



THE BIOTECHNOLOGY  
EDUCATION COMPANY®



Edvo-Kit #

**956**

Edvo-Kit #956

## **Bioremediation By Oil-Eating Bacteria**

### **Experiment Objective:**

In this exploration, students experimentally determine the optimum conditions for microbes to break down the components of oil. They will determine the number of active microbes in the sample using the standard bacterial plate counts and the tetrazolium indicator.

**See page 3 for storage instructions.**

# Table of Contents

	Page
Experiment Components	3
Experiment Requirements	3
Background Information	4
Experiment Procedures	
Experiment Overview	7
Module I: Preparing the OEM Starter Culture	8
Module II: Analyzing the Growth of OEMs in Response to Various Environmental Conditions	9
Module III: Standard Plate Count	11
Module IV: Tetrazolium Testing	13
Study Questions	14
Instructor's Guidelines	15
Pre-Lab Preparations	19
Experiment Results and Analysis	23
Study Questions and Answers	24

Safety Data Sheets can be found on our website: [www.edvotek.com/Safety-Data-Sheets](http://www.edvotek.com/Safety-Data-Sheets)

## EDVO-TECH Service

**1.800.EDVOTEK**

Mon. - Fri. 8am-5:30pm EST



### Please Have the Following Info:

- Product Number & Description
- Lot Number on Box
- Order/Purchase Order #
- Approx. Purchase Date

Fax: 202.370.1501 • [info@edvotek.com](mailto:info@edvotek.com) • [www.edvotek.com](http://www.edvotek.com)

**[www.edvotek.com](http://www.edvotek.com)**

- Online Catalog
- Order Products
- Experiment Protocols
- Tech Support
- Resources!



1.800.EDVOTEK • Fax 202.370.1501 • [info@edvotek.com](mailto:info@edvotek.com) • [www.edvotek.com](http://www.edvotek.com)

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 1989-2015 EDVOTEK, Inc., all rights reserved. 956.150925

## Experiment Components

### Component

- Oil-eating microbes (powder)
- Growth media
- Petri plates
- 1N HCl
- 1M NaOH
- Tetrazolium Indicator Powder
- pH paper
- 10 ml pipet
- Transfer pipets
- Individually wrapped inoculating loops

### Check (✓)

☐  
☐  
☐  
☐  
☐  
☐  
☐  
☐  
☐  
☐

Experiment #956 is designed for 10 groups.

**Storage:**  
Store entire experiment in the refrigerator.

## Requirements

- Shaking platform or stir plate (with stir bar)
- Glassware
- Various cooking oils such as vegetable, canola, olive, peanut, etc.
- Balance
- Distilled Water
- Pipet pumps
- Spectrophotometer (optional)

EDVOTEK and The Biotechnology Education Company are registered trademarks of EDVOTEK, Inc.

1.800.EDVOTEK • Fax 202.370.1501 • [info@edvotek.com](mailto:info@edvotek.com) • [www.edvotek.com](http://www.edvotek.com)

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 1989-2015 EDVOTEK, Inc., all rights reserved. 956.150925



## Background Information

Potentially harmful chemicals are introduced into our environment on a daily basis. Some of the chemicals that contaminate our soil and groundwater result from the breakdown of compounds found in nature. Ammonia, for example, is a naturally occurring chemical byproduct from the bacterial breakdown of nitrogen-rich plant and animal materials. Unfortunately, human activities such as pollution, waste disposal, and industrial spills often augment natural contaminants and can lead to significant ecological harm. In addition, human activity alone is responsible for certain contamination in the environment. Both the presence and the concentration of contaminants pose a threat to the environment, making the process of monitoring and decontaminating the environment essential.

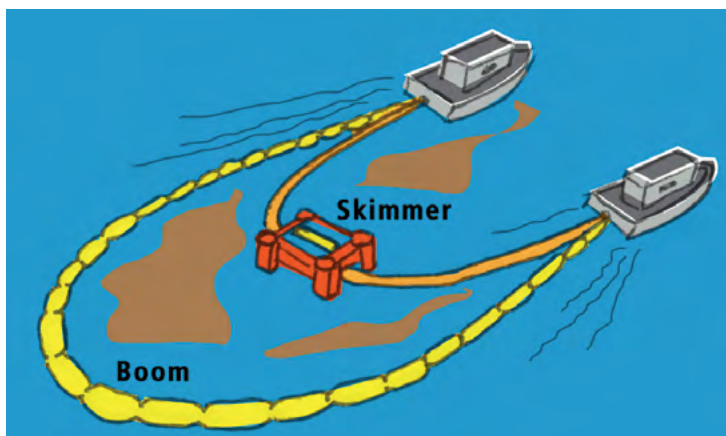
To prevent contaminants from entering the food chain, naturally occurring microorganisms remove organic waste, heavy metals, and crude oil from the environment. These microorganisms have even evolved to degrade man-made waste like pesticides, herbicides, and detergents. Just like our bodies generate chemical energy from the food we eat to survive, these microbes make the energy necessary to live and to multiply by breaking down the chemical bonds in environmental pollutants.

One major environmental hazard is crude oil. Many industrialized nations rely upon crude oil for products like plastics, fuel and asphalt, and it is vital to many industries. Although the technology exists to extract, refine, and transport oil safely around the world, millions of gallons of oil are unintentionally released into the environment each year. These disastrous events pose a threat to humans and the natural environment, leading to billions of dollars in cleanup and restoration (Figure 2). For example, the Deepwater Horizon oil spill devastated the Gulf of Mexico in April 2010. This oil rig, located just 42 miles off the coast of Louisiana, exploded during a routine drilling operation on the ocean floor. Over 200 million gallons of oil spilled into the ocean over the course of 87 days. A more recent spill occurred on January 17th, 2015, when a pipeline leaked 50,000 gallons of crude oil into the Yellowstone River. The local farming and ranching communities were warned not to ingest the tap water due to elevated levels of the volatile organic compound benzene.

