Coliform Analytical Methods and Data Application in Wastewater and Recreational Waters

March 15rd, 2017

1:00 PM – 3:00 PM ET

Today's webcast is the result of collaboration between the WEF Laboratory Practices Committee and the American Public Health Laboratories

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APHL ASSOCIATION OF PUBLIC HEALTH LABORATORIES

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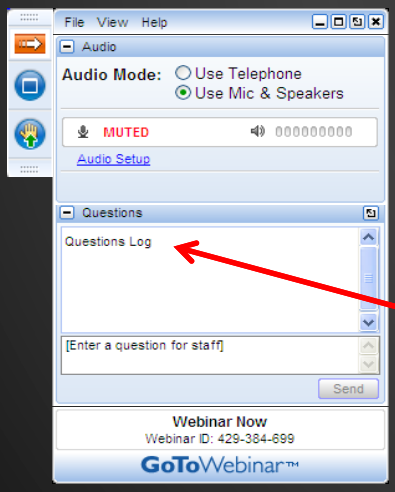
How to Participate Today



- **Audio Modes**
  - Listen using Mic & Speakers
  - Or, select "Use Telephone" and dial the conference (please remember long distance phone charges apply).
- **Submit your questions using the Questions pane.**
- **A recording will be available for replay shortly after this webcast.**

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Today's Moderator

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Coliform Analytical Methods and Data Application in Wastewater and Recreational Waters

Anthony Russo, Chief of Biological Services, Bureau of Laboratories, PA DEP

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Coliforms & Hygienic Significance

- Initially thought to indicate fecal contamination and serve as surrogate for Salmonella.
- Coliforms became an indicator of sanitary conditions in dairy, food and water commodities.
- It is now known that many of the coliforms can be found also in plants and the environment, thus a positive coliform test does not necessarily indicate fecal contamination.
- Monitoring of coliforms was legislated many years ago to protect public health in drinking and recreational water usages. Laws are still active today in PA.

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Coliform as Indicator Bacteria

- Easy to detect
- A variety of bacteriological media are used to detect coliforms in water
- Some strains can be further classified as "fecal coliforms"
- Indicator organisms provide insight to the history of a sample
- Presence may indicate presence of pathogens and other deleterious agents to human health.

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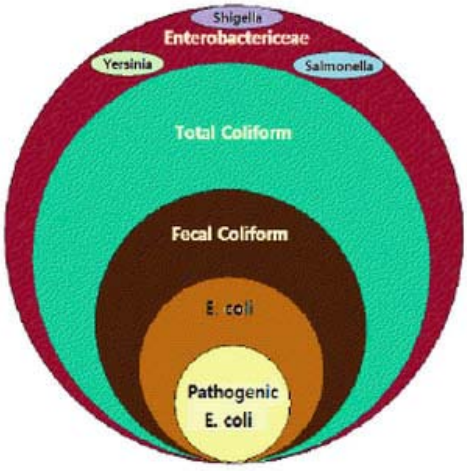
Family Enterobacteriaceae- Significant Sanitary Indicators



- 20 genera belong to this family
- Biochemically & genetically related to one another
- Including *E. coli* and all members of the coliform group
- Also includes known pathogens *Salmonella*, *Shigella*, *Yersinia* & ECO157:H7
- This group is used as sanitary indicators in the EU

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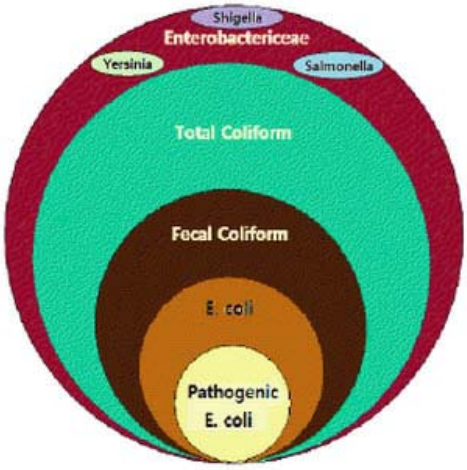
Total Coliform Bacteria- Sanitary Indicators in Water



- 4 genera belong to the **total coliform** group: *Citrobacter*, *E. coli*, *Enterobacter* and *Klebsiella*
- Has admirably served to protect public health although has limitations
- Easily distinguishable from other microorganisms due to unique ability to ferment lactose.
- Easily detected & enumerated in a relatively short time.
- Able to survive as well as the associated pathogenic organisms in water.

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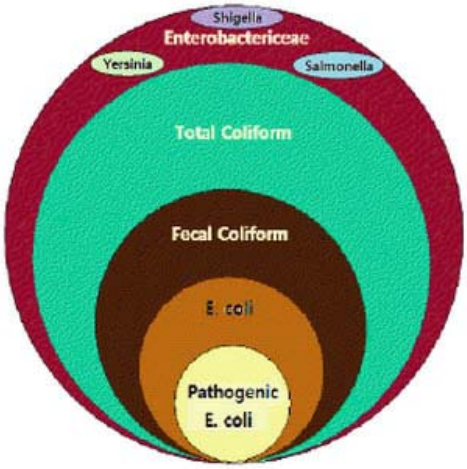
Fecal Coliform- Better Indicator of Fecal Contamination



- **Fecal coliforms** are a subset of the total coliform group
- Has the same properties as the coliform group except fermentation is able to proceed at 44.5° C which is called thermotolerant.
- Considered a better indicator of fecal contamination than coliform
- Serves as good indicator of bio-solid composting due to its temperature threshold.

