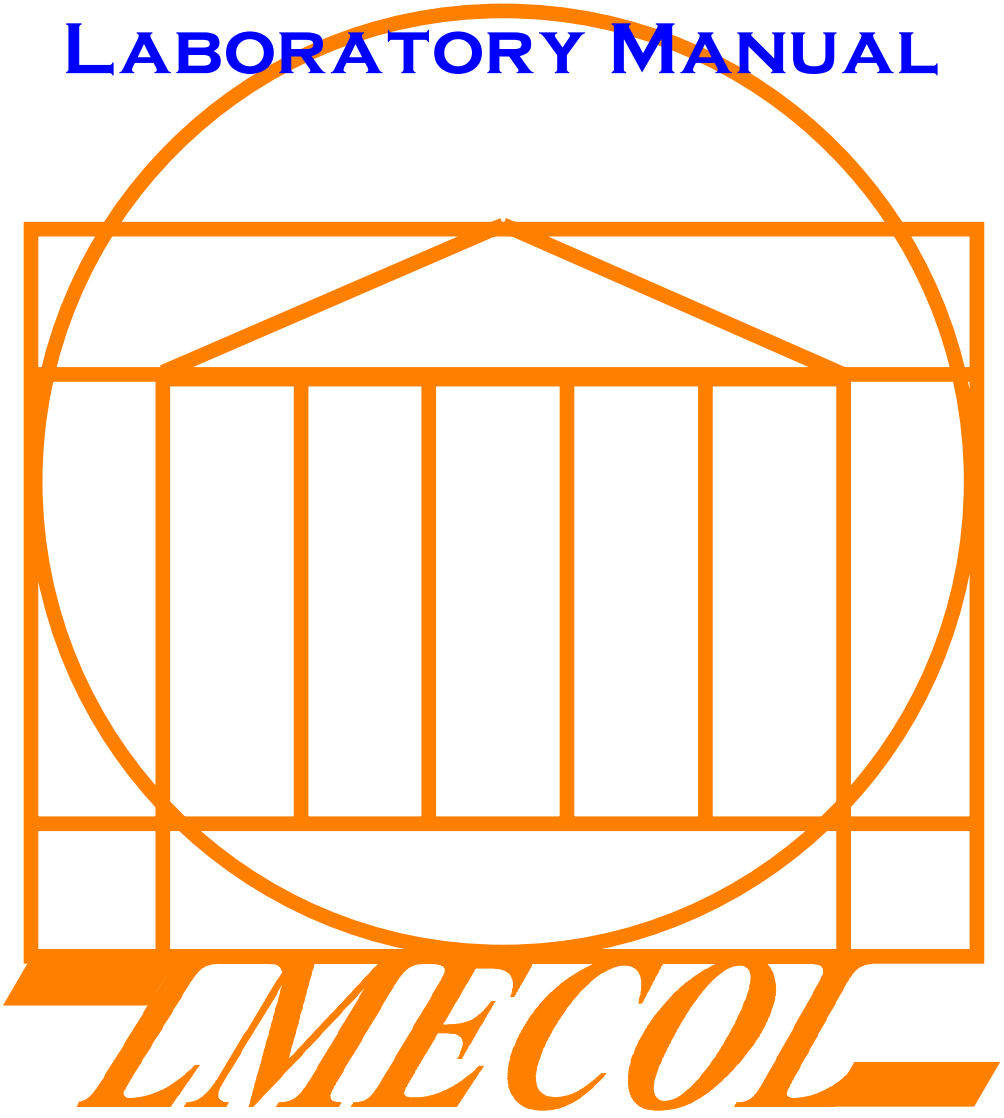
EVSC 523

Microbial Ecology

Laboratory Manual



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A. GENERAL INFORMATION

The aim of this laboratory course is to expose students to basic laboratory techniques used in the microbiological sciences; most of the exercises involve hands-on approaches to be performed by each student. Unlike many other laboratory methods, microbiological laboratory techniques require a high degree of organizational skill, coordination, and quickness of work. With some patience and practice, you will be able to master all of these aspects. Hopefully, by the end of the semester, you will have discovered that microorganisms not only have fascinating personalities, but also make for excellent laboratory pets that are fun to play with.

There are a number of reference manuals that may be of special use to the new microbiologist. Some especially helpful ones are:

Seeley, H. W., P. J. Vandemark, and J. J. Lee. 1990. Microbes in Action: A Laboratory Manual of Microbiology. W. H. Freeman & Co., New York. ISBN: 0716721007.

Gerhardt, P., R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed). 1981. Manual of methods for general bacteriology. American Society for Microbiology, Washington, D. C. ISBN: 0914826301.

Pepper, I. L., C. P. Gerba, and J. W. Brendecke. 1995. Environmental Microbiology : A Laboratory Manual. Academic Press, San Diego, CA. ISBN: 0125506554.

Claus, G. W. and W. G. Claus. 1989. Understanding Microbes: A Laboratory Textbook for Microbiology. W. H. Freeman & Co., New York. ISBN: 071671809.

B. LABORATORY SAFETY

Here are a few general, common sense rules about working in the microbial lab:

1) Never eat, drink, or store food in the lab.

2) Wash your hands thoroughly with soap and water after you get done working in the lab.

3) Never pipette microbial cultures, or any chemicals by mouth!

4) Place contaminated materials in the **proper** disposal receptacles.

5) Before and after each exercise, wipe bench tops with bleach or disinfectant.

6) When indicated, wear gloves and/or lab coats to avoid contamination.

7) Please report any spills or accidents.

Use your common sense in applying these rules. Please also keep in mind that the microbial lab is a **research lab**. **Do not remove any items from any of the lab benches**; work areas will be designated and you should stay within these areas. Obviously, there are space limitations and we will have to work together in a coordinated fashion to make do with the available space. **Do not take any items from drawers and/or cabinets**! All the necessary items for the exercises will have been prepared prior to each lab session. **Non-compliance to these basic rules may result in dismissal from the lab!**

Furthermore, we work a number of hazardous compounds, radioactive material, and equipment that can cause serious injury if misused. The TA's authority in the laboratory is absolute. Willful ignorance of a directive that affects the safety of any person or equipment will be grounds for dismissal from the lab and recording of a failing grade. Additional action may also be taken as necessary.

C. ASEPTIC TECHNIQUE

Aseptic technique is the summary term for precautionary laboratory techniques used to avoid microbial contamination during manipulations of culture and sterile culture media. Aseptic technique requires some preparatory work prior to the experiment (i.e., autoclaving of vessels, media, etc.), as well as proper handling of instruments throughout the actual experiment. During the course, you will become familiar with certain sterile techniques as they apply to the various experiments. Here is some general information about sterilization and aseptic technique:

1) Most sterilization of materials will be done by autoclaving in pressurized steam. The autoclave settings will be 121°C and 15 psi. Liquids (broth media, agar media) and containers holding liquids (dilution tubes and bottles) should be autoclaved for 15-20 minutes. Never fill a flask more than 2/3 full; the flasks will boil over in the autoclave if they are too full. **Use the liquid cycle with slow exhaust to avoid over boiling!**

2) "Dry" materials (pipets, spatulas, etc.) should be wrapped or the openings covered (empty flasks, filter funnels, etc.) with aluminum foil prior to autoclaving. Be sure to mark packages to avoid opening of the "business" end of pipets, thus exposing them to the air and potential contamination. **Use the dry cycle with fast exhaust for these materials!**

3) If liquids are being autoclaved in screw-top vessels, do not tighten the cap. The high pressure may cause the vessel to burst. Tighten the cap, then back it off 1/4 to 1/2 turn.

4) All manipulations of media, samples, sampling instruments, etc. must be done using aseptic techniques. This means only sterile glassware, pipettes, forceps, spatulas, etc. must be used. While glassware is sterilized by autoclaving, metal objects (i.e. forceps, spatulas) are sterilized for each use by dipping them into ethanol followed by ignition of the ethanol by passing the object through a burner flame. **Prior to use, let the object cool down! Microorganisms are heat sensitive!**

5) Each time a sterile package or container is opened, there is a risk of contamination. Therefore, do not leave sterile material open to the air for any longer than is necessary. Never let a sterile object touch anything that is not sterile or not meant to remain uncontaminated. Never lay sterile objects on the benchtop. **The key rule in aseptic technique is "WHEN IN DOUBT, THROW IT OUT"**.

6) Work quickly and carefully when inoculating, spreading or streaking plates. Shield the surface of the plate as much as possible with its cover. Do not breathe on the culture plate during spreading. Likewise, avoid touching the inside of the plate. Always flame inoculating loops and the neck of the culture tube prior to transfer of bacterial cultures. After completion of the transfer, briefly flame the neck of the culture tube before you replace the cap or plug.

1. DILUTION AND SPREAD PLATES, STREAK PLATES AND STAINS

A. DILUTION AND SPREAD PLATE PROCEDURES

Due to their small size, microbes can occur in great numbers in a given sample. One milliliter of a typical sediment sample may contain between 10^6 to 10^9 microorganisms, and maximum concentrations may reach 10^{12} bacteria/ml. In order to examine microbial samples, one needs to physically separate the microorganisms to manageable levels. This is done in a stepwise fashion using the dilution method (see Figure 1).

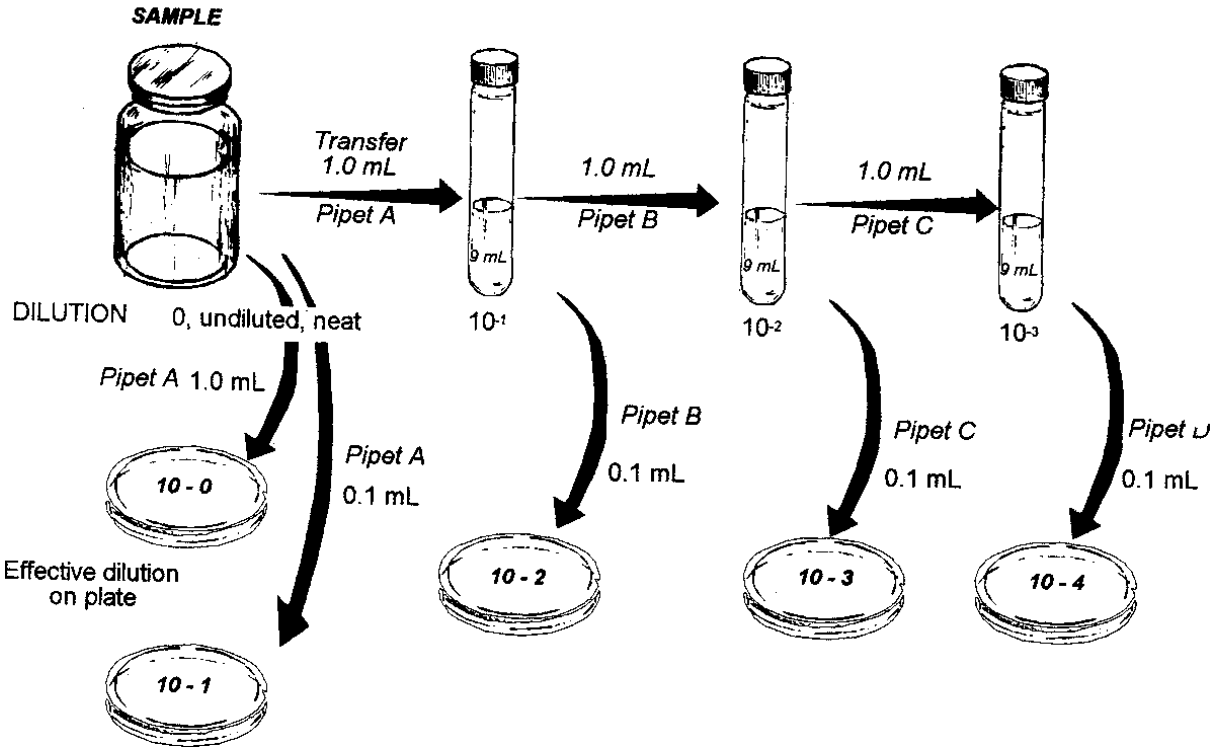

Figure 1. Dilution series for the spread plate technique. Each effective dilution represents the fraction of one milliliter of the original sample that is on the plate. To get the number of organisms in the sample, divide the number of colonies appearing on the plate by the dilution factor. Figure is redrawn from Seeley, Vandermark and Lee using elements scanned from the original.

The general idea of this method consists of introducing known "amounts of microbes" into dilution blanks of known volume. We will use this technique for the cultural enumeration of soil and water microbes on spread plates, i.e. we will "count" the physical manifestations of individual microorganisms (microbial colonies) that were cultured on solidified growth medium, which will enable us to estimate the number of bugs per ml of soil or water suspension.

The diluent used should reflect the environment from which the samples were collected. For example, freshwater and sediment samples may be diluted with distilled (or deionized) water, although some investigators prefer to use a buffer solution of 0.85 % NaCl (physiologic saline) to prevent any possible cell lysis due to osmotic stress. Marine samples should be diluted in a solution that approximates the salinity of the environment from which the samples were collected.

The culture (spread) plates used in this exercise contain a layer of solidified, sterile nutrient agar. All you need to know about this particular growth medium is that it contains essential nutrients that enhance the metabolism and growth of a wide range of microorganisms. However, this medium is by no means ideal for all the organisms (e.g., nitrifiers) present in your water or soil sample. Obviously, it would be very difficult to formulate such a complete growth medium.

Water Samples

1) Mark four dilution tubes with your dilution strength, 10^{-1} through 10^{-4}.

2) With a sterile pipette transfer 1.0 ml from the water sample into the dilution tube marked 10^{-1}.

3) Make 10-fold dilutions of the sample (9 ml diluent + 1 ml sample) to 10^{-4}. Remember to use a new, sterile pipette between each dilution and to mix the dilution tubes thoroughly each time.

4) Label two replicate plates for each dilution you intend to plate out. For example, label the plate receiving the 10^{-2} subsample "10^{-2}". **Put all the necessary marks (i.e., sample type, replicate number, dilution, initials) on the bottom of the dish!!** Also, make sure the plates are labeled with the volume of subsample actually placed on the plate.

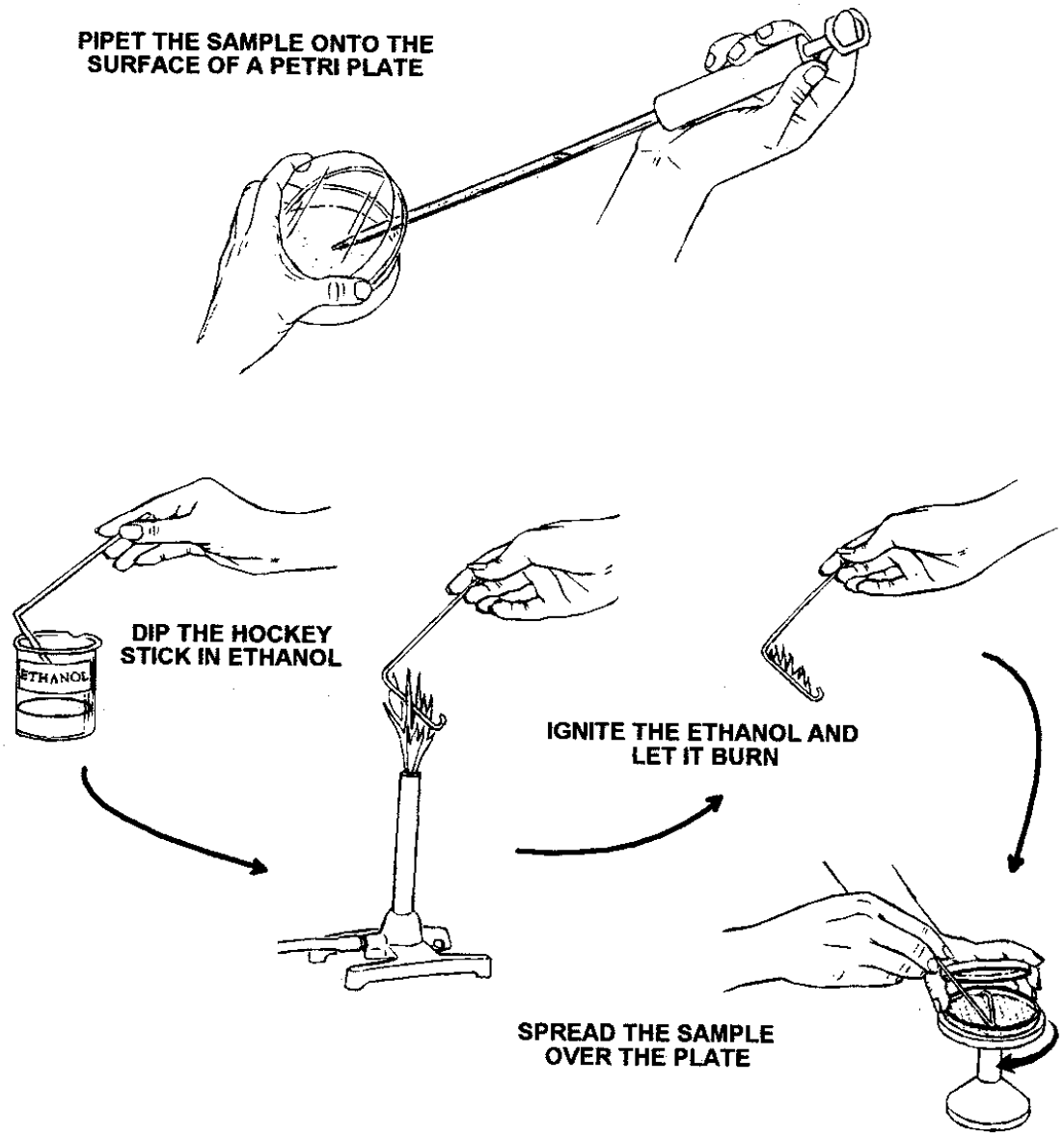

Figure 2. Technique for spreading samples on agar media in the spread-plate method. Figure redrawn from Seeley, Vandemark, and Lee using elements scanned from the original.

5) Using a sterile 1.0 ml pipette, and starting with the most dilute solution, pipette 0.5 - 0.1 ml onto the center of an appropriately marked plate. If you start with the most dilute solution, there is no need to change pipettes as you remove samples from the most dilute to the most concentrated.

6) Flame-sterilize a glass "hockey stick" and carefully spread the sample drop around the plate until you feel "resistance" to the spreading motion and the culture medium becomes "more sticky". Avoid touching or breathing on the inside of the plate while spreading. Protect the plate with the plate cover.

7) The same hockey stick may be used on plates representing the same dilution without resterilizing it, but make sure it gets sterilized between dilution samples.

8) Invert the plates (to avoid condensation on top of the culture medium) - the writing on the plate bottoms should face up! - and incubate them at room temperature for 48 hours.

Sediment Samples

1) Flame sterilize a clean spatula.

2) Weigh out 1.0 gram of sediment or soil and add it to a 99 ml dilution blank. Save several grams of the sample for oven drying to determine the dry weight of sample added to the bottle.

3) Shake the bottle vigorously for about two minutes.

4) Make 10-fold dilutions from the 1/100 dilution bottle.

5) Proceed as you did when diluting and plating water samples.

Plate Counting and Calculations (important for next week)

After incubation, the plates will be analyzed. Analysis, in this case, means simply counting all of the colony forming units (CFU). The assumption that we have to make for this procedure is that each CFU originated from one individual microorganism. To get reliable results with the spread plate method, count only those plates that have between 30 - 300 CFUs. For ease of counting, mark the plate into quadrants and count each quadrant separately. As you count colonies, mark them off with a Sharpie to avoid repeated counting.

Based on the plate counts, you will have to do some simple calculations to estimate the number of bacteria in one ml or one gram of your sample. The calculation is as follows:

1) Divide the number of CFUs counted by the dilution factor and adjust for the amount of sample actually plated. Report the number of colonies as CFU/ml.

$$\text{CFU/ml} = \text{counts}/(\text{dilution factor} \times \text{amount of sample plated})$$

2) Water samples are always reported on a volumetric basis, while soil or sediment samples may be reported either on a volumetric or a weight basis. After weighing, dry the spare soil sample overnight at 105°C and reweigh. Adjust your calculations accordingly.

3) Estimate the number of microorganisms in your total sample.

B. STREAK PLATES

The streaking of microbes onto culture plates is a useful method to isolate pure bacterial strains from mixed cultures. The general idea of this method is the physical separation of individual cultures by dragging progressively smaller "amounts of microorganisms" across a culture plate. Again, the assumption is that one CFU represents an individual microbe.

In this exercise, you will be provided with a mixed culture of soil microorganisms. With a sterile inoculating loop, take a loopfull of material containing microorganisms from one particular colony and streak the microbes according to the following procedures (see also Figure 3):

1) Lift the lid of the plate and gently streak the loop across the surface of the medium near the edge of one quadrant of the plate.

2) Dip the inoculating loop into ethanol and flame until red-hot. Allow loop to cool.

3) Drag the loop once through the previously streaked area and repeat the streaking in the neighboring quadrant.

4) Repeat steps 2) and 3) until you have streaked at least three quadrants.

5) Repeat the streaking procedure with another colony from the plate containing the mixed culture.

6) Incubate plates upside down at room temperature for 48 hours.

7) After incubation, inspect the streak plates and describe the colony morphology with the help of the information in Appendix 1.

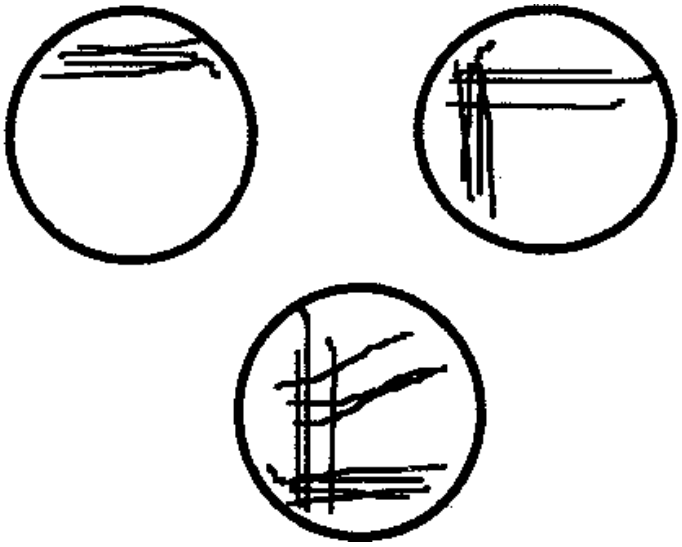

Figure 3. Pattern of streaking used to isolate colonies. Other patterns are often used, as well.

