A Comparison of Methods for Enumeration of Bacteria from Natural Water Samples: A Multi-Week Laboratory Project

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Introduction

As part of a multi-week laboratory project in Microbial Ecology (BIO 300 level), students enumerate bacteria from natural water samples.

Goals for Student Learning:

- Learn to work in teams on a complex laboratory project.
- Learn to work on a laboratory project for which lab manual-style, step-by-step directions are not provided. (Students work from *Standard Methods For The Examination of Water and Wastewater*.)
- Learn to function independently in a microbiology laboratory setting in which materials such as pre-prepared culture media are not provided.
- Understand different methods of bacterial enumeration:
  1.) *viable* count: heterotrophic plate count
  2.) *viable* count: most probable number (MPN)
  3.) *direct total count*: epifluorescence microscopy
- Learn to closely evaluate and critically analyze data about bacterial numbers.
- Learn to ask meaningful comparative questions. [Students evaluate water from the Chester River, a tidal tributary of the Chesapeake Bay. They may chose to compare water from different locations within the river or water from the river and from a different source (e.g. groundwater).]

FIG. 1: Chester River (Maryland Eastern Shore) and its connection to the Chesapeake Bay. Image from Google Earth.

Direct Total Microbial Count using Epifluorescence Microscopy

Water Sample Mixed Vigorously with Vortex Mixer

⬇

Sample Diluted in Sterile Phosphate Buffer (PB) (if necessary)

⬇

Sample or Diluted Sample Mixed with an Equal Volume of Fluorochrome [Acridine Orange, 0.1%] for Approximately 2 min. PB added for volume.

⬇

A Minimum of 2 mL Filtered through a Black Polycarbonate Filter (Nuclepore™ or Isopore™, pore size 0.2 micron, diameter 25mm)

⬇

Phosphate Buffer Filtered to Wash Acridine Orange from Filter

⬇

Filter Removed, Air-Dried for Approximately 2 mins., and Cut into Quarters

⬇

One Filter Piece Placed onto a Drop of Low Fluorescing Immersion Oil on Slide

⬇

Immersion Oil Added to the Top of the Filter

⬇

Cover Glass Added

⬇

Sample Viewed (1000x) and Bacteria in Fields Counted

⬇

Number of Bacteria per mL Calculated Based on Dilutions, Volume Filtered, Average Number of Bacteria per Field of Known Size

Samples not rapidly processed can be preserved with 5% (w/v) glutaraldehyde in phosphate buffer (9:1 sample volume: fixative volume).

Stained, filtered samples can be stored in the dark for weeks before viewing.

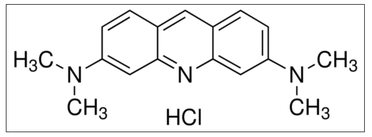

FIG. 2: Acridine Orange (*N,N,N′,N′*-Tetramethylacridine-3,6-diamine). Image from sigma.com.

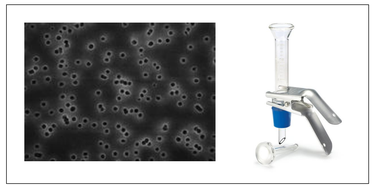

FIG. 3: Black Polycarbonate Filter (left) and Filter Support (right). Image from millipore.com.

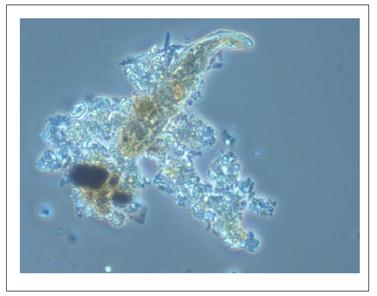

FIG. 4: Chester River water viewed by phase contrast microscopy (400x). High concentration of particulates limits ability to view bacteria.

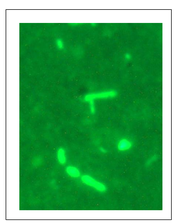

FIG. 5: Chester River bacteria filtered through a black polycarbonate filter and stained with Acridine Orange (1000x). Filtration places the cells in one plane and the fluorochrome increases contrast.

Sample Learning Questions:

What are reasons why some bacteria that can be viewed by microscopy do not grow in culture? Explain "the great plate count anomaly."

How might it be determined whether a bacterium that does not grow in culture is alive or dead?

Are all bacteria able to be trapped by the 0.2 micron filter and therefore visible by microscopy?

When a stained sample is viewed by microscopy, are all bacteria identified as bacteria and thus counted?

How could the direct total count procedure be modified to count only a particular type of bacterium?

How could the MPN method be modified to count heterotrophs rather coliforms?

What are the detection limits of Colilert and the other procedures used?

Heterotrophic Plate Count

Sample Diluted (1:10 Dilution Series) in Sterile 0.1% Peptone Water or Buffered Dilution Water (Hach)

⬇

Sample Processed by Either:

- **Pour Plate Method**
- **Spread Plate Method**
- **Membrane Filter Method (rarely used)**

⬇

Sample Plated on Either:

- **Plate Count Agar**
- **m-HPC agar**
- **R2A agar**
- **NWRI Agar**

⬇

Plates Incubated (Students choose incubation conditions and time.)

⬇

Colonies Counted and used to Determine Colony Forming Units (CFU)/mL

Detection of Coliforms and the Coliform *E. coli* with Colilert

Colilert® (IDEXX) Reagent Packet added to 100 mL Water Sample (or diluted sample)

⬇

Sample w/ Dissolved Colilert® Reagent Poured into Quanti-Tray 2000

⬇

Sample dispersed into wells and Quanti-Tray sealed with a Quanti-Tray sealer

⬇

Tray incubated at 35°C for 24 hours

⬇

Coliforms: Number of Yellow Wells (large, small) Counted and Compared to IDEXX MPN Table to Determine Number per 100 mL

⬇

***E. coli*: Quant-tray Illuminated with UV lamp and Number of Fluorescent Wells (large, small) Counted and Compared to IDEXX MPN Table to Determine Number per 100 mL**

FIG. 6: Colilert assay showing sample bottle, reagent packets, Quanti-Trays with positive wells (yellow, coliforms; fluorescent, *E. coli*),Quanti-Tray sealer, and UV lamp.

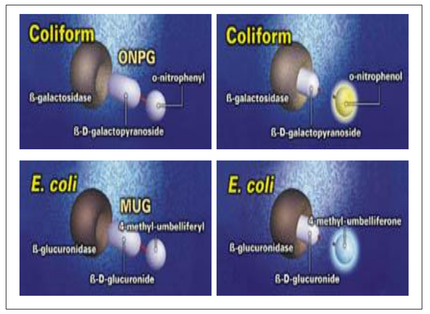

FIG. 7: Coliforms produce ß-galactosidase which hydrolyzes ONPG to produce o-nitrophenol (yellow). *E. coli* produces ß-glucuronidase which hydrolyzes MUG to form 4-methylumbelliferone, which has a blue fluorescence. Image from idexx.com.

References

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