EXPERIMENT 8

BACTERIOLOGICAL ANALYSIS OF WATER

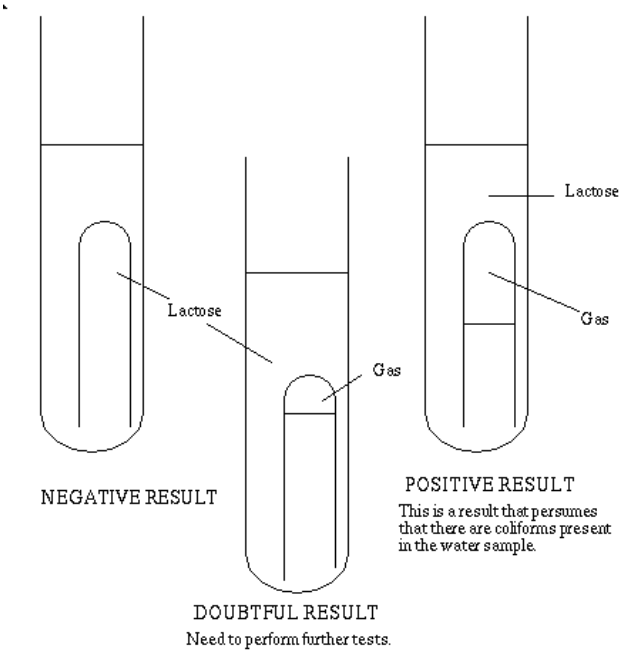
All natural waters contain bacteria. The aerobic gram negative rods of the genera *Pseudomonas, Alcalignes* and *Flavobacterium* as well as others are common in water. Many of these bacteria are capable of growing on a wide variety of single carbon sources. That is, they are able to grow on a medium containing only mineral salts and one carbon source as the sole organic nutrient supply. In such a medium all other nutrients are inorganic. Some members of the genus *Pseudomonas* can grow on as many as 90 different sole carbon sources: the epitome of nutritional versatility. Also, *Pseudomonas* contains species that produce fluorescent pigments that are water soluble and diffuse into the medium. The property of producing water soluble fluorescent pigments and the ability to grow on single carbon sources can be taken advantage of to enumerate (titer) the number of fluorescent pseudomonads by the **Multiple Tube Technique** (see below).

All natural waters can also be populated by transient bacteria. Among these are the human pathogens that gain entry to water from fecal contamination. Thus contaminated water is a potential transmitter of any of a number of intestinal diseases. The direct isolation of intestinal pathogens is impractical; instead public health inspectors determine the number of **indicator bacteria**. *Escherichia coli* which is in the large intestine of virtually all people has been used as the indicator of human fecal contamination of water and food. Tests for the presence of this organism (and closely related types generally known as **coliforms**) utilize either a multiple tube technique (see below) or direct plating onto differential media.

Multiple Tube Techniques utilize selective and differential liquid media into which multiple aliquots of serial dilutions are inoculated. The advantage of this technique is that it will detect organisms a small titers, much less than one per ml which would otherwise require inoculation of large volumes. The multiple tube techniques yield the statistically derived **Most Probable Numbers** of organisms per aliquot (usually 100 ml) of water. If large volumes of water have to be sampled by direct plating procedures, they must first be aseptically filtered onto a sterile bacteriological membrane filter which can then be placed directly onto an agar plate containing the appropriate differential medium.

In this experiment, three sets of tubes will be inoculated with a ten fold difference in inoculum volume between each set: one set of three tubes will be inoculated with 10 ml per each tube, one set will be inoculated with 1 ml per each tube, and the last set will be inoculated with 0.1 ml per each tube. After appropriate incubation, the tubes are then examined for the diagnostic reaction: gas production for coliforms and fluorescent pigments for pseudomonads. Each set is scored for the number of positive tubes (note some tubes may have growth but not a positive diagnostic reaction) and the score of all three sets is then used with the **Most Probable Number Table** in Appendix IV to determine the number of each group (coliforms or fluorescent pseudomonads) per 100 ml of water.