Due to the extensive environmental damage from the crude oil, various strategies are used in concert to remediate lost oil depending upon the impact of the spill. Often, the first methods involve physical removal of the oil using booms and skimmers. Booms prevent oil from spreading beyond the spill site by containing it within a dedicated space. Skimmers are then used to collect the oil



**Figure 1:** Oil Spills are harmful for marine life (photo credit NOAA and Georgia Department of Natural Resources, CC-BY 2.0)



**Figure 2:**  
Oil Spill response methods - booms and skimmers

## Background Information, continued

contained within the booms (Figure 2). Another physical method used involves burning the spilled oil on a body of water before it reaches the coast. Chemical dispersants can be sprayed across an oil spill, where they break large oil slicks into smaller droplets that are more easily cleared from the water's surface.

Unfortunately, physical cleanup strategies are laborious and expensive. The equipment used for physical cleanup is very expensive and does not recover all the crude oil dumped into the ocean. Furthermore, certain chemical dispersants may be dangerous for the marine environment. These chemicals harm marine life by causing mutations in their DNA, resulting in deformities like crabs without claws, fish without eyes, and shrimp with tumors. However, the greatest limitation of using these traditional strategies is that they do not completely remove the oil from the contaminated site.

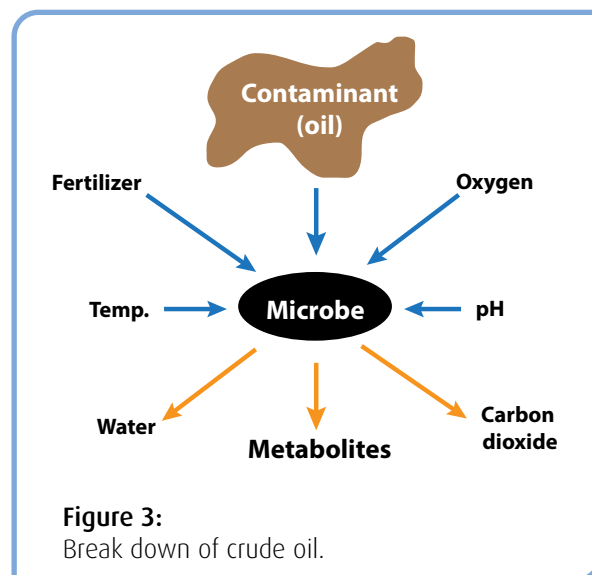
Realizing the need to minimize the overall environmental and economic impact, scientists and environmentalists worked together to develop a safer alternative known as bioremediation. This strategy uses oleophilic ("oil-loving") bacteria and other microorganisms (yeast and fungi) to break down and cleanup the byproducts of various manufacturing and chemical industries. This biological response method is not only safe and inexpensive, but also maximizes the removal of oil when coupled with physical and chemical strategies.

Oleophilic microbes (a.k.a. Oil Eating Microbes or OEMs) are normally found in marine environments where they feed upon the variety of compounds that make crude oil. It is a complex mixture of hydrocarbons – chemicals composed of carbon and hydrogen. There are three basic types of hydrocarbons: straight chains, branched chains, and six-membered rings. These microbes separate hydrocarbons into fatty acids, which are further split into carbon atoms to be reused by the bacteria in metabolism. Thus, oil is broken down into basic, non-toxic elements – metabolites, carbon dioxide and water (Figure 3).

In nature, these communities of microorganisms work together to help process low levels of contamination over time. An example of an OEM that has been doing its biological janitorial work in the Gulf of Mexico is *Alcanivorax*, which consumes a broad range of hydrocarbon alkanes. *Alcanivorax* is generally found in low numbers in the Gulf of Mexico; in the wake of oil spills, the population increases significantly, ensuring the health of the marine and coastal environment. From our perspective, oil-eating microbes like *Alcanivorax* clean up the environment; from the microbes' perspective, they are consuming oil to live and to grow. The oil spilled in coastal areas would persist if it were not for the bacteria and other microorganisms breaking down excess crude oil.

Although naturally occurring microorganisms decompose small amounts of oil and other environmental contaminants, they are not plentiful enough to handle the amount of waste found in large spills. In this situation, scientists supplement the spill zone with a concentrated culture of live and active oil-eating microbes in a process called bio-augmentation. After being mass-produced in industrial fermenters, these cultures are released into the contaminated body of water to help the indigenous microbial population process the oil contamination.

Like all microorganisms, OEMs require a hospitable environment to thrive and to mitigate the environmental damage. For optimal activity, OEMs should be cultured at a temperature between -2°C and 60°C and a pH between 5.5 and 10. Furthermore, lack of oxygen and nutrients can inhibit bioremediation by OEMs. Since these conditions are essential for bioremediation, scientists will often enrich water with



## Background Information, continued

oxygen and nutrients in a process called biostimulation. This technique revives dormant microbes, allowing them to once again feed on the spilled oil.

In this inquiry-based experiment, students will simulate the bioremediation process using OEMs to digest food oils. Students empirically determine the optimal growth conditions for the OEMs by changing growth conditions like temperature, pH or bacterial concentration. The freeze-dried OEM mixture included with this experiment contains bacteria from the *Pseudomonas*, *Flavobacterium*, *Arthobacter*, and *Azotobacter* genera. Although this bacterial consortium is best suited for fresh water “oil spills”, we encourage students and teachers to try increasing concentrations of salts to mimic a marine environment.

