

Algae Culture for Freshwater Mussel Propagation



**A Companion Manual to the
Online Video Tutorial**



Introduction

The production and delivery of a nutritious food source is a critical step in any freshwater mussel propagation program. The marine bivalve industry has tried many food types over the years, but no suitable food resource has been found that replaces live phytoplankton. The following pages are not intended to be a stand-alone guide to algae culture but are designed to be a companion manual to the online video tutorial “Algae Culture for Freshwater Mussel Propagation”.

This manual describes the algal culture methods designed by Drs. David and Pam Orcutt under contract with the White Sulphur Springs National Fish Hatchery (US Fish and Wildlife Service). The method uses small stock cultures to inoculate successively larger cultures until a sufficient amount of algae is cultured to supply the hatcheries needs.

Sterile Technique Standard Methods

It is very important when culturing algae for freshwater mussel propagation to work with pure cultures (pure starter cultures can be obtained from a variety of commercial sources). Unfortunately, the world around us is covered with microorganisms. Algae cells and bacteria cells are even carried on dust particles in the air. In order to protect sterile media, plates, slants, flasks, and carboys from these microorganisms, we must practice sterile (aseptic) technique at all times. This simply means that sterile surfaces or sterile media must be protected from contamination by microbes in the air or residing on non-sterile surfaces. In sterile technique, only sterile surfaces touch other sterile surfaces and exposure to the air is kept to an absolute minimum.

To maintain sterile cultures always do the following:

- 1) Thoroughly clean and autoclave all glassware
- 2) Autoclave or filter sterilize all media
- 3) Wipe down all work surfaces with alcohol
- 4) Use sterile pipets and loops for transferring cultures into fresh media
- 5) Do not touch sterile surfaces with your hands or any other non-sterile utensil
- 6) When possible, always use the laminar flow hood when transferring cultures.

The lab, where you conduct media preparation and algae transfers, should be kept as clean as possible. Counter tops, the inside of the hood and floors should be cleaned and vacuumed at least weekly, if not more frequently. The door to the lab should be kept closed at all times.

Getting Started

Glassware Cleaning

All glassware used in algae culture must be VERY clean. Wash all glassware with a detergent that is good at removing grease (e.g. Dawn). Using the appropriate size brush, scrub all surfaces inside and out. If a piece of glassware is particularly dirty or has algae that has dried to the surface, is it best to let it soak in soapy water for an hour or more. After cleaning, rinse each piece of glassware in tap water at least 4 times inside and out. A final rinse should be done with distilled water. Invert the clean glassware on a wire rack to dry, making sure it is in a position where all water will drain out and it will dry completely.

After cleaning and drying, glassware should be sterilized in an autoclave. Glassware to be sterilized in the autoclave should be covered with a piece of heavy duty aluminum foil large enough to cover down the outside of the glassware at least one inch. Place a small piece of autoclave tape on each foil cover and mark the day, month and year using a black marker.

Bottles should be capped **loosely**. A piece of dated autoclave tape should be placed on the cap reaching to the bottle neck. Test tubes to be used for algae culture slants should be handled in the same manner.

Flasks for growing stock cultures (500mL and larger) need to have a foam stopper, appropriately sized for the flask, inserted and covered with a large piece of foil in such a way as to not compact the foam stopper. Again a piece of dated autoclave tape is put on the foil top.

Bubbling tubes, feeding tubes, forceps and other assorted items should be placed in the appropriate sized autoclaveable bag and dated.

Sterile Distilled Water

Sterile distilled water should be used for all algae culture purposes. To make sterile distilled water, simply add distilled water to 1L media bottles, leave the caps loose, and place a piece of dated autoclave tape on the cap. Autoclave and then allow to cool completely before tightening the lids.

Autoclave

Please follow your manufacturer specifications for sterilizing glassware and liquid media in an autoclave. The extremely high temperatures and pressures in an autoclave can be very dangerous if not operated properly.

Laminar Flow Hood

A laminar flow hood is important for any algae culture lab. As with the autoclave, please follow your manufacturer specifications for operation and maintenance of the laminar flow hood.

Follow the instructions below to prepare the laminar flow hood before working with algae cultures.

1. Clean the inside of the Laminar Flow Hood thoroughly with 70% isopropanol
2. Once clean, turn on the UV light and blower and allow to sterilize for at least 15 minutes prior to use
3. Once sterile, leave the blower on and turn off the UV before working under the hood.

Media preparation

There are a large number of algae growth media formulas available from commercial vendors. Below is the growth media formula used at White Sulphur Springs National Fish Hatchery.

