Biotech in Your Backyard:
Experiments from Backyard to Classroom

Laboratory Manual
Version 1.0
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Jessica Halliley
James Hewlett

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Contributors to the text of this manual include: Jessica Halliley, Virginia Balke, Heather Bock, James Hewlett

Biotech in Your Backyard: Experiments from Backyard to Classroom

Introduction

Finding new ways to engage high school students in the science classroom can be a difficult task. *Biotech in Your Backyard: Experiments from Backyard to Classroom* aims to introduce high school science teachers to new techniques and concepts in biotechnology that can be incorporated for their students using real-world applications found in their own backyard. In this workshop, we introduce freshwater sponges as an ideal classroom experimental model; a simple organism that can be harvested from local streams, easily and economically stored and cultivated in the classroom, and versatile enough to demonstrate many general scientific principles from genetics and taxonomy to conservation and toxicology. In this workshop, we use the method of DNA Barcoding to identify species of sponges found in the Northeastern United States, where there is a gap in the knowledge of freshwater sponge species in this area. Submitting these results to the Sponge Barcoding Project (www.spongebarcoding.org), a global database for the study of Porifera across the world, helps advance education and research in this field. Identifying sponges in your area and adding to this database would be a great classroom project with wide-ranging real-world applications for students. Additionally, using freshwater sponges creates the opportunity to introduce your students to an array of biotechniques such as the use of dissecting and slide microscopes, pipetting and aliquoting, PCR, DNA sequencing and gel electrophoresis.

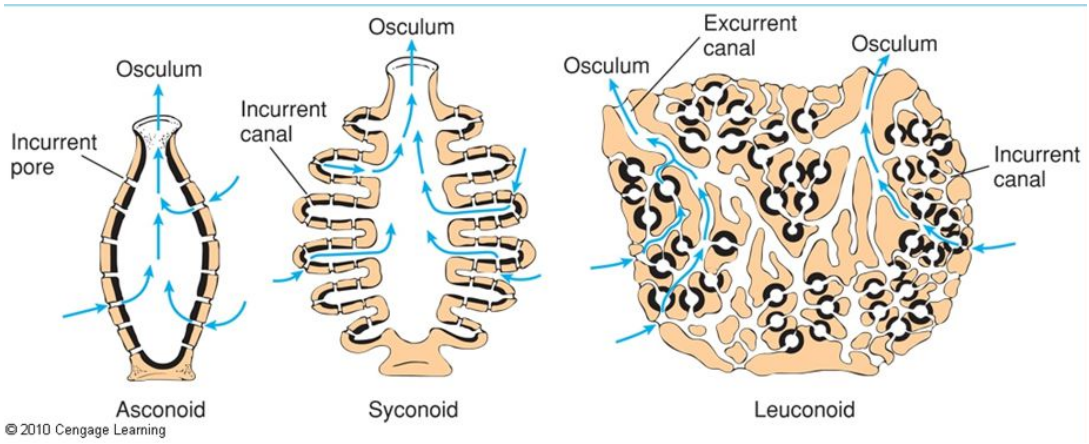
Biotech in Your Backyard also introduces a module on using wild mushrooms as a source of a biofuel-producing enzyme, cellobiase. Using a simple colorimetric assay kit, we will assess and compare the ability of different wild species of mushrooms to break down cellulose into glucose for the purpose of generating biofuels. This module presents a great opportunity to expose students to concepts such as enzyme kinetics and biochemistry, fermentation and biomanufacturing, and energy and environmental sustainability. This module lends itself naturally to extension experiments using simple fermentation techniques to generate a bioethanol product for further testing.

With *Biotech in Your Backyard*, the real-world questions assessed using the simple experimental platforms introduced in this workshop have the ability to capture students' imaginations and excite intellectual curiosity in ways that can enrich the classroom experience for both teachers and students.

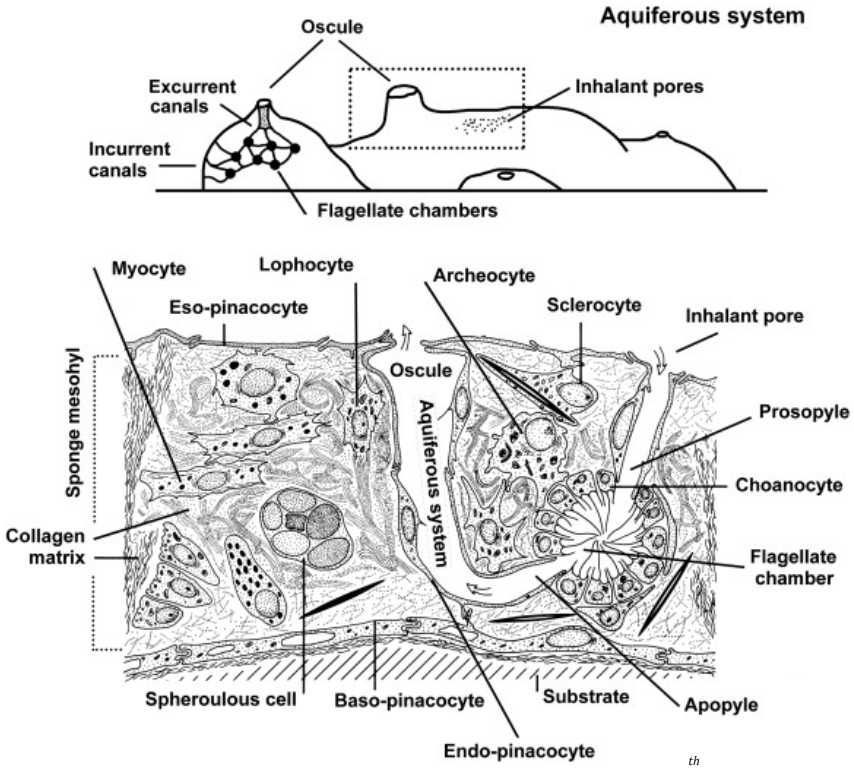
Freshwater Sponges: An Ideal Classroom Experimental System

Background

In aquatic ecosystems, sponges (phylum Porifera) carry out important functions by filtering microorganisms from vast volumes of water, hosting complex microbiomes including symbiotic organisms, and providing nutrition to a wide range of predators. There are estimated to be between 5,000 and 10,000 species of sponge worldwide with a small percentage of them living in freshwater environments. In North America, there are fewer than 30 freshwater species found in streams, rivers, ponds, and lakes attached to substrate surfaces such as rocks, branches, and aquatic plants. Since most sponge research has focused on marine sponges, there are many questions still to be asked and answered regarding freshwater sponges.

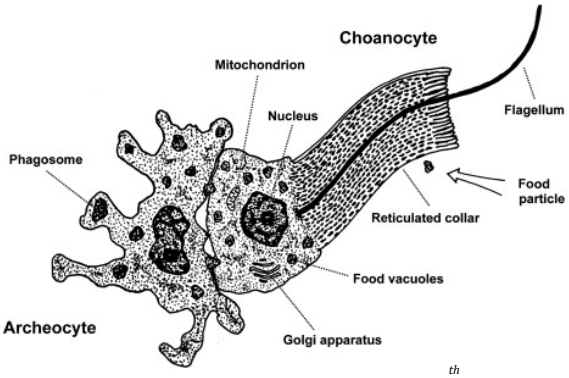


Sponges are an ancient group of sessile animals lacking a nervous system, tissues, and organs. The sponge body consists of three main layers, the pinacoderm, the choanoderm, and the mesohyl. The pinacoderm is formed by thin cells, pinacocytes, which act as a "skin." Pinacocytes are also capable of phagocytosis of particulates that are too large to enter the sponge body and are involved in attachment to the substrate. Interspersed through the pinacoderm are ostia, or openings, formed by porocytes. The mesohyl is a proteinaceous layer containing collagen, spicules, and motile, totipotent archaeocytes. The mesohyl acts as a "skeleton", supporting the



©Thorp and Covich's Freshwater Invertebrates (4th Ed., 2015)

sponge body.
Spicules can be formed from calcium carbonate or silica, providing structural support and protection from predators. Choanocytes form the choanoderm and are responsible for water filtration and feeding.
They possess a collar of microvilli surrounding a central flagellum. Through coordinated beating of their flagella, the choanocytes create a current that pulls water into the sponge through the ostia, allowing microplankton and organic debris to be caught in the collar. The food particles are engulfed through phagocytosis and passed to archaeocytes that distribute the nutrients to other cells throughout the sponge. The water then leaves the sponge through a larger opening, the osculum. Depending on the arrangements of choanocytes and canals, there are three main body plans, asconoid, syconoid, and leuconoid, the most complex. In the leuconoid body plan, the choanocytes are clustered in choanocyte chambers. The water enters an extensive system of incurrent canals which slows the current down for efficient collection of food particles. The water then enters excurrent canals before exiting through the osculum.



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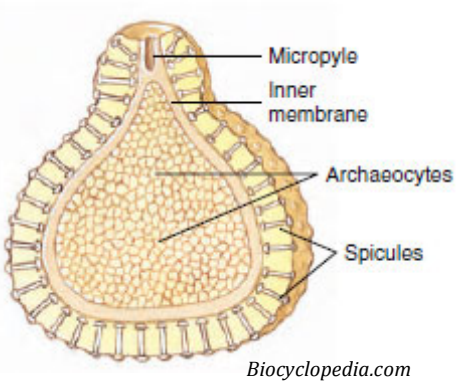
Sponges can reproduce both sexually and asexually with significant variation among species. Sexually produced larvae have flagella to move through the water. Asexual reproduction may occur through fragmentation or budding. Many species of freshwater sponges asexually produce gemmules to survive through harsh environmental conditions such as the freezing conditions of winter. Gemmules consist of thesocytes (a type of archaeocyte) surrounded by a tough coating of spongin (collagen microfibrils) and gemmuloscleres (spicules). The shape of the gemmuloscleres is species-unique and can be used for identification. After gemmulation is complete, the sponge body disintegrates, leaving the gemmules trapped in the skeletal material. Gemmules may be distributed in the environment by being consumed by animals such as ducks or by being dislodged.



Biocyclopedia.com

Hatching or germination of gemmules occurs in a very defined manner and is triggered by temperature and light. The thesocytes emerge from a small hole (foramen or micropyle), begin to divide, and attach to the substrate. Within a few days, a leuconoid species such as *Ephydatia muelleri* will form a structure in which choanocyte chambers, canals, and osculum can be observed pumping water through the sponge body. The ability to store gemmules long term in a laboratory setting and ease of hatching, makes freshwater sponges an excellent candidate organism for experiments investigating the effects of water conditions and environmental pollutants.

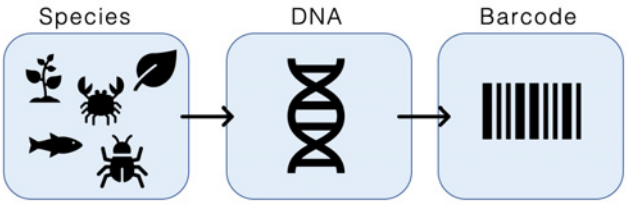
Marine sponges are hosts to a rich community of microbial symbionts, both intra- and extracellular. The composition of the sponge microbiome differs from the bacterial population of

the surrounding water and some bacteria are unique to sponges. Eukaryotic algae species are intracellular symbionts and photosynthesis can provide up to 80% of the required nutrients. Much less is known about the microbiome of freshwater sponges. *Chlorella*, a small, unicellular green alga, has been found as an intracellular symbiont in freshwater sponges. The conditions required for the formation of the relationship are not fully understood.

There is much to still be explored in the world of freshwater sponges. State surveys of sponges are often decades old, and populations of sponges are not always stable. Although gross distribution maps of sponge are available, minimal information is available about specific locations or optimal environmental conditions that support sponge growth. Development of a national network of sponge researchers investigating location along with water quality and substrate characteristics will advance our knowledge of freshwater sponge biology.

Barcoding Freshwater Sponges

DNA Barcoding



In order to identity an organism by DNA barcoding, DNA is first extracted from an organism, the barcode sequence is amplified using PCR and then sequenced. Because we want to simply amplify and sequence the DNA without having to clone it, it is important to use a haploid gene. Mitochondrial DNA is only inherited from the mother, and thus all the genes on the mitochondrial DNA are haploid. Also, there are multiple copies of the circular DNA molecule per mitochondria, and many mitochondria per cell so the copy number of mitochondrial genes is higher than nuclear genes. Thus the amplification can be done with limited amounts of tissue.

Mitochondrial genes have a low level of intraspecies diversity and a high level of interspecies diversity, which make them useful for differentiating species based on DNA sequence differences. We will be amplifying part of the cytochrome c oxidase (CO1) gene located on mitochondrial DNA. This gene has been accepted by scientists as the standard gene to be used for all animal barcoding studies (Herbert *et al.*, 2003). For the barcode PCR, we use what are known as "universal primers" which are designed to recognize conserved areas in the CO1 gene in many invertebrate species. Because the primer sequences will not be an exact match to the CO1 target sequence in all invertebrate species, the PCR reaction is performed at a low annealing temperature. This should allow primers that are not an exact match to still anneal well enough to form a stable duplex for the PCR reaction.

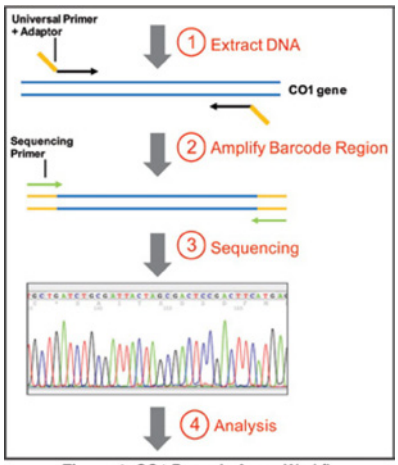


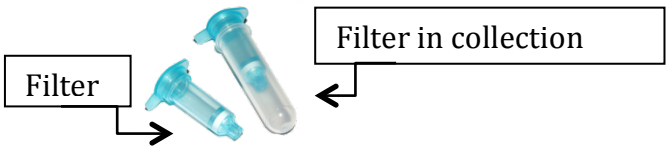
Figure 1: CO1 Barcode Assay Workflow

After running the PCR, some of the PCR product is run on a gel to confirm that the reaction was successful, and the product is the expected size of 660-680 base pairs. The remainder of the PCR sample is then cleaned up to remove the free nucleotides, primers, and enzyme and it is then sent for Sanger sequencing. The sequencing results are analyzed using free bioinformatics programs and databases.

DNA Extraction using the QIAGEN DNeasy Blood and Tissue Kit

Protocol:

1. Using a clean micropipette, transfer 10 gemmules into a 1.5mL tube.
2. Microcentrifuge the 1.5mL tube containing the gemmules for 10 seconds.
3. Using a micropipette, remove any liquid being careful not to disturb the gemmules.
4. Add 60 µL of ATL buffer to the tube.
5. Using a blue pestle, carefully crush the gemmules avoiding spilling any buffer.
6. Add 60 µL of ATL buffer to the tube.
7. Add 20 µL of proteinase K to the microfuge tube.
8. Place your tube on the heat block that is set to 56^{o}C. Incubate for 2 hours. After the 2 hour incubation, your instructor will remove it and place it in the freezer for use in the next lab meeting.
9. Collect your gemmule digestion from the instructor.
10. Ensure that the tube lid is closed and vortex the tube containing your digested tissue for 20 seconds.
11. Add 200 µL of AL and vortex for 5 seconds. (ensure that the tube lid is closed)
12. Add 200 µL of ethanol and vortex for 5 seconds. (ensure that the tube lid is closed)
13. Place a column in a collection tube. Label the lid of your tube. Pipette all of the liquid from your gemmule tissue onto this column.



14. Centrifuge at 8,000 rpm for 1 minute. Ensure that the centrifuge is balanced and the plastic lid is on. This allows the DNA to bind to the filter column.
15. Place the filter column into a new collection tube and discard the old collection tube. **Keep the filter column**, this has collected the DNA from your gemmules.
16. Add 500 µL of AW1.

17. Centrifuge for 1 minute at 8,000 rpm. Ensure that the centrifuge is balanced and the plastic lid is on.

18. Place the filter column into a new collection tube and discard the old collection tube. **Keep the filter column**, this has collected the DNA from your gemmules.

19. Add 500 µL of AW2.

20. Centrifuge for 3 minutes at 13000 rpm. *Note the change in centrifuge speed!* Ensure that the centrifuge is balanced and the lid is on.