Coliform Analysis. Coliforms are able to ferment lactose to acid and gas. The medium used in the multiple tube technique for coliforms is **Lauryl SO_4 Lactose Broth** and is placed in tubes containing little upside down tubes (Durham tubes). If coliforms are inoculated into this medium, they can ferment lactose producing acid and gas (Figure 1.). The gas will be seen in the Durham tubes: this is a positive **Presumptive Test** for coliforms. Lauryl SO_4 Lactose broth contains the detergent lauryl SO_4 which retards the growth of Gram positive bacteria.



RESULTS OF THE DURHAM TUBES OF LACTOSE BROTH

Figure 1. Presumptive test is positive if gas can be seen in Durham tube

It is possible that bacteria other than *Escherichia coli* can give a positive Presumptive Test. There are two different fermentation patterns: the Classical Mixed Acid fermenters and the Butane Diol fermenters; both produce soluble end products and gas (CO_2 and H_2). The Classical Mixed Acid fermenters like *E. coli* ferment sugars to a variety of acids and only small amounts of soluble neutral end products. The Butane Diol fermenters like *Enterobacter aerogenes* ferment sugars to a small amounts of mixed acids and larger amounts of neutral end products: butane diol (same as 2,3 butylene glycol), ethanol. Two points are important: classical mixed acid fermenters do not produce butane diol and butane diol fermenters produce very small amounts of acids.

The American Public Health Association has developed Standard Methods to determine if *E. coli* is actually present. The Presumptive Test is the first followed by the Confirmative Test, the Completed Test and the IMViC tests. The **Confirmative Test** consists of streaking a positive Presumptive tube (gas production) onto an **Eosin Methylene Blue Agar** (EMB Agar) and an **EC+MUG agar** (Escherichia *coli* with 4-methylumbelliferyl-β-D-glucuronide agar) plate. EMB agar contains lactose and the dyes Eosin Y and Methylene Blue.
When *E. coli* grows on EMB it ferments so much acid that the two dyes precipitate out in the colony producing a **metallic green sheen** appearance (Figure 2a.). A positive Confirmative Test is then the presence of green sheen colonies on EMB streaked from a positive Presumptive Test.

