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Isolation of green algal symbionts from freshwater sponges and subsequent reinfection of sponge tissues

April Hill¹, Ivy Nguyen¹, Malcolm Hill¹¹Department of Biology, Bates College, ME, USA**1** Works for me dx.doi.org/10.17504/protocols.io.bmuzk6x6

April Hill

ABSTRACT

A variety of green algal species form intracellular symbioses with freshwater sponges. These sponges and their algal symbionts play important roles in freshwater ecosystems and provide a model for asking questions about freshwater endosymbioses involving heterotrophic animal hosts in mutualistic relationships with photosynthesizing algae. The freshwater sponge *Ephydatia muelleri*, for example, has a fully sequenced chromosomal level genome as well as many features that make it amenable to cellular and molecular studies. Here, we provide a simple protocol for isolating green algae from freshwater sponge host tissues. In most cases, microalgae can be cultured outside of the host in commercially available algal medium and on agar substrates. We also describe methods for infecting algal-free sponges hatched from gemmules to establish stable symbioses in juvenile sponges.

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IMAGE ATTRIBUTION

From: Freshwater hosts and their green algal symbionts: a tractable model to understand intracellular symbioses. Hall, et al., 2020. <https://doi.org/10.1101/2020.08.12.247908>

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MATERIALS TEXT

Field collection bags or bottles
 Razor blades (for scraping sponge off of rocks if necessary)
 Cooler with ice packs (for transporting sponge tissue to lab)
 Petri dishes or finger bowls
 Distilled water
 Forceps
 Hydrogen peroxide (H₂O₂)
 Small mortar & pestle
 Modified Bold 3N Medium (UTEX)
 Bold Modified Basal Freshwater Nutrient Solution (Sigma Life Science)
 Microcentrifuge tubes (sterile)
 Mini-microfuge (tap spinner)
 Microcentrifuge
 Sterile growth flasks and glass culture vials
 Rotating platform
 Fluorescent grow lights or growth chamber with lights
 Modified Bold 3N Medium agar plates (15 g agar/Liter of media)
 Sterile loops
 Dissecting and light microscope
 Spectrophotometer (e.g., Spec 200)
 hemacytometer
 tissue culture dishes

Strekal's medium:

Strekal TA, McDuffett W. 1974. Factors affecting germination, growth, and distribution of the freshwater sponge, *Spongilla fragilis* Leidy (Porifera). *Biological Bulletin* 146:267-278.

For M-medium see Rasmont R. 1961. Une technique de culture des éponges d'eau douce en milieu contrôlé. *Annales de la Société Royale Zoologique de Belgique* 91:147-156.

We use 1 mM CaCl₂·H₂O, 0.5 mM MgSO₄·7H₂O, 0.5 mM NaHCO₃, 0.05 mM KCl, 0.25 mM Na₂SiO₃·9H₂O.

We generally make up the Strekal's or M-medium as a 5X or 10x stock. When diluted to 1X the pH should be around 7.2.