C. DIFFERENTIAL STAINS

Preliminary microscopic identification of microorganisms is usually based upon gross colony morphology and the manner in which the bacteria react to staining procedures. All microbiological stains have one feature in common: coloration is due to the presence of chromophore groups that have conjugated double bonds. The chromophores bind with cells due to ionic (most common mode of binding), covalent, or hydrophobic interactions. Ionizable dyes can be further subdivided into basic dyes and acidic dyes. The basic dyes have positively charged chromophores that bind to negatively charged cell surfaces. These are the most common microbiological dyes (Methylene Blue, Crystal Violet, Safranin). Acidic dyes have negatively charged chromophore groups (-COOH, -OH) that interact with positively charged structures on the cell surface.

On a functional level, stains are divided into either simple or differential stains. Simple stains involve one single staining agent that produces similar results for different microorganisms. Simple stains are mainly basic stains (Crystal Violet, Methylene Blue) and they are used to microscopically determine microbial shape and size. Differential stains, on the other hand, involve treatment of the bugs with several different stains. Microorganisms are divided into separate groups based on their particular staining properties.

Probably the most common differential stain is the Gram stain, discovered by Christian Gram in 1883. Its diagnostic value, however, is restricted to prokaryotes with cell walls; for these microorganisms, the resulting Gram reaction is either positive (cells retain blue Crystal Violet stain) or negative (cells take on red Safranin counterstain). Nearly all the bacteria can be subdivided into these two subgroups on the basis of their Gram reaction. A battery of diagnostic tests and elaborate identification schemes (dichotomous keys) are available to further identify the microorganisms in question.

We will perform Gram stains on the strains that you have isolated with the streak plate technique. Sample preparation for staining purposes involves heat fixation of bacterial smears.

Bacterial Smears and Heat Fixation of Smears (see also Figure 4)

1) Mark a clean microscope slide with a Sharpie and place one drop of deionized water in the middle of the slide.

2) With a flame-sterilized inoculation loop, grab one isolated colony from your streak plate and mix the bugs with the water on the slide.

3) Allow the water to air-dry on the slide.

4) With forceps, pick up the microscope slide by one corner and pass it several times **over** the flame of a Bunsen burner. Do not touch the heated microscope slide unless you like to burn your fingers!

5) Let the slide cool down. You should now have a slide that looks like it has some specs of dirt on it.

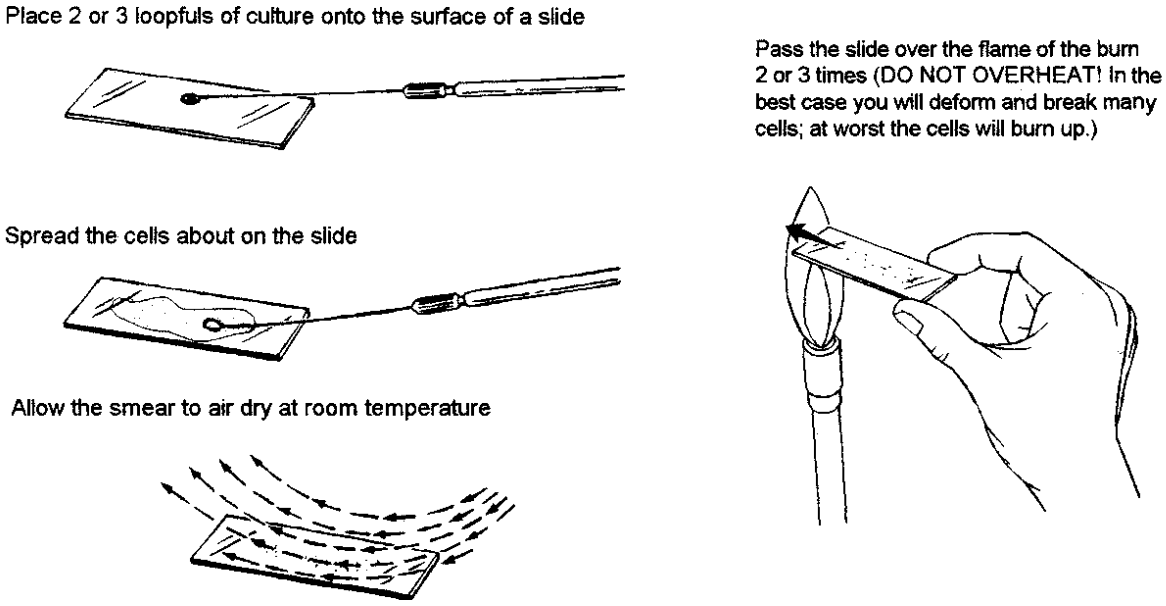

Figure 4. Heat fixing a smear of a culture. If cells are taken from a slant or plate, mix them into 2 or 3 drops of filtered distilled water or saline on the slide. Figure made from scanned images from Seeley et al.

The Gram Stain (see also Figure 5)

1) Cover the heat-fixed smear with Crystal Violet and let sit for 30 seconds.

2) **Gently** rinse off the Crystal Violet with deionized water. Use a squirt bottle for this.

3) Cover the smear with Gram's Iodine for 30 seconds. Gram's Iodine acts as a mordant, fixing the Crystal Violet to the cell walls of the microorganisms.

4) **Gently** rinse off the Iodine with ethanol. The alcohol acts as a decolorizer; Gram positive bacteria are unaffected by this step; Gram negative bacteria have the Crystal Violet washed off by the alcohol.

5) **Gently** wash the alcohol off the smear with deionized water.

6) Cover the smear with Safranin for 30 seconds.

7) Wash once again with deionized water and carefully blot the slide dry without wiping off the fixed bacteria.

Microscopic Observations

1) Add **one** drop of immersion oil to the top of your fixed, stained sample.

2) If necessary, shift the 100× microscope objective into the viewing position.

3) Put the slide in the slide holder and raise the stage until the tip of the objective is immersed into the oil.

4) Adjust the focus using the coarse and the fine adjustment knobs.

5) Try to focus on a few cells rather than the entire field of vision.

6) Describe the Gram reaction of your sample, the bacterial shape, relative size (small, very small, etc.) of your bugs, and the colony appearance (e.g., clumped vs. small groups or pairs of bacteria). Use the information from Appendix 1.

7) In case you do not see anything, here are some typical problems:
   - too small a sample used for preparation of the smear.
   - heating the smear too much, which causes the cells to burn off.
   - washing the smear too rigorously during staining.
   - wiping cells off the slide when blotting it dry.

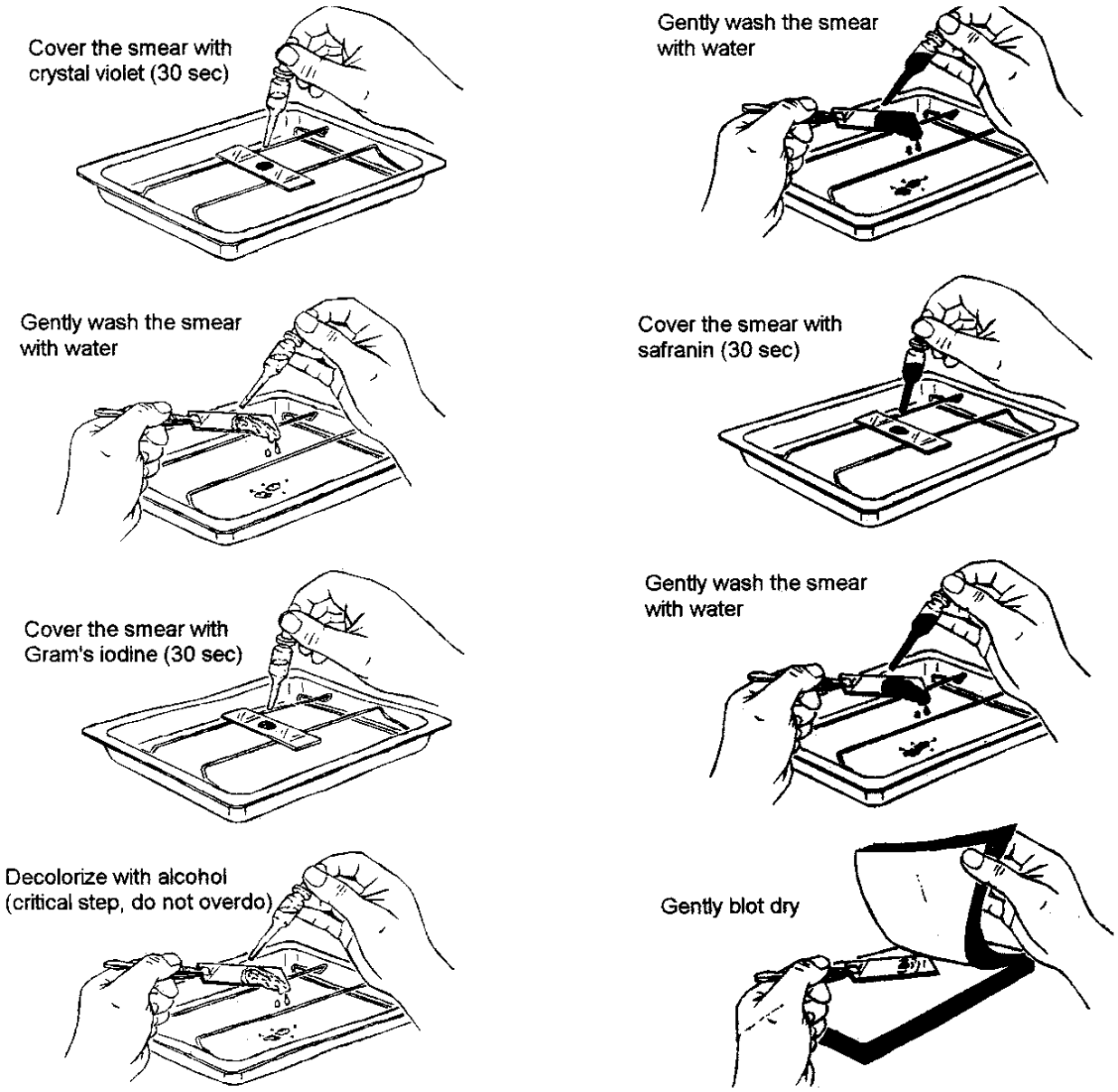

Figure 5. Gram staining. Figure taken from Seeley et al.

APPENDIX 1

A. CELLULAR MORPHOLOGY

Shape: cocci, coccoid, coccoid-bacillary, filaments, commas, spirals, pleomorphic, rods, etc.

Axis: straight or curved

Size: *Overall:* minute, small, medium, large
Length: short, medium, long, filament
Breadth: thin, medium, thick

Sides: parallel, ovoid (bulging), concave, irregular

End: rounded, truncate, concave, pointed, feathery

Arrangement: singly, pairs, chains, tetrads, groups, clusters, packets, chinese letters, etc.

Pleomorphic forms: variations in size and shape, clubs, citron, filamentous, branched, fusiform, giant swollen forms, shadow forms

Spores: central, terminal, sub-terminal, round, oval, swelling or not swelling the rod

Staining (Gram's): negative, positive, variable, evenly, irregularly, unipolar, bipolar, beaded, barred, variation in depth, granules

B. COLONIAL MORPHOLOGY

Size: punctate, 0.5 mm, larger sizes designated as 1.0 mm, 1.5 mm, 2.0 mm, etc.

Shape: circular, irregular, rhizoid, filamentous

Surface elevation: flat, raised, low convex, convex, pulvinate, umbonate, convex-papillate

Edge: entire, undulate, lobate, erose

Internal: curled, filamentous, granular

Surface: smooth, rough, rugose (wrinkled), contoured (an irregular, smoothly undulating surface, like that of a relief map), granular (fine, medium, coarse), papillate, dull, glistening

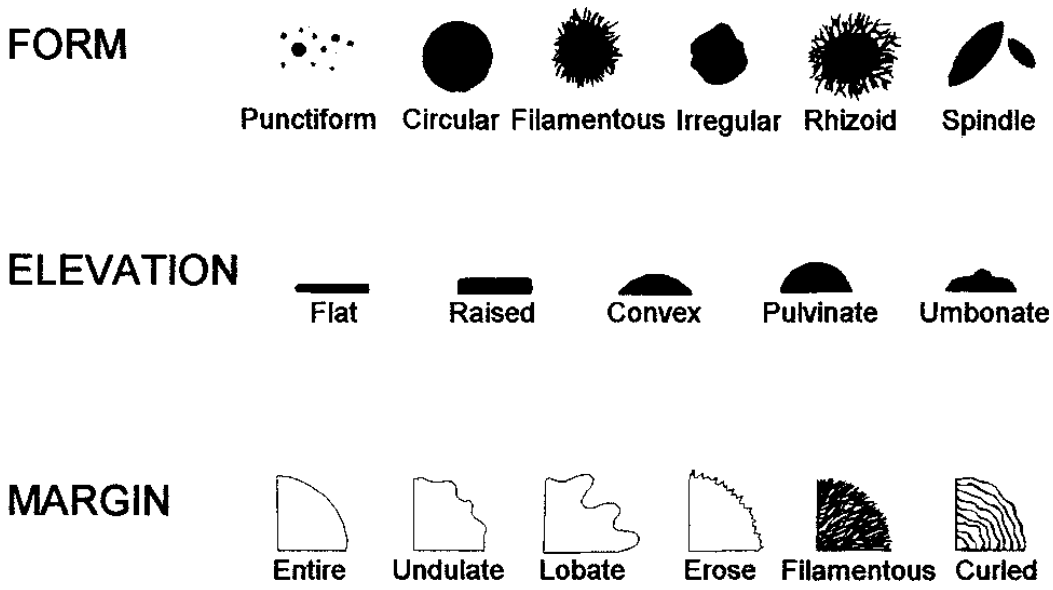

Figure 6. Variation in forms, elevations, and margins of bacterial colonies. Redrawn from Smibert and Kreig, in Gephardt et al., 1981.

Optical characteristics:

opaque - not allowing light to pass through
translucent - allowing light to pass through without allowing complete visibility of objects seen thru the colony
opalescent - resembling the color of an opal
iridescent - exhibiting changing rainbow colors in reflected light
dull - not glossy or glistening
glistening - glossy, not dull

Consistency:

butyrous - growth of butterlike consistency
viscid - growth follows the needle when touched and withdrawn
membranous - growth thin, coherent, like a membrane
brittle - growth dry, friable under the platinum needle

Emulsifiability: homogeneous, granular or membranous suspension

Pigmentation of growth: white, buff, light yellow, straw yellow, deep yellow, pink, red, etc.

2. ACRIDINE ORANGE DIRECT COUNTS OF BACTERIA IN WATER AND SOIL SAMPLES

A. INTRODUCTION

Enumeration of microorganisms in environmental samples is an issue central to many applications in microbial ecology. Due to the microscopic dimensions and the abundance of microorganisms in the environment, cultural enumeration techniques (i.e., spread plates) have approached the problem indirectly, counting visible manifestations (colonies) of cells rather than individual cells directly. As you experienced in the previous lab, the analytical accuracy of the spread plate method is confounded by vague definitions of what actually constitutes a colony forming unit, and, more importantly, by the assumption that each counted colony originated from one individual cell. Microscopic examinination of microbial samples offers an important alternative to the cultural enumeration method. Hobbie et al. (1977) pioneered the Acridine Orange Direct Count (AODC) method for the enumeration of microbes in aquatic and soil samples. In this method, a sample containing microorganisms is stained with Acridine Orange (a fluorescent stain) and filtered through a specially-treated polycarbonate filter membrane with pore openings in the submicron range. While the pore openings allow filtrate containing submicron particles to pass through, they impede the passage of bigger microorganisms (which get trapped on top of the filter). The filter with the stained, trapped microorganisms is then examined under high magnification with a UV-light equipped microscope. Either by itself, or in conjunction with the viable plate count method, the AODC technique has become one of the most widely used enumeration methods in environmental microbiology.

The staining action of Acridine Orange (AO) arises from its reaction with the nucleic acid material present in cells. While DNA typically stains green, RNA will be stained orange. Given these different staining reactions, associated with the different nucleic acids, there is some controversy as to whether the AO stain can actually distinguish between live and dead cells. As with several other techniques, a disadvantage of the AODC method is that the AO stain is lethal to the microrganisms; the sample cannot be recovered after analysis.

In this exercise, you will re-analyze the water/sediment sample, which you used in the previous exercise, using the AODC method. This will enable you to compare and evaluate the results from the two techniques.

B. PREPARATIONS

1. Filters

The filters used for this exercise are polycarbonate Nuclepore filters (0.2 μm pore size, 25 mm diameter) pre-dyed with Irgalin Black. Treatment with the Irgalin Black dye eliminates autofluorescence of the filter.

2. Acridine Orange Stain

The AO stain is made up by dissolving 0.1 % (w/v) Acridine Orange in 2 % formaldehyde. Formaldehyde is usually bottled as a 37 % solution (= 100 % formalin); therefore, to make 100 ml of 2 % formaldehyde, use 5.4 ml of the 37 % formaldehyde stock solution. The AO/formaldehyde solution should then be filtered through a 0.2 μm filter.

When working with the Acridine Orange stain, it is highly advisable to wear gloves. The stain is mutagenic and possibly carcinogenic! Dispose of AO wastes in the proper hazardous waste containers!

3. Dilution Blanks

To perform any necessary dilution of your samples, you will be supplied with filter sterilized water (passed through a 0.2 μm filter). If you determine from microscopic examination that a serial dilution is appropriate, use the supplied acid-washed reagent tubes and proceed as you learned in the previous exercise. If you need to preserve your dilution samples for more than 5-6 hours, you should add formaldehyde to your sample to a final concentration of 2 %. For example, to a 20 ml sample, add 1.1 ml of filtered formaldehyde.

C. STAINING PROCEDURE

Before you run any samples, you will have to examine the dfH_2O that you use during the filter operation. Prepare a blank slide as outlined below and look for contamination. If there are > 5 cells/field, you will have to filter a fresh batch of water and you will have to filter the AO stain once more.

1) Rinse a clean reagent tube three times with dfH_2O. Then mix 5 ml of deionized, filtered water, 0.5 ml AO stain and 0.1 - 0.2 ml of your original, vortexed sample (the total volume in the tube should be approximately 5 ml, with a ratio of AO: dfH_20 = 1:10). Note the time when adding the AO stain to your sample. Vortex gently for 30 seconds.

2) Let the solution stain for 3 minutes (maximum).

3) While the solution is staining, assemble the filter tower. Place a gasket on the nylon frit, followed by a Nuclepore filter membrane (shiny side up), and then the second gasket. Carefully screw the filter tower onto the filter base, while holding the filter/gasket assembly in place.

4) With a sterile pipette (which can be reused if kept in the flask containing dfH_2O) add several drops of dfH_2O on top of the filter and check for leaks.

5) Connect the filter apparatus to the vacuum aspirator on the faucet.

6) Add your sample and filter with a gentle vacuum.

7) When the sample has been filtered, add a rinse solution (dfH_2O) to the tower, rinsing the sides of the tower well. When the last of the solution has filtered, break the vacuum **first**, to prevent backwashing, and then turn the water off.

8) With forceps, peel the filter off the filter base and place on a clean glass slide (shiny side up). Put one drop of immersion oil on the filter, followed by a cover slip. Add one more drop of immersion oil on top of the cover slip. This preparation will last several hours at room temperature and much longer with refrigeration.

D. COUNTING WITH THE MICROSCOPE

As mentioned above, enumeration of the microbes present in the sample is done by viewing the stained Nuclepore filter with an oil immersion objective, while illuminating the sample with UV light.

1) Place the slide with the stained filter in the slide holder on the stage of the microscope. Swing the oil immersion objective into position.

2) Raise the stage with the coarse adjustment knob until the oil on top of the slide touches the objective. Continue to slowly raise the stage until you see a "blue flash of light"; this marks the appropriate position at which the slide can be viewed. All you need to do now is focus with the fine adjustment knob.

3) In order to focus, move the slide from side-to-side or up and down until you see an area that is brighter than the surrounding area.

4) Focus on the bright area.

5) Once in focus, look at the eyepiece micrometer. The field delineated by the micrometer is your orientation for counting. There are 10× 10 squares in the field. Use these squares as counting guides. Be consistent in counting bugs that sit directly on a line.

6) Most of the bacteria will fluoresce a pale green. Occasionally a few will be orange, red or yellow. In general count all particles that look like bacterial cells. Bacteria may be rods, spheres, or spirals. The cells will always be much smaller than the counting grid.

7) After counting one field of 100 squares, randomly move the slide to another position without looking through the ocular (to avoid cheating). Continue counting until you have scored at least 5 fields. If the five fields tally 200 cells or more, stop counting. If you have counted fewer than 200 cells, continue counting until you have counted > 200 cells. Fields with < 20 cells, or with > 200 cells should not be counted. In this situation you will have to adjust your dilution accordingly.

E. Calculation of Bacteria in the Sample

In order to calculate the number of bacteria per ml of sample, use the following formula:

$$\text{Bacteria/ml} = \frac{(\text{total area})/(\text{area/field}) \times (\text{cells/field})}{\text{volume filtered} \times \text{dilution factor}}$$

where:

total area = total area of stained filter = 314 mm^2
area/field = area of one field as defined by the eyepiece micrometer = 0.008649 mm^2
cells/field = number of cells counted averaged over the number of fields counted
volume filtered = amount of sample filtered onto filter

F. AODC of Bacteria for Sediment Samples

Preparation of stains and diluent are the same as described for the analysis of water samples.

However, the staining procedure requires some additional steps:

1) A minimum of two subsamples should be prepared from each sediment sample. Place the freshly collected, wet sediment into a blender that had been rinsed three times with dfH_2O. Save some of the wet sediment and determine the dry weight of the subsample.

2) Add 100 ml of dfH_2O and blend at high speed for 1 minute.

3) Remove 0.5 ml of the suspension and place it in a tube with about 4.5 ml of dfH_2O and 0.5 ml of AO stain. Stain for 3 minutes.

