E. coli Analysis in Wastewater by Quanti-Tray, Method 9223 B
Amy Staley
Alloway

E. coli happens

**E. coli**
- a species of bacteria within the fecal coliform group
- dominant bacteria found in waste of humans and warm-blooded animals.
- produce a positive total coliform response
- possess an enzyme called (ß-glucoronidase) which releases fluorogen that is detected using a 365 nm UV lamp.

**Fecal Coliform Group**
- group of total coliform bacteria found in intestinal tracts of warm-blooded animals.

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**E. coli**

• Why test for **E. coli** and not just Fecal Coliforms?
  - Because we are now required to.
  - As NPDES permits are renewed **E. coli** will be added.
  - Fecal coliform requirements are being phased out and **E. coli** limits and monitoring requirements are being put in place
  - **E. coli** has been shown to be a better predictor of the potential for impacts to human health from exposure to waste effluent and surface waters which contain wastewater effluent.

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**E. coli as an indicator organism**

• Determines the sanitary quality of water
• Impossible to test for ALL pathogenic microorganisms, so test for easily detectable indicator organisms.
• Hundreds of **E. coli** strains
  - Most are non pathogenic (some beneficial)
  - Some pathogenic strains
• Although generally not pathogenic, their presence indicates a pathway for human pathogen (ex. Viruses, bacteria) to enter the water source.

**Escherichia coli (E. coli)**

• Ideal indicator organism for testing water for fecal contamination
  - Ability to survive for brief periods outside of the body
  - Other fecal coliforms can arise from environmental factors (not always a result of fecal contamination) ex. Klebsiella spp.
E. coli as an indicator organism

- Not all E. coli comes from humans
  - Different strains from different species (e.g., Humans, birds, cows, etc.)
  - Most harmful pathogenic strain, E. coli O157:H7, found in cow intestines
    - e.g., Food poisoning
    - Can NOT be detected using standard fecal coliform methods.
  - Differentiation may be necessary to pinpoint source of contamination
    - Performed by specialized labs.

Escherichia coli (E. coli)

- Primary Body Contact Recreation: E. coli is a more accurate indicator of fecal contamination than the fecal coliform group.
  - A positive relationship exists between E. coli density in recreational waters and numbers of observed gastrointestinal illnesses.
  - Lack of a positive relationship between fecal coliform group and gastrointestinal illness.
- Ohio Revised Code 3745-1-07 identifies criteria for recreation uses
  - Applies to inside and outside the mixing zone
  - Applies at all times during recreation season

3745-1-07 has updated classifications

- Class A primary contact – paddling streams, public/private access points, frequent recreational activity
- Class B primary contact – occasional primary contact, non paddling, non bathing waters, occasional recreational activity
- Class C primary contact – infrequent primary contact, wading, very small drainage area (<3.1 square miles)
- Secondary contact recreation – minimal exposure potential
  - Insufficient depth to provide full body immersion
  - Rarely used for recreation

Escherichia coli (E. coli)

- EPA approved methods for testing for E. coli in wastewater include:
  - Membrane filter (MF)
    - EPA Method 1603 (m-TEC media)
    - HACH Method 10029 (mColiblue 24 media)
  - Multiple tube/multi-well procedures
    - Standard Methods 9223 B (Enzyme Substrate)
    - Quanti-Tray and Quanti-Tray/2000

Enzyme Substrate Methods

- Enzyme based methodologies detect both total coliforms and E. coli simultaneously:
  - Easy, rapid, accurate
  - Widely accepted as standard for microbiological analysis of water.
- Enzymes for Quanti-Tray method: Colilert, Colisure, Colilert-18
Traditional media provides a nutrient rich environment that supports the growth of both target and non-target organisms, when non targets grow and mimic target organisms false positives occur. Growth of non targets can also suppress target organism and give false negative in traditional media.

To suppress non target organisms, traditional media often include high levels of salts, detergents and other selective agents which may inadvertently suppress target organisms and give false negatives.