A tetrazolium indicator dye is used to detect active OEMs while culturing the bacteria in different growth conditions. This indicator is colorless in its native form, but it is rapidly converted into a red dye by metabolically active bacteria. Since only live and active bacteria can perform this reaction, tetrazolium allows for the quantification of live bacteria within a solution. Scientists have used tetrazolium to detect active bacteria in different environmental samples including soil, groundwater, and aquatic environments. In this experiment, the indicator provides a simple and fast way to detect the bacteria at work instead of waiting weeks for the oil to disappear. Finally, students will quantify the number of live OEMs within each sample by analysis with the standard plate count and use these values to determine optimal conditions for OEM bioremediation.





## Experiment Overview

### EXPERIMENT OBJECTIVE:

In this exploration, students experimentally determine the optimum conditions for microbes to break down the components of oil. They will determine the number of active microbes in the sample using the standard bacterial plate counts and the tetrazolium indicator.

### LABORATORY SAFETY:

Be sure to READ and UNDERSTAND the instructions completely BEFORE starting the experiment. If you are unsure of something, ASK YOUR INSTRUCTOR!

1. Wear gloves and goggles while working in the laboratory.
2. Exercise caution when working in the laboratory – you will be using equipment that can be dangerous if used incorrectly.
3. Wear protective gloves when working with hot reagents like boiling water and melted agarose. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
4. The bacteria used in this experiment are not considered pathogenic. Regardless, it is good practice to follow simple safety guidelines in the handling and disposal of materials contaminated with bacteria.
  - A. Wipe down the lab bench with a 10% bleach solution or a laboratory disinfectant.
  - B. All materials, including petri plates, pipets, transfer pipets, loops and tubes, that come in contact with bacteria should be disinfected before disposal in the garbage. Disinfect materials as soon as possible after use in one of the following ways:
    - Autoclave at 121° C for 20 minutes. Tape several petri plates together and close tube caps before disposal. Collect all contaminated materials in an autoclavable, disposable bag. Seal the bag and place it in a metal tray to prevent any possibility of liquid medium or agar from spilling into the sterilizer chamber.
    - Soak in 10% bleach solution. Immerse petri plates, open tubes and other contaminated materials into a tub containing a 10% bleach solution. Soak the materials overnight and then discard. Wear gloves and goggles when working with bleach.
  - C. Wear gloves, and at the end of the experiment, wash hands thoroughly with soap and water.



### LABORATORY NOTEBOOKS:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

#### Before starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

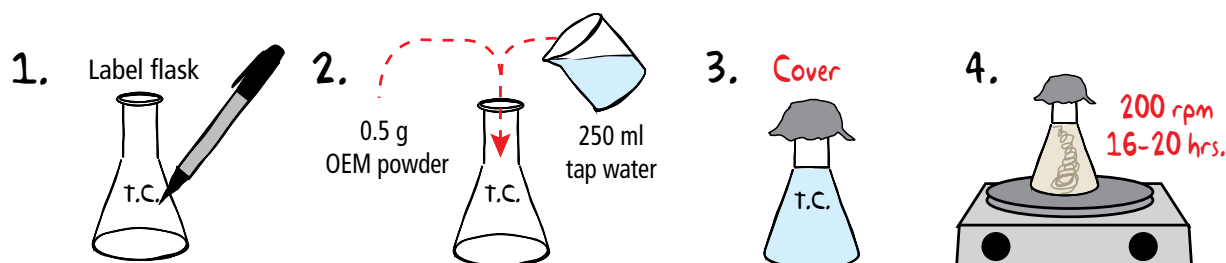
#### During the Experiment:

- Record your observations.

#### After the Experiment:

- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

## Module I: Preparing the OEM Starter Culture



1. **LABEL** a 500 ml flask with your name or group number.
2. **ADD** 0.5 g of OEM powder to 250 ml of tap water in a 500 ml flask.
3. **COVER** the neck of the flask with a lid or aluminum foil.
4. **INCUBATE** the culture at room temperature for 16-20 hours or until the solution appears milky. During this incubation, the solution should be stirred at 200 rpm using a clean stir bar. (Alternatively, the culture can be placed on a shaking platform.)
5. After the incubation, **PROCEED** to Module II: Analyzing the Growth of OEMs in Response to Various Environmental Conditions

**NOTE:**

Two student groups will share 250 ml starter culture. See the instructor's guide (page 16) for information on setting up cultures of other sizes.



**Wear gloves  
and safety goggles**



## Module II: Analyzing the Growth of OEMs in Response to Various Environmental Conditions

---

In this module, students will design experiments to determine the optimum conditions for microbes to break down oil. While we provide four different experimental setups in this section, students should not be limited to these options. For all conditions, students should create a Water Control sample (OEMs plus water) and an Oil Control sample (OEMs plus water and oil) that are kept at room temperature.

### **Suggestion 1: What is the Optimum Temperature for OEM Growth?**

While room temperature has been effective for culturing the OEMs, what would be the effect of changing the temperature on microbial growth? If an incubator or a refrigerator is readily available, try incubating the culture at different temperatures.

### **Suggestion 2: What is the Optimum pH for OEM Growth?**

While OEMs can grow over a wide range of pH values, what is the optimal pH? In this experiment, students can use dilute NaOH (a base which raises the pH) and dilute HCl (an acid which lowers the pH) to adjust the pH of the OEM growth medium used. Be sure to use pH paper to measure the pH of the cultures before performing the experiment.

### **Suggestion 3: What is the optimum salinity for OEM growth?**

Although the OEMs included with this kit are best suited for fresh water, can they break down oil in brackish or salt water? Sea water has a salinity of about 3.5%, with the main dissolved salt being NaCl. In this experiment, students can use NaCl to adjust the salinity of the OEM growth medium.

### **Suggestion 4: What effect do nutrients have on OEM Growth?**

The addition of nutrients (biostimulation) enhances the ability of OEMs to break down oil. For this experiment, students can add a small amount of different microbial nutrients to their cultures to observe the effects of these additives on OEM activity. Alternatively, they can try to determine the optimal level of a specific nutrient. If the appropriate equipment is available, students can also experiment with aeration.