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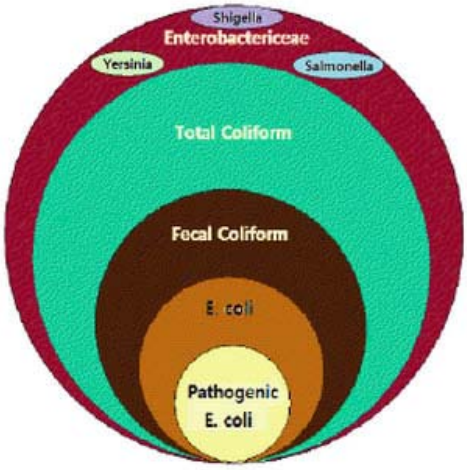
E. coli- Best Indicator of Fecal Pollution



- *E. coli* is present in feces of all mammalian species at high concentrations
- Can survive in water for weeks but will not thrive outside host
- Useful tool to indicate fecal pollution of water systems
- Gram (-) cell wall is heat and chemical labile
- The organism can be distinguished from other fecal coliforms by the lack of urease and presence of β-glucoronidase enzymes
- Most *E. coli* are innocuous but there are 6 pathogenic strains: STEC, ETEC, EPEC, EAEC, EIEC, DAEC

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DEPARTMENT OF ENVIRONMENTAL PROTECTION

- PA DEP monitoring program is based on needs of clients (enforcement bureaus, state parks, contracts …)
- Key criteria is compliance testing for the protection of public & environment
- Indicator organism monitoring most beneficial for public health protection
- May be dependent on special funding requirements such as EPA, USGS …

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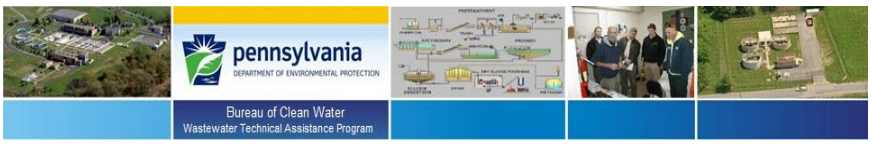
Bacteriological Tests for Indicator Organisms

Target	Reference Method	Method Technology	Comments
Total coliform	SM9222B SM9221B, D SM9223B	Membrane Filtration Multiple Tube Ferment Enzyme Substrate Test	Rapid Labor intensive Minimal QC
Fecal coliform	SM9222D SM9221B, E SM9223B	Membrane Filtration Multiple Tube Ferment Enzyme Substrate Test	Rapid Labor intensive Minimal QC
E. coli	EPA 1603 SM9221B, F SM9223B	Membrane Filtration Multiple Tube Ferment Enzyme Substrate Test	Rapid Labor intensive Minimal QC

Drinking Water

- **PA Statute:** Public Water Systems must test water for compliance to EPA's Revised Total Coliform Rule: if total coliforms are present, must confirm for *E. coli*. FR10269, Feb 13, 2013, volume 78
- **Regulation:** Total coliform <1/100ml; must verify *E.coli* if positive for total coliform.
- **Regulatory Action:** Non-potable, boiled water advisory
- **Analytical Methods:** SM9222B, SM9223B, SM9221B, F



Sewage Treatment Plant Effluents

- **PA Statute:** NPDES Permit
- **Regulation:** Fecal coliform <200 cfu/100ml geometric mean
- **Regulatory Action:** Remedial action to correct discharge
- **Analytical Methods:** SM9222D, SM9223B, SM9221B, E

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Watershed Monitoring Program

- **PA Statute: 25** PA Code § 93.7, Table 3
- **Regulation:** Fecal coliform <200/100ml geometric mean
- **Regulatory Action:** Impair waterway, correct source
- **Analytical Methods:** SM9222D, SM9223B, SM9221C,E

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PA State Parks Bathing Beaches

- **PA Statute: 28** PA Code § 18.28
- **Regulation:** *E. coli* <235 cfu/100ml single sample, 126 cfu/100 ml geometric mean
- **Regulatory Action:** restrict usage until correction
- **Analytical Methods:** EPA 1603, SM9221C, F

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Swimming & Spray Pools

- **PA Statute: 28** PA Code § 18.27
- **Regulation:** Total coliform <3 cfu/100ml
- **Regulatory Action:** restrict usage until correction
- **Analytical Methods:** SM 9222B, SM9221C,

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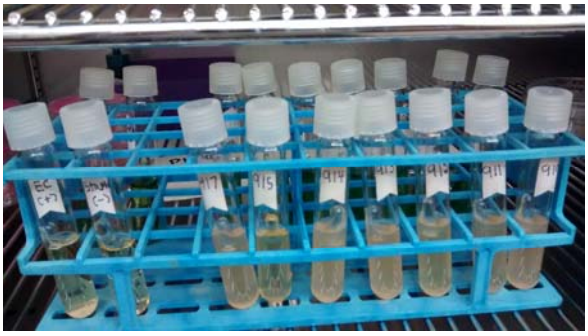
Multiple Tube Fermentation

- SM 9221B, C determines Total coliform MPN
- SM 9221D determines Total coliform Presence/Absence
- SM9221C, E determines Fecal coliform MPN
- SM9221C, F determines *E. coli* MPN

