This lesson is part of the Khaled bin Sultan Living Oceans Foundation's *Mangrove Ecology Curriculum*. It was developed by the Khaled bin Sultan Living Oceans Foundation and North Carolina State University. The lesson has been designed for secondary school students, but can be adapted for other uses.

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About Khaled bin Sultan Living Oceans Foundation
The Khaled bin Sultan Living Oceans Foundation was incorporated in California as a 501(c)(3), public benefit, Private Operating Foundation in September 2000. The Living Oceans Foundation is dedicated to providing science-based solutions to protect and restore ocean health through research, outreach, and education. The educational goals of the Khaled bin Sultan Living Oceans Foundation and development of this are generously supported by Prince Khaled bin Sultan of Saudi Arabia.

About North Carolina State University
North Carolina State (NC State) was founded with a purpose: to create economic, societal and intellectual prosperity for the people of North Carolina and the country. NC State is a pre-eminent research enterprise that excels in science, technology, engineering, math, design, the humanities and social sciences, textiles and veterinary medicine.

Dear Educator,

The Khaled bin Sultan Living Oceans Foundation and North Carolina State University Department of Applied Sciences have partnered to develop a lesson plan about mangrove disease. The lesson provides students with the latest science on mangrove disease and enables them to participate in hands-on research.

Around the world mangroves are in peril. Much of the decline of mangrove forests has been due to human destruction, including overexploitation, coastal development, changes in water flow, oil spills, and marine debris. In the Bahamas and greater Caribbean, there is great concern because large areas of mangroves have completely died off. As a result, scientists have been researching these areas in hope of finding out what is causing the die-off, so that damage to critical mangrove habitats can be mitigated.

Dr. Ryann Rossi first became interested in mangrove die-offs throughout the Bahamas when she was getting her doctorate at North Carolina State University. In 2013, Dr. Rossi started researching various mangrove die-off areas in the Bahamas. She found that mangroves are dying due to the combined effects of plant pathogens, herbivory, and altered abiotic factors, possibly due to humans.

In 2017, Dr. Rossi and the Khaled bin Sultan Living Oceans Foundation partnered to develop this lesson plan allowing high school students to participate in citizen science. This lesson plan is part of the Khaled bin Sultan Living Oceans Foundation's *Mangrove Ecology Curriculum*, which was developed for the Mangrove Education and Restoration programs. These programs aim to increase environmental awareness and restore mangrove forests in the Caribbean. For more information about the Mangrove Education and Restoration Programs go to *https://www.lof.org/education/mangrove-education-and-restoration/*.

In this lesson students will collect, process, and analyze potentially diseased red mangrove leaves. Then they determine which pathogens are present and explore what could be causing the disease.

Our Best,

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LESSON 8 TEACHER'S NOTES

AUTHOR

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LEARNING OBJECTIVES

- Locate areas around the world where mangrove disease has been discovered.
- Define microbiology.
- Describe the cause of plant disease.
- Differentiate between the main types of plant pathogens.
- Illustrate the structures of a fungus.
- Interpret the Disease Triangle Diagram.
- Define epidemic and host.
- Develop a hypothesis
- Record location, date, and time.
- Determine the latitude and longitude of diseased mangroves using a GPS unit or other electronic device.
- Make observations about the mangrove forest.
- Identify red mangrove trees.
- Record results in data tables.
- Recognize and randomly sample potentially diseased red mangrove leaves.
- Label and photograph mangrove leaf samples.
- Recognize red dead mangrove trees.
- Illustrate lesions on potentially diseased mangrove leaf.
- Dissect a red mangrove leaf.
- Culture a red mangrove leaf.
- Differentiate between cultured bacterial and fungal growth.
- Make observations about bacterial and/or fungal culture.
- Categorize fungal growth by genera.
- Examine bacterial and/or fungal culture using a microscope.
- Illustrate a microscopic image of a cultured sample.
- Compare fungal and bacterial colonies from different diseased mangrove leaves.
- Conclude how disease affects the mangrove ecosystem and its biodiversity.
- Determine other research or experiments that need to be conducted in order to understand disease in mangroves.
- Research the causes of disease in mangroves.

KEYWORDS

- Agar
- Bacterium
- Citizen Science
- Culture Medium
- Disease
- Epidemic
- Fungus
- Geographic Location
- Geographic Positioning System (GPS)
- Host
- Hypha
- Hypothesis
- Microbiology
- Mycelium
- Nematode
- Observation
- Pathogen
- Pathology
- Random Sampling
- Red Mangrove
- Spore
- Virus

MATERIALS

- GPS or electronic device
- Digital camera or electronic device
- Ziploc bag
- Permanent marker
- 250 mL distilled water
- 9.25 g Potato Dextrose Agar (PDA) media
- Pressure cooker or nonstick pot to boil water
- Foil
- Stirring rod or spoon
- 3.5 mL Hydrochloric acid (HCl), (Optional)
- Petri dish (60 mm)
- Graduated cylinder
- Gloves
- Hand sanitizer
- Rubbing alcohol
- Mason jar or lab grade glassware (if using pressure cooker)
- Vacuum grease (if using pressure cooker)
- 4 L distilled water (if using pressure cooker)
- Protective gloves or potholder
- Paper towel or towel
- Potentially diseased red mangrove leaf
- Sterile paper towel (covered in foil)
- Scissor or scalpel
- Bleach solution
- Alcohol burner
- Tweezer or forcep
- Saran wrap or parafilm
- Tape or permanent marker
- Whatman FTA™ plant card
- Small hammer, screw driver handle, or pestle
- Ziploc or paper envelope
- Dissecting microscope
- **Mangrove Disease PowerPoint** with presentation notes
- **Lesson 8: Outbreak Investigation** student worksheet
- **Finding Diseased Red Mangrove Leaves** video
- **Preparing Agar Petri Dishes** video
- **Plating Red Mangrove Leaf** video
- **Appendix A: Instructions for Using Offline Maps**
- **Appendix B: Field Data Collection Instructions**
- **Appendix C: Preparing Agar Dishes & Bleach Solution**
- **Appendix D: Plating Mangrove Leaf**
- **Appendix E: Mangrove Leaf Disease Identification Guide**

EXTENSIONS

- Ask students to analyze data and write a scientific report using their results from this activity.
- Assign students a specific plant fungus to research.
- Instruct students to create an awareness flyer or brochure about mangrove disease. Then, organize a public forum.

STANDARDS

Ocean Literacy Principles and Fundamental Concepts (Grades 9-12)

- Principle 5.B.11: Ocean ecosystems are connected to each other in a macro food web. Over time, organisms move from one ecosystem to another as they grow, migrate, and die. Changes in an ecosystem or an organism may have unpredictable effects on other ecosystems.
- Principle 6.D: The exponential growth of human populations, together with technical advances, have exacerbated changes in the ocean and atmosphere.
- Principle 6.E: Achieving sustainability of the diverse ecosystems and resources in the ocean depends upon collective and individual action based on scientific research and exploration.
- Principle 7.A.1: There are many opportunities for ocean exploration, which can lead to hypothesis-generated science.
- Principle 7.B.5: Exploring the ocean involves global participation because there is only one ocean that connects and sustains all life.

PROCEDURE

PART 1: FIELD DATA COLLECTION

A. PREPARATION:

1. In case of inclement weather, have a backup plan.
2. Make sure to follow all school or education facility policies.
3. When first discussing the field trip, be sure to cover the following safety procedures:
   a. Everyone will have a buddy. You are to stay with that buddy at all times.
   b. Do not wander from the path or away from your instructor(s).
   c. Wear comfortable, close-toed walking shoes that could possibly get dirty and/or wet.
   d. Bring water on the hike. It will be hot, and everyone needs to stay hydrated.
   e. There will probably be bugs, so bring bug spray.
   f. Wear sunscreen. Hats are allowed, as well.
   g. Do not touch anything unless your instructor asks you to.
4. Inform parents/guardians of the field trip and get all permission slips and/or liability waivers signed. Send parents a description of the field trip and a checklist of items that the students will need to bring the day of the field trip.
5. Bring a fully stocked first aid kit on the mangrove field trip.
6. In case of emergency, make sure that at least two adults have a way to communicate (such as a cell phone). Make sure to share this contact information with the school or education facility.
7. Print and laminate **Appendix A: Instructions for Using Offline Maps** (optional) and **Appendix B: Field Data Collection Instructions**.
8. If using **Appendix A: Instructions for Using Offline Maps**, make sure to charge electronic devices prior to the field trip and complete step A in *Appendix A*.
9. Create supply kits. This will lessen the amount of time that you have to spend handing out the supplies at the field trip site. A supply list can be found in **Appendix B: Field Data Collection Instructions**. The supplies listed in *Appendix A* will make one kit.
10. Decide if the students are going to collect leaves individually or in groups.
11. Find a mangrove forest that has numerous red mangrove trees.

B. IN CLASS:

1. Present **Mangrove Disease PowerPoint** *Part 1: What is Disease?*. There are teacher notes that go along with the presentation.
2. Watch **Finding Diseased Red Mangrove Leaves** video to determine how to find potentially diseased red mangrove leaves.
3. During the presentation, explain to students that they will take notes on *Part 1: What is Disease?* section of their **Lesson 8: Outbreak Investigation** student worksheet.
4. Before going on the field trip, ask each student to develop and write down their research hypothesis in the space provided on their **Lesson 8: Outbreak Investigation** student worksheet.

