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Extremophiles in My Backyard? Enhancing Analytical and Math Skills with a Simple Enquiry Based Lab

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What lives in compost? What survives extreme conditions such as hydrothermal vents? We harness that curiosity in a guided inquiry-based laboratory exercise that promotes critical analysis and reinforces math skills. In this authentic research, students explore the relationship between physico-chemical characteristics and diverse microbial community of a natural environment. Using selective and differential media, dilution, viable counts, and the scientific method, they enrich thermophiles from compost. In collaborative exercises, they collect, evaluate, and analyze numerical data, and present their findings in scientific format. This flexible, easily adaptable model has proven to be invaluable in contextualizing science in our classrooms

Keywords: Extremophiles, thermophiles, authentic research, critical thinking, math skills, compost, Enrichment of thermophiles from compost, authentic research

Introduction

Enrichment of Thermophilic Microorganisms from a Compost Sample

Most microbiology labs teach the concepts of selective and differential media, enumeration of microorganisms, microbial diversity, and complexity of metabolic pathways. Students often understand these concepts as discrete units and the intricate crosslinking of concepts and skills is sometimes lost. The goal of this exercise is to tie together multiple concepts and competencies in the same way that real life research tasks do. It works on the principle that students do their best work when they are challenged with a task that is a little beyond their comfort level. This authentic research also provides students with a taste of the rigor required for and the unpredictability of results experienced in real scientific experiments.

This experiment was designed for biology majors taking an upper division microbiology lab course. As such, prior to this activity, students in our lab will have completed a unit on microbial physiology, gaining a thorough understanding of selective and differential media. Through other unrelated experiments in the lab, students will have practiced serial dilutions and plating, with particular reference to the standard methods of calculating culture concentrations. However, I have also conducted this exercise with high school students after leading them through a basic primer on media and dilutions. The techniques in this experiment are very simple to execute. Students with a basic knowledge of sterile technique, plating methods, serial dilutions, and simple math, can complete the actions involved. The concepts of enrichment and the mathematical calculations involved are more complex and require higher level thinking on the part of the students and greater clarity in teaching by the instructors.

The most important factor in the success or failure of this experiment is ensuring the preparedness of the students prior to beginning the work. Appendix A and the worksheet in Appendix B provide information on selective and differential media and a worksheet for students to test themselves on the application of these concepts. The introductory material in the Student Outline section of this paper and Appendix C provide background information on enumeration and enrichment concepts and a worksheet for students to practice them. The amount of time devoted to these activities and lessons depends on the level of prior exposure to the concepts in other course units. The most commonly used equation in calculating culture concentrations is given below. Additional equations for assessing the success of the enrichment are given in the Student Outline and use this calculation as the basis. Appendices D, E, and F provide sample worksheets for counting colonies, calculating concentrations, and calculating fold-enrichment.

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Concentration of stock =

$$C = \frac{\text{Colony number}}{\text{Plating volume in ml}} \times \text{Cumulative Dilution Factor}$$

The stock refers to any undiluted starting material such as a liquid sample or a dry soil or compost sample. The concentration is thus derived in units of cfu/ml or cfu/g. The cumulative dilution factor (CDF) is the final dilution factor obtained in the diluted sample used for plating purposes. For example, the sixth tube of a 1:10 serial dilution is the 1×10^{-6} dilution and the CDF is 1×10^{6}.

Set up of the laboratory materials is simple, with the greatest time commitment being allotted to pouring the required media plates. A list of the materials necessary, their distribution across the lab days, and information on purchasing and preparing the media is provided in Appendix G. The experiment itself takes four lab days, not including the preliminary preparation or any time used after completion to discuss data and/or write a report on the experiment. The average time spent on each task is 30 – 60 minutes. A brief time line of the experiments is provided in Table 1.

Table 1. Timeline of the enumeration and enrichment experiment.

Lab Day	Activities	
	Baseline Enumeration	**Enrichment**
Prior to start of experiment	Prepare students on concepts of dilution math, selective and differential media, simple enumeration, and enrichment	
Lab day 1	Suspend sample, serially dilute, plate and incubate	Inoculate control and enrichment media with sample
Lab day 2	Check colonies for countability	Serially dilute control and enrichment, plate, incubate
Lab day 3	Complete colony counts and calculations	Check colonies for countability. Count if possible
Lab day 4		Complete counts and calculations.
After completion of experiment	Check all calculations, share and assess data, complete statistical analyses, and discuss outcomes	

The student outline will provide an introduction to the concepts of enumeration and enrichment and a step-by-step description of an enrichment assay for thermophiles from a compost sample. The same principles can be applied to virtually any enrichment exercise. Appendix H (Chilukuri, 2012) is a short chapter on bacterial survival in extreme conditions.

Student Outline

Introduction

Natural communities are almost never composed of monoclonal cultures of any organism. The large number of physical, chemical, and biological factors involved in creating an environment also produces a plethora of microniches that are occupied by microbes. Given the vast diversity of microbial metabolic capabilities and the potential for interactions among the microbes, it would be interesting to find out what kinds of microbes live in these microniches and what kinds of metabolic activities they are capable of. There are many approaches to answering these questions including metagenomics, metabolomics, proteomics, and transcriptomics. There are also a vast number of natural environments that can be explored. This exercise uses compost as an example of a complex environment and enrichment as one approach to study its microbial community. The principles expressed in this exercise can be equally applied to other environments, with the necessary modifications to accommodate the differences in environmental factors. Some of the fundamental questions one may ask about any environment include:

- How many microbes are in the sample and what are they?
- What can these organisms do?
- Which kinds of organisms specialize in each activity?
- What kinds of physical and chemical conditions are they adapted to or are able to survive?
- How can we enrich or isolate organisms of a particular desired characteristic from a mixed natural population?

Soil is a complex mixture of minerals and decaying organic matter that serve as nutrients for a vast number of organisms, particularly microbes (Slonczewski and Foster, 2009). Soils range in quality from the humus-rich mixtures found in tropical rainforests to the arid low organic soils found in desert climates. The breakdown of organic compounds from plants and animals, primarily by microbes such as fungi and bacteria, recycles precious nutrients back into the soil, and allows the growth of producers, thus supporting food and energy webs. While not all soils are naturally blessed with these recycled nutrients, composting provides a simple way to amend soils with natural materials. Composting is simply the process of decomposing organic matter through the action of microbes that occur naturally in soils and compost is the resulting product. At its most basic level, composting consists of accumulating a wetted heap of leaves, twigs, and other organic material, generally in a corner of a garden or in a pit and allowing it to break down naturally over a period of time. Mixing soil into this heap provides a source of microbial decomposers.

More complex composting methods have evolved that speed up the composting process and more reliably produce compost that can serve as fertilizer, mulch, aerator, or natural pesticide. Such methods carefully combine high carbon organic compounds (brown materials such as dried leaves, twigs) with high nitrogen organic compounds (green plant materials, fruits, vegetables) in favorable ratios (C:N ratios about 30:1). These compost heaps are periodically mixed to aerate the material, allowing the growth of microbes such as fungi and bacteria (including actinomycetes), and increasing the rate of decomposition. Temperatures within a compost heap can reach 50-70°C, and the pH can range from 5.5 to 8.0, depending on the composition of the material and the types of microbial activity. The high temperatures have the beneficial effect of killing or discouraging the growth of pathogens and weed seeds. The variety of organic substrates and the changing conditions obtained in a compost heap promote metabolic diversity in its microbes and serve as an interesting source for enrichment studies.

Enumeration

By the end of this exercise, you should be able to:

- Describe how to enumerate microorganisms from an environmental sample such as compost.
- Devise a method to enumerate microorganisms with specific physiological capabilities.
- Devise a method to enumerate microorganisms that are adapted to specific environmental conditions such as temperature and pH.

The word "enumerate" simply means to list, count, or tally a number of items. Compost is likely to host a variety of bacterial and fungal strains, of varying adaptation to temperature and pH extremes and with varying physiological capabilities. When enumerating these organisms, it is thus possible to approach the problem from several different perspectives, depending on the question that is being asked.

One way to enumerate the microorganisms would be through microscopic examination of compost suspensions and a simple count of all microbes thus observed. One advantage of this method is that, done correctly, it is likely to produce the most accurate estimate of the total concentration of microbes in a sample. However, many compost microorganisms share common morphological traits such as shape and size so that such an examination may be interesting without being informative as to the type and variety of microbes.

A second method of enumeration is by plating the sample on non-selective agar media and counting (enumerating) the colonies that grow. This type of enumeration is useful in establishing a baseline of the concentration of various viable microbes in

the compost sample. However, it restricts the information to only those organisms that can grow in the medium and under the conditions used in the experiment. Organisms occurring at very low frequencies in the compost or those that have very specific growth requirements may be missed by this method. Moreover, different microbes can exhibit the same colony morphology so that it is hard to determine whether we are seeing many representatives of the same organism or fewer representatives of many types of organisms. Thus, this method would always underestimate the number and diversity of organisms in the sample, often by a very large margin. However, this method does allow us to visualize some of the compost microbes and to utilize the isolated organisms for further experimentation.

A third method of enumeration would be to subject the sample to a particular physical or chemical condition (a selective pressure) and only count the microbes that grow or survive. For example, if the compost sample is subjected to high temperature, it is likely that only thermophilic and thermoduric organisms are likely to survive. With the right plating conditions, it is possible to limit the enumeration to only these two categories of organisms and to eliminate all psychrophiles and mesophiles.

A fourth method of enumeration would be to separate organisms by physiological function and only count those organisms capable of a particular physiological activity. For example, a compost sample may be plated on a rhodamine plate and only the colonies that are positive for lipid degradation can be enumerated.

