Road, Homewood, AL 35209-6961	**Monroe County Environmental Laboratory at the Frank E. VanLare Waste Treatment Plant** Drew Smith, Mary Merner, Dave Spanganberg, Stephen Bland 1574 Lakeshore Blvd, Admin Bldg #7, Rochester, NY 14617
Bay County Laboratory Services Division Carol Monti and Anna Wright 3420 Transmitter Road, Panama City, FL 32404	**Nova Biologicals, Incorporated** Paul Pearce, Brenda Bates, Donna Reioux, Amber Sutton 1775 East Loop 336, Suite 4, Conroe, TX 77301
City of Everett Water Pollution Control Facility Jeff Wright and Tim Rickman 4027 4th Street S.E., Everett, WA 98205	**Orange County Utilities Central Laboratory** Terri Slifko, Shelley Patterson, Vanessa Perez, Scott Rampenthal, Theresa Slifko 9124 Curry Ford Road, Orlando, FL 32825
County Sanitation Districts of Los Angeles County (JWPCP – WQL) Kathy Walker, Debra Leachman, Mark Patterson 24501 S. Figueroa Street, Carson, CA 90745	**St. George Regional Water Reclamation Facility Laboratory** Leslie Wentland and Amy Howe 3780 South 1550 West, St. George, UT 84790
Edge Analytical Larry Henderson, Kent Oostra, Shannon Kizer 805 West Orchard Drive, Suite 4, Bellingham, WA 98225	**SVL Analytical, Incorporated – Microbiology Laboratory** Linda Johann 2195 Ironwood Court, Suite C, Coeur d'Alene, ID 83814
Energy Laboratories, Incorporated – Casper Branch Sheryl Garling, N. Lou Miller, Sherri L. Boatman, Randy Ogden 2393 Salt Creek Highway, Casper, WY 82601	**Universal Laboratories** Carol Kleemeier, Stacie Splinter, Linda McFarland 20 Research Drive, Hampton, VA 23666
Environmental Protection Agency, National Risk Management Research Laboratory Mark Meckes, Laura Boczek, Cliff Johnson 26 West Martin Luther King Drive, Cincinnati, OH 45268	**Wichita Water and Sewer Wastewater Laboratory** Becky Gagnon and Karen Roberts 2305 East 57th Street South, Wichita, KS 67216
Environmental Science Corporation Rodney Shinbaum, Kim Johnson, Rachel Freeman 12065 Lebanon Road, Mt. Juliet, TN 37122	**Verification laboratory:** **Orange County Sanitation District, Environmental Sciences Laboratory** Charles McGee and Kim Patton 10844 Ellis Avenue, Fountain Valley, CA 92708

SECTION 4.0 DATA REPORTING AND VALIDATION

4.1 Data Reporting

Laboratories submitted the following data to CSC for review and validation:

- Completed cover sheet with sample collection and QC information
- Completed sample-specific data reporting forms
- Documentation of any additional information that would assist in evaluating the data

4.2 Data Validation

Data review checklists were used to ensure that each data package was complete and to ensure that each sample result met the study-specific and method-specific requirements. Items reviewed for each sample included the following:

- Confirmation that original forms were submitted
- Confirmation that all QC checks were performed and exhibited the appropriate response
- Confirmation that all method-specific incubation times and temperatures were met
- Confirmation that all media and reagents were used within expiration dates
- Confirmation that all calculations were correct

This process was performed independently by two data reviewers, each of whom entered the results into separate spreadsheets designed for data review and validation for this study. The results were compared to verify consistency and identify potential data entry errors.

The following issues were encountered during laboratory analyses:

Laboratory 3 (Methods 1680 and 1681, Class A, composted and Class B, aerobically digested sewage sludge): Eighteen of the isolates submitted were from spiked samples. As a result data were considered invalid and not included in subsequent data analyses.

Laboratory 14 (Method 1680, Class B, anaerobically digested sewage sludge): One hundred of the isolates submitted were from spiked samples. As a result data were considered invalid and not included in subsequent data analyses.