C. WHILE ON FIELD TRIP:

1. Inform students to bring two pencils and their **Lesson 8: Outbreak Investigation** student worksheet. While on the fieldtrip students will fill in *Part 2: Field Data Collection, Table A*.
2. Students need to be able to identify red mangrove trees. Review the characteristics of a red mangrove tree (prop and drop roots; leaf shape, color, and texture; red tannin under bark).
3. Explain why scientists randomly sample. Tell students the number of leaves that you want each student or group to collect. Instruct students to randomly sample potentially diseased red mangrove leaves.
4. Hand each group a supply kit. Explain to students that they are responsible for their supplies and when they are finished, they need to double check that all of the supplies are put back in their supply kit. There

is a checklist in **Appendix B: Field Data Collection Instructions**.

5. Instruct students to follow the instructions on **Appendix B: Field Data Collection Instructions**.
6. Prompt students to fill out *Table A* on **Lesson 8: Outbreak Investigation** *Part 2: Field Data Collection* student worksheet.

NOTE: If your school does not have a GPS device(s), you can use a smart phone or tablet to mark the GPS location using offline maps. You do not need the internet to use these maps. One example of an app that utilizes offline maps is Google Maps. For instructions on how to use Google Maps, see **Appendix A: Instructions for Using Offline Maps**. No matter which device you choose to use, make sure to protect your device(s) from the different environmental conditions (water and heat) that are present in the mangroves by equipping your device(s) with the appropriate hardware.

PART 2: SAMPLE PROCESSING

A. PREPARATION:

1. Print and laminate **Appendix D: Plating Mangrove Leaf** and **Appendix E: Mangrove Leaf Disease Identification Guide**.
2. Create supply kits. A supply list can be found in **Appendix D: Plating Mangrove Leaf**. This will lessen the amount of time that you spend handing out the supplies in the classroom.
3. Prepare Potato Dextrose Agar (PDA) petri dishes and bleach solution. Instructions can be found in **Appendix C: Preparing Agar Dishes & Bleach Solution**.

B. IN CLASS:

1. Present **Mangrove Disease PowerPoint** *Part 2: Mangrove Isolations*. There are teacher notes that go along with the presentation. Make sure to emphasize the laboratory/facility safety rules.
2. Instruct students to fill out *Part 3: Sample Processing* on their **Lesson 8: Outbreak Investigation** student worksheet while presenting.
3. Hand out **Appendix D: Plating Mangrove Leaf**. Instruct students to use this procedure to plate their red mangrove leaves.
4. Provide students with an agar petri dish(es). Remind students that they are not to open the petri dish until they are ready to insert their leaf pieces into the agar.
5. After completing this part of the lesson, make sure that the samples are stored at room temperature. Do not allow students to open the petri dishes until after 3 days, 1 week, and 2 weeks when they make observations. This will help prevent contamination in the samples.
6. Allow the samples to culture for a minimum of one week, but preferably two. Ideally, students should look at samples after 3 days, and 1 and 2 weeks. At each time interval, students should fill in *Table C* on their **Lesson 8: Outbreak Investigation** student worksheet. Make sure that the students rewrap the petri dishes in saran wrap or parafilm.
7. When the samples have been cultured for two weeks, present **Mangrove Disease PowerPoint** *Part 3: Sample Analysis*. There are teacher notes that go along with the presentation.
8. Afterwards, ask students to follow steps listed in *Part 4: Sample Analysis, After 2 Weeks* on their **Lesson 8: Outbreak Investigation** student worksheet and fill in the information. Provide each group with **Appendix E: Mangrove Leaf Disease Identification Guide**.
9. After the experiment is complete, soak the petri dishes in a 10% bleach solution for 24 hours to kill all of the microbes growing in the agar. Then dispose of the petri dishes.
10. Ask students to complete their **Lesson 8: Outbreak Investigation** student worksheet and answer the remaining questions.
11. Please see the Mangrove Detectives website (*www.mangrovedetectives.org*) for more information about where to send the FTA™ cards.

ATTRIBUTIONS

Appendix E:

NOTE: Photos are listed per category and attributed in order from left to right.

Penicillium

- © 2017 Ryann Rossi
- Culture plate of penicillium mould, London, England By Wellcome Images [CC BY 4.0 (*https://creativecommons.org/licenses/by/4.0*)], 17 October 2014 via Wikimedia Commons. *https://commons.wikimedia.org/wiki/File:Culture_plate_of_penicillium_mould,_London,_England_Wellcome_L0059175.jpg*.
- Penicillia on Petri dish By Convallaria majalis [CC BY-SA 4.0 (*https://creativecommons.org/licenses/by-sa/4.0*)], 2 October 2017 via Wikimedia Commons. *https://commons.wikimedia.org/wiki/File:Penicillia_on_Petri_dish.jpg*.

Pestalotiopsis

- All photos: © 2017 Ryann Rossi

Unknown

- © 2017 Ryann Rossi
- *Aspergillus fumigatus* By Dr. David Midgley [CC BY-SA 2.5 (*https://creativecommons.org/licenses/by-sa/2.5*)], 28 July 2006 via Wikimedia Commons. *https://commons.wikimedia.org/wiki/File:Aspergillus_fumigatus.jpg*.
- *Verticillium theobromae* culture By Annabel [CC BY-SA 3.0 (*https://creativecommons.org/licenses/by-sa/3.0*)], 29 January 2017 via Wikimedia Commons. *https://commons.wikimedia.org/wiki/File:Verticillium_theobromae_culture.jpg*.

Bacteria

- Bacteria on agar plate By HansN [CC BY-SA 3.0 (*https://creativecommons.org/licenses/by-sa/3.0*)], 18 December 2012 via Wikimedia Commons. *https://commons.wikimedia.org/wiki/File:Bacteria_on_agar_plate.jpg*.
- *Actinomyces spp* 01 By US gov [Public domain], 27 March 2007 via Wikimedia Commons. *https://commons.wikimedia.org/wiki/File:Actinomyces_spp_01.jpg*.
- Contamination on agar plate By Garnhami [CC BY-SA 4.0 (*https://creativecommons.org/licenses/by-sa/4.0*)], 10 February 2016 via Wikimedia Commons. *https://commons.wikimedia.org/wiki/File:Contamination_on_agar_plate.jpg*.

PRESENTATION NOTES

Slide 1: Part 1 – What is Disease?

Slide 2: Mangrove Disease

- What is disease? **Disease** is a condition of a plant or animal that impairs normal body structure or function.
- Throughout the world, mangrove disease has been documented by scientists in all mangrove species. Plant disease has been found in Australia, Puerto Rico, and South Africa but all from different pathogens.
- In the Marls, which is on the western side of Abaco, Bahamas, fisherman noticed a die-off of mangroves, particularly red mangroves. Scientist Dr. Ryann Rossi from North Carolina State University started studying the die-off in 2013. She hypothesized that there were probably multiple stressors acting in coordination that might have led to the mangrove die-off. One of those factors she believed to be a pathogen that resides on the leaves of mangroves.

Slide 3: What is Microbiology?

- The word *microbiology* originates from the words *Micro*- meaning small, -*Bio*- meaning life, and –*Ology* meaning the study of. Therefore, the definition of the word **microbiology** is the study of microscopic organisms.

Slide 4: Cause of Plant Disease

- The main cause of plant disease is from **pathogens** or infectious agents.
  - The word *pathology* originates from the words *Path*- meaning suffering and –*Ology* meaning the study of. Therefore, the definition of the word **pathology** is the study of diseases.

Slide 5: Main Types of Plant Pathogens

- There are four main types of plant pathogens (though there are others):
  1. **Fungus** (plural fungi): eukaryotic organisms that includes unicellular microorganisms such as yeast, mold, and mushrooms. They are classified in the kingdom Fungi.
  2. **Bacterium** (plural bacteria): Prokaryotic microorganisms or unicellular organism in the domain Bacteria. Most bacteria are beneficial; however, some are harmful.
  3. **Virus**: A small infectious agent that replicates inside a living organism.
  4. **Nematode**: Roundworms which are classified in the phylum Nematoda.

Slide 6: Structure of Fungi

- Identifying Structures:
  1. **Hypha** (plural hyphae): Branching filaments that make up the mycelium of a fungus.
  2. **Spore**: Reproductive structures.
  3. **Mycelium** (plural mycelia) A network of branching hyphae.

Slide 7: Identifying Bacteria

- There may be bacterial contamination in the samples. Bacteria contamination will appear slimy and they will often have satellite colonies or little colonies all over the petri dish rather than continuous growth, like fungi.

PRESENTATION NOTES

Slide 8: What is Needed for Disease to Occur?

- Disease is often not an isolated event, especially in nature. Often it spreads and becomes an **epidemic** or a widespread occurrence of a disease. The Disease Triangle Diagram shows that there are three elements that cause an epidemic:
  1. A susceptible **host** or organism that supplies food, resources, and acts as a substrate for a disease
  2. The presence of a pathogen
  3. A conducive environment (e.g. high temperatures, direct sunlight, too much rainfall)
- If one of these criteria are not met, then the disease cannot occur.