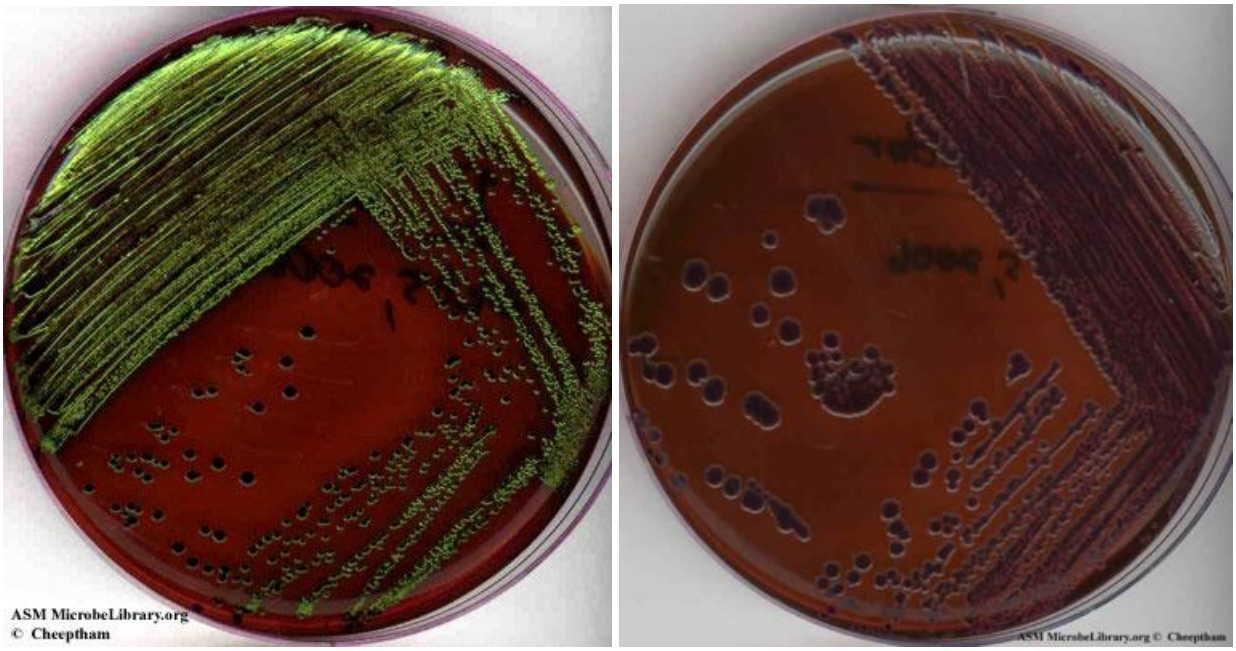


Figure 2a. *E. coli* and *Enterobacter aerogenes* on EMB agar plates (the formation of green sheen colonies is a positive confirmative test for *Escherichia coli*).

EC + MUG agar contains 4-methylumbelliferyl-β-D-glucuronide (MUG) which is converted into 4-methylumbelliferone (MUB), a fluorogenic compound, by β-glucuronidase (GUD) – an enzyme present in *E. coli*. MUB fluoresces under long wave UV light (Figure 2b). The activity of β-D-glucuronidase is a highly specific characteristic of most strains of *E. coli*.

Figure 2b. *Enterobacter aerogenes* and *Escherichia coli* on EC+MUG agar observed under long wave UV light (bluish fluorescence indicate the presence of MUB)

The Completed Test is the inoculation of **Phenol Red Lactose Broth** with a green sheen colony from the Confirmative Test. A positive Completed Test is the production of acid and gas in this medium (Figure 3.). This tests only one biochemical trait: the fermentation of lactose. Thus, additional biochemical tests are usually done from EMB colonies: the **IMViC** tests. The green sheen colony is inoculated into Tryptone broth for the **Indole** test, MRVP broth for the **Methyl Red** and **Voges Proskauer** tests and a Simmons Citrate slant for the **Citrate** test.

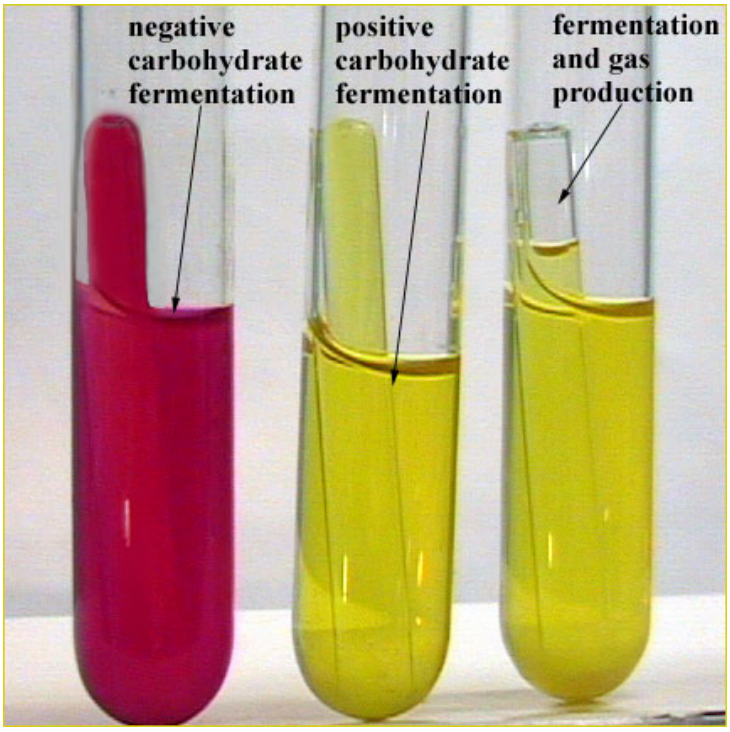


Figure 3. The production of acid and gas in Phenol Red Lactose Broth is a positive Completed Test

The **Indole Test** (Figure 4.) tests for the ability of the bacterium inoculated into Tryptone broth to produce Indole from the amino acid tryptophan. Tryptone broth is the tryptic digest of casein and contains a good quantity of tryptophan. After growth in Tryptone broth, the Indole test reagent is added; if Indole has been produced, a red color will be seen.