21. Place the filter column into a new collection tube and discard the old collection tube. **Keep the filter column**, this has collected the DNA from your gemmules.

22. Centrifuge for 1 minute at 13,000 rpm to remove any remaining buffer. Ensure that the centrifuge is balanced and the lid is on.

23. Place the column in a new 1.5 mL microfuge tube.

24. Discard collection tube.

25. Add 50 µL of AE buffer to the **center** of the filter column (not near the side).

26. Let sit for 5 minutes.

27. Centrifuge for 1 minute at 8,000 rpm to elute the DNA from the filter column. Ensure that the centrifuge is balanced and the lid is on.

    Note: You will not be able to close the lid of the 1.5 ml microcentrifuge tube over the top of the filter column. When you place the tube with the column into the centrifuge, point the tube lid into the center of the centrifuge so that the centrifuge cap will fit.

28. The flowthrough that has collected in the microcentrifuge tube contains your gemmule DNA. You want to save this. Remove the filter column and close the lid to the microcentrifuge tube. **Save the Flowthrough – this contains the DNA.** You will use this DNA to set up the PCR reaction.

29. Calculate the DNA concentration using the Nanodrop.

Nanodrop Instructions

Your instructor will blank the nanodrop for you:

You will start here:

Measuring a Sample

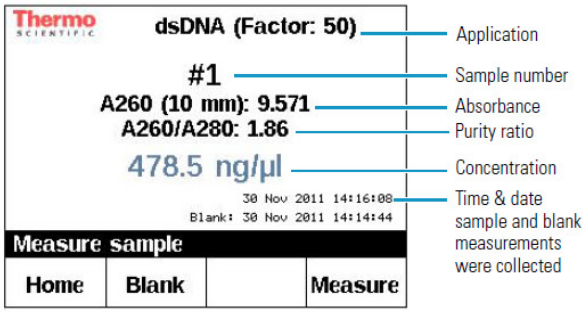
Pipette 1-2 µl of sample onto lower pedestal and press **Measure**.

Note A fresh aliquot of sample should be used for each measurement.

Although it is not necessary to blank between each sample, it is recommended that a new blank be taken every 30 minutes when measuring many samples.

Note When measuring more than one sample, be sure to wipe the upper and lower pedestal before loading the next sample.

Figure 7. Sample measurement screen



Taking Measurements

Blanking the Instrument

1. Select the assay type from the **Home** screen.
2. Establish a blank by pipetting 1-2 µl of the blanking buffer onto the bottom pedestal, lower arm and press **Blank**.
3. When measurement is complete, raise the arm and wipe buffer from both upper and lower pedestals using a dry laboratory wipe.
4. Confirm blank measurement by pipetting a fresh aliquot of blanking buffer onto the bottom pedestal, lower the arm and press **Blank**.
5. When measurement is complete, raise the arm and wipe buffer from both the upper and lower pedestals using a dry laboratory wipe.

DNA Concentration _____ng/ul

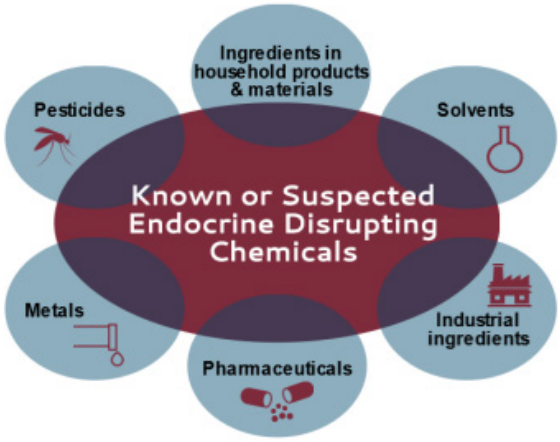
A260/280 purity ratio_____

A DNA concentration of more than 10ng/ul is good, 30ng/ul or higher is ideal. The A260/208 purity ratio should be close to 2.0. If your DNA concentration is higher than 30 ng/ul, use $C_1V_1 = C_2V_2$ to make a 20 ul dilution of your DNA sample.

Experimental Design and Freshwater Sponges as Bioindicators for Environmental Toxicology

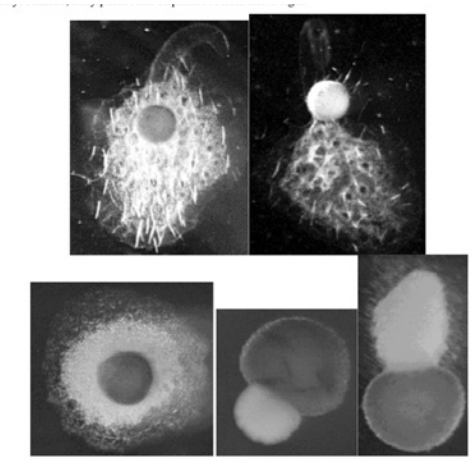
Background

Many human activities generate waste, and waste accumulation can cause significant environmental problems. Some pollutants are especially problematic because of the severe effects they have on wildlife and human health even at low concentration. One class of pollutants that is particularly dangerous includes chemicals known as endocrine disruptors (ED). These compounds can interfere with the body's endocrine system and produce adverse developmental, reproductive, neurological, and immune effects.



The list of ED compounds is long, and includes various pharmaceuticals, dioxin and dioxin-like compounds, polychlorinated biphenyls, DDT and other pesticides, and plasticizers such as bisphenol A (BPA). Given the ubiquity of plastics, that last entry is important - plasticizers are required to make material more malleable, flexible, and durable. They are everywhere in our environment (e.g., the "new car smell" includes plasticizers). BPA is used to make shatter-resistant and clear polycarbonate plastic bottles, eyeglasses, sports safety equipment, etc. BPA is also found in baby bottles, sippy cups, teethers, water bottles, food storage containers, and the lining of many food and beverage cans. BPA has been linked to a variety of health issues, including hormonal and developmental problems (e.g., lowered fertility, increased endometriosis, elevated rates of some cancers). Infants and young children are likely to be at most risk from exposure to chemicals like BPA. In 2012 the U.S. Food and Drug Administration banned the sale of baby bottles that contain BPA but has since concluded that BPA does not pose dietary risk to adults, a position consistent with that of the World Health Organization. Manufacturers responded to consumer concerns about BPA after several studies found the chemical mimics estrogen and could harm brain and reproductive development in fetuses, infants and children. Thirteen states and the District of Columbia have enacted restrictions since 2009 (http://www.ncsl.org/research/environment-and-natural-resources/policy-update-on-state-restrictions-on-bisphenol-a.aspx).

Recently, it has been described that BPA is also used in thermal receipt paper commonly used in retail stores. Handling this receipt paper before eating can expose you to ingesting low levels of BPA. Additionally, large amounts of receipt paper end up in freshwater streams and waterways due to pollution, contaminating the water and



Top row: sponges hatched in spring water or low concentration treatments and exhibit 'normal' development with well developed water canals and oscula.
Bottom row: abnormal growth in response to ethyl benzene and bisphenol-A (BPA)
Hill et al. 2002

possibly affecting the wildlife within. Stock BPA for testing is difficult to obtain as it is a tightly regulated substance, however one way to test BPA exposure on freshwater sponges is to soak thermal receipt paper in sponge media (stream water or 1x Strekal's) and observe the morphologic and developmental effects on plated gemmules.
Another ED pollutant commonly found in fresh waterways is the hormone estrogen. Most commonly released as a byproduct of the birth control pill, estrogen is not removed by water treatment facilities and ends up in streams and lakes in sometimes potentially unsafe concentrations. Studies have shown that male fish experience reproductive dysfunction when exposed to low levels of estrogen during their development.

In this lab, you will conduct experiments with freshwater sponges, an important invertebrate that plays essential ecological roles in freshwater ecosystems. Two ED treatment options to consider are birth control (synthetic progesterone and estradiol) and BPA thermal receipt paper. Stock solutions have been prepared and you have been provided with 6 well plates with which you will design your experiment with the proper variables and controls. Use the following questions to help develop your experimental design.

Questions to consider when developing a project:

- What biological processes am I interested in exploring relative to ED exposure?
  - Growth?
  - Behavior?
  - Metamorphosis/development?
  - Fecundity or reproductive capacity?

- What comparisons do I want to make and how does this play into my ability to ensure suitable replication?

- Are my potential dosage rates biologically relevant? How many treatment levels (ie different dosage rates) do I want to consider, and how will this affect my ability to ensure adequate replication?

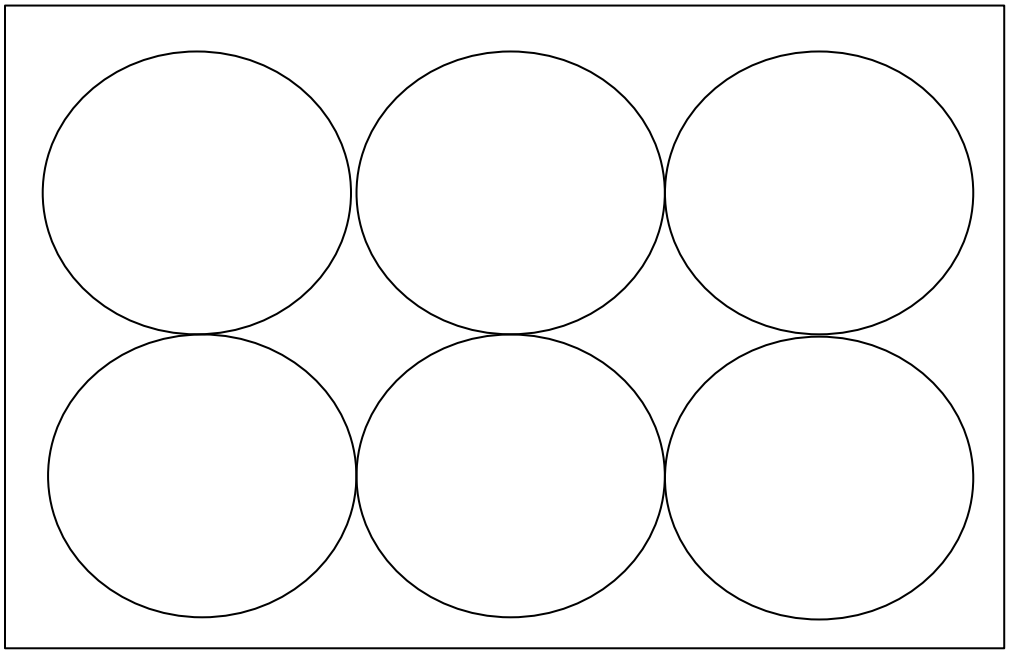
- What are the proper controls to ensure the effects observed can be attributed to the ED treatment?

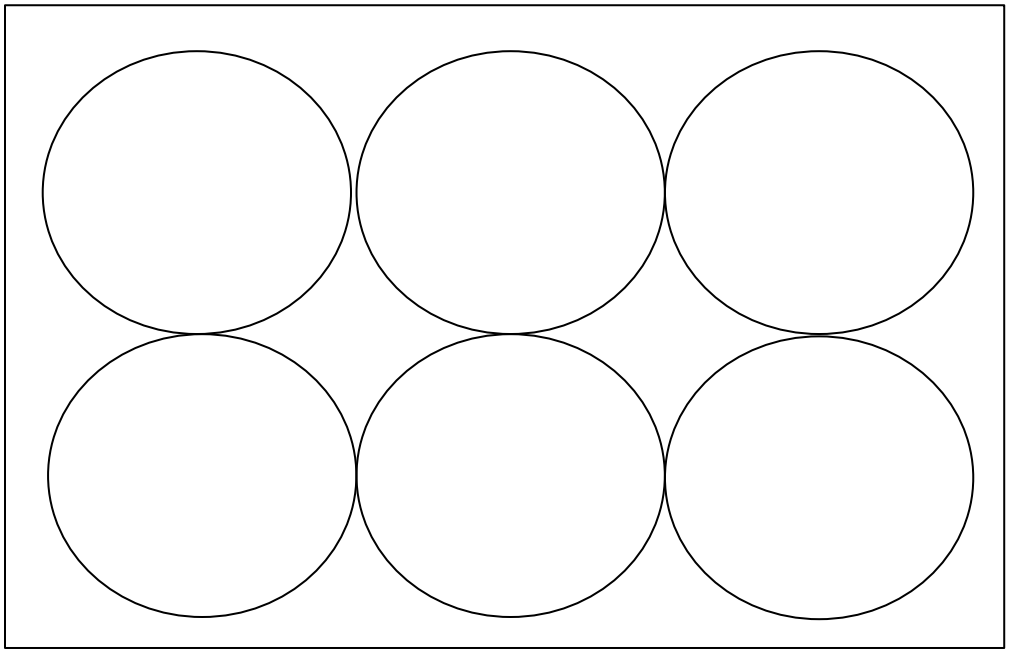
- Describe your hypothesis:

- Describe the methodological approach you intend to use, including discussion of replication, timeline, schedule of data collection, and necessary equipment.

Experimental Worksheets

Date:
Specimen:
Treatment(s):





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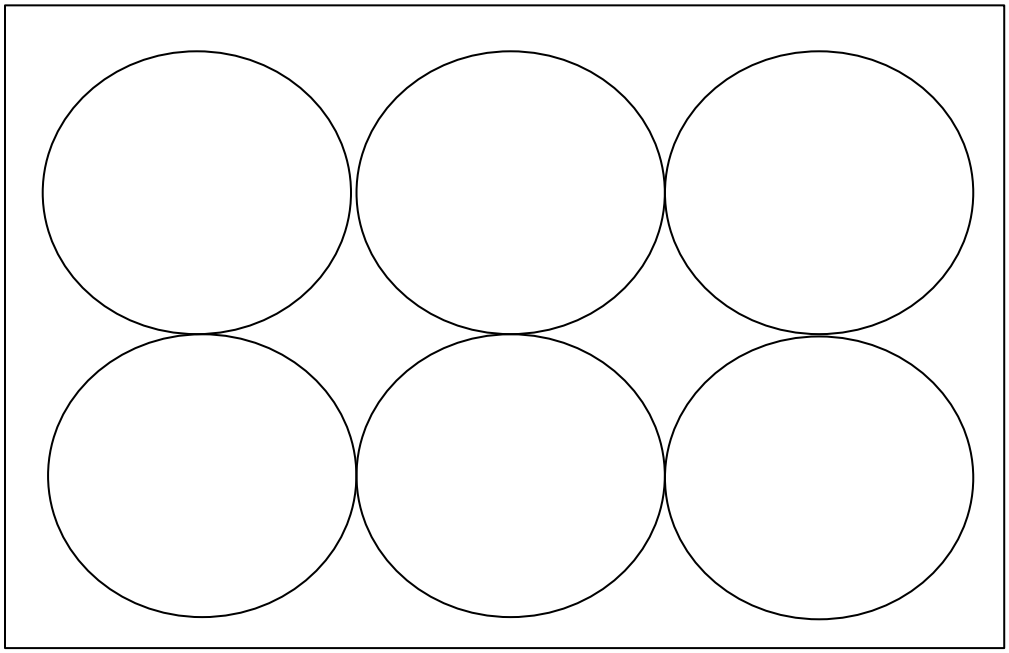

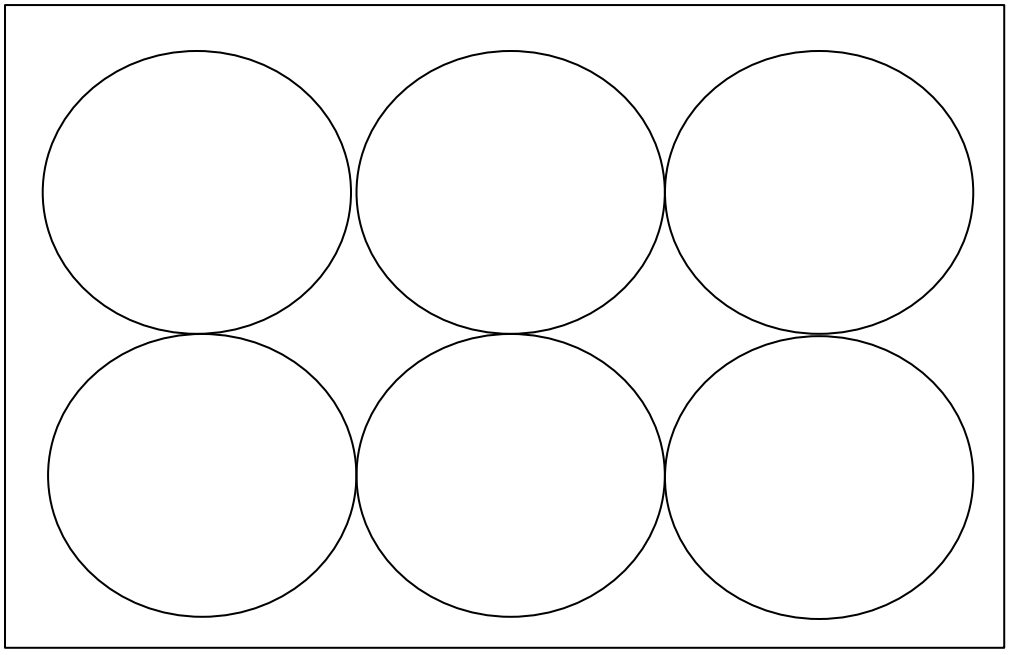

Results Worksheets

Date:
Specimen:
Treatment(s):

Attach photos or Draw Sponge Morphology After Treatments and Describe your Results.