As you can see, the third and the fourth method of enumeration are somewhat related. No method is perfect and most of the methods are likely to underestimate the numbers. Nevertheless, a fair amount of interesting information may be gathered from such experiments. In our experiment we will measure using the second and third methods.

Outline of the Enumeration Process

In this exercise, you will begin with a gram of compost that is suspended and serially diluted in sterile saline. You will use the same compost sample and the same serial dilutions to enumerate microorganisms at two levels: enumeration of viable microorganisms and of microbes adapted to an extreme condition.

Enumeration of viable bacteria: A simple enumeration of the culturable bacteria in the compost sample. For this part of the experiment, serial dilutions of the compost suspension are plated on Tryptic Soy Agar (TSA) plates. TSA is a rich medium and allows rapid growth of bacteria.

Enumeration of bacteria capable of surviving extreme conditions: Enumeration of compost microorganisms that survive a set of extreme conditions such as low or high pH, low or high temperature, low or high salt conditions, etc. We will pick one extreme condition and try to find out the percentage of the total microorganisms that can survive this condition. For this part of the experiment, the same compost suspension is plated under the extreme conditions. For example, to determine how many bacteria survive high temperature, the dilutions should be plated on TSA plates and incubated at high temperature. To determine the percentage of acidophiles, the dilutions should be plated on low pH TSA plates and incubated at room temperature. Why do we use TSA here? Why would we use high temperature for thermophiles and room temperature for acidophiles? Why would we expect to find thermophiles or acidophiles in compost?

Enrichment

By the end of this exercise, you should be able to:

- Understand the physical and chemical parameters of an environment
- Relate the parameters to physiological capabilities of the microorganisms in the environment
- Relate the physiological capabilities to selective and/or differential media and conditions
- Understand the concept of selective pressure and apply it to the problem of enriching for particular types of organisms
- Set up an enrichment for a target group of organisms
- Determine the correct control conditions needed for each step
- Calculate the fold enrichment of target organisms

Enrichment simply means the increase in proportion of one component in a mixture when compared to the proportion of other components in the same mixture. These proportions are most commonly expressed as a percentage of the whole.

Example of Enrichment (Non-biological)

To illustrate the idea of enrichment in a non-biological context, let us first take a look at the purification of iron from iron ore. The process uses two known properties of iron, its density and its magnetic quality.

Iron deposits are found all over the world in the form of iron ores of varying quality embedded in sedimentary rocks. The most common iron ores are typically a mixture of iron minerals such as magnetite (oxidized iron), siderite (carbonates of iron), grunerite (silicates of iron), etc., mixed with quartz and other minerals from the rocks in which the ore is embedded. The amount of iron in the ore in its most raw state can be as little as 15%. Thus, if you measured out 1000 g of the iron ore, it might contain as little as 150 g of iron. This kind of iron ore is usually put through several purification (enrichment) steps. If the main

iron mineral is magnetite, it has magnetic properties, so the first step might be to break the ore up into small particles and use a powerful magnet to separate the magnetite from the quartz and other non-magnetic minerals. Magnetite can have as much as 60% iron so that 1000 g of magnetite would contain 600 g of iron. But there is still 40% impurity content which needs to be removed. The magnetite is then put through the next enrichment step using another property of iron, that it is denser than the impurities and therefore heavier. The magnetite enriched from the last step is put through a blast furnace, whereupon the melted iron sinks to the bottom and the impurities float on top and can be removed. The resulting iron is called cast iron and is about 95% pure so that 1000 g of cast iron has 950 g of iron and 50 g of impurities.

Let us calculate the enrichment of iron as a result of this process:

Step 1: Raw ore: The first form of iron ore had 150 g iron/1000 g ore = 15% purity of iron

Step 2: Separated magnetite: This form has 600 g iron/1000 g magnetite= 60 % purity of iron

Step 3: Cast iron: This form has 950 g iron/1000 g cast iron = 95% purity of iron

Note that in each case, the purity is calculated as a percentage so that the purity at one step can be easily compared to the purity at another step. In this enrichment or purification process, the percentage of the iron steadily increases. The fold enrichment (times enrichment) can be calculated thus:

Between the raw ore and the final product:

$$\text{Fold enrichment} = \text{Step 3 purity divided by Step 1 purity}$$

$$= \frac{95\%}{15\%} = 5.66 \text{ fold enrichment.}$$

This means that the iron is 5.66 times more concentrated at the end of the process than as raw ore. You can also calculate the fold enrichment between steps if necessary.

Enrichment of Microbes

Let us examine the same idea from a biological perspective. The main difference between a biological sample and iron is that the iron does not grow and multiply but the microbes do. The word "enrich", in the microbiological context, involves growing mixed microbial samples in media or growth conditions that favor the rapid growth of one organism or type of organism, while other organisms in that sample may die, or survive but be unable to grow under those conditions. The nutrient, chemical, or physical condition that restricts the growth of some organisms in the mixture is referred to as the selective pressure. At the end of a successful enrichment experiment, representatives of one organism or a limited group of organisms outnumber all other types of microbes in the culture.

For example, if a soil sample is grown in minimal media containing citrate as the sole carbon source, it is possible that over time, the number of organisms that CAN utilize citrate as carbon source will outnumber those that cannot utilize citrate. This would happen if those organisms that cannot utilize citrate either die or simply not grow, while those that do utilize citrate increase in numbers. Thus the selective pressure here is the use of citrate as the sole carbon source.

What would you expect to find if you were to plate the enriched culture on the same selective or differential medium that you plated your original (unenriched) sample on for the enumeration?

- Would you expect the percentage of organisms that can utilize citrate to have gone up, stayed the same, or gone down?
- Remember that due to microbial growth in the flask, a decrease in the number of types of organisms can occur simultaneously with an increase in total cell numbers in the enrichment flask.

Outline of the Enrichment Process

a. Identify the environmental sample to enrich from: Usually this is an environment with specific chemical or physical properties that make it interesting to you. For e.g., hydrothermal vents, compost heaps, soil, pond water, etc. We will be working with a compost sample taken from the community garden close to our lab.

b. Identify the target organisms: based on the characteristics of the environment you are studying or the type of physiological function you need, decide what type of organism you are trying to enrich. For e.g., thermophiles, acidophiles, nitrate reducers, etc. We will be enriching for thermophiles from our compost sample.

c. Identify the selective pressure that can be used: What physical or chemical condition will promote the growth of the target organism while restricting the growth of non-target organisms? For e.g., high temperature incubations for enriching thermophiles from a compost sample

d. Establish the baseline: Before setting up the enrichment, determine the concentration of your target organisms in your environmental sample and calculate the percentage of total microbes in the pre-enrichment sample. For e.g., calculate the percentage of compost organisms that are thermophiles in a sample that has not been manipulated in any way.

e. Set up the enrichment: Add your sample to the appropriate medium and incubate it under the appropriate conditions to encourage the growth of the target organisms while limiting the growth of non-target organisms. For e.g., add your compost sample to TSB and incubate at 65°C to encourage the growth of thermophiles and discourage the growth of non-thermophilic organisms. Also, by comparing the growth of the target organisms in the absence and presence of a selective pressure, you must establish that any enrichment has occurred as a RESULT of the selective pressure and not independently of any selective pressure. This means that you will need to set up a control flask with Tryptic Soy Broth (TSB) and compost that is incubated at the same time as the enrichment flasks but without ANY selective pressure.

f. Assess the enrichment: Count the target and non-target organisms in the enrichment flasks after incubation: Make serial dilutions of your control flask and each of the enrichment flasks and plate them on the appropriate media. To determine what media you should use, ask yourself the following question: what kinds of organisms might be present in the flask and on which media and incubation conditions will they grow? For e.g., in the TSB flasks at 65°C, what types of organisms are likely present: thermophiles, which can grow actively at the high temperature, and thermoduric organisms, which can survive the high temperature but cannot grow, i.e., multiply at the higher temperatures. How can you count the thermophiles? Plate the different sample dilutions on TSA and incubate the plates at 65°C. Will the thermoduric organisms grow actively enough to make colonies on this plate? Probably not, as they are not thermophilic. How can you count the thermoduric organisms? Plate the same dilutions on TSA plates and incubate them at temperatures lower than the thermophilic range, e.g. room temperature.

g. Calculations to assess the enrichment: Enumerate the target and non-target organisms in the enrichment. To calculate the fold enrichment of the target organism, compare the percentage of thermophiles before enrichment and after enrichment.

First Day of the Enrichment Exercise:

1. Establishing the baseline: Enumerating total bacteria and thermophiles in 1 g of compost.
2. Setting up the enrichment: Inoculating the control and enrichment flasks and incubating them.

Materials

- A single compost sample will be provided by the teaching assistant (TA)

Establishing the baseline: (per group of four)

- One bottle sterile saline buffer
- Large sterile test tubes
- For enumerating viable non-thermophilic bacteria
  - Five TSA plates – for all bacteria, to be incubated at room temperature
- For enumerating thermophiles
  - Three TSA plates to be incubated at 65°C
- Setting up the enrichment (per classroom)
  - One flask of TSB to be incubated at 65°C (Enrichment)
  - One flask of TSB to be incubated at room temperature (Control)

Procedure

Establishing the Baseline

1. Add 1 g of compost to 9 ml of sterile buffered saline and mix the suspension well. This will be the 1×10^{-1} dilution of the compost sample. Use the dilution to set up your serial dilutions as soon as possible.
2. Check the dilution charts (below) for this exercise. Note the range of dilutions that your group will use. Make the entire dilution series before you start plating. Remember to set up and label all your plates you begin your dilutions. Work rapidly to avoid prolonged maintenance of the diluted cultures prior to plating.
3. Set up test tubes with 9 ml sterile saline. The number of tubes you need will depend on the extent of dilution you require. Check the charts below.