Colilert:
- Ability to detect either presence/absence or to enumerate organisms.
  - Detects a single, viable organism per sample
- Suppression of non-coliforms
  - Ex. Antibiotics
  - Suppresses up to 2 million heterotrophs per 100 ml

Benefits of Quanti-Tray
- Detects down to one organism per 100 mL
- No dilutions (for counts to 200/100mL or 2,419/100 mL)
- Results in 24 hours
- No confirmation necessary
- If no dilutions are used: No glassware to purchase and clean

**Equipment Needed for Method 9223 B**

**Autoclave**
- Sterilize TSB media for 15 minutes at 119º - 121
- Sterilize blank water
  - < 500 ml = 30 mins
  - > 500 ml = 45 mins

**Refrigerator**
- Storage of reagents at 0º-5º C
  - TSB media
  - Bacterial cultures
  - ex. Kwik Stiks
Equipment

**Oven**
- Sterilize measuring glassware for 2 hrs. at 180º C
- Graduated cylinders
- Supplies: aluminum foil

**Incubator**
- Incubate Quanti-Trays for E. coli analysis at 35ºC +/- 0.5ºC for indicated amount of time

Equipment

**Testing Supplies**
- Quanti-Tray sealer and rubber inserts
- Quanti-Tray (51 wells) range: 1-200 MPN/100 mls
- Quanti-Tray 2000 (97 wells) range: 1-2419 MPN/100 mls

**Testing Supplies (cont.)**
- Pre-sterilized clear sample bottles with dechlorination chemicals
- Squeeze bottles for blank water
- Enzymes - ex. Colilert
- Long wave UV lamp

Equipment

**Measuring Items**
- Sterile graduated cylinders
- Sterile pipettes
- Balance for weighing dry media
  - if preparing TSB

**Misc. Items**
- Pipette washer
- Conductivity meter
  - monthly QC of blank water
- pH meter
  - for checking pH of TSB
Reagents and Standards

- **Colilert**
  - Snap packs for sample size 100 mL
  - Sample turns yellow when total coliform bacteria are present and fluoresces blue to indicate the presence of *E. coli*

- **Distilled Water/RODI Water**
  - Do NOT use phosphate buffered rinse water with this method

- **pH Buffers**
  - For calibration of pH meter used for monthly QC of RODI water

- **DPD reagent packets**
  - For determination of residual chlorine (monthly QC on RODI water)

- **Conductivity Standard**
  - For calibration of conductivity meter used for monthly QC of RODI water

- **Bacterial Cultures**
  - QC for media
  - Ex: Microbiologic Kwik Stiks

- **TSB media (tryptic soy broth)**
  - QC for bottles
  - Can be purchased premade or as a dry media

- **Autoclave Biological Indicator Checks**
  - QC for Autoclave

- **Clorox Bleach**
  - Disinfection of counter and spills

- **Colilert comparator**
  - Pre-dispensed in either types of Quanti-Tray
  - Used for determination of positive result

Sample Collection

- Samples to be representative of the water being tested
  - Use aseptic technique for collection

- Keep sample container closed until collection begins
  - Avoid contact with inside of bottle and/or cap

- Collect directly into sterile container containing de-chlorinate agent
  - Do not rinse the bottle

- Leave air space to allow for mixing

- If not analyzed immediately:
  - Cool sample to <10°C

- Ideally analysis within 2 hours of collection is preferred

- Sample must be analyzed within 6 hours of collection.
  - New EPA amendment states lab can analyze within 8 hours of collection if sample is not able to be delivered to lab within the 6 hour hold time.
Procedure

• Turn on Quanti-Tray Sealer
  - Warm up time approx. 10 mins.

• Vigorously shake water sample bottle 25 times within 7 seconds.
  - Interval between shaking and measuring the test portion should not exceed 3 minutes.

Procedure

Aseptically remove lid and adjust sample volume to the calibrated 100 ml line on sample container: (this is for use of 100 mls of sample)

 Procedure

Need Dilutions?

Dilutions may also be used in which case you do NOT need to pour off excess water.

Test requires the use of 100 ml of sample:
  • Ex. 1:10 dilution; use 10 ml sample: 90 ml blank water
  • Final results must be multiplied by the applicable dilution factor.