Laboratory 20 (Method 1680, Class B, anaerobically digested sewage sludge): Three of the isolates submitted were from spiked samples. As a result data were considered invalid and not included in subsequent data analyses.

Laboratory 25 (Method 1681, Class A, anaerobically digested sewage sludge): All mEndo plates submitted to verification were not sealed prior to shipment; during transit plates opened resulting in contamination. None of the plates were submitted to verification.

Laboratory 29 (Method 1681, Class A, composted sewage sludge): Only spiked samples were submitted to verification. As a result, data were considered invalid and not included in subsequent data analyses.

Although growth from 996 tubes (positive and negative) were submitted by the participating laboratories, only 731 were actually verified due to issues noted above, and/or because no growth was observed on submitted mEndo plates.

SECTION 5.0 RESULTS

This section includes verification results of unspiked sewage sludge samples which were analyzed for fecal coliforms using Methods 1680 and 1681 and submitted to verification. Only valid results are included in this section; a detailed description of data invalidation information is included in Section 4.0.

The false negative rate was adjusted based on the number of "true" negatives [clear tubes (not submitted for confirmation)] because it can be assumed that all clear tubes did not include the target organism. However, the false positive rate could not be adjusted in a similar manner, because no assumption can be made about whether the target organism was in tubes that appeared to be positive.

5.1 Method 1680 Class A Sewage Sludge Results

False positive and false negative confirmation rates for Class A matrices analyzed for fecal coliforms using Method 1680 are summarized in **Table 4**.

Table 4. Laboratory–Specific False Positive and False Negative Rates for Method 1680 Class A Matrices

Lab	Matrix (digested sewage sludge)	False Positive Assessment			False Negative Assessment		
		Typicals Submitted	Number of False Positives	False Positive Rate (%)	% Atypicals Submitted[a]	Number of False Negatives	False Negative Rate (%)[b]
3	Compost	8	0	0	0/221	0	0
15					11/320	2	0.6
7	Heat–Dried	25	0	0	0/80	0	0

a The false negative rate was adjusted based on the number of "true" negatives [clear tubes (not submitted for confirmation)]
b The false negative confirmation rate = (false negatives)/(total number of atypicals); e.g., 2/320 = 0.63%

5.2 Method 1680 Class B Sewage Sludge Results

False positive and false negative confirmation rates for Class B matrices analyzed for fecal coliforms using Method 1680 are summarized in **Table 5**.

Table 5. Laboratory–Specific False Positive and False Negative Rates for Method 1680 Class B Matrices

Lab	Matrix	False Positive Assessment			False Negative Assessment		
		Typicals Submitted	Number of False Positives	False Positive Rate (%)	% Atypicals Submitted[a]	Number of False Negatives	False Negative Rate (%)[b]
3	Aerobic	7	3	42.9	0/233	0	0
12		24	5	21	25/81	22	27.2
24		24	4	16.7	0/149	0	0
27		22	1	4.6	12/238	10	4.2
1	Anaerobic	21	0	0	0/172	0	0
9		25	3	12	6/212	6	2.8
14		49	6	12	0/131	0	0
18		24	4	17	7/117	6	5.1
20		15	1	6.7	12/179	12	6.7
28		21	0	0	15/118	0	0
22	Thermophilic	50	0	0	0/175	0	0

a The false negative rate was adjusted based on the number of "true" negatives [clear tubes (not submitted for confirmation)]

b The false negative confirmation rate = (false negatives)/(total number of atypicals); e.g., 22/81 = 27.16%

5.3 Method 1681 Class A Sewage Sludge Results

False positive and false negative confirmation rates for Class A matrices analyzed for fecal coliforms using Method 1681 are summarized in **Table 6**.

Table 6. Laboratory–Specific False Positive and False Negative Rates for Method 1681 Class A Matrices

Lab	Matrix	False Positive Assessment			False Negative Assessment		
		Typicals Submitted	Number of False Positives	False Positive Rate (%)	% Atypicals Submitted[a]	Number of False Negatives	False Negative Rate (%)[b]
3	Compost	8	0	0	0/233	0	0
13		25	6	24	4/152	2	1.3
23		10	10	100	0/58	0	0

a The false negative rate was adjusted based on the number of "true" negatives [clear tubes (not submitted for confirmation)]

b The false negative confirmation rate = (false negatives)/(total number of atypicals); e.g., 2/152 = 1.32%

5.4 Method 1681 Class B Sewage Sludge Results

False positive and false negative confirmation rates for Class B matrices analyzed for fecal coliforms using Method 1681 are summarized in **Table 7**.