4. From the 1 x 10^{-1} dilution provided by the TA, do serial dilutions, 1 ml in 9 ml to get 1 x 10^{-2}, 1 x 10^{-3}, 1 x 10^{-4}, 1 x 10^{-5}, 1 x 10^{-6}, 1 x 10^{-7}, etc.
5. Plate 0.1 ml of the appropriate dilutions as indicated for your group in Table 2 below.
6. Use the spread plate method of plating.
7. Incubate the plates at the temperature indicated. You will check the plates regularly for three lab periods, as it is difficult to predict the growth rate of organisms from a natural sample.

IMPORTANT: Note that the plates and the incubation temperatures are very specific for enumeration and it is important that you know in advance which plates you should use and at what temperature you will incubate the plates. Plates incubated at high temperature should be placed in a protective sleeve and a beaker of water should be placed in the incubation tray to prevent drying. High temperature plates MUST be removed from the incubator in 18 – 24 hours. Plating assignments may change; check with your TA before plating.

Table 2. Plating assignments for establishing the baseline

Type of medium	Incubation temperature	Group number	Dilutions to plate	What you are counting
TSA	Room temp	All groups	1 x 10^{-2}, 1 x 10^{-3}, 1 x 10^{-4}, 1 x 10^{-5}, 1 x 10^{-6}	All viable non thermophilic bacteria
TSA	65°C	All groups	1 x 10^{-1}, 1 x 10^{-2}, 1 x 10^{-3}	Thermophilic bacteria

Setting up the Enrichment:

Add 1 g of compost to a 100 ml flask containing 100 ml of TSB. Incubate the flask at 65°C for 24 hours. Then, subculture the enrichment by transferring 1 ml of the growth in the flask to a new TSB flask after 24 hours and re-incubate at 65°C.

Second Day of the Enrichment Exercise:

1. Establishing the baseline: enumeration of total bacteria and thermophiles (colony counts)
2. Assessing the enrichment: Serial dilution and plating to get thermophile and thermoduric organism numbers.

<u>General instructions</u>

1. First observe and record the number and descriptions of the different colony types.
2. Then, count and record the total number of colonies on each plate.
3. Use tables provided in addition to taking careful notes in your lab notebook so that the data can be recorded in a systematic manner.
4. If the colonies are still very small, re-incubate original plates for further observations. Plates that show dense growth should be refrigerated for possible future observation.
5. Calculate the concentration only from plates with countable colonies (remember that countability is defined as having 30-300 colonies per plate.
6. Report the concentration to the TA for entry in the class spreadsheet.
7. You will not use your data only in the final calculations for the lab report. Take a look at the data in the class spreadsheet. Other groups have entered their data too. Remember all of you plated dilutions from a suspension of the same compost sample. Do a preliminary assessment of the concentrations to see if the numbers are in the same range. Talk to your TA or instructor about outliers in the data.

Procedure:

Establishing the baseline: Count the colonies and calculate the concentrations. Follow the guidelines given above. You have been provided two tables, Table 3 for a 48-hour count and Table 4 for a 1-week count in case some colonies are too small to count on the first day.

Table 3. The 48-hour enumeration of non-thermophilic bacteria.

TSA plate		
Colony #	**Dilution factor**	**Concentration (cfu/ml)***

* Remember to incorporate the CDF and the plating volume when calculating concentrations.

Table 4. The 1-week enumeration of non-thermophilic bacteria.

TSA plate		
Colony #	**Dilution factor**	**Concentration (cfu/ml)***

* Remember to incorporate the CDF and the plating volume when calculating concentrations.

IMPORTANT: For TSA plates at high temperature (65°C)

Colonies on this plate often grow very rapidly and some will swarm over other colonies. Show your plates to the TA or instructor and ask them for any specific instructions on counting. Because of the rapidity of growth, the plates are usually removed from the incubator in 18-24 hours. At the end of the incubation period, count the colonies on your plates and record the numbers in Table 5 below:

Table 5. The 24-hour enumeration of thermophilic bacteria

TSA plate at 65°C		
Colony #	**Dilution factor**	**Concentration (cfu/ml)**

Calculations:

At this point, you have already calculated the concentrations of bacteria and thermophilic bacteria. During lecture and in the lab, we will discuss the significance of these numbers and how to calculate percentages. Take a few minutes to think about and decide which of these numbers serve as control data and for what purpose. If you don't know, discuss this with your partners, the TA, or the instructor. Make sure you do this calculation accurately as you will be using this information in the Enrichment report!

1. Calculate the average concentration of non-thermophilic bacteria from the class spreadsheet and enter it here:

2. Calculate the average concentration of thermophilic bacteria from the class spreadsheet and enter it here:

3. Concentration of total bacteria in the sample =

   Concentration of non-thermophilic bacteria + concentration of thermophilic bacteria =

4. Calculate the percentage of thermophilic bacteria in your sample in the space below:

   Baseline % of thermophilic bacteria =

$$\frac{\text{concentration of thermophiles}}{\text{concentration of thermophiles}+\text{concentration of non-thermophiles}} =$$

Assessing the Enrichment

On this lab day, you will plate serial dilutions of the control flask and the enrichment on the media described below. This will allow you to determine the extent of the enrichment with the selective pressure. Follow the plating chart in Table 6 to determine which dilutions you will plate

Materials

- The subcultured control flask incubated at room temp (Control)
- The subcultured thermophile enrichment flask incubated at 65°C
- Sterile saline

Plating from Control Flask (Per Group of Four Students)

- Four TSA plates to be incubated at room temp
- Four TSA plates to be incubated at 65°C

Plating from Thermophile Enrichment Flask (Per Group of Four Students)

- Seven TSA plates

IMPORTANT: Note that the plates and the incubation temperatures are very specific for each flask and it is important that you know in advance which plates you should use and at what temperature you will incubate the plates. Plates incubated at high temperature should be placed in a protective sleeve and a beaker of water should be placed in the incubation tray to prevent drying. High temperature plates MUST be removed from the incubator in 18 – 24 hours. Plating assignments may change; check with your TA before plating

Table 6. Plating assignments for enrichment.

Enrichment/ Control	Group Number	Plate medium	Dilutions to plate	Incubation temp
Control (TSB at room temp)	All groups	TSA	1 x 10^{-5}, 1 x 10^{-6}, 1 x 10^{-7}, 1 x 10^{-8}	Room temp
	All groups	TSA	Undiluted, 1 x 10^{-1}, 1 x 10^{-2}, 1 x 10^{-3}	65°C
Enrichment for thermophiles (TSB at 65°C)	All groups	TSA	1 x 10^{-2}, 1 x 10^{-3}, 1 x 10^{-4}, 1 x 10^{-5}	65°C
	All groups	TSA	Undiluted, 1 x 10^{-1}, 1 x 10^{-2}	Room temp

Third Day of the Enrichment Exercise:

On this day, you will count the colonies on each plate, determine if you need to re-incubate for more accurate numbers, and complete your calculations to determine if enrichment occurred, and if so, what fold enrichment you obtained. At the end of the incubation period, count the colonies on the plates as described below. If the colonies are really small on any of the plates, check with your instructor to see if it might be worth waiting an extra lab day before counting.

Record the results in the Tables 7 and 8. Once you have completed the calculation of the concentrations and entered the data in the class chart, you can proceed to the next steps, calculating the percentage of target organisms and then determining the extent of enrichment.

1. Control Flask

On your TSA, RT plates: To enumerate microbes growing without selective pressure, count all bacterial colonies

On your TSA, 65°C plates: To enumerate thermophiles growing in the absence of selective pressure, count all bacterial colonies. Watch for swarmers growing over non-swarmers.

2. Thermophile Enrichment Flask

On your TSA, 65°C plates: To enumerate thermophiles growing in the presence of selective pressure, count all bacterial colonies. Watch for swarmers growing over non-swarmers.

On your TSA, RT plates: To enumerate thermoduric colonies that may have survived but not grown in the presence of selective pressure, count all bacterial colonies.

Table 7. Colony counts of organisms plated from Control flask (TSB at RT).

TSA plates at RT: non-thermophiles growing in absence of selective pressure		
Colony #	**Dilution factor**	**Concentration (cfu/ml)**
TSA plates at 65°C: thermophiles growing in the absence of selective pressure		
Colony #	**Dilution factor**	**Concentration (cfu/ml)**

Table 8. Colony counts of organisms plated from enrichment flask (TSB at 65°C).

TSA plates at RT: non-thermophiles growing in absence of selective pressure		
Colony #	**Dilution factor**	**Concentration (cfu/ml)**
TSA plates at 65°C: thermophiles growing in the absence of selective pressure		
Colony #	**Dilution factor**	**Concentration (cfu/ml)**

Calculations:

1. Calculate the average concentration of non-thermophilic bacteria in the control flask from the class spreadsheet and enter it here:

2. Calculate the average concentration of thermophilic bacteria in the control flask from the class spreadsheet and enter it here:

3. Calculate the percentage of thermophilic bacteria in your control in the space below:

   % of thermophilic bacteria in Control =

   $$\frac{\text{concentration of thermophiles}}{\text{concentration of thermophiles+concentration of non-thermophiles}} \text{ x } 100 =$$

4. Calculate the average concentration of thermophilic bacteria in the enrichment flask from the class spreadsheet and enter it here:

5. Calculate the average concentration of non-thermophilic (thermoduric) bacteria in the enrichment flask from the class spreadsheet and enter it here:

6. Calculate the percentage of thermophilic bacteria in your enrichment flask in the space below:

   % of thermophilic bacteria in Enrichment =

   $$\frac{\text{concentration of thermophiles}}{\text{concentration of thermophiles+concentration of non-thermophiles}} \text{ x } 100 =$$

Next, determine whether or not any enrichment of the thermophiles was solely due to the selective pressure. To do this, first establish whether or not there was enrichment in the ABSENCE of selective pressure.