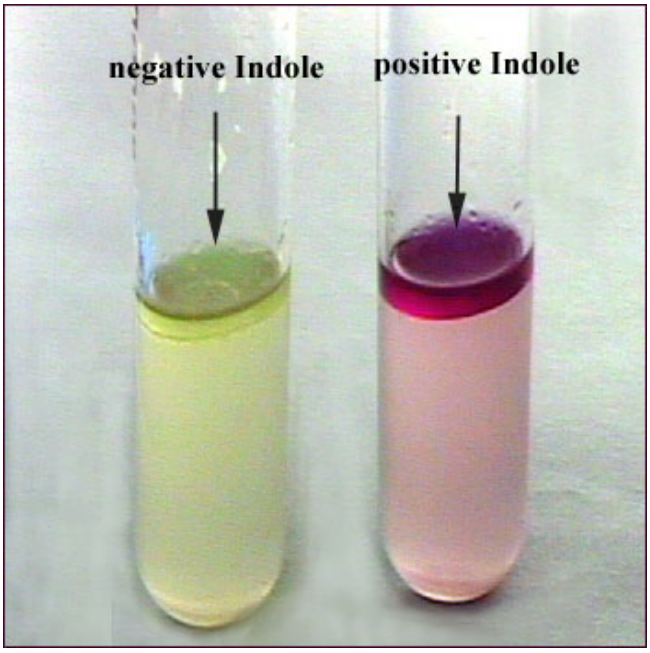


Figure 4. Production of red color after addition of Indole reagent is Positive Indole test

The **Methyl Red** and **Voges Proskauer** tests are done in the same medium: MRVP that is a glucose-peptone medium that contains phosphate buffer. After growth of the bacteria in this medium, half of the culture is poured into another tube and each test is preformed separately. To one half of the culture, the Methyl Red test is done by adding a few drops of the dye methyl red. Methyl red is red in acidic conditions and yellow in neutral conditions (note that this is the opposite of Phenol Red used earlier) (Figure 5.). A positive test is a red color indicating the production of enough acid to overcome the phosphate buffer.

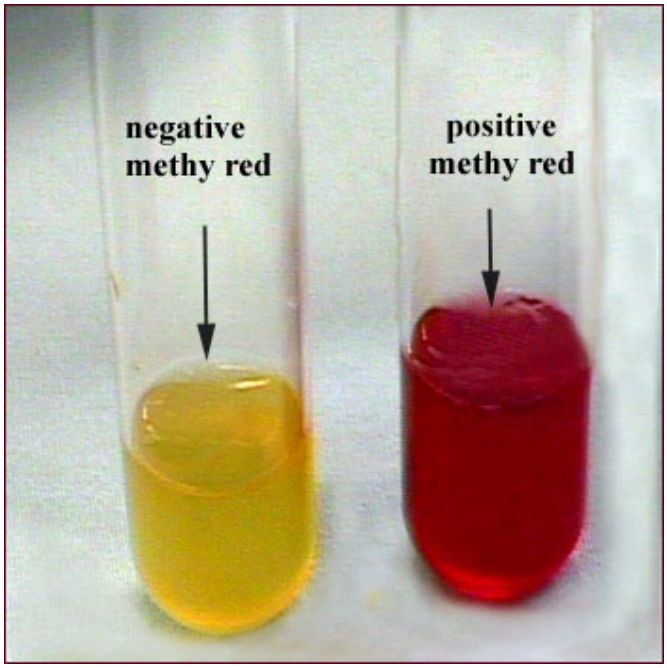


Figure 5. Methyl red is red in acidic conditions (positive test) and yellow in neutral conditions (negative test)

The **Voges Proskauer** reaction tests for the presence of acetoin (acetyl methyl carbinol) which is an intermediate in the butane diol fermentation. This test must be done after only 24 hours of growth; if done later all the acetoin will be converted to butane diol for which there is no easy color test. The two Voges Proskauer reagents are added and a positive test is the production of a wine-red color after vigorous shaking (Figure 6.). This reaction requires oxygen and the tube should be agitated (to get oxygen into the fluid) at intervals for 10 to 20 minutes.

