EXPERIMENT 8

Water Bacteriology

INTRODUCTION

Natural waters contain bacteria. The aerobic gram negative bacillus of the genera *Psedomonas, Alcalignes*, and *Flavobacterium* are common in natural waters. Many of these bacteria are able to grow on a wide variety of single carbon sources. Single carbon medium are those that contain only mineral salts and one carbon source as the sole *organic* nutrient, and other *inorganic* ingredients.

Some species of the genus *Pseudomonas* are extremely versatile and can survive on as many as 90 varying sole carbon sources. The *Pseudomonas* genus also includes species that produce **water-soluble** fluorescent pigments that diffuse into the medium (Figure 1). The ability to grow on wide varieties of single carbon sources, and the ability to produce water-soluble fluorescent pigments is often taken advantage of to count or enumerate the number (or titer) of *Pseudomonas* by using the Multiple Tube Technique, as we will see later.

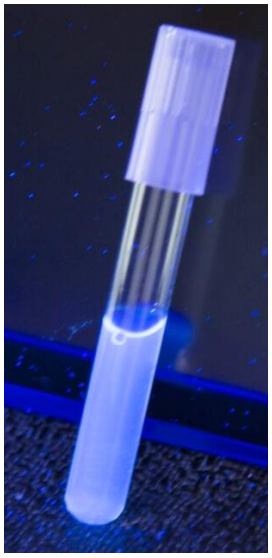

Figure 1. Asparagine broth inoculated with *Pseudomonas fluorescens* under UV light; observe the fluorescence emitted from the water-soluble fluorescent compound.

Among commonly occurring bacteria are transient bacteria that can often populate natural waters. These bacterial species are often a result of human pollution being introduced into the water system. A major component of that human pollution is fecal matter. When fecal contamination is introduced into waterways, if and when the contaminated water is used, potential infectious diseases may emerge and can be transmitted. Among the commonly introduced infectious contaminants are: *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Salmonella*, *Shigella dysenteriae*, and *Vibrio cholera*. In South Florida even our aquifers, that are several hundred feet below sea level, contain wide varieties of naturally occurring bacteria including those introduced through human and animal fecal waste.

The presence of *E. coli* in water is the direct indication of fecal contamination, as these bacteria are the common inhabitants of the intestine. Thus, public health inspectors often use *E. coli* as **indicator bacteria**. Microbiologists determine the Coliform count to determine the quantity of this intestinal bacterium in water. **Coliform** bacteria are those that are gram negative facultative anaerobic bacilli, and non-spore forming. Coliform bacteria include *E. coli*, *Enterobacter aerogenes*, and *Klebsiella*. *Escherichia coli* is a coliform that is able to ferment lactose at 44.5°C and produce acid and gas as end products and we can use this ability for identification. To test and confirm the presence of these microorganisms, we often use a series of tests in order: the **Presumptive test**, the **Confirmative test**, and the **Completed test**.

The Presumptive Test

The presumptive test uses the **Multiple Tube Technique**. This technique uses selective and differential liquid media into which multiple aliquots of serial dilutions of a water sample are inoculated. The medium is selective in that it contains Lauryl Sulfate (SO_4) that inhibits the growth of gram positive bacteria. This technique is very helpful as it can detect very small titers of microorganisms, where a large volume of water is not required. The multiple tubes technique yields the statistically derived **Most Probable Number** of microorganisms per aliquot of water. Since coliform bacteria ferment lactose, the presumptive test uses the medium Lauryl Sulfate Lactose Broth. The lactose broth is placed in tubes containing Durham tubes, which are smaller tubes that are placed upside down within the larger tube (Figure 2). If a sample contains coliform bacteria and is inoculated in these tubes, the bacteria will ferment the lactose and produce acid and gas. The gas produced by the bacteria is captured within the Durham tubes and results in a positive **Presumptive Test**.

In this lab, we will be using the medium Asparagine *broth* for the presumptive test and *agar* for the confirmative test for *Pseudomonas* species. Asparagine is an organic amino acid used as the sole carbon source in media directed towards the growth of *Pseudomonas* species. When *Pseudomonas* is present in a water sample inoculated into a multiple tube technique containing Asparagine broth, the bacteria will secrete (release) fluorescent compounds into the medium. Under UV lamp, the presence of *Pseudomonas* is established with a blue fluorescence in broth and diffused in agar. This tests for the natural occurrence of *Pseudomonas* species in your environmental sample.