Procedure

• Aseptically add 1 packet of Colilert reagent to the 100 ml test bottle
  - If sample "flashes" blue: excessive chlorine and invalid for analysis

• Re-cap the bottle and shake until reagent is mostly dissolved.
• Label back of tray with sample ID and dilution used

Procedure

• Use one hand to hold open the Quanti-Tray or Quanti-Tray/2000
  - Well side is facing the palm of the hand.

• Squeeze upper part of tray so it bends toward the palm.

• Gently pull foil tab to open the tray.
  - Avoid touching inside of tray or foil tab.

• Pour 100 ml sample into the tray.

Procedure

• Tap small wells 2-3 times to release air bubbles.
• Place tray with sample into rubber insert so that wells sit within the cutouts

• Place rubber insert on the input shelf of sealer.
• Slide rubber insert with tray into the sealer
Procedure

• Once sealed, incubate the tray/trays for 24 hrs at 35 +/- 0.5 º C

• After 24 hrs, if fluorescence is questionable, incubate for an additional 4 hrs.

Counting and Calculations

• Quanti-Tray (51 wells) and Quanti-Tray/2000 (97 wells)

  Counting Ranges:
  Quanti-Tray: max. of 200 MPN/100 mls sample
  Quanti-Tray 2000: max. of 2,419 MPN/100 mls sample

• Count both small and large yellow wells.
  Use color comparator to confirm positive result.
  Document these as total coliform positive.

Counting and Calculations

• Use the UV lamp to check for fluorescence.
  If no wells fluoresce, negative for E. coli
  If wells do fluoresce, positive for E. coli
  • Count small and large fluorescing wells
  • Refer to table for MPN

Counting and Calculations

Quality Control

Daily QC
  Method Blank
  • Once per batch (every 10 samples)

Duplicate
  • One sample per batch

Incubator Temperature checks
  • Twice daily - 4 hours apart

Refrigerator Temperature
  • Once per day
## Quality Control

### Autoclave Biological Indicator Checks
- **Monthly QC**
- **Reagent Water Analysis (Blank Water)**
- **Residual Chlorine**
- **Conductivity**
- **pH**

### Autoclave Biological Indicator Check

<table>
<thead>
<tr>
<th>Month</th>
<th>Analyst</th>
<th>Date</th>
<th>Time</th>
<th>Equipment</th>
<th>Date</th>
<th>Time</th>
<th>Color</th>
<th>Pass or Fail</th>
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</table>

### Autoclave Timer Calibration

1. Check the conductivity electrode with RO/DI water.
2. Clean the Beaker & Solution in a beaker to submerge the electrode.
3. Read the conductivity of the solution while gently swirling.
4. Adjust the meter to read the known value by using the up and down arrows.
5. Rinse the electrode with RO/DI water.

### Autoclave External Thermometer Calibration

1. Place the external thermometer in water at the desired temperature.
2. Record the temperature reading on the thermometer.
3. Adjust the thermometer to read the known value.
4. Rinse the thermometer with RO/DI water.

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### Bacteriology Distilled Water pH & Chlorine Check

- **Monthly QC**
- **Determination of pH**
- **Determination of Residual Chlorine**
- **DPD control #: Form 5971-1**

<table>
<thead>
<tr>
<th>Date performed</th>
<th>Analyst</th>
<th>pH Obtained</th>
<th>pH Limits</th>
<th>Buffer control</th>
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### Bacteriology Distilled Water Conductivity Check

- **Monthly QC**
- **Frequency:** Monthly
- **Conductivity:**

### Bacteriology Distilled Water Conductivity Check

<table>
<thead>
<tr>
<th>Date performed</th>
<th>Analyst</th>
<th>Conductivity</th>
<th>Limits</th>
<th>Control # for KCl Solution</th>
<th>Meter/Electrode Serial #</th>
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### Reagent Number Autoclave Date Date/Time Color Pass or Fail

### Instructions:
- Perform once per month.
- Run one indicator through an autoclave sterilization cycle.
- Compress the plastic vial.
- Incubate at 57°C for 48 hours.
- Document the color of the indicator after 48 hours.
- Document "Pass" if the purple color is present without any cloudiness.
- Document "Fail" if the yellow color is present or if cloudiness is present.
- Notify the Laboratory Manager immediately if the test fails.
- Items autoclaved in a cycle that fails this test should not be used.