ABSTRACT

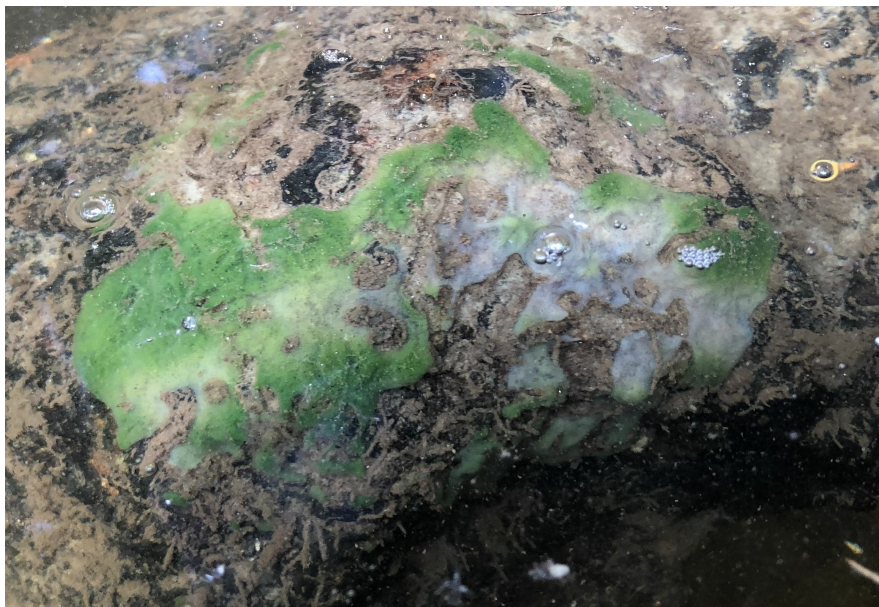
A variety of green algal species form intracellular symbioses with freshwater sponges. These sponges and their algal symbionts play important roles in freshwater ecosystems and provide a model for asking questions about freshwater endosymbioses involving heterotrophic animal hosts in mutualistic relationships with photosynthesizing algae. The freshwater sponge *Ephydatia muelleri*, for example, has a fully sequenced chromosomal level genome as well as many features that make it amenable to cellular and molecular studies. Here, we provide a simple protocol for isolating green algae from freshwater sponge host tissues. In most cases, microalgae can be cultured outside of the host in commercially available algal medium and on agar substrates. We also describe methods for infecting algal-free sponges hatched from gemmules to establish stable symbioses in juvenile sponges.

BEFORE STARTING

If necessary, secure a permit for collecting freshwater sponges

Algal cell isolation

- 1 Identify algal-bearing sponges in the field.



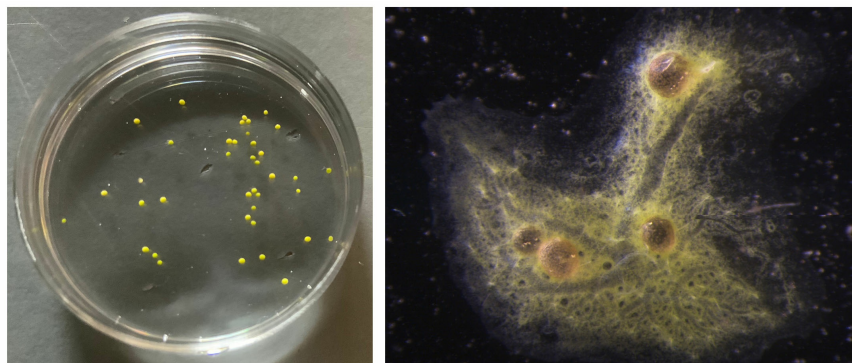
Freshwater sponge *Ephydatia muelleri* with green algal symbionts. Lisbon, ME

- 1.1 Obtain a small piece of freshwater sponge containing green algal symbionts. Excise a small piece (~0.5 cm) for subsequent work. Keep a small piece of sponge tissue for DNA barcoding the sponge and algal species. We recommend using CTAB to isolate genomic DNA and then barcoding with ITS1 and ITS2 sequences of rDNA. Identification of the sponge species is also possible if gemmoscleres from the gemmule are available.



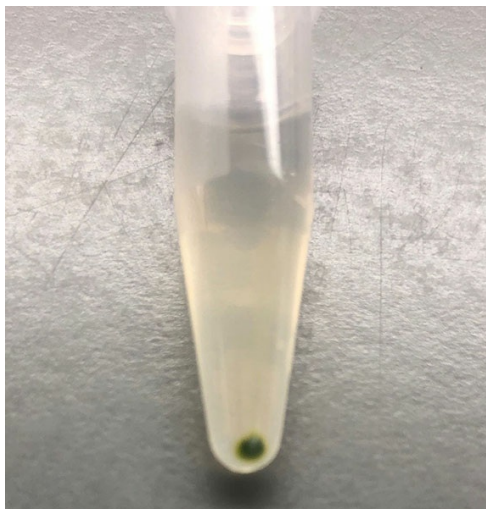
Explant of *Ephydatia muelleri* bearing green algal symbionts

- 1.2 An alternative method to isolate algae from freshwater sponge hosts involves obtaining green gemmules if they are available in the host tissue. Not all gemmules harbor algae, but algae may be isolated from green gemmules if they can be found. Separate approximately 20 green gemmules from the adult tissue for subsequent steps. Finally, it is also possible to hatch green gemmules and isolate algae from juvenile sponges. Protocols for gemmule separation and hatching can be found at dx.doi.org/10.17504/protocols.io.863hzgn.