## Module II: Analyzing the Growth of OEMs in Response to Various Environmental Conditions

**1. Label**

**2. Add**  
25 ml Starter culture

**3. Add**  
0.5 ml Tetrazolium

**4. Add**  
Reagents for experiment

**5. Add**  
1 ml food oil

**6. Incubate**  
20-24 hours

**7. Observe**

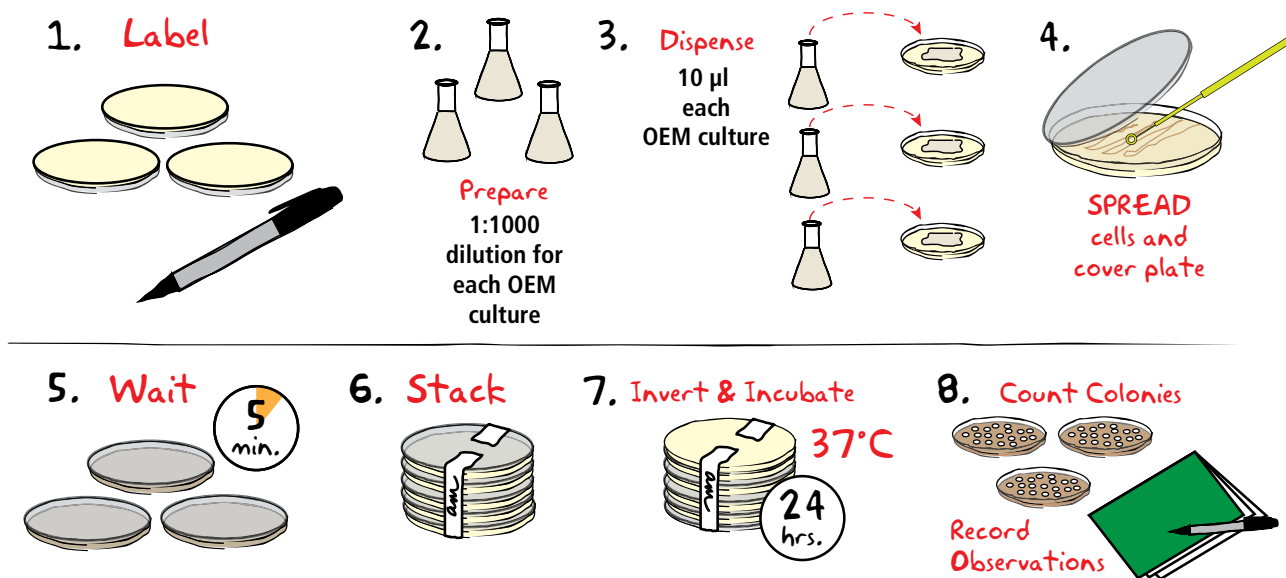
**8. Incubate**  
24 hours if necessary

- LABEL** the culture vessels for each different condition being tested. Be sure to label one vessel "Water Control" and a second vessel "Oil Control".
- DISPENSE** 25 ml aliquots of starter culture into the labeled vessels. Be sure to keep culture stirring while dispensing the samples.
- ADD** 0.5 ml of tetrazolium indicator to each sample. **MIX** well.
- ADD** any reagents necessary to perform the experiments.
- ADD** 1 ml of food oil to all of the samples except the Water Control.  
**Note:** Oil is viscous. Use care while measuring to ensure accurate volumes.
- INCUBATE** samples at room temperature for 20-24 hours. If testing the effects of temperature on OEM growth, incubate the samples at the appropriate temperature.
- After the incubation, **OBSERVE** and **RECORD** the appearance of the culture. **PROCEED** to Module III: Standard Plate Count.
- If a color change in the Oil Control sample is not obvious after 24 hours (milky-white to red), **INCUBATE** the samples for an additional 24 hours before proceeding to Module IV: Tetrazolium Absorbance Testing.



Sample	Culture	Tetrazolium Indicator	Food Oil	Additional Reagents
Water Control	25 ml	0.5 ml	-----	-----
Oil Control	25 ml	0.5 ml	1 ml	-----
Experimental Samples	25 ml	0.5 ml	1 ml	Varies

## Module III: Standard Plate Count



The Standard Plate Count allows us to measure oil degradation by determining live, healthy bacteria present in the culture. First, a dilution of each control and experiment culture is prepared. The dilutions are plated on LB-agar plates and allowed to grow overnight. The next day, the colonies formed on the agar plate are counted and multiplied by the dilution factor. This calculation determines the concentration of the culture in colony-forming units (CFU) per milliliter. In general, favorable environmental conditions should have a higher CFU than the controls. Unfavorable conditions will have a lower CFU than the controls.

1. **LABEL** the bottom of one LB-agar plate for each culture.
2. **PREPARE** a 1:1000 dilution of each OEM culture. Please note that the bacteria may settle to the bottom of the flask. Be sure to mix the culture well before removing cells for dilution.
3. **DISPENSE** 10 µl of each dilution onto the labeled agar plate.
4. **SPREAD** the cells over the entire plate with a sterile inoculating loop. Make sure the cells have been spread over the entire surface of the plate. **COVER** the plate. **REPEAT** with the other cultures. Be sure to use a fresh, sterile loop for each plate.
5. **WAIT** five minutes for the cell suspension to be absorbed by the agar.
6. **STACK** the plates on top of one another and **TAPE** them together. **LABEL** the plates with your initials or group number.
7. **PLACE** the plates in the inverted position (agar side on top) in a 37° C bacterial incubation oven for overnight incubation (18-24 hours).
8. **REMOVE** plates after the overnight incubation. **COUNT** and **RECORD** the number of colonies on each plate.



Wear gloves  
and safety goggles

### Module III: Standard Plate Count

**RECORD** your observations in Table 1. Make note of size, color, and appearance of colonies. Be sure to count each colony on a dilution plate if possible. If your plate has too many colonies to easily count, try dividing the plate into sections. Count the number of colonies in that section, then multiply by the number of sections.