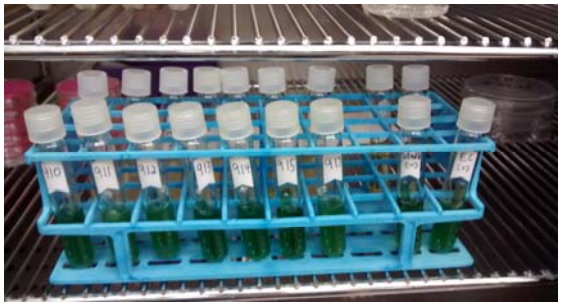
Present/Absent

9221 B. Presumptive Test for Total Coliform



- Transfer sample portions to individual tubes of Lauryl Tryptose broth (LTB). Incubate at 35 ± 0.5ºC for 24 ± 2hrs - 48 ± 3hrs.
- Lactose fermented to gas (CO_2) = positive reaction

9221 B. Confirmed Test for Total Coliform



- Transfer all presumptive tubes showing growth, gas production, or acidic reaction to Brilliant Green Lactose Bile Broth tubes (BGBB) with a sterile loop. Incubate at 35 ± 0.5ºC for 24 ± 2hrs - 48 ± 3hrs.
- Formation of gas in any amount in the inverted tube constitutes a positive confirmed test. Calculate the MPN value from the number of positive BGBB tubes from the following chart.

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9221 C. Estimation of Coliform Density

- Select highest dilution that yields positive results in all five tubes (no lower dilution giving any negative results) and the two next succeeding higher dilutions. The index depicted in example A is 5-1-0 starting at the 1 ml sample volume.
- If the lowest dilution tested has less than five portions with a positive results, select it and the two next succeeding higher dilutions.

Example	Sample volume ml					Combination of Positives	MPN/100ml
	10	1	0.1	0.01	0.001		
A	5	5	1	0	0	5-1-0	330
B	4	5	1	0	0	4-5-1	48
C	0	0	1	0	0	0-0-1	1.8

- Record the coliform concentration as the Most Probable Number (MPN) /100 ml.

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9221 E. Fecal Coliform Procedure

- Transfer all presumptive fermentation tubes showing any amount of gas, growth or acidity within 48 hrs. of incubation to the fecal coliform test.
- Gently roll presumptive fermentation tubes. Using a sterile loop transfer growth from each presumptive tube to EC broth. Incubate EC broth tubes in a water bath at 44.5 ± 0.2 ºC for 24 ± 2 hrs.
- Interpretation: Gas production with growth in an EC broth within 24 ± 2 hrs. or less = positive fecal coliform result.
- Calculate MPN from the number of positive EC broth tubes as described in 9221 C.
- When using only one tube for subculturing from a single presumptive bottle, report as presence or absence of fecal coliforms.

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9221 F. *E. coli* Procedure

- Transfer all presumptive fermentation tubes showing any amount of gas, growth or acidity within 48 hrs. of incubation to EC-MUG broth .
- Gently roll presumptive fermentation tubes. Using a sterile loop transfer growth from each presumptive tube to EC-MUG broth. Incubate EC-Mug broth tubes in a water bath at 44.5 ± 0.2 ºC for 24 ± 2 hrs.
- Interpretation: Examine all tubes exhibiting growth for fluorescence using a long-wavelength UV lamp. The presence of bright blue fluorescence = positive result for *E. coli*.
- Calculate MPN from the number of positive EC-MUG broth tubes as described in 9221 C.
- When using only one tube for subculturing from a single presumptive bottle, report as presence or absence of *E. coli*.

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Media Quality Control

- Place all EC-MUG broth tubes in water bath within 30 minutes after inoculation. Maintain sufficient water depth in water bath incubator to immerse tubes to upper level of the medium.
- Check each new batch of tubed medium with an *E. coli* culture and a negative species such as *Staphylococcus aureus* before using for sample testing. Control organisms must be ATCC, American Type Culture Collection. Record the QC results in the Bacteriological Media Quality Control Record form in the Bacteriological Media Quality Assurance Records laboratory notebook. If the media QC is not acceptable, discard the batch and re-prepare from an unopened bottle of dehydrated media.
- A positive control consisting of a known *E. coli* MUG positive culture, a negative control consisting of a thermotolerant *Klebsiella* (MUG negative) culture and a un-inoculated medium control should be used to interpret the results and avoid confusion of weak auto-fluorescence of the medium of a positive reaction.
- Laboratory and analyst competency should be demonstrated before official analytical results are reported. Initial Precision & Recovery validates the lab's ability to conduct the assay. Initial Demonstration of Capability studies by each analyst verifies their capability to correctly perform the assay. On-going demonstrations should be conducted annually by each analyst.