4) Proceed with the remainder of the procedure as outlined for water samples. Counting and calculations are the same as before except that the dilution factor will be different for soil samples. Furthermore, the volumetric term in the denominator should be replaced by a weight (gram) term that has been adjusted based on the soil dry weight.

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Bowden, W. B. 1977. Comparison of two direct-count techniques for enumerating aquatic bacteria. Applied and Environmental Microbiology. 33:1229-1232.

Daley, R. J. and J. E. Hobbie. 1975. Direct counts of aquatic bacteria by a modified epifluorescence technique. Limnology and Oceanography. 20:875-882.

Hobbie, J. E., R. J. Daley, and S. Jasper. 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Applied and Environmental Microbiology. 33:1225-1228.

DATA ANALYSIS – CULTURAL ENUMERATION OF BACTERIA AND AODC METHOD

In this lab report, you should compare the results of the AODC and the spread plate technique. Make sure to briefly summarize what we set out to do with this lab, and include answers to the questions outlined below. For both methods, include a table of raw data and calculate the average bacterial concentration in your soil or water sample.

Spread Plates

Calculate the concentration of bacteria in CFU/ml (or per gram) for each of the "countable" plates you obtained. Report these values and the average. How close were the replicas? What does this tell us about using this technique to quantify the number of cells in a sample?

Acridine Orange Direct Counts

Using the following equation, calculate the concentration of cells from your AODC data:

$$\text{cells/ml} = \frac{((\text{total area of filter})/(\text{area/field})) \times (\text{cells/field})}{\text{vol. filtered} \times \text{dilution factor}}$$

where the total area of the filter is 314 mm^2, the area/field is 0.008649 mm^2, and "cells/field" is the ***average*** number of cells per field The "dilution factor" is the actual proportion (e.g. 1/100 or 10^{-2} – rather than "-2").

Again, how close were the replicas? Did the technique give you more or less consistent results than the spread plate method?

How close were the estimates of abundance between the two techniques? Give a few reasons why they might be different. Discuss the advantages/disadvantages of each of the two enumeration methods. In what situations would each method be useful? How does the data obtained from each method differ?

3. DETERMINATION OF HETEROTROPHIC ACTIVITY

A. INTRODUCTION

Counting microbes in a sample, using the methods you have learned previously, often provides somewhat incomplete information - with respect to questions of ecological importance. The ecological significance of microorganisms in the environment has to be evaluated in terms of **metabolic activity** of particular microorganisms. Given such information, one could, for example, evaluate the relative effects of different environmental factors on a microbial community, or the physiological response of microorganisms to certain environmental conditions.

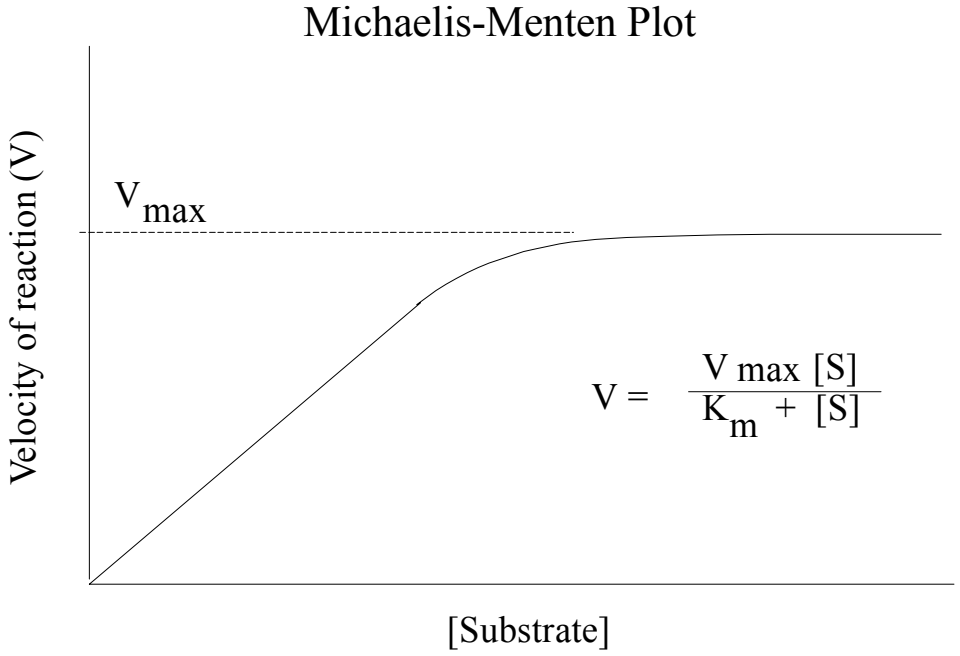
The advent of **radioisotope labeling** in the early 1940s has provided microbial ecologists with highly sensitive tools to estimate microbial activity both *in situ* and *in vitro*. The basic concept underlying microbial radioisotope work is rather simple and elegant: a sample containing microorganisms is incubated with radioactively labeled compounds, the cells are collected and then analyzed for the amount of incorporated radioactivity.

The radioisotope method is extremely sensitive in that even minute amounts of incorporated radiolabel can be detected. Furthermore, depending on the problem at hand, the method can be "customized" by employing different isotopes (i.e., ^{14}C-, ^{35}S-, or ^{3}H-) to examine particular processes (e.g., sulfate reduction), as well as by radiolabeling specific atoms within a molecular compound (which is very helpful for the examination of metabolic pathways). While the cell-free, *in vitro* radioisotope method has mainly been applied by medical microbiologists interested in the elucidation of microbial metabolic pathways, the *in situ* technique has been extensively used by microbial ecologists and ecologists in general. Despite some inherent limitations, microbial ecologists commonly apply this method for the examination of environmental samples.

In this laboratory exercise, you will be introduced to a basic *in situ* application of this method. You will be supplied with bacteria from two sources, and based on the incorporation of radiolabel, you will estimate **uptake** of the label by the microorganisms. Uptake is the summary term attached to the combined processes of **assimilation** and **respiration**. Assimilation refers to the metabolic processes by which the compound containing the radioisotope is oxidized by the microorganisms and used as a growth nutrient (i.e., label is retained as cellular material or "biomass"). Respiration, on the other hand, is the term used to describe the process during which the compound serves as an energy source and organic carbon is converted to CO_2.

The data generated in this exercise will also serve to introduce you to microbial **uptake kinetics**. Uptake kinetics can be equated with enzyme kinetics since substrate uptake is controlled by cellular enzyme complexes. The velocity of substrate uptake is a function of the speed at which available enzyme complexes react with the substrate. Microbial uptake processes can be described by saturation kinetics: initially, substrate is taken up by the enzymes at reaction velocities that are proportional to the concentration of the substrate. Once the available enzyme sites reach saturation, the reaction velocity slows down and eventually reaches steady state conditions. At this point, velocity also becomes independent of substrate concentration.

In order to compare enzyme kinetics from different samples, it is convenient to use graphical representations. There are several ways of plotting these processes. The most conventional approach is to portray enzyme kinetics in a Michaelis-Menten plot (resulting in the typical exponential shaped curve). The height and steepness of the Michaelis-Menten curve are defined by two parameters, respectively: $\mathbf{v_{max}}$ (the maximum velocity that can be obtained by enzyme binding to a particular substrate), and $\mathbf{k_m}$ (the concentration of substrate at which the enzyme reaction velocity = 1/2 v_{max} (1/2 substate saturation)).



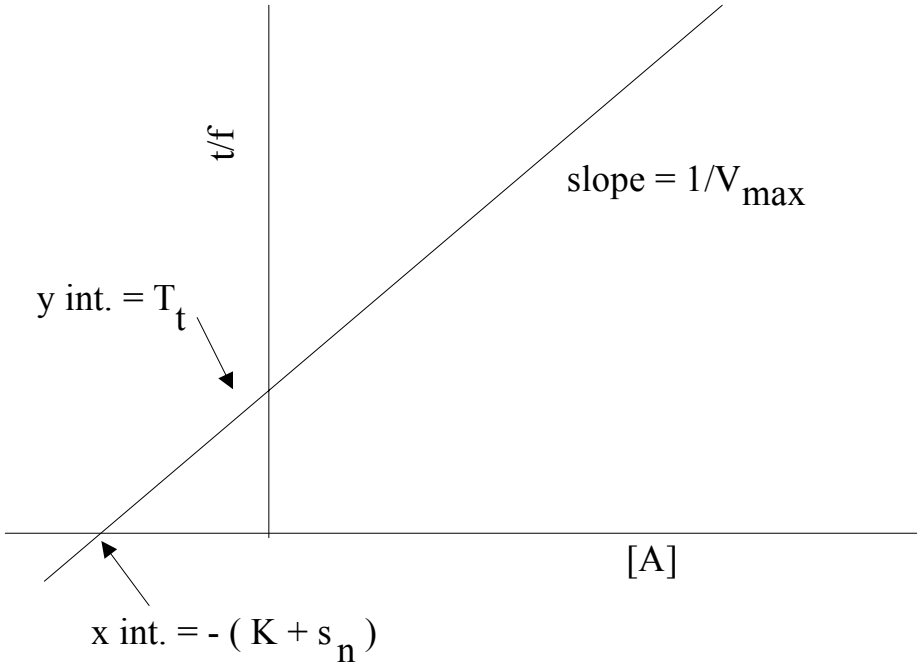
Other graphical representations of the same process take this information and establish linear relationships between the different parameters. The outcome is a more easily plotted straight line. Please keep in mind that the ultimate motivation for all these different graphical representations is to determine v_{max} and k_m.

A **Modified Lineweaver-Burke** plot will result in a straight-line relationship (that is if the experiment works out and the data conform to saturation kinetics assumptions); the axes of the graph are:

- t / f, where t = incubation time and f = fraction of isotope taken up
- [A], where A = concentration of compound added during incubation

Modified Lineweaver-Burke

$$t/f = \frac{1}{V_{max}}[A] + \frac{K + s_n}{V_{max}}$$



The character of the straight line describing enzyme kinetics can then be expressed by the slope of the resulting line, and its x- and y- intercepts:

slope = $1/v_{max}$

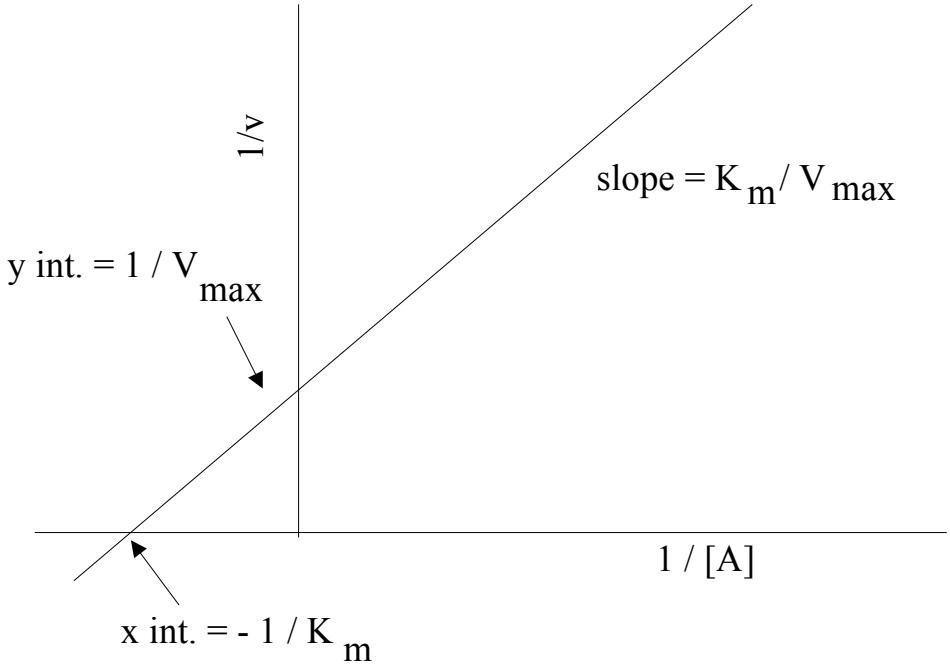
x-intercept = $-(k + S_n)$, where k = transport constant
and S_n = the natural substrate concentration

y-intercept = $T_t = (k + S_n)/v_{max}$, where T_t = turnover rate constant

Estimates of v_{max} and k_m can also be obtained from a Lineweaver-Burke plot.

Lineweaver-Burke

$$\frac{1}{V} = \frac{K_m}{V_{max}} \left\{ \frac{1}{[A]} \right\} + \frac{1}{V_{max}}$$



B. GENERAL INFORMATION ABOUT RADIOISOTOPES

The radioisotope that we will use in this exercise is ^{14}C. ^{14}C is a ß emitter that emits particulate radiation in the form of a high speed electron that is ejected from the isotope's nucleus. Particulate radiation is a form of radiation that has mass (as opposed to emitting massless, electromagnetic radiation of high energy light waves) and as such has a finite range. The maximum range of ß particles in the air is about 22 cm. Due to its low energy ^{14}C is barely able to penetrate human skin, but it is still considered externally hazardous. ^{14}C does represent a high internal hazard as its energy is directly deposited into sensitive organs. The half life of ^{14}C, which is the time required for the isotope to decay to 50% of its activity, is 5730 years.

Activity of radioisotopes is expressed in units of Curie (Ci), after one of the most important early researchers of radioactivity, Marie Curie. In order to calculate heterotrophic activity from our experiment (which will be expressed as moles of radiolabeled ^{14}C incorporated into the cells), you will have to know these conversions:

$$1 \text{ Ci} = 3.7 \times 10^{10} \text{ atoms degrading/second}$$
$$1 \text{ } \mu\text{Ci} = 2.22 \times 10^{6} \text{ atoms degrading/minute (dpm)}$$
$$1 \text{ } \mu\text{Ci/ml} = 3.7 \times 10^{4} \text{ atoms degrading/second/ml}$$

You will also need to know the specific activity of the compound you are using – the number of mCi/mmoles of substrate.

C. SOME PRECAUTIONARY NOTES ON THE HANDLING OF RADIOISOTOPES

Before you get ready to perform the experiment, please be sure that you have prepared yourself appropriately. The handling of radioactive substances requires some precautionary effort in order to avoid contamination. Make sure to read through the following list **before** you start with the procedure:

- *Do not pipette by mouth!*
- *Nobody will be allowed in the work area unless they are wearing a lab coat **and** gloves!*
- *Do not bring pencils or notebooks into work area. Once a utensil has been brought into the work area, it is considered contaminated!*
- *Avoid touching your face, hair, or other body parts when handling the radioisotope. Be aware of your habits, i.e. do not adjust your glasses, etc.*
- *Work quickly without rushing*
- *Have all containers clearly marked **before** you proceed with the actual experiment*
- *Notify TA of any spills!*
- *Dispose off any radioactive wastes in the designated waste containers!*
- *Monitor work area after experiment by doing wipes*
- *Wash your hands thoroughly after you are done!*

D. PROCEDURE

1. Preparations

All the preparations for this exercise were completed prior to class. Two fresh microbial samples were obtained. The radioisotope for this exercise has been diluted from a stock solution of ^{14}C labeled acetate by addding 10 µL of acetate to 2 ml of filtered DIW. The resulting solution has a concentration of about 5 µCi/ml.

2. Incubation

Standards:

1) Prepare 8 scintillation vials and familiarize yourself with the operation of the micropippeter.

2) Place 10 µL of diluted ^{14}C-acetate in each of two labeled scintillation vials. Fill the vials with scintillation cocktail.

3) Repeat step 1) with 20, 40, and 80 µL diluted acetate.

Samples (the same procedure applies to both water samples):

1) Prepare all flasks and rubber stoppers.

2) Per dilution you will need 10 replicate flasks and 10 rubber stoppers (or 5 per sample site). All of these rubber stoppers need to be outfitted with the small plastic wells (used to measure mineralization).

3) Add 10 ml of sample to each labeled uptake flask. With a micropipetter, add 10 µL of diluted acetate to each flask. Close flasks with rubber stoppers. Record time.

4) To flasks 4 and 5 add 0.10 ml phenethylamine into the small plastic cups (designate a syringe with a short needle for this purpose).

5) Kill flasks 4 and 5 by adding 1 ml of 2N H_2SO_4 to the sample (designate a syringe with a long needle for all handling of acid).

6) Repeat steps 1) - 5) with 20, 40, and 80 µL ^{14}C acetate.

7) Incubate flasks at room temperature for exactly 2 hours. At the end of the incubation, kill the live flasks (#1-3) by adding 1 ml of 2N H_2SO_4. Record the time.

3. Analysis

Mineralization (M):

1) After killing the live flasks (after the 2 hour incubation time), add 0.10 ml of phenethylamine to the small plastic cups to trap CO_2 (designate a syringe with a short needle for this task). Record time and incubate for 45 minutes.

2) Remove the plastic cups carefully from all (#1-5) flasks by cutting off the cup-part with scissors (results from flasks 4 and 5 will serve as a control). Do this carefully to avoid spilling the phenethylamine. Place the cup in a labeled scintillation vial, and fill the vial with scintillation cocktail.

Assimilation (A):

1) Filter the contents of the uptake flasks through a 0.2 µm membrane filter. Rinse the flasks with DIW and filter the rinse; finally rinse the tower. If the sample is hard to filter, filter only 2 ml of the sample and adjust your calculations accordingly.

2) Place the filters in labeled scintillation vials and fill with cocktail.

4. Counting

A short explanation of the counting procedure

Counting of radioactivity in the samples is done by an instrument called a scintillation counter. This instrument can detect low ß emitters by registering the emitted electron (which are converted to photons by the scintillation cocktail) energy as activity. The activity of a sample is expressed as:

disintegrations per minute (dpm) = counts per minute (cpm)/ counting efficiency

Counting efficiency is a function of the quench correction factor (H#) that is individualized for each sample and is reported by the scintillation counter. The counting efficiency varies somewhat with different isotope / scintillation counter combinations.

Procedure

1) Place the vials in the liquid scintillation counter, preceded by the appropriate control tower. An explanation of the machine settings will be made.

2) Be sure to terminate your samples with a **STOP** tower!

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APPENDIX 1

Wright and Burnison article

DATA ANALYSIS – DETERMINATION OF HETEROTROPHIC ACTIVITY

A. CONSTRUCTING A QUENCH CURVE

In this assignment, you will be analyzing the data we obtained for the heterotrophic uptake of ^{14}C labeled acetate. The raw data consist of H# and CPM for each sample. CPM stands for "counts per minute" and represents the number of light emissions that the scintillation counter recorded per minute for each sample. The H# is a quench correction factor that is individualized for each sample and describes the amount of counting error that occurred for each sample.

Using a "quench curve," you can determine, for each sample, the counting efficiency using the H#. The quench curve is simply a linear relationship between H# and the counting efficiency for a number of standards. Using the data given below, plot a quench curve and perform a linear regression on the data.

H #	Counting efficiency
1.1	0.9678
52.1	0.9278
110.3	0.9291
154.7	0.9026
178.4	0.8780
182.6	0.8808
213.5	0.8516
273.2	0.7547
283.8	0.7356
310.8	0.6871

In your lab report, show a graph of the quench curve and report the equation and R^2 you obtain from the linear regression. Then use this equation on our experimental samples to convert each H# to counting efficiency.

B. CONVERTING CPM TO DPM

Use the quench curve equation to convert the H# for each sample to counting efficiency. Once you have determined the counting efficiency, you can convert each measure of CPM to DPM:

disintegrations per minute (DPM) = counts per minute (CPM) / counting efficiency

C. CORRECTING OUR UPTAKE ESTIAMTES USING THE KILLED CONTROLS

When the ^{14}acetate is incubated with the bacterial samples, there are several places it can go. It can be taken up by the bacteria and used for energy. In this case it is respired and converted to $^{14}CO_2$ (it is Mineralized) and becomes trapped in the headspace above the sample. We used the phenethylamine solution to further trap all the CO_2 from the headspace in a liquid form. Then we measured the radiation associated with the phenethylamine solution in the small plastic cup. The bacteria may also retain some of the ^{14}C in their bodies by converting it to biomass. We refer to this portion as having been Assimilated. This was quantified by filtering the aqueous solution onto 0.2 μm filters (the same size we used to trap the bacteria when we did the AO staining). We then used the scintillation counter to see how much radiation was present in the bacteria on the filters.

It is also possible that radiolabel could become adhered to the outsides of bacterial cells, stick to our filter, contaminate our phenethylamine cup, breakdown chemically... you get the point. Since our goal is to examine total bacterial Uptake (U = M + A), we want to eliminate some of these potential sources of error. To do so, we kept a set of "killed" controls where we incubated the ^{14}acetate with a dead bacterial sample (these are the samples to which we added H_2SO_4 at the start of the experiment). Since the bacteria were dead, these samples should contain no ^{14}C that has been assimilated or mineralized by active bacterial growth - only ^{14}C that is the result of "experimental error."

The amount of radiation quantified in these killed controls can then be subtracted from our experimental samples to remove some of this error. However, the amount of ^{14}C that contributes to such errors is partly dependent upon the amount ^{14}C available. This is why killed controls were maintained at each acetate concentration. Make sure to subtract the amount of

activity (in DPM) from each sample of the same respective type (meaning subtract the mineralization error from each mineralization sample and subtract the assimilation error from each assimilation sample).

D. Determining carbon uptake

You can now convert your DPM per 10 ml of sample to a mass of carbon assimilated or mineralized. Useful conversions include:

$$1 \text{ Ci} = 3.7 \times 10^{10} \text{ atoms degrading per second}$$

$$1 \text{ } \mu\text{Ci} = 2.22 \times 10^{6} \text{ atoms degrading per minute (dpm)}$$

You will also need to know the specific activity of the ^{14}carbon we used – this is the amount radioactivity per mole of substrate (e.g., mCi/mmol or μCi/μmol).

Total carbon uptake is then equal to assimilation + mineralization. Calculate uptake for each bacterial sample at each ^{14}C concentration. Reaction rate can then be determined based upon incubation time.