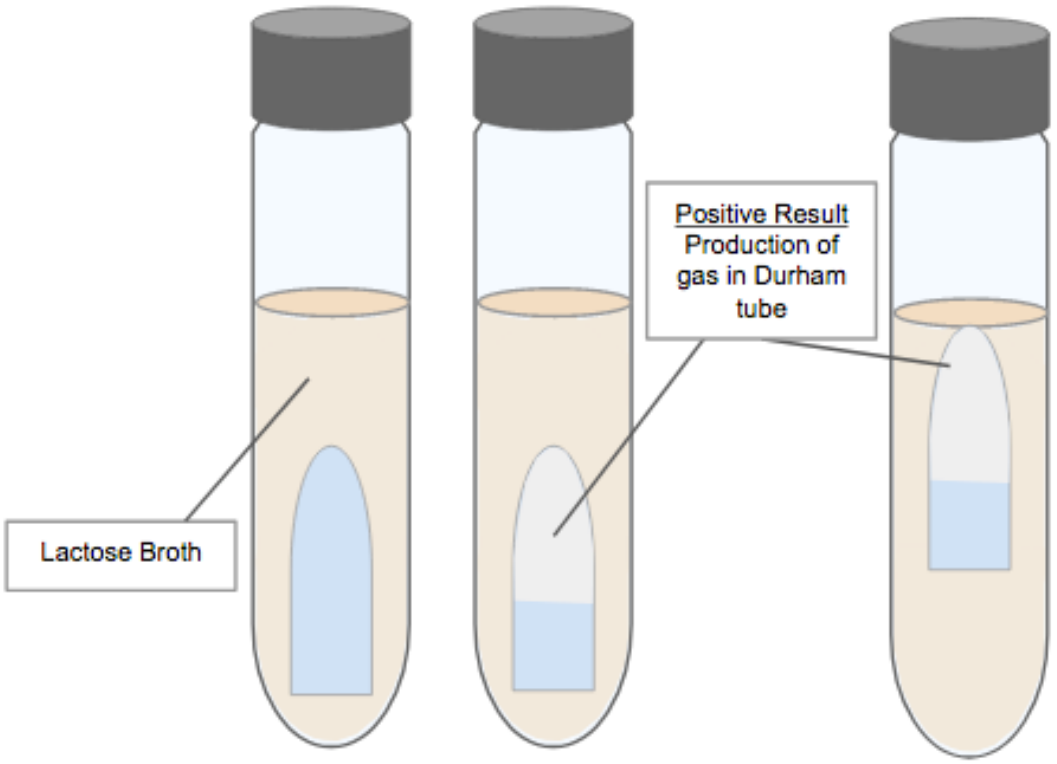

Figure 2. Diagram showing the test results for a Presumptive test with E. coli inoculated in Lauryl Sulfate Lactose Broth

The Confirmative Test- Coliform

The confirmative test is performed on a positive tube from the presumptive test in order to further verify the coliform contamination in the water sample. This test consists of streaking a positive presumptive sample from the tube onto an **EMB Agar** (Eosin Methylene Blue Agar) and an **EC+MUG agar** plate. EMB agar contains lactose and the dyes Eosin Y and Methylene Blue. When E. Coli is growing on EMB, it ferments the lactose and the two dyes are absorbed into the colony producing a metallic green sheen appearance for a positive EMB confirmative test (Figure 3a). EMB agar is selective and differential where the dye combination inhibits the growth of gram positive bacteria and the lactose differentiates between lactose fermenters and non fermenters.

EC+MUG agar plates contains 4-methylumbelliferyl-B-D-glucuronide (MUG) that is converted as an end product to 4-methylumbeliiferone (MUB) by the enzyme present within *E. coli*, B-glucoronidase (GUD). The activity of the enzyme B-glucuronidase is a highly specific characteristic of most strains of *E. coli*. The compound produced by E. coli, MUB, is a water-soluble, fluorescent compound

that diffuses into the agar. When under a long wave UV lamp, you can observe the fluorescence of a positive confirmative EC+MUG plate (Figure 3b).