7. Calculate the fold enrichment (FE) in the control flask (no selective pressure) using the equation given below. What results do you expect in each case? What value indicates that enrichment has occurred? What does the FE value in your control flask tell you? Was there enrichment?

   $$\frac{\text{\% thermophilic bacteria in control flask [without selective pressure]}}{\text{\% baseline thermophiles}} \text{ x } 100 =$$

   Next, determine whether there was any enrichment in the flasks WITH the selective pressure.

8. Calculate the fold enrichment in the enrichment flasks (with selective pressure) using the equations given below. What results do you expect? What value indicates that enrichment has occurred? What does the FE value in your enrichment flask tell you? Was there enrichment?

$$\frac{\text{\% thermophilic bacteria in control flask [WITH selective pressure]}}{\text{\% baseline thermophiles}} \times 100 =$$

How do the FE values tell you whether or not any enrichment was DUE to the selective pressure? What does it mean if the FE in the control is less than that in the enrichment flask? Equal to that in the enrichment flask? More than that in the enrichment flask?

Some Things to Think About:

- Did the visual examination of the various plates (colony colors, shapes, swarmers, etc) show any differences before and after enrichment? If yes, what does this imply? If no, why might this be so?
- What additional information would you like to obtain about the organisms in your enrichment? How would you go about getting that information?
- If you were able to get information about their identities (perhaps through the use of metagenomics techniques), how would that be helpful?
- If you were interested in isolating some of these organisms, what techniques might you try?
- How might the organisms in your enrichment be useful for research or for commercial purposes?

Design an Enrichment Experiment:

Imagine that you are working with mud from a pond in a volcanic park such as Lassen. The mud smells strongly of H_2S and the pH is about 2.0. The mud is taken from the bottom of the pond and the temperature there is about 85°C. Leaves that fall in from local trees very rapidly break down in the mud. You think there may be some interesting microorganisms there that might have commercial use.

Ask yourself some questions:

- What physical, chemical, or physiological features of this environment strike you as interesting or unusual?
- How might these features affect the physiological capabilities or adaptations of the microorganisms that live in the mud?
- How can you use these capabilities to design an enrichment exercise?
- What kind of media or incubation conditions might you use?
- Why might these organisms be of interest to you or for commercial reasons?

Materials

Materials required for each lab day have been listed in the student protocol. Appendix G provides a summary of the materials, organized by lab day.

Notes for the Instructor

1. Preparing for the Module

Discussion on the Concept of Enrichment

In addition to walking students through the vocabulary of the experiment, it was very profitable to go through the iron purification example provided in the background information, before discussing enrichment of live organisms. An important concept to emphasize is the importance of calculating the proportions of target and non-target organisms as percentages. The discussion should explore the concept that in the process of enrichment, a decrease in the number of types of organisms (due to selective pressure) could occur simultaneously with an increase in cell numbers due to microbial growth. This is then connected to a discussion of the need for control conditions, the type of controls to use, and the type of calculations required.

Designing an Enrichment Study:

This part of the exercise often simultaneously proves to be the most challenging, the most enjoyable, and works best when done in groups. There is a great deal of flexibility in how to incorporate this into the curriculum. This can be done as a theoretical exercise assigning a different environment to each group or by having your whole class work on different aspects of a single environment of interest. It can be done as a Think-Pair-Share exercise, a homework assignment, a presentation, or a class discussion. An example of a practice worksheet used in group discussions is given in Appendix C. The objectives of this part of the exercise were to enable the students to:

1. Understand the physical and chemical parameters of the environment
2. Relate the parameters to physiological capabilities of the microorganisms
3. Relate the physiological capabilities to selective and/or differential conditions and media
4. Determine the correct control conditions needed for each step of the process

This experiment readily lends itself to adaptation to a variety of samples and/or target organisms. Before you begin the experiment, you would need to make some decisions based on the number of students, the amount of time you wish to spend on the unit, and the amount of media you can afford. Possible scenarios include:

A. Use a single sample, a single set of target organisms, and whole class works on the same enrichment (e.g. enriching acidophiles from a compost heap). Time, preparation, and resource savings.

B. Use a single sample and enrich for different target organisms. This allows students to explore different aspects of the same environment.

C. Allow students to select their own samples and design their own experiment. This requires more materials and takes more time to put together but is truly invaluable as a way to promote independent analytical thinking.

2. Conducting the Experiment

The details of the experiment are provided in the student handout and include:

a. Establishing the baseline: Serial dilution of the sample and plating on the appropriate media. This step gives them a snapshot of the sample before any selective pressure is used. Depending on available resources and your approach (see options A, B, and C above), each student group may plate an entire range of dilutions in duplicate or be assigned overlapping sets of dilutions to collectively cover the entire range. Incubation times may vary.

b. Setting up the enrichment: Plan this to allow one subculturing step to ensure the dilution of any carbon source or other component found in the compost sample.

c. Assessing the enrichment: The concentration of organisms can be unpredictable and thus require plating a wider range of dilutions to obtain countable colonies. If resources are an issue, it works well to have multiple groups work on the same sample to obtain replicate data points

d. Data collection, calculation, and evaluation: This is possibly the most error-prone part of the proceedings but with care, it is possible to obtain clean and usable data. TAs are a good resource to use to check calculations in the lab notebooks before sharing with the rest of the class. It is recommended that you provide an Excel spreadsheet that is formatted to complete the calculations when students enter the dilution factor and colony number. This gives students the opportunity to verify their non-computerized calculations. This step also provides opportunity to discuss data reliability and ways to increase accuracy of data collection. A simple comparison of the percent thermophiles before and after enrichment reveals whether or not the enrichment is effective. An increase in the percentage indicates that the selective pressure serves to reduce the viability of the non-target organisms. However, the calculations are not considered complete until the students satisfied themselves (and the reader) that the

enrichment did not occur in the absence of selective pressure. This is accomplished by calculating the fold enrichment values for the control flask and the enrichment flask. In a successful enrichment experiment, the FE value for the control flask will be zero or low and not approach or exceed the FE value for the enrichment flask.

e. Discussions on the effectiveness of the experimental design: Once the data has been analyzed and students have come to a conclusion regarding the effectiveness of the enrichment (or the different enrichments), it was a natural progression to discuss the different approaches, to evaluate their effectiveness, and to explore other options. When time permitted an intensive discussion, it was often the most invigorating part of the project as students were able to communicate with their peers in an informed manner on a scientific subject.

Special Considerations

The type of target organism and the type of selective pressure affects the success of the enrichment. For example, enrichments with extreme conditions such as temperature, pressure, osmolarity, or pH as the selective pressure had the greatest probability of enriching specific organism types. Using macronutrients as the sole available carbon source as a selective pressure was less effective, resulting either in no enrichment or very poor enrichment. However, it was not part of our game plan to have all the enrichments in the class be successful. This module worked best when we had some enrichments that worked very well (enrichment for thermophiles) and some that either did not work or that worked poorly. Enrichments that "failed" were often better material for the discussions that followed, as students deconstructed the failed experiments, looked for alternative approaches to achieve enrichment, or found alternative sources of the same target organisms.

Assessment

This module offers many opportunities for formative and summative assessments. Peer instruction questions using clickers, mock enrichments, sample calculations, and other forms of assessment were used either as low stakes or zero stakes formative assessments. Exam or quiz questions and an end of project lab report provided opportunities for summative assessments. The lab report is partly an analysis of the experimental design (supported by an analysis of the data obtained) and partly a proposal for an enrichment designed and outlined by the individual student. Of these summative assessments, the quizzes tested knowledge (lowest Bloom level), the midterm tested both knowledge and analytical skills (mid to higher Bloom level), and the lab report tested at the level of synthesis, the highest Bloom level (Crowe et al, 2008).

How My Students Have Benefited

To evaluate the benefit of this module in the overall development of the student, I compared their performance on assessments of similar level before and after the enrichment exercise. In the two years that we have used this module, I have observed an increase in math skills, analytical and reasoning skills, and an increased confidence in their ability to propose a new experiment. This module provides an opportunity for students to understand that research is never as clean as the tailored lab exercises they are accustomed to, and to contemplate the possibility that "failure" in research can be a stepping-stone to more critical thinking and more creative approaches to problem solving. I have also presented the results of my evaluation of their data analysis and critical thinking skills in posters at the 2012 ABLE and ASMCUE meetings. More details on some of their improvements were presented as the PowerPoint presentation at the ABLE 2013 meeting. A copy of the presentation can be provided upon request.

In addition to the tangible improvements in skills, students derived great satisfaction from the examination of the different types of organisms they "discovered" in the enrichment process. Included in this category were different types of colony morphology (colors, shapes, textures), swarming motility, antibiotic producers, fluorescent and/or bioluminescent bacteria, and bacteria with various physiological capabilities including casein, starch, and lipid hydrolyzers, acidophiles, alkaliphiles, and thermophiles. An extension of the enrichment exercise was the identification of thermophilic and non-thermophilic antibiotic producers in soil and compost. These antibiotic producers were identified using the metagenomics process outlined in the PowerPoint presentation and were the source of much discussion on both method and results. Student responses to this exercise were largely positive as seen from their responses to Clicker questions at the end of the experiment. I have included one email from a student that encapsulates the various comments I have received from students during and after the course.