Table 7. Laboratory–Specific False Positive and False Negative Rates for Method 1681 Class B Matrices

Lab	Matrix (digested sewage sludge)	False Positive Assessment			False Negative Assessment		
		Typicals Submitted	Number of False Positives	False Positive Rate (%)	% Atypicals Submitted[a]	Number of False Negatives	False Negative Rate (%)[b]
3	Aerobic	8	1	12.5	0/239	0	0
8		25	0	0	0/144	0	0
1	Anaerobic	22	2	9.1	5/191	0	0
5		25	1	4	11/103	10	9.7
11		24	3	12.5	20/184	2	1.1
19		23	0	0	5/183	3	1.6
26		24	0	0	14/53	4	7.6

a The false negative rate was adjusted based on the number of "true" negatives [clear tubes (not submitted for confirmation)]
b The false negative confirmation rate = (false negatives)/(total number of atypicals); e.g., 10/103 = 9.71%

5.5 Alkaline–Stabilized Results

False positive and false negative confirmation rates for alkaline–stabilized matrices analyzed for fecal coliforms using both Methods 1680 and 1681 are summarized in **Table 8**. Due to the variability of treatment within alkaline–stabilized matrices, results are not categorized by Class (A or B).

Table 8. Laboratory–Specific False Positive and False Negative Rates for Alkaline–Stabilized Matrices

Lab	Method	False Positive Assessment			False Negative Assessment		
		Typicals Submitted	Number of False Positives	False Positive Rate (%)	% Atypicals Submitted[a]	Number of False Negatives	False Negative Rate (%)[b]
2	1680	9	1	11.1	3/25	2	8
22		50	0	0	0/23	0	0
2	1681	11	1	9.1	2/52	1	1.9

a The false negative rate was adjusted based on the number of "true" negatives [clear tubes (not submitted for confirmation)]
b The false negative confirmation rate = (false negatives)/(total number of atypicals); e.g., 2/25 = 8.00%

5.6 Overall Results by Matrix and Method

Overall false positive and false negative confirmation rates for fecal coliforms using both Methods 1680 and 1681 are summarized in **Table 9**.

Table 9. Overall False Positive and False Negative Rates for Methods 1680 and 1681 by Matrix

Matrix[a]	Method	False Positive Assessment			False Negative Assessment		
		Typicals Submitted	Number of False Positives	False Positive Rate (%)	% Atypicals Submitted[b]	Number of False Negatives	False Negative Rate (%)[c]
Aerobic	1680	77	13	16.9	37/701	32	4.6
	1681	33	1	3	0/383	0	0
Alkaline–Stabilized	1680	59	1	1.7	3/48	2	4.2
	1681	11	1	9.1	2/52	1	1.9
Anaerobic	1680	155	14	9	40/929	24	2.6
	1681	118	6	5.1	55/714	19	2.7
Compost	1680	8	0	0	11/541	2	0.4
	1681	43	16	37.2	4/443	2	0.5
Heat–Dried	1680	25	0	0	0/80	0	0
Thermophilic	1680	50	0	0	0/175	0	0

a Aerobic, anaerobic and thermophilic matrices refer to specific digester process products
b The false negative rate was adjusted based on the number of "true" negatives [clear tubes (not submitted for confirmation)]
c The false negative confirmation rate = (false negatives)/(total number of atypicals); e.g., 32/701 = 4.56%

5.7 Overall Results by Method

Overall false positive and false negative confirmation rates for fecal coliforms using both Methods 1680 and 1681 are summarized in **Table 10.**

Table 10. Overall False Positive and False Negative Rates for Methods 1680 and 1681 by Method