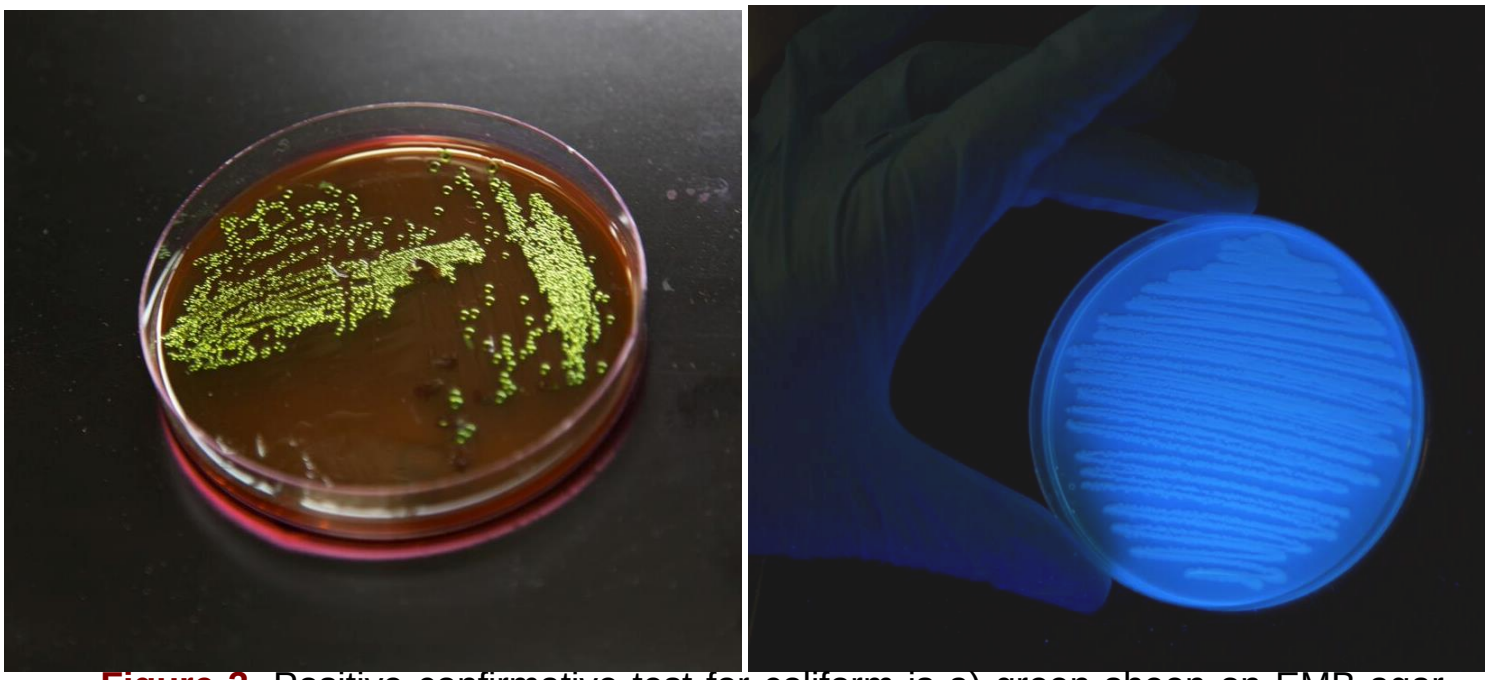

Figure 3. Positive confirmative test for coliform is a) green sheen on EMB agar plate, and b) fluorescence from EC+MUG agar plate both inoculated with *E. coli.*

The Confirmative Test- Pseudomonas

A positive confirmative test for *Pseudomonas* is the production of a fluorescent pigment that diffuses through the agar medium and observable under UV light.

The Completed Test

The completed test is performed in order to have a definite result in determining the presence of fecal coliform in water samples. The completed test uses the medium **Phenol Red Lactose Broth**. The completed test is done by taking a positive green sheen colony from the confirmative test and inoculating it into this broth containing a Durham tube. The broth contains Phenol red that is red in neutral conditions and turns yellow in acidic conditions. A positive completed test is the production of acid and gas from the fermentation of lactose by *E. coli*. As a result of the presence of *E. coli,* the phenol red lactose broth will turn yellow and the Durham tube will collect gas (Figure 4).