Slide 9: Mangrove Disease in the Bahamas

- The Marls is not the only place that Dr. Ryann Rossi has found pathogens. Other locations on Abaco include Camp Abaco, Cherokee, Sandy Point, Crossing Rocks, Hills Creek, Treasure Cay, and Snake Cay. Additionally, she has found pathogens on other Bahamian islands such as Grand Bahama and San Salvador.

Slide 10: Field Data Collection Instructions

- Review **Appendix B: Field Data Collection Instructions** with students.

Slide 11: Mangrove Disease Activity

- What do diseased mangrove leaves look like? Some common symptoms are:
  - Necrotic tissue (dead tissue) - This is referred to as a lesion.
  - Yellowing tissue
  - Discolored veins
  - Striping appearance (yellow stripes running through the leaf)

Slide 12: Finding Diseased Red Mangrove Leaves Video

Slide 13: Part 2 – Mangrove Isolations

Slide 14: Plating Red Mangrove Leaf Video

Slide 15: Sample Size

- Using scissors or a scalpel, cut the leaf at the margin of the lesion and the green leaf. The leaf piece should contain a small portion of the lesion and adjacent green tissue. Leaf pieces should be no bigger than .5 cm x .5 cm. Following the same instructions, cut a different area of the leaf that is diseased. If there isn't another diseased section, cut another piece from the same section.

Slide 16: Leaf Sterilization

- Sterilize both leaf pieces by placing them into a bleach solution for one minute. This prevents contamination from microbes living on the surface of the leaf. The microbe causing the lesion has penetrated the leaf, so it will still grow when plated on the agar.

Slide 17: Sterilize Tweezers

- While leaf pieces are sterilizing, sterilize the tweezers. Dip the tweezers in rubbing alcohol and then move the tweezers back and forth through the flame for 5 seconds.

Slide 18: Dry Leaf Pieces

- Remove one paper towel from the foil.

PRESENTATION NOTES

- Use tweezers to transfer leaf pieces from the bleach solution to a paper towel. Blot the water off the leaf pieces but do not touch the paper towel or leaf pieces.

Slide 19: Insert Leaf Pieces into Agar

- When dry, use sterile tweezers to place leaf in the agar petri dish. Make sure the piece is inserted into the agar so that it is slightly buried, not just laying on top. **NOTE**: Sometimes it is easiest to pierce the surface of the agar with the tweezers first and then insert the leaf pieces into the slit in the agar.

Slide 20: Seal Petri Dish

- Close the petri dish lid and wrap it with saran wrap or parafilm to help prevent contamination.
- Store the petri dishes in a plastic container at room temperature.
- Monitor the samples for fungal growth in three increments after 3 days, and 1 and 2 weeks.
- Record data in *Table C* on the **Lesson 8: Outbreak Investigation** student worksheet.

Slide 21: Part 3 – Sample Analysis

Slide 22: *Pestalotiopsis*

- *Pestalotiopsis* is a genus of fungi, which is why it is italicized. Fungal colonies that appear to be white and fluffy are likely to be *Pestalotiopsis*. If there are black dots present on top of the white fluffy fungus, then you can be certain you have a species of *Pestalotiopsis*. The black dots are *Pestalotiopsis'* spores.

Slide 23: *Penicillium*

- *Penicillium* is also a genus of fungi. Fungal colonies that appear to be olive green and densely compacted are likely to be *Penicillium*.

Slide 24: Unknown Colony

- This is unknown fungal colony. It appears to be light gray and fluffy.

Slide 25: Bacterial Contamination

- Bacteria contamination will appear slimy and wet. It can cover the whole petri dish, or can grow in many, small colonies called satellite colonies.

Slide 26: Practice Identification 1

- Some samples might have multiple fungal and/or bacterial colonies present on the same petri dish.
- In this petri dish, there are three different colonies present. The one on the far left is a bacterial contamination. The second colony (top, middle) is *Penicillium* identified by its dense fibers and olive green coloration. The third colony on the far right is *Pestalotiopsis*, which is identified by the white and fluffy appearance.

Slide 27: Practice Identification 2

- Again, there are three different colonies present. The bottom left colony is a bacterial contamination because it appears slimy, green, and dense. The top right colony is likely *Penicillium* because of its green coloration and dense fibers. The colony on the right is white and fluffy and is likely *Pestalotiopsis.*

PRESENTATION NOTES

ATTRIBUTIONS

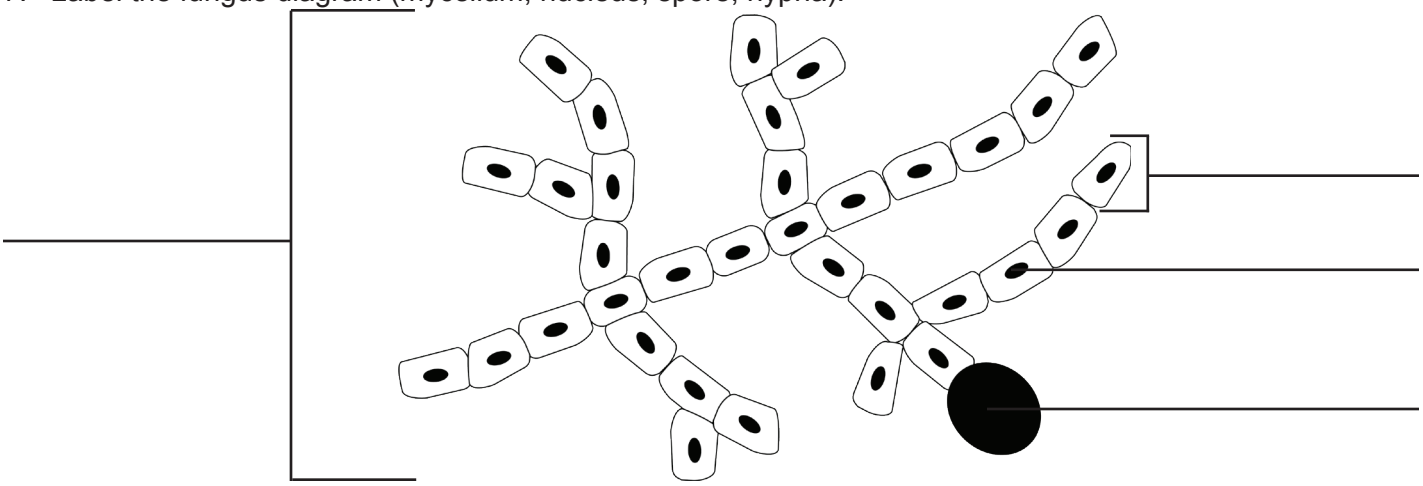
- Plant Disease Triangle By Earlycj5 (Own work) [CC BY-SA 3.0 (*http://creativecommons.org/licenses/by-sa/3.0*)] 25 February 2008 via Wikimedia Commons. *https://commons.wikimedia.org/wiki/File%3APlant_Disease_Triangle.png*.
- Striping: Photo by Yue Jin. [*http://www.ars.usda.gov/is/graphics/photos/jun06/d517-1.htm* Image Number D517-1] {PD-USGov-USDA-ARS} 26 September 2006 via Wikimedia Commons. *https://commons.wikimedia.org/wiki/File:Stripe_rust_on_wheat.jpg#file*.
- Yellowing: By Babahu [CC0 (*https://creativecommons.org/share-your-work/public-domain/cc0/*)]13 June 2012 via Wikimedia Commons. *https://upload.wikimedia.org/wikipedia/commons/b/b7/Yellow_Mosaic_Virus_on_yellow_squash_leaf.jpg*.
- Discolored Veins: Taro Vein chlorosis By Scot Nelson [CC By-NC-SA 2.0 (*https://creativecommons.org/licenses/by-nc-sa/2.0/*)] June 2013 via Flickr. *https://www.flickr.com/photos/scotnelson/8981028008/*.

Khaled bin Sultan Living Oceans Foundation

LESSON 8 OUTBREAK INVESTIGATION

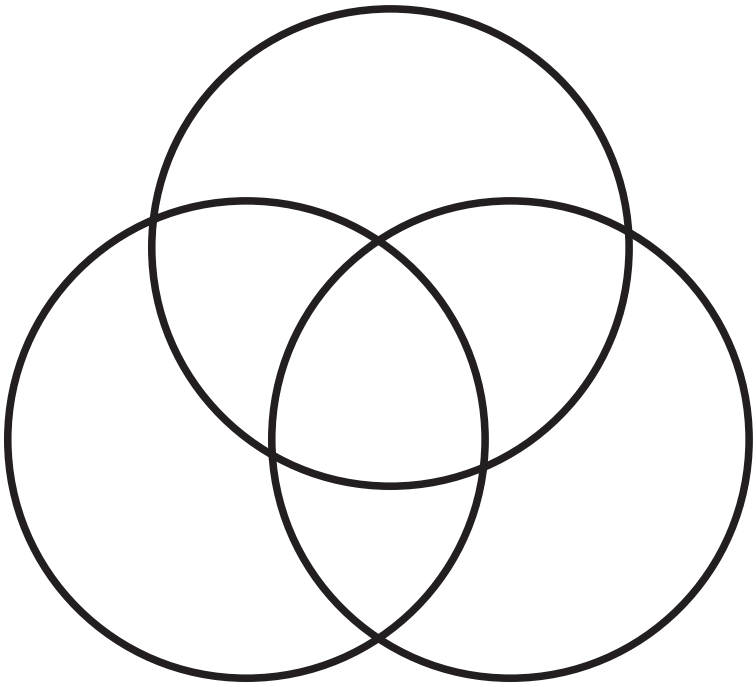
PART 1: WHAT IS DISEASE?