E. Graphing the results

You should generate two graphs for each sample. The first should be a simple Michaelis-Menten plot of velocity of reaction (rate) verses concentration of compound added during incubation. It is possible that the graph may be linear, displaying only the region where rate is determined by substrate concentration and the enzyme is not yet saturated. The second graph should be a Wright-Hobbie plot (a modified Lineweaver-Burke) relating t/f (incubation time/fraction of isotope taken up) verses added concentration of substrate. f is calculated as:

$$f = [\text{assimilation } (\mu\text{mol}) + \text{mineralization } (\mu\text{mol})]/ \mu\text{mol added}$$

From this graph you can determine the turnover time (the y-intercept) and V_{max}.

Summarize your findings in a table that reports V_{max}, turnover time, R^2 for the Wright-Hobbie plot, and the growth efficiency for each sample. Growth efficiency is calculated as assimilation/total uptake and describes how much of the labeled substrate actually goes into building more biomass (relative to the substrate that is "lost" to CO_2).

F. DISCUSSION

Write a brief discussion of the results you obtained. Compare things like V_{max} and T_t between the different bacterial samples and discuss what these parameters mean. Does what you found make sense? Finally, discuss the limitations of the heterotrophic activity measurement. What are some important assumptions in this technique?

4. COMPARING MICROBIAL COMMUNITIES IN AQUATIC HABITATS

A. INTRODUCTION

There are a number of methods for determining characteristics and capabilities of natural aquatic microbial populations. Some are more qualitative, some fairly quantitative. Certain methods examine total numbers/biomass of microbes; others examine viability on an individual cell basis, while still others determine characteristics of a whole community such as rates of community metabolism, uptake of a certain substance, or community genetic relatedness. These measures are often correlated; however, they are distinct and cannot be used interchangeably.

Microbial populations vary greatly in number and diversity, often over small areas. This patchiness means that it is very difficult to extrapolate from one or few samples to an entire environment, though replication helps to give some idea of the variability present. Directly counting cells by epifluorescence microscopy is a fairly reliable method for getting at the total number of cells in an aquatic sample. However, with this approach, no information is gained about the viability of the cells present, and, without detailed visual analysis, no direct information about biomass is obtained. Viable cultural counts, by plating on nutrient-containing agar media, are a method for evaluating the concentration of viable/living and functioning cells. Unfortunately, this is a fairly restrictive technique and typically only 0.1 - 1 % of the cells in an environmental sample will grow in culture. Many environmental microbes may be unable to survive the plating process, while the media may select many others out, and still others may be dormant or grow at such a slow rate as to be invisible on the plates. Viable counts vary depending on the plate medium, with fewer numbers obtained for more restrictive media. Community heterotrophic activity may be estimated by the rate of uptake of ^{14}C-labeled substrate. This method will give the approximate rate of community respiration and substrate uptake, but does not quantify the number of cells that are active. Also, for environmental samples, the substrate used is different from the natural organic matter substrate, and so "heterotrophic activity" gives a measure of potential rather than actual metabolism.

Taken together, these three methods can tell us a great deal about the quantity and metabolic activities of a microbial community. We have spent the last few weeks in lab mastering these techniques, and learning the limitations and applicability of each. This week we will combine these measures to study the activities of the microbial communities in the pond and stream on the Pace Estate. We will also collect and preserve samples to analyze using molecular genetic techniques; the goal of this will be to compare microbial community structure at each site and to evaluate overall community similarity.

B. SCHEDULE OF ACTIVITIES

Week 1	Sample collection, spread plating, and heterotrophic activity * will preserve samples for AODC and molecular genetic analysis
Week 2	AODC and DNA extraction
Week 3	DNA quantification and PCR (RAPD) setup
Week 4	Agarose gel electrophoresis and analysis of molecular genetic data

C. COLLECTION OF SAMPLES

Replica water column samples will be collected from four different sites on the Pace Estate using 250 ml polyethylene sampling bottles. You should take note of the physical characteristics of each site (light availability, relative flow rate, organic matter availability, turbidity, etc.) and the approximate distances between sampling points. At each site, we will also measure pH, dissolved oxygen, and the temperature of the water.

D. DIRECT COUNTS AND VIABLE CULTURAL COUNTS

Total bacterial abundance will be estimated using acridine orange direct counts (preserve samples for counting next week). Viable counts will be determined using three separate agar media and the spread plate technique. Three types of media will be used, each differing in the availability of organic carbon; all media will contain sufficient trace nutrients (N, P, and S) for microbial growth. The agar-based media to be used are:

PTYG (peptone-tryptone-yeast extract-glucose)
R2A (contains no glucose, does contain dextrose, yeast-extract, peptone, and tryptone)
glucose medium (glucose is the only available carbon source)

For each sample, replicate spread plates should be prepared for each medium at 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions. This adds up to 8 plates per sample, per media type (or 24 plates per sample). Plates will then be incubated at 30°C and analyzed for colony forming units (CFUs) after 3 days.

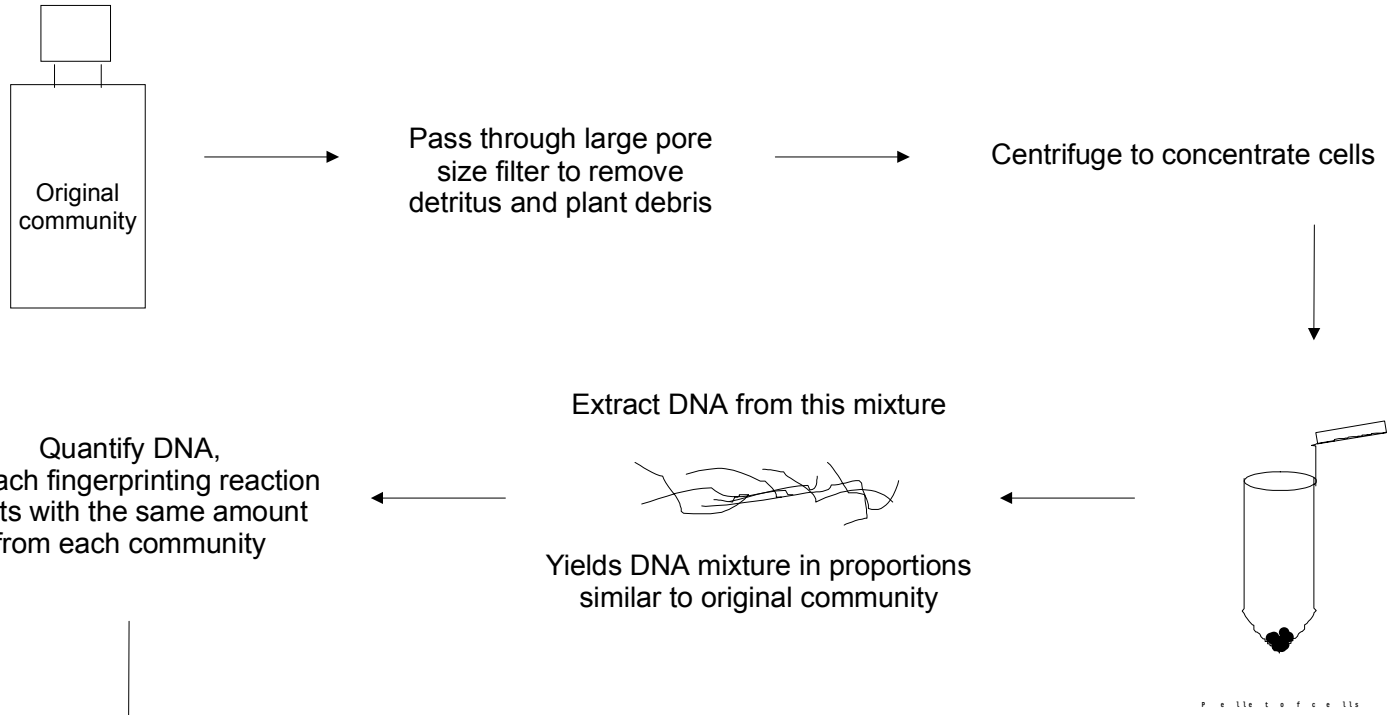
E. HETEROTROPHIC UPTAKE

For each sample, three flasks will be established as 10 ml incubations with 0.1 μCi of ^{14}C-glucose. One flask will serve as a killed control and the other two will serve as replicas for determining assimilation, respiration, and total uptake (using a 2 hour incubation period) as previously described.

F. DNA EXTRACTION AND COMMUNITY FINGERPRINTING

For each sample, approximately 200 ml of water will be centrifuged to concentrate out the microbial community for analysis. These samples will be stored in the freezer until DNA extraction and PCR can be performed. The steps involved with this DNA fingerprinting analysis are: (1) extracting DNA, (2) confirming DNA yield and quality with agarose gel electrophoresis, (3) PCR (RAPD) to generate the community fingerprints, and (4) agarose gel electrophoresis to visualize the community fingerprints.

SCHEMATIC OF DNA EXTRACTION AND COMMUNITY FINGERPRINTING



DATA ANALYSIS
COMPARING AQUATIC MICROBIAL COMMUNITIES

You are expected to write a full lab report for this exercise including: abstract, introduction, methods, results, discussion, bibliography. Also, make sure you include the data (preferably in the form of tables of averages and error estimates), an explanation of your calculations, and graphical representations of your results. In your methods section, you may be very brief. First describe the sampling locations. Then, rather than regurgitating the protocols given, simply tell the methods used (e.g., "Acridine orange direct counts were used to determine total microbial abundance (Hobbie et al. 1977). One milliliter samples were stained for each filter and five fields were counted per slide.") Elaborate more **only** if significant changes were made from the standard laboratory protocol.

A. CALCULATIONS

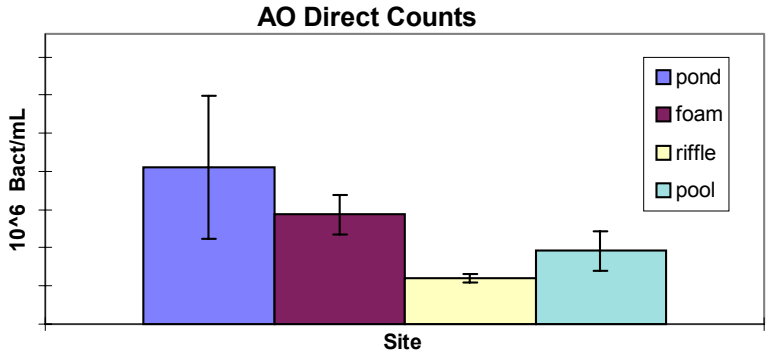
AODC, spread plate, and heterotrophic activity data should be analyzed using your previous lab handouts as a guide. For heterotrophic uptake, the H# and cpm will be reported – you should use the same quench curve as last week (the same regression line) to convert this information to dpm. Tabulate "^{14}glucose uptake" for each live sample. You should also report the "efficiency" for each sample. Efficiency can be calculated as:

$$\text{Efficiency} = \text{assimilation} / (\text{assimilation} + \text{mineralization})$$

and describes how "efficiently" the microbes were able to process the substrate into biomass.

B. GRAPHING

The first graphs you make should summarize the results. Plot each quantitative measure by site (plot the average and include error bars to show the variability in your replicas (see example below)). This means three graphs: AO abundance verses site, ^{14}glucose uptake verses site, and CFU (colony counts) verses site.



The next set of graphs should compare the plate counts with the direct counts. This means three graphs, one for each medium, plotting CFU verses abundance (AO). You should have eight points on your graph (one for each replica (× 2) for each site (× 4)).

The last set of graphs should compare CFU verses ^{14}glucose uptake. This can be done either with three different *types* of points one graph (each representing a different media), or three graphs.

C. Discussion

In your discussion and analysis, be creative, looking for meaningful relationships when comparing across methods and between sites. A few questions are listed below to help you get started. The ecology of the situation should be discussed thoroughly, and this should NOT be simply a comparison of methods. Be sure to discuss (in your introduction) what you expected to find. For example, you might anticipate that numbers and activity are would increase in the organic matter rich pond relative to the higher velocity stream…you might expect glucose uptake rate to be highly correlated with growth on glucose plates… you might expect community structure (DNA fingerprints) to be very similar among all of the stream samples, compared to the pond samples… and so on…. With regards to the DNA fingerprinting, simply discuss which communities appear more similar, and how this correlates to the results from other analyses; you are not expected to perform any calculations or statistical evaluation of that data.

Did ^{14}C glucose uptake differ across the sites and was this difference significant?

Did total abundance differ across sites?

What about the cultural counts? Within the cultural counts, were different numbers obtained depending upon the growth medium? What can you infer from these differences?

Looking across the different methods, were similar patterns observed (e.g. highest glucose uptake with the higher bacterial abundance)?

Is there any correlation between the direct counts and the cultural counts (plot AO vs. CFU)?

Overall, which sites were similar and did that follow what you expected? Why? How can you interpret the results you obtained in light of your site descriptions (e.g. morphological differences between sites, relative velocity of the water (which would influence the settling of organic material for microbial degradation), available sunlight…)?

5. DNA EXTRACTION FROM ENVIRONMENTAL SAMPLES

A. BACKGROUND

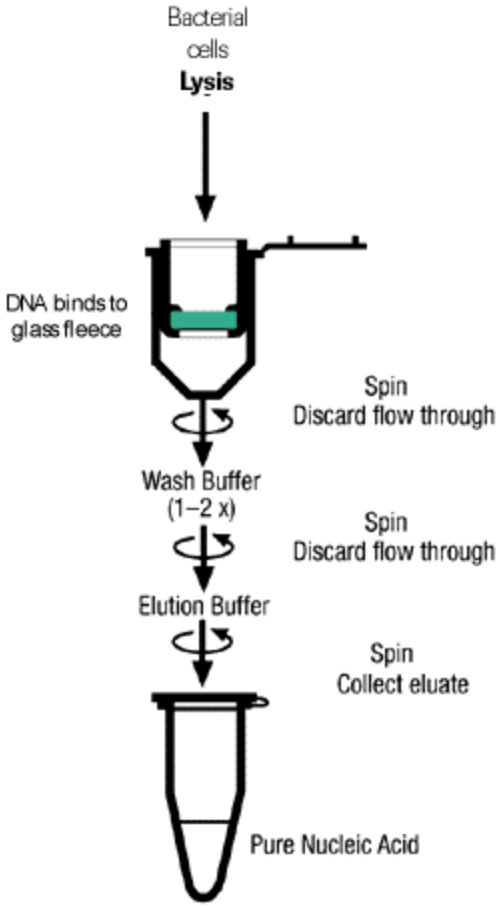
Preparation of nucleic acids is the first step in most molecular biology studies and all recombinant DNA techniques. The extraction of nucleic acids from biological material requires cell lysis, inactivation of cellular nucleases (a nuclease is an enzyme that can breakdown DNA or RNA), and separation of the desired nucleic acid from cellular debris. Conventional methods usually employ a lysis procedure that is rigorous enough to fragment the complex starting material (e.g., bacterial cell membranes) and inactivate nucleases, yet gentle enough to preserve the target nucleic acid.

Traditional methods for purifying DNA from cell extracts are often combinations of extraction, precipitation, chromatography, centrifugation, electrophoresis, and affinity separation. Most of these methods have the drawback that they require the extensive handling of toxic chemicals (e.g., phenol or ethidium bromide), need expensive equipment (e.g., ultracentrifuges), and are very time consuming. DNA extraction and purification from environmental samples are further complicated because it is often difficult to obtain enough material for analysis (e.g., it may be necessary to filter large quantities of water to gather enough bacterial cells) or it may be difficult to separate the organisms of interest from their environmental matrix (e.g., collecting fungi from soil or sediment). We will concentrate the microbial community from our water samples using centrifugation.

In our extraction procedure, we will depend on the tendency of DNA to adsorb to silica (glass) in the presence of a chaotropic salt. A chaotropic agent is a chemical that can disrupt the hydrogen bonding structure of water - in concentrated solutions, these compounds can denature proteins (examples include: sodium iodide, guanidine thiocyanate, or guanidine hydrochloride). This tendency was discovered by Vogelstein and Gillespie (1979) who found that DNA fragments adsorbed to powdered flint glass in the presence of saturated NaI. Later work showed other nucleic acids adsorbed to glass in the presence of other chaotropes. Different types of nucleic acids adsorb more or less tightly to glass depending on the ionic strength and the pH of

the surrounding solution. A low salt buffer or water can then be used to elute the nucleic acids from the glass.

For extracting the DNA from our samples, we will use a special plastic filter tube, with glass fiber fleece immobilized as the filter, to purify the DNA. Only the nucleic acids will adsorb to the glass filter, other molecules (e.g., proteins, sugars, lipids) will pass through. After washing away these impurities, we will release the DNA from the filter using a special buffer. The figure below shows a generalized example:



More specifically, the cells will be lysed during a short incubation with proteinase K in the presence of a chaotropic salt (guanidine HCl), which immediately inactivates all nucleases. Cellular nucleic acids will then bind selectively to the glass fiber fleece in the special centrifuge tube. The nucleic acids will remain bound while a series of rapid "wash-and-spin" steps is used to remove contaminating molecules. Finally, low salt elution will be used to remove the nucleic acids from the glass fiber fleece.

B. MATERIALS REQUIRED (Boehringer Mannheim's High Pure PCR Template Preparation Kit)

Binding Buffer with 6 M guanidine HCl

Proteinase K, lyophilized (dissolve in 4.5 ml double-distilled H_2O before use).

Wash Buffer (add 80 ml absolute ethanol to wash buffer before use).

Elution Buffer (10 mM Tris buffer, pH 8.5; 40 ml)

Filter Tubes

Collection Tubes

Absolute ethanol

PBS buffer (phosphate buffered saline)

Isopropanol

Lysozyme

C. PROCEDURE

1. Cell lysis and nuclease inactivation

(1) Collect sample by low speed centrifugation (3000 × g for 5 min). Remove supernatant and resuspend cell pellet in 200 µl PBS.

(2) And 5 µl of lysozyme (10 mg/ml in 10 mM Tris-HCl, pH 8.0) and incubate 15 minutes at 37° C.

(3) Add 200 µl binding buffer (green cap) and 40 µl proteinase K (reconstituted). Mix immediately and incubate at 72° C for 10 minutes.

(4) Add 100 µl of isopropanol and mix well.

(5) Pipette the sample into the upper reservoir of a combined Filter Tube-Collection assembly.

(6) Centrifuge for 1 minute at 8000 rpm in the tabletop centrifuge.

(7) Discard the flowthrough and collection tube.

2. "Wash and spin" steps to remove cellular debris

(8) Combine the filter tube with a new collection tube.

(9) Optional: Add 500 µl of Inhibitor Removal Buffer (black cap) to the upper reservoir. Centrifuge for 1 minute at 8000 rpm.

(10) Discard the flowthrough and collection tube.

(11) Combine the filter tube with a new collection tube.

(12) Add 500 µl of Wash Buffer (blue cap) to the upper reservoir. Centrifuge for 1 minute at 8000 rpm.

(13) Discard the flowthrough and collection tube.

(14) Combine the filter tube with a new collection tube.

(15) Add 500 µl of Wash Buffer (blue cap) to the upper reservoir (second wash). Centrifuge for 1 minute at 8000 rpm.

(16) Discard the flowthrough, recombine the filter tube and collection tube. Centrifuge for 10 seconds at max. speed (14,000 rpm) to remove residual Wash Buffer.

3. Elution of nucleic acids

(16) Discard the collection tube and insert the filter into a clean 1.5 ml reaction tube.

(17) Add 200 µl of prewarmed (70° C) Elution Buffer to the top of the filter tube. Centrifuge for 1 minute at 8000 rpm. The microcentrifuge tube now contains the eluted DNA. The DNA may then be stored in the freezer (-15° C to -25° C) for later analysis.

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6. AGAROSE GEL ELECTROPHORESIS

A. BACKGROUND

Gel electrophoresis is a method of separating chemical compounds and molecules by their size and charge. The substances being separated are placed in wells in an agarose gel and subjected to an electrical field. Negatively charged molecules move towards the positive anode, and positively charged molecules move towards the negative anode. Longer or larger molecules have difficulty traveling through the gel; they become entangled in the gel matrix. Shorter or smaller molecules migrate through the agarose matrix faster and thus travel farther in a given time period. Similarly sized fragments travel at relatively the same speed and form a tight "band" of material.

This week, we will introduce the concept of gel electrophoresis and use it to determine the quantity and quality of the DNA you just extracted. We will be analyzing the samples we extracted last week as well as a number of standards (of known DNA concentration). By comparing the brightness of the sample DNA with the standards, we will approximate the concentration of DNA in our samples. We will also use electrophoresis to visualize our fingerprinting results in a few weeks.

The activities are divided into three parts:

1. Preparing an agarose gel
2. Loading DNA samples and running an agarose gel
3. Staining the DNA in an agarose gel

B. PREPARING AN AGAROSE GEL

Different types and concentrations of media can be used to make a gel. The concentration and type of media will affect the gel's pore size and ability to separate similarly sized fragments. Agarose gels separate DNA fragments differing by a hundred or more base pairs, while polyacrylamide gels can separate DNA fragments differing by a single base pair. Well forming combs are inserted into the gel media as it cools and solidifies; after the gel solidifies, the combs

are removed. The gel will be placed into an electrophoresis chamber, covered with a buffer solution, and DNA samples will be loaded directly into the wells.

DNA gels are made of agarose, a highly purified agar, which is heated and dissolved in a buffer solution. The agarose molecules form a matrix with pores between them. The more concentrated the agarose, the smaller the pores. We will be using a 1% agarose gel (1 gram of agarose per 100 ml of buffer) because we are looking at large DNA fragments thousands of base pairs in length. A 2% agarose gel separates DNA fragments that differ in length by as few as 50-100 base pairs.

Before beginning, double check the size of the gel you are preparing. All volumes and weights are given for a 35 ml gel.

1. Weigh 0.35 grams of agarose powder and place it in a 125 ml flask.

2. Add 35 ml of 1X TBE (Tris-Borate-EDTA) buffer to the flask. Swirl to mix the solution.

3. Place the flask in the microwave. Heat on high until the solution is completely clear and no small floating particles are visible (about 2 minutes). Swirl the flask frequently to mix the solution and prevent the agarose from burning.

   * Do not allow the agarose to boil over.
   * Use hot mitts when handling the flask because it will be very hot.

4. Cool the solution to 55°C before pouring the gel into the plastic casting tray. Higher temperatures will warp (and destroy) the plastic tray.

