Marine Microbes: Culturing the “Unculturable”

Grade Level: Middle or High School
Subjects: Marine Science, Ecology, Chemistry, Math, Writing
Duration: Three 45-minute class periods
Group Size: 3-4 students
Setting: Indoors

NC Essential Standards:
Bio.2.1.1. Analyze the flow of energy and cycling of matter (water, carbon, nitrogen and oxygen) through ecosystems relating the significance of each to maintaining the health and sustainability of an ecosystem.

Bio.2.1.3. Explain various ways organisms interact with each other (including predation, competition, parasitism, mutualism) and with their environments resulting in stability within ecosystems.

Objective:
To culture marine bacteria while considering microbial growth requirements, symbioses, microbial food web interactions, and human impacts on marine organisms.

Key Concepts:
- The abundance and diversity of bacteria in the ocean
- The challenges and methods of studying marine bacteria
- The requirements for microbial growth and how to culture microorganisms in the lab
- The roles of heterotrophic bacteria in marine ecosystems: nutrient recyclers, trophic links, and positive & negative interactions with other organisms

Materials/Resources:
- Heating plate or microwave
- Large glass flask, beaker, or mixing bowl
- Oven mitt
- Muffin baking tray(s)
- Foil muffin tins
- Plastic sandwich bags (tip: the kind with the fold-over closure work better than ziplock)
- Plastic wrap
- Gelatin
- Sugar
- Seawater (or estuarine water)
- Distilled Water
- Table salt
- Pipettes or plastic droppers
- Plastic cell spreaders a.k.a. “hockey sticks” (e.g. VWR item #89042-021) (tip: if cell spreaders are not available sterile popsicle sticks could be used to spread samples on plates)
- Gloves
- Microscopes (if available)

Background:
Bacteria are some of the most diverse and numerous organisms on earth. They are found everywhere – from deep-sea hydrothermal vents to the open ocean to hot springs and wastewater treatment plants. They carry out important ecosystem functions by cycling carbon and nutrients in the
environment, and are trophic links in global food webs. Some bacteria also co-exist with other organisms in mutualistic or commensal symbioses, while others act as parasites and pathogens. Despite their global distribution and incredible abundance, it is extremely difficult to grow, or culture, environmental microbes on growth medium in the laboratory. For example, only an estimated 1% of bacteria present in the ocean can be grown in the lab. If we cannot grow them, how do we know they are there? Examining a sample of seawater under the microscope and plating the same amount of sample on an agar plate will result in about 100 times more bacteria cells observed microscopically than colonies (which are assumed to grow from a single cell) counted on the agar plate. Scientists refer to this conundrum as “The Great Plate Count Anomaly.”

Why aren’t bacteria growing in the laboratory? It is likely that many bacteria will not grow because microbiologists are unable to adequately reproduce one or more important aspects of the bacteria’s natural environment in the growth medium. Often, the “non-growers” require growth factors produced by other “helper” bacteria. These growth factors are necessary for the growth of the “non-growers,” but the bacteria themselves cannot make these on their own. Examples include vitamins like B$_{12}$ and enzymes that break down harmful waste products (Figure 1). Microbial growth may also be inhibited in lab cultures when many different kinds of bacteria are competing for the same nutrients, or when culturable bacteria produce antibiotics that kill microbes around them.

In this activity, students will try their hand at culturing heterotrophic bacteria from tap water and seawater. They will consider what components are necessary for different bacteria to grow in the lab.

**Figure 1.** Model of mechanisms of co-culture helping. Unculturable bacteria are schematically represented in the center of the figure, while known and potential helpers are arrayed around the periphery. Arrows indicate positive growth effects; stopped lines indicate inhibitory growth effects. Dashed arrows and inhibition lines indicate effects caused by as-yet-unidentified factors, while solid symbols indicate known factors. (Top right) Hydrogen peroxide (and possibly other forms of reactive oxygen species) can prevent the growth of sensitive bacteria. Helper organisms can protect against this effect by removing the oxidative stress, allowing the growth of the sensitive bacteria. (Bottom right) Helper bacteria can provide amino acids, vitamins, carbon sources, and other common nutrients that are often included in rich laboratory medium. (Bottom left and top left) Depictions of growth factors and stress-relieving effects yet to be discovered. Adapted from Stewart E J J.
Lesson Plan:

A. Class I – Student Activity

1. Discuss with students the basic requirements for microbial growth (elements C, N, P, O; light requirements if any; temperature ranges and other abiotic factors) and the challenges of culturing marine microbes despite their ubiquity and abundance in marine ecosystems.

2. Lead the class in a brainstorming session on possible natural growth substrates, species interactions, and anthropogenic compounds that might influence microbial growth in the ocean.