1. _____ is a condition of a plant or animal that impairs normal body _____ and _____.
2. List countries where plant disease has been documented: _____ _____
3. The word microbiology originates from:
   "Micro–" meaning _____ and "–bio–" meaning _____ and "–ology" meaning _____
   Therefore the word microbiology means _____.
4. The word pathology originates from:
   "Path–" meaning _____ and –"ology" meaning _____
   Therefore the word pathology means_____.
5. _____ is an infectious agent.
6. There are _____ main types of plant pathogens:
   a. _____are eukaryotic organisms that includes _____microorganisms such as yeast, mold, and mushrooms. They are classified in the _____.
   b. _____ are _____microorganisms or unicellular organism in the domain Bacteria. Most bacteria are _____; however, some are _____.
   c. A _____ is a small _____agent that replicates inside a living organism.
   d. _____ are roundworms that are classified in the _____.
7. Label the fungus diagram (mycelium, nucleus, spore, hypha).



8. Sometimes, _____ contamination may occur in samples. This contamination will appear _____ and they will often grown in little _____ all over the petri dish.

9. Disease often spreads and becomes a widespread occurrence called an_____. There are three elements that cause an epidemic. Label the Disease Triangle Diagram below.



A _____ is an organism that supplies food, resources, and acts as a substrate for disease.

10. List where plant disease has been documented in the Bahamas: _____

_____

11. There are four common symptoms that diseased mangroves have:

a. _____
b. _____
c. _____
d. _____

PART 2: FIELD DATA COLLECTION

Hypothesis:

_____

_____

_____

INSTRUCTIONS:

1. Get supply kit from your teacher.
2. Follow instructions listed on **Appendix B: Field Data Collection Instructions**.

TABLE A:

Mangrove Location Name:			Date:		Time:						
Leaf ID #	Position		Observations	Diseased Leaves in Area						# Dead Trees at the Site
	Latitude (N or S)	Longitude (E or W)		0	1	2-10	11-30	31-50	>50	

PART 3: SAMPLE PROCESSING

INSTRUCTIONS:

1. Label your Leaf ID # on the line provided.
2. Draw your leaf in *Table B* below. Color in the areas of the leaf that are diseased. It is okay to use pen or pencil.

TABLE B:

Leaf ID #_____

3. Follow instructions listed on **Appendix D: Plating Mangrove Leaf**.

PART 4: SAMPLE ANALYSIS

INSTRUCTIONS – AFTER 3 DAYS:

1. After you remove the saran wrap or parafilm, make sure not to touch or breath on the agar or you may contaminate your sample.
2. After 3 days, retrieve your sample. Is there any growth taking place? In *Table C*, write "Y" for yes or "N" for no. If you answered yes, describe the colony opacity, color, and description in *Table C*.
3. When you are finished with your sample, wrap your petri dish in saran wrap or parafilm before storing.

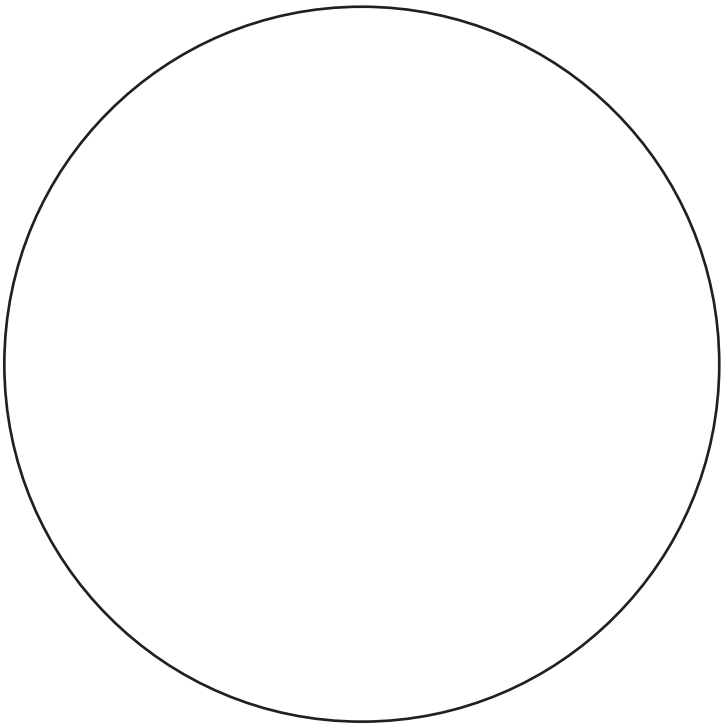
INSTRUCTIONS – AFTER 1 WEEK:

1. After you remove the saran wrap or parafilm, make sure not to touch or breath on the agar or you may contaminate your sample.
2. After one week, retrieve your sample. Is there any growth taking place? In *Table C*, write "Y" for yes or "N" for no. If you answered yes, describe the colony opacity, color, and description in *Table C*.
3. When you are finished with your sample, wrap your petri dish in saran wrap or parafilm before storing.

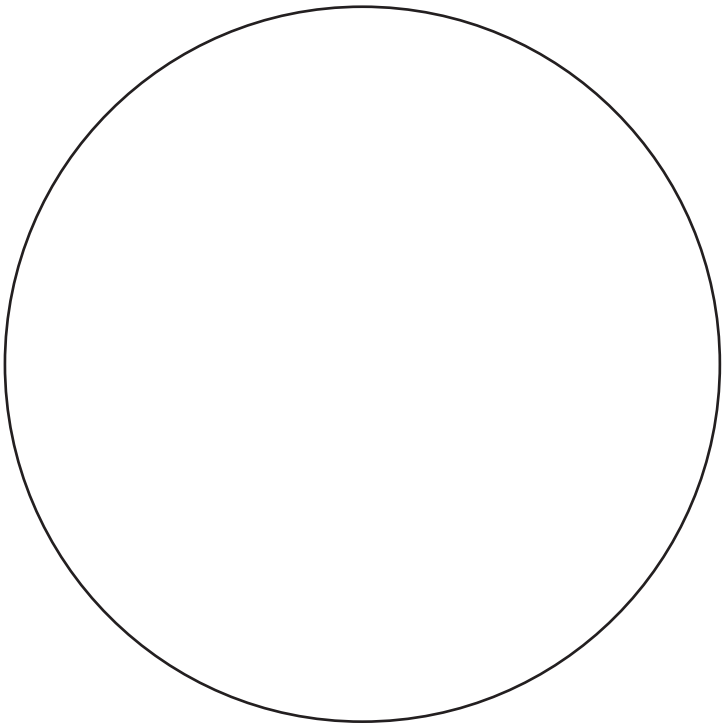
INSTRUCTIONS – AFTER 2 WEEKS:

1. After two weeks, retrieve your sample. Is there any growth taking place? In *Table C*, write "Y" for yes or "N" for no.
2. Next, remove the saran wrap or parafilm.
3. Remove the lid from your petri dish. Using **Appendix E: Mangrove Leaf Disease Identification Guide**, fill in the following information in *Table C*:
   a. Identify whether your sample is a bacterial or fungal colony. Fungal growth will appear fuzzy and fluffy and should have strands of hyphae. Bacterial growth will look slimy and wet in addition to being mostly flat on the surface of the agar. **NOTE**: Bacterial contamination and fungal growth can both be present.
   b. Next, make observations about your sample. What is the opacity of your sample (translucent, opaque, or transparent)?
   c. What is the color of your sample?
   d. Describe the colonies. Make sure to be descriptive. Act as though you have to explain how your colonies look to someone who hasn't seen it before. **NOTE**: There can be multiple colonies present.
   e. Using **Appendix E: Mangrove Leaf Disease Identification Guide**, identify the colony.
6. Place your sample under a microscope. In the area below, draw what you see. If present, label hyphae, mycelium, and spores of fungi.

Agar Sample #1



Agar Sample #2



7. Collect an agar petri dish.
    a. Write your name and date on the outside of the FTA™ card.
    b. Scoop a 1-2 cm size piece of agar that contains fungal tissue (e.g. hyphae or spores) from your petri dish. This is typically a different color than the agar.
    c. Open the FTA™ card.
    a. Choose a circle and write your name and date on the outside of the FTA™ card.
    b. Place the agar and tissue inside one of the four circles on the FTA™ card.
    c. Close the FTA™ card.
    d. Apply moderate pounding or pressure to the FTA™ card using a blunt object (e.g. small hammer, screw driver handle, pestle).
    e. Open the FTA™ card and remove the large residue from it. Place the residue back in your petri dish.

8. Upload your data to *https://mangrovedetectives.org/lessons/*.

TABLE C:

Time	Growth (Y or N)	Bacterial or Fungal	Colony Opacity (Transparent, Opaque, Translucent)	Colony Color	Colony Description	Colony(s) Present
3 days						
1 week						
2 weeks						

INSTRUCTIONS: Answer the following questions.