F/2 Modified Guillard's Medium

Part A – contains trace nutrients and vitamins. This must always be filter sterilized.

Part B – contains primary nutrients but no silicate.

Part C – contains silicate.

For Concentrated F/2:

1. Using a 1 gallon glass jug and 1L graduated cylinder measure out 3L distilled H₂O into glass jug (jug and graduated cylinder do not need to be sterile). Place a piece of tape on jug and mark 3L level. Remove about 1L H₂O from jug by pouring into a 1L flask or graduated cylinder.
2. Add a stir bar and place jug on stirrer/hotplate with heat on 3.
3. With funnel in the top of the jug slowly add the contents of one pack of **Part B**. It will take a while to get all added. Make sure what you have added goes into solution before adding more. Rinse bag with a little distilled H₂O and add to jug.
4. Remove stir bar and add distilled H₂O to bring total volume in jug to your 3L mark.
5. Using the 1L graduated cylinder, dispense 980mL of F/2 into each of 2 - 1L bottles. Then dispense the remaining L into 2 – 500mL bottles, adding approximately 490mL to each. If there is extra F/2 divide it between the 2 – 1L bottles. (It is better to store in smaller volumes. This decreases the times each bottle is opened and lessens the chance of contamination).
6. Label bottles with contents and date and place a strip of autoclave tape over corner of label. Add strip of autoclave tape over cap and bottle.
7. Autoclave bottles on slow exhaust and wait until autoclave has cooled to below 100 degrees F to open door (helps prevent any boiling over and loss of media)
8. Allow bottles to cool enough to be held in your hand before adding vitamins (**Part A**). Bottles can also be refrigerated as is and the vitamins added later. Do not tighten caps completely until totally cool or cap liners may be pulled out.
9. Measure total volume of **Part A**, (this should be about 6mL). Under the laminar flow hood, filter sterilize all of **Part A** into sterile capped test tubes and then using aseptic technique add 1/3 of total **Part A** (should be about 21.3mL) to each of the 2 sterile 1 L bottles of F/2 and 1/6 of total **Part A** (should be about 10.6 mL) to each of the 2 – 500mL bottles of F/2.
10. Add the words plus (+) vitamins to the bottle label.
11. Cap tightly and refrigerate bottles in the dark (helps prevent degradation of vitamins).
12. Use at the rate of 1mL/L to make up final solution for carboys, flasks or carboy tubes.

For F/2 with silicate:

1. Add concentrated silicate at the rate of 0.66mL/L to make carboys, flasks or carboy tubes. Use aseptic technique in hood. Silicate will precipitate so shake well before use.

Essential Nutrients for F/2

Dr. Orcutt found commercially available F/2 to be lacking in a few key ingredients or essential nutrients. The recipes for these essential nutrients are given below.

Essential Nutrients 1 (EN1)

In 1L Bottle

33.6 g - H_3BO_3
50.0 g - $MgSO_4 \cdot 7H_2O$
5.0 g - $KH_2PO_4 \cdot 7H_2O$
175.0 g - $NaNO_3$

In 3L Glass Jug

100.8 g - H_3BO_3
150.0 g - $MgSO_4 \cdot 7H_2O$
15.0 g - $KH_2PO_4 \cdot 7H_2O$
525.0 g - $NaNO_3$

1. Add approximately 800mL distilled H_2O to a 1L bottle or 2,500mL to 3L Glass Jug.
2. Add stir bar and place on stirrer/hot plate. Set heat on 3-4 and stir.
3. Weigh out each ingredient and add to the bottle in the order listed above. Make sure each is dissolved before the next ingredient is added. Add each slowly so they dissolve completely.
4. Remove stir bar and using distilled H_2O bring volume in bottle to 1L using calibrations on bottle.
5. Label with contents and date. Place strip of autoclave tape over one corner of label. Cap loosely, place strip of autoclave tape over lid to bottle and autoclave on slow exhaust.
6. Wait until autoclave temperature is 100 degrees F before opening door to prevent boiling over.
7. Cool completely, then tighten cap and store. Does not need to be refrigerated.

USE AT THE RATE OF 1mL/L IN FINAL MEDIA SOLUTION

Essential Nutrients 2 (EN2)

In 0.5L Bottle

25.0 g CaCl₂

In 3L Glass Jug

150.0 g CaCl₂

1. Add approximately 400mL distilled H₂O to a 500mL bottle or 2500mL to 3L glass jug.
2. Add stir bar and place on stirrer.
3. Weigh CaCl₂ and slowly add to bottle while stirring. Don't add all at once but a little at a time until all is in solution.
4. Remove stir bar and using distilled H₂O bring volume in bottle to 500mL (or 3L) using calibrations on bottle.
5. Label with contents and date. Place strip of autoclave tape over one corner of label. Cap loosely, place strip of autoclave tape over lid to bottle and autoclave on slow exhaust.
6. Wait until autoclave temperature is 100 degrees F before opening door.
7. Cool completely, then tighten cap and store. Does not need to be refrigerated.