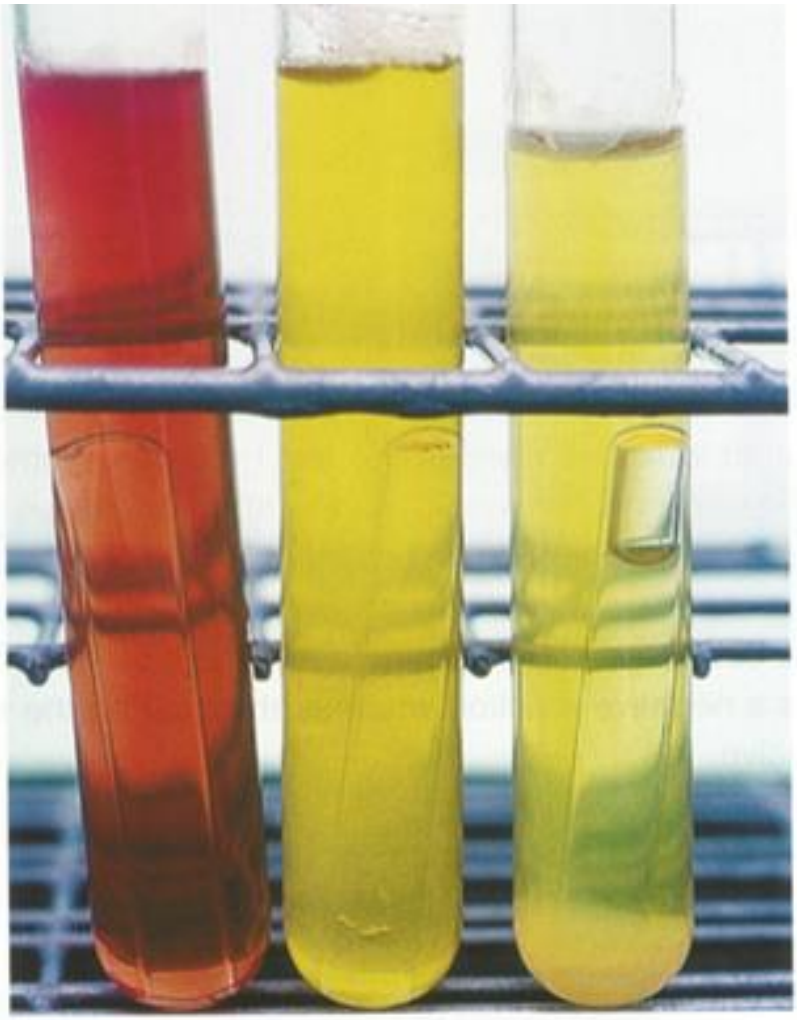

Figure 4. Production of acid and gas is a positive result in the Completed test using Phenol Red Lactose Broth. Red color in the first tube is a negative result for lactose fermentation, while the second tube with yellow is positive for lactose fermentation but not gas production, only the third tube, with both production of acid and gas is a positive result. Reference Pollack et al. 2002.

Materials WEEK 1:

1. **(3) Double Strength Lauryl SO4 Lactose Broth tubes with Durham tubes**
2. **(6) Single Strength Lauryl SO4 Lactose Broth tubes with Durham tubes**
3. **(3) Double strength Asparagine Minimal Broth tubes**
4. **(6) Single strength Asparagine Minimal Broth**
5. **95ml dilution blank (95ml 0.1% peptone in square dilution bottles**
6. **Sterile 10ml and 1 ml pipettes**
7. **Lake water or Ocean water sample (in 500ml flask)**
8. **Incubator at 37°C**
9. **Incubator at 44.5°C**
10. **(3) 9ml Dilution blanks (0.1% peptone) 16X150mm tubes**
11. **(4) Nutrient Agar Plates**
12. **Glass spreader in a beaker with 95% alcohol**

Materials WEEK 2:

1. **(1) EMB agar plate**
2. **(1) EC+MUG agar plate**
3. **Long wave UV lamp**
4. **Asparagine minimal Agar plate**
5. **Catalase and Oxidase Reagent**
6. **Gram Stain Kit**

Materials WEEK 3:

1. **(3) Phenol Red Lactose Broth tubes – 4ml (13X10mm tube)**
2. **Catalase and Oxidase Reagent**
3. **Long wave UV lamp**

Procedures for WEEK 1:

Part I. Multiple Tube Technique;
The Presumptive Test

1. Determine what group you are designated: Diluted (DI) or Undiluted (UD). **Make sure all of your plates and tubes** are labeled with your group designations.