1. What could potentially be causing mangrove disease?

2. Describe symptoms of the infected leaves. Refer to your notes to recall the symptoms of plant disease. What was the most common symptom that you encountered?

3. Compare fungal colonies that grew from different leaves. Do any look similar? Do they look different? Explain.

4. Does the bacteria in your culture(s) cause plant disease? Explain.

5. Was your hypothesis correct? Why or why not? Explain.

6. In what ways does disease affect the mangrove ecosystem ?

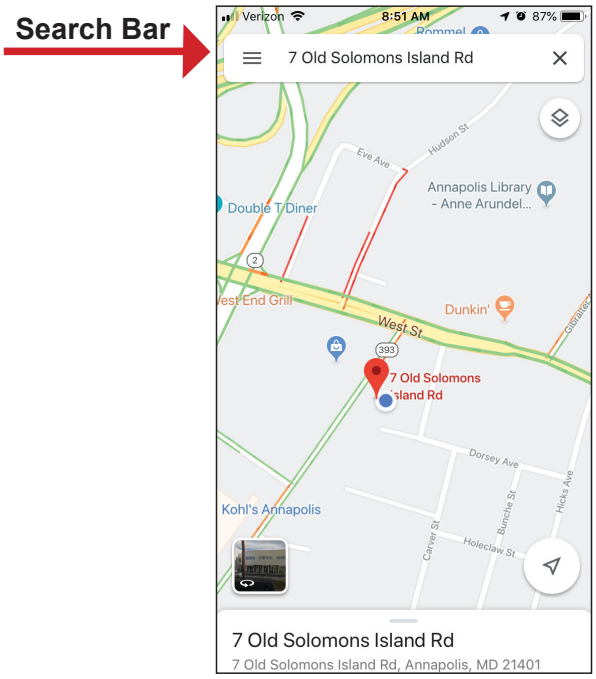
7. How can disease affect biodiversity?

8. What other experiments/research do you need to conduct to understand mangrove disease?

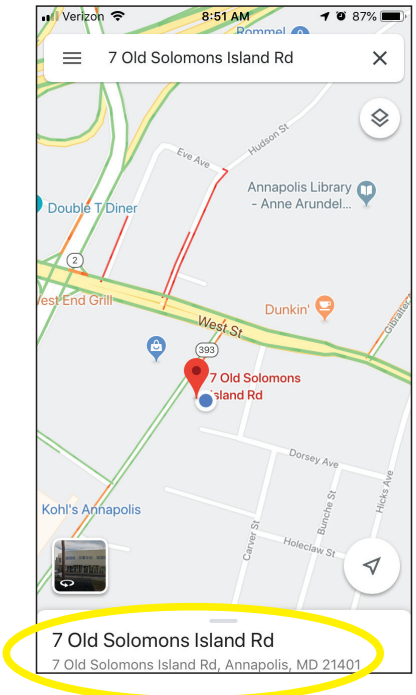
INSTRUCTIONS FOR USING OFFLINE MAPS

A. DOWNLOAD AN OFFLINE MAP:

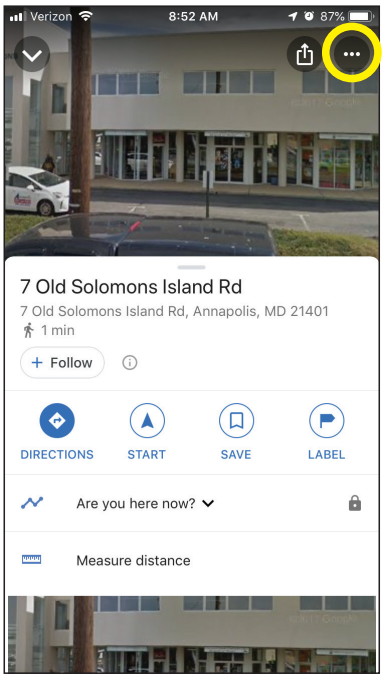
1. Establish an internet connection on your electronic device.
2. If Google Maps is not already downloaded on your device, then download the app.
3. Sign-in to Google Maps or setup login information.
4. In the search bar, enter the city or island name nearest to the location of the mangrove forest you will be visiting.



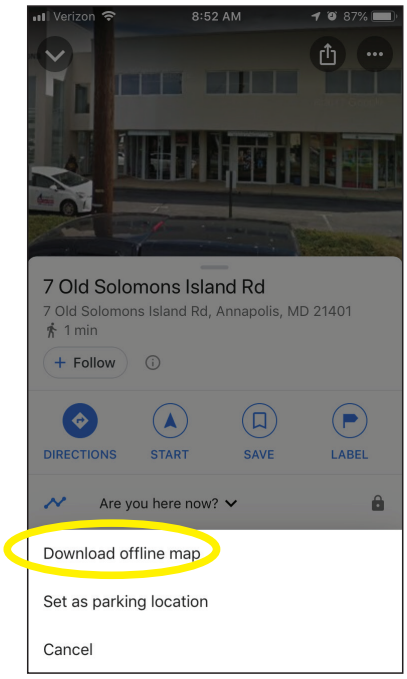
5. At the bottom of the screen, tap the name of the location that you just entered.



6. Then, tap the symbol that is circled (in yellow) in the upper right-hand corner of the screen.

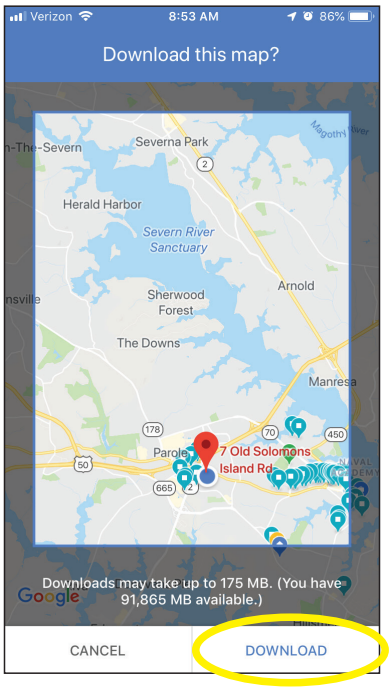


7. Select “Download offline map.”

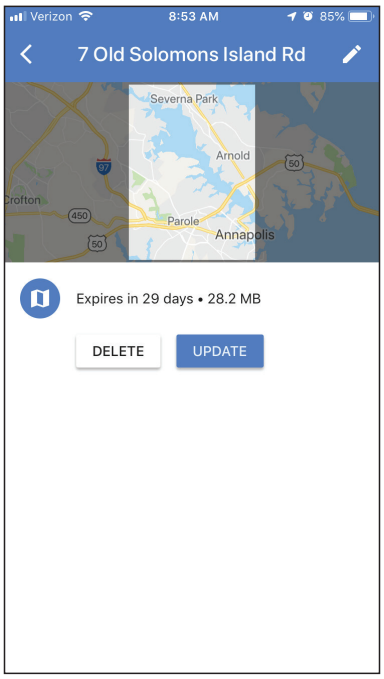


8. Using two fingers, zoom in and out to select the mangrove area. It’s better to capture a larger area that includes the area surrounding the mangroves.

9. Select “Download.”

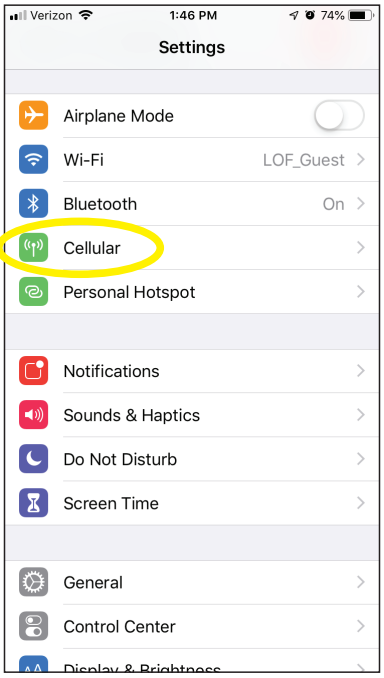


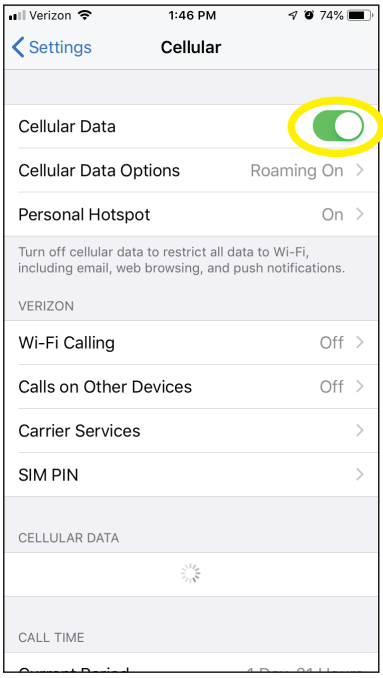
10. The map will be downloaded and saved offline for 30 days.