Excerpt from a student letter: "Upon my return to San Diego, I have taken a volunteer position at the UCSD-Student Run Free Clinic which has been rewarding and a great learning experience. I also received an internship with a hematologist oncologist. I am currently writing a few case reports, one of which is an interesting case involving thymic carcinoma. I am also developing my own research project. In keeping in form, I have an interview with an infectious disease doctor this week to discuss a typical workday, challenges, etc. All of these wonderful opportunities have taken persistence and determination, but it was also the confidence I gained in your lab. In your lab, I was able to really understand the research process itself, how to ask the question, organize my data, and more importantly how to do proper research queries and examine those queries. In the wise words of Benjamin Franklin, 'Tell me and I forget, teach me and I may remember, involve me and I learn.' In your class, I was involved every step of the way".

Lab Safety

Compost samples may have a lower risk of containing pathogens due to the high temperatures reached within a compost heap. However, no environmental sample can ever be completely risk free and therefore the following rules are strictly enforced in our microbiology lab:

1. All students receive both standard lab and microbiology lab safety training through a safety video and a safety talk. All students sign a document outlining the safety regulations and agreeing to live by the rules. TAs discuss relevant components of the safety training at the start of each lab day.
2. Students are required to treat all microbes, whether identified or unidentified with the same care, as they would treat a potential pathogen.
3. Students do not work with the enrichment samples until they have completed the standard microbial physiology unit during which they receive extensive training in aseptic technique and safety procedures.
4. All materials used in our lab are pre-approved by the Environmental Health and Safety office at our University.
5. A Biohazard Use Authorization is in place for all lab courses at our University
6. Students are required to use full Personal Protective Equipment including full-length clothes to cover their legs, lab coats, closed toed shoes, goggles or safety glasses, and safety gloves.
7. Students are strongly encouraged to inform us of any conditions that would compromise their safety in our lab including but not limited to recent injury, allergies, pregnancy, or health conditions involving a compromised or suppressed immune system.

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Appendix A

Selective and Differential Media

Expected Outcomes of this Experiment

By the end of this exercise, you should be able to:

- Distinguish among general-purpose, selective, differential, and enriched media
- Predict the selective or differential effect of media ingredients given information on the microorganisms involved
- Describe the different degrees of hemolysis and how they can be distinguished
- Discuss the need or use of different categories of media

In their natural environments, microorganisms are often highly adapted to the microniches they occupy, living in a wide range of physical and chemical conditions. As a result, the nutritional needs of individual microbes can be very different from each other. In any attempt to grow all the microbes from a natural environment, no single medium can hope to satisfy all the needs of every individual organism. In that sense, therefore, almost any medium is sure to inhibit the growth or some organisms and no medium is truly universally suitable. The basic requirements for growth include trace elements, some salts, vitamins, a carbon source, and a nitrogen source. Organisms differ widely in the particular carbon and nitrogen sources they can use, which growth factors they can make, and which elements are essential for their normal growth.

General-purpose media or permissive media such as Trypticase Soy Agar (TSA) or Nutrient Agar (NA) contain a rich mixture of nutrients including amino acids, simple sugars, salts and water, thus permitting the growth of many different non-fastidious or semi-fastidious organisms. Fastidious organisms are those have complicated nutritional requirements and need special media to permit optimal growth. Semi-fastidious and non-fastidious organisms either have fewer such requirements or a wider range of nutrient sources they can utilize. These two general-purpose media are also described as undefined or complex because they use complex ingredients such as yeast extract or hydrolysed milk protein in which the exact amounts of each of the nutrients cannot be precisely predicted from one batch to the next. Defined media, by contrast, are those in which the quantity of each nutrient is known. General-purpose media are often used to maintain standard laboratory cultures or to grow mixed cultures of organisms with differing nutritional needs. In this lab, we will be using general-purpose media for most experiments, including those involving the enumeration of microbes from compost.

While general-purpose media are advantageous for microbial work, they are not necessarily suitable for all purposes. Any complex environment contains a wide variety of microorganisms, some in large numbers, others in very low concentration. Organisms that grow slowly or those that are present in low numbers in these mixed cultures are generally harder to isolate by either the streak plate method or by dilution and plating using the spread plate method. In the case of the streak plate method, organisms that are in low concentration in the sample would have the greatest chance of being found in the first quadrant. However, this is also the part of the plate with the greatest density of inoculation and growth here is confluent. Thus it would be impossible to identify and subculture the organism of interest from this region. The lower the concentration of the desired organism in the mixed culture, the lower the probability of its being found isolated in the other quadrants. Similarly, if we were to try to isolate the same organism by doing a serial dilution and plating the dilutions, the organism is most likely to be growing on the plates with the most colonies and less likely to be found on the more diluted plates. In either the streak or the spread plate, the presence of large numbers of non-target organisms (organisms we are not interested in isolating) reduces our chances of isolating the organism of interest.

One way of dealing with this situation is to find a way to eliminate the other organisms, thus making it easier to isolate the one you want. For e.g. if you know that your organism is resistant to heat, you can heat your sample up, killing those organisms that are heat sensitive and then plate the sample. Since only the survivors will grow, the relative concentration of the desired organism increases and the colonies of the desired organism are less likely to be obscured by colonies of the non-target organisms. An alternative method would be to use a medium (for streaking or spreading) that contains an ingredient that inhibits the growth of at least some of the non-target organisms but allows the growth of the target organism. Such a medium would be known as a selective medium, since it selects against non-target organisms, thus reducing their concentration. Please note that it is very rare that selective media select for the organism of interest, so that it is by a process of eliminating other organisms that you have made it easier to isolate the organism of interest. Many ingredients are known to provide a selective function including various dyes, antibiotics, and other inhibitory chemicals. An example of a selective medium is the MacConkey agar, which contains two ingredients – crystal violet and bile salts – that inhibit the growth of Gram positive organisms. Thus, if you were to plate a mixed culture of Gram positive and Gram negative organisms, only the Gram negative organisms would grow on this medium.

The level of selectivity of media can range from slight selectivity to stringent selectivity. NA and TSA are both rich, complex, general-purpose media. However, TSA has a greater variety of and a richer composition of the numerous factors required for growth and allows the growth of semi-fastidious organisms as well as non-fastidious organisms where the less rich NA me-

dium is less well suited to semi-fastidious microbes while supporting a great many non-fastidious microbes. Thus NA could be considered to be slightly selective compared to TSA. The presence of inhibitory chemicals such as antibiotics renders a medium more highly selective, particularly if resistance to the antibiotic is limited to few organisms in the mixed cultures.

A mixed culture contains microorganisms with different physiological capabilities and a general-purpose medium would not be useful in distinguishing among them. If however, an ingredient were added to the medium that changes color (or another easily distinguished property) only when a particular physiological activity occurred, it would be easier to identify which of the colonies have this property. A medium that contains such an ingredient is known as a differential medium as it allows us to differentiate among organisms visually based on physiological function. For example, a simple swab of the throat and plating of the organisms picked up by the swab may yield colonies of a variety of organisms including several strains of *Streptococcus, Enterococcus, Staphylococcus*, and *Neisseria*, among others. If the purpose of the swab were to identify whether or not the person has strep throat or a different infectious disease, it would be necessary to identify the different organisms. One step in this identification is to determine whether or not the organism is capable of hemolysis or the breakdown of red blood cells. The medium best suited to determine this is blood agar, a rich medium to which defibrinated sheep or horse blood has been added, rendering the medium bright red. Since this medium contains whole red blood cells, the hemolytic ability of the colonies can be determined by a simple visual examination of the agar around the colony. *Streptococcus pyogenes*, the organism that causes strep throat, is capable of β-hemolysis or the complete lysis of the red blood cells. This activity produces a complete clearing around the colony. Other strains of *Streptococcus* such as *S. pneumoniae* or *S. viridans* produce only partial hemolysis or α-hemolysis resulting in a green or greenish brown halo around the colony, while others such as *Streptococcus salivarius* or *Enterococcus faecalis* show no hemolysis (γ-hemolysis) resulting in no alteration of the agar around the colony. In this course, you will use blood agar plates as well as other differential media including MacConkey agar, starch agar, rhodamine agar, X-gal plates, etc. Please note that some of these media require the addition of a reagent after incubation and growth of the organisms while others do not.

Blood agar is a differential medium that is not selective, that is, it allows all sorts of bacteria to grow on it whether or not they are capable of hemolysis. In fact, blood agar is considered to be an enriched medium as the addition of whole blood allows the growth of fastidious as well as non-fastidious organisms. Media may also be selective and differential as seen in the MacConkey medium. As noted previously, this medium is a selective medium due to the presence of crystal violet and bile salts. The presence of lactose and a pH indicator allow the differentiation between Gram-negative organisms that are capable of lactose fermentation from those that lack this function. This medium and others like it (e.g. Levine EMB) are useful in enumerating coliform bacteria, common gut dwelling bacteria whose presence is used as an indicator of fecal contamination of food or water. Coliforms are defined as facultatively anaerobic Gram negative bacteria capable of fermenting lactose in the presence of bile to produce acid and gas. The selective nature of the MacConkey medium limits the growth to Gram-negative bacteria alone while the fermentation of lactose in the medium and the color change in the pH indicator from the resulting acid production helps distinguish coliform from non-coliform Gram negative organisms.

It is thus seen that different types of media have varied function in microbiology including

1) enumeration of microorganisms
2) isolation of specific microorganisms
3) quality control
4) prevention of contamination.

It is part of the microbiologist's job to determine which medium will best suit the experimental purpose, whether it is a general-purpose, selective, differential, enriched, or defined medium or any combination of the above.

Appendix B

Selective and Differential Media Worksheet

You have been given a mixture of 3 organisms: each shares some characteristics with one other. The goal is to separate them into individual monocolonal colonies. You may find that you need 2 or more steps to adequately separate all three. Know/show how you can confirm each organism once you have isolated colonies.

Assume similar colony size, shape, and texture when grown in optimal condition in each case. Optimal temp same if not otherwise indicated.

Table 9. Problem 1.