3. Discuss the difference between seawater and artificial seawater, both of which are used in laboratories to grow marine microbes.

<table>
<thead>
<tr>
<th>Nutrient medium</th>
<th>Strengths</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seawater</td>
<td>● Salt, nutrient, &amp; organic matter concentrations are what microbes encounter in nature</td>
<td>● Unknown concentrations of salt, nutrients &amp; organic matter</td>
</tr>
<tr>
<td></td>
<td></td>
<td>● Growth inhibiting contaminants may be present</td>
</tr>
<tr>
<td></td>
<td></td>
<td>● Levels of organic matter, vitamins and trace metals may be too low for growth</td>
</tr>
<tr>
<td>Artificial seawater</td>
<td>● Known concentration of media components</td>
<td>● Does not contain vitamins, organic matter, and/or trace metals that may be required for growth (unless these are added separately)</td>
</tr>
</tbody>
</table>

4. Each student should write hypotheses on their handout or in their lab notebook about what type of marine bacterial growth they expect to observe on the seawater agar plate and artificial seawater plate with or without the addition of sugar.

B. Class II - Advanced Preparation

Make seawater and artificial seawater agar plates; this should be done ahead of time by the instructor. Prepared plates can be stored in the refrigerator for up to one week.

For the seawater plates:
1. Place foil muffin cups into the muffin tray(s)
2. Mix 1 cup seawater with 3 Tbs. gelatin in a glass flask or mixing bowl
3. Cover the vessel loosely with a damp paper towel and heat the mixture to boil in the microwave or on a stir plate (tip: check that the gelatin is dissolving by swirling or stirring the mixture at 1 minute intervals)
4. Carefully remove the vessel from heat using an oven mitt; allow to cool 15 minutes (tip: the media should still be hot [45-55°C] but cool enough to pour without the oven mitt)
5. Pour the seawater agar into each muffin cup so that they are about 1/3 full
6. Cover loosely with plastic wrap and allow plates to cool and solidify; this will take at least one hour (tip: condensation will likely form on the plastic wrap; be sure to recover the muffin tray with fresh plastic wrap before placing in the refrigerator for storage)

For the artificial seawater plates:
1. Place foil muffin cups into the muffin tray
2. Mix 1 cup distilled water with 3 Tbs. gelatin and 1 Tbs. table salt (you are making a solution that is approximately 35 ppt NaCl, which is about the average salinity of the ocean)
3. Cover the vessel loosely with a damp paper towel and heat the mixture to a boil in the microwave or on a stir plate (tip: check that the agar is dissolving by swirling or stirring the mixture at 1 minute intervals)
4. Carefully remove the vessel from heat using an oven mitt; allow to cool 15 minutes (tip: the media should still be hot [45-55°C] but cool enough to pour without the oven mitt)
5. Pour the seawater agar into each muffin cup so that they are about 1/3 full
6. Cover loosely with plastic wrap and allow plates to cool and solidify; this will take at least one hour (tip: condensation will likely form on the plastic wrap; be sure to recover the muffin tray with fresh plastic wrap before placing in the refrigerator for storage)

C. Class II – Student Activity

1. Provide each student group with a small cup of freshly collected seawater (or estuarine water) and one seawater and one artificial seawater agar plate
2. Emphasize the importance of not touching the plates with bare hands to avoid contamination by bacteria residing on the students' bodies or in the classroom environment
3. Wearing gloves, students will add 100μl of seawater (a drop approximately the size of a dime) to each agar plate using the dropper or pipette
4. Distribute the seawater evenly across the plate using the cell spreaders or popsicle sticks by pushing the water to the edges of the plate in a circle or while spinning the muffin tin slowly around (tip: a video of the technique for spreading plates can be found here: [http://www.youtube.com/watch?v=1gs4JQ4B1TU](http://www.youtube.com/watch?v=1gs4JQ4B1TU))
5. Students should label 2 plastic bags with their names, date, and growth substrate. Have the students carefully remove the plates from the muffin tin holders and slide the agar into the appropriately labeled bag
6. Store the agar plates in the dark, at room temperature, for one to two weeks (tip: this may be a good time to teach or remind students about microbial growth rates and lifestyles)

D. Class III – Student Activity

1. After one to two weeks students will examine the microbial growth on their medium plates
2. On the provided handout, students should record the number of colonies on each plate and draw the different colony morphologies they observe (tip: observations can be made with the naked eye or under magnification, using a microscope)
3. Have the students compare and contrast the number and diversity of bacteria they have cultured on the different nutrient media.
4. Have the students calculate their isolation efficiencies using an assumed average of $10^6$ bacterial cells per milliliter seawater

\[
\text{Percent of the total community isolated (isolation efficiency)} = \left( \frac{\# \text{ colonies on plate}}{0.1 \text{ mL seawater plated}} \right) \times 10^6 \times 100
\]