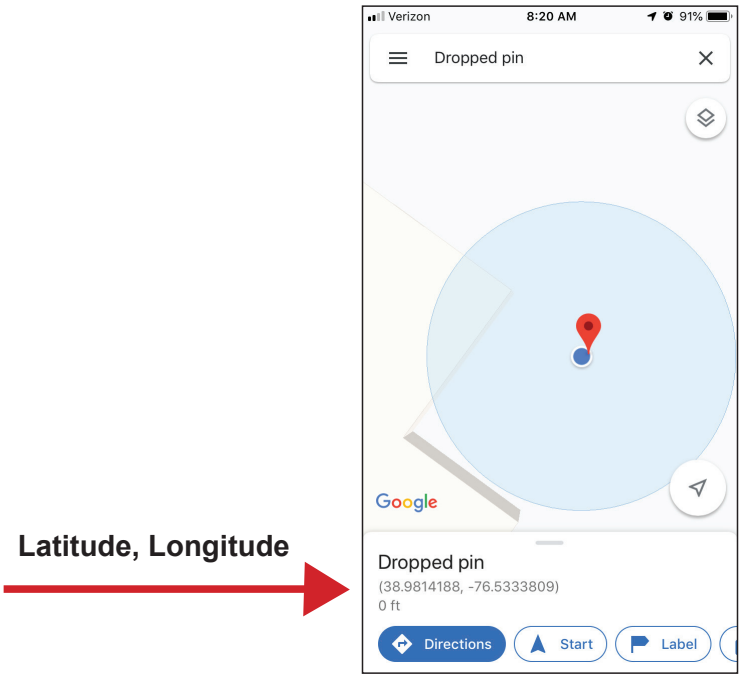
B. USE AN OFFLINE MAP:

1. If applicable, turn off data on the electronic device.
   a. Go to "Settings."
   b. Select "Cellular."
   c. Turn off the "Cellular Data" by pushing the toggle (will turn gray when off).



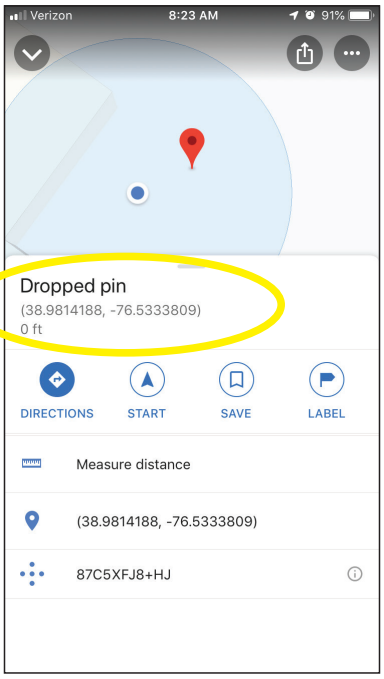


2. Open Google Maps app.
3. Use Google Maps as you normally would.
4. When you need to find your GPS coordinates, use two fingers to zoom in to your location, which is represented by a blue dot. Make sure to scroll in close to the blue dot.
5. Drop a pin over the blue dot. To do so, hold your finger over the blue dot to drop a pin.

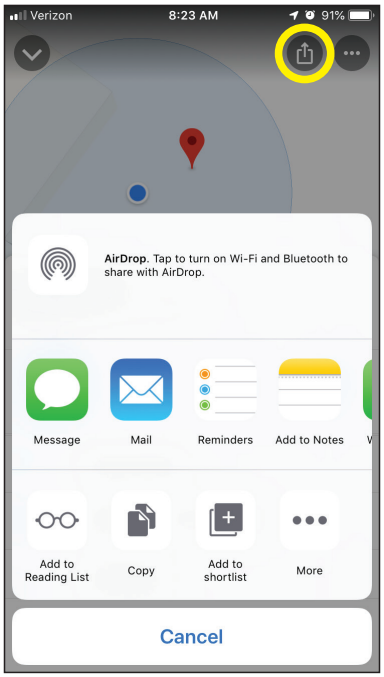


6. Record the GPS location (latitude and longitude) in *Table A* on your **Lesson 9A: Secret Agents – Investigating Insect Vectors** student worksheet. See the above image for the location of latitude and longitude.

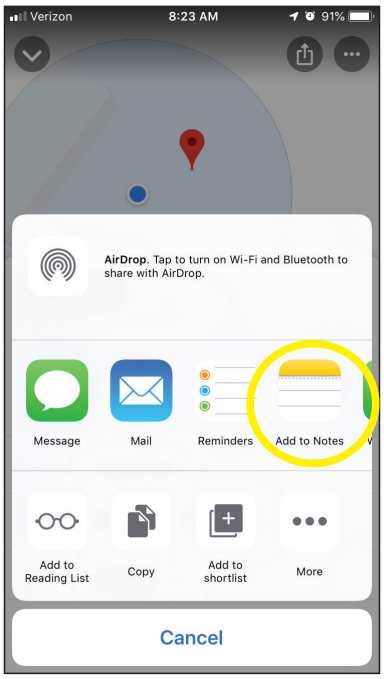
7. To save a pin:
   a. For Apple devices:
      i. At the bottom of your screen, use your fingers to tap the GPS location.



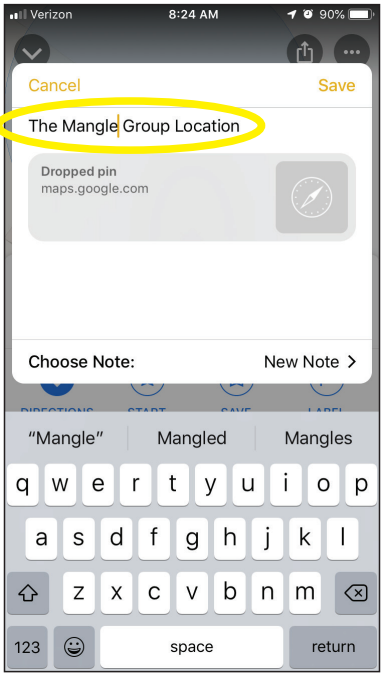
      ii. Select the share button circled in yellow.



iii. Select “Add to Notes.”



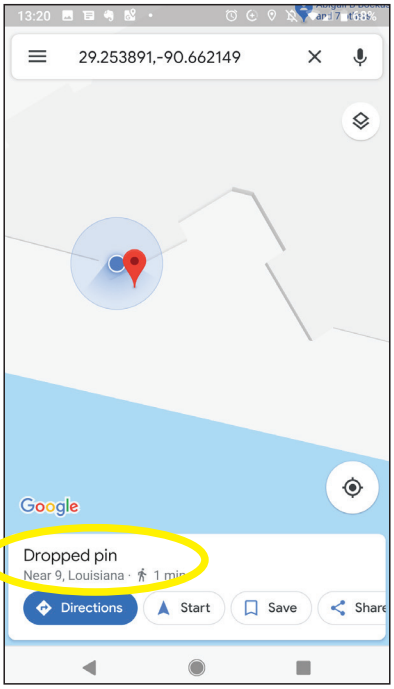
iv. Label the pin with your group information or your name.



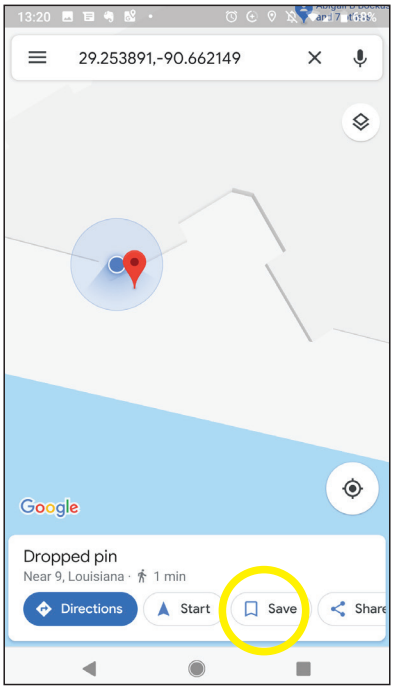
v. Select “Save.”

b. For Android devices:

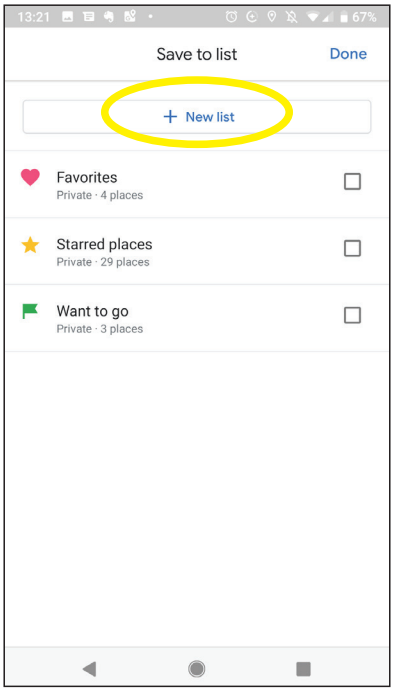
i. Tap the location of the place.



ii. Tap “Save.”



iii. Click on “New List.”



iv. Type your group information or your name into the text field.

v. Hit “Create.”