Organism	Character 1	Character 2	Character 3
A	Gram negative	Lactose fermenter	Alpha hemolytic
B	Gram negative	Lactose fermenter	Beta hemolytic
C	Gram positive	Lactose fermenter	Beta hemolytic

Solution:

Table 10. Problem 2.

Organism	Character 1	Character 2	Character 3
A	Acidophile	White	Temp range 20-38°C
B	Acidophile	Yellow	Temp range 40-55°C
C	Acidophile	Yellow	Temp range 20-38°C

Solution:

Table 11. Problem 3.

Organism	Character 1	Character 2	Character 3
A	High salt adapted, low salt sensitive	White	Opt temp 25°C
B	High salt sensitive	Red	Opt temp 25°C
C	High salt sensitive	White	Opt temp 55°C

Solution:

Table 12. Problem 4.

Organism	Character 1	Character 2	Character 3
A	Amp sensitive	Gamma hemolytic	White
B	Amp resistant	Alpha hemolytic	White
C	Amp sensitive	Alpha hemolytic	White

Solution:

Table 13. Problem 5.

Organism	Character 1	Character 2	Character 3
A	Gram positive	Neutrophile	Amp resistant
B	Gram positive	Neutrophile	Amp sensitive
C	Gram negative	Acidophile	Amp resistant

Solution:

Key to Sample Problems

You have been given a mixture of 3 organisms: each shares some characteristics with one other. The goal is to separate them into individual monocolonal colonies. You may find that you need 2 or more steps to adequately separate all three. Know/show how you can confirm each organism once you have isolated colonies.

Assume similar colony size, shape, and texture when grown in optimal condition in each case. Optimal temp same if not otherwise indicated

Table 13. Problem 1 solution.

Organism	Character 1	Character 2	Character 3
A	Gram positive	Neutrophile	Amp resistant
B	Gram positive	Neutrophile	Amp sensitive
C	Gram negative	Acidophile	Amp resistant

Solution: Plate on blood agar. The alpha hemolytic strain would be A, confirm by plating on MacC. It should grow and show fermentation. Pick several colonies of the beta hemolytic strains and streak them on TSA and MacC. The strains that grow on MacC are B, the ones that don't are C.

Table 14. Problem 2 solution.

Organism	Character 1	Character 2	Character 3
A	Acidophile	White	Temp range 20-38°C
B	Acidophile	Yellow	Temp range 40-55°C
C	Acidophile	Yellow	Temp range 20-38°C

Solution: Grow the mixture on low pH plates at 25°C. This eliminates B. Pick the remaining two colonies based on color – A is white and C is yellow. Plate the mixture on low pH plates at 55°C. This eliminates A and C. B should be yellow.

Table 15. Problem 3 solution.

Organism	Character 1	Character 2	Character 3
A	High salt adapted, low salt sensitive	White	Opt temp 25°C
B	High salt sensitive	Red	Opt temp 25°C
C	High salt sensitive	White	Opt temp 55°C

Solution: Grow the mixture on High salt plates at 25°C. This should eliminate B and C. A should make white colonies. Plate the mixture on low salt plates at 25°C and 55°C. The 25°C plate should eliminate both A and C, the 55°C plate should eliminate both A and B.

Table 16. Problem 4 solution.

Organism	Character 1	Character 2	Character 3
A	Amp sensitive	Gamma hemolytic	White
B	Amp resistant	Alpha hemolytic	White
C	Amp sensitive	Alpha hemolytic	White

Solution: Plate all on blood agar plates. Pick the Gamma hemolytic strain – it should be A. Confirm on Amp plates- it should not grow. Pick several alpha hemolytic strains and streak them on both Amp+ and Amp- plates. B should grow and C should not.

Table 17. Problem 5 solution.

Organism	Character 1	Character 2	Character 3
A	Gram positive	Neutrophile	Amp resistant
B	Gram positive	Neutrophile	Amp sensitive
C	Gram negative	Acidophile	Amp resistant

Solution: Plate the mixture on MacC. That would eliminate A and B. Confirm C by plating it on neutral and low pH plates. Should grow at low pH and not at neutral. Plate mixture on neutral pH plates, should eliminate C. Pick several colonies and plate on Amp+ and Amp – plates. A will grow on both. B will only grow on Amp-

List of Microbiological Media

- Blood Agar plates
    - Tryptic Soy Agar + 5% Sheep Blood
    - Shows hemolysis if present

- MacConkey Agar
    - This medium allows Gram negative organisms to grow
    - Inhibits growth of Gram positive organism
    - Lactose fermenters form pink-red colonies
    - Non lactose fermenters form clear white colonies

- Tryptic Soy Agar
    - Allows growth of variety of microorganisms
    - pH at 25°C is 7.3
    - Contains 0.5% NaCl

- Tryptic Soy Agar + 5% NaCl

- Tryptic Soy Agar pH 5

- Tryptic Soy Agar pH 8

- Tryptic Soy Agar +100 mg/ml Ampicillin

Appendix C

Worksheet for Pre-enrichment Exercise

Part A. Understanding Selective Pressure

Pick either question 1 or question 2 to answer:

1. A body of water at pH about 9.4, periodically gets an influx of acidic waste at pH 3 from a pulp mill
   - What is the range of pH that one might find at different parts of the water body?
   - What are the three main types of organisms that you might find in the water body? Where? E.g. acidophile, etc
   - Pick one of the three types of organisms and tell me what selective pressure you would apply to kill or prevent the growth of the other two types. What medium type might you use and how would you alter it to kill two types and allow one to grow?

2. A body of water at an average temperature of 10°C, periodically gets an influx of hot water (60°C) from the cooling towers of a local power plant
   - What is the range of temperature that one might find at different parts of the water body?
   - What are the three main types of organisms that you might find in the water body? E.g. mesophile, etc
   - Pick one of the three types of organisms and tell me what selective pressure you would apply to kill or prevent the growth of the other two types. What medium type might you use and how would you alter conditions to kill two types and allow one to grow?

Part B. Selective Pressure Involving Nutrients

This one might be a little harder. Pick either question 1 OR question 2 to answer:

1. A very oligotrophic water body (very low organic carbon levels) periodically gets an influx of cellulose waste from a pulp mill.
   - In terms of cellulose hydrolysis, what are the two main types of organisms in the water body?
   - If you wanted to enrich only one of the two types, which type would it be easier to enrich and how would you do it? What medium type might you use and how would you alter it to kill one type and allow the other to grow?

2. A very oligotrophic water body (very low organic carbon levels) periodically gets an influx of oil from a corn oil factory.
   - In terms of lipid hydrolysis, what are the two main types of organisms in the water body?
   - If you wanted to enrich only one of the two types, which type would be easier to enrich and how would you do it? What medium type might you use and how would you alter it to kill one type and allow the other to grow?

Can you envision a slightly different situation where the chemical that comes in is not a nutrient but a toxin? How would the questions change?

Part C. Working with Complex Environments

This is closer to what you might find in nature where there is more than one variable (changing condition) to consider. Choose any 1 scenario below and see if you can work out an enrichment plan. Questions are listed below options 1, 2, and 3

1. Mud from different parts of a pond in a volcanic park. Lots of leaves have fallen in the mud and animals that fall in die there. The mud smells of H_2S (high sulfur concentration) and the pH is about 2.0. The mud bubbles and the temperature is about 85°C at the bottom and 50°C at the surface.
2. Water under the polar ice cap is at a temperature of -13°C, is hypersaline (very high salt concentration), very high dissolved oxygen levels, and high levels of dissolved organic carbon (mostly protein and lipid)
3. Mud outside dairy farm, receives hot, alkaline waste (60-70°C, pH 9) from dairy. Contains large amounts of lactose and milk proteins.

Pick one of the 3 samples listed above and answer the following questions:

a. What physical, chemical, or biological features of this environment strike you as interesting or unusual?

b. How might these features affect the physiological capabilities or adaptations or survivability of the microorganisms that live in the mud?

c. Pick any one type of organism that you would like enrich. How can you use the information above to set up a medium and incubation such that you get one organism or type of organism to grow where the others die or fail to grow

- What kind of media might you use?
- What incubation conditions might you use
- What is the “selective pressure” that you are using? (What condition are you using that is restricting the growth of non-target organisms)

d. If you wanted to be sure that any enrichment that occurs is due to the selective pressure, what control incubation would you set up?

e. Why might these organisms be of interest to you – for research or commercial purposes

Worksheet for Pre-enrichment exercise

Part A: Understanding selective pressure

Pick **either** question 1 **OR** question 2 to answer:

1. A body of water at pH about 9.4, periodically gets an influx of acidic waste at pH 3 from a pulp mill

    - What is the range of pH that one might find at different parts of the water body? Anywhere from pH 3 to pH 9.4, dependending on the influx location + buffering properties of the body of water.
    - What are the three main types of organisms that you might find in the water body? Where? E.g. acidophile, etc
      Acidophile – near influx of acid (low pH)
      Neutrophiles – intermediate distance from influx (neutral pH)
      Alkaliphiles – far away from acid influx (high pH)
      Needed to verify nomenclature
    - Pick one of the three types of organisms and tell me what selective pressure you would apply to kill or prevent the growth of the other two types. What medium type might you use and how would you alter it to kill two types and allow one to grow?
      You could have a medium buffered to a low pH, allowing the acidophiles to grow and hindering the growth of the neutrophiles and alkaliphiles.

2. A body of water at an average temperature of 10C, periodically gets an influx of hot water (60C) from the cooling towers of a local power plant

    - What is the range of temperature that one might find at different parts of the water body?
      10C - 60C, depending on location of influx
    - What are the three main types of organisms that you might find in the water body? E.g. mesophile, etc
      Psychrophile – far away from influx
      Mesophile – intermediate distance from influx
      Thermophile – near/at influx
      Needed to verify nomenclature
    - Pick one of the three types of organisms and tell me what selective pressure you would apply to kill or prevent the growth of the other two types. What medium type might you use and how would you alter conditions to kill two types and allow one to grow?
      You could incubate a medium at high temperature to facilitate thermophile growth and retard psychrophiles and mesophiles

Figure 1. Sample of a student answer sheet for pre-enrichment exercise, part A.