5. While the mixture cools, assemble the gel tray. Place the plastic comb in the slots on the side of the gel tray. The comb teeth should not touch the bottom of the tray.

6. Pour the agarose mixture into the gel tray until the comb teeth are immersed about 6 mm or 1/4" into the agarose. Push any bubbles to the side farthest from the wells.

7. Allow the agarose gel to cool until solidified. The gel will appear a cloudy white color and will feel cool to the touch (about 20 minutes). Gels can be stored, wrapped in plastic wrap, in the refrigerator for a few days.

C. Loading an Agarose Gel

Prior to loading the gel, the DNA sample of interest must be mixed with a loading dye. The loading dye contains glycerol, which will make your DNA denser, so that it will sink into the wells. The dye molecules also provide a visual tracking method so you know how far the DNA (which is not visible) has traveled through the gel.

1. After the gel has cooled, remove the comb from the wells by pulling straight up on the comb. Be careful not to tear the wells as you remove the comb. Remove the rubber walls from both ends of the gel tray.

2. Place the gel tray in the gel box with the wells closest to the negative (black) electrode.

3. Add enough 1X TBE buffer to fill the buffer tank and submerge the gel about 1/4 inch.

4. On a piece of wax paper, for each of your samples, mix 2 μL of gel loading dye with 10 μL of your DNA solution. Mix the solution by pipetting the dye up and down into the 10 μL DNA sample.

   * Make sure that you record that order in which you load your samples.

5. Pipette the first sample, usually a reference sample such as a 1 kb ladder, into the first well. Keep the tip of the pipettor ABOVE the bottom of the well. The DNA will sink into the well because it has been mixed with loading dye. If you puncture the bottom of the well your DNA run out the bottom of the gel into the buffer tank. Continue until all of your samples have been loaded into the gel.

* Molecular biologists often use a size standard called a 1 kb DNA ladder. The DNA ladder produces several different sized fragments or bands and can be used to estimate the size of an unknown DNA fragment.

D. Running an Agarose Gel

The buffer solution contains ions that conduct electrical charges. The charges travel through the gel because it also contains ions (it was prepared with the same buffer). DNA is negatively charged and migrates toward the positive anode. The speed with which the DNA moves through the gel is determined by the size of the DNA fragment and the voltage. DNA will migrate faster at higher voltages, although lower voltages provide better resolution between similar sized fragments (the bands are less blurry).

1. Carefully close the top of the gel box. Plug the leads into the voltage supply. The black lead is the negative lead and should be plugged in closest to the wells. The red lead is the positive lead and should be plugged in furthest from the wells (the DNA will move from black to red)

2. Turn on the power source and run the gel at between 80-120 volts until the loading dye has traveled 1/2 - 3/4 of the way down the gel (about 30-45 minutes). You can check that everything is set up and running correctly by looking for bubbles coming from the electrode closest the wells.

3. After running the gel, turn off the power supply. Unplug the leads and the power supply before opening the gel box. If necessary, the gel may be wrapped in plastic wrap and stored overnight until it can be stained.

E. Staining the DNA in an Agarose Gel

Ethidium bromide is commonly used to stain agarose gels because it is highly sensitive to DNA and is visible under ultraviolet light. Ethidium bromide is a mutagen and a carcinogen so it should only be used with extreme care – gloves must be worn at all times. Other dyes are also used to stain DNA (e.g., Methylene blue); however they usually require the presence of higher quantities of DNA to be visible.

1. Place the gel in a plastic container that is a little larger than the gel itself. Add enough EtBr solution to cover the gel about 1/4". Stain the gel for 20-30 minutes. Gently rock the container every 10 minutes or so to move the dye across the gel.

2. Using a funnel, carefully pour off as much of the EtBr solution as possible back into the bottle (the EtBr may be reused several times).

3. Optional - Rinse the gel in running tap water. Let gel soak covered with water for 10 minutes. Rock the tray occasionally to help destain the gel. Repeat 3-4 times. The DNA bands will become more distinct as the gel destains. If necessary, you may continue to destain the gel overnight in a small amount of water; the gel will destain too much if left in a large amount of water. Cover the staining tray to prevent evaporation.

After staining, the DNA in the gel may be visualized using the UV light box (DO NOT LOOK AT THE UV LIGHT) and photographed using the Polaroid camera.

F. NOTES TO HELP WITH INTERPRETING THE RESULTS

A single band near the wells is characteristic of whole, uncut genomic DNA.

A smear of DNA down the lane represents large pieces of genomic DNA that are partially degraded (broken down) into thousands of smaller fragments or "cut" genomic DNA.

Discrete bands represent a group of DNA fragments that are similar in length.

Darker bands have more DNA.

No bands are present when the concentration is either very low or DNA is not present. This may occur if the well was punctured while loading the DNA.

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7. DNA FINGERPRINTING OF MICROBIAL COMMUNITIES

A. BACKGROUND

The abundance and diversity of microorganisms in the environment is enormous. As a result, it is not possible to get a complete sense of the relative numbers and identities of the constituent organisms in a microbial community, nor is it possible to thoroughly understand the function of each individual organism type, the specific interactions that may exist between populations, or the independent influences organisms may have on ecosystem processes. This inability to completely categorize the constituents of a community has hampered the efforts of microbial ecologists to investigate concepts such as microbial diversity and community similarity.

While macro-organism ecology typically sets out to examine what individual species do in an ecosystem, such an approach is impossible in microbial systems. Since it has been estimated that between only 1 and 5% of the microorganisms on earth have even been classified and named, approaches to community ecology based on individual species abundance and species composition are not reasonable. Microbial ecologists have attempted this, sampling, culturing, identifying, and enumerating what organisms they can in an area; however, it is well documented that cultural techniques are both selective and unrepresentative of the total microbial community. Current studies propose that only 0.1-1% of microorganisms in the environment can be cultured in the lab.

Understanding the full extent of the relationships within a microbial community, and between the community and its surroundings, requires a technique that allows scientists to evaluate attributes for the entire community without relying upon microbial growth for detection. This has lead to the development of several procedures that use whole-community samples for analysis of microbial community structure. Much of this research has focused on the analysis of whole-community DNA samples and several new molecular genetic approaches have recently emerged.

In particular, the use of PCR (see appendix 1 and 2) has revolutionized the study of microbial community ecology. PCR is an enzymatic reaction that allows repeated amplification of DNA *in vitro*, producing large amounts of DNA for analysis starting from as little as a single molecule. Replication is directed by using two oligonucleotide primers that hybridize to opposite strands of DNA and flank the target DNA region of interest.

PCR-based approaches to characterizing microbial communities can take two directions: [1] the use of specific primers to amplify exact portions of a populations' DNA or [2] arbitrarily primed PCR (AP-PCR) approaches that use small primers of random sequences to direct amplification. These shorter sequences can anneal at specific, "randomly" distributed sites within a DNA sample, producing a series of amplification products. The raw data from AP-PCR consist of a series of bands on an agarose gel, each corresponding to a certain size DNA fragment (amplified by the PCR).

In this project, RAPD (a type of AP-PCR) will be used to study microbial community structure. Randomly amplified polymorphic DNA (RAPD) is a specialized form of PCR, differing from normal PCR in that only one 10 bp primer, with a random sequence, is used. This single short primer can anneal specific spots, randomly distributed within a sample of DNA. Since the primer can anneal at several sites, it is possible to obtain several amplification products from a sample. Such fingerprints can be used to identify pure cultures of bacteria at the species or stain level. In this experiment, we will be using these fingerprints in a slightly different fashion – instead of comparing isolates, we will be comparing entire communities to see how different their fingerprints are. Communities with more similar fingerprints are, overall, more similar in community structure (distribution and identity of communities members).

The methodology for producing a DNA fingerprint is similar to that of normal PCR and involves three basic steps (denaturation, annealing, and extension). The materials for RAPD are the same as for normal PCR expect one 10 bp primer is used instead of a pair of longer primers. The RAPD technique was chosen for use in this study because of its ease and high resolution. RAPD is limited, however, in the amount of data obtained with each fingerprint. Usually, only 10 - 15 "bands" are produced per primer. This means that several different RAPD fingerprints (each generated using a different primer) are necessary if one wants to perform a statistical analysis of the data.

B. COMPONENTS OF A PCR REACTION

Taq DNA polymerase	The enzyme that replicates the DNA.
$MgCl_2$	Supplies the enzyme with a cation (Mg^{2+}) it requires to function.
dNTPs	Deoxyribonucleotides. It is a mixture that contains the A, T, G, and C that will be used to build the new DNA.
Primer	We will add one 10 bp primer to each reaction tube to direct the PCR. The sequence of this primer is (essentially) random and is not designed to match up with specific organisms, groups of organisms, or genes.
Template DNA	The target DNA we want to amplify. In our case, we will add an aliquot from the community DNA we extracted last week.
PCR Buffer	Provides the correct pH and ionic environment for the reaction.
Water	Enough water is added to the reaction to dilute all of the components to the correct concentration.

C. NOTES ON SETTING UP THE PCR REACTION

Contamination - PCR is very sensitive to contamination (any type of DNA (human, plant, bacterial, fungal) can be amplified using this procedure)), so gloves should be worn throughout the experiment. Similarly, pipette tips should be changed between each step. Be especially careful when using the class's reagents - try not to contaminate the stock solutions.

Temperature - Many of the components of PCR (in particular the enzyme and the dNTPs) are temperature sensitive. This means that they have a limited lifetime out of the freezer. Taq is especially sensitive, and should be kept in the freezer until right before use – and returned there immediately after.

Because of the temperature constraints, do all of your work on ice. Work quickly, but carefully. Make sure that you have done all of your calculations before you get out your reagents.

Mixing - It is important that all of the components in the PCR be well mixed (gentle vortexing is appropriate). HOWEVER, enzymes may lose their structure with this much agitation, so you should vortex mix all of the components except the Taq; then add the Taq and mix gently by tapping the tube with your finger.

D. RAPD RECIPE

Deionized water	12.3 µl
10X PCR buffer	2.5 µl
$MgCl_2$ (25 mM solution)	1.5 µl
Primer (2 uM stock)	2.5 µl
dNTP mixture *	1.0 µl
Taq (1 unit)	0.2 µl
DNA solution	5.0 µl
	25.0 µl total volume

*the dNTP mixture contains all four nucleotides (A, T, G, and C), at a concentration of 2.5 mM each.

Mixing these reagents in such small volumes could lead to a great deal of variability between our PCR reactions. Instead of preparing a separate set of reagents for each sample we want to analyze, it is much more accurate (and convenient) to work with a "master mix." For example, suppose you wanted to fingerprint 10 communities, the only component that would differ in the RAPD reaction for each community would be the DNA solution. A master mix (without DNA) could be prepared by multiplying the reagent quantities above by 10 (perhaps by 12 so that there is enough extra for pipetting error), mixing well, and then dispensing 20 µl of it to each PCR tube. The DNA for each community can then be added separately.

E. THERMAL CYCLING PARAMETERS

After mixing the PCR reactions and the template DNA, the reaction is placed in a thermal cycler, which carefully controls the temperature in the tube allowing the PCR to take place. The thermal cycling parameters for RAPD are 45 cycles of: 1 minute at 94°C (denaturing), 1 minute at 36°C (annealing), and 2 minutes at 72°C (extension). This profile takes approximately 5 hours on the machine, after which time the samples may be stored in the freezer until analyzed by agarose gel electropheresis.