Results Worksheets

Date:
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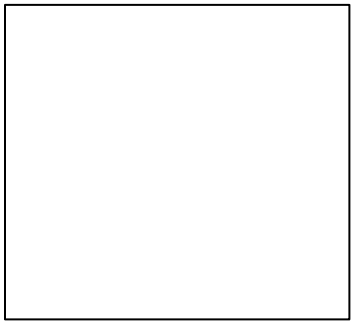
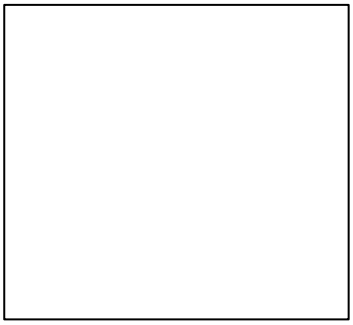
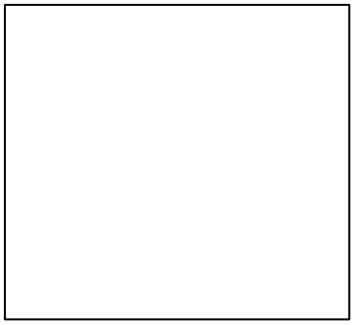
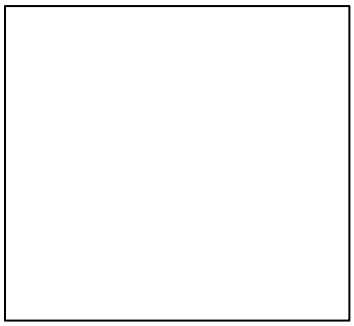
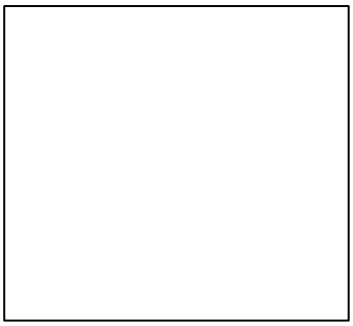
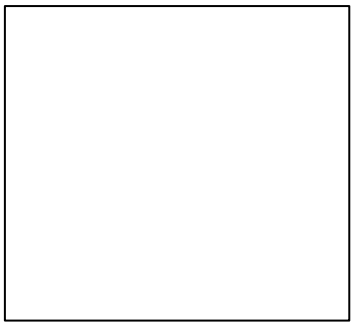
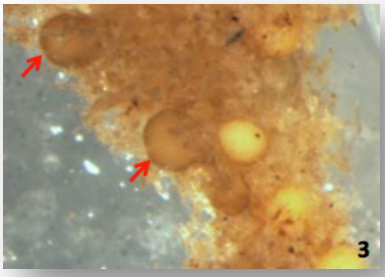







Freshwater Sponge Gemmule Harvest and Hatching; In Vitro Care

Background

Gemmules are important to the dormant life-phase of freshwater sponges, and are typically found in Northeastern climates during the winter months when stream water temperatures decrease below 4°C. Sponges can be found in shallow running streams near inlets or outlets, and prefer shaded microhabitats such as under stones or wood pieces. When collecting sponge and gemmule samples, use a sharp stone or stick to scrape the tiisue and gemmules off of the substratum to which they are adhered and place in a 50mL conical tube or other jar or vial with a small amount of stream water for it to float in. Give each sample you find a sample number and record the location you found it in. Tissue and gemmule samples should be stored in unfiltered stream water at 4°C in the dark (wrapped in tin foil) for at least 1 year without needing to refresh the storage water. Gemmules will stay dormant as long as they are kept dark and cold until you are ready to hatch them.

When ready to hatch gemmules for your experiment, the gemmules need to be isolated from the sponge tissue and washed to remove contaminants and prepare the spongin coat for hatching. The following handbook will walk you through picking and washing gemmules for hatching. The preferred medium for freshwater sponge growth is 1x Strekal's solution, however at a minimum, filtered stream water from the site of sponge sample collection may be used to wash and culture instead.

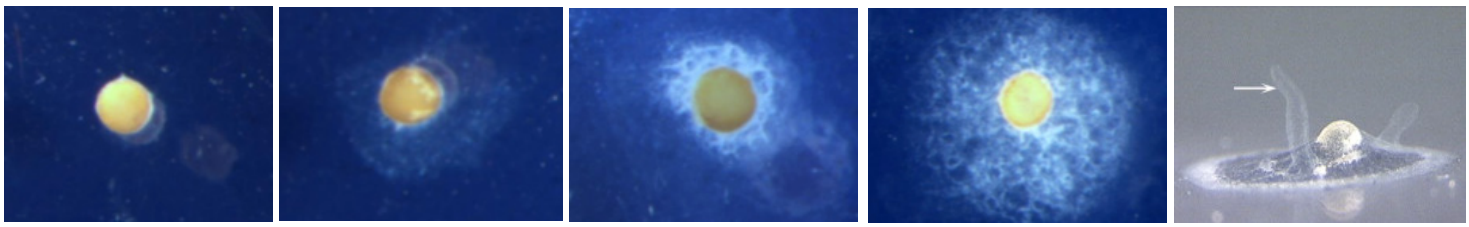


Hill et al. University of Richmond 2014

Once washed gemmules have been plated in petri dishes or 6 well plates, they should be left at room temperature in the dark for 2-3 days to hatch and begin attachment. Place your sample dishes or plates underneath a cardboard box to keep them dark. Sponges should be kept in the dark when you are not observing them. The medium (1x Strekal's solution, filtered stream water, or treatment media) should be changed every 1-2 days after the sponge cells have attached to the bottom of the dish. To change the medium, tilt the dish or plate to one side and aspirate out all of the old medium being careful not to disturb the attached sponge. Once the old media is removed, new room temperature medium can slowly and gently be added to the dish being careful not to disturb the attached sponge.

New sponges can survive *in vitro* for weeks, however long-term growth is typically not successful under these short-term growth conditions.

Hill et al. University of Richmond 2014



Stage 1 Stage 2 Stage 3 Stage 4 Stage 5

January 2014

Pick, Wash and Hatch Freshwater Sponge Gemmules Handbook

For

RNAi Treatment
Chemical inhibition
Other uses

NSF/EDEN Grant number IOS # 0955517

Required Equipment

Sample	
Maternal tissues containing gemmules	
Solutions	
Strekal's Medium	10 x 15mL
Strekal's Medium	14,5mL
Strekal's Medium	1mL/well
Hydrogen peroxide 30 wt % in water	500µL
Tools	
Recipient with ice	x2
Little Petri dish	x1
Microscope	x1
Teasing needle	x2
15mL Falcon conical tube	x1
Pipette (P1000)	x1
12-well Plate	
Circle cover glass (Ø18mm)	1/well

Sample storage

Sponges samples were collected near Prince William Forest National Park during late fall/early winter and stored in sampling water or cold Strekal's media for one year or more at 4°C and preserved from the light. Gemmules are aerated week and water replaced monthly.

Streakal's Medium

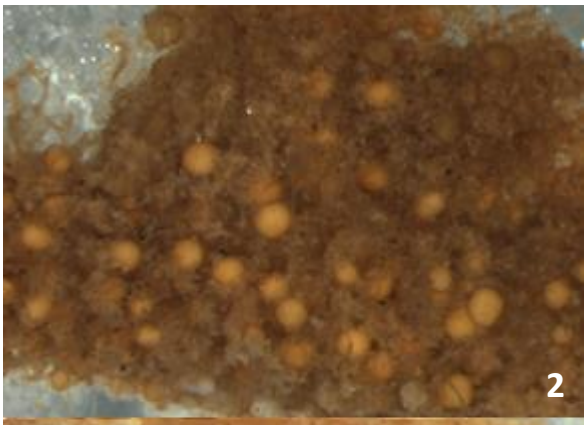
Strekal's Medium is an aqueous solution enabling gemmules attachment (Strekal and McDiffit, 1974) . 10X Strekal's Medium contains 0.9mM of $MgSO_4$-$7H_2O$, 0.5mM $CaCO_3$, 0.1mM Na_2SiO_3-$9H_20$ and 0.1mM KCl.

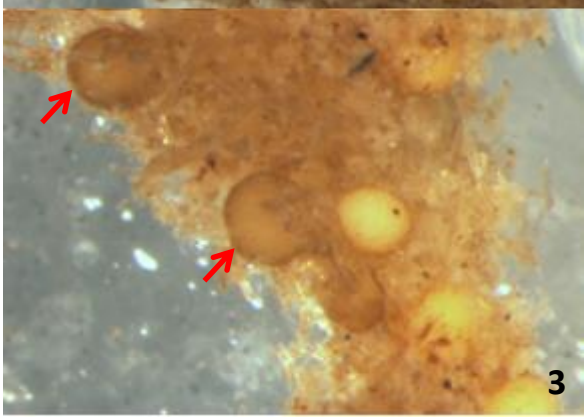
Pick gemmules

Important note before starting, all steps should be done on ice and keep Strekal's Medium cold during the procedure.

1. **Remove a small piece of maternal tissue from sampling water and pull it in a little Petri dish containing cold Strekal's Medium.**

2. **Using the microscope and teasing needles, carefully collect gemmules from maternal tissue (Pictures 1 and 2).**
   Sometimes, some gemmules coats are empty (picture 3) and do not contain cells. Squeezing them can confirm if the gemmule coat has cells on the inside, but will lead to popping the gemmule. A trained eye will lead to the ability to differentiate.





3. **Change Strekal's solution to remove all remaining maternal tissues and empty gemmules.**

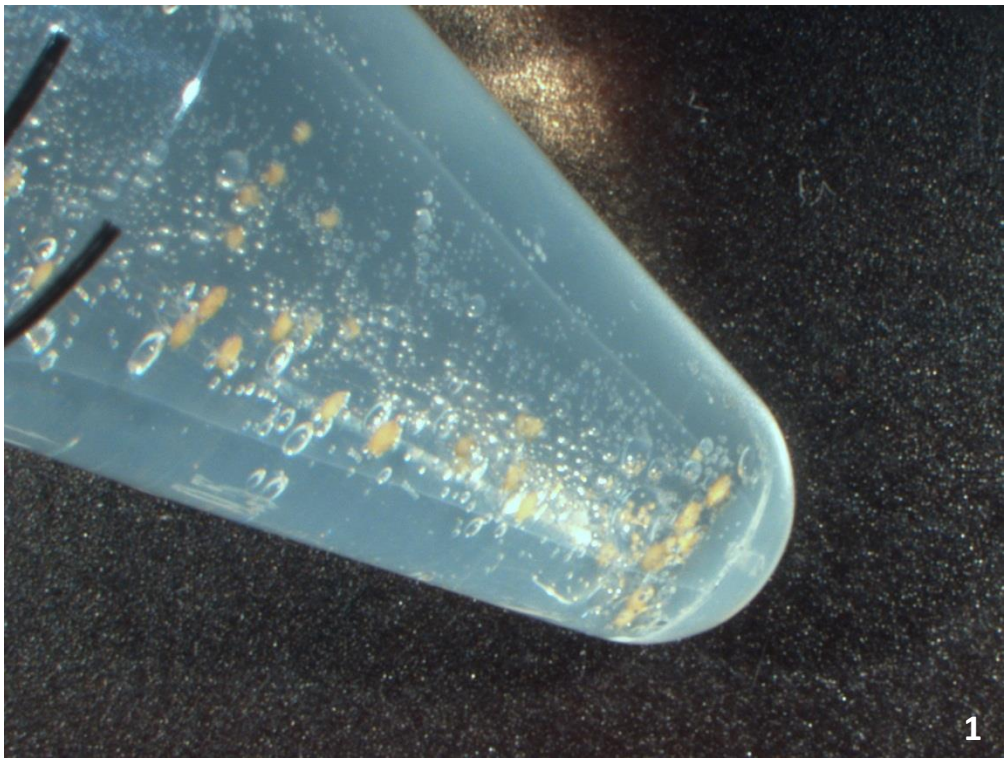
Wash gemmules

1. **Prepare hydrogen peroxide solution in a 15mL Falcon conical tube by diluting 500µL of 30% hydrogen peroxide solution in 13.5mL.**

2. **Gather gemmules by executing circles with the Petri dish.**
   Centripetal force will regroup gemmules in the center of the Petri dish.

3. **Pipette gemmules in 1mL of Strekal.**
   At least, adding 1mL of Strekal will permit to reach a hydrogen peroxide concentration of 1%.

4. **Add gemmules to the falcon containing the hydrogen peroxide solution and invert tube every minutes during 3 minutes.**
   This step permit removal of algae and to weaken gemmules membrane. Bubbles should appear (Picture 1).


1

5. **Help gemmules to go down by softly slapping the Falcon.**
   Some of them will not go down because of bubbles, don't worry and continue the procedure.

6. **Remove the solution by inverting the Falcon above the Petri dish, rapidly pick up the few gemmules from the Petri dish, pull them with others in the falcon, fill it with Strekal and invert it immediately several times.**
   Once at Falcon tip, gemmules stay there when you invert it (Picture 2).


2

7. **Change the solution using the same method and incubate gemmules 3 minutes. Invert falcon every minutes.**
   All gemmules which are still floating can be removed at this step.

8. **Proceed to step 7. 6 or 8 times more.**

9. **Gemmules are washed and ready to use. They can also be stored at 4°C at this time for later use.**

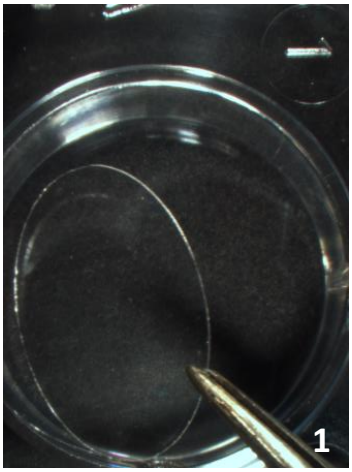
Hatch gemmules

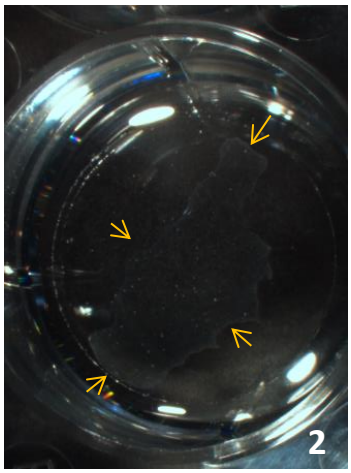
1. **Remove gemmules and Strekal's from ice. And let them reach room temperature.**
   During this time, proceed to next steps

2. **Open a 12-well plate and put a circular cover glass in each well (Picture 1).**

3. **Add 1mL of Strekal's Medium in each well.**

4. **Remove any air between well and cover-glass with a pipette tip (Picture 2 and 3).**
   Cover-glasses tend to float.