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Media Preparation Quality Control

COMMONWEALTH OF PENNSYLVANIA
DEPARTMENT OF ENVIRONMENTAL PROTECTION
BUREAU OF LABORATORIES

Biological Media Quality Control Record
*See Abbreviation Key on Inside Cover

Media Unique Identifier	pH	Date incubated Initials	Sterility Blank	Positive Control Organism*	Positive Control Result*	Negative Control Organism*	Negative Control Result*	Date read Initials
2-6-17 TSB CB	7.31	2-6-17 CB	NG	E.Coli	GROWTH	—	—	2-7-17 CB
2-7-17 LTB CB	6.86	2-7-17 CB	NG	E.coli	GROWTH GAS	staph	NG	2-8-17 CB
2-7-17 BHIA CB	7.38	2-7-17 CB	NG	E.coli	GROWTH	N/A	—	2-8-17 CB
2-10-17 PCA PK	7.08	2-10-17 PK	NG	SA	GROWTH PK	N/A	—	2-11-17 PK
2-13-17 TRYPTONE H2O CB	7.47	2-13-17 CB	NG	E.col.	GROWTH RED RING 53 hr. TNTC	ent. Aerog	RED RING NO RING	2-14-17 CB
2-13-17 MENDO CB	7.30	2-13-17 MC	NG	E.coli	GROWTH	staph	NG	2-14-17 MC
2-13-17 MFC CB	7.60	2-13-17 MC	NG	E.coli,	42 colonies TNTC	ent. Aerog	NG	2-14-17 MC
2-13-17 BHI-B CB	7.40	2-13-17 CB	NG	E.coli,	GROWTH	N/AO	—	2-14-17 CB
2-16-17 mTEC TR	7.44	2-16-17 TR	NG	E.Coli	GROWTH TNTC	ENT aerogenes	NG	2-17-17 MC
2-21-17 MFC CB	7.56	2-21-17 TR	NG	E.coli	TNTC 35	ent. Aerog.	NG	2-22-17 CB
2-21-17 M-ENDO CB	7.30	2-21-17 TR	NG	E.coli	TNTC 45	staph	NG	2-22-17 CB
2-21-17 PCA TR	7.04	2-21-17 TR	NG	E.coli	30 CFU TNTC	N/A	—	2-23-17 TR
2-22-17 PCA CB	7.17	2-22-17 CB	NG	E.coli	GROWTH	N/A	—	2-23-17 CB
2-23-17 ILTB TR	6.91	2-23-17 TR	NG	E.coli	G. w/ G. TNTC	staph	NG	2-25-17 MC
2-23-17 II LTB TR	6.85	2-23-17 TR	NG	E.coli	G. + G. TNTC	staph	NG	2-25-17 MC
VOID — 2 TR								

Reviewed: [illegible] Date: 2-27-17
Section 1 Page 19 B of 50

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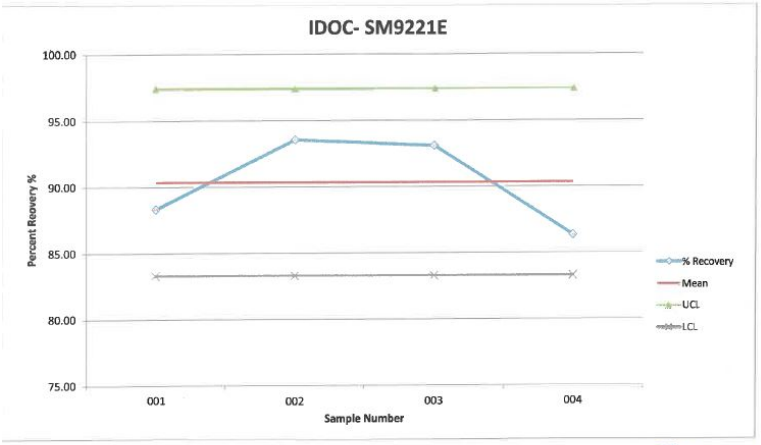
Analyst Capability (IDOC)

INITIAL DEMONSTRATION OF CAPABILITY
Fecal Coliform by Multiple Tube Fermentation, SM 9221B and E

Assayed on 2/1/2016



Reviewed by TRuggio Date 2-5-16

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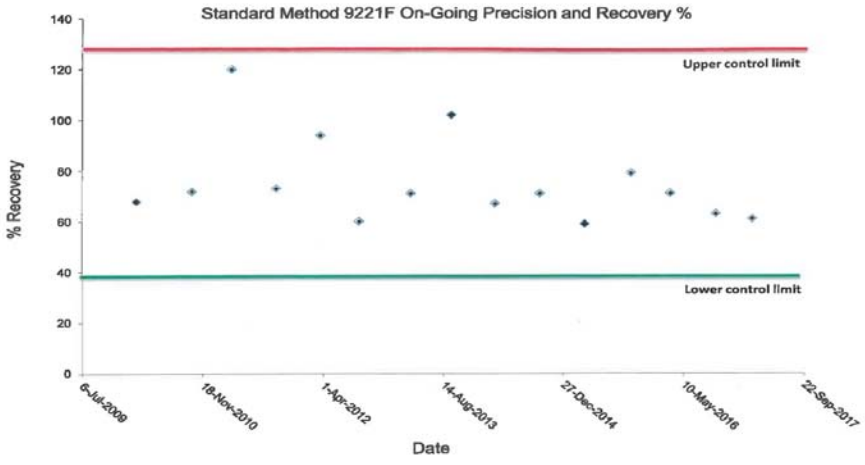
Lab Capability (OPR)



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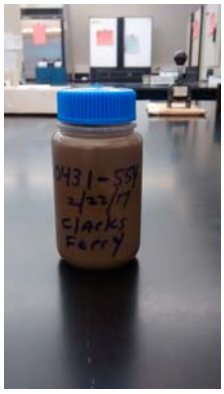
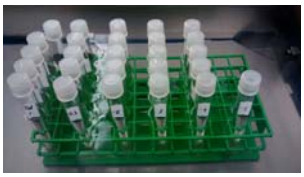
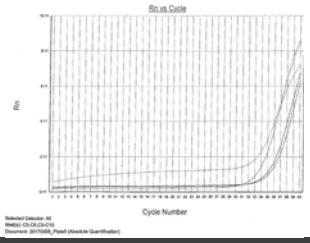
Example of Which Method to Use



Which methods should we use???