TABLE 1: STANDARD PLATE COUNT		
Culture	Observations (Number, size, appearance of colonies)	CFU/ml
Oil Control Sample		
Water Control Sample		
Experimental Sample 1		
Experimental Sample 2		
Experimental Sample 3		

Calculate the Colony Forming Units (CFU) per ml using the formula below:

$$\frac{\text{\# of Colonies}}{\text{Dilution factor} \times \text{Volume plated (mL)}} = \text{CFU/mL}$$

## Module IV: Tetrazolium Testing

The tetrazolium indicator dye (added in Module II) is used to detect live and active OEMs while culturing the microbes in different growth conditions. This indicator is colorless in its native form, but it is rapidly converted into a red dye by metabolically active bacteria. The color change allows for the quantification of live bacteria within a solution.

After 24 hours, observe the color and turbidity (cloudiness) of the cultures. Note these observations in the table below. If a color change is not obvious in the Oil Control sample after 24 hours, incubate the samples for an additional 24 hours before measuring the absorbance.

TABLE 2: TETRAZOLIUM TESTING		
Culture	Observations (color, turbidity)	Absorbance at 500 nm (optional)
Oil Control Sample		
Water Control Sample		
Experimental Sample 1		
Experimental Sample 2		
Experimental Sample 3		

As an optional STEM activity, we recommend measuring and recording the absorbance of each sample containing the tetrazolium indicator.

1. **TRANSFER** 1-2 ml of water into a cuvette. (Exact volume will be dependent on your spectrophotometer.)
2. **BLANK** your spectrophotometer with water.
3. **TRANSFER** 1-2 ml of culture from the Oil Control tube into a cuvette. Be sure to avoid the oil layer.
4. **READ** absorbance at 500 nm. To obtain accurate absorbance readings, **DO NOT WAIT** for the cells to settle down in the cuvette.
5. **RECORD** the absorbance reading.
6. **REPEAT** steps 3-5 with the Water Control sample and each experimental sample. Be sure to avoid the oil layer.
7. **GRAPH** the values to determine the best growth condition for the OEMs.



Wear gloves and  
UV safety goggles

## Study Questions

---

1. Define oleophilic microbes and bioremediation.
2. Describe the process for cleaning up an oil spill using oil eating bacteria.
3. What three things do OEMs need to work successfully? What other parameters affect growth and efficacy?
4. What are the benefits of using OEMs if the environment contains organisms that can clean up oil spills naturally?



# Instructor's Guide

## **GUIDELINES FOR PERFORMING INQUIRY-BASED EXPERIMENTS**

In this experiment, students will test various parameters to determine the optimum conditions for microbes to break down the components of oil. The following guidelines are provided to help instructors prepare for this experiment.

Preparation For:	What to do:	When:	Time Required:
<b>Module I: Preparing the OEM Starter Culture</b>	Prepare starter culture	24 hours before performing Module II.	20 min.
<b>Module II: Analyzing the Growth of OEMs in Response to Various Environmental Conditions</b>	Conceptualize experiment and gather reagents.	Any time before performing Module II.	Varies by student group
	Prepare tetrazoleum reagent	One day before performing the experiment.	20 min.
<b>Module III: Standard Plate Count</b>	Pour plates	One week to one day before performing the experiment.	60 min.
<b>Module IV : Tetrazolium Testing</b>	Warm up spectrophotometer (optional)	Before the class period.	Refer to instrument for guidelines

### **EDVO-TECH Service**

**1.800.EDVOTEK**

Mon. - Fri. 8am-5:30pm EST

#### **Please Have the Following Info:**

- Product Number & Description
- Lot Number on Box
- Order/Purchase Order #
- Approx. Purchase Date

Fax: 202.370.1501 • [info@edvotek.com](mailto:info@edvotek.com) • [www.edvotek.com](http://www.edvotek.com)



**[www.edvotek.com](http://www.edvotek.com)**

- Online Catalog
- Order Products
- Experiment Protocols
- Tech Support
- Resources!



1.800.EDVOTEK • Fax 202.370.1501 • [info@edvotek.com](mailto:info@edvotek.com) • [www.edvotek.com](http://www.edvotek.com)

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 1989-2015 EDVOTEK, Inc., all rights reserved. 956.150925



## Pre-Lab Preparations

### MODULE I: PREPARING THE OEM STARTER CULTURE

The OEM powder must be rehydrated in water and cultured at room temperature for ~24 hours before beginning the inquiry experiments. This incubation period allows the dormant microbes to begin to grow and multiply. During this incubation, we recommend stirring the solution at 200 rpm using a clean stir bar. Alternatively, the culture can be placed on a shaking platform. Sediment may be observed at the bottom of the culture vessel. This is normal and does not affect the results.

Each student group can prepare their own starter cultures, or the instructor can prepare enough for the class. All glassware should be thoroughly washed with soap and water and triple rinsed before using. Regular tap water should be used to prepare the starter culture.

We recommend splitting the class into 10 student groups. Each group will require at least 125 ml of the starter culture, which will be split into five 25 ml samples. This represents a water control (bacteria and water), an oil control (bacteria, water and oil), and three experimental samples. If students wish to test more than three conditions, the volume of starter culture should be adjusted accordingly.

This kit includes enough OEM powder to make about 5 L of bacterial culture. In general, 2 g of OEM powder should be dissolved in 1 L of tap water. The table below suggests several different ways to prepare starter depending on which glassware is available in the classroom. By maintaining the 2 g OEM to 1 L water ratio it is possible to scale the starter culture based on the number of student groups per class and the number of conditions being tested by each group.