Left: green gemmules removed from freshwater sponge (*Ephydatia muelleri*) host. Right: juvenile *Ephydatia muelleri* with green algal symbionts.

- 2 Wash the tissue or gemmules in 1% H₂O₂ by shaking gently for several seconds at room temperature followed by at least 5 washes in sterile distilled water, M-medium, or Strekal's medium.
- 3 Place tissue or gemmules in a clean mortar & pestle.
- 4 Grind tissue/gemmules in a small volume (~0.5 mL) of sterile algal culturing media. We have had the most success with Modified Bold 3N Medium (UTEX), but many strains also grow fine in Bold Modified Basal Freshwater Nutrient Solution (Sigma Life Science).
- 5 Transfer the solution of ground sponge tissue with algae to a microfuge tube.
- 6 Tap spin for 5 seconds in a mini-centrifuge to remove spicules and large debris.
- 7 Transfer the supernatant to new microcentrifuge tube. Tap spin again for 10 seconds to remove excess animal cells.
- 8 Transfer the supernatant to a new microcentrifuge tube and spin the remaining solution at 5000 rpm for 1 minute to pellet the algal cells. Some sponge cells will remain in the mixture, but these cells will not grow in the algal culture media.
- 9 Resuspend the pellet (it should be green), add 0.5 mL of media, spin again at 5000 rpm for 1 min, and remove supernatant.



Green algal cell pellet.

- 10 Resuspend the algal cell pellet in ~0.5mL of media and transfer to a 125 mL sterile culture flask containing ~30 mL of sterile media.
 - 10.1 Place algae under grow lights (we use plant grow lights on a 16 hr light; 8 hr dark cycle) at room temperature. Shake/mix cultures once per day or place on a rotating platform.
 - 10.2 Algal grow should be obvious within one week. Depending on the state of the initial inoculate, contaminating organisms may be present. Further purification of algal cultures can be achieved using the plating technique (see below).



Algal cultures isolated from freshwater sponge hosts.

Purification of Algal Cultures

- 11 To obtain pure algal cultures, initial cultures can be streaked on agar plates made with Modified Bold 3N Medium (15 g agar/L).

11.1 Prepare a dilution series of algal culture (log phase stock, 1:10, 1:100) in culture media and spread 100 μ L of each on three plates. Grow under lights at room temperature for several days to a week.

11.2 Alternatively, use a sterile loop to streak algae onto agar plates. Grow as above.



Green algae extracted from freshwater sponge plated for single colony isolation.

- 12 Use a dissecting microscope to visualize algal colonies. In some cases, there will be bacteria growing alongside or even around the algae. In this case, it may be necessary to use antibiotics if pure algal strains are needed, however, some prefer not to eliminate the commensal bacteria. We find that many strains of algae will grow well with and without the bacteria.

12.1 Select individual colonies to inoculate into 10 mL of Modified Bold 3N Medium (UTEX) in glass culture vials.

Determination of algal cell densities

- 13 Algal cell densities from cultures can be empirically determined from liquid media by direct cell counts using a hemacytometer. A faster method of determining cell density involves the use of OD (i.e., absorbance) readings at 425 nm or 675 nm (depending on algal pigment properties). To use OD readings, one must first create a standard curve for each algal isolate by regressing OD against known cell densities. Once a standard curve is created for each algal strain, a simple linear equation can be used to estimate cell density based on OD.