FIELD DATA COLLECTION INSTRUCTIONS

SUPPLY CHECKLIST:

- □ GPS or electronic device
- □ Ziploc bag
- □ Permanent marker

INSTRUCTIONS:

1. In *Table A*, record the mangrove location name, and the date and time that you started your survey.
2. Randomly sample potentially diseased red mangrove leaves. Remember that these leaves will have necrotic lesions on them that are visible on both sides of the leaf.
3. Use the GPS or electronic device to find the latitude and longitude of the tree that the diseased leaf was on. Record this information in *Table A*. Don't forget to record whether its north or south and east or west.
4. Place the leaf in a Ziploc bag and with a permanent marker label the bag with the date, you or your group's name, and leaf ID (e.g. ME1, ME2).
5. In *Table A*, write down any observations that you can about the site. Remember an observation is when you draw a conclusion based off of one of the five senses: sight, sound, touch, taste, and smell. For example, are the mangroves located close to a road? Near a development? Is it flooded or dry?
6. In a 5 x 5 meter squared area where you found your leaf (with the tree at the center of the 5 x 5 meter squared area), conduct a visual survey for disease. In *Table A*, check the box that corresponds to the number of diseased leaves that you see (0, 1, 2-10, 11-30, 31-50, or >50).
7. In the same 5 x 5 meter squared area, list approximately how many dead trees that you see? Record this information in *Table A*. If there are none, write zero.
8. Make sure that the leaves you collected are stored in the Ziploc bag and that the bag is tightly sealed. We do not want to risk spreading the disease.
9. After you have finished sampling, make sure that all of your supplies listed are returned to the container.

PREPARING AGAR PETRI DISHES & BLEACH SOLUTION

PART 1A: PREPARING POTATO DEXTROSE AGAR (PDA) MEDIA ON STOVETOP

SUPPLIES:

- ☐ **Preparing Agar Petri Dishes** video
- ☐ 250 mL distilled water
- ☐ 9.25 g Potato Dextrose Agar (PDA) media
- ☐ Non-stick pot
- ☐ Stirring rod or spoon
- ☐ 3.5 mL Hydrochloric acid (HCl), (Optional)
- ☐ Petri dish (60 mm)
- ☐ Graduated cylinder
- ☐ Gloves
- ☐ Hand sanitizer
- ☐ Rubbing alcohol

INSTRUCTIONS:

1. Watch the **Preparing Agar Petri Dishes** video.
2. To sterilize the area, wipe down the table with rubbing alcohol.
3. Measure 250 mL of distilled water.
4. Mix 9.25 g of PDA with distilled water in a sterilized, non-stick pot.
5. Boil for approximately 15 minutes stirring constantly so that the agar doesn't burn. The mixture will become thick when it's ready to be plated. The viscosity is similar to sugar when it is boiled down. To test, dip the stirring rod or spoon into the mixture. Pull it back out and let the mixture drip into the pot. If the mixture almost forms a thread, then it is done cooking.
6. Allow this mixture to cool for approximately 10 minutes.
7. Put on gloves.
8. Add 3.5 mL of HCl to the PDA solution and swirl. If you do not have HCl, skip this step.
9. Gently pour the PDA solution into the petri dishes. Pour enough to cover the bottom of the dish in a thin layer, but do not fill the agar to the brim of the petri dish.
10. After pouring, immediately cover each petri dish with the lid.
11. Gently swirl petri dishes to ensure even distribution of agar.
12. Let the liquid solidify on the tabletop for at least 30 minutes.
13. With the covers on, turn each petri dish upside down (agar side up) and transfer them to the refrigerator until further use. This prevents moisture from building up on the PDA which could be a source of contamination.

    NOTE: The agar will be enough to create 30 petri dishes (60 mm).

PART 2B: PREPARING POTATO DEXTROSE AGAR (PDA) MEDIA IN PRESSURE COOKER:

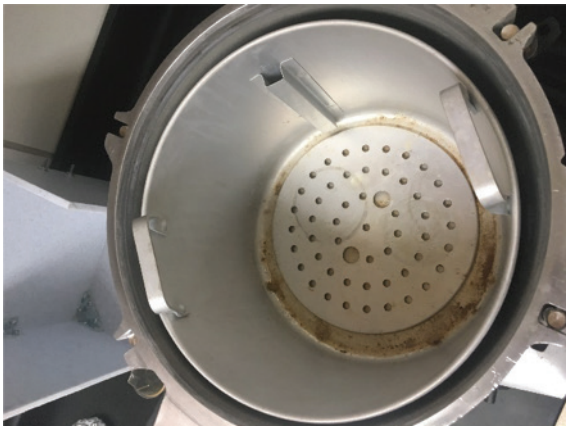
SUPPLIES:

- ☐ 250 mL distilled water
- ☐ 9.25 g Potato Dextrose Agar (PDA) media
- ☐ Pressure canner/cooker
- ☐ Stirring rod or spoon
- ☐ Mason jar or lab grade glassware (dependent on the size of the pressure canner/cooker)
- ☐ Foil
- ☐ Vacuum grease
- ☐ 4 L distilled water
- ☐ Petri dish (60 mm)
- ☐ Graduated cylinder
- ☐ Rubbing Alcohol
- ☐ Protective gloves or potholder

INSTRUCTIONS:

1. To sterilize the area, wipe down the table with rubbing alcohol.
2. Measure 250 mL of distilled water.
3. Mix 9.25 g of PDA with distilled water in a clean, mason jar or lab grade glassware.
4. Place foil over the top of the jar or glassware. This prevents contamination and leakage of the solution in the pressure cooker.
5. Prepare the pressure cooker. See the image below for an example of a tabletop pressure cooker.

   a. Remove the cover of the pressure cooker by turning the wingnuts in a counterclockwise motion. Always undo 2 opposite wingnuts at a time. Turn the cover top and remove it from the pot.
   b. Remove the inner container pot (photo on the left). The photo on the right doesn't contain it.

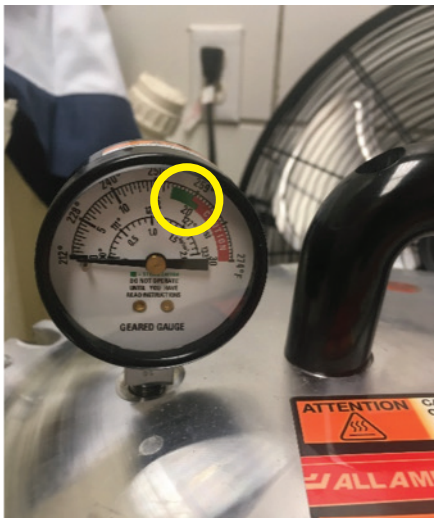


c. Before heating, apply vacuum grease to the top edges of the pot to help ensure a good seal.
d. Fill the pot with 4 liters of distilled water.
e. Place the glassware that contains the agar solution into the inner container pot.
f. Place cover on with the air exhaust tube (left image) inside of its designated chamber (right image) inside the pot.


g. Open the control valve on top of the cover. This allows steam to escape.

h. Twist the lid to the right to secure it in place and then tighten opposing wingnuts 2 at a time.
i. Flip the on switch to begin the boiling process
j. Allow the machine to boil in this state until the open control valve steams (~20-30 minutes). Once steam is present, wait 5 minutes before closing the valve.

k. Allow ~30 minutes for the Geared Steam Gauge to approach 17-21 PSI (the green zone circled in yellow). Once this PSI is reached, adjust the thermoswitch control to between 4 and 5 PSI. This will help keep the pressure cooker in the green zone.



l. Allow an additional 30 minutes to completely sterilize the solution. Be sure to check the steam gauge to ensure it is in the green zone.

m. After 30 minutes, turn the pressure cooker off.

n. Allow the pressure cooker too cool for 20 minutes, and then open the control valve switch. Be sure the stem gauge is between 0-3 PSI before opening the control valve. Open the control valve switch slowly and note that steam will be hot. Wear protective gloves or a potholder.

o. Once the steam gauge reads 0 PSI, carefully unlatch the lid by loosening opposite wingnuts. Twist top and remove lid.

p. Wearing protective gloves or a pot holder, remove the glassware or mason jar.

6. Gently pour the PDA solution into the petri dishes. Pour enough to cover the bottom of the dish in a thin layer, but do not fill the agar to the brim of the petri dish.
7. After pouring, immediately cover each petri dish with the lid.
8. Gently swirl petri dishes to ensure even distribution of agar.
9. Let the liquid solidify on the tabletop for at least 30 minutes.
10. With the covers on, turn each petri dish upside down (agar side up) and transfer them to the refrigerator until further use. This prevents moisture from building up on the PDA which could be a source of contamination.

    NOTE: The agar will be enough to create 30 petri dishes (60 mm).

PART 2: PREPARING BLEACH SOLUTION

SUPPLIES:

- □ Bleach
- □ Distilled water

INSTRUCTIONS:

If using 5% bleach:

- To create a 5% solution, mix 300 mL of bleach to 1,700 mL of water.

If using 8% bleach:

- To create an 8% solution, mix 200 mL bleach to 2,800 mL of water.