Number of groups	Glassware to use	Number of flasks	Volume of Water (L)	Amount of OEM (g)
8	2 L flask	1	1	2
10	500 ml flask	5	0.25 L per flask	0.5 g per flask
10	250 ml flask	10	125 ml per flask	0.25 g per flask

## Pre-Lab Preparations

### MODULE II: ANALYZING THE GROWTH OF OEMS IN RESPONSE TO VARIOUS ENVIRONMENTAL CONDITIONS

#### Experimental Design

In the student protocol, we present four different scenarios that can be tested. However, we highly recommend that students should form a hypothesis and determine which conditions would test that hypothesis. Before performing Module II, the teacher should ensure that any additional reagents are available.

**Each Group should receive:**

- 125 ml OEM starter culture
- 5 small culture vessels
- 5 ml oil
- 3 ml Tetrazolium Indicator Solution
- Reagents necessary to test hypothesis.

#### Preparing the OEM Cultures

Each group should receive 125 ml starter culture, which is enough for each student group to set up five samples – the oil control, the water control, and three experimental conditions. The starter culture should be aliquoted into smaller volume vessels (small flasks or tubes, covered beakers, baby food jars, or small plastic food vessels).

#### Selecting An Appropriate Oil Source

Student groups may choose to explore whether the OEMs can metabolize different types of oils. Olive oil, corn oil, vegetable oil and mineral oil have been used in our laboratory with success. Each group will need at least 4 ml oil for the experiment. We recommend providing each group with about 5 ml of oil to account for pipetting difficulties.

#### Preparation of the Tetrazolium Indicator

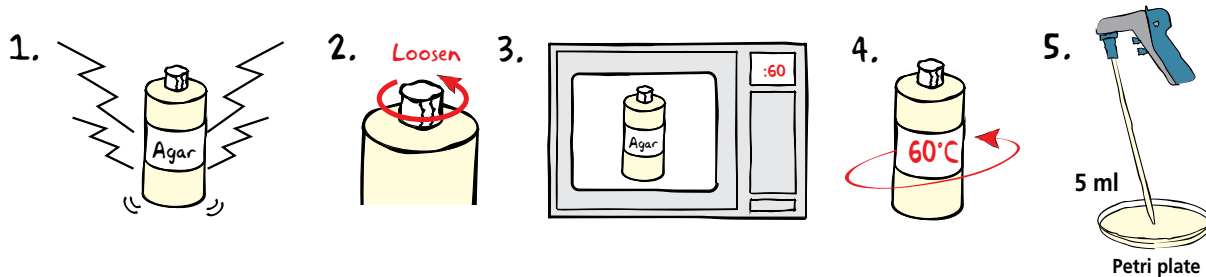
Prepare the Tetrazolium Indicator one day before performing Module II.

1. Add the entire amount of Tetrazolium indicator powder to 30 ml of distilled water in a flask. Mix well.
2. Store the indicator in the refrigerator overnight. We recommend wrapping the flask in foil to protect the indicator solution from light.
3. Before performing the experiment, dispense 3 ml of the indicator into tubes for each of the groups. Alternatively, the stock solution can be placed at a classroom pipetting station for students to share.

## Pre-Lab Preparations

### MODULE III: STANDARD PLATE COUNT

#### Agar Plate Preparation for Standard Plate Count



Sterile agar plates should be prepared at least one day before the laboratory experiment.

1. **BREAK** solid ReadyPour™ LB Agar into small chunks by vigorously squeezing and shaking the plastic bottle.
2. **LOOSEN**, but DO NOT REMOVE, the cap on the ReadyPour™ Agar bottle. This allows the steam to vent during heating. **CAUTION:** Failure to loosen the cap prior to heating may cause the bottle to break or explode.
3. **MICROWAVE** the ReadyPour™ Agar on high for 60 seconds to melt the agar. Carefully **REMOVE** the bottle from the microwave and **MIX** by swirling the bottle. Continue to **HEAT** the solution in 30-second intervals until the agar is completely dissolved (the amber-colored solution should be clear and free of small particles).
4. **COOL** the ReadyPour™ Agar to 60° C with careful swirling to promote even dissipation of heat.
5. Using a sterile 10 ml pipet, **POUR** 5 ml of the agar into 60 x 15 mm petri plates provided.
6. **COVER** and **WAIT** at least twenty minutes for the LB-agar plates to solidify. For optimal results, leave the plates at room temperature overnight.
7. **STORE** plates at room temperature for no more than two days. Plates should be inverted and placed in a sealable plastic bag to ensure that they do not dry out.



Wear Hot Gloves and Goggles during all steps involving heating.

#### NOTE:

This kit includes enough media to make 20 plates. Additional plates (Cat. #633) and Ready-Pour™ LB (Cat. #615) can be purchased from EDVOTEK®.

**NOTE:** If plates are prepared more than two days before use, they should be allowed to dry at room temperature overnight (step 6) and then stored inverted in a plastic bag in the refrigerator (4°C). Remove the plates from the refrigerator and warm in a 37°C incubator for 30 minutes before use.

#### Quick Reference: Pouring LB Agar Plates

- Use a sterile 10 ml pipet with a pipet pump to transfer the designated volume of medium to each petri plate. Pipet carefully to avoid forming bubbles.
- Rock the petri plate back and forth to obtain full coverage.
- If the molten medium contains bubbles, they can be removed by passing a flame across the surface of the medium.
- Cover the petri plate and allow the medium to solidify.

## Experiment Results and Analysis

### CONTROLS FOR INQUIRY EXPERIMENTS

#### NOTES:

Only one set of control experiments is presented in this section. These results are representative of the controls for all four modules. In the following experiments, we used 1 ml of vegetable oil as a food source for 25 ml OEM culture. The starter culture was prepared by culturing 2 g OEM in 1 L water and incubating for 16-20 hr at room temperature.

Several colony morphologies may be present on the plate because the OEM powder contains four different species of bacteria.