Method	Matrix[a]	False Positive Assessment			False Negative Assessment		
		Typicals Submitted	Number of False Positives	False Positive Rate (%)	% Atypicals Submitted[b]	Number of False Negatives	False Negative Rate (%)[c]
1680	Aerobic	77	13	16.9	37/701	32	4.6
	Alkaline–Stabilized	59	1	1.7	3/48	2	4.2
	Anaerobic	155	14	9.	40/929	24	2.5
	Compost	8	0	0	11/541	2	0.4
	Heat–Dried	25	0	0	0/80	0	0
	Thermophilic	50	0	0	0/175	0	0
	Overall	374	28	7.5	91/2474	60	2.4
1681	Aerobic	33	1	3	0/383	0	0
	Alkaline–Stabilized	11	1	9.1	2/52	1	1.9
	Anaerobic	118	6	5.1	55/714	19	2.7
	Compost	43	16	37.2	4/443	2	0.5
	Overall	205	24	11.7	61/1592	22	1.4

a Aerobic, anaerobic and thermophilic matrices refer to specific digester process products
b The false negative rate was adjusted based on the number of “true” negatives [clear tubes (not submitted for confirmation)]
c The false negative confirmation rate = (false negatives)/(total number of atypicals); e.g., 32/701 = 4.56%

SECTION 6.0 ASSESSMENT OF FALSE POSITIVE AND FALSE NEGATIVE CONFIRMATION RATES: DISCUSSION

Participating laboratories streaked growth from 996 (767 typical and 229 atypical) tubes for isolation on mEndo plates and submitted to verification. Of the 996 mEndo plates submitted, 731 (579 typical and 152 atypical) were actually verified; 265 plates submitted were not confirmed through biochemical verification (see discussion in Section 4.2). Since "true" negative (clear/no growth) tubes observed during the study could not be submitted to confirmation, the false negative confirmation rate was adjusted to include the number of "true" negatives [clear tubes (not submitted for confirmation)] because it can be assumed that all clear tubes did not include the target organism. However, the false positive rate could not be adjusted in a similar manner, because no assumption can be made about whether the target organism was in tubes that appeared to be positive.

Although the false negative rates were adjusted to include the number of "true" negatives [clear tubes (not submitted for confirmation)], a significant number of negative tubes (turbidity and no gas) submitted to verification verified as false negatives (82/152). This indicates that laboratories may be incorrectly reading the tubes. In some instances, gas production indicating a positive result may be very weak (especially as sample volume analyzed is decreased) and may be recorded incorrectly as a negative. To improve future performance, analysts should verify ten percent of positive and negative tubes for each matrix type at a minimum of once per month to increase proficiency.

Results of the analyses and summary observations for each individual method and matrix are provided below.

6.1 Method 1680 Confirmation Rates for Class A Matrices

False positive and false negative rates for Class A matrices (Compost and Heat–Dried) using Method 1680 were very low. False positive rates were 0% for both matrices and false negative rates ranged from 0% to less than 1%.

Composted Matrices

- The false positive rate for Laboratory 3 was 0%; the false negative rates for Laboratories 3 and 15 were 0% and less than 1%, respectively. Two laboratories analyzed composted Class A matrices resulting in two valid data sets.

Heat-Dried Matrix

- The false positive and false negative rate for Laboratory 7 was 0%. One laboratory analyzed a Class A heat-dried matrix resulting in a single valid data set.

6.2 Method 1680 Confirmation Rates for Class B Matrices

False positive rates for Class B matrices using Method 1680, ranged from 0% - 43%. False negative rates were relatively low across all matrices for 10 of 11 laboratories, ranging from 0% - 7%. However, Laboratory 12 had a false negative rate of 27%.

Aerobically Digested Matrices

- False positive rates for Laboratories 3, 12, 24, and 27 were 43%, 21%, 17%, and 5%, respectively, with false negative rates of 0%, 27%, 0%, and 4%, respectively. Although Laboratory 3 observed a false positive rate of 43% compared to the other laboratories, only seven plates/isolates were

submitted to verification. Therefore the rate occurred based on only three false positives. Four laboratories analyzed aerobically digested Class B matrices resulting in four valid data sets.