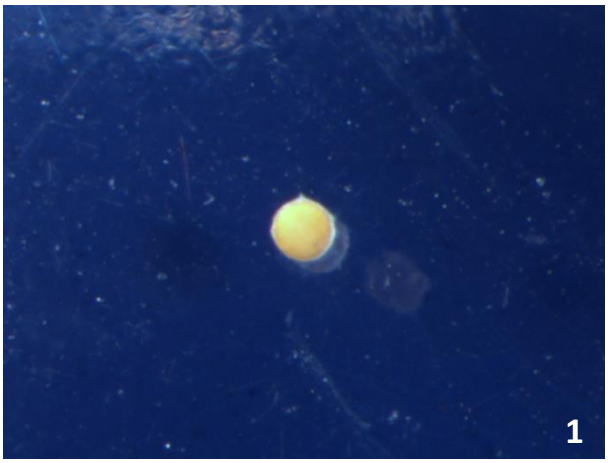


5. **Once gemmules and Strekal's reached room temperature, place 3-4 gemmules in each well.**
   Try to separate gemmules one from an other and from cover-glass edge.

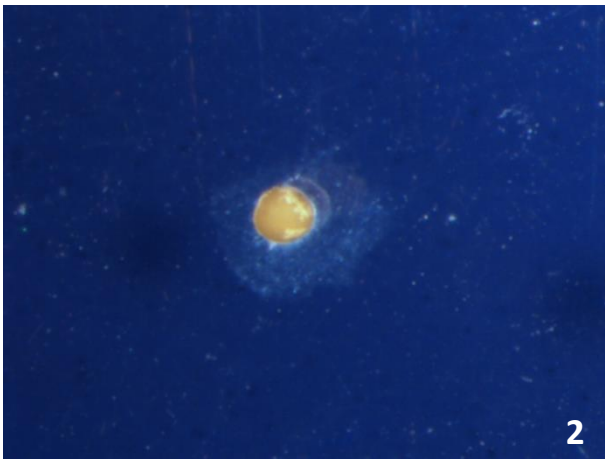
6. **Let gemmules to hatch for 2-3 days at room temperature in the dark.**

7. **Treat them at the desire stage (Pictures 1 to 6) (see Y27632 and RNAi treatment handbook).**
   Even without treatment, change the solution every 24h after attachment.

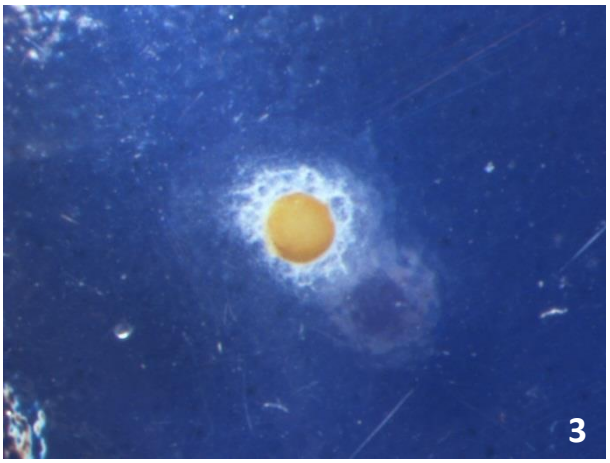
Stage 1



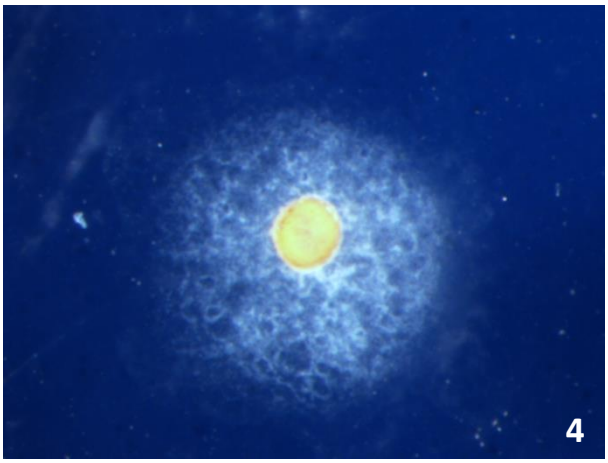
Stage 2



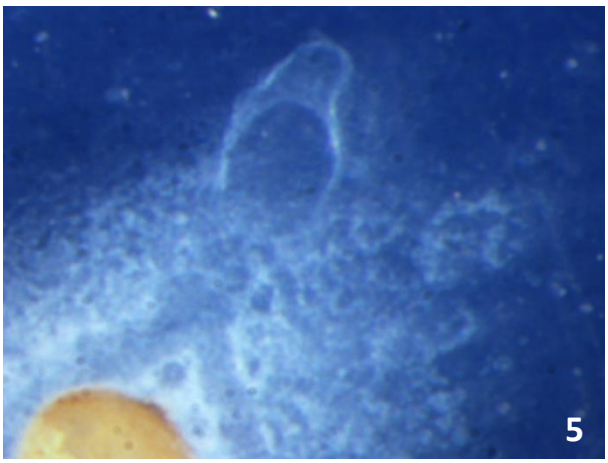
Stage 3

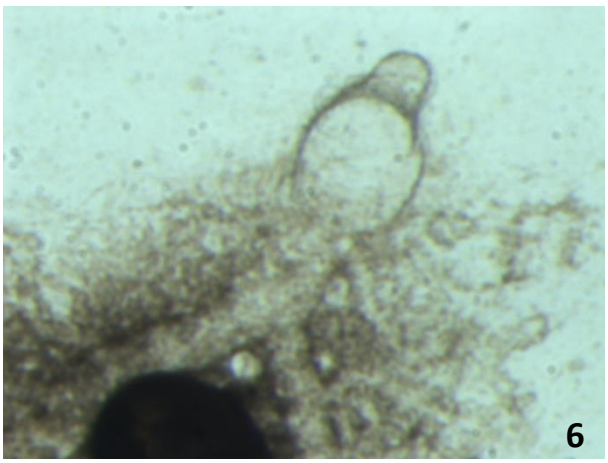


Stage 4



Stage 5





April Hill, *Department of Biology, University of Richmond, Richmond, VA, USA (ahill2@richmond.edu)*

Quentin Schenkelaars, *Institut Méditerranéen de Biodiversité et d'Ecologie marine et continentale (IMBE), Aix Marseille-Université, Marseille, France (quentin.schenkelaars@univ-amu.fr)*

Fernando Gomez, *Department of Biology, University of Richmond, Richmond, VA, USA (fgomez@richmond.edu)*

Chelsea Hall, *Department of Biology, University of Richmond, Richmond, VA, USA (chelsea.hall@richmond.edu)*

Laura Fierro, *Institut Méditerranéen de Biodiversité et d'Ecologie marine et continentale (IMBE), Aix Marseille-Université, Marseille, France (laura.fierro@univ-amu.fr)*

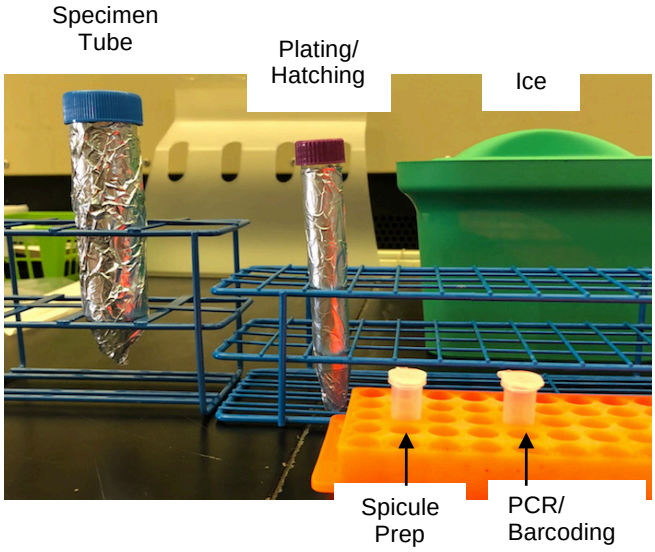
Emmanuelle Renard, *Institut Méditerranéen de Biodiversité et d'Ecologie marine et continentale (IMBE), Aix Marseille-Université, Marseille, France (emmanuelle.renard@univ-amu.fr)*

Carole Borchiellini, *Institut Méditerranéen de Biodiversité et d'Ecologie marine et continentale (IMBE), Aix Marseille-Université, Marseille, France (carole.borchiellini@univ-amu.fr)*

NSF/EDEN Grant number IOS # 0955517

Picking and Cleaning Gemmules

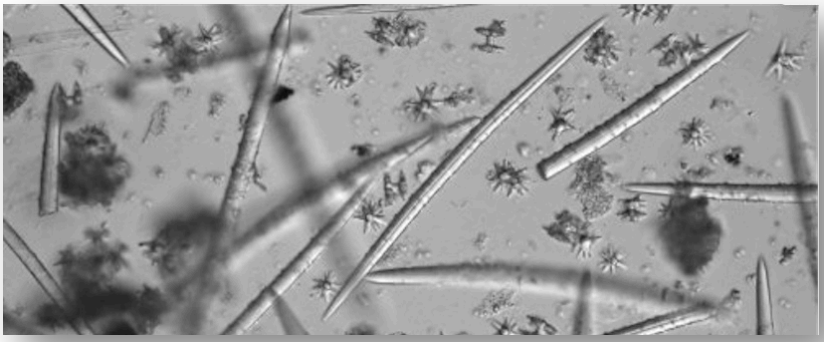
What you need for this workshop:



- PCR/Barcoding – one 1.5mL tube with 10 gemmules in Strekal's
- Spicule prep – one 1.5mL tube with 10-15 gemmules and a small amount of tissue (if present) in Strekal's
- Plating/hatching – 30-40 gemmules in 15mL tube in Strekal's, wrapped in foil
- Remaining gemmules and tissue stay in 50mL tube in Strekal's, wrapped in foil

Keep all samples on ice at all times!

Using Spicules to Identify Freshwater Sponge Species



Ephydatia mulleri

Spicules are microscopic elements that make up the skeleton of the sponge and the hard shell of gemmules. Spicules are made of silica, the same material found in glass and sand. Large spicules are called megascleres and smaller spicules are called microscleres or gemmuloscleres. The different shapes of spicules very greatly among different species of sponge, so visualizing these microscopic skeletal pieces can help identify your sponge specimen.

Sponge Spicule Preparation

Protocol:

1. Separate out 10-15 gemmules and a small (4mm X 2mm) amount of tissue with forceps. Using a p1000 micropipettor, transfer them to a 1.5 mL microcentrifuge tube. Let the gemmules and tissue settle to the bottom of the tube and, using a p1000 micropipettor, remove as much of the water as possible without disturbing the sample. Spin briefly in a microcentrifuge at high speed if needed.

2. Add 1 mL of bleach to the microcentrifuge tube(s), close the tube(s), and mix by inverting several times.

3. Place the microcentrifuge tube(s) on a rocker and let rock overnight at room temperature for digestion.

4. After digestion, let the spicules settle to the bottom of the microcentrifuge tube(s). *This may take 5-10 minutes for all of the particles to completely settle to the bottom of the tube(s).*

5. Using a p1000 micropipettor, remove 90% of the bleach solution from the tube(s) and discard, being careful not to draw at the very bottom of the tube where the spicules have settled. Remove the bleach solution slowly so as not to disturb the spicules.

6. Add 1 mL of diH_20 to the microcentrifuge tube(s), do not mix so as not to break the fragile spicules.
7. Let the spicules settle to the bottom. Using a p1000 micropipettor, remove 90% of the diH_20 in the tubes and discard, being careful not to draw at the very bottom of the tube where the spicules have settled. Remove the diH_20 slowly so as not to disturb the spicules.

8. Repeat steps 5-6 2x more with fresh diH_20.

9. After performing 3 washes with diH_20, let the spicules settle to the bottom. Using a p1000, remove 90% of the diH_20, being careful not to draw at the very bottom of the tube where the spicules have settled, and discard. Add 1 mL of 100% ethanol to wash. Repeat this wash once more, letting the spicules settle to the bottom of the tube before removing and discarding 90% of the first ethanol wash. Add 1 mL of fresh 100% ethanol.

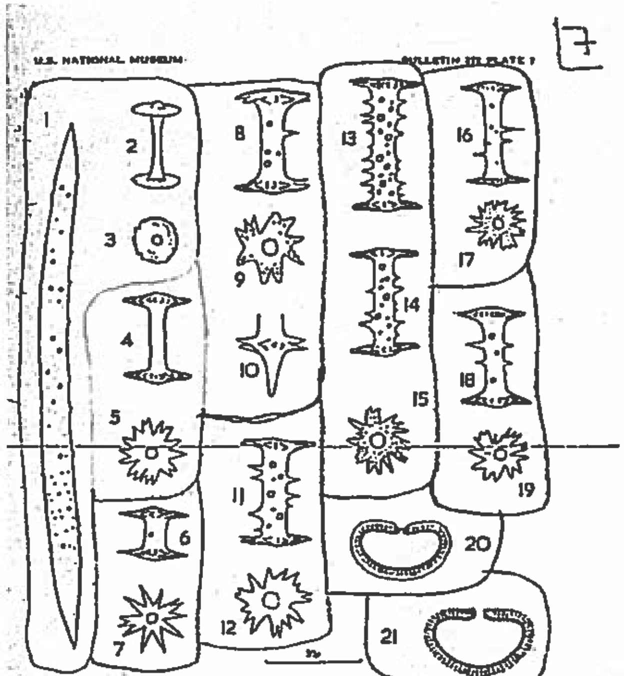
10. To prepare sample(s) to view, remove about 75% of the remaining ethanol from the tube(s) after letting the spicules settle to the bottom, and discard.

11. Prepare and label microscope slides for each sample.

12. Let the spicules settle to the bottom of the microtube(s). Using a disposable pipette, draw up a small amount of sample from the very bottom of the tube where the spicules have settled. Holding pipette over the microscope slide, let the spicules settle to the tip of the pipette for 5-10 seconds, and place 2 drops of sample on the center of the microscope slide. Return the remaining sample to the same microtube.

13. Let the ethanol dry from the slides completely before viewing the samples under a microscope. Observe the samples under 4X, 10X and 40X objectives; *do not view under immersion oil unless the slides have been mounted with a cover slip.*

14. Draw, photograph and measure all types of spicules found. For reference when naming types of spicules, use the *Thesaurus of Sponge Morphology*.

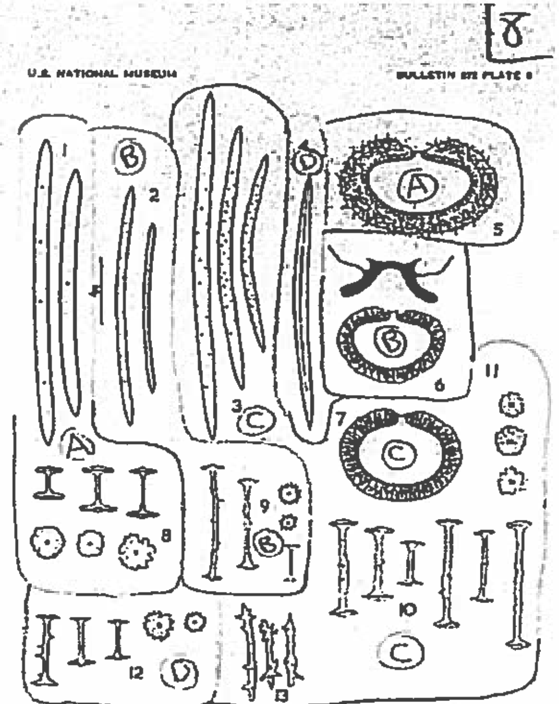


FIGURES 1-21.—1, Megasclere, *Ephydatia milleri*; 2, 3, gemmoscleres, *E. milleri*; 4, 5, gemmoscleres, *E. fluviatilis*; 6, 7, gemmoscleres, *E. muelleri*; 8, 9, gemmoscleres, *E. fortis*; 10, gemmosclere, *E. fortis* with freak rotule; 11, 12, gemmoscleres, *E. robusta*; 13, longer gemmosclere, *E. ramsayi*; 14, shorter gemmosclere, *E. ramsayi*; 15, rotule of gemmosclere, *E. ramsayi*; 16, 17, gemmoscleres, *E. facunda*; 18, 19, gemmoscleres, *E.* [illegible]; 20, gemmule, *E. meyeni*, optical sect.; 21, gemmule, *E. ramsayi*, optical sect.