PA DEP Sanitarian suspected facility was illegally dumping human waste behind facility. Fecal coliform count = 1,700,000 MPN/100ml. Human gene copies= 21,900/ml, total gene copies= 1,100,000/ml

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Questions?

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Defined Substrate Technology for Testing Waste and Recreational Waters

Gil Dichter, World Wide Technical Support Manager
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IDEXX

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Objectives

- Introduction to Indicator Bacteria
- Test Methods for Waste and Recreational Water
  - *E.coli*, fecal coliforms & enterococci
- Quality Control
  - Defined Substrate Technology (DST) and accessories
  - Micro Lab requirements
- CFU vs. MPN

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Introduction to Indicator Bacteria

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E. coli (non-pathogenic)

- Major inhabitant of gastrointestinal tract in warm-blooded animals, birds and humans
- Shed in feces at high levels
- Thus, true indicator of fecal contamination
- Gram negative
- Thermotolerant
- Does not occur naturally in the environment

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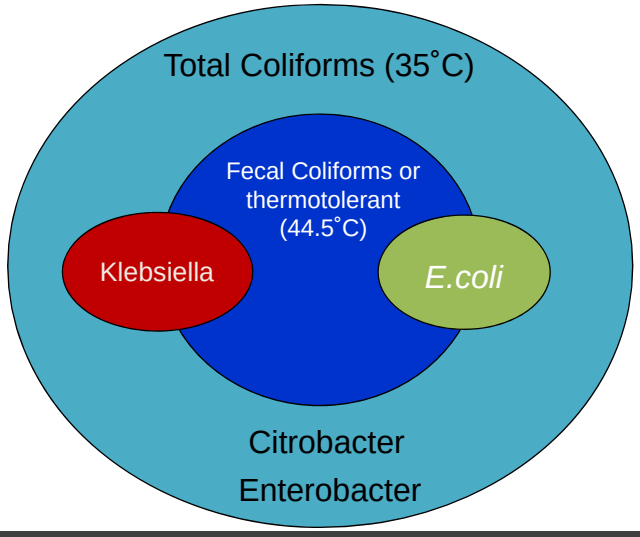
Coliform Bacteria Group



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Enterococcus vs. Fecal Streptococcus

- Enterococci are the key subset of fecal streptococcus
- Fecal Streptococcus
  - 1) Enterococcus spp.:
    - E. faecalis, E. faecium, E. gallinarum, E. avium, E. durans, E. casseliflavus
  - 2) Non-enterococci streptococcus
    - S. bovis, S. equinus
- Defined as gram +, catalase -, grows in 6.5% saline, 40% bile salts, and at 10ºC and 45ºC.
- Occurs in the intestine of humans and animals
- Some strains are found in the environment

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Requirements for an Indicator Organism

- Present when pathogens are present
- Absent in uncontaminated waters
- Present in higher numbers than pathogens in contaminated water
- Better survival in water than pathogens
- Easy and Safe to analyze
- Rapid results
- Inexpensive
- Accurate

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Test Methods for Wastewater

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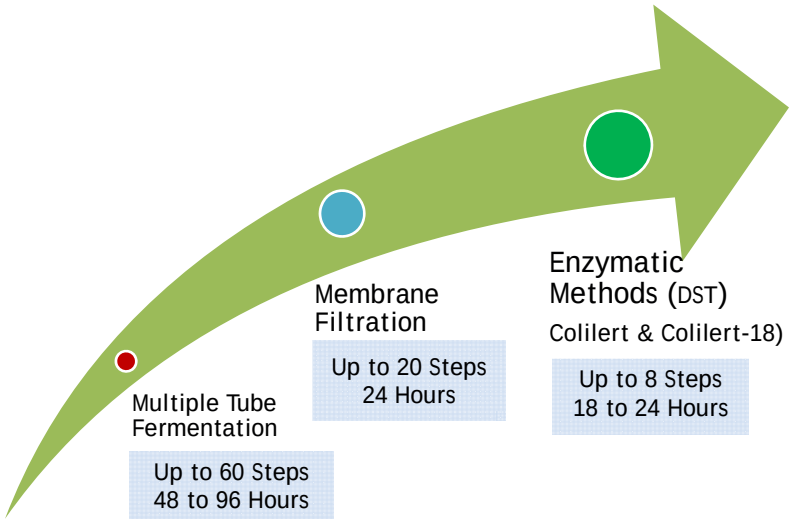
Drinking Water & Wastewater Methods – Evolution Over Time



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Colilert, Colilert-18 and Enterolert