Part B: Selective pressure involving nutrients

This one might be a little harder. Pick **either** question 1 **OR** question 2 to answer:

1. A very oligotrophic water body (very low organic carbon levels) periodically gets an influx of cellulose waste from a pulp mill
   - In terms of cellulose hydrolysis, what are the two main types of organisms in the water body

     Don't know the specific names —

     Organisms that can or cannot use cellulose as a carbon source.

   - If you wanted to enrich only one of the two types, which type would it be easier to enrich and how would you do it? What medium type might you use and how would you alter it to kill one type and allow the other to grow?

     - Enrich for the cellulose hydrolyzers
     - Use a medium that has cellulose as the only source of carbon

2. A very oligotrophic water body (very low organic carbon levels) periodically gets an influx of oil from a corn oil factory
   - In terms of lipid hydrolysis, what are the two main types of organisms in the water body

     Don't know specific names —

     Organisms that can or cannot hydrolyze lipids

   - If you wanted to enrich only one of the two types, which type would it be easier to enrich and how would you do it? What medium type might you use and how would you alter it to kill one type and allow the other to grow?

     - Grow in media with high lipid content and check for hydrolysis?
     - Enrich for lipid hydrolyzers

* Format {

Can you envision a slightly different situation where the chemical that comes in is not a nutrient but a toxin? How would the questions change?

Part C: Working with complex environments.

Figure 2. Sample of a student answer sheet for pre-enrichment exercise, part B.

Appendix D

Figure for Practicing Colony Counting

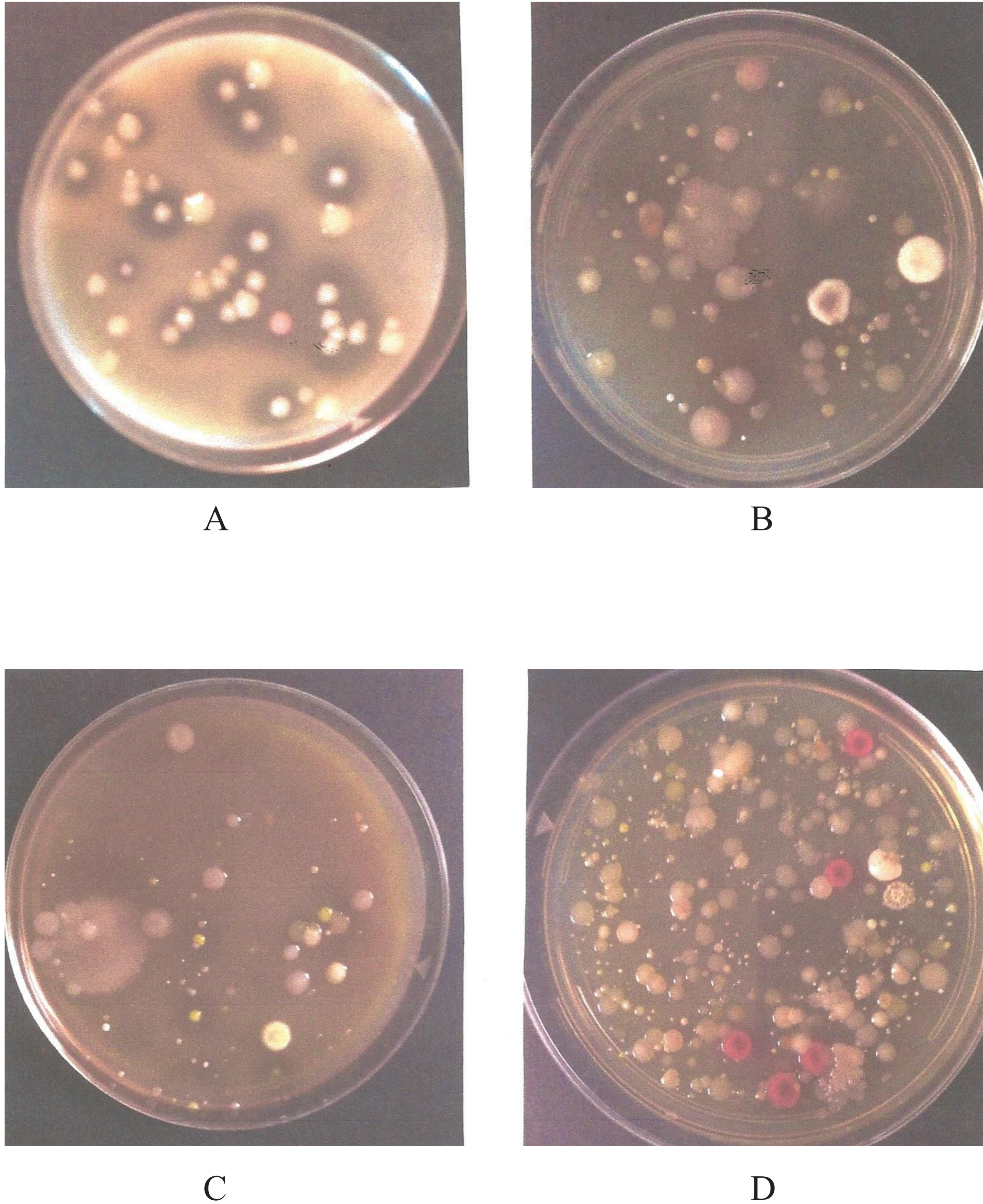

Figure 3. Practicing Colony Counting. Panels A-D show the results from plating serial dilutions of the casein hydrolyzer enrichment flask on Minimal media + Skim milk agar (panel A) or dilutions of the Control flask on TSA. All plates were incubated at room temperature. More information on the dilutions and plating volumes used is provided in Appendix E.

Appendix E

Plating and Counting exercise

Plating

One gram of a compost sample was added to 100 ml of TSB and incubated overnight at 65°C. This is the enrichment for thermophiles. Given the time constraints, we have not subcultured the sample or set up the control incubations. This exercise is simply so that you may experience a part of the experiment that the students complete in lab. In this exercise, we will only do the dilutions and plating for the enrichment. Participants will work in pairs and will be designated a group number. The required dilutions for even and odd numbered groups is given below.

Take 1 ml of the enrichment; make serial dilutions in sterile saline and plate 0.1 ml of the designated dilutions on TSA plates by the spread plate method. Work quickly to minimize the amount of time the sample spends at room temperature.

Table 18. Required dilutions for even and odd number groups.

Enrichment/Control	Group Number	Plate Medium	Dilutions to Plate	Incubation temp
Enrichment for thermophiles	***Even # groups***	TSA	1 x 10^{-2}, 1 x 10^{-3}, 1 x 10^{-4}, 1 x 10^{-5}	65°C
	Odd # groups	TSA	Undiluted, 1 x 10^{-1}, 1 x 10^{-2}	Room temp

Colony Counts

You have been given a sheet with photos (PDF of Appendix D provided as attached figure) of plates from the baseline, control, and casein hydrolysis enrichment experiments in my class. Thus, you are seeing pretty much what my students see when they get their incubated plates to count. Points to keep in mind:

- The "Countable" range of colony numbers is 30 – 300 colonies on a single plate. Use this information to decide which plates are "countable"
- Plate A represents colonies obtained when 0.1 ml of a 1 x 10^{-6} dilution of the enrichment sample was plated on MM+ SM and incubated at RT. The photo shows both casein hydrolyzer and non-hydrolyzer colonies. Hydrolyzers are readily recognizable by the clear zone of hydrolysis. Count hydrolyzers and non-hydrolyzers separately.
- Plates B, C, D represent colonies obtained when 0.1 ml of the 1 x 10^{-6}, 1 x 10^{-6}, and 1 x 10^{-5} dilutions respectively of the control sample were plated on TSA and incubated at RT. Keep in mind that part of what the students are checking is whether the serial dilutions were done accurately.
- You may count as many plates as you would like and calculate the concentrations using the equation:

$$\text{Concentration of undiluted culture} = \frac{\text{Colony number}}{\text{Plating volume in ml}} \times \text{Dilution factor}$$

- Enter the data below:

Table 19. Colony count data table.

Plate	Colony number	DF	Concentration (cfu/ml)
A: Hydrolyzers			
A: non-hydrolyzers			
B			
C			
D			

Appendix F

Sample Enrichment from One Section of BIMM 121, Winter 2013

Table 20. Enrichment for thermophiles.

Type of Bacteria	Media and Temperature	Average Concentration (cfu/ml)	% Target Organism	Fold Enrichment
Baseline = Compost sample suspended in sterile saline, serially diluted and plated				
Non-thermophilic	TSA at Room Temp	1.91E+05		
Thermophilic	TSA at 65°C	2.83E+03	1.46E+00 (1.461%)	
Enrichment = 1 g compost sample in TSB at 65°C, subcultured, serially diluted and plated				
Non-thermophilic	TSA at Room Temp	3.33E+00		
Thermophilic	TSA at 65°C	1.47E+06	1.00E+02 (99.99%)	6.84E+03 (6840)
Control = 1 g compost sample in TSB at RT, subcultured, serially diluted and plated				
Non-thermophilic	TSA at Room Temp	1.11E+08		
Thermophilic	TSA at 65°C	1.40E+05	1.26E-01 (0.1264%)	1.28E-05 (less than 1)

Calculations: Do this for Each Condition (Baseline, Enrichment, Control)

Concentration of total bacteria in the sample =

Concentration of non-thermophilic bacteria + concentration of thermophilic bacteria =

Calculate the percentage of thermophilic bacteria in your sample in the space below:

$$\text{\% of thermophilic bacteria} = \frac{\text{concentration of thermophiles}}{\text{concentration of thermophiles+concentration of non-thermophiles}} =$$

Calculating the fold enrichment: follow the example given at the beginning of this chapter

$$\text{Fold enrichment of thermophiles} = \frac{\text{\% thermophilic bacteria in enrichment (or control)}}{\text{\% baseline bacterial thermophile}} =$$

Table 21. Enrichment for casein hydrolyzers using minimal media + skim milk (MM + SM).