E. talaensis

Ephydatia

E. japonica (pg. 88-89)



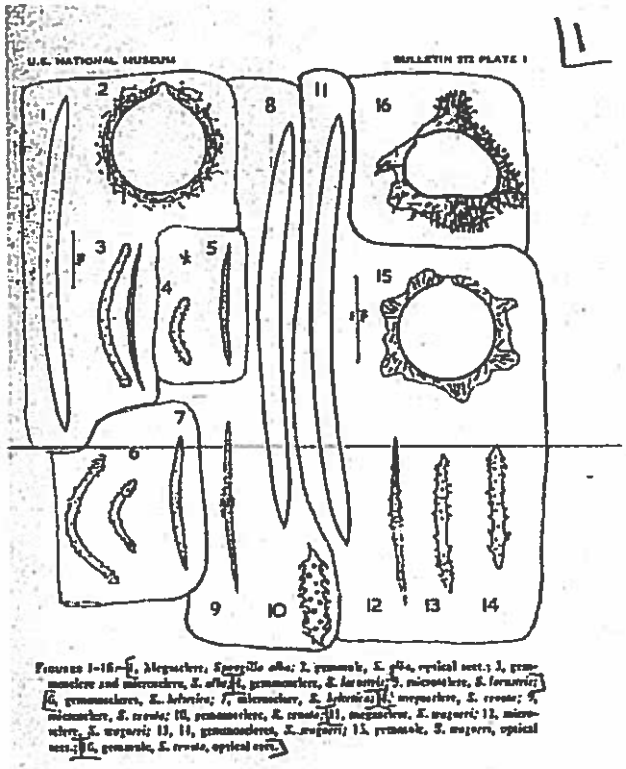
FIGURES 1-13.—1, Megasclere, *Heterorotula capewelli*; 2, megasclere, *H. nigra*; 3, megasclere, *H. multidentata*; 4, megasclere, *H. multiformis*; 5, gemmule, *H. capewelli*, optical sect.; 6, gemmule, *H. nigra*, optical sect. and enlarged foramen tube; 7, gemmule, *H. multidentata*, optical sect.; 8, range of gemmoscleres, *H. capewelli*; 9, range of gemmoscleres, *H. nigra*; 10, range of gemmoscleres, *H. multidentata*; 11, rotules of gemmoscleres, *H. multidentata*; 12, range of gemmoscleres, *H. multiformis*; 13, malformed gemmoscleres, *H. multidentata*, probably a result of hybridization.

Heterotula

*only in Australia + New Zealand

A: H. capewelli
B: H. nigra
C: H. multidentata
D: H. multiformis

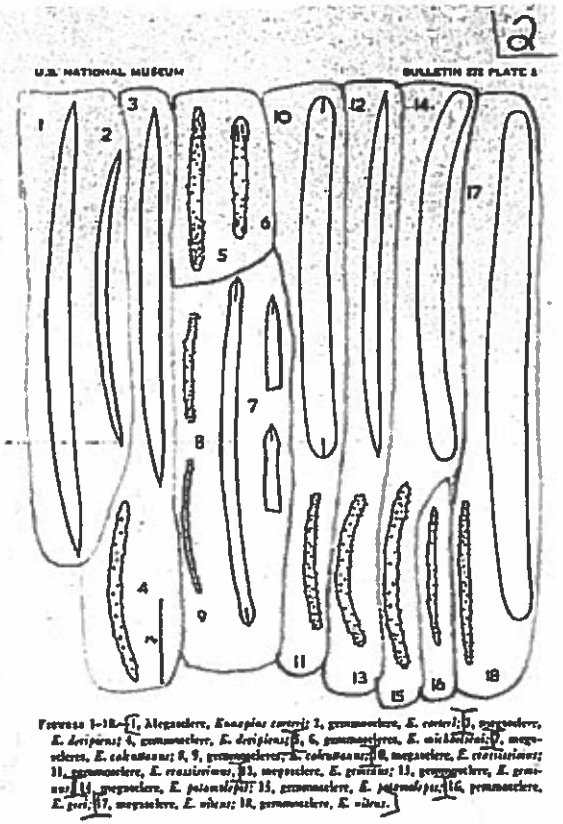
hispid



Spongilla

* may or may not be in range

- Spongilla asphaera (pg. 14-15)

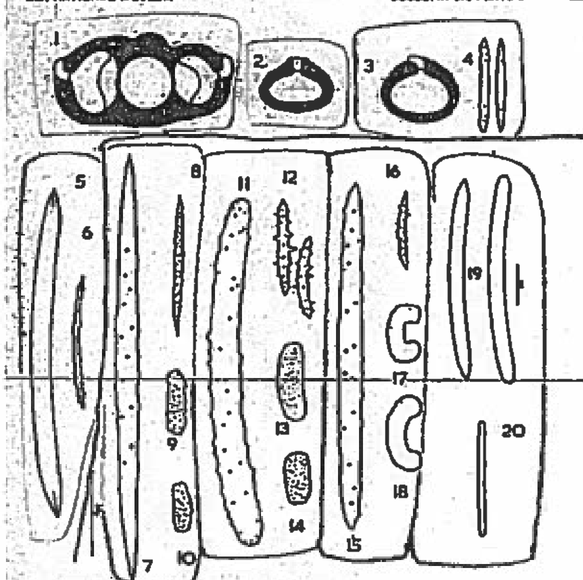


Eunapius

- Eunapius iglouiformis (pg. 31-32)

3

U.S. NATIONAL MUSEUM BULLETIN 272 PLATE 3

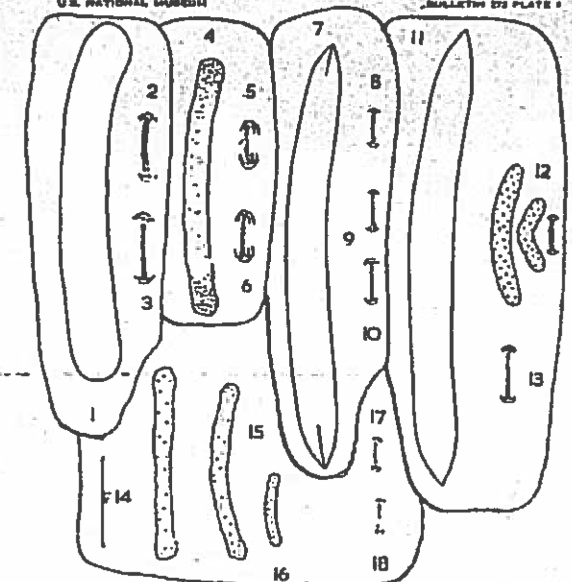


FIGURES 1-20.—1, Gemmules, *Eunapius fragilis*, optical sect.; 2, gemmule, *E. carteri*, optical sect.; 3, gemmule, *E. sinensis*, optical sect.; 4, gemmoscleres, *E. sinensis*; 5, megasclere, *E. geeri*; 6, gemmosclere, *E. geeri*; 7, megasclere, *Stratospongilla sumatrana*; 8, microsclere, *S. sumatrana*; 9, 10 gemmoscleres, *S. sumatrana*; 11, megasclere, *S. indica*; 12 microscleres, *S. indica*; 13, 14, gemmoscleres, *S. indica*; 15, megasclere, *S. gravelyi*; 16, microsclere, *S. gravelyi*; 17, 18, gemmoscleres, *S. gravelyi*; 19, megascleres, *S. clementis*; 20, gemmosclere, *S. clementis*.

Eunapius/Stratospongilla

4

U.S. NATIONAL MUSEUM BULLETIN 272 PLATE 4

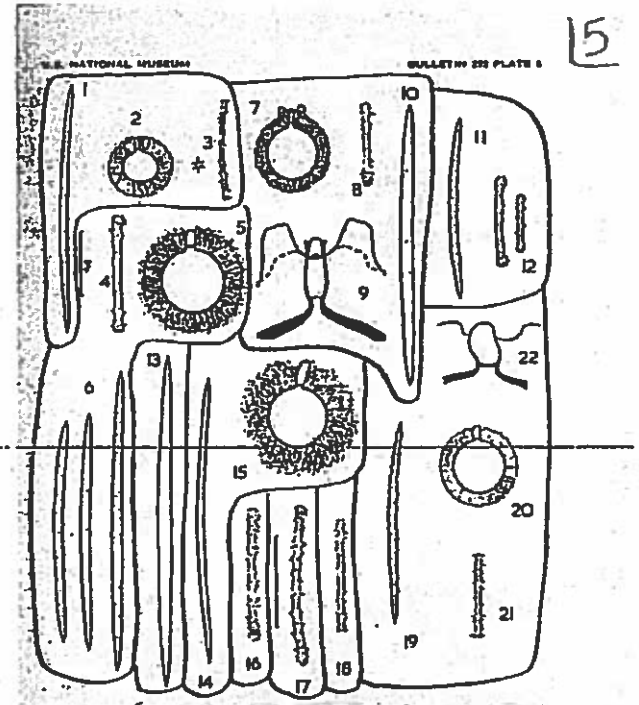


FIGURES 1-18.—1, Megasclere, *Corvospongilla lapidosa*; 2, 3, microscleres, *C. lapidosa*; 4, megasclere, *C.* [illegible]; 5, 6, microscleres, *C.* [illegible]; 7, megasclere, *C. coacteri*; 8-10, microscleres, *C. coacteri*; 11, megasclere, *C.* [illegible]; 12, gemmoscleres and microsclere, *C.* [illegible]; 13, microsclere, *C. ultima*; 14, 15, megascleres, *C. scabrispiculis*; 16, gemmosclere, *C. scabrispiculis*; 17, 18, microscleres, *C. scabrispiculis*.

Corvospongilla

*: not found in USA

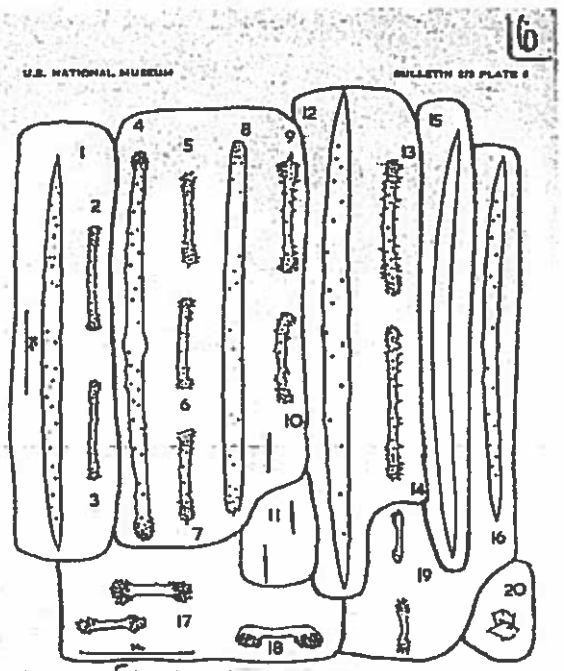
15



FIGURES 1–22.—[1, Megasclere, *Radiospongilla crateriformis*, Indies; 2, gemmule, *R. crateriformis*, India; 3, gemmuscleres, *R. crateriformis*, India; 4, gemmuscleres, *R. serpentina*; 5, gemmule, *R. serpentina*, apical rect.; 6, megasclere, *R. serpentina*; 7, gemmule, *R. brewsterensis*, apical rect.; 8, gemmuscleres, *R. brewsterensis*; 9, enlarged gemmule pores tube, *R. brewsterensis*; 10, megasclere, *R. brewsterensis*; 11, megasclere, *Spongilla multispiculifera*; 12, microsclere, *S. multispiculifera*; 13, megasclere, *S. reticulata*; 14, megasclere, *S. proliferens*; 15, gemmule, *S. proliferens*, apical rect.; 16, gemmuscleres, *S. reticulata*; 17, gemmuscleres, *S. verrucosa*; 18, gemmuscleres, *S. proliferens*; 19, megasclere, *Radiospongilla cenotensis*; 20, gemmule, *R. cenotensis*, apical rect.; 21, gemmuscleres, *R. cenotensis*; 22, enlarged gemmule pores tube, *R. cenotensis*.]

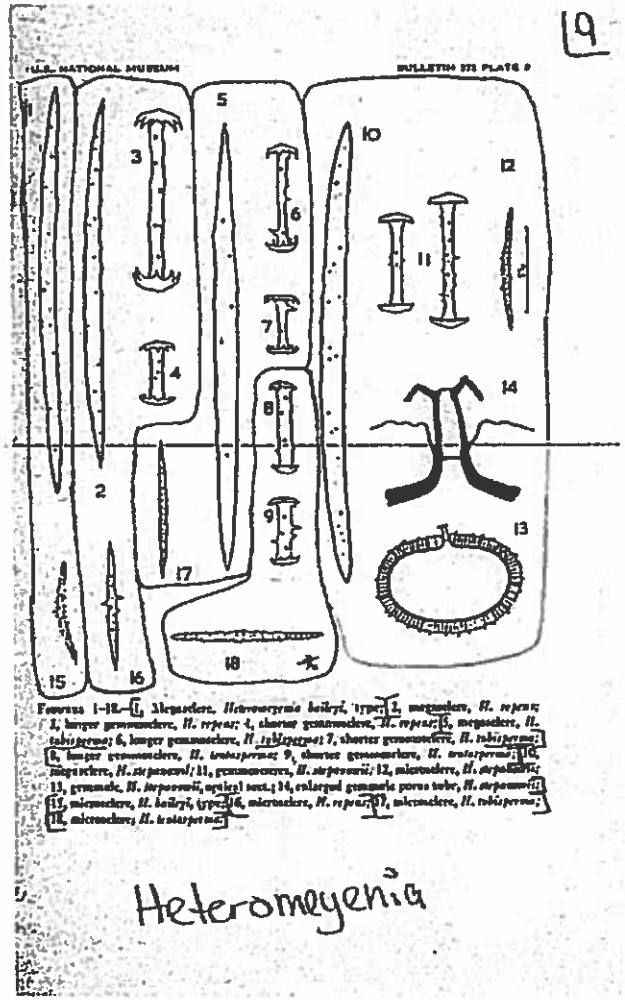
Radiospongilla

16



FIGURES 1–20.—[1, Megasclere, *Radiospongilla crateriformis*, Indonesia; 2, 3, microscleres, *R. crateriformis*, Indonesia; 4, megasclere, *R. indica*, India; 5–7, gemmuscleres, *R. indica*, India; 8, megasclere, *R. indica*, Indonesia; 9, 10, gemmuscleres, *R. indica*, Indonesia; 11, microscleres, *Pectispongilla aurea*; 12, megasclere, *Radiospongilla cenotensis*; 13, 14, gemmuscleres, *R. cenotensis*; 15, megasclere, *Pectispongilla aurea*; 16, megasclere, *P. subspinosa*; 17, 18, gemmuscleres, *P. aurea*; 19, gemmuscleres, *P. subspinosa*; 20, spherasters, *P. stellifera*.]