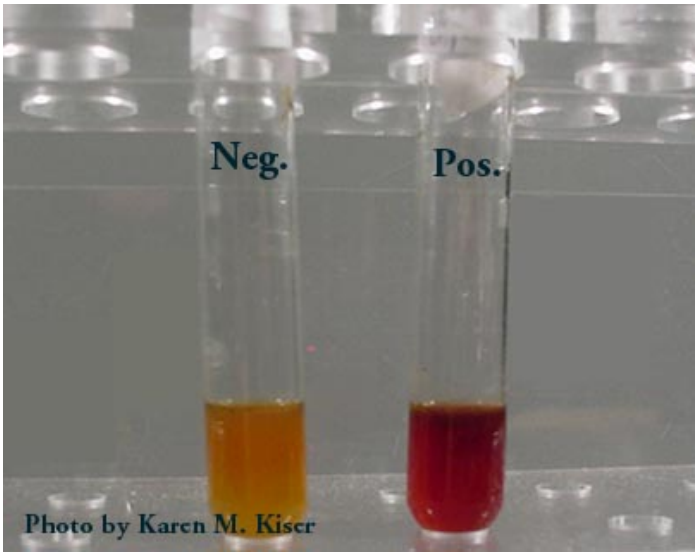


Figure 6. Production of wine-red color after addition of two Voges Proskauer reagents is a positive test

The **Citrate Test** tests the ability to grow on citrate as a sole carbon source. The medium contains the dye bromo-thymol blue which is blue in the alkaline and yellow in the acid. At neutral pH the dye is half yellow and half blue making a green color (Figure 7.). The green color or the uninoculated medium is an optical illusion; there is no green! Citrate is supplied to the medium as sodium citrate, as the organism uses citrate, sodium ions remain in the medium making the medium basic and therefore a blue color develops as the citrate is utilized.

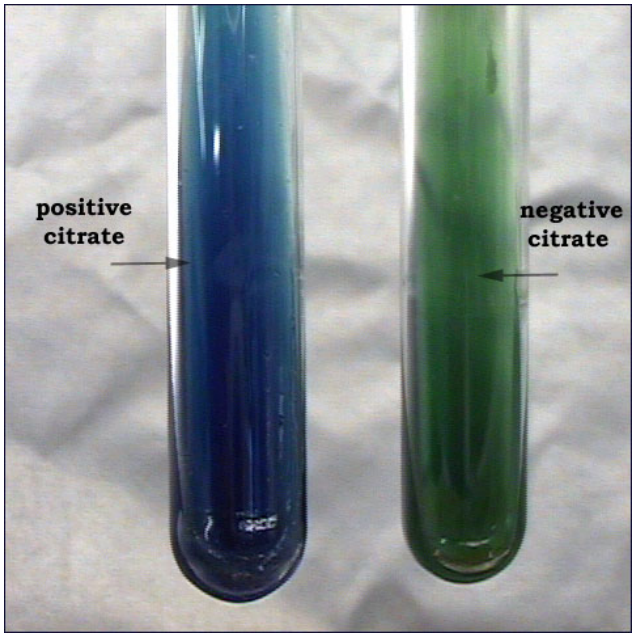


Figure 7. Appearance of blue color is positive citrate test

E. coli (green sheen colonies) produces a ++-- IMViC. Thus it is positive in the Indole and Methyl Red tests and negative in the Voges Proskauer and Citrate tests. Another coliform (positive Presumptive test), *Enterobacter aerogenes* produces pink colonies with a purple center on EMB and a --++ IMViC. Other bacteria that can grow on the EMB plates from positive Presumptive tests produce different IMViC reactions and pink, purple or colorless colonies on EMB.

MATERIALS

First Lab Period (Do As Pairs)

1. 3 double strength Lauryl SO_4 Lactose Broth tubes - 10 ml in 18x200 mm tubes with Durham tubes.
2. 6 single strength Lauryl SO_4 Lactose Broth tubes - 10 ml in 16x150 mm tubes with Durham tubes.
3. 3 double strength Asparagine Minimal Broth tubes - 10 ml in 18x200 mm tubes.
4. 6 single strength Asparagine Minimal Broth tubes - 10 ml in 16x150 mm tubes.
5. Sterile 500 ml flask with cotton stopper.
6. Sterile 10 ml and 1 ml pipettes.
7. Incubators at 37^{o}C
8. Waterbath at 44.5^{o}C
9. 95 ml dilution blanks (95 ml 0.1% peptone in square dilution bottles) for half of the pairs.
10. 9 ml dilution blanks (0.1% peptone) in 16x150 mm tubes.
11. 4 Nutrient Agar plates.
12. Glass spreader and a beaker 1/3 filled with alcohol.