Additional Resources:
- [http://serc.carleton.edu/microbelife/marine/index.html](http://serc.carleton.edu/microbelife/marine/index.html)
- [http://www.eoearth.org/article/Marine_microbial_loop](http://www.eoearth.org/article/Marine_microbial_loop)
Name:

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Hypotheses:

Date of culturing:

Date of counting:

Observation notes and colony counts

Seawater

Artificial Seawater

Plate drawing:

Plate drawing:

Compare and contrast results:
Optional Activity: Part 2 - Culturing the “Unculturable”

Objective: Find out what components are necessary for an artificial growth medium and try to come up with additional growth substrates that might help even more bacteria grow.

Class 1: Student activity

1. Lead the class in a brainstorming session on possible natural growth substrates that might enhance the growth of bacteria on artificial growth medium.
2. Have each group select four growth substrates to test (Possible substrates: seaweed, lotion, sunscreen, sugar, protein shake, etc). At least one group should test the controls (the natural seawater and artificial seawater plates)
3. Discuss what the control growth substrates will be in your experimental design. What are the strengths and limitations of each control?
4. Each student should write hypotheses on their handout or in their lab notebook about what type of growth they expect to observe on the different substrates

Following the Section B, C, D in the above lesson plan.

To make seawater plates:
7. Place foil muffin cups into the muffin tray(s)
8. Mix 1 cup seawater with 3 Tbs. gelatin in a glass flask or mixing bowl
9. Cover the vessel loosely with a damp paper towel and heat the mixture to boil in the microwave or on a stir plate (tip: check that the agar is dissolving by swirling or stirring the mixture at 1 minute intervals)
10. Carefully remove the vessel from heat using an oven mitt; allow to cool 15 minutes (tip: the media should still be hot [45-55°C] but cool enough to pour without the oven mitt)
11. Pour the seawater agar into each muffin cup so that they are about 1/3 full
12. Cover loosely with plastic wrap and allow plates to cool and solidify; this will take at least one hour (tip: condensation will likely form on the plastic wrap; be sure to recover the muffin tray with fresh plastic wrap before placing in the refrigerator for storage)

To make artificial seawater plates:
7. Place foil muffin cups into the muffin tray
8. Mix 1 cup distilled water with 3 Tbs. gelatin and 1 Tbs. table salt (you are making a solution that is approximately 35ppt NaCl)
9. Cover the vessel loosely with a damp paper towel and heat the mixture to a boil in the microwave or on a stir plate (tip: check that the agar is dissolving by swirling or stirring the mixture at 1 minute intervals)
10. Carefully remove the vessel from heat using an oven mitt; allow to cool 15 minutes (tip: the media should still be hot [45-55°C] but cool enough to pour without the oven mitt)
11. Pour the seawater agar into each muffin cup so that they are about 1/3 full
12. Cover loosely with plastic wrap and allow plates to cool and solidify; this will take at least one hour (tip: condensation will likely form on the plastic wrap; be sure to recover the muffin tray with fresh plastic wrap before placing in the refrigerator for storage)

To make plates with added growth substrates:
1. If you are using sugar, chemicals or other uniform growth substrates that will dissolve or mix easily, follow the protocol for making artificial sweater plates with the following amendment: add 1 Tbs. growth substrate to the mixture in step 2 and continue with steps 3-6 as above.
2. If you are using algae, nori, fish bait, or other dense growth substrate proceed as follows:
3. Add the substrate to a pot of boiling water.
4. Boil for 3 minutes.
5. Pour off most of the water so that about 1/2 to 1 cup of liquid remains.
6. Add the boiled substrate and liquid to a food processor or blender and process the substrate into a puree.
7. Follow the protocol for making artificial sweater plates with the following amendment: add 2 Tbs. of the pureed growth substrate to the mixture in step 2 and continue with steps 3-6 as above.
**Name:**
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**Hypotheses:**

**Date of culturing:**

**Date of counting:**

**Observation notes and colony counts for Controls**

<table>
<thead>
<tr>
<th>Seawater</th>
<th>Artificial Seawater</th>
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<tbody>
<tr>
<td>Plate:</td>
<td>Plate:</td>
</tr>
</tbody>
</table>
Observation notes and colony counts for Additional Growth Substrates

Seawater
Additional Substrate:

Artificial Seawater
Additional Substrate:

Compare and contrast results between controls (seawater & artificial seawater plate) and plate
with additional substrate. Did the addition of substrate enhance, inhibit or exhibit no effect on the growth of bacteria?