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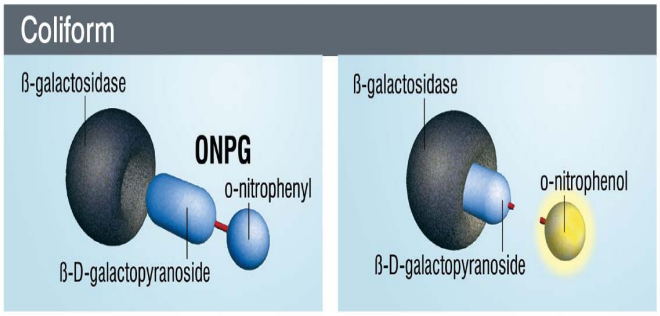
Colilert & Colilert-18 Defined Substrate Technology for Total Coliforms or Fecal Coliforms



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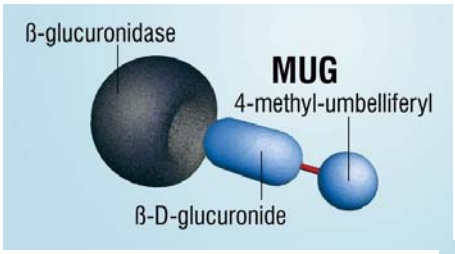
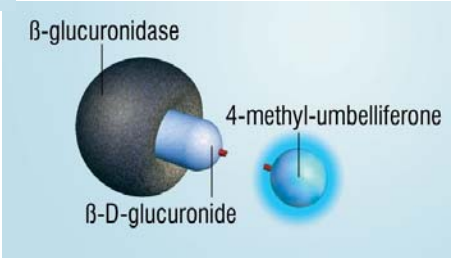
Defined Substrate Technology

Colilert/Colilert-18 for E. coli





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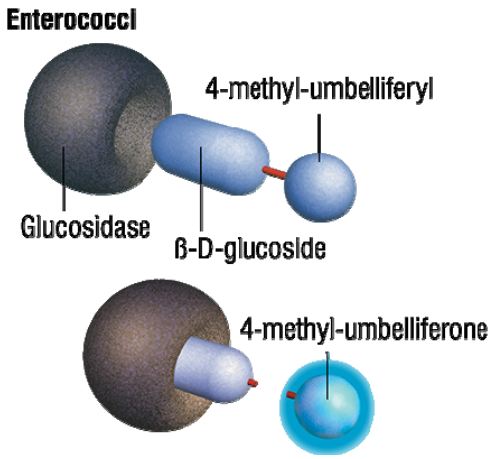
Defined Substrate Technology

Enterolert



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Quantification for Waste and Recreational Waters

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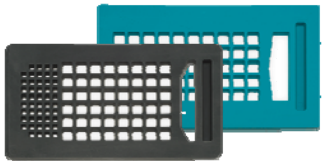
Quantification Tools

51 well Quanti-Tray

97 well Quanti-Tray/2000



Quanti-Tray Rubber Inserts

Quanti-Tray Sealer PLUS

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Colilert and Colilert-18 Procedure for Quantification

1

Add reagent to the sample

2

Pour into Quanti-Tray or Quanti-Tray/2000

3

Seal using Quanti-Tray Sealer PLUS

4

Incubate at 35ºC +/- 0.5ºC for 18 or 24 hours*

*dependent on specific test

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Colilert-18 Incubation- Water Bath

- Water Bath for Fecal Coliform Testing with Colilert-18 at 44.5±0.2ºC for 18 Hours

Colilert and Colilert-18 Comparator

- Comparator is used when reading results
- Comparator provides the minimum yellow (and fluorescence) for positive results

Colilert and Colilert-18 Reading Results

Yellow color indicates positive for Total and Fecal Coliforms

Fluorescence indicates positive for *E. coli*.

Read *E. coli* under a 365nm long wavelength 6 watt UV light

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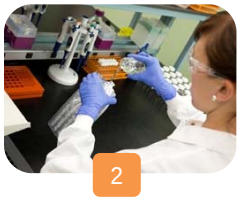
Enterolert Procedure for Quantification*

Add reagent to the sample



Pour into Quanti-Tray or Quanti-Tray/2000

Seal using Quanti-Tray Sealer PLUS

Incubate at 41ºC +/- 0.5ºC for 24 hours

Count fluorescent wells and refer to MPN table

*1:10 dilution necessary for marine waters

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MPN Tables for Quanti-Tray

51 well Quanti-Tray MPN

No. of + Wells	MPN per 100mL Sample
0	<1
15	17.8
35	59.1
51	>200.5

Quanti-Tray 2000

No. of Large + Wells	No. of Small + Wells	MPN/ 100mL Sample
10	2	13.2
30	12	64
47	40	509.9

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Certificates of Quality

- Obtain from Vendors to show Quality
- Important to have a copy for your records and audits
- Should indicate all the testing performed for the specific product and lot

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Enzyme Substrate Coliform Test- Colilert

- Quanti-Tray Sealer - Dye test

- Quanti-Trays - Sterility

- UV Lamp
- 365-366 nm (long) 6 watt

Colilert & Colilert-18 QC Testing

- Media is purchased directly from vendor
- The products should be tested upon receipt in the lab
  - Colilert (example)
    - Positive control
    - Negative control
    - Sterility test
  - Vessels
    - Thiosulfate content
    - 100 mL line
    - Sterility
    - Non-fluorescing

Micro Lab QC Requirements

- Avoid contamination when testing: Use Aseptic Techniques
  - Disinfect bench tops prior to testing.
  - Wear disposable gloves and lab coat.
  - Do not touch any surfaces that are sterile.
  - Keep sterile plates, bottles, pipettes closed until ready to use.
  - No food or drink in the laboratory