2. For the undiluted groups, the water sample will be used directly without diluting. For the diluted groups: make a 1:20 dilution of the water sample by pipetting 5ml into a sterile 95ml dilution blank.

3. Using your designated water sample (diluted or undiluted) inoculate 10ml each into THREE double strength Lauryl SO_4 Lactose Broth tubes and into THREE double strength Asparagine Minimal Broth tubes.

4. Inoculate 1 ml into of your sample into THREE single strength Lauryl SO_4 Lactose Broth tubes and into THREE single strength Asparagine Minimal Broth tubes.

5. Inoculate 0.1ml of your sample into THREE single strength Lauryl SO_4 Lactose Broth tubes and into THREE single strength Asparagine Minimal Broth tubes.

6. If your group is “Diluted” incubate all of your Lauryl SO_4 Lactose Broth tubes at 37°C

7. If your group is “Undiluted” incubate all of your Lauryl SO_4 Lactose Broth tubes at 44.5°C

8. Incubate all of your Asparagine plates at 30°C.

Part II. Lake Water Viable Titer

1. Obtain all of your nutrient agar plates and peptone blank test tubes and label them according to your serial dilution.

2. Grab your original undiluted lake water sample and pipette 0.1ml onto 1 Nutrient Agar plate.

3. Prepare a 1:10 of the *original* lake water sample by adding 1ml of the lake sample into the first 9ml blank.

4. Pipet 1ml from this newly inoculated peptone blank and transfer it into your next peptone blank.

5. Again, remove 1 ml from this new dilution and place it into your final peptone blank.

6. From each of your dilutions, pipet 0.1ml onto the three respective Nutrient Agar plates.

7. Use a sterile glass spreader, making sure to sterilize between plates, and spread the inoculum evenly over the agar surface.

8. Incubate all of your Nutrient Agar plates at 30°C.

Procedures for WEEK 2:

Part I. The Confirmative Test; Most Probable Number

1. Obtain all of your previously labeled plates and tubes. Observe the Lauryl SO_4 Lactose Broth tubes. Count the number of positive tubes in each set of tubes inoculated with different volumes.

2. Use the Most Probable Numbers table to calculate the MPN titer.

3. Observe the growth in the Asparagine Minimal Broth tubes under a long wave UV lamp. Count the number of fluorescent tubes and use the MPN table to calculate the MPN titer.

4. Use a positive Lauryl SO_4 Lactose Broth tube that contained GAS and inoculate an EMB and EC+MUG plate using the Streaking for Isolating method.

5. Use a positive fluorescent Asparagine Minimal broth tube and inoculate an Asparagine minimal Agar plate by streaking for isolation.

6. Incubate your EMB plate at 37°C, the EC+MUG at 44.5°C, and the Asparagine Minimal Agar plate at 30°C.

Part II. Lake Water Viable Titer

1. Obtain all of your nutrient agar plates an count the colonies on each agar plate. Choose the best countable plate and calculate the total viable titer.

2. Choose and label **THREE** different and well-separated colonies on the back of the agar plate.

3. Perform Gram staining and catalase and oxidase on each colony. Record your data in your worksheet.

Procedures for WEEK 3:

Part II. The Completed Test

1. Obtain all of your incubated plates: EMB, EC+MUG, and Asparagine Minimal Agar.

2. Observe the EMB for presence of green sheen colonies. Identify two different bacterial colonies on the plate including the green sheen colony and label them on the plate.

3. Gram-stain and perform catalase and oxidase on all three colonies.
4. Observe your EC+MUG plate under UV light for presence of fluorescence.