USE AT THE RATE OF 0.5mL/L IN FINAL MEDIA SOLUTION

****NOTE**** EN1 and EN2 cannot be made in one bottle. Adding CaCl₂ to essential nutrients 1 causes a precipitate that will not go back into solution.

For Concentrated F/2 silicate:

1. In a one gallon jug with the 3L volume marked, similar as is done for F/2 preparation, bring volume up to 3L, mark this level if not already marked and then pour off about 500mLs of water.
2. Add stir bar. Add the packet of silicate gradually and mix until dissolved. Can add heat if needed.
3. Remove stir bar and bring total volume in jug to 3L mark. Mix well.
4. Pour into two 1L bottles and two 500mL bottles.
5. Label bottles with contents and date (Silicate conc. date). Place a strip of autoclave tape over corner of label.
6. Cap **loosely**, add autoclave tape to cap and bottle and autoclave on slow exhaust.
7. Cool **completely**, tighten cap and store. Does not need to be refrigerated.
8. **Use at the rate of 0.66mL/L to make up carboys or flasks using sterile technique in hood. Silicate will precipitate so shake well before using.**

Algae Slant Culture

Backup schedule

Healthy stock algae slant cultures should be transferred to fresh media every two weeks. If cultures do not look good, they may need to be transferred earlier.

Backup protocol: (See video)

1. Using a sterile loop, transfer algae from the stock culture to a fresh, sterile agar slant
2. Streak the algae back and forth across the slant from top to bottom
3. Using a new sterile loop repeat the steps above for all species in culture
4. Leave the cap loose to allow for gas exchange
5. Place fresh cultures back in incubator

F/2 Agar Slant Preparation

With silicate:

500mLs agar will make about 28 slants of 18mLs each using 25mm tubes.

1. Weigh out 7.5g agar.
2. Add agar to a 1L flask containing 400mL distilled water (measure amount as calibrations on flasks are not accurate)
3. Add stir bar and heat on 5 stirring until agar has dissolved. Watch this as it gets hotter as it will boil over if just left alone.
4. Add an additional measured 100mL distilled water to give total volume of 500mL.
5. Cover flask top with foil, autoclave tape strip and autoclave on slow exhaust.
6. Allow autoclave to cool to 80 before opening.
7. Have sterile 25mm tubes in a rack in hood with tape moved to side and caps loose.
8. Also have an extra empty rack for 25mm tubes and a stirrer/hot plate. If you work quickly the hot plate will not be needed.
9. Label extra rack with media type and date, then position rack so tubes will slant at the desired angle (two sharpie pens placed under front edge of rack works well).
10. When agar has cooled but still liquid and can be held in your hand, aseptically add 0.5mLs essential nutrients A, 0.25mL essential nutrients B and 0.33mL concentrated silicate and 0.5mL F/2 concentrate + vitamins.
11. If **F/2 without silicate** is desired just leave out the silicate above.
12. If using stirrer/hot plate keep on low to prevent solidification. Keep temp as low as possible as excess heat destroys vitamins.
13. With a sterile 25mL pipette aseptically add 18mLs agar liquid to each sterile tube.
14. Cap loosely and put in slanted rack.
15. Allow to solidify for several hours or overnight.
16. Leave slants out at room temperature 2-3 days then check for sterility and allow to dry somewhat. Once they are deemed sterile tubes can be **capped tightly** and put in rack in refrigerator.

WHEN THE TERM ASEPTIC IS USED THIS MEANS USE STERILE TECHNIQUE IN A CLEAN ENVIRONMENT SUCH AS A LAMINAR FLOW HOOD AND USE STERILE GLASSWARE AND SUPPLIES.

Flask Culture

Back-up Schedule

Healthy stock algae cultures in flasks should be transferred to a new flask with fresh media every two weeks. Flask to flask transfer should be limited to 4 generations so new flask cultures should be started directly from slants every 2 months. If flask cultures do not look good it may be necessary to start new cultures from slants earlier. Flask cultures are used for starting fresh carboys so make sure to have enough flask cultures for each species in culture to accommodate both the carboy back-up schedule and the flask back-up schedule.

Flask Culture Maintenance

Flask cultures should be stirred vigorously twice per day to resuspend any settled algae.