A red color may be observed in the Water Control sample, even though the bacteria do not have an added food supply. This results from nutrients left over from the initial culture.



*Bacteria/water control*  
CFU –  $1.2 \times 10^7$



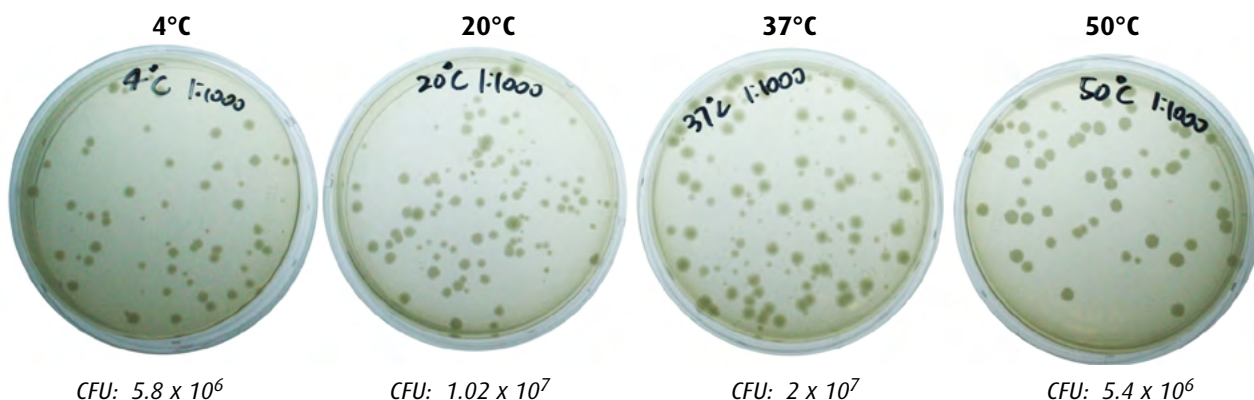
*Bacteria/water/oil control*  
CFU –  $1.02 \times 10^7$

### INQUIRY EXPERIMENT 1:

#### WHAT IS THE OPTIMAL TEMPERATURE FOR OEM GROWTH AND ACTIVITY?

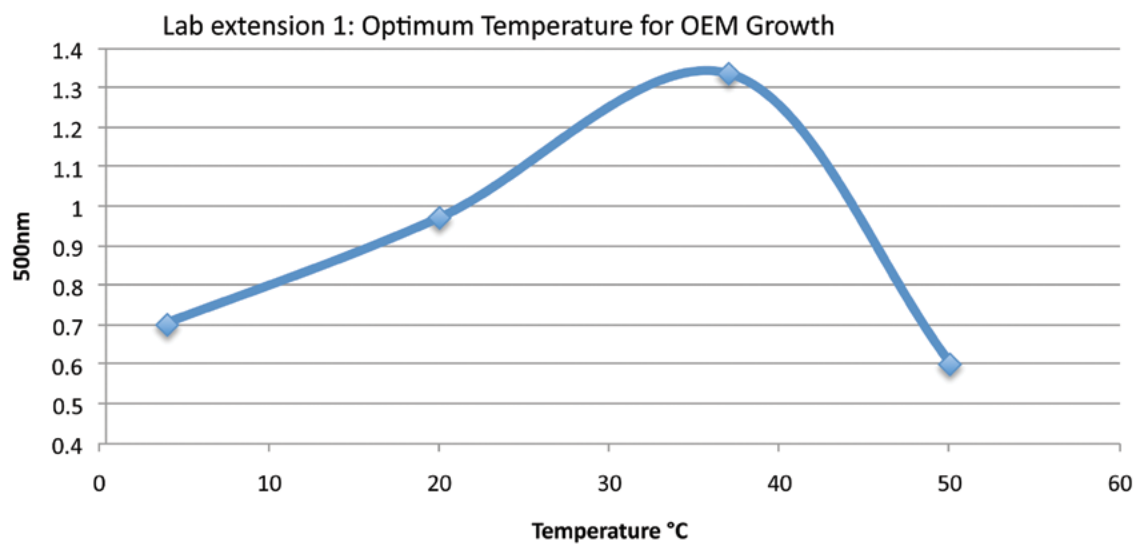
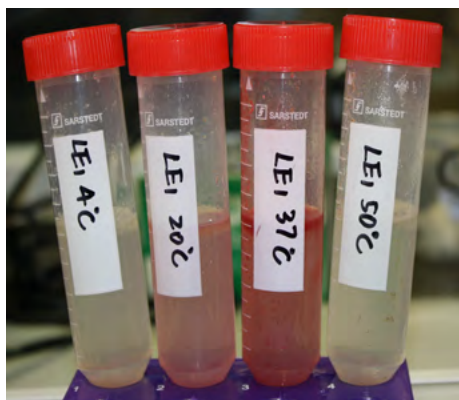
In this module, students are encouraged to choose three to four temperature conditions to test. The temperatures tested by EDVOTEK® were 4°C, 20°C, 37°C, and 50°C. It is important that temperatures are maintained accurately throughout the experiment. An incubation oven is recommended for the higher temperature conditions. A refrigerator can be used for the 4°C and 20°C temperature conditions.