5. Choose a fluorescent colony from the EC+MUG plate and Gram stain and catalase and oxidase as well.

6. Observe your Asparagine Minimal agar plate under UV.

7. Gram stain, oxidase and catalase the positive colony from this plate.

8. Label all of your Phenol Red Lactose broth tubes with: 1) positive colony from EMB, 2) Positive colony EC+MUG, 3) Negative colony from EMB

9. From your colonies inoculate these cells into separately labeled Phenol Red Lactose Both tubes and incubate at 37°C.

REPORT 1

Environmental Bacterial Biochemistry

REPORT 8

Part I. Presumptive Test

Directions: Include your results from the multiple tubes technique below. Circle your respective lake water sample letter and whether your group was diluted or undiluted.

Lake Analyzed: A B C

Dilution: DI UD

Inoculum Volume (ml)	Number of Positive Tubes (our of 3)	
10		
1		
0.1		
Fecal Coliform (DI) MPN		**/100ml**
Total Coliform (UD) MPN		**/100ml**

Fluorescent Pseudomonads

Inoculum Volume (ml)	Number of Positive Tubes (our of 3)	
10		
1		
0.1		
Fluorescent Pseudomonads MPN		**/100ml**

Part II. Lake Water Total Viable Titer

Directions: Include your results from the Nutrient agar plates used to calculate lake titer.

Dilution	CFU per plate
Undiluted	

10^{-1}		
10^{-2}		
10^{-3}		
Total Titer:		

Calculation of Viable Titer

Part III. Microscopic Observations
Directions: Include your results from your gram staining and oxidase and catalase tests below using your Nutrient Agar plates.

Colony	Colony Morphology	Cellular Morphology	Catalase	Oxidase	Gram
1					
2					
3					

Include a **SKETCH** of each of your colonies below.

Colony 1

Colony 2

Colony 3

Part IV. Questions

Directions: Answer the following questions based on your results from the presumptive test and counting the total viable titer.

1. Using your results from the Nutrient Agar Lake plates that you gram stained and performed the oxidase and catalase tests on: What type of bacteria were apparent in the lake water tested?

2. According you your MPN numbers for total coliform, fecal coliform, and fluorescent pseudomonads, which bacterial Genus predominates in the lake water sample you measured? Why do you think this is so?

3. What percent of the total bacteria count in the lake is fecal?

What percent of the total bacteria in the lake are Pseudomonads?
What percent of the total coliform bacteria are fecal?
Show ALL of your calculations.

a. %Fecal of total bacteria_____

b. %Pseudomonads of total bacteria _____

c. %Fecal of total coliforms_____

4. Which Dilution onto the Nutrient Agar gave you the best countable plate? Why were the other dilution plates not used to count total titer?

5. According to your results for all three colonies and Oxidase and Catalase tests, what can you conclude about the bacteria type that you isolated form the lake?

REPORT 2

Environmental Bacterial Biochemistry

REPORT 8

Part I. Microscopic Observations; Confirmative Test
Directions: Include a SKETCH of your gram results from your chosen EMB, EC+MUG, and Asparagine Minimal Agar plate colonies.

EMB+

EMB-

EC+MUG

Asparagine

Part II. Microscopic Observations (continued)
Directions: Include your results from your gram staining and oxidase and catalase tests below from your EMB, EC+MUG, and Asparagine Minimal Agar plates.

Colony Source	Colony Morphology	Cellular Morphology	Catalase	Oxidase	Gram
EMB+					
EMB-					
EC+MUG					
Asparagine					

Part I. The Completed Test
Directions: Indicate which colonies showed production of acid and/or gas in the Phenol Red Lactose Broth tubes by placing a + or -.

Organism	Acid	Gas
Colony 1		
Colony 2		
Colony 3		

Part III. Questions (5 points)
Directions: Answer the following questions based on your results from the Confirmative test and the Completed test.

1. Was the confirmative test positive/negative for fecal coliform? Explain how you derived your answer using both plates EMB and EC+MUG plates.

2. Was the confirmative test positive/negative for *Pseudomonas*? Explain how did you derive your answer using the Asparagine Minimal Agar plate.

3. Is the completed test positive for lactose fermentation? Which colony produced a positive result, and how do you know?

4. Which test do you think is the most conclusive in indicating the presence of either fecal coliform bacteria and/or Pseudomonads, the presumptive, confirmative, or the completed? Why?

5. How do the bacteria you isolated in the second part of the experiment differ than those you isolated in the first part of the experiment? Include gram stain, catalase, oxidase, and colony morphology results.