Micro Lab QC Requirements - Incubators

- Incubator units must have an internal temperature monitoring device
- Thermometers should be placed on the top and bottom shelves of the incubator, immersed in liquid (except for electronic thermometers)
- Calibration-corrected temperature should be recorded for each thermometer being used twice per day when incubator is in use
- Temperature readings separated by at least 4 hours
- Documentation should include the date/time of reading, temperature, and technician's initials

Micro Lab QC Requirements - Thermometers

- Glass, dial, or electronic thermometers - graduated in 0.5˚C increments
- For refrigerators; graduated in 1˚C
- The calibration of glass and electronic thermometers are checked annually against a National Institute of Standards and Technology (NIST) traceable reference thermometer
- If a thermometer differs by more than 1˚C from the reference thermometer, it should be discarded

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MPN vs. CFU

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MPN vs. CFU

- Is there a difference?
  - Simple answer is no!
  - It is based on the method used
  - It is the label or unit associated with the numerical result.
  - Reported as MPN/100 mL or CFU /100 mL based on the method used for testing between the 2 reported units.
  - Both methods have a lower and upper 95% confidence limit.

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Confidence Intervals

- The width of the 95% CI provides a useful measure of the uncertainty of the mean.
- The wider the 95% CI, the less certainty can be placed on how accurately it estimates the true value of the concentration.

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Coliform Analytical Methods and Data Application in Wastewater and Recreational Waters

Membrane Filtration Technology

-Akin Babatola, MS Molecular Biology

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Membrane Filtration Technology for Coliform Analyses

- Principle and Scope of Method
- Basic Instrumentation
- Considerations for Method choices
- Quality Control in Method
- Relative Advantages

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Membrane Filtration Technology for Coliform Analyses

- Principle and scope:
  - Membrane Filtration Technique gives a direct count of coliforms present in a sample of water.
  - A measured volume of water is filtered, under vacuum, through a cellulose acetate membrane of uniform pore diameter (usually 0.45 µm.)
  - Bacteria are retained on the membrane which is then placed on a suitable selective medium in a sterile petri-dish
  - The petri-dishes are then placed in an incubator or water bath at the specified temperature for the test.
  - If coliforms are present in the water sample, characteristic colonies form that can be counted directly.
  - Theoretically each colony arose from one viable cell only.

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Membrane Filtration Technology for Coliform Analyses: Basic Instrumentation

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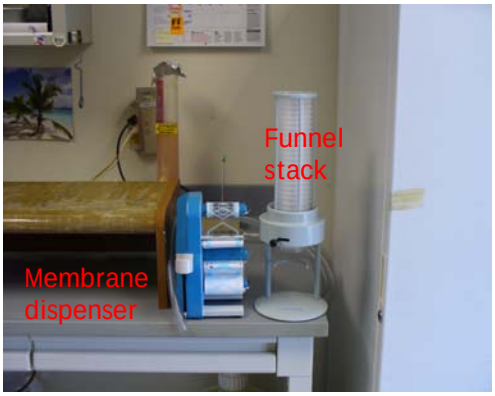
Membrane Filtration Technology for Coliform Analyses



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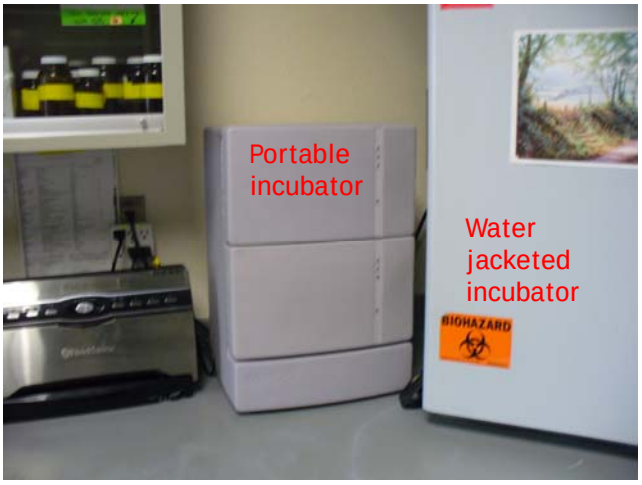

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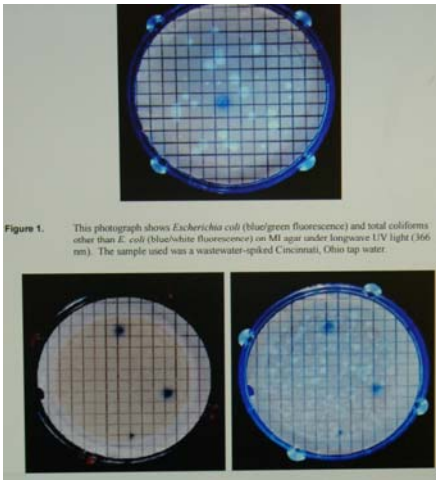

Figure 1. This photograph shows *Escherichia coli* (blue/green fluorescence) and total coliforms other than *E. coli* (blue/white fluorescence) on MI agar under longwave UV light (366 nm). The sample used was a wastewater-spiked Cincinnati, Ohio tap water.