#### Standard Plate Count



## Experiment Results and Analysis

### INQUIRY EXPERIMENT 1: TETRAZOLIUM ABSORBANCE TESTING





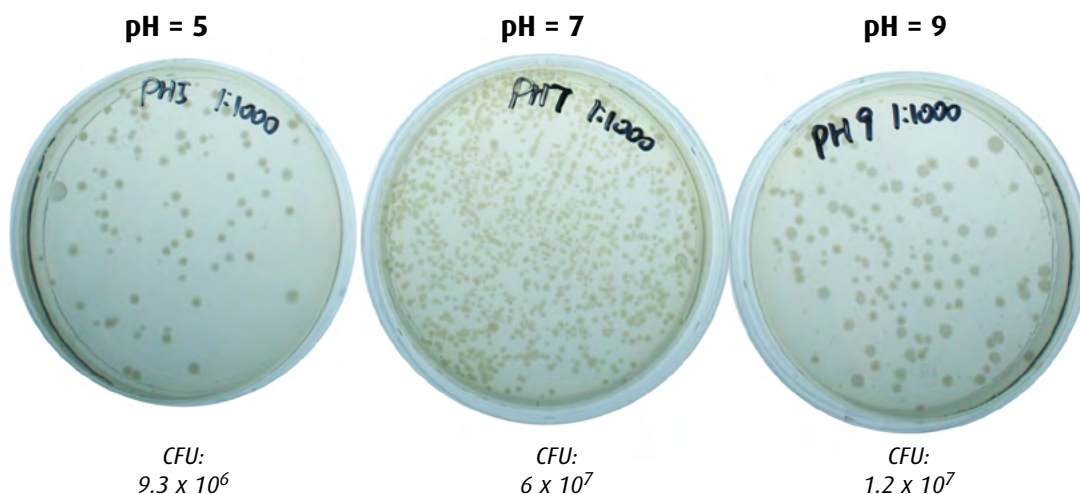
## Experiment Results and Analysis

### INQUIRY EXPERIMENT 2:

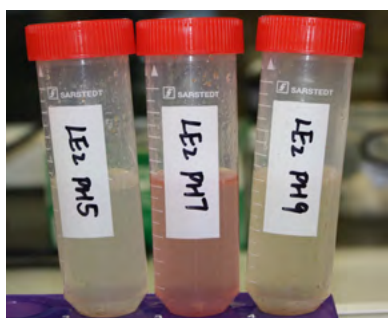
#### WHAT IS OPTIMAL PH FOR OEM GROWTH AND ACTIVITY?

In this module, students are encouraged to choose three to four pH values to test. The pH values tested by EDVOTEK® are 5, 7, and 9. Diluted NaOH and HCl (included with kit) should be used to adjust the pH of their cultures. The included pH paper should be used to measure the final pH of the cultures.

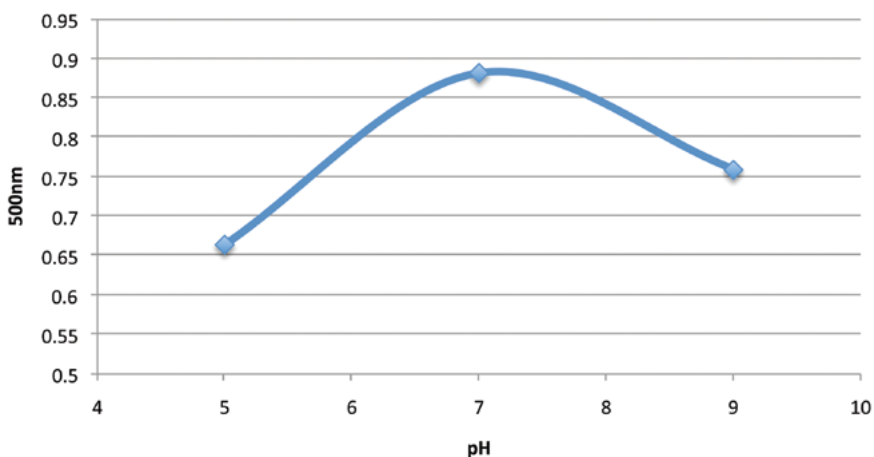
#### Standard Plate Count



#### TETRAZOLIUM ABSORBANCE TESTING



#### Lab Extension 2: Optimum pH for OEM Growth



## Experiment Results and Analysis

### INQUIRY EXPERIMENT 3: WHAT IS THE OPTIMUM SALINITY FOR OEM GROWTH?

In this module, students are encouraged to research the salinity of water in order to test its effects on OEM activity. Students can adjust the salinity of the OEM culture using NaCl. The salinity values tested by EDVOTEK® were 1.75 g/L NaCl and 3.5 g/L NaCl (seawater).

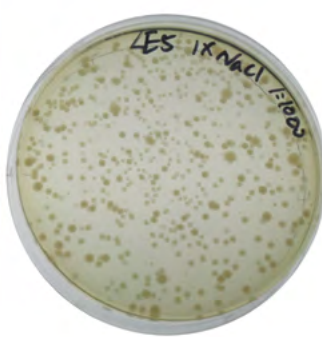
#### Standard Plate Count

1.75 g/L NaCl



CFU:  $5.44 \times 10^7$

3.5 g/L NaCl



CFU:  $4.5 \times 10^7$

#### Tetrazolium Testing



### INQUIRY EXPERIMENT 4: WHAT IS THE EFFECT OF ADDING NUTRIENTS TO STIMULATE OEM GROWTH AND ACTIVITY?

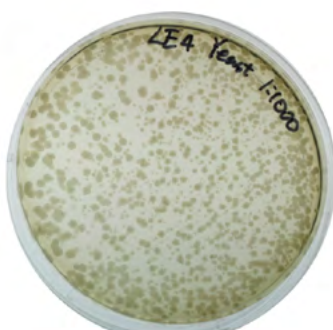
In this module, students are encouraged to research microbial nutrients and to explore the effects of these nutrients on OEM activity. Students can design experiments to test several different nutrients or different concentrations of the same nutrient. Glucose (0.5 g) and yeast extract (0.5g) were tested in the EDVOTEK® lab. Please note that the addition of some nutrients may change the color of the culture (for example, yeast extract darkens the milky-white culture to a golden brown color).

Glucose



CFU:  $1.2 \times 10^7$

Yeast



CFU:  $6.2 \times 10^7$

#### Tetrazolium Testing



**Please refer to the kit  
insert for the Answers to  
Study Questions**