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### Quality Control

### Autoclave Biological Indicator Checks

- **Monthly QC**
- **Reagent Water Analysis (Blank Water)**
- **Residual Chlorine**
- **Conductivity**
- **pH**

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1. Check the conductivity electrode with RO/DI water.
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3. Read the conductivity of the solution while gently swirling.
4. Adjust the meter to read the known value by using the up and down arrows.
5. Rinse the electrode with RO/DI water.

### Autoclave External Thermometer Calibration

1. Place the external thermometer in water at the desired temperature.
2. Record the temperature reading on the thermometer.
3. Adjust the thermometer to read the known value.
4. Rinse the thermometer with RO/DI water.

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### Bacteriology Distilled Water pH & Chlorine Check

- **Frequency:** Monthly
- **Determination of pH**
- **Determination of Residual Chlorine**
- **DPD control #: Form 5971-1**

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### Bacteriology Distilled Water Conductivity Check

- **Frequency:** Monthly
- **Conductivity:**

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### Quality Control

### Autoclave Biological Indicator Checks

- **Monthly QC**
- **Reagent Water Analysis (Blank Water)**
- **Residual Chlorine**
- **Conductivity**
- **pH**

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### Autoclave Timer Calibration

1. Check the conductivity electrode with RO/DI water.
2. Clean the Beaker & Solution in a beaker to submerge the electrode.
3. Read the conductivity of the solution while gently swirling.
4. Adjust the meter to read the known value by using the up and down arrows.
5. Rinse the electrode with RO/DI water.

### Autoclave External Thermometer Calibration

1. Place the external thermometer in water at the desired temperature.
2. Record the temperature reading on the thermometer.
3. Adjust the thermometer to read the known value.
4. Rinse the thermometer with RO/DI water.
Quality Control

**Autoclave Timer Check**  
**WWTP**  
**Frequency:** Quarterly

1. Determine correction setting for autoclave timer as outlined below.
2. Set autoclave timer to operate for 50 minutes.
3. Use a lab clock as a reference and record autoclave timer reading after 15, 30, and 45 minutes.
4. Complete calculations in table below and post instructions to obtain desired exposure on autoclave.

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Timer Setting</th>
<th>Timer Reading</th>
<th>Column C</th>
<th>Column D</th>
<th>Column E</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 minutes</td>
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<td>30 minutes</td>
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<td>45 minutes</td>
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</table>

- **Column C:** Autoclave timer reading
- **Column D:** 50 - Column C
- **Column E:** Column D ÷ Column A

5. Complete documentation in bacteriology log book.

**Average Ratio:**

Setting is obtained by multiplying the desired exposure time by the average ratio.

**Autoclave Thermometer Calibration**

**Frequency:** Quarterly

1. Place the calibrated maximum registering thermometer in the autoclave.
2. Run a 15 minute cycle using slow exhaust and monitor the exterior thermometer for the maximum reading during the cycle. Record maximum external reading below (°F).
3. After the cycle is completed record the maximum internal temperature on the maximum registering thermometer.
4. Add the correction factor for the maximum reading thermometer to obtain corrected maximum temperature. See thermometer calibration for correction factor.
5. Convert the internal temperature from °C to °F.

<table>
<thead>
<tr>
<th>Maximum registering thermometer reading (°C)</th>
<th>Correction for Max. registering thermometer (°F)</th>
<th>Corrected maximum temperature in autoclave (°F)</th>
<th>°F = (1.8)(°C) + 32</th>
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</table>

6. Label the external thermometer on the autoclave with the correction factor.

**Bacteriology RO/DI Water Contamination Check**

**Frequency:** Annually

<table>
<thead>
<tr>
<th>Metal Limit (mg/L)</th>
<th>Result</th>
<th>Pass / Fail</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd, Cr, Cu, Pb, Ni, Zn</td>
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</tbody>
</table>

**Balance Service Check**

- **Outside Contractor**

**Maximum Registering Thermometer (MRT) Calibration**

**Frequency:** Annually

1. Place the reference NIST calibrated MRT in a 25 ml graduated cylinder containing 10 mL reagent water.
2. Place the daily working MRT thermometer in the same 25 ml graduated cylinder containing 10 mL reagent water.
3. Run a 15 minute cycle using slow exhaust.
4. After the cycle is complete and pressure is @ 0 psi, open the autoclave door and remove the graduated cylinder containing the MRT's.
5. After five minutes record the temperature of each MRT below.
6. Calculate the correction factor for the daily working MRT thermometer.
7. Label the daily working MRT with the correction factor, date calibrated, and analyst initials.
8. Apply correction factors to every temperature documented.