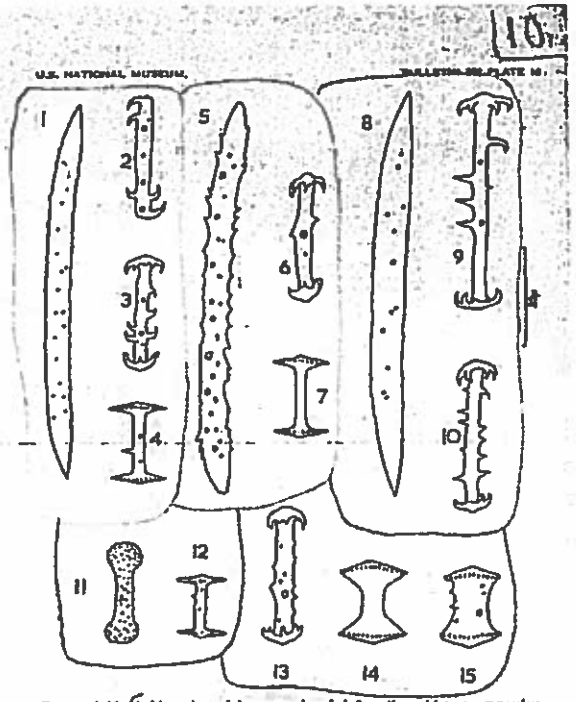
Radiospongilla
Pectispongilla



9

Heteromeyenia

· H. latitenta - pg. 111 *

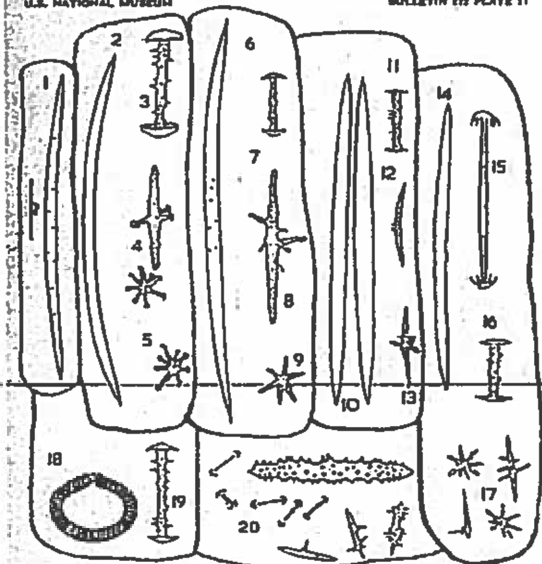


10

Anheteromeyenia
Heteromeyenia

11

U.S. NATIONAL MUSEUM BULLETIN 272 PLATE 11



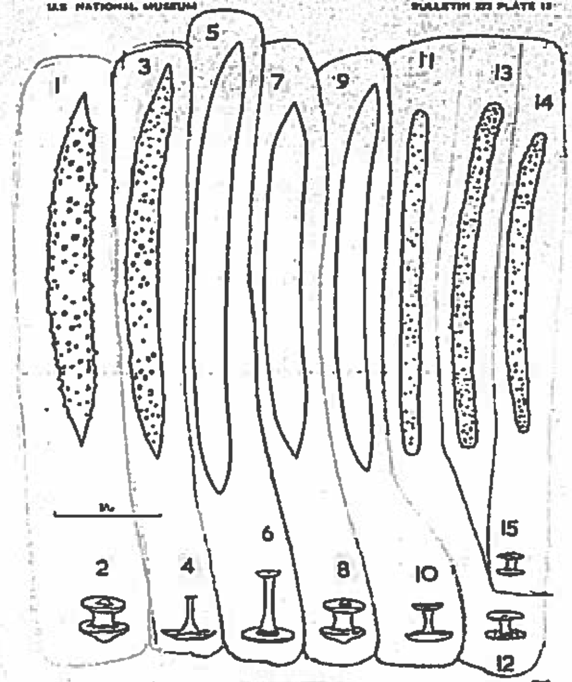
FIGURES 1-20.—1, Megasclere, *Umborotula bogorensis*; 2, megasclere, *Dosilia plumosa*; 3, gemmoscleres, *D. plumosa*; 4, oxeate microsclere, *D. plumosa*; 5, stellate microsclere, *D. plumosa*; 6, megasclere, *D. palmeri*; 7, gemmosclere, *D. palmeri*; 8, oxeate microsclere, *D. palmeri*; 9, stellate microsclere, *D. palmeri*; 10, megasclere, *D. brouni*; 11, gemmosclere, *D. brouni*; 12, 13, microscleres, *D. brouni*; 14, megasclere, *D. radiospiculata*; 15, longer gemmosclere, *D. radiospiculata*; 16, shorter gemmosclere, *D. radiospiculata*; 17, range of microscleres, *D. radiospiculata*; 18, gemmule, *Umborotula bogorensis*, optical sect.; 19, gemmosclere, *U. bogorensis*; 20, range of spicular components, *Spongilla cenotensis*.

· Umborotula *not found in USA
Dosilia
Spongilla

· Corvomeyenia everetti – (pg. 125-126)

12

U.S. NATIONAL MUSEUM BULLETIN 272 PLATE 12

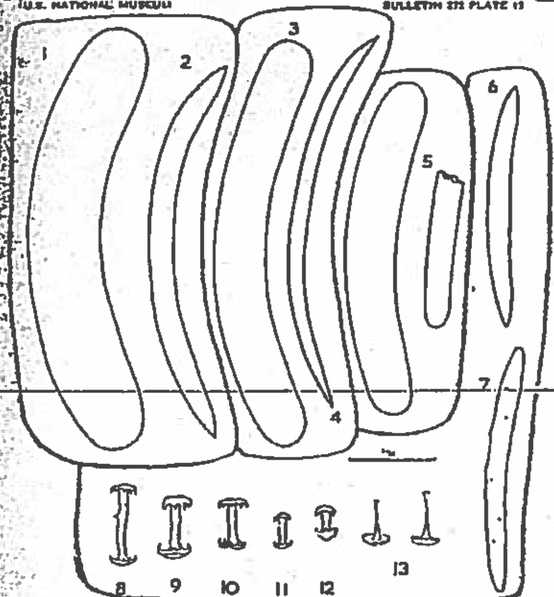


FIGURES 1-15.—1, Megasclere, *Trochospongilla horrida*; 2, gemmosclere, *T. horrida*; 3, megasclere, *T. pennsylvanica*; 4, gemmosclere, *T. pennsylvanica*; 5, megasclere, *T. pandula*; 6, gemmosclere, *T. pandula*; 7, megasclere, *T. leidyi*; 8, gemmosclere, *T. leidyi*; 9, megasclere, *T. latouchiana*; 10, gemmosclere, *T. latouchiana*; 11, megasclere, *T. philottiana*; 12, gemmosclere, *T. philottiana*; 13, megasclere, *T. philottiana* var. *javanensis*; 14, megasclere, *T. philottiana* var. *tanghaensis*; 15, gemmosclere, *T. philottiana* var. *tanghaensis*.

Trochospongilla

13

U.S. NATIONAL MUSEUM — BULLETIN 272 PLATE 13

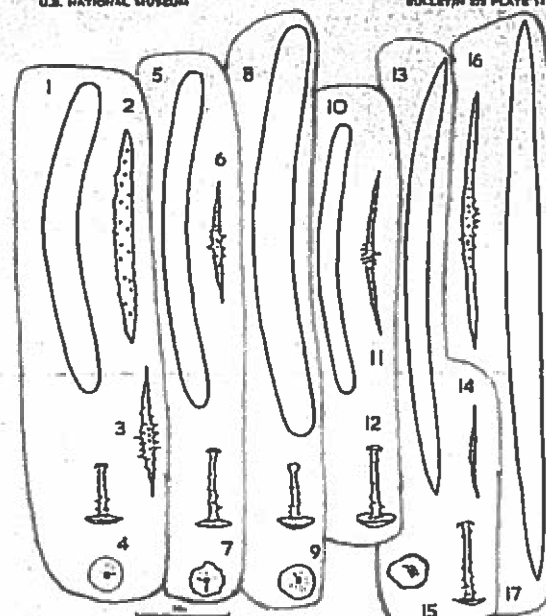


FIGURES 1–13.—1, Former megasclere, *Uruguaya corallioides*; 2, slender megasclere, *U. corallioides*; 3, [illegible] megasclere, *U. macandrewi*; 4, slender megasclere, *U. macandrewi*; 5, megasclere, *U. repens*; 6, 7, megasclere, *Acalle recurvata*; 8–12, birotulate gemmoscleres found in slide of type, *A. recurvata*; 13, tubelliform gemmoscleres of inner series, *A. recurvata*.

Uruguaya * not found in USA
Acalle *

14

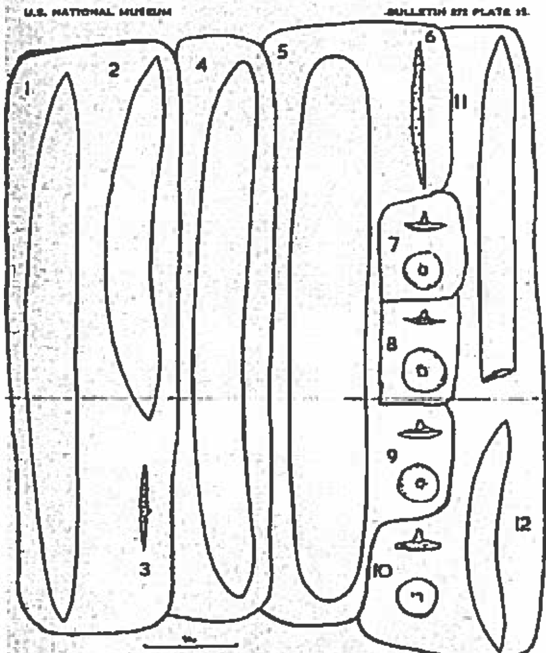
U.S. NATIONAL MUSEUM — BULLETIN 272 PLATE 14



FIGURES 1–17.—1, Normal megasclere, *Metania reticulata*; 2, unusual megasclere, *M. reticulata*; 3, microsclere, *M. reticulata*; 4, gemmosclere, *M. reticulata*; 5, megasclere, *M. vesparia*; 6, microsclere, *M. vesparia*; 7, gemmosclere, *M. vesparia*; 8, megasclere, *M. pottsi*; 9, gemmosclere, *M. pottsi*; 10, megasclere, *M.* [illegible]; 11, microsclere, *M.* [illegible]; 12, gemmosclere, *M.* [illegible]; 13, megasclere, *M. vesparioides*; 14, microsclere, *M. vesparioides*; 15, gemmosclere, *M. vesparioides*; 16, microsclere, *M. rhodesiana*; 17, megasclere, *M. rhodesiana*.

Metania * not found in USA

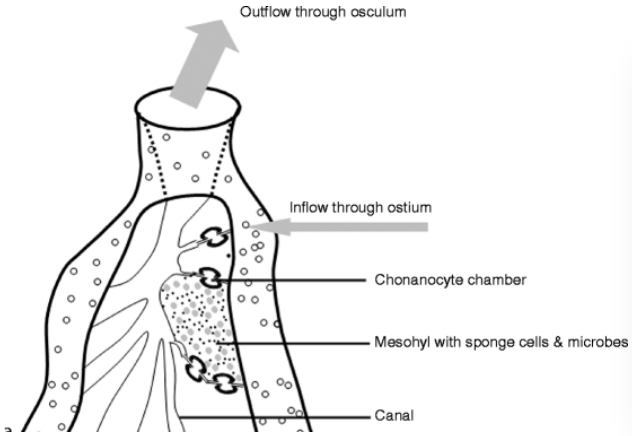
15



FIGURES 1-12.—1, Longer megasclere, *Drulia browni*, type; 2, shorter megasclere, *D. browni*, type; 3, microsclere, *D. browni*, type; 4, megasclere, *D. grayi*; 5, megasclere, *D. cristata*; 6 microsclere, *D. cristata*; 7, gemmosclere, *D. browni*; 8, gemmosclere, *D. grayi*; 9, gemmosclere, *D. cristata*; 10, gemmosclere, *D. batesii*; 11, longer megasclere, *D. batesii*; 12, shorter megasclere, *D. batesii*.

Drulia *not found in USA

Observing Freshwater Sponge Morphology and Function: Dye Pumping



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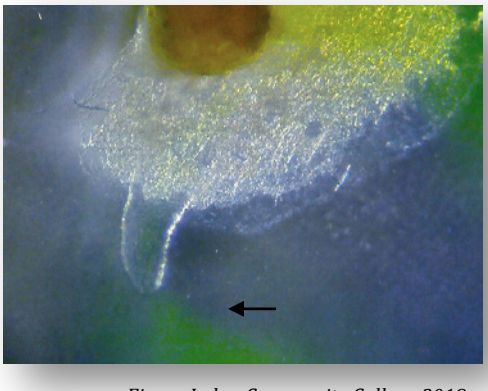
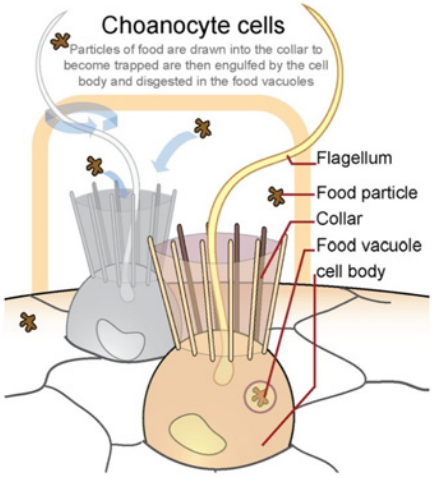


Finger Lakes Community College 2018

Sponges pump water through their ostia and canals to facilitate feeding and exchange of oxygen and nutrients. Feeding is controlled by contracting both inhalant and exhalant chambers and canals to move water through and out through the osculum. The pumping rate of sponges is impressive – large sponges can pump about 1000 times its own volume of water over 24 hours, and large sponge communities act as very efficient natural biofilters.

You can visualize this pumping process by adding a non-toxic dye to the media close to the sponge body.



©CK-12 Biology Advanced Concepts

Dye Pumping Procedure:

1. Dilute a small piece (1 cm^3) of Bright Dyes tracer dye tablet (Kingscote Chemicals, #101101) in 250 mL of dH_20.

2. Place your petri dish or 6 well plate containing your stage 4+ sponge samples under a dissecting microscope. Focus in on the osculum of the sponge sample.

3. Using a p10 micropipettor and tip, draw up 5-10 µL of diluted dye solution into the microtip. If using a disposable pasture pipette, draw up just a small drop of dye into the tip and hold.

4. While looking under the microscope, carefully place your microtip containing dye directly next to the sponge body on the opposite side of the osculum. Slowly expel a minute amount of dye into the media next to the sponge and withdraw your tip.
5. Watch carefully as the sponge takes up the dye through its ostia and into its choanocyte chambers and canals, finally pumping the dyed water out through the osculum.

6. If you do not observe dye being pumped out through the osculum, it is possible that the sponge has not matured enough to have opened its osculum and you should try again the next day.

7. If you plan to repeat the dye pumping experiment or to save your sponge specimen for future experiments, remove the dyed media and replace it with fresh Strekal's or filtered stream water.

PCR of Sponge Stem Cells for Genotyping

Protocol:

Your instructor has prepared a mastermix with all the components required for the polymerase chain reaction (PCR).

1. Your instructor will pipet 47.5 µL of the mastermix into a PCR tube.

2. Label the PCR tube you receive from your instructor.

3. Add 2.5 µL of your DNA to the PCR tube.

4. Close the PCR tube. Be careful that you do not squeeze the tube sides as they will crack.

5. Place your PCR tube on the class rack that is on ice.

6. Place your remaining DNA back on ice.

PCR Conditions

	Temperature	Time	Cycles
Denature	95°C	1 minutes	1
Annealing	95°C	45 seconds	35
	51°C	45 seconds	
	72°C	60 seconds	
Final Extension	72°C	15 minutes	1
Hold	4°C	∞	N/A

Notes

- The expected PCR product is 660-680 base pairs

- We order the primers from IDT (http://www.idtdna.com/site) and then store the stocks at 100 uM in water. The primer stock is diluted to 10 uM for use in the PCR reaction. For the PCR reaction, we use 2X GoTaq Green mix from Promega. It is relatively inexpensive and already has a gel loading dye in the master mix.

- The sequences of the primers, which amplify a 660-680 bp fragment of the COX1 gene, are shown in the table below:

Primer	Name	Sequence
Forward	COX1-R1	5'- TGTTGRGGGAAAAARGTTAAATT -3'
Reverse	COX1-D2	5'- AATACTGCTTTTTTTGATCCTGCCGG -3'

PCR mastermix

- Below is the mastermix reaction for a single PCR reaction. The total reaction volume per reaction will be 50 µL with the sponge DNA added.

Components of and respective volumes for a single PCR reaction:

Volume (µL)	Component	Stock Concentration (µM)
25	2x GoTaq green	na
2.5	forward primer	10
2.5	reverse primer	10
17.5	sterile water	na

(OPTIONAL)
PCR Clean Up Protocol

1. Add 225 µL of buffer PB to a 1.5 mL microcentrifuge tube and label the tube with your sample name.
2. Add 45 µL of PCR product to the microcentrifuge tube containing the tube containing buffer PB.
3. Close the lid and vortex for 5 seconds.
4. Place a QIAquick column into a 2 mL collection tube. Label the tube with your group name.
5. Add the entire sample (about 270 µL) to the column.
6. Close the lid and centrifuge for 1 minute at 13,000 rpm.
7. Discard the flow-through by dumping into a waste beaker and place the QIAquick column back into the same 2 mL collection tube. Keep the filter column, this has collected all the DNA from your PCR reaction.
8. Add 750 µL buffer PE to the QIAquick column.
9. Centrifuge for 1 minute at 13,000 rpm.
10. Discard the flow-through into the waste beaker and place the QIAquick column back into the same 2 mL collection tube.
11. Centrifuge the QIAquick column once more in the 2 mL collection tube for 1 minute at 13,000 rpm. This will remove residual Wash Buffer.