Second Lab Period (Do Individually)

1. Levine EMB Agar plate.
2. EC+MUG Agar plate
3. Long wave UV lamp.
4. Asparagine Minimal Agar plate.
5. BHI Slant cultures of *Escherichia coli* and *Enterobacter aerogenes*.
6. Catalase and Oxidase test reagents.

Third Lab Period (Do Individually)

1. 3 Phenol Red Lactose Broth tubes - 4 ml 13x100 mm tube.
2. 3 Tryptone Broth tubes - 3 ml 13x100 mm tube.
3. 3 MRVP Broth tubes - 4 ml 13x100 mm tube.
4. 3 Simmons Citrate slants.
5. Catalase and Oxidase Reagents.

Fourth Lab Period (Do Individually)

1. Indole Test reagent.
2. Methyl Red.
3. Voges Proskauer reagents I and II.
4. Clean 13x100 mm culture tubes.

PROCEDURE

First Lab Period (Pairs)

1. Take a sterile 500 ml flask and collect a sample of lake water: remove the closure and plunge the flask down into the lake so that only a small amount of surface water enters the flask.

2. Some groups will use the lake water directly. Others will make a 1:20 dilution of the lake water by aseptically pipetting 5 ml to a sterile 95 ml dilution blank. Other variations are important here: the instructor will direct groups so that all the lakes will be sampled and which groups will make the 1:20 dilution. Be sure to read over the experiment in advance and mark all your tubes and plates.

3. Using the water sample (or dilution) inoculate 10 ml each into three double strength Lauryl SO_4 Lactose broth tubes and into three double strength Asparagine Minimal broth tubes.

4. Using the water sample (or dilution) inoculate 1 ml each into three single strength Lauryl SO_4 Lactose broth tubes and three single strength Asparagine Minimal broth tubes.

5. Using the water sample (or dilution) inoculate 0.1 ml each into three single strength Lauryl SO_4 Lactose broth tubes and three single strength Asparagine Minimal broth tubes.
6. Depending upon your group, incubate the Lauryl SO_4 Lactose broth tubes at either 44.5^{o}C or

37°C. All the Asparagine Minimal broth tubes should be incubated at 30°C.

7. Prepare a 1:10 dilution of the original lake water. Inoculate 0.1 ml onto each of two Nutrient Agar plates from this dilution. Inoculate 0.1 ml of lake water onto each of two Nutrient Agar plates. The inoculum on these plates must be spread with a sterile glass spreader so that it is evenly distributed over the surface. This is the **spread plate** technique; the instructor will demonstrate alcohol flaming of glass spreaders so that they do not get hot! Incubate these plates at 30°C.

Second Lab Period (Individually)

1. Observe the Lauryl SO_4 Lactose broth tubes. Count the number of positives in each set. Use the Most Probable Number table to calculate the coliform MPN titer.

2. Using the growth in a positive Presumptive Test tube (Lauryl SO_4 Lactose Broth containing **GAS** in the Durham tube, inoculate an EMB agar plate and an EC+MUG agar plate by streaking for isolation. Incubate the EMB plate at 37°C and the EC+MUG plate at 44.5 °C

3. Observe the Asparagine Minimal broth tubes with a partially darkened room and a long wave UV lamp. Count the number of positives in each set. Use the Most Probable Number table to calculate the fluorescent pseudomonad MPN titer.

3. Using a positively fluorescent tube, inoculate an Asparagine Minimal Agar plate by streaking for isolation. Incubate the plate at 30°C.

4. Count the colonies on the Nutrient Agar spread plates. Calculate the total viable titer (CFU) of the lake.

5. Choose 3 different, well isolated colonies and mark their location on the back of the plate. Describe each colony and Gram stain the cells from each colony.