**MRT Calibration**

**Frequency:** Annually

<table>
<thead>
<tr>
<th>NIST Reference MRT Serial Number</th>
<th>Thermometer Ser. No.</th>
<th>NIST Reading</th>
<th>Test Reading</th>
<th>Correction °C</th>
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</table>

Completed By: Date:

**QC per each new lot prior to use**

Sample bottle sterility checks: each new lot
- **Use TSB media (Tryptic Soy Broth)**
  - Test 1% of each box received for growth
Quality Control

• QC per each new lot *prior to use*

  **TSB media check**
  1 positive control (using E. coli),
  1 negative control (no inoculation)

Quality Control

Colilert check
(Each new lot received)

Inoculation with 3 control bacteria:
One control bacteria must be E. coli
  total coliform (+), E. coli (+)
One control bacteria can be
  Pseudomonas aeruginosa (or other non-coliform)
  total coliform (-), E. coli (-)
One control bacteria can be
  Klebsiella pneumoniae (or other coliform)
  total coliform (+), E. coli (-)

Quality Control

**TSB Bottle Sterility Check**

**WWTP**

<table>
<thead>
<tr>
<th>Reagent Number</th>
<th>Reagent Number</th>
<th>Date</th>
<th>Time</th>
<th>Date</th>
<th>Time</th>
<th>Pass or Fail</th>
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</table>

Instructions:
1. Aseptically transfer 25 ml of single strength TSB to a sample container using a sterile pipette.
2. Incubate the sample bottle at 35 ± 0.5 C for 24 hours and check for growth.
3. Growth will be indicated by even the slightest turbidity in the TSB.
4. If the sample container is opaque, the TSB must be poured into a glass vessel after incubation in order to look for turbidity.
5. Document "Pass" if no turbidity is detected.
6. Document "Fail" if turbidity is detected. Notify the laboratory director immediately.

Quality Control

**Form 155-0**

**TSB Media Positive Check**

**WWTP**

Frequency: Each New Lot

**Positive Control Procedures**
1. Before using each new lot of TSB media, it must be checked for positive growth.
2. For the positive control check, use the E. coli microorganism from Fisher (23-0035004).
3. Take one E. coli pellet and transfer it to a bottle containing 99 ml of sterile phosphate buffer water (that has been slightly warmed). Ensure the pellet is dissolved.
4. Incubate bottle for 30 minutes at 35 C.
5. Remove from incubator and shake vigorously.
6. Using a sterile loop, transfer one loop of the above solution to a sterile sample bottle containing 25 mls of TSB.
7. Swirl the sterile loop in the TSB media.
8. Transfer the bottle with the TSB to the incubator and incubate for 24 hours at 35 ± 0.5 C.
9. Growth will be indicated by even the slightest turbidity in the TSB.
10. Document "Pass" if turbidity is detected.
11. Document "Fail" if turbidity is not detected.
12. The TSB must Pass (show signs of turbidity). If it does not, notify the laboratory supervisor immediately and contact the supplier of the TSB. The TSB must not be used if it fails this check.

**TSB Media**

<table>
<thead>
<tr>
<th>Lot Number</th>
<th>Reagent Number</th>
<th>Date</th>
<th>Date</th>
<th>Date/Time</th>
<th>Date/Time</th>
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</table>

**Analyst**

**Result**

**Quality Control Thoughts**

• Without quality control is your data defensible?
• What is the "true" overall cost of quality?
• Alloway is a full service laboratory and we are committed to helping you.
  – At Alloway we can help you:
    – Set up your lab for E. coli
    – Train your analysts
    – Perform many of the required QC for E. coli testing