Anaerobically Digested Matrices

- False positive rates for Laboratories 1, 9, 14, 18, 20, and 28 were 0%, 12%, 12%, 17%, 7%, and 0%, respectively, with false negative rates of 0%, 3%, 0%, 5%, 7%, and 0%, respectively. Six laboratories analyzed anaerobically digested Class B matrices resulting in six valid data sets.

Thermophilically Digested Matrices

- The false positive and false negative rate for Laboratory 22 was 0%. One laboratory analyzed a Class B thermophilically digested matrix resulting in a single valid data set.

6.3 Method 1681 Confirmation Rates for Class A Matrices

False positive rates for Class A matrices using Method 1681 varied from a low of 0% to a high of 100%. In contrast, the false negative rates were consistently low with rates ranging from 0% - 1%.

Composted Matrices

- False positive rates for Laboratories 3, 13, and 23 were 0%, 24%, and 100%, respectively, with false negative rates of 0%, 1%, and 0%, respectively. The high false positive confirmation rates observed for Laboratories 13 (24%) and 23 (100%) may be due to the composted matrices evaluated. Materials used in the composting process may cause tubes to appear turbid and produce air bubbles when agitated. Three laboratories analyzed composted Class A matrices resulting in three valid data sets.

6.4 Method 1681 Confirmation Rates for Class B Matrices

False positive rates for Class B matrices using Method 1681 ranged from 0% - 13%. False negative rates ranged from 0% - 10%.

Aerobically Digested Matrices

- False positive rates for Laboratories 3 and 8 were 13% and 0%, respectively, with false negative rates of 0% and 0%, respectively. Two laboratories analyzed aerobically digested Class B matrices resulting in two valid data sets.

Anaerobically Digested Matrices

- False positive rates for Laboratories 1, 5, 11, 19, and 26 were 9%, 4%, 13%, 0%, and 0%, respectively, with false negative rates of 0%, 10%, 1%, 2%, and 8%, respectively. Five laboratories analyzed anaerobically digested Class B matrices resulting in five valid data sets.

6.5 Methods 1680 and 1681 Confirmation Rates for Alkaline–Stabilized Matrices

For alkaline–stabilized matrices using Method 1680, false positive and false negative rates were relatively low ranging from 0% - 11% and 0% - 8%, respectively. False positive and false negative rates for Method 1681 were also relatively low at 9% and 2%, respectively.

Alkaline–Stabilized Matrices Analyzed Using Method 1680

- False positive and negative rates for Laboratories 2 and 22 were 11% and 0% and 8% and 0%, respectively. Two laboratories analyzed alkaline–stabilized matrices resulting in two valid data sets.

Alkaline–Stabilized Matrices Analyzed Using Method 1681

- The false positive and negative rate for Laboratory 2 was 9% and 2%, respectively. One laboratory analyzed an alkaline–stabilized matrix resulting in a single valid data set.

SECTION 7.0 CONCLUSIONS

The objective of this study was to assess false positive and false negative confirmation rates for fecal coliform Methods 1680 and 1681 as they were not characterized based on biochemical verification during the validation study for these methods in sewage sludge.

In this study EPA has characterized the false positive and false negative rates for Methods 1680 and 1681 for fecal coliforms in Class A and B sewage sludge across multiple laboratories and sewage sludge matrices. During the study, 23 participating laboratories streaked growth from 996 (767 typical and 229 atypical) tubes for isolation on mEndo plates and submitted the plates to the verification laboratory for confirmation between February 2006 and August 2006.

<u>False Positive Rates</u>
Overall false positive rates for Method 1680 ranged from 0% - 17% for all six matrices (aerobically digested, alkaline–stabilized, anaerobically digested, composted, heat–dried, and thermophilically digested).

Overall false positive rates for Method 1681 were relatively low for three of the matrices (aerobically digested, alkaline–stabilized, and anaerobically digested) ranging from 3% - 9%. The false positive rate for composted matrices was considerably higher, at 37%. Since materials used in the composting process may cause tubes to appear turbid (not due to growth) and may also produce air bubbles (not gas) when agitated, it is recommended that laboratories characterize each composted matrix evaluated in-house to reduce the number of false positives and increase analyst proficiency.