6. Perform the oxidase and catalase test on each of the 3 colonies.

Third Lab Period (Individually)

1. Observe the EMB plate (Confirmative Test) for the presence of green sheen colonies. Mark well isolated colonies of the major types of colonies on the plate, describe each and Gram stain each one. If you have a positive (green sheen colony) one student of the pair will use that for the steps on section 3 below; the other student will use another colony type. If you do not have a green sheen colony use the two major colony types that are composed of Gram negative rods. Each student is responsible for one complete set of diagnostic media.

2. Observe the EC+MUG plate under the long wave UV light to check for fluorescent colonies (make sure to look thru the protective glasses). Gram stain the colony and follow the step on section 3 below.

3. Inoculate cells from the colonies on the EMB plate and EC+MUG plate into Phenol Red Lactose broth, Tryptone Broth, MRVP broth, and onto Simmons Citrate slant.

4. The instructor will inoculate known cultures of *Escherichia coli* and *Enterobacter aerogenes* into the media listed in step 3.

5. Observe the Asparagine Minimal Agar plate in normal room light and under the long wave UV lamp. Describe the major colony types and Gram stain each.

6. Perform the oxidase and catalase test on the fluorescent colony.

Fourth Lab Period (Individually)

1. Perform the **Indole Test** on the Tryptone broth tubes: add 3 drops of Indole Test reagent. Shake and record the color. Red color is positive. This color may fade with time.

2. Pour one half of the MRVP medium into a clean culture tube. Perform the **Methyl Red Test** on one half of the culture by adding 2 to 3 drops of Methyl Red to the tube. Shake and record the color. Red is a positive test.

3. To the other half of the MRVP culture perform the **Voges Proskaeur Test**: add 3 drops of Voges Proskaeur reagent I and mix, then add 3 drops of Voges Proskaeur reagent II and mix vigorously for 1 minute. Allow the tube to stand for 10 to 20 minutes. Development of a pink to wine red color is positive.

4. **Citrate Test**. Observe the Simmons Citrate slant: a blue color is positive, green is negative.

DAILY LAB REPORT: Water Bacteriology - 1 **Name: _____**

COLIFORM ANALYSIS

Lake Analyzed: _____

Presumptive Test (Lauryl SO_4 Lactose Broth) Incubation Temperature:_____

Inoculum Volume	**Number Positive** (Gas in Durham Tube)	
10 ml	_____	
1 ml	_____	**MPN: _____** Total Coliforms/100 ml
0.1 ml	_____	**MPN**: _____ Fecal Coliforms/100ml

Fluorescent Pseudomonads

MPN Analysis

Inoculum Volume	**Number Positive** (fluorescent)	
10 ml	_____	
1 ml	_____	**MPN:** Fluor._____ Pseudomonads/100 ml
0.1 ml	_____	

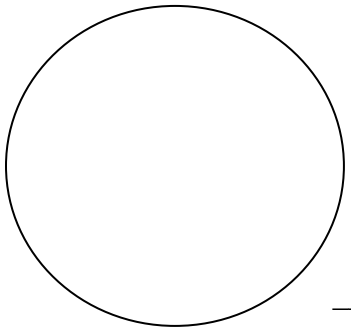
VIABLE TOTAL TITER of LAKE _____

Dilution	**CFU/plate**
Undiluted	_____, _____
1:10	_____, _____

Calculation of Viable Titer:

Colony 1: Description: _____
_____

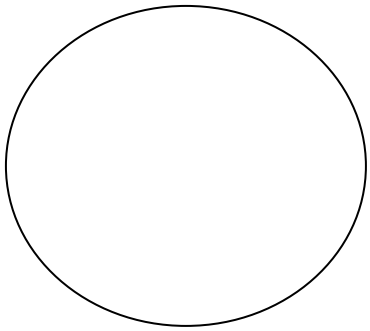
Gram Stain:



_____X

Colony 2: Description:_____
_____

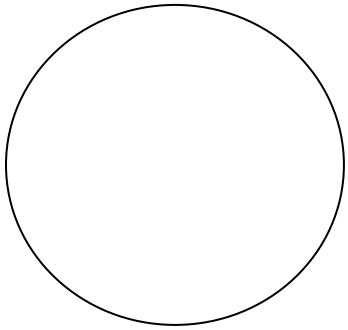
Gram Stain:



_____X

Colony 3: Description: _____
_____

Gram Stain:



_____X

Colony No.	Oxidase	Catalase
1	_____	_____
2	_____	_____
3	_____	_____

List of Conclusions Report #1:

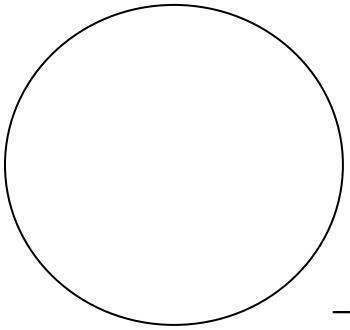
DAILY LAB REPORT: Water Bacteriology - 2 **Name: _____**

COLIFORM ANALYSIS

Confirmative Test (EMB plate)

Colony 1: Description: _____
_____

Gram Stain:



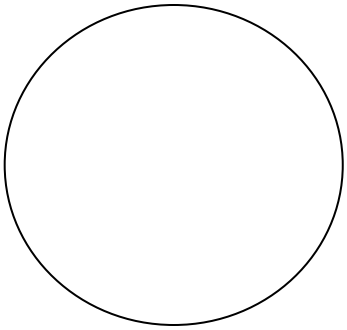
_____X

Catalase test _____

Oxidase test _____

Colony 2: Description: _____
_____

Gram Stain:



_____X

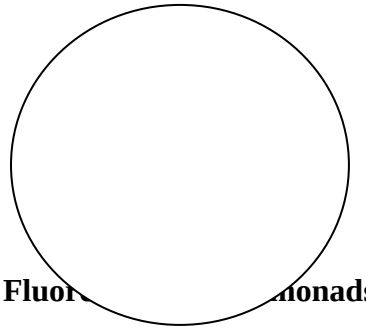
Catalase test _____

Oxidase test _____

Confirmative Test (EC+MUG plate)

Colony 3: Description: _____
_____

Gram Stain:



_____X

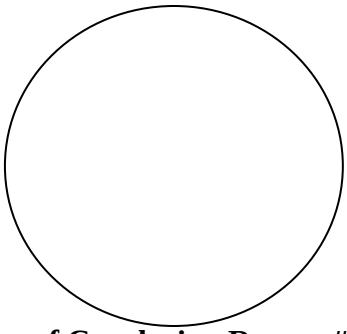
Catalase test _____

Oxidase test _____

Fluor[illegible]monads: (Asparagine Minimal Agar Plate)

Colony 1: Description: _____
_____

Gram Stain



_____X

Catalase test _____

Oxidase test _____

List of Conclusion Report # 2

DAILY LAB REPORT: Water Bacteriology - 3 **Name: _____**

COLIFORM ANALYSIS

Completed Test: (Phenol Red Lactose Broth)

Organism	**Acid**	**Gas**
Colony 1	_____	_____
Colony 2	_____	_____
Colony 3	_____	_____

IMViC Test:

Organism:	**Indole**	**Methyl Red**	**Voges Proskauer**	**Citrate**
Colony 1	_____	_____	_____	_____
Colony 2	_____	_____	_____	_____
Colony 3	_____	_____	_____	_____

List of Conclusions Report #3:

COMPARISON
Your Data and Class Data
of
BACTERIA IN
SOIL vs AIR vs WATER

Your DATA:

Environment	Viable Titer	#Gram Positive			#Gram Negative		
		RODS	COCCI	TOTAL	RODS	COCCI	TOTAL
SOIL (Exp. 4)							
AIR (Exp. 7)	Not done						
WATER (Exp. 9)							

Class DATA:
Put your data on the black board with all other groups, then calculate the Average Viable Titer and the Percent of each morphological type.

Environment	Average Viable Titer	%Gram Positive			%Gram Negative		
		RODS	COCCI	TOTAL	RODS	COCCI	TOTAL
SOIL (Experiment 4)							
AIR (Experiment 8)	Not Done						
WATER (Experiment 9)							

Questions:
1. Why is it that one Gram type predominates in air and another type in water?
2. Why is this different in soil?
3. Is there a large difference in titer between soil and water? Why is this so?
4. If we had done a viable titer of air, what would you expect it to be?