12. Place the QIAquick column into a clean 1.5 mL microcentrifuge tube. Label the side of the tube with your group name.

13. Add 50 µL buffer EB to the center of the column.

14. Incubate at room temperature for 1-3 minutes.

15. Centrifuge for 1 minute at 13,000 rpm.

Note: You will not be able to close the lid of the 1.5ml microcentrifuge tube over the top of the filter column. When you place the tube with the column into the centrifuge, point the tube lid into the center of the centrifuge so that the centrifuge cap will fit.

16. After the spin is complete, measure your DNA concentration on the Nanodrop.

DNA Concentration _____ng/ul

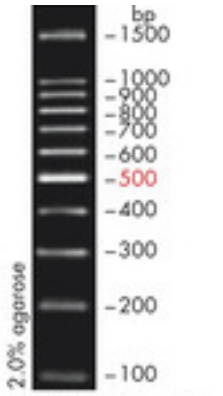
A260/280 purity ratio_____

Need 20 ng of sample for sequencing. Divide 20 ng by the concentration of your sample to get the volume of sample that needs to be sequenced. Bring total volume to 10 µL in a PCR tube using nuclease free water.

DNA Gel Electrophoresis of PCR Product

Protocol:

1. Obtain a flask with 0.8% agarose and pour a gel for electrophoresis.
2. Wait for the gel to solidify (this should take 10-15 minutes). During this time you will practice loading a gel.
3. Place the gel into a electrophoresis chamber and cover with 1x TAE buffer.
4. Load 5 µL of ladder, GelPilot 100bp Plus, into the first lane.
5. Load 5 µL of PCR product into the third lane.
6. Place the lid on the gel box.
7. Connect the gel box to the power source.
8. Run your gel for 30 minutes 150mV (Set timer for 30 minutes on power supply so gel run will stop automatically).
9. Turn off the power source and unplug the gel lid.
10. Carefully lift the gel tray out of the buffer. Hold the gel from the ends so that it does not slide out of the tray.
11. Take your gel to the Bio-Rad gel imager and follow instructions to capture an image of the PCR product.



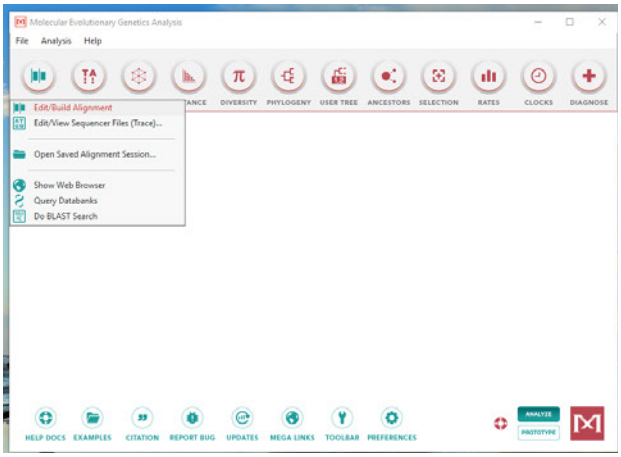
GelPilot 100 bp Plus Ladder (100) (cat. no. 239045)

Using MEGA10 to Analyze Sequences from Freshwater Sponges

Protocol:

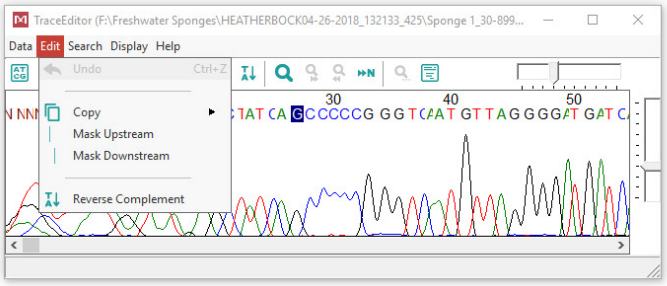
1. Load the latest version of MEGA on your computer. http://www.megasoftware.net/ There is no cost associated, but you will need to register.

2. Open MEGA and in the drop-down menu under Align, select Edit/View Sequencer Files. Select the forward sequence file (.ab1) that you wish to view.

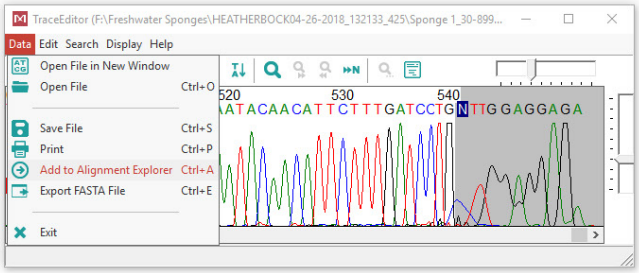


3. Make sure that the chromatogram looks clean without overlapping peaks as shown on the left. If the chromatogram appears similar to the one on the right, do not continue with the analysis.

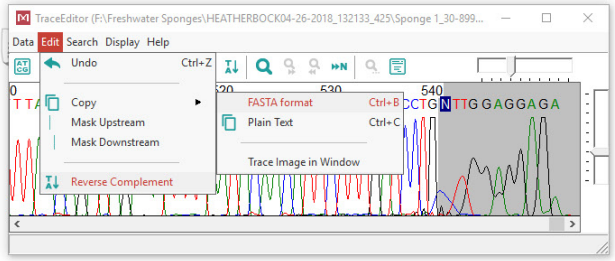
4. Observe the 5' chromatogram. Select a base (highlighted in blue below) where the peaks start appearing very clearly. In the drop-down menu under Edit, select "Mask Upstream." Repeat the process for the 3' end of the sequence, except select "Mask Downstream." You should be able to get 500-550 clean bases.



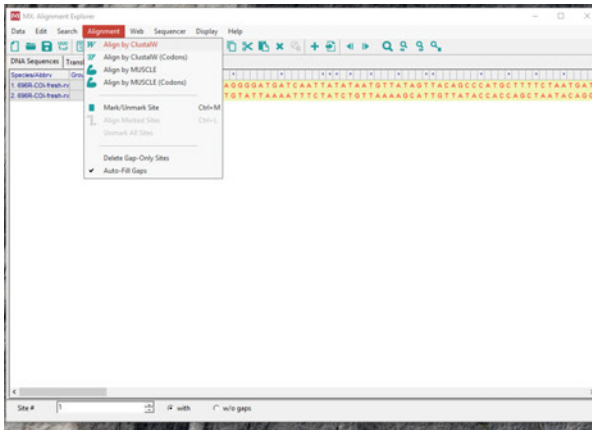
5. After the 5’ and 3’ regions are masked, from the dropdown menu under Data select “Add to Alignment Explorer.” Click “OK” in the pop-up window.



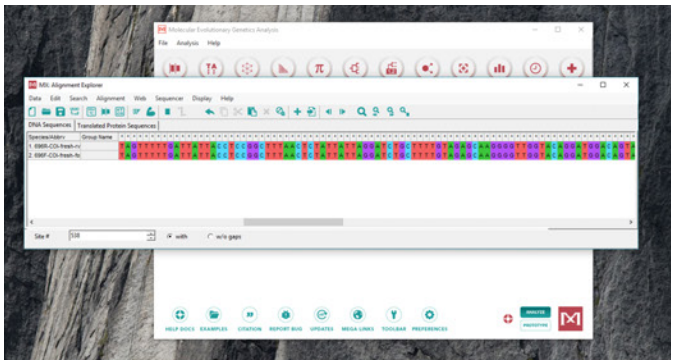
6. Repeat the previous steps for the reverse sequence **with the following modification**. In the drop-down Edit menu, select “Reverse Complement” prior to adding to Alignment Explorer. For aligning the sequences, the two reads must be in the same direction.



7. The two sequences should be aligned to confirm the sequence. Select both sequences (will be highlighted in yellow). In the Alignment drop down window select Align by ClustalW or Align by MUSCLE. Use the default values.

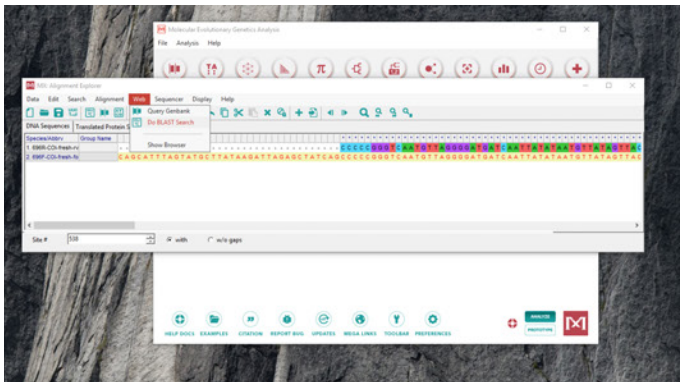


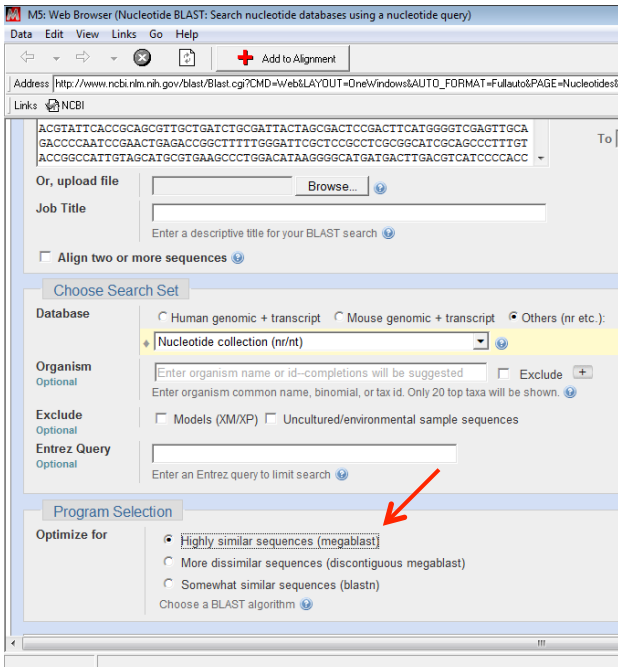
8. When the sequences are aligned, the boxes above the sequences will be filled with a symbol. Scan across the overlapping sequences to look for any mismatches between the two sequences.

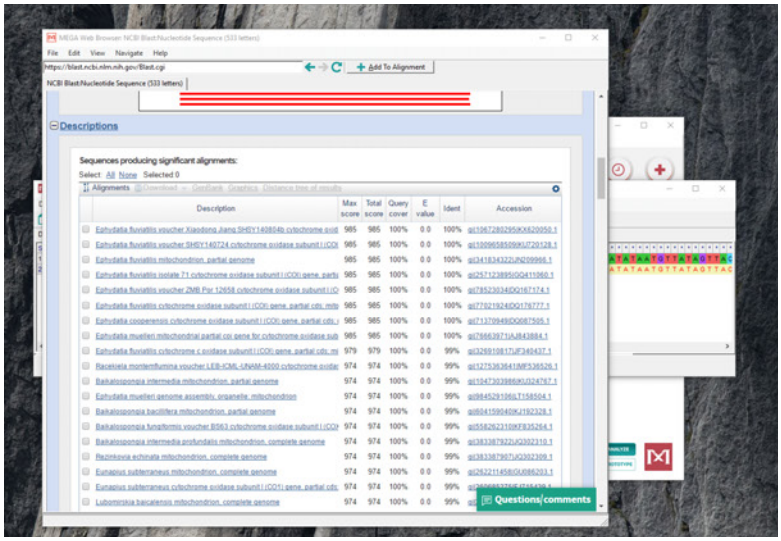


9. If any bases mismatch, look at the two TraceEditor windows to determine the correct base. In the example below, the forward sequence has only one G and the reverse sequence has two Gs. By looking at the TraceEditor windows, it can be clearly seen that there are two Gs present in the reverse sequence. Therefore, another G is inserted into the forward sequence.

10. In the Alignment Explorer window, select the entire sequence by clicking on the name to the left. In the drop down menu for Web, select Do BLAST Search. The sequence will be automatically entered into the Query Box at NCBI. Click the button next to Optimize for Highly similar sequences. Click the BLAST button and wait patiently for the hits to be returned.



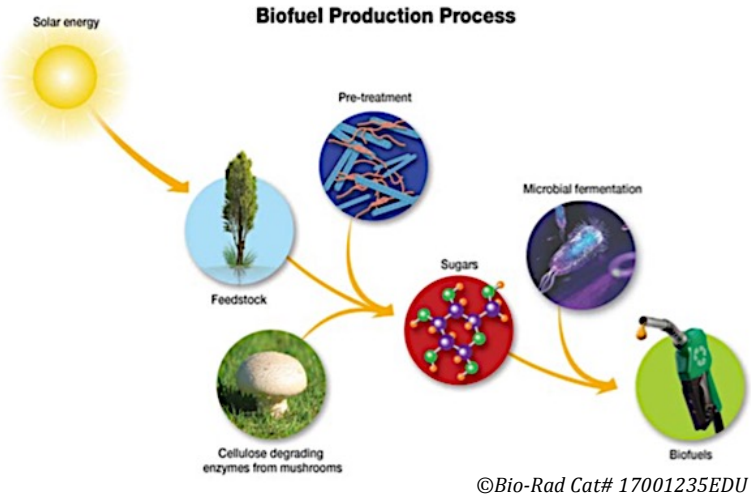

M5: Web Browser (Nucleotide BLAST: Search nucleotide databases using a nucleotide query)
Data Edit View Links Go Help
Add to Alignment
Address http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Web&LAYOUT=OneWindow&AUTO_FORMAT=Fullauto&PAGE=Nucleotides&
Links NCBI
ACGTATTCACCGCAGCGTTGCTGATCTGCGATTACTAGCGACTCCGACTTCATGGGGTCGAGTTGCA
GACCCCAATCCGAACTGAGACCGGCTTTTTGGGATTCGCTCCGCCTCGCGGCATCGCAGCCCTTTGT
ACCGGCCATTGTAGCATGCGTGAAGCCCTGGACATAAGGGGCATGATGACTTGACGTCATCCCCACC
To
Or, upload file
Browse...
Job Title
Enter a descriptive title for your BLAST search
Align two or more sequences
Choose Search Set
Database
Human genomic + transcript
Mouse genomic + transcript
Others (nr etc.):
Nucleotide collection (nr/nt)
Organism
Optional
Enter organism name or id--completions will be suggested
Exclude
Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown.
Exclude
Optional
Models (XM/XP)
Uncultured/environmental sample sequences
Entrez Query
Optional
Enter an Entrez query to limit search
Program Selection
Optimize for
Highly similar sequences (megablast)
More dissimilar sequences (discontiguous megablast)
Somewhat similar sequences (blastn)
Choose a BLAST algorithm


Descriptions
Sequences producing significant alignments:
Questions/comments

Biofuels: Powering our Future from Backyard to Classroom

Background

Adapted from Bio-Rad Biofuel Enzyme Reactions Kit for AP Biology: A ThINQ! Investigation, Instructor's Guide. Cat # 17001235EDU ***Explorer.bio-rad.com***



©Bio-Rad Cat# 17001235EDU

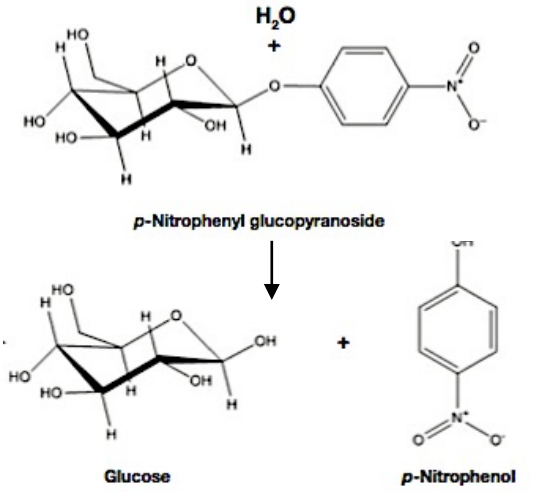
Biofuels currently account for a small portion of transportation fuels used in the US, however advances in biofuel technologies, as well as concerns about climate change and fuel sustainability, are prompting broader interest in the biofuel industry. Cellulosic biofuel is an efficient, more sustainable fuel to replace petroleum. The enzyme cellulase is important in the process of making cellulosic biofuel, and the enzyme reaction is simple to set up and produces reliable, quantifiable data. Also, the option to source cellulase from wild mushrooms lends the experimental narrative well to the high school classrooms, giving the opportunity to introduce biofuel theory and environmental conservation together.