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APPENDIX 1
~~~ WHAT THE HECK IS PCR? ~~~

From: http://falcon.cc.ukans.edu/~jbrown/pcr.html

Polymerase chain reaction (PCR) is a technique that is used to amplify the number of copies of a specific region of DNA, in order to produce enough DNA to be adequately tested. This technique can be used to identify with a very high-probability, disease-causing viruses and/or bacteria, a deceased person, or a criminal suspect. In order to use PCR, one must already know the exact sequences which flank (lie on either side of) both ends of a given region of interest in DNA (may be a gene or any sequence). One need not know the DNA sequence in-between. The building-block sequences (nucleotide sequences) of many of the genes and flanking regions of genes of many different organisms are known. We also know that the DNA of different organisms is different (while some genes may be the same, or very similar among organisms, there will always be genes whose DNA sequences differ among different organisms - otherwise, would be the same organism (e.g., same virus, same bacterium, an identical twin; therefore, by identifying the genes which are different, and therefore unique, one can use this information to identify an organism).

A gene's building-block sequence is the precise order of appearance, one after the other, of 4 different components (deoxyribonucleotides) within a stretch of DNA (deoxyribonucleic acid). The 4 components are: Adenine, Thymidine, Cytosine, and Guanine, abbreviated as: A, T, C and G, respectively (a 4-letter alphabet). The arrangement of the letters (one after the other) of this 4-letter alphabet generates a "sentence" (a gene sequence). The number of letters in the sentence may be relatively few, or relatively many, depending on the gene. If the sentence were 1000 letters-long, the sequence would be said to be 1 kilobase (1000 bases).

As an example:

ATATCGGGTTAACCCCGGTATGTACGCTA would represent part of one gene. DNA is double-stranded (except in some viruses), and the two strands pair with one another in a very precise way. EACH letter in a strand will pair with only one kind of letter across from it in the opposing strand: A ALWAYS pairs with T; and, C ALWAYS pairs with G across the two strands.

So:

TTAACGGGGCCCTTTAAA.....TTTAAACCCGGGTTT

Would pair with:

AATTGCCCCGGGAAATTT.....AAATTTGGGCCCAAA

Now, let's say that the above sequences "flank" (are on either end of..) the gene, which includes a long stretch of letters designated as: .....

These are known, absolutely identified to be, the sequence of letters which ONLY flank a particular region of a particular organism's DNA, and NO OTHER ORGANISM'S DNA. This region would be a target sequence for PCR.

The first step for PCR would be to synthesize "primers" of about 20 letters-long, using each of the 4 letters, and a machine which can link the letters together in the order desired - this step is easily done, by adding one letter-at-a-time to the machine (DNA synthesizer). In this example, the primers we wish to make will be exactly the same as the flanking sequences shown above. We make ONE primer exactly like the lower left-hand sequence, and ONE primer exactly like the upper right-hand sequence, to generate:

TTAACGGGGCCCTTTAAA.....TTTAAACCCGGGTTT
AATTGCCCCGGGAAATTT.....>

and:

<.....*TTTAAACCCGGGTTT*
AATTGCCCCGGGAAATTT.....AAATTTGGGCCCAAA

Now, the ..... may be a very long set of letters in-between; doesn't matter. If you look at this arrangement, you can see that if the lower left-hand primer sequence (italics) paired to the upper strand could be extended to the right in the direction of the arrow, and the upper right-hand sequence paired to the lower strand could be extended to the left in the direction of the arrow (remembering that the ..... also represent letters, and opposite pairing will ALWAYS be A to T and C to G), one could successfully exactly duplicate the original gene's entire sequence. Now there would be four strands, where originally there were only two. If one leaves everything in there, and repeats the procedure, now there will be eight strands, do again - now 16, etc. therefore, about 20 cycles will theoretically produce approximately one million copies of the original sequences (2 raised to the 20th power).

Thus, with this amplification potential, there is enough DNA in one-tenth of one-millionth of a liter (0.1 microliter) of human saliva (contains a small number of shed epithelial cells), to use the PCR system to identify a genetic sequence as having come from a human being! Consequently, only a very tiny amount of an organism's DNA need be available originally. Enough DNA is present in an insect trapped within 80 million year-old amber (fossilized pine resin) to amplify by this technique! Scientists have used primers that represent present-day insect's DNA, to do these amplifications.

Here is how PCR is performed:

First step: unknown DNA is heated, which causes the paired strands to separate (single strands now accessible to primers).

Second step: add large excess of primers relative to the amount of DNA being amplified, and cool the reaction mixture to allow double-strands to form again (because of the large excess of primers, the two strands will always bind to the primers, instead of with each other).

Third step: to a mixture of all 4 individual letters (deoxyribonucleotides), add an enzyme which can "read" the opposing strand's "sentence" and extend the primer's "sentence" by "hooking" letters together in the order in which they pair across from one another - A:T and C:G. This

particular enzyme is called a DNA polymerase (because makes DNA polymers). One such enzyme used in PCR is called Taq polymerase (originally isolated from a bacterium that can live in hot springs - therefore, can withstand the high temperature necessary for DNA-strand separation, and can be left in the reaction). Now, we have the enzyme synthesizing new DNA in opposite directions - BUT ONLY THIS PARTICULAR REGION OF DNA.

After one cycle, add more primers, add 4-letter mixture, and repeat the cycle. The primers will bind to the "old" sequences as well as to the newly synthesized sequences. The enzyme will again extend primer sentences ... Finally, there will be PLENTY of DNA - and ALL OF IT will be copies of just this particular region. Therefore, by using different primers that represent flanking regions of different genes of various organisms in SEPARATE experiments, one can determine if in fact, any DNA has been amplified. If it has not, then the primers did not bind to the DNA of the sample, and it is therefore highly unlikely that the DNA of an organism that a given set of primers represents, is present. On the other hand, appearance of DNA by PCR will allow precise identification of the source of the amplified material.

APPENDIX 2
POLYMERASE CHAIN REACTION - XEROXING DNA

National Center for Human Genome Research, National Institutes of Health. "New Tools for Tomorrow's Health Research." Bethesda, MD: Dept. of Health and Human Services, 1992.
http://www.accessexcellence.org/AB/IE/PCR_Xeroxing_DNA.html

Who would have thought a bacterium hanging out in a hot spring in Yellowstone National Park would spark a revolutionary new laboratory technique? The polymerase chain reaction, now widely used in research laboratories and doctor's offices, relies on the ability of DNA-copying enzymes to remain stable at high temperatures. No problem for Thermus aquaticus, the sultry bacterium from Yellowstone that now helps scientists roduce millions of copies of a single DNA segment in a matter of hours.

In nature, most organisms copy their DNA in the same way. The PCR mimics this process, only it does it in a test tube. When any cell divides, enzymes called polymerases make a copy of all the DNA in each chromosome. The first step in this process is to "unzip" the two DNA chains of the double helix. As the two strands separate, DNA polymerase makes a copy using each strand as a template.

The four nucleotide bases, the building blocks of every piece of DNA, are represented by the letters A, C, G, and T, which stand for their chemical names: adenine, cytosine, guanine, and thymine. The A on one strand always pairs with the T on the other, whereas C always pairs with G. The two strands are said to be complementary to each other.

To copy DNA, polymerase requires two other components: a supply of the four nucleotide bases and something called a primer. DNA polymerases, whether from humans, bacteria, or viruses, cannot copy a chain of DNA without a short sequence of nucleotides to "prime" the process, or get it started. So the cell has another enzyme called a primase that actually makes the first few nucleotides of the copy. This stretch of DNA is called a primer. Once the primer is made, the polymerase can take over making the rest of the new chain.

A PCR vial contains all the necessary components for DNA duplication: a piece of DNA, large quantities of the four nucleotides, large quantities of the primer sequence, and DNA

polymerase. The polymerase is the Taq polymerase, named for Thermus aquaticus, from which it was isolated.

The three parts of the polymerase chain reaction are carried out in the same vial, but at different temperatures. The first part of the process separates the two DNA chains in the double helix. This is done simply by heating the vial to 90-95 degrees centigrade (about 165 degrees Fahrenheit) for 30 seconds.

But the primers cannot bind to the DNA strands at such a high temperature, so the vial is cooled to 55 degrees C (about 100 degrees F). At this temperature, the primers bind or "anneal" to the ends of the DNA strands. This takes about 20 seconds.

The final step of the reaction is to make a complete copy of the templates. Since the Taq polymerase works best at around 75 degrees C (the temperature of the hot springs where the bacterium was discovered), the temperature of the vial is raised.

The Taq polymerase begins adding nucleotides to the primer and eventually makes a complementary copy of the template. If the template contains an A nucleotide, the enzyme adds on a T nucleotide to the primer. If the template contains a G, it adds a C to the new chain, and so on to the end of the DNA strand. This completes one PCR cycle.

The three steps in the polymerase chain reaction - the separation of the strands, annealing the primer to the template, and the synthesis of new strands - take less than two minutes. Each is carried out in the same vial. At the end of a cycle, each piece of DNA in the vial has been duplicated.

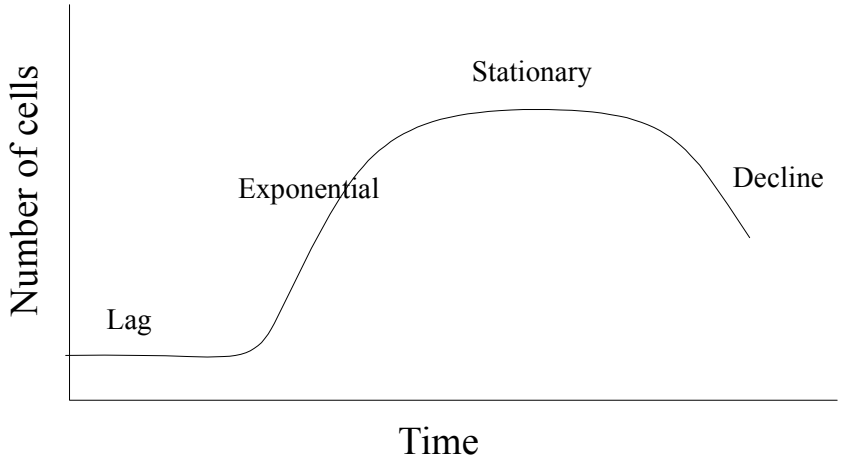
But the cycle can be repeated 30 or more times. Each newly synthesized DNA piece can act as a new template, so after 30 cycles, 1 billion copies of a single piece of DNA can be produced! Taking into account the time it takes to change the temperature of the reaction vial, 1 million copies can be ready in about three hours.

8. BACTERIAL GROWTH CURVE MEASUREMENTS

A. INTRODUCTION

Bacteria are capable of very rapid growth. Since they usually reproduce by binary fission, two become four become eight become sixteen and so on. The generation or doubling time can be as little as twenty minutes. Thus, if growth continues without check, they will rapidly cover the earth. This of course does not happen, with bacteria or with any other organism. Natural checks on numbers exist. Something slows growth as population density rises, until the number of beasts no longer increases. The limiting " something " may be competition for food, secretion of a toxic compound, injury of individuals by others, cannibalism, or something else. This is an active area of ecological research, and examples can be found of each of these limiting conditions.

When a tube of sterile liquid medium is inoculated with bacteria, the numbers of bacteria in the tube change in a predictable way over time. At first, the numbers do not change much, increasing slightly, if at all. This lag phase is followed by a period during which abundance increases exponentially. Then, for a period of time, the abundance levels off (stationary phase) until the population in the tube eventually declines in size. These phases of population growth are often represented graphically as below.



The **lag phase** is a time when cells increase in size and when the amounts of intracellular macromolecules increase. Inducible enzyme synthesis is expected during this phase. This phase is skipped if cells actively growing in a particular medium are used to inoculate a second culture in the same type of medium (why?).

The **exponential phase** is a time of balanced growth, meaning that cell components are produced at rates such that their relative proportions do not change. The cells divide at a constant rate which depends upon both the organism and the environmental conditions. It is this phase of growth used in calculations of a culture's maximal growth rate. The **stationary phase** is a time when the number of cells remains roughly constant as the rates of production and loss are equal. Its length varies. The **decline phase** is when cell numbers decrease. In a closed system it may result from exhaustion of nutrients, accumulation of waste, or by other less simply defined mechanisms. In this exercise we will record the growth curves for liquid medium cultures of several bacteria of environmental origin. We will also calculate their maximal rates of growth during exponential phase.

B. GROWTH MEASUREMENT

Within limits, the light absorbed or reflected by a bacterial suspension is proportional to the concentration of cells in the culture. Thus, we can use absorbance measured with a spectrophotometer as a measure of cell concentration. Note that this will yield relative numbers only. This is enough for calculating a generation or doubling time, however, calculating the growth rate in # of cells per unit of time would require correlating absorbance values with bacterial counts (made at the same time).

Two different types of bacteria will be used in this experiment; each will be grown on several different media types, varying in glucose concentration. Absorbance measurements will be made until the cultures reach stationary phase. This will take about three days, so we will take turns and be willing to lose data by not sampling at night. We will use a spectrophotometer to measure absorbance values. Its use entails:

1) Turn on and let warm × 30 minutes.
2) Set wavelength.

3) Insert sterile medium blank and set the zero absorbance.
4) Insert sample and read absorbance.
5) Re-blank intermittently.

Specific instructions and data sheets will be placed next to the spectrophotometer, as will standard and semi-log graph paper. Our aim will be to record and graph the data points as they are measured.

C. DOUBLING TIME CALCULATION

The maximal growth rate for each bacterium / broth combination can be determined from our data. This can be done most easily by inspection by using the semi-log plot. The time required for the, absorbance value to double during the exponential growth phase is the doubling time. This can be determined more precisely from the equation developed below:

Let: m_1 = amount of bacteria at time t_1

m_2 = amount of bacteria at time t_2

g = doubling time

n = # of cell divisions in time $t_2 - t_1$

t = time

With reproduction by binary fission,

$$m_2 = m_1 \times 2^n \quad \text{and} \quad g = (t_2 - t_1)/n$$

so $m_2 = m_1 \times 2^{(t2 - t1)/g}$

so $\ln m_2 = \ln m_1 + (t_2 - t_1)/g \times \ln 2$

$(\ln m_2 - \ln m_1)/\ln 2 = (t_2 - t_1)/g$

$g = (\ln 2 \times (t_2 - t_1))/(\ln m_2 - \ln m_1)$

We can insert absorbance values for m_1 and m_2 and solve for the doubling time.

*Note that this calculation yields only the maximal growth rate under the conditions specified. This tells us precious little about the behavior of the beasts in nature. We will discuss in class the limitations of this approach. We will also touch upon the differences between exponential and logistic models of population growth.

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APPENDIX 1
DERIVATION OF THE UNIVERSAL GROWTH EQUATION

DATA ANALYSIS – GROWTH CURVES

In this experiment, we monitored the growth of a two different microorganisms to see how it varied with different carbon substrate concentrations. Bacteria were grown in a minimal liquid media (which provided nitrogen, phosphorus, and other such nutrients) with varying amounts of glucose added. The four treatments included:

0, 0.1, 1, 10 g glucose added per liter

Absorbance (at 590 nm) was used as a proxy for cell abundance.

Plotting the growth curve:

Conceptually, describe the four stages of growth and briefly outline what is occurring in each. Then, plot your own growth curves as:

1. absorbance verses cumulative time
2. ln absorbance verses cumulative time (plot #1 but on a semilog axis)

for each organism at each concentration (total = 16 graphs!). Alternately, you may put both organisms on one graph for a total of eight.

From a visual inspection of these plots, summarize (in table form) the approximate length of the lag phase, the exponential phase, and the stationary phase for each organism/[glucose] combination. Additionally, report the maximum absorbance reached. Then discuss:

> *Did you observe a lag phase in any of the treatments? What does the lag phase represent? How can you explain any difference between treatments and/or organisms?*
>
> *How did the length of the exponential phase change between treatments/organisms and on what might this depend?*

How did the maximum absorbance differ between treatments/organisms? What about the length of the stationary phase? Did this differ between glucose treatments? between organism types?

Did any of the populations decline and, if so, what might have caused this?

What things might be limiting growth in our experimental system? Are these the same things that would limit growth in nature?

Determining μ:

Select the time period during which exponential growth seems to be occurring. **We will be using this subset of data,** and the universal growth equation (which only applies during this time), to determine the growth rate constant (μ) and the generation time of the organisms.

Recall equation (8) from the U.G.E. derivation:

$$\mu t = \ln M - \ln M_0 \qquad (8)$$

Rearranging the equation in y = mx + b form:

$$\ln M = \mu t + \ln M_0 \qquad (8a)$$

This means that if you plot ln M (in our case ln (absorbance)) verses t, the slope of the line is μ. Perform a linear regression on these points and report the μ and R^2 for each [glucose]/organism combination.

What does μ represent? Why might μ differ between the different carbon treatments? between the different organisms?

Generate a Monod plot:

We anticipate that the rate at which a population grows may depend upon the amount of substrate available. When nutrients are very scarce, the populations will grow very slowly and have a long generation time. When nutrients are more abundant, the growth rate may be much faster and the doubling time much shorter.

Make a Monod plot for each organism to show how it responded to the changing availability of glucose. Plot μ verses [substrate]. Can you estimate μ_{max} from this graph? What does μ_{max} represent and how does it change between organisms?

Determining generation time (= doubling time):

Starting with the universal growth equation we know:

$$M = M_o e^{\mu t}$$

However, for "doubling time" we are looking to see at what time the mass (M) will be double the initial mass (M_0).

$$M = 2M_o$$

Substituting into U.G.E. yields:

$$2M_o = M_0 e^{\mu t}$$

$$2 = e^{\mu t}$$

$$\ln 2 = \mu t$$

Since we are looking for "generation time," (g) the equation can also be written as:

$$g = (\ln 2)/\mu$$

Use this equation to determine the generation time for each organism in each media.
Discuss what “generation time” or “doubling time” means. On what might it depend in nature? How did it vary in our experiment?

Conclude your report with a brief discussion of what growth curves such as ours (batch cultures in highly manipulated laboratory environments) can actually tell us about what is happening in the environment

9. GROWTH OF MICROORGANISMS AND PRODUCT FORMATION (THE BEER LAB)

A. BACKGROUND

ATP-producing processes in which organic compounds serve as both the terminal electron acceptor (TEA) and the electron donor are called fermentations. The reaction pathway known as glycolysis is central to most fermentations. Glycolysis is the process, diagrammed in Figure 1, by which glucose is converted to pyruvate. Enzymes which catalyse this sequence of reactions are listed in Table 1.

The pyruvate produced by glycolysis has many potential fates (Figure 2). In aerobic organisms, pyruvate is processed through respiration to CO_2. In anaerobic processes, or under oxygen-deprived conditions in aerobic organisms, pyruvate is converted into lactate (cause of sore muscles in humans), or ethanol and CO_2. Since ethanol and lactate are organic compounds serving as TEA, both of the latter processes are fermentations. A well-known application of the process of alcoholic fermentation is the brewing of alcoholic beverages (see also Figure 2 for other bacterial fermentation pathways).

In the brewing of beer, yeast (single-celled fungi) ferment sugars furnished by the hydrolysis of polysaccharides present in grain (malted barley). The yeast used for beer can be of two types, *Saccharomyces cerevisiae* (top fermenting, used in the production of ales) or *Saccharomyces uvarum* (bottom fermenting, used for making lagers). *Saccharomyces* are very efficient at alcoholic fermentation of sugars, but are facultative aerobes. In the presence of oxygen, they use respiration to extract as much energy as possible from pyruvate. In the absence of O_2, they perform the less energetically lucrative fermentation process and convert pyruvate to ethanol and CO_2.

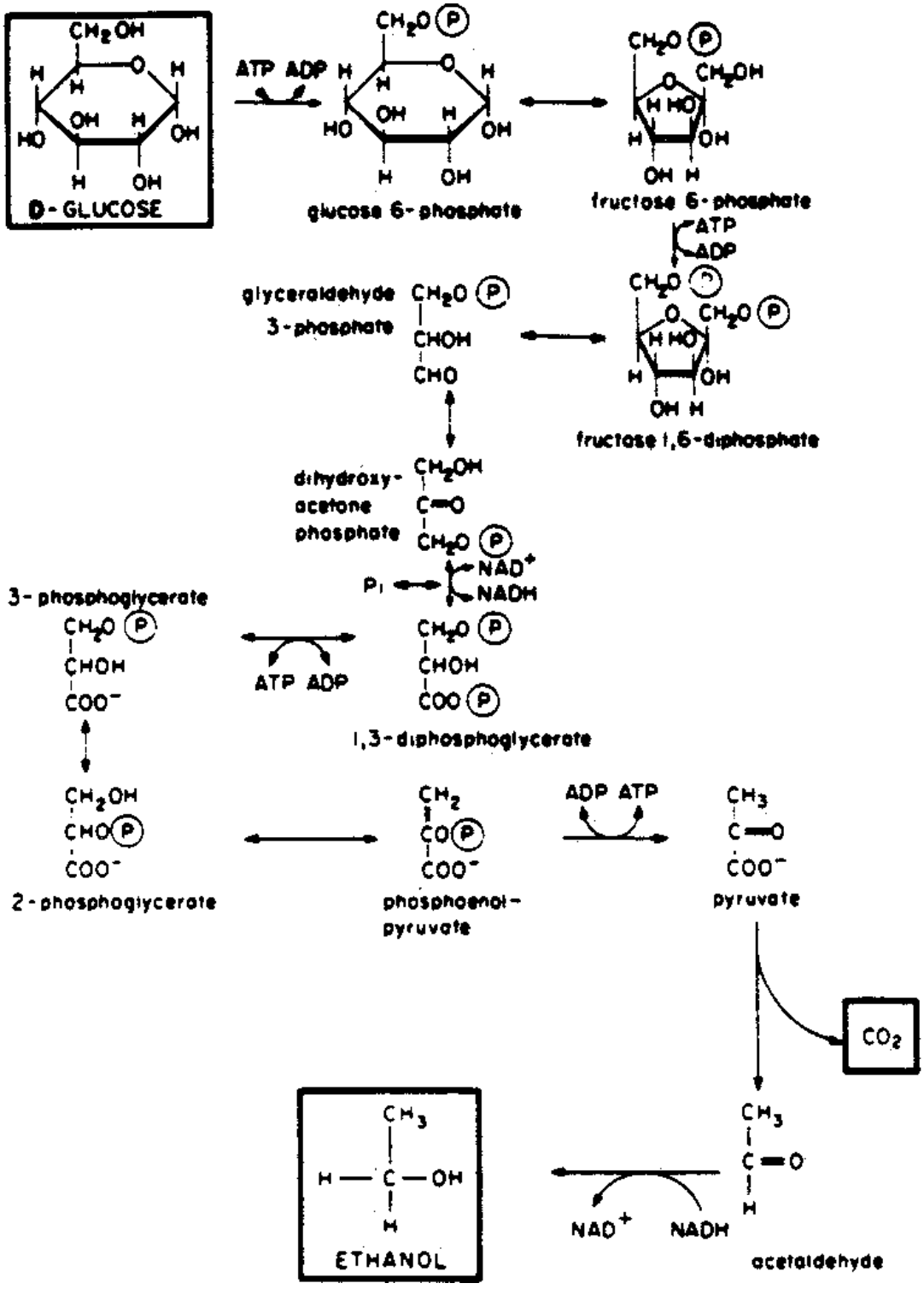

Figure 1. The glycolytic pathway.

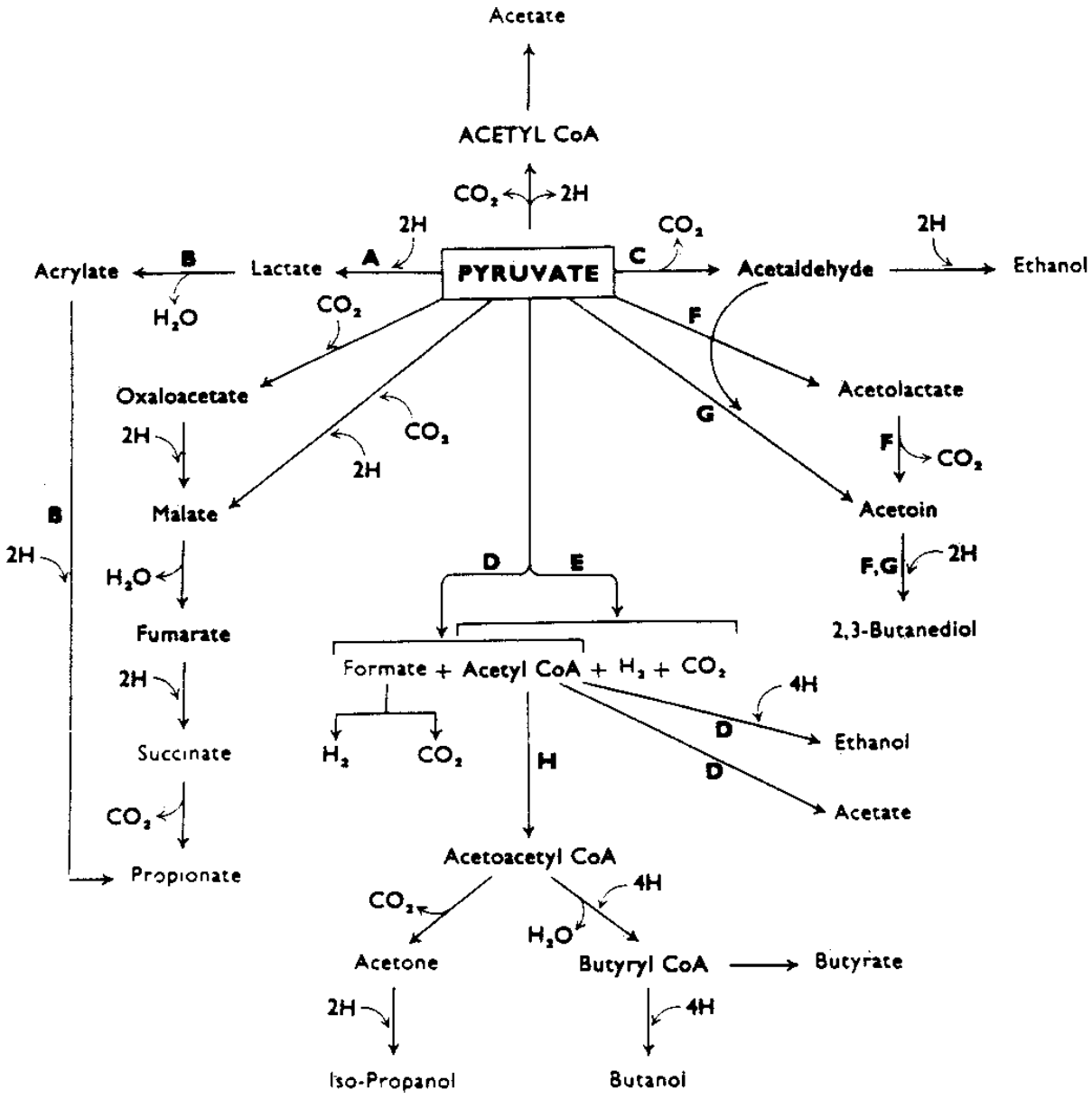

Figure 2. Bacterial fermentation products of pyruvate.

Table 1. Enzymes involved in the catalytic conversion of glucose to pyruvate.

Reaction Number	Reaction catalyzed	Name of enzyme involved	Cofactors
1	Glucose + ATP → glucose 6-phosphate + ADP	Hexokinase	Mg^{2+}
2	Glucose 6-phosphate ⇆ fructose 6-phosphate	Glucosephosphate isomerase	
3	Fructose 6-phosphate + ATP → fructose 1, 6-diphosphate + ADP	Phosphofructokinase	Mg^{2+}
4	Fructose 1,6-diphosphate ⇆ glyceraldehydes 3-phosphate + dihydroxyacetone phosphate	Aldolase	$Zn^{1=}$ (enzyme-bound)
5	Dihydroxyacetone phosphate ⇆ glyceraldehyde 3-phosphate	Triosephosphate isomerase	
6	glyceraldehyde 3-phosphate + NAD^+ + Pi ⇆ 1, 3-diphosphoglycerate + NADH	Glyceraldehyde 3-phosphate dehydrogenase	
7	1, 3-Diphosphoglycerate + ADP ⇆ 3-phosphoglycerate + ATP	Phosphoglycerate kinase	Mg^{2+}
8	3-Phosphoglycerate ⇆ 2-phosphoglycerate	Phosphoglycerate mutase	2, 3-diphosphoglycerate
9	2-Phosphoglycerate ⇆ phosphoenolpyruvate + H_2O	Enolase	Mg^{2+}
10	Phosphoenolypyruvate + ADP → pyruvate + ATP	Pyruvate kinase	
11	Pyruvate → acetaldehyde + CO_2	Pyruvate decarboxylase	Thiamin pyrophosphate; Mg^{2+}
12	Acetaldehyde + NADH ⇆ ethanol + NAD^+	Alcohol dehydrogenase	Zn^{1+} (enzyme-bound)

During the primary fermentation of beer, yeast go through three phases of growth. While there is O_2 available, the yeast grow rapidly using respiration, which produces CO_2, but no alcohol. After O_2 is depleted, the yeast switch to alcoholic fermentation, which releases less energy for growth and produces a harmful byproduct - ethanol. Once they exhaust the sugar source, the yeast go dormant and settle to the bottom of the container. Primary fermentation of beer takes 4 to 7 days, and will be performed and monitored in this lab. The "ripening" of the beer, or secondary fermentation occurs after bottling. Additional sugar is added to the beer shortly before bottling, resulting ultimately in elevated alcohol levels and carbonation of the beverage. Secondary fermentation can take several weeks to months (depending on the recipe).

At the first week's lab meeting, the beer will be made and placed in the primary fermenter. Samples will be removed during the next 7 days for later analysis. Four variables will be monitored. The specific gravity of the beer solution will be measured because sugars in solution have a specific gravity greater than that of water and pure alcohol has a specific gravity less than that of water. This means that a decrease in the specific gravity of the solution should be observed as the yeast convert sugars to alcohol. The abundance and biomass of the yeast population, alcohol level, and glucose concentration will also be measured for each of the samples.

B. SCHEDULE

Week 1:	make beer, demonstrate sampling and set up sampling schedule.
Week 2:	strain and bottle beer. Perform yeast cell counts and filter samples preserved with formaldehyde for dry weight determination.
Week 3:	do glucose and ethanol assays on the samples preserved with phosphoric acid.

C. FERMENTATION LAB, WEEK 1

Recipe for European Ale

Ingredients:

3.3	lbs	Plain Light Malt Extract
2	lbs.	Spray Dried Malt Extract
1/3	oz	Irish Hops
1	oz	Bittering Hops
5	oz	priming sugar
1	package beer yeast	

Procedure:

Before starting all materials (fermentor, hydrometer, tubing…) must be sterilized by rinsing with 10% bleach and filtered deionized water.

1) Add two gallons (7.6 liters) to a large pot (12 quart capacity) and bring to boil. Remove from heat. Slowly pour in malt extract (3.3 lbs (one tin)) while stirring constantly. NOTE: Malt extract will pour easier if you first place the tin in a pan of boiling water.

2) Add the 2 lb. bag of dried malt, again stirring constantly.

3) Bring this mixture (called *wort*) to a slow boil. Boil for 35 minutes, stirring occasionally (watch closely as it may boil over). Add the 1 oz. of bittering hops and the 1/3 oz. of Irish Moss directly into the wort and allow to slow boil 15 more minutes.

4) Pour the wort into a sterilized 6.5 gallon fermenter.

5) Add 3 gallons (11.4 liters) of cold water. Snap on the sterile lid and fill the airlock halfway with water.

6) Let the wort cool to 70°F (= 21°C). To speed up the cooling process, fermentor may be placed in a tub of cold water.