PLATING MANGROVE LEAF

SUPPLIES:

- ☐ Potato Dextrose Agar (PDA) petri dish
- ☐ Potentially diseased red mangrove leaf
- ☐ Rubbing alcohol
- ☐ Paper towel or towel
- ☐ Hand sanitizer
- ☐ Sterile paper towels (covered in foil)
- ☐ Scissor or scalpel
- ☐ Bleach solution
- ☐ Alcohol burner
- ☐ Tweezer or forcep
- ☐ Saran wrap or parafilm
- ☐ Tape or permanent marker
- ☐ **Lesson 8: Outbreak Investigation** student worksheet

INSTRUCTIONS:

1. Now that you are back in the classroom, take a photo of the potentially diseased leaf.
   a. Place an individual leaf on a piece of white paper with the top side of the leaf facing upwards.
   b. Assign the leaf with a unique identification (ID) label. **NOTE**: The labels should be consecutive (e.g. ME1, ME2, ME3).
   c. In pencil, write the ID and the word “upperside” (for upperside of the leaf) next to the leaf.
   d. For scale, position the metric side of a ruler next to the leaf, but not covering the ID label or word “upperside.” See the image below for an example.

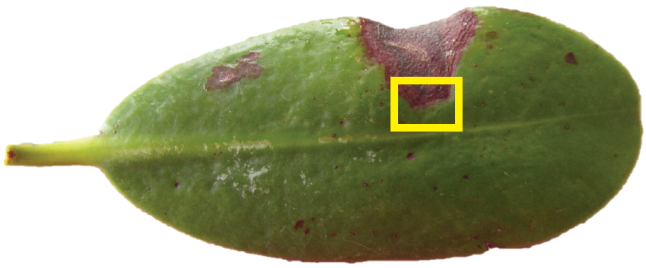
   e. Use a camera to take a photo of the upperside of the leaf. Hold the camera approximately 3 inches above the leaf and center the camera horizontally over the leaf. Make sure that the image is not blurry and take the photo.
   f. Remove the leaf and flip the piece of paper over. Write the ID label and the word “underside” (for underside of the leaf) next to the leaf.

g. Flip the leaf over so that the underside of the leaf is facing upwards. For scale, position the metric side of a ruler next to the leaf, but not covering the ID label or world "underside." See the image below for an example.

h. Use a camera to take a photo of the underside of the leaf. Hold the camera approximately 3 inches above the leaf and center the camera horizontally over the leaf. Make sure that the image is not blurry and take the photo.

i. If you have more than one leaf, repeat step a-h.

2. Draw your red mangrove leaf in *Table B* of your **Lesson 8: Outbreak Investigation** student worksheet. Color the areas of the leaf that are potentially diseased. It is okay to use pen or pencil.
3. When you have received your petri dish make sure to leave the lid on. Do not touch or breath on the agar at any time during this process because it can contaminate your sample.
4. Make sure that the agar side of the petri dish is facing upwards. Using a piece of tape or a permanent marker, label petri dish with your name and Leaf ID # (e.g. ME1, ME2).
5. Add rubbing alcohol to a paper towel or towel. Wipe down the surface where you will be working to sterilize the area.
6. Sanitize hands with hand sanitizer or put on gloves.
7. Using scissors or a scalpel, cut the leaf at the margin of the lesion and green leaf. The leaf piece should contain a small portion of the lesion and adjacent green tissue. Leaf pieces should be no bigger than .5 cm x .5 cm. See image below for clarification.



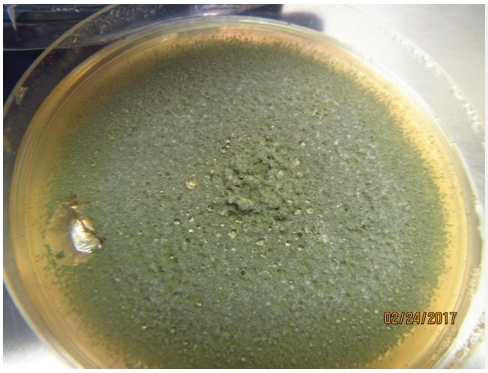
8. Repeat step 7. If there is a different area of the leaf that is diseased, use this section.
9. Sterilize both leaf pieces by placing them into a bleach solution for one minute. This prevents contamination from microbes that might be living on the surface of the leaf. The microbe causing the lesion has penetrated the leaf, so it will still grown when plated in the agar.
10. While leaf pieces are sterilizing, sterilize the tweezers. Dip the tweezers in rubbing alcohol and then move the tweezers back and forth through the flame for 5 seconds.
11. Remove one paper towel from the foil.

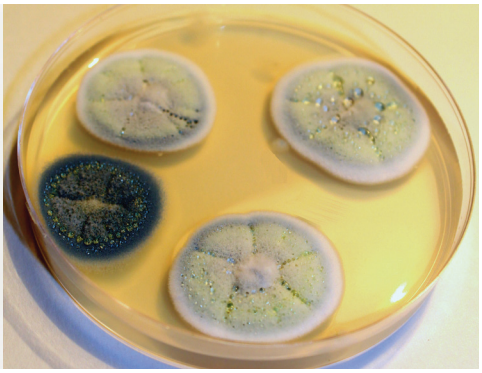
12. Use tweezers to transfer leaf pieces from the bleach solution to a paper towel. Blot the water off the leaf pieces but do not touch the paper towel or leaf pieces.
13. When dry, use sterile tweezers to place leaf in the agar petri dish. Make sure the piece is inserted into the agar so that it is slightly buried, not just laying on top. **NOTE**: Sometimes it is easiest to pierce the surface of the agar with the tweezers first and then insert the leaf pieces into the slit in the agar.
14. Close the petri dish lid and wrap it with saran wrap or parafilm to help prevent contamination.
15. Store in a plastic container at room temperature and monitor your sample for fungal growth after 3 days, one week, and two weeks.
16. After you have finished sampling, make sure that all of your supplies listed are in the container.

MANGROVE LEAF DISEASE

PENICILLIUM

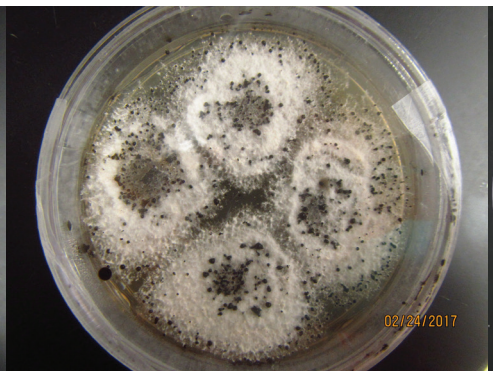
This fungal colony appears to olive green and densely compacted.

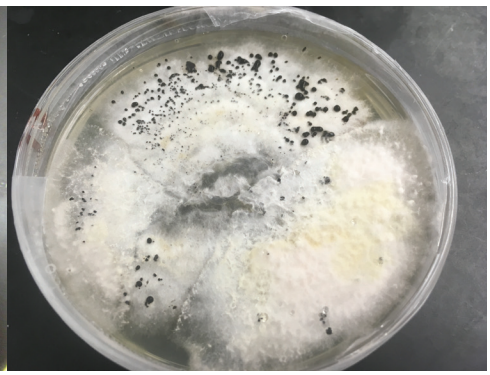


PESTALOTIOPSIS

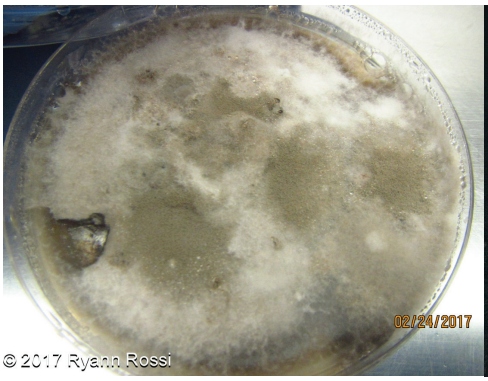
This fungal colony appears to be white and fluffy. Sometimes there are black dots present on top. These are spores.

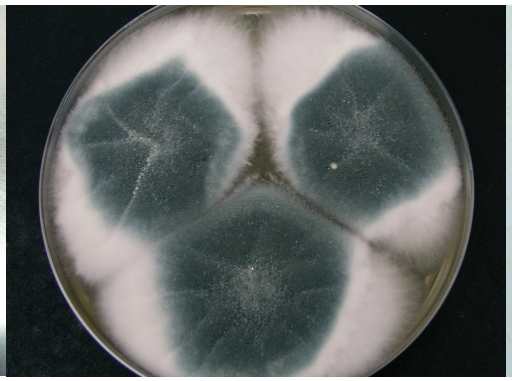
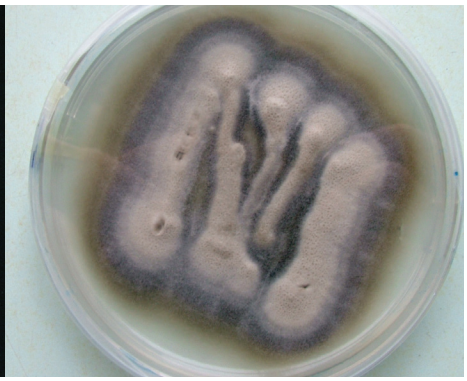




UNKNOWN

This fungal colony is light gray and fluffy.



MANGROVE LEAF DISEASE

BACTERIA

Bacteria contamination will appear will appear slimy and wet. It can cover the whole petri dish, or can grow in many small colonies.