The biofuel industry uses cellulases to convert the cellulose in plant cell walls into sugars such as glucose that can then be converted to ethanol and other fuels by microbial fermentation. The ethanol or other fuels can in turn be used alone in certain engines on in combination with gasoline to power car, truck and airplane engines.

A plant's biomass is mostly cell wall material. Plant cell walls are made up of a variety of polysaccharides and other compounds, but the primary component is cellulose. Cellulose is made up of a very long chain of glucose molecules. Each cellulose molecule is attracted to other cellulose molecules by the hydrogen bonds that form between their respective glucose molecules. These attractions form cellulose microfibrils made up of 60 to 80 individual strands of cellulose.

For cellulosic biofuel production, lignins must be removed because they inhibit enzymatic activity of cellulases. Lignin is a highly complex aromatic macromolecule found in high quantities in secondary cell walls of fibrous and woody plant tissue in close association with cellulose. Once the lignin is removed, the cellulose is more exposed and can me more readily broken down. Generally lignins are removed by a combination of chemical and mechanical processes involving heat and pressure.

Cellobiase breaks down cellobiose, a disaccharide made up of 2 glucose molecules connected by a 1,4 β-glucoside linkage. The breakdown of cellobiose by cellobiase is the final step in producing glucose from cellulose. Using kit #17001235EDU, the enzymatic reaction can be visualized thanks to the addition of a substrate coupled to a colorimetric indicator. Instead of the natural cellobiase substrate, cellobiose, the kit includes an artificial substrate, p-Nitrophenyl glucopyranoside. This substrate is composed of a β-glucose covalently linked to a molecule of Nitrophenol. When the bond connecting these two molecules is cleaved by cellobiase, the p-Nitrophenol is released creating a yellow colored solution. The intensity of the color is correlated to the amount of glucose released and the reaction can be detected visually by comparing the deepness of the yellow color to a set of standards of known concentration of p-Nitrophenol by eye or by using a spectrophotometer to produce more accurate, quantitative results.



©Bio-Rad Cat# 17001235EDU

Using kit #17001235EDU, you can extract natural cellobiase from wild mushrooms found in your backyard, in order to compare the cellobiase activity in different mushroom species and optimize reactions for maximum glucose production. This set of experiments are useful for teaching experimental design, variables and controls, enzymatic reactions, biofuel theory and environmental conservation. A natural continuation of the set of experiments provided here would be to demonstrate fermentation with yeast and glucose to produce bioethanol.

Enzyme-Substrate Reactions: Cellobiase Extraction from Wild Mushrooms

To extract the enzyme, cellobiase, that will break down the substrate p-Nitrophenyl glucopyranoside to release the glucose product and indicator, p-Nitrophenol, follow the following enzyme extraction protocol for each muchroom type you would like to test, individually.

Adapted from Bio-Rad Biofuel Enzyme Reactions Kit for AP Biology: A ThINQ! Investigation, Instructor's Guide. Cat # 17001235EDU ***Explorer.bio-rad.com***

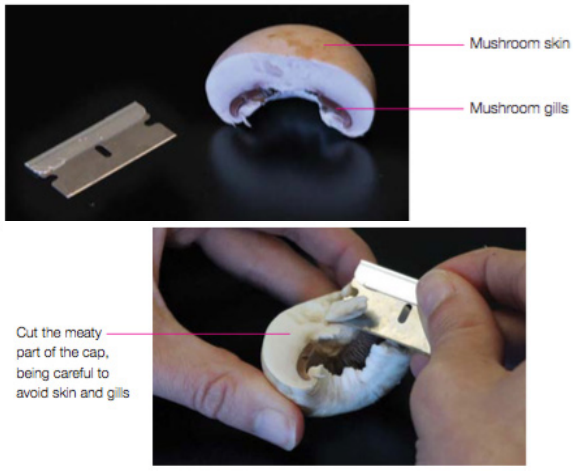
Protocol:

** Note: wild mushrooms can be difficult to identify visually, and some are poisonous. For safety reasons, do not eat any wild-sourced mushrooms.*

1. Using a sterile razor or scalpel, carefully remove the stem of your mushroom if you are using one with a woody stem like button or shitake. You will not need to remove the stem if you are using a soft-stemmed mushroom like oyster.

2. Carefully cut through the center of the cap of the mushroom or the thickest section. Trying to avoid the mushroom skin and gills, carefully cut out the internal flesh of the cap.

3. Using a laboratory scale, weigh approximately 1g of mushroom flesh in a weigh boat and then transfer to a mortar.



©Bio-Rad Cat# 17001235EDU

4. Add 2 mL of extraction buffer to the mortar for every gram of mushroom.

5. Using a pestle, grind your mushroom to produce a slurry – a semiliquid mixture.

6. Strain the solid particles out of the slurry and scoop the slurry into a 1.5 mL microcentrifuge tube using a scoupula. Pellet the solid particles by spinning in a microcentrifuge at top speed for 2 min. If you do not have a centrifuge, use a piece of cheesecloth to squeeze the extract into a 1.5 mL microtube. (Note: If you use the cheesecloth method, use 2g of mushroom and 4 mL of extraction buffer.)

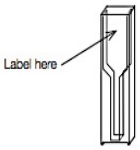
7. You will need at least 250 µL of extract to perform the enzymatic reactions in the next protocol.

Enzyme-Substrate Reactions: Enzyme Kinetics and Glucose Formation

To test the extracted cellobiase enzyme kinetics for glucose formation from different wild mushroom species, following the following protocol for each mushroom type, individually.

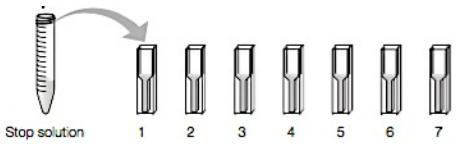
Adapted from Bio-Rad Biofuel Enzyme Reactions Kit for AP Biology: A ThINQ! Investigation, Instructor's Guide. Cat # 17001235EDU ***Explorer.bio-rad.com***

Protocol:



1. Writing on the upper part of the cuvette face, label 7 disposable cuvettes 1-7.

2. Using a micropipette and tips or disposable pasture pipette, pipette 500 µL of Stop Solution into each of the cuvettes 1-7.

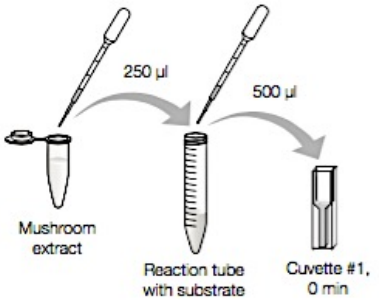


3. Obtain a 15 mL conical tube and label it with the mushroom type you are using. Using a clean pipette, pipet 3 mL of Substrate into the tube.

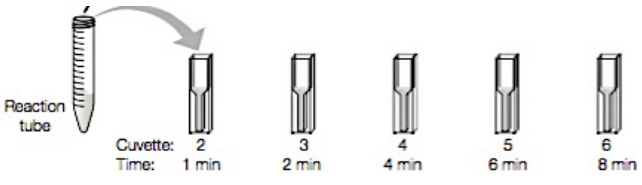
4. From the stock Substrate tube, pipet 450 µL of substrate and 50 µL of Extraction Buffer into cuvette #7 only. Set aside for analysis. This cuvette will act as a control during your experiment because it does not contain mushroom extract.

5. **Ready your timer.**

6. Using a clean pipette, pipet 250 µL of mushroom extract into your 15 mL conical tube labeled with your mushroom type. This is now referred to as the reaction tube. Pipet the liquid up and down to mix, then quickly pipet 500 µL of this reaction mixture into cuvette #1. **Start your timer.**



7. At the times indicated in the table below, remove 500 µL of reaction mixture containing mushroom extract and substrate from the reaction tube and add it to the appropriately labeled cuvette that contains stop solution.



Time	Cuvette
0 min	1
1 min	2
2 min	3
4 min	4
6 min	5
8 min	6

8. Once all reaction times are completed, read the results either by comparing by eye to standard cuvettes, or using a spectrometer.

Results: Use one of the methods below to determine the amount of product formed from your enzymatic reaction(s).

Qualitative Determination of the Amount of Product Formed (no spectrophotometer needed):

1. Locate the 5 cuvettes of standards labeled S1-S5. Their concentrations are noted in the table below. Hold the cuvettes against a white background to compare your one control and 6 reaction cuvettes to each standard. Record in the table below the standard that is most similar to each of your experimental cuvettes.

Standard	Amt. of p-Nitrophenol (nmol)		Time (min)	Cuvette	Standard Most Similar	Est. Amt. of p-Nitrophenol (nmol)	
S1	0		0	1			
S2	12.5		1	2			
S3	25		2	3			
S4	50		4	4			
S5	100		6	5			
			8	6			
			8	7, control			

Quantitative Determination of the Amount of Product Formed (spectrophotometer needed)

1. See the instructions provided for *Measuring the Amount of p-Nitrophenol Produced Using SmartSpec Plus Spectrophotometer*. Be sure the spectrophotometer is turned on and warmed up in advance.

2. Locate the 5 cuvettes of standards S1-S5. Their concentrations are noted in the table below. Blank your spectrophotometer at 410nm with the cuvette labeled S1.

3. Measure and record the absorbance at 410nm for the remaining standards in the table below. Use this information to generate a standard curve that will allow you to calculate and amount of product formed.

4. After recording your standard absorbance values, re-blank the spectrophotometer with cuvette #1.

5. Measure the absorbance of your enzyme-catalyzed reaction cuvettes (#1-6) and your control cuvette (#7) at 410nm and record your results in the table below. You will then use the information in an excel worksheet provided to you to determine the amount of product (p-Nitrophenol/glucose) formed.

Standard	Absorbance at 410nm	Amount of p-Nitrophenol (nmol)		Time (min)	Cuvette	Absorbance at 410nm
S1		0		0	1	
S2		12.5		1	2	
S3		25		2	3	
S4		50		4	4	
S5		100		6	5	
				8	6	
				8	7, control	

Field Work: Freshwater Sponge and Wild Mushroom Specimen Collection

Specimen #	Location	Description	Identification

** Note: wild mushrooms can be difficult to identify visually, and some are poisonous. For safety reasons, do not eat any wild-sourced mushrooms.*

Field Work: Freshwater Sponge and Wild Mushroom Specimen Collection

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Supply/Equipment List for Freshwater Sponge Protocols

Pick/Wash/Hatch Protocol		
Supply Item	**Vender**	**Catalog number**
10x Strekals Solution		
0.9mM of MgSO4 -7H2O (Magnesium sulfate)	VWR	BDH9246-500G
0.5mM CaCO3 (Calcium carbonate)	VWR	97061-150
0.1mM Na2 SiO3 -9H20 (Sodium metasilicate nonahydrate)	VWR	IC19138280
0.1mM KCl (Potassium chloride)	VWR	BDH9258-500G
Cold 1x Strekals		
30% Hydrogen peroxide	VWR	BDH7690-1
Ice box	VWR	102093-896
Ice		
60mm Petri dishes	VWR	25384-090
Dissecting Microscope	VWR	470315-512
Forcep (2 per person)	VWR	89259-944
15mL conical tubes	VWR	21008-216
p1000 pipette	VWR	76177-990
p1000 pipette tips	VWR	83007-386
6 well plates	VWR	10861-554
Aluminium Foil		
Cardboard box to cover plates		
Weigh boats or other way to hold ice under microscope		
Waste beaker		

DNA Extraction		
Supply Item	**Vender**	**Catalog number**
DNeasy Blood & Tissue Kit	QIAGEN	69504
Microcentrifuge	VWR	97058-930
p200	VWR	76177-988
p200 tips	VWR	37001-524

p1000	VWR	76177-990
p1000 tips	VWR	83007-386
Vortex	VWR	10153-834
1.5mL microcentrifuge tubes	VWR	10025-726
Pestal, 1.5mL	VWR	KT749521-1500
Nanodrop	Thermo	ND-LITE

PCR		
Supply Item	**Vender**	**Catalog number**
PCR Kit	Promega	M7122
Primers Forward	IDT	Fw: 5' CAGGCATGATAGGTACAGCATTTAG 3'
Primers Reverse	IDT	Rev: 5' CTCCYCCAGCAGGATCAAAG 3'
Nuculase-Free Water	VWR	98062-790
PCR tubes	VWR	20170-010
p10	VWR	76322-132
p10 filter tips	VWR	53509-138
p200	VWR	76177-988
p200 filter tips	VWR	82003-192
PCR machine	BIO-RAD	1861096

PCR Clean Up		
Supply Item	**Vender**	**Catalog number**
QIAquick PCR Purification Kit	QIAGEN	28106-50
Ethanol (96-100%)		
1.5mL microcentrifuge tubes	VWR	10025-726
microcentrifuge	VWR	97058-930
p200	VWR	76177-988
p200 filter tips	VWR	82003-192
p1000	VWR	76177-990
p1000 filter tips	VWR	83007-386
Nanodrop	Thermo	ND-LITE

Gel Electrophoresis		
Supply Item	**Vender**	**Catalog number**
Agarose	BIO-RAD	1613101EDU
Electrophoresis Rig	BIO-RAD	1640302
GelPilot 100bp Plus Ladder	QIAGEN	239045
Loading dye (if not in PCR product)		
p20	VWR	76180-016
p20 tips	VWR	76322-134
Sybrasafe	Invitrogen	S33102
10x TAE	BIO-RAD	1610743

Sequencing		
Supply Item	**Vender**	**Catalog number**
Primers Forward	IDT	Fw: 5' CAGGCATGATAGGTACAGCATTTAG 3'
Primers Reverse	IDT	Rev: 5' CTCCYCCAGCAGGATCAAAG 3'
Nuculase-Free Water	VWR	98062-790
PCR tubes	VWR	20170-010
p10	VWR	76322-132
p10 tips	VWR	53509-138
p2.5	VWR	76180-014
p2.5 tips	VWR	76322-528

Supply Item	**Vender**	**Catalog number**
Spicule Prep		
Slide Microscope	VWR	89404-470
Glass slides	VWR	16004-370
Slide coverslips	VWR	48376-049
Bleach		
p1000	VWR	76177-990
p1000 tips	VWR	83007-386
microcentrifuge	VWR	97058-930

1.5mL tubes	VWR	10025-726
DI water		
Tube rocker	VWR	10159-756
Ethanol		
p200	VWR	76177-988
p200 tips	VWR	37001-524

Biofuel Enzyme Supply/Equipement List

Materials	Vendor	Catalog #
Biofuel Enzyme Reactions Kit for AP Biology	Bio-Rad	17001235EDU
Enzyme, Cellobiase		
Substrate, p-Nitrophenyl glucopryansoide 90mg		
Standard, p-Nitrophenol 1mM		
Stop Solution, Carbonate buffer pH 9.5		
Resuspension buffer, Sodium Acetate buffer pH 5		
Extraction buffer, Tris MgCl2 Triton X-100 pH 7.2		
Disposable plastic transfer pipettes		
1.5 mL microcentrifuge tubes		
15 mL conical tubes		
1.5 mL disposable cuvettes		
Instruction manual		
Mushroom Habitat Flash Cards	Bio-Rad	
Experimental Design and Planning Worksheet	Bio-Rad	
20 Questions to Master Inquiry	Bio-Rad	
500 mL bottle		
200 mL bottle		
100 mL bottles		
150 mL bottle		
50 mL conical tubes	VWR	10160-140
Serologic pipettors	VWR	29442-430
Pipettor tips	VWR	83007-376
Graduated cylinders	VWR	65000-006
Deionized or distilled water		
Lab tape	VWR	89097-920
Balance	VWR	11379-234
Parafilm	VWR	52858-000
Razor blades or scalpels	VWR	82029-858

Mushrooms		
Spectrophotemeter	Bio-Rad	1702525EDU
Water bath	VWR	89501-464
Ice buckets	VWR	89198-950
Thermometers	VWR	89095-622
Microcentrifuge	VWR	BKA46472
Kim wipes	VWR	21905-026
Mortar and pestles	VWR	89038-148
Markers or marking pens	VWR	95042-566
Beakers	VWR	414004-149
Stopwatch or timers	VWR	62344-641
Heat blocks	VWR	10153-318

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