7) When the temperature reaches 70°F, sterilize the hydrometer and take your starting gravity reading. Then sprinkle the package of yeast directly onto the work. Replace the lid.

8) Place fermentor in warm area (preferable 70 - 75°F). Within 12 to 36 hours, you should notice bubbles coming through the airlock. This is a sign of active fermentation during which yeast consume sugars, producing alcohol and carbon dioxide. This fermentation should continue for three to seven days (confirm that it is done by monitoring the specific gravity). At this point the beer is ready to be bottled.

9) Using the syphon package, transfer the beer from the fermenter to the bottling bucket. Heat approximately one pint of beer in a pan and dissolve into it the 5 oz. of priming sugar.

10) Once sugar is dissolved, add this mixture back into the bottling bucket and stir to disperse the sugar evenly. Use the syphon to fill the sterilized bottles to within 1" from the top.

11) Cap the bottles and store at 70 - 75°F (in the dark) for the next ten days. At this point the beer should be carbonated. For proper aging and flavor, store another 30 days.

Yield:	5 gallons
Starting gravity:	1.039 - 1.044
Final gravity:	1.005 - 1.010
Alcohol by volume:	4 - 5 %

Sampling procedure

1) Label 2 clean 60 ml plastic sample bottles and 2 clean vials with the appropriate sample designations (i.e. T = 3.5-1 and T = 3.5-2, etc.), written on the label tape.

2) Place the stopper with the fermentation lock on a paper towel, NOT the lab bench! Open the fermentor for as short a time as possible.

3) Using a 50 or 100 ml pipette, remove as much beer from the fermentor in order to fill the hydrometer tube. Wipe the bottom and sides of the stopper with 70% ethanol just before resealing the fermentor.

4) Read the specific gravity and record this on the log sheet, along with the date, time (both the time of day and the time elapsed since fermentation began), and the sample number.

5) Measure 70 ml of beer from the hydrometer tube into a graduated cylinder. Discard the remainder of the sample.

6) Remove 10 ml from the graduated cylinder with a **sterile** pipette and place into one of the properly marked, clean vials. Pour the remaining 60 ml of beer into the appropriately labeled sterile 60 ml sample bottle.

7) Preserve: 60 ml beer sample with 2 ml (37% formaldehyde)
10 ml beer sample with 2.5 ml of 10% H_3PO_4.

8) Open up the fermentor again and repeat steps 2) through 7).

9) Invert bottles gently to mix sample and preservative.

10) Rinse the large pipette (used for sample withdrawal from fermentor), the graduated cylinder and the hydrometer tube with DIW. Place all but the pipette back next to the fermentor in the Soils Lab. Leave the aluminum foil case and used pipette on the lab bench back in the Microbial Lab.

11) Place the sample bottles and vials in the laboratory refrigerator.

D. FERMENTATION LAB, WEEK 2

Bottling and secondary fermentation

Boil the appropriate amount of corn sugar (recipe) in water. Pour this into a sterilized >5 gallon bucket. Siphon beer from the fermentor into the bucket, leaving as much of the yeast sediment as possible in the fermentor. Siphon the beer/corn sugar mixture into clean, sterilized bottles, leaving about an inch of air space in each bottle. Cap bottles. Allow to age for several weeks.

Yeast enumeration, using a haemacytometer

1) Gently invert the sample bottle a few times to resuspend the settled yeast cells.

2) For the 0 - 20 hour and the 70 - 170 hour samples try an undiluted sample at first. If necessary, make a 10^{-1} dilution in PBS (phosphate buffer solution) in a test tube. For the other samples, start with a 10^{-1} dilution and increase the dilution if necessary. If your dilution is incorrect, make it stronger or weaker.

3) Rinse and dry the haemacytometer and its cover slip, being very careful not to scratch them.

4) Place the cover slip over the grid on haemacytometer. Fill the haemacytometer chamber using a Pasteur pipette. Be sure to keep track of the sample number.

5) Examine samples under 40× dry objective. Count five of the medium-sized (edged by a double line) squares (a total of 80 tiny squares). There is no need to record the number of yeast cells per square, just the cumulative count. Since the yeast cells were collected while they were budding, use your best judgment and be consistent in determining if a budding cell should be counted as a new individual (i.e., count as individual if the budding cell is half as large or larger than the mother cell).

6) Given that the medium-sized squares are 0.2 mm on a side, and that the chamber is 0.1 mm deep, calculate the number of cells per ml in each beer sample using the following expression:

$$\text{No. cells/ml} = \frac{C \times 1000\ mm^3}{L \times D \times W \times S} \times \text{dilution factor}$$

where: C = total # of cells counted
L = length of field (0.2 mm)
D = depth of chamber (0.1 mm)
W = width of field (0.2 mm)
S = # of fields counted

Determination of Yeast Dry Weight

1) Take aluminum folders containing glass fiber filters (type A/E) from the desiccator. Assign numbers to the filters and weigh the filters one at a time on the Mettler balance in the front room of the microbial lab. Record the numbers and their appropriate tare weights on the log sheet.

2) Gently invert the sample bottle containing 60 ml of beer sample a few times to resuspend settled yeast cells throughout the sample.

3) Assemble the filter apparatus.

4) Filter 15 or 20 ml of beer sample (as much as will filter) onto the appropriate glass fiber filter. Record the identity of the sample and the amount filtered next to the appropriate filter number and tare weight on the log sheet. Rinse the filter tower well with fDIW before filtering the next sample.

5) Return each filter to its appropriate aluminum folder and place in basket to be oven dried.

6) Repeat step 4) and 5) for all of the other beer sample.

7) The filters will be in a desiccator next to the Mettler in two days. Reweigh them on the same balance.

8) For each replicate calculate the dry weight of yeast cells per ml of beer. Average the two replicates.

E. FERMENTATION LAB, WEEK 3

Glucose analysis

Consider the following two reactions:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose-6-P} + \text{ADP} \qquad (1)$$

$$\text{Glucose-6-P} + \text{NAD} \xrightarrow{\text{G-6-PDH}} \text{6-Phosphogluconate} + \text{NADH} \qquad (2)$$

Reaction (1), the hexokinase-catalysed phosphorylation of glucose, is the first step of glycolysis as performed by the yeast during fermentation. Unlike glycolysis, in reaction (2), the glucose-6-phosphate is oxidized to 6-phosphogluconate in the presence of NAD by the catalyst glucose-6-phosphate dehydrogenase (G-6-PDH). During reaction (2), NAD is reduced to NADH in amounts equimolar to the quantities of converted glucose-6-phosphate. The increase in absorbance of light at 340 nm wavelength, which is due to the production of NADH, is directly proportional to the initial concentration of glucose in the sample.

1) Prepare 0.05 dilutions (0.5 ml of sample to 9.5 ml DIW) of each H_3PO_4-preserved beer sample. **Do not shake the bottles, you want as yeast-free a sample as possible**.

2) Zero the spectrophotometer with a DIW blank.

3) Run triplicates of rehydrated glucose reagent blank (NAD, ATP, hexokinase, and G-6-PDH enzyme).

4) Read and record at A_{340} for the triplicate blanks of glucose reagent.

5) For each replicate beer sample dilution, add 20 µl (20 µl = 0.020 ml) of sample to a test tube containing 3.0 ml glucose reagent. Incubate at room temperature for at least 5 minutes.

6) After the 5 minute incubation, read and record the A_{340} for the samples using clean, dry cuvettes.

7) Rinse and dry the cuvettes using DIW.

8) Calculate the glucose concentration as g of glucose per L beer and convert the answer to moles of glucose (you will need this conversion to answer one of the assigned problems). **Remember the initial dilution of the beer sample with phosphoric acid (10 ml of beer preserved in 2.5 ml H_3PO_4):**

$$\frac{\text{g glucose}}{\text{Liter beer}} = \frac{(dA)\,(V_t)\,(180.16\ \text{g/moles})}{(CF)\,(V_S)} \times \text{dilution factor}$$

where dA = change in absorbance (sample - glucose blank avg.)
V_t = total volume in test tube (ml)
$MW_{Glucose}$ = 180.16 g/moles
CF = millimolar absorptivity correction factor (L/mmol) = 6.22×10^3
V_S = sample volume (ml)

Ethanol analysis (using gas chromatography)

1) Using a 0.2 mm syringe filter, filter 5 ml of each phosphoric acid-preserved sample into appropriately labeled scintillation vials.

2) As a group, run a series of 2 ml injections of alcohol concentration standards (1, 2, 4, 6, 10%) into the gas chromatograph (GC). Time the peaks as they come off for each alcohol concentration in the standard.

3) Each individual will run several of the ethanol standards (make sure all standards get done at least twice) and several beer samples.

4) Construct a standard curve using the mean peak areas of the replicate standard measurements (integrator units). Construct a standard curve by plotting the EtOH% vs. Peak Area.

5) Calculate values for the ethanol concentrations of the beer samples using the standard curve. You will each need to construct your own EtOH standard curve and calculate the ethanol levels in each of the beer samples with the regression of this line.

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APPENDIX 2
THE HOMEBREW EXPERIENCE

From: http://www.brewguys.com/FirstBatch.htm

Homebrewing is an easy, fun, and rewarding hobby. It's great to be able to brew beer to your own tastes. In fact, with a little patience, you can not only duplicate your favorite brand, but develop your own style of beer.

As you explore The Home Brew Experience, you will learn more about ingredients, equipment and methods used in making beer.

Beer is made by extracting the essence from malted (germinated) grains such as barley. Hops are added to flavor and help preserve the beer. Then yeast is added. It is the yeast that converts the sugars from the grains into alcohol. This process must take place in a sealed container to prevent spoilage of the beer. It takes about 1-2 weeks. The beer is then aged for another 1-8 weeks before it is ready to drink.

Today it is easier than ever to do this right in your own kitchen. Modern equipment and ingredients make it simple enough so that anyone can make not just good, but great beer, better than most you can buy.

The basic steps are outlined below. You may have been told of other methods elsewhere, and certainly there are other methods that will work well for you, but these steps have always worked well for us.

1) The Equipment you need
2) The Ingredients you need
3) The Method you use

- Sanitizing
- Cooking
- Siphoning
- Conditioning
- Bottling
- Kegging
- Pouring

THE EQUIPMENT YOU NEED

Before you can brew great beer, you need the right equipment. We suggest that you start simple. The first thing you need is a brewpot. You may already have a pot that will do. It should hold at least three gallons. Next, you need a fermenter. For a typical five gallon batch, your fermenter should hold six gallons or more to allow space for a foam that forms during the vigorous fermentation. A glass carboy, or food grade plastic bucket is most commonly used. Your fermenter also needs an airlock to allow the CO2 to escape while keeping air out. A siphon is needed to transfer beer from the fermenter when it is ready, without mixing air into it. A bottling bucket, while not absolutely needed, makes the process much simpler. A bottling bucket is similar to a fermenter except that it has a spigot at the bottom to allow you to fill the bottles directly, making the process quicker and less messy. You also need a capper to seal your bottles. Bottles and caps or a keg are also needed to store and serve your beer.

THE INGREDIENTS YOU NEED

Beer is traditionally brewed from four ingredients: water, barley, hops, and yeast. Other ingredients used in some beers include fruit, honey, wheat, rice, and corn.

The Homebrewer may choose to simplify the process by using concentrates such as malt extracts to replace the grains; this is most likely what you will choose to do for your first batch. You have the choice of either using pre-determined recipe kits or you can mix and match different ingredients and be creative. Malt Extract is an essential ingredient in all homebrewed beers, except those that are brewed entirely from grain. It is made up of concentrated sugars (and other substances) yielded from mashed malted barley (and/or wheat). When reconstituted in your brewpot it is essentially the same as if you took the time to mash the grains yourself. The first decision a brewer must make when choosing malt extract depends on the type of beer they will be brewing. The types of extract available are: Light, Extra Light, Amber, Dark, Extra Dark, and Wheat.

The simplest method is to use syrups in the color you intend your final beer to be.

Brewers of Amber and Dark beers can choose whether to add color and flavor their beers solely from extracts or through the addition of flavor grains. After experimenting with a variety of styles and brands you will notice subtle differences that will allow you to more closely tailor your beer to your desired results. For instance, certain varieties may offer a fuller body while others will ferment more cleanly, it really depends on the characteristics you are looking for when you choose an extract.They include everything you need to make 5 gallons of authentic beer in the style of your choice. They range from American light to Irish Stout. Each kit's recipe includes easy to understand instructions written with the beginner in mind. And now on to your first batch!

THE METHODS YOU SHOULD FOLLOW

Once you have assembled the necessary equipment and ingredients you are ready to begin. I recommend that you set aside at least a couple of hours for the process.

SANITIZING

The first thing you need to do is to sanitize everything that will come in contact with your unfermented beer. To do this you need a sanitizer such as B-Brite or chlorine bleach. Immerse everything in a solution of 2 teaspoons (about 2 caps) of bleach to five gallons of water, or follow the directions that come with your sanitizer. It takes time for the sanitizer to do its job. For this reason you should allow you equipment to soak in the sanitizing solution for at least a 1/2 hour.

Next you must rinse everything to remove remaining sanitizer. Add 3 1/2 gallons of water to your fermenter and seal it with the fermenter's lid or a rubber stopper. This should be done as soon as possible before you begin cooking your wort.

COOKING

Add two gallons of cold water to your brew-pot and bring to a boil.

If you are using any flavor grains in your recipe, add them to the cold water and remove them at the first sign of boiling. The grains can be easily removed if they are first placed in a grain bag, alternately they may be strained out with a colander. If you are using dry malt it works better if it is added to cold water and then is brought to a boil. If it is added to boiling water it will tend to clump up and be difficult to work with.

ADDING MALT SYRUP

Once the water is boiling (make sure there is head room to put in all the ingredients), add your malt syrup or extract kit. If you are adding corn sugar add that too now. Bittering hops are necessary if you are using unhopped malt extract. They can improve the character of hopped extract and kits as well and need to boil for a long time in order to reap their full benefits. Hops act as a flavoring agent, as well as, aid in beer-foam head retention. They too should also be added now.

STIRRING THE WORT

Stir well until the extract is thoroughly dissolved.

You should now boil your wort for one hour, although this is not strictly necessary when using hopped extract or a extract kit, it will help to stabilize the flavor of your beer. A good rolling boil is considered ideal, but you must watch your brewpot carefully to prevent a boil over, as this can seriously affect the quality of your final brew, not to mention making a sticky mess on the top of your stove. Some brewers will add additional hops during the boil, particularly if they are making a hoppy brew.

A STARTER SHOWING SIGNS OF LIFE

To insure a quick starting, healthy fermentation, you should hydrate your yeast. This is done by adding a few tablespoons of your boiling wort to 1 cup of cool water, and then adding your yeast packet and covering. Within a short time, you should see your yeast come back to life. It should look something like the picture above.

ADDING FLAVOR HOPS

During the last 5 to 10 minutes of the boil the homebrewer may choose to add flavor hops. These hops add a different quality to the beer than the bittering hops you may have added earlier. They impart a flavor that can be spicy or flowery or herbal in nature, depending on the variety used. Crushed fruit such as cherries, raspberries or blueberries, etc., etc., etc. may also be added at this point. Additional hops may be added to brewpot at the end of the boil and steeped for 2 to 3 minutes. Again these hops serve a different purpose from those added earlier. Hops added at this time contribute to the pleasant aroma that is the mark of a fine brew.

After the boil, let the mixture cool a bit and carefully open your fermenter and add the boiled wort and place the lid or stopper on top. Make sure there is cool water already in a carboy if that is what you are using. **NOTE** ADDING ANY HOT LIQUID TO A CARBOY MIGHT SHATTER THE GLASS IF THAT LIQUID HASN'T BEEN COOLED ADEQUATELY. At this point you may want to remove a small amount of beer to and take a hydrometer reading, this will tell you the starting gravity of your beer. By comparing readings taken now and at bottling time, you can determine the alcohol content of your beer. More information on hydrometers and hydrometer readings is available in Brewing Finesse.

PITCHING THE YEAST

When the temperature has dropped to about 75 degrees F. add your yeast. Now assemble your airlock, fill with water, and insert it into the hole at the top of your fermenter.

ASSEMBLING AND INSERTING 3-PIECE AIRLOCK

In about 12 to 24 hours some signs of life should be present. Your airlock should be bubbling, and if you sniff the gas coming from the airlock it should smell yeasty or beerlike. If all is not well you can try kick-starting your beer by adding yeast nutrient and additional yeast. More troubleshooting information is available in Brewing Finesse.

After a week or so, (longer for strong beers) your fermentation should subside, this may occur in only one or two days, if so don't be concerned, your beer should still turn out fine. When the bubbles in your airlock appear only once a minute or their production seems to have stopped, your beer is ready to move to the next stage. If these signs are not apparent, a hydrometer may be useful in helping to determine whether your beer is ready, you do this by checking its specific gravity.

SIPHONING

When your beer is finished fermenting it is time to move it into another vessel, be it a carboy, bottles, or a keg. When transferring the beer it is important not to mix air into the beer, as this can cause it to have a stale flavor. Therefore, you must use a siphon to move your brew.

STARTING THE SIPHON

To start your siphon place the container holding your beer higher than the container you will be transferring your beer into.

Fill the siphon tubing with water hold it in a "U" shape and place one end in each container (make sure your hands are clean). As you bring the lower piece of hose down to the siphon bucket, the beer will be pulled through the tube.

Gravity will start the flow, and the beer will follow the water into the new vessel. If your siphon needs to be restarted, follow the same procedure. DO NOT use your mouth for suction, as this will most likely ruin your beer. At the bottom of your original container there may be a layer of sediment. This is mostly dead yeast. You should try to leave this behind by not allowing the suction end of the tubing into it, as it can add undesirable flavors to your beer.

CONDITIONING

You may choose now whether you want to bottle your beer or move it to a carboy for additional aging. Some beers such as those that are high in alcohol, very dark, or still cloudy will benefit from extended conditioning. Any beer that will be aged for more than a couple of weeks before bottling will need to be moved off of the yeast (called racking) that has settled at the bottom of the fermenter to prevent your beer from becoming yeast bitten

Before moving your beer into a carboy, you must sanitize the carboy and siphon first. Follow the same procedure you used to sanitize your fermenter.

Now siphon the beer into the sterilized carboy. Then seal the carboy with a stopper and add the airlock. Place the carboy in a cool, dark place until you are ready to bottle or keg it.

BOTTLING

You will need about 55-60 bottles and caps to bottle a 5 gallon batch. You may not need all of them but having a few extra bottles and caps is better than having extra home-brew and nothing to put it in. Bar bottles work well, and some unusually shaped bottles may not work with some capers.

SANITIZING BOTTLES

As with all stages in the brewing process, sanitation is extremely important. before using your bottles they, and everything else that will come in contact with your beer must be thoroughly sanitized (including the caps). Follow the steps outlined above.

In order for your beer to be carbonated you must use priming sugar, to do this:

PREPARING PRIMING SUGAR

Dissolve 3/4 cup corn sugar or 1 1/4 cup dry malt in 1 quart of water and bring it to a boil. Priming your mixture causes the carbonation and final pressurization needed in the bottles to take place.

BEER BEING SIPHONED

Siphon your beer into your bottling bucket following the steps above.

While the siphon is flowing, slowly add the boiling mixture to the bottling bucket.

FILLING A BOTTLE

Now you are ready to begin bottling. Fill each bottle, leaving about an inch of headroom. This head room allows room for the carbon dioxide to expand and create a proper seal.

CAPPING

When your bottles are filled, cap them and place them in a cool dark place for at least 10 days (longer for dark or strong beers)

Chill and Enjoy!

KEGGING

If you prefer, you may keg your beer instead of bottling it. Kegging does require additional equipment, but it offers added convenience over bottling.

You must, of course sanitize your keg and anything else that will come in contact with your beer. If you are using a plastic keg, then follow the sanitizing procedures outlined above. If you are using a metal keg you need to use iodine to sterilize your keg as bleach and other oxidizing sanitizers may react with the metal in your keg.

Prepare your priming sugar by dissolving it in water and bringing it to a boil.

Siphon your beer into the keg and add the dissolved sugar as you go.

Seal the keg and place it in a cool place for two weeks.

Tap and Enjoy!

POURING

If you bottled your beer then you will notice that there is some sediment at the bottom of each bottle. This is mostly spent yeast, and you should avoid mixing it into your beer as it will cloud it and add a bitter, yeasty flavor to your beer. Each step in racking reduces the amount of sediment in the bottles. Sometimes it is possible to virtually remove all the sediment. However, since sediment is common to homebrewed beverages, it is recommended that you should pour your beer slowly, in one smooth motion, stopping before you pour the yeasty dregs into your glass. You should also start pouring slowly, so that you can see how foamy your beer is and adjust to bring about the proper head. You can pour it slowly down the side of the glass for bubbly beers or down the middle of the glass if it is less bubbly. This may take a little practice, but the results are worth it. ©

DATA ANALYSIS – "THE BEER LAB"

This exercise requires a full lab write up, brief but in journal style format. A data file containing all the necessary information will be posted. Include (in the appropriate sections):

1. What did we do and why?

2. Briefly define: hops, malt, primary fermentation, secondary fermentation, facultative anaerobe

3. Make graphical representations of:
   (a) number of yeast verses time
   (b) dry weight of yeast verses time
   (c) concentration of glucose verses time
   (d) adjusted specific gravity verses time
   (e) EtOH conc. verses time (one calculated from specific gravity and another from the GC readings)

Adjusting Specific gravity: the scale used to measure specific gravity was calibrated for 60°F. Use the following information to correct for our measured temperatures:

Temperature (°F)	Correction
50	-0.0005
60	0
70	0.001
77	0.002
84	0.003
95	0.005

e.g., suppose we read 1.100 at 84°, using the correction factor of "0.003" for this temperature means the actual specific gravity was 1.100 + 0.003 = 1.103

Calculating Alcohol Content: First use the following scale to convert from our specific gravity readings to potential alcohol content.

Potential Alcohol:	Specific Gravity:
0	1
1	1.007
2	1.0145
3	1.022
4	1.029
5	1.036
6	1.044
7	1.051

Remember, these values represent "potential alcohol content". To determine the actual concentration you must subtract each reading from our initial reading.

For example:

1st reading	7%
2nd reading	4%
Alcohol content	3% at time 2

For the alcohol content at time 3, take (1st reading - 3rd reading) etc.

4. Explain the relative shapes of the curves graphed in #3. Do they follow your expectations? Justify why or why not. Can you draw any conclusions by looking across the different plots? Which, if either, of the two yeast measurements do you think is more reliable? Which of the two glucose estimates do you consider dependable?

5. What processes influence the specific gravity of the beer during primary fermentation? Why are brewers interested in monitoring it and how could the specific gravity be controlled?

6. Yeast cells are **not** obligate anaerobes. Given your knowledge about metabolic processes, discuss what may have occurred during sampling of the fermenting beer and how sampling might have affected the final product?

7. Based on the recipe we used to brew the beer and the following equation:

$$C_6H_{12}O_6\,(\text{glucose}) \longrightarrow 2\ CH_3CH_2OH\ (\text{ethanol}) + 2\ CO_2$$

Calculate the maximum amount (both in grams and liters) of CO_2 and ethanol produced during primary fermentation. Relate these theoretical calculations to those obtained analytically (i.e. compare potential alcohol content from the calculation with potential content as measured from the hydrometer, then compare these with our actual alcohol content).

Assume that one molecule of maltose equals two molecules of glucose. Further assume that the yeast ferment sugar with a 45% efficiency and that the malt inputs were all 80% fermentable sugars.

The molecular weights are: glucose = 180.16 g/mol, ethanol = 46.07 g/mol, carbon dioxide = 44.0 g/mol, and maltose = 342.3 g/mol. Additionally, the density of ethanol is 0.79 g/ml, the density of CO_2 is 0.00198 g/ml, and 1 gallon = 3.7588 L.

10. PUBLIC HEALTH MICROBIOLOGY

A. INTRODUCTION

Throughout history, humans have tended to contaminate their water supplies with excretory waste. This has caused health problems when contaminated water is imbibed by other people who are unfortunate enough to live downstream. We now know of many bacteria (as well as viruses and protozoa) that are the causal agents of waterborne diseases. Several enteric pathogens, which cause gastrointestinal disease when ingested, are *Salmonella* spp., *Shigella* spp., and enteropathogenic *Escherichia coli.* Microorganisms that cause water contamination problems in swimming pools, hot tubs, etc. are *Staphylococcus aureus* and *Pseudomonas aeruginosa*. For the purpose of this lab we will concern ourselves primarily with enteric pathogens.

Detection of water contamination caused by microbial vectors involves the principle of **indicator organisms**. This principle is motivated by the fact that the variety of water-contaminating microorganisms (protozoa, viruses, bacteria) is simply too great to allow screening for individual pathogenic microbes. It would be just too difficult and time-consuming to look for these microorganisms on an individual basis. Indicator organisms are the "surrogates" that are screened for instead. These organisms are other, both pathogenic and non-pathogenic microbes, that are also associated with the intestinal tract. Detection of indicator organisms in a water sample therefore strongly suggests that the water was contaminated with organisms of intestinal origin.

There are several objectives to keep in mind when choosing a good indicator organism:

1) The indicator organism must be more numerous and hardy than the pathogen.

2) It must always be associated with the material of interest.

3) It must be possible to detect low numbers that are in some kind of proportion to the substance being detected.

4) The organism must be easy to detect (morphologically and biochemically).

5) The organism must survive longer than the pathogen (i.e., there are many questions about the appropriate use of *E. coli* as indicator organisms in coastal and marine environments due to evidence that *E. coli* survival may be drastically shortened in saline waters).

6) The organism must not proliferate in the environment.

7) There must be no other source of association for the indicator organism.

The most widely used group of indicator organisms is the coliform group of organisms. For the purpose of water bacteriology, this group is defined as all the aerobic and facultative aerobic, Gram negative, non-sporeforming, rod-shaped bacteria that ferment lactose with gas formation within 48 hours at 35°C. Please keep in mind that this definition is operational, rather than taxonomic. Most, but not all, members of this functional group are of intestinal origin. Applied to the examination of water samples, however, it turns out that the coliform organisms are almost always members of the enteric bacterial group. It should also be pointed out that the coliform group includes organisms not only associated with human but also with other warm-blooded animal wastes (e.g., bovines, swine, etc.).

The coliform indicator organisms are conveniently subdivided into three functional groups: **total coliforms** (TC), **fecal coliforms** (FC), and **fecal streptococci** (FS). Did you know that 30% of the dry weight of human feces is composed of bacteria and that there are 10^{11} cells/gram of fecal material (this includes bacteria, fungi, protozoa, and viruses)?

1. Total coliforms (TC)

These organisms are the lactose-fermenting members of the family *Enterobacteriaceae*. They are widely distributed in nature and are native to the gut of warm-blooded animals. Their role in the gut is to aid in the digestion and synthesize vitamins. TC are used as an indicator of possible enteric pathogens such as viruses, protozoa, and bacteria. They are Gram negative rods measuring 2 mm by 0.5 mm, and ferment lactose with the production of acid and CO_2. Because they can ferment as well as respire, they are considered facultative anaerobes. Members of this group include *E. coli, Enterobacter, Klebsiella*, and *Serratia*. Many of these organisms are found associated with plants and soil.

2. Fecal coliforms (FC)

This group is functionally defined to include those TC that can withstand heat shock to 44.5°C and grow at that temperature. This group defines the level of warm-blooded animal fecal contamination of a water sample (only fecal coliforms from the intestines of warm-blooded animals can tolerate elevated temperatures). The FC test is used to evaluate the quality of recreational and shellfish-bed waters, where higher total coliform levels make a TC test less useful. FC make good indicator organisms as their die-off curve is similar to that of the pathogen *Salmonella typhimurium* (see Figure 1). Note also that the permissible numbers for FC are less than those for TC (Table 2).

3. Fecal streptococci (FS)

Organisms in this third group are Gram positive cocci and native to the guts of warm-blooded animals. They are generally considered non-pathogenic and include *Streptococcus fecalis, S. fecalis* var liquefaciens, *S. fecalis* var zymogenes, *S. durans*, *S. faecium, S. bovis*, and *S. equinus*. Their main utility is to indicate the source of pollution (human vs. livestock vs. poultry) by calculating the ratio between fecal coliforms and fecal strep (FC/FS). This ratio has been increasing in popularity as an indicator in stream pollution studies (see Table 3). FS are also used to indicate contamination in brackish and marine waters. In salt water, these bacteria die back at a slower rate than the FC, providing a more reliable indicator of possible recent pollution.

There are two types of procedures used to detect coliforms in water samples: the **most-probable-number** (MPN) method and the **membrane filter** (MF) procedure. We will use the MF procedure to detect contamination in the water samples you collected for this exercise. In this technique, the sample of water is passed through a sterile membrane filter (0.45 μm pore size) and the bacteria are trapped on the surface. The filter is then placed in a Petri dish containing an appropriate sterile culture medium for incubation. The culture medium is highly selective for coliform organisms that grow with distinct morphologies and colorations.

A note on sample collection: the source water should be between pH 4-9. Samples should be taken using a sterile container, stored in the cold, and analyzed within 6 hours of collection. The information given in the tables and figures for this exercise are national guidelines, local standards may vary.

B. PROCEDURE ***ASEPTIC TECHNIQUE IS NECESSARY FOR THIS EXERCISE**

1) Before you begin with the actual exercise, spend a minute consulting planning out the proper dilutions for your water sample for the determination of TC and FC. FS dilutions will be the same as for TC. Figure out how much sample you will need for all your plates (three replicates per dilution) and plan your dilution volumes accordingly.

2) Prepare appropriate dilutions, using sterile dilution blanks of the provided phosphate buffer solution (PBS).

Filter the samples according to the procedure below, filtering the most dilute sample first, and so on, filtering the most concentrated sample last.

3) Rinse the filter tower with 70% ethanol, then with sterile water.

4) Place a sterile Metricel GN-6 filter (0.45 μm) on the tower.

5) If the sample volume is less than 20 ml, add 10 ml of sterile PBS to the tower, pour in your sample and swirl gently to mix. This insures an even sample distribution on the filter.

6) Apply vacuum, then rinse the sides of the filter tower with sterile PBS.

7) Break the vacuum and remove the filter with flame-sterilized forceps and place in an appropriately labeled petri dish. **Please note that there are three different kinds of plates: TC, FC, and FS plates**.

8) Place the different plates in the appropriate basins. These plates will be incubated as follows: TC plates will be incubated at 35°C for 24 hours. FS plates will be incubated at 35°C for 48 hours. FC plates will be incubated at 44.5°C for 24 hours. FC plates must go into the 44.5°C incubator within 20 minutes of filtration, so that the heat shock will discourage growth of other coliforms on the plates.

9) Counting proceeds as for spread plates (one of the dilutions should be countable). FC and TC plates can be counted at 24 hours, FS plates at 48 hours. **Do not wait more than 48 hours to count any of the plates**.

10) The colonies you want to count as the different coliforms are:

TC - colonies are pink to dark red with golden green metallic sheen.
FC - blue colonies.
FS - pink to dark red colonies

11) Calculations: Coliform levels in water are expressed as cells per 100 ml. Calculate the number of indicator organisms per ml of sample as for the spread plate exercise and multiply by 100 for comparison with the standards in Table 2.

TABLE 1

Pathogenic Microorganisms Associated with Fecal Material

Microorganism	Disesase
Salmonella typhi	Typhoid fever
Salmonella paratyphi	Paratyphoid fever
Salmonella schottmulleri	Paratyphoid fever
Salmonella hirshfeldi	Paratyphoid fever
Salmonella dysentariae	Bacillary dysentery
Salmonella typhimurium	Gastroenteritis
Salmonella enteritis	Gastroenteritis
Shigella dysentariae	Bacillary dysentery
Shigella flexneri	Shigellosis
Shigella boydii	Shigellosis
Shigella sonnei	Shigellosis
Vibrio cholerae	Cholera
Leptospira icterohemorrhagiae	Leptospirosis
Entamoeba histolytica	Amoebic dysentery
Polioviruses	Poliomyelitis
Hepatitis A virus	Hepatitis

TABLE 2

Recommended Limits of Coliforms per 100 ml water (U.S. Public Health Service)

Type of Water	**Total Coliforms**		**Fecal Coliforms**	
	Desirable	Permissible	Desirable	Permissible
Potable	0	1	0	0
Primary contact (swimming)	< 1,000	< 2,400	< 200	< 1,000
Secondary contact (boating/fishing)	< 5,000	< 10,000	< 1,000	5,000
Treated sewage effluent	*Coliform levels should not exceed those of water receiving the discharge*			

TABLE 3

Number of Fecal Indicators per Gram of Feces

Animal	**FC** $\times 10^6$	**FS**	**FC/FS**
Man	13.00	3.00	4.4
Sheep	16.00	38.00	0.4
Cow	0.23	1.30	0.2
Poultry	0.29	2.80	0.1
Pig	3.30	84.00	0.04

Bureau of Water Hygiene, EPA Cincinnati, Ohio

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DATA ANALYSIS - PUBLIC HEALTH MICROBIOLOGY

First, calculate the coliform counts/100 ml of sample for each of the dilutions you ran for TC and FC. Calculate the FC/FS ratio. Discuss your results, and the "safety" of your water sample; be sure to answer the following questions:

1. In the grand scheme of things, what is the significance of a positive test result for total coliforms (TC)? What about for fecal coliforms (FC)?

2. Why would one test for FS and what information does that provide in addition to that afforded by FC counts?

3. From the sample data in the table provided, calculate the FC/FS ratios. Based on the results and the information in Table 3 of the lab handout, (a) match each site with the appropriate sample location and (b) discuss possible sources of fecal pollution for each contaminated sample.

Site	Sample	TC	FC	FS
A	1	0	0	3200
	2	0	0	2600
	3	0	0	3800
B	1	960	0	200
	2	840	0	100
	3	1040	0	300
C	1	17000	2400	8900
	2	22000	2500	10400
	3	37000	2100	12100

Sampling locations:

- stream near a cow pasture
- outdoor swimming pool (untreated since early fall)
- well from the Pace estate (historical contamination is expected)

11. MOLECULAR DETECTION OF PATHOGENS

12. FOOD MICROBIOLOGY

A. INTRODUCTION

Food spoilage caused by microorganisms is a topic of great concern for both consumers and public health agencies (you might recall the recent outbreak of enteric disease caused by a rare strain of *E.coli* associated with hamburger meat sold by a national fast food chain). Due to the omnipresence of microorganisms, it is virtually impossible to keep perishable foodstuffs free of microorganisms for prolonged periods of time. Throughout history, people have invented methods of food processing that allow us to eliminate, or temporarily control, microorganisms in order to increase the shelf life of various foods. The pasteurization of milk is one such preservation method. In this exercise, the effects of pasteurization on bacteria present in the milk will be demonstrated by estimating the concentrations of microorganisms associated with both raw, untreated, and pasteurized milk.

Milk, which contains carbohydrates, fat, minerals, vitamins, and proteins, and has a pH of approximately 6.8, is highly susceptible to degradation by various species of microorganisms. Contrary to popular belief, milk is never quite sterile. Even in the udder of a healthy cow, milk becomes contaminated by bacterial cocci that are present in the milk ducts or in the reservoir of the udder. Bacterial numbers vary from a few to a few hundred, depending on the health and environment of the animal. During the actual milking operation and the subsequent handling of the milk, further microbial contamination is virtually unavoidable.

The microbial load of the milk can be reduced or eliminated by means of pasteurization or heat sterilization. However, it is important to note that the keeping quality of the final milk product depends, ultimately, on how successfully the reintroduction of microorganisms can be prevented and how their growth and multiplication are inhibited.

B. METHODS USED FOR THE PROCESSING OF MILK AND DAIRY PRODUCTS

Pasteurization uses high temperatures to eliminate disease-causing organisms and reduce microbial populations present in the milk. This technique was first developed in the 1860s by the French bacteriologist, Louis Pasteur, to control the problem of wine spoilage. During the last century, the pasteurization methods have been refined substantially. During conventional **low-temperature holding (LTH) pasteurization** (the method that we will be using in this exercise) milk is maintained at 62.8°C for 30 minutes. In the **high-temperature, short-time (HTST)** process, milk is held at 71°C for 15 seconds, followed by rapid cooling of the milk. Finally, milk can also be treated at 141°C for 2 seconds for **ultra-high-temperature (UHT)** processing. Such shorter term processing has the advantage that more of the original flavor of the milk is maintained. The technique of heat sterilization also relies on high, prolonged processing temperatures to eliminate all living microorganisms. While some milk products are heat sterilized, this method is mainly applied for the preservation of canned goods.

Using the relationship between time of heating and decline of microorganisms in the processed food item, the index of **thermal death time (TDT)** was developed. TDT is the time it takes, at a given temperature, to completely destroy all the microorganisms in a sample. To better understand and predict the effects of heat on microorganisms, the **D value** was developed. This is the time required to cause a one-log decrease, or a 90% reduction, at a given temperature. From the D value, one can determine the microbial population remaining in the product after any heating time. We will determine the $\mathbf{D_{62.8}}$ value for the LTH pasteurization of milk samples by monitoring the remaining microbial population during the processing of the milk.

C. DETERMINATION OF THE MICROBIAL CONTENT OF THE MILK

In order to determine the microbial content of milk, several standard methods have been developed. In addition to the **Standard Plate Count** (sound familiar?), **direct microscopic observation** of milk bacteria, **coliform counts**, tests for specific **microbial pathogens**, and a **dye reduction test** are most commonly applied. For the purposes of this laboratory exercise, we will perform both the Standard Plate Count and the Resazurin reduction test.

1. Standard Plate Count

Despite its inherent limitations, the Standard Plate method has evolved as the method of choice in the testing of milk. Although more time-consuming and more costly than other enumeration methods, this technique is especially suited to determinations where bacterial densities are low. Consequently, when testing pasteurized milk, this method is used not only for bottled milk but also to assess the efficiency of pasteurization and to detect sources of contamination at successive stages of milk processing. In addition, this method is used for the inspection of retail raw milk, particularly that of higher grade (the grade given to a particular milk is based on the viable bacteria that are detected by the Standard Plate Count; see also Table 1). As the most suitable method to detect viable bacteria in exceptionally low-count milk, the Standard Plate Method is the basic procedure approved by the American Association of Medical Milk Commissions for analysis of samples of certified milk.

In addition to being a highly sensitive method, the Standard Plate Count technique also lends itself to the detection of specific functional groups of bacteria associated with dairy products. The specific bacteria that we will be concerned with in this exercise are **thermophilic** and **psychrophilic** bacteria in the pasteurized milk. **Thermophilic** (heat-loving) bacteria are those bugs that can grow at temperatures of 55°C. Many of these bacteria are facultative and can grow at 37°C or lower. Usually thermophiles are spore-forming bacilli that enter milk from various sources on the producing farm. For example, repasteurization of milk will favor proliferation of thermophilic bacteria. **Psychrophiles**, on the other hand, are those bacteria that are capable of relatively rapid growth at low temperature (5° to 7°C). These bacteria, therefore, present a major problem in the refrigerated storage of many dairy products. Psychrophiles are the bacteria responsible for spoilage of both raw and pasteurized milk that keep in your refrigerator for prolonged periods of time without opening. The bacteria most commonly encountered among psychrophiles belong to the genera *Pseudomonas, Achromobacter, Flavobacterium, and Alcaligenes*.

2. Resazurin reduction method

Another common, low-tech method used in the determination of the bacterial load in milk and other dairy products is the **Resazurin reduction method**. This method indirectly measures bacterial densities in milk samples in terms of the time interval required for a dye-milk mixture with a characteristic blue color to turn white. Based on the length of time required for the color change, the milk can be then grouped into classes and grades. The dye-reduction method depends on the **metabolic activity** of bacteria associated with the milk. As the bacteria grow, they consume dissolved oxygen, which in turn lowers the **redox potential** of the dye-milk mixture. Resazurin is used as a redox indicator, which is highly susceptible to changes in the dissolved oxygen concentration of the milk. As oxygen is consumed by the bacteria, the resazurin dye is reduced to Resofurin which is indicated by a gradual color change from blue to pink; this is followed by further reduction to Dihydroresofurin. This second reductive step is characterized by a fading of the pink color to white.

The Resazurin dye test can be applied to both refrigerated raw and pasteurized milk. In order to hasten bacterial oxygen consumption, the dye-milk mixture is kept in a 37°C water bath throughout the incubation.

The relationship between reduction and bacterial load is generally such that reduction time is inversely proportional to the bacterial content. Based on this relationship then, milk testers do either one or both of the following:

- *to classify samples into two or more major groups, according to appreciable differences in reduction times*
- *to use descriptive grading terms to indicate whether or not a milk sample is "acceptable".*

For the purposes of this exercise, we will report the average **Resazurin Reduction Times (RRT)** for replicate dye-milk samples without attempting to discern a quantitative and/or qualitative relationship with the Standard Plate Count.

TABLE 1

Dairy Product Standards recommended by the American Public Health Association

MILK GRADE	RAW MILK	PASTEURIZED MILK	
	Standard Plate Count	Standard Plate Count	Coliforms
A (prior to mixing with other milk)	100,000	20,000	10
A (after mixing with other milk)	200,000	20,000	10
B	600,000	40,000	10
C	>1,000,000	40,000	>10
Ice Cream	----------	50,000	10

D. PROCEDURE

We will use two milk samples for this exercise. One of the samples comes from a cow that recently gave birth to a calf. This cow lives on a family farm and the produced milk is strictly used for consumption on the farm. The second milk sample is from a dairy farm.

For this exercise, we will examine both milk samples for their bacterial load. We will subsample the milk before, during, and after pasteurization at 62.8°C using the outlined methods. Due to time constraints we will examine the two milk samples as a group rather than as individuals. Be sure that you familiarize yourselves with each of the different procedures even if you do not work on a particular procedure.

Standard Plate Count

1) Using aseptic technique prepare three dilutions (10^{-2}, 10^{-3}, and 10^{-4}) for each of the two milk samples. Use sterile Phosphate buffer solution (PBS) to prepare the needed dilution blanks.

2) Label petri plates as needed for your samples.

3) Per dilution, pipette **1.0 ml** into the appropriately labelled petri dishes. **DO NOT TOUCH THE INSIDE OF THE PETRI DISH WITH YOUR PIPETTE TIP AND/OR YOUR FINGERS! PLACE THE TUBES HOLDING THE LEFT-OVER DILUTED MILK IN THE ICE BATH.**

4) Add enough melted agar medium to cover the bottom half of the petri dish to an approximate depth of 0.5 cm.

5) As each plate is poured, thoroughly mix the medium with the test portions in the petri dish. Do this by **gently** rotating and tilting the dish.

6) Prepare three replicate plates per dilution for each of the two milk samples.

7) After the medium has solidified in the plates, put the plates in the incubator and incubate at **35°C for 48 hours**.

8) Count the number of colonies on the agar plates for each of the dilutions and record the results. Keep in mind that countable plates are plates that have between 30 to 300 colonies present.

9) Calculate Standard Plate Counts per ml by multiplying the averaged numbers of colonies for each dilution with the reciprocal of the dilution used:

Counts/ml = average number of colonies/dilution factor

Resazurin Reduction test

1) For this part of the exercise you will have to use the portions of the two milk samples that have been cooled at 18°C for the past 20 hours. Before you remove the cooled milk from its storage, prepare all necessary test tubes to minimize the amount of time the cooled milk is kept at room temperature prior to the procedure.

2) Label three replicate screw cap test tubes for each of the two milk samples.

3) With a pipette, transfer 1.0 ml of the sterile Resazurin dye solution into each of the test tubes.

4) Rapidly transfer 10 ml of cooled milk to each test tube, cap the tubes and slowly invert the tubes three times to mix the contents.

5) Put the tubes into the 35°C incubator and record time as beginning of incubation.

6) Check for dye reduction (color change from blue to pink to white) every 15 minutes for the next 2 hours. Record your results.

7) Describe the reduction process in a qualitative manner by noting the time of major color changes.

Pasteurization of milk samples

1) As a group, designate and prepare one sterile screw-capped test tube as the "pilot" test tube.

2) For each milk sample, label four replicate screw-capped test tubes.

3) Using aseptic technique, pipette 6.0 ml of raw milk into each test tube as well as into the pilot test tube.

4) Insert a thermometer into the pilot test tube and place all the test tubes containing 6.0 ml raw milk, as well as the diluted milk samples that were used to prepare the Standard Plate Counts into the pasteurizing bath. The temperature of the water should be as close as possible to 62.8°C. The test tubes should be immersed so that the water line is approximately 1.5 in. above the level of the milk in the tubes.

5) Monitor the temperature of the milk in the pilot tube. When it has reached 62.3°C, start timing the holding period and expose the samples to pasteurizing temperature for 30 minutes.

6) Designate one of the replicate tubes for subsampling every 5 minutes by pipetting 1.0 ml into an appropriately labelled petri dish. Add melted agar to the test portions and proceed as outlined above.

7) At the end of the 30 minutes holding period, immediately place the tubes in an ice bath and cool tubes to 10°C.

8) Using the diluted milk samples, prepare three replicate pour plates per dilution for each of the two milk samples. Place these plates in the incubator and incubate at 35°C for 48 Hours.

9) Calculate the bacterial load after pasteurization as above.

10) From the three remaining test tubes containing 6.0 ml of raw milk, determine the Thermophilic and Psychrophilic Bacteria Count. Do this by preparing two sets of three replicate pour plates.

11) Incubate one set of replicate plates at **55°C for 48 hours**.

12) Place the other set of replicate plates into the refrigerator and incubate at **5° to 7°C for 7-10 days**.

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