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Membrane Filtration Technology for Coliform Analyses: Considerations for Method choices

Multiple fermentation tube technique	Membrane filter technique
Slower: requires 48 hours for a positive presumptive	More rapid: quantitative results in about 18 hours
More labor-intensive	Less labor-intensive
Requires more culture medium	Requires less culture medium
Requires more glassware	Requires less glassware
More sensitive	Less sensitive
Result obtained indirectly by statistical approximation (lower precision)	Results obtained directly by colony count (higher precision)
Not readily adaptable for use in the field	Readily adapted for use in the field
Applicable to all types of water	Not applicable to turbid waters
Consumables readily available in most countries	Cost of consumables is high in many countries; NOT in the US.
May give better recovery of stressed or damaged organisms in some circumstances	
Reference: http://www.who.int/water_sanitation_health/resourcesquality/wqmchap10.pdf	

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Membrane Filtration Technology for Coliform Analyses

- Quality Control:
- 1. Test sterility integrity checks:
- **Filter**: Incubate one or more membrane filters on plates, for 24 hours at 35°C.
- **Phosphate-Buffered Dilution Water:** Filter a 50-mL volume of sterile dilution water before beginning the sample filtrations and a 50-mL volume of dilution water after each batch. Place the filters on plates, and incubate the plates for 24 hours at 35°C.
- **Agar or Broth**: Place one or more agar plates or broth pad plates in the incubator for 24 hours at 35°C. Broth pad plates should be incubated *grid-side up*, not inverted like the agar plates.
- Absence of growth indicates sterility of the filter membranes; Dilution water and plates.

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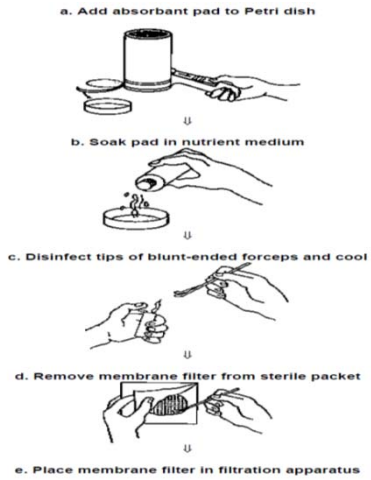
Membrane Filtration Technology for Coliform Analyses



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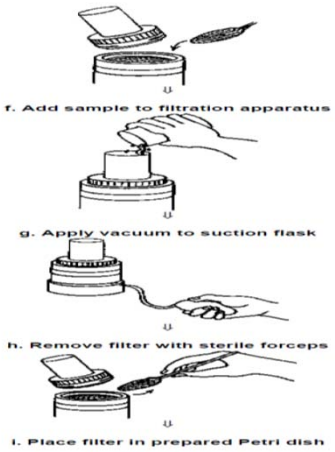

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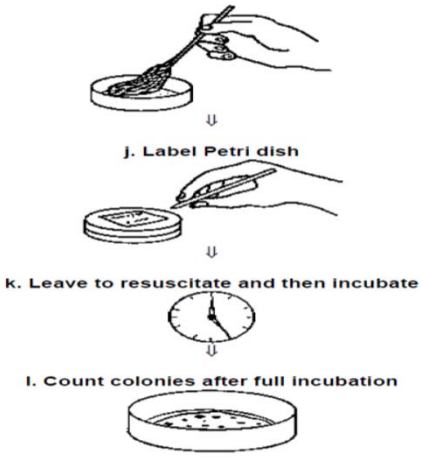
Membrane Filtration Technology for Coliform Analyses



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Membrane Filtration Technology for Coliform Analyses

- Considerations for Method choices in the USA:
  - Cost: Comparable or better than competitors
  - T-A-T: 24 hours per test
  - Precision: Each colony derives from single viable cells; not derived from probabilistic calculations.
  - Portability: From filtration to incubation, all testing components are portable in the US; Testing can begin in the field.
  - Matrix Considerations:Not recommended for turbid samples and samples with high particulate content.

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Membrane Filtration Technology for Coliform Analyses

Table 10.10 Colony characteristics following analysis by the membrane filtration method

Medium	Colony characteristics	
	Total coliforms at 35 or 37 °C	Thermotolerant coliforms at 44 or 44.5 °C
Lactose TTC agar with Tergitol 7	Yellow, orange or brick red coloration with yellow central halo in the medium under the membrane	Same as total coliforms at 35 or 37 °C
Lactose agar with Tergitol 7	Yellow central halo in the medium under the membrane	Same as total coliforms at 35 or 37 °C
Membrane enriched Teepol broth	Yellow colour extending on to the membrane	Same as total coliforms at 35 or 37 °C
Membrane lauryl sulphate broth	Yellow colour extending on to the membrane	Same as total coliforms at 35 or 37 °C
Endo agar or broth	Dark red colour with golden-green metallic sheen	(not applicable)
LES Endo agar	Dark red colour with golden-green metallic sheen	(not applicable)
MFC medium	(not applicable)	Blue colonies

Source: Adapted from ISO, 1990a

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Membrane Filtration Technology for Coliform Analyses

- Additional Resources:
- **1. USEPA Method 1604**: Total Coliforms and *Escherichia coli* in Water by Membrane Filtration Using a Simultaneous Detection Technique (MI Medium)
- **2. Water Quality Monitoring:** A practical guide UNEP/WHO: ISBN O 419 21730 (Chapter 10) 1996
- **3. A Comparison of Methods…Solutions v12 (2) April/May 2005.**

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