<u>False Negative Rates</u>
Overall false negative rates were very low for Method 1680, ranging from 0% - 5% for all six matrices. In addition, overall false negative rates for Method 1681 were also very low, ranging from 0% - 3% for aerobically digested, alkaline–stabilized, anaerobically digested, and composted matrices.

Although the false negative rates were adjusted to include the number of "true" negatives [clear tubes (not submitted for confirmation)], a significant number of negative tubes (turbidity and no gas) submitted to verification verified as false negatives (82/152). This indicates that laboratories may be incorrectly reading the tubes. In some instances gas production may be very weak (especially as sample volume analyzed is decreased) and may be recorded incorrectly as a negative.

<u>Analyst Proficiency Recommendations</u>
The high false positive and negative rates observed for some method/matrix combinations may be due to lack of familiarity with the matrices. To ensure that analysts are familiar with the different matrices evaluated for fecal coliforms using Method 1680 and/or Method 1681, analysts should verify a minimum of ten percent of all positive and negative tubes for each matrix evaluated to increase proficiency.

SECTION 8.0 REFERENCES

8.1 USEPA. 2005. Assessment of the Effects of Holding Time on Fecal Coliform and *Salmonella* Concentrations in Biosolids. EPA-821-R-04-029.

8.2 USEPA. 2006. Assessment of the Effects of Holding Time on Fecal Coliform and Salmonella Concentrations in Biosolids. EPA-821-R-07-003.

8.3 USEPA. 2005. EPA Method 1680: Fecal Coliforms in Sewage Sludge (Biosolids) by Multiple-Tube Fermentation using Lauryl Tryptose Broth (LTB) and EC Medium

8.4 USEPA. 2005. EPA Method 1681: Fecal Coliforms in Sewage Sludge (Biosolids) by Multiple Tube Fermentation using A-1 Medium

8.5 Gilbert, R.O. 1987. Statistical Methods for Environmental Pollution Monitoring. Van Nostrand Reinhold, New York.

SECTION 9.0 ACRONYMS

LTB	Lauryl tryptose broth
LIA	Lysine iron agar
MPN	Most probable number
MSRV	Modified semisolid Rappaport-Vassiliadis medium
QC	Quality control
RSD	Relative standard deviation
SAS	Statistical analysis software
SD	Standard deviation
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TSI	Triple sugar iron agar
XLD	Xylose-lysine desoxycholate agar

Appendix A:

Verification Instructions

Sewage Sludge Holding Time Study
Verification Instructions for False Positive/False Negative Rates
Method 1680 and 1681: Fecal Coliforms (Unspiked samples only)

Note: Verification of positive tubes and negative tubes with growth from unspiked samples will only be performed during the holding time study - not during preliminary analyses.
Remove 6 hour and 24 hour EC and A-1 tubes from refrigerator.

- For each matrix and method, submit growth from 25 positive tubes and 25 negative tubes with growth to verification.
- For each positive and negative tube being verified, streak growth from tubes for isolation onto m-Endo plates and incubate at 35.0°C ± 0.5°C for 24 ± 2 hours.
- Label each m-Endo plate with corresponding sample ID and tube dilution and number (e.g. Rep. #1, tube #3, 0.1 dilution).
- After 24 ± 2 hours, remove m-Endo plates from incubator.
- To prepare the m-Endo plates for shipping to the verification laboratory, wrap the edges of the plates with parafilm and wrap the stack of plates associated with each sample with bubble wrap.
- Place the plates in a cooler lined with a trash bag and surround the plates with blue ice. Seal the cooler with shipping tape.
- ***NOTE:*** Please ship the plates on the same day that they are removed from the incubator and contact us on the day of shipment via phone/email/or fax:

  Darcy Gibbons: 703-461-2308, dgibbons3@csc.com
  Mary Smith: 703-461-2058, msmith247@csc.com
  Fax: 703-461-8056

- **Use provided FedEx mailing documents to ship the coolers to the verification laboratory** (Orange County Sanitation District, Attn: Charlie McGee).