Type of Bacteria	Media and Temperature	Average Concentration (cfu/ml)	% Target Organism	Fold Enrichment
Baseline = compost sample suspended in sterile saline, serially diluted and plated				
Total bacteria*	MM + SM at RT	1.91E+05		
Casein hydrolyzers	MM + SM at RT	3.62E+04		
Enrichment = 1 g compost sample in MM + SM at RT, subcultured, serially diluted and plated				
Total bacteria*	MM + SM at RT	1.59E+07		
Casein hydrolyzers	MM + SM at RT	4.88E+06		
Control = 1 g compost sample in TSB at RT, subcultured, serially diluted and plated				
Total bacteria*	MM + SM at RT	9.51E+07		
Casein hydrolyzers	MM + SM at RT	3.09E+07		

Calculations: Do this for each condition (Baseline, Enrichment, Control)

Concentration of total bacteria* in the sample =

Concentration of non-hydrolyzers bacteria + concentration of hydrolyzers bacteria =

Calculate the percentage of thermophilic bacteria in your sample in the space below:

$$\text{\% of casein hydrolyzer bacteria} = \frac{\text{concentration of hydrolyzers}}{\text{concentration of hydrolyzers}+\text{concentration of non-hydrolyzers}} =$$

Calculating the fold enrichment: follow the example given at the beginning of this chapter

$$\text{Fold enrichment of hydrolyzers} = \frac{\text{\% hydrolyzing bacteria in enrichment (or control)}}{\text{\% baseline bacterial hydrolyzers}} =$$

Appendix G

Recipes and Ordering Information for Media

All media ordered from Thermo Fisher Scientific
Customer Service: +1 800-766-7000
Web Support: +1 877-885-2081

TSA + Tryptic Soy Agar (Reference no. BD Difco 236920)
Directions :
Suspend 40 g in 1 liter dH_2O
Mix , heat while stirring until completely dissolved
Autoclave 121°C for 15 min
Temper to 55°C
Pour 25 ml per plate

TSB= TRYPTIC Soy Broth (Reference No. BD 211825)
Directions:
Suspend 30 g in 1 liter dH_2O
Mix, heat while stirring until completely dissolved
Autoclave 121°C for 15 min.

Table 22. Supplies for Thermophiles in my Compost for a class of 24 students., 12 group 2 students per group.

Day 1			
Materials	**Comment**	**Materials/ grp**	**Total needed**
Compost Sample	1 g		1
TSB Room Temp	Broth (250 flask with 100 ml broth)		1
TSB 65°C	Broth (250 flask with 100 ml broth)		1
Tryptic Soy Agar (TSA)	Plates	8	96
MacConkey Agar	Plates	4	48
0.9% Sterile Saline	100 ml per bottle	1	12
Sterile test tubes		8	96
Bacterial Spreaders		1	12
Turn Table		1	12
Bunsen Burner		1	12
Ohaus Balance			3
Weigh Boat			1
65°C incubator			1

Day 2			
Materials	**Comment**	**Materials/ grp**	**Total needed**
Colony Counters	Observe plates/Count Colonies		3
Tryptic Soy Agar (TSA)	Plates	15	180
0.9% Sterile Saline	100 ml per bottle	1	12
Sterile test tubes		8	96
Bacterial Spreaders		1	12
Turn Table		1	12
Bunsen Burner		1	12
65°C incubator			1

Day 3			
Materials	**Comment**	**Materials/ grp**	**Total needed**
Colony Counters	Observe Plates and Count Colonies		3

Appendix H

Extreme Conditions: The Effect of Environmental Variability on Bacterial Survival

Expected Outcomes of this Section:

By the end of this reading, you should be able to:

- Explain the terms “range”, “optimum”, “extreme conditions”, etc
- Explain terms specific to each physical and chemical condition, e.g. acidophilic, acidoduric, alkaliphile, etc for pH, psychrophile, mesophile, thermophile, etc for temperature.
- Explain how to determine the salt, temperature, and pH tolerances of an organism.
- Demonstrate how extreme conditions can be used in separating organisms based on their specific adaptation.

Microbes live in close contact with their environment and are subject to the vagaries of changing physical and chemical factors. The ability to adapt to variations in the myriad conditions that influence their niches dictates the survival of the microbial strain. This experiment focuses on the effect of pH, temperature, and salt concentration. In other experiments in the course, you will examine the effect of oxygen levels and antimicrobial agents on the growth and distribution of bacteria.

The Effect of Temperature on Microbial Growth

Unlike mammals, which are capable of homeostasis, the temperature of a microbial cell is at the mercy of the environment. A patch of soil in your garden may shift from freezing temperatures at night to warm conditions during the day. If the growth rate (the ability to divide) is taken as a measure of the temperature sensitivity of an organism, the optimum growth temperature is the temperature at which the growth rate is highest. It is not surprising that the optimum growth temperature of a microbe is closely related to that of its natural habitat. The maximum growth temperature and the minimum growth temperature are the highest temperature and lowest temperature respectively, at which growth is possible. Different microbes have different degrees of tolerance to heat; some have a narrow range (between minimum and maximum growth temperature) indicating a high degree of sensitivity while others are more forgiving of temperature changes.

Organisms are classified according to the temperature range of their optimum growth temperature. Most commonly studied microorganisms grow at moderate temperatures with optimum growth temperatures between 28°C and 38°C. These organisms are called mesophiles; those that grow best at temperatures below 16°C are known as psychrophiles, and those at temperatures above 65°C as thermophiles. Microbes near the deep-sea hydrothermal vents are even known to thrive at temperatures close to 100°C (hyperthermophiles), the boiling point of water. The mesophilic temperature range is 20°C to 45°C, that of thermophiles is 45°C to 80°C, and that of psychrophiles is below 20°C. These ranges are approximate and slightly different values are obtained from different sources.

The growth ranges of microbes are of interest from many perspectives. Many pathogenic mesophiles have an optimum temperature of 37°C – normal human temperature. Raising temperatures high enough to kill mesophilic spoilage bacteria or maintaining food in the refrigerator at temperatures low enough to prevent their growth reduces food spoilage. However, psychrophilic spoilage bacteria do exist, accounting for the fact that food is not exempt from spoiling at low temperatures. Conversely, preserving milk by converting it to yogurt requires the activity of a high thermophilic bacterial strain *Streptococcus thermophilus*.

The Effect of Solute Concentrations on Microbial Growth

Microbes may be loosely described as membrane bound bags of fluid. The cytoplasm of a microbe contains a variety of dissolved materials, the concentrations of which are often higher than those obtained in their environments. Conversely, microbes may live in environments in which the concentration of some solutes is much higher than the intracellular concentration. It is therefore necessary to understand how differences between intracellular and extracellular solute concentrations may affect a microbial cell. Taking the example of a microbial cell in a freshwater environment, it is likely that the total solute concentration inside the cell is higher than that in the water. The intracellular and extracellular spaces are separated by a selectively permeable membrane, the cell membrane, which allows water to flow from a region of low solute concentration to a region of high solute concentration, a phenomenon known as osmosis. In this example, the higher solute concentration inside the cell creates osmotic pressure and the higher the intracellular solute concentration, the greater the osmotic pressure would be.

The effect of the movement of water due to osmotic pressure would be to cause an influx of water if a cell is placed in a medium of lower solute concentration and an efflux of water if a cell is placed in a medium of higher solute concentration. The influx of water and the rapid increase in cell volume could result in a rupture or explosion of the cell wall, leakage of cell material, and death of the cell, a phenomenon known as **plasmoptysis**. When bacterial cell death does occur this condition would be considered to be bacteriocidal. Conversely, an efflux of water from the cell causes shrinkage of the cell, concentration of

cellular contents, disruption of the cell wall-cell membrane interaction, and a reduction in metabolic activity due to the reduced intracellular water content. This condition is known as plasmolysis and may prevent cell growth even if it does not kill the cell (bacteriostatic).

The food industry harnesses these phenomena in food preservation strategies. Water can be removed from plant and animal tissues in a number of ways, including drying (beef jerky), adding sugar (jams and jellies), and salting (salted meat). In each case, the dehydration of the tissue promotes the plasmolysis of any bacterial cells in the food and by preventing metabolic activity, controls the spoilage of the food. However, it should be noted that many yeasts and molds (fungi) are more tolerant of high osmotic pressure and may continue to grow on or in food that has been treated in this manner.

The Effect of pH on Microbial Growth

The pH of an environment changes when acid is produced or removed. Thus milk becomes more acidic as bacteria ferment lactose and produce lactic acid. Soil pH decreases due to the decomposition of woody plant materials and the consequent release of humic acids and increases when alkaline minerals such as limestone leach into it. Like most organisms, microbes have a definite range of pH values that they can tolerate. We use this on a daily basis, preserving food through acidification by microbial fermentation (kimchi, sauerkraut, pickles, yogurt). The low pH kills or prevents the growth of spoilage bacteria. It should be noted, however, that the very process of preserving food through microbial activity often requires microbes that are themselves acid tolerant or acid loving. For example, the formation of yogurt from milk requires two bacterial strains *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, both of which are resistant to low pH.