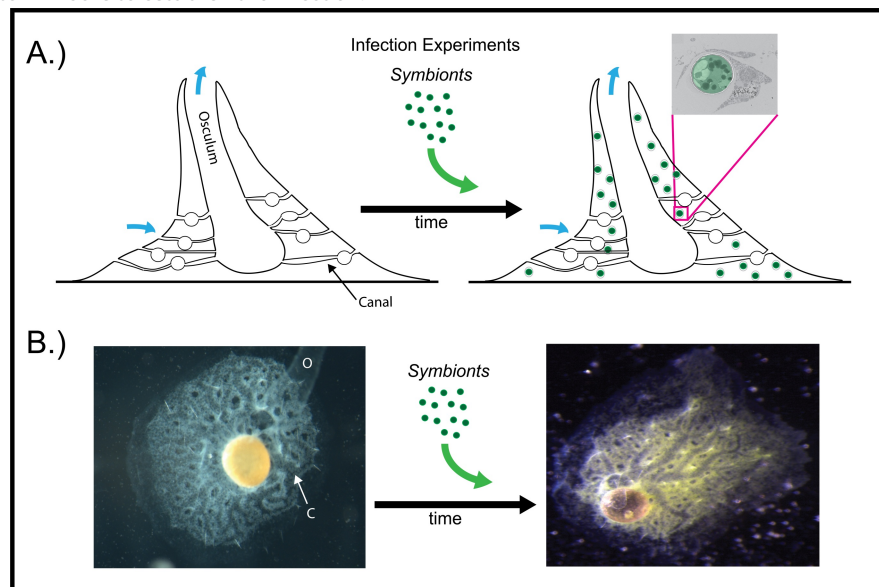
Infection of freshwater sponges with microalgae

- 14 Refer to "Hatching and freezing gemmules from the freshwater sponge *Ephydatia muelleri*" [dx.doi.org/10.17504/protocols.io.863hzgn](https://doi.org/10.17504/protocols.io.863hzgn) for protocols on cleaning and hatching gemmules. This protocol will assume that Stage 5 juvenile sponges can be obtained.

14.1 Hatch gemmules in 6 or 12 well tissue culture microplates or in individual tissue culture dishes in the dark at room temperature for two days (or longer depending on gemmule hatching). Replace half of culture media (Strekal's or M-Med) daily after hatching. Sponges should always be immersed/covered

by culture media and monitored for growth stages until Stage 5.

- 14.2 When sponges have reached Stage 5 juveniles with functioning oscula, they can be infected with microalgae. Refresh the media right before infection and then carefully pipette the determined number of algal cells around the base and above sponges in each culture dish or well. Obtain early log phase microalgal cultures for the algal line of interest.
- 14.3 Place infected sponges under grow lights (e.g., 16 hours light:8 hours dark) for at least 4 hours and up to 24 hours to establish the infection.




Ephydatia muelleri infected with *Chlorella*-like green algal symbionts. From <https://doi.org/10.1101/2020.08.12.247908>.

- 14.4 Wells/dishes can be washed several times with culture media to remove excess and dead algae after the infection is established. Sponges not infected with algae can continue to grow in the dark to inhibit growth of any possible algae that were present in the gemmules. We routinely grow sponges that are free of algae (as determined by fluorescent microscopy and/or qPCR).
- 14.5 Infected sponges can be grown for up to a week post infection under grow lights. Sponges can be re-infected every 24 hours after gentle washing in Strekal's or M-medium if desired.

Post-infection experimentation

- 15 Algal infected sponges can be used for a variety of experiments including DNA and RNA isolation for qRT-PCR or transcriptome sequencing, confocal and other fluorescent microscopy, or TEM.
 - 15.1 Symbiotic sponges to be used for subsequent RNA isolation can be gently scraped off the bottom of the culture dish using the end of a p1000 filtered tip and transferred to a microfuge tube. Excess media should be carefully removed and replaced with RNAlater storage solution. Sponges can be held at 4°C overnight and then excess RNAlater is removed before storage at -80°C until further use.
 - 15.2 For imaging by confocal or other fluorescent microscopy, media should be replaced with 4% paraformaldehyde made in 1/4 Holfreter's solution (HS) directly into culture dishes (sponges can be grown on sterile coverslips or on 35 mm MatTek dishes with mounted coverslips. Fix tissue overnight at



4°C followed by at least 3 washes in 1/4 HS the next day before staining and viewing.