Back-up Protocol: (see video)

Stepping up from slant culture to flask culture

Starting 500mL and 1000mL Flask Cultures from a Slant

ALL OF THE FOLLOWING SHOULD BE DONE ASEPTICALLY IN THE LAMINAR FLOW HOOD

1. Look in the refrigerator for a bottle of diluted F/2 + vitamins + Essential nutrients + whatever else you need. If there is not a bottle already made you first will need to make up this media in the quantity that you need. It is typically made up in either 1L or 500mL bottles.
2. Start with a bottle of sterile water. Pick one that has as close to 1L (or 500mL) of water as possible so your dilution factor will be correct (we use 1mL/L of most stock solutions)
3. Add to the 1L bottle of sterile water, 1mL of concentrated F/2 + vitamins (500mL bottle will get 0.5mL of same concentrate stock) stock solution. If there is not a bottle of this made up take a bottle of concentrated F/2 and aseptically add the appropriate amount of sterile vitamins. (21.4mL/L or 10.7mL/500 mL) If there are not any sterile vitamins made up you will need to filter sterilize some.
4. Next add 1 mL stock sterile essential nutrients solution 1 (EN1) and 0.5 mL sterile essential nutrients solution 2 (EN2).
5. If you need silicate add 1mL of sterile stock silicate solution.
6. Have at least 1 sterile 500mL flask that has a stopper with a hole for each new culture you want to prepare. Usually make 2 flasks per algae species.
7. After aseptically removing the foil covering the stopper, aseptically place a sterile 1 mL pipette through the hole in the stopper and insert into the flask until the tip touches the bottom of the flask. Take care not to touch the top of the stopper with anything until this is done.
8. Aseptically remove the stopper with bubbling tube and pour in at least 300mL (for 500mL flasks) or 700mL (for 1000mL flasks) of whatever nutrient solution you want to use. Replace the stopper and bubbling tube. Do this for each culture you want to inoculate. Label each flask.
9. To transfer from a stock slant culture use a sterile loop and get as much of the culture in the **large** loop as possible then place the loop into the media in the flask and stir around to remove as much of the culture as possible. You can also try rubbing the loop end against the side of the flask below the media level to try and remove more culture from the loop.
10. Put the flask in an incubator and aerate.

Starting 500mL and 1000mL Flask Cultures from a Flask

To transfer from a stock flask culture to a new flask, swirl the stock culture to make sure all the culture is in solution and none remains on the bottom of the flask. With a sterile pipette remove 5mL of stock culture and add to media in the new flask. You should be able to see a faint green tint to the media in the new flask (if you can't see green color add more of the flask culture, 5mL at a time until you see a faint green color in the new flask). It is always better to add too much rather than too little.

1. Swirl the new flask to mix well and then put the flask in the small incubator and attach the bubbling tube to one of the empty air tubes in the incubator. Unclip the closure on the tube to allow air to flow and bubbling to begin.
2. Once all new flasks are inoculated and placed in the incubator and bubbling, place a piece of cardboard on the top shelf to shield the new cultures from the intense light. Leave the cardboard in place for 3-4 days until the culture has had a chance to begin growing and has started to darken slightly, then remove the cardboard and allow cultures to continue to grow until desired density is achieved.

Carboy Culture

Back-up Schedule

Healthy stock algae cultures in carboys should be transferred to a new carboy with fresh media every two weeks. Carboy to carboy transfer should be limited to 4 generations so new carboy cultures should be started directly from flasks every 2 months. If carboy cultures do not look good it may be necessary to start new cultures from flasks earlier.

Carboy cleaning, sterilization, and inoculation procedures

1. Used carboys should be washed with a brush and soap and water to remove **ALL** traces of algae. We are using a color code system on the carboys. Put only the color indicated algal species in the coded carboys when inoculated to help cut down on cross contamination.
2. After washing, rinse each carboy 5-6 times with tap water.
3. Add 25mL Clorox to the carboy and fill up with water. Water should fill the carboy neck so that when the cap is put on, water will completely contact the cap surface. Put clean cap on carboy and let sit overnight. Carboys can be filled this way and sit for some time if not needed immediately.
4. **To use carboy for algal culture** place in hood and using aseptic technique remove 4,400mL water/Clorox solution from the carboy to give you a total volume of 15L. Replace cap to maintain a sterile carboy. This can just be rested on top and not snapped down.
5. Weigh out 3.86 gm Sodium Thiosulfate and aseptically add to the carboy.
6. Using aseptic technique add one tube of sterile F/2 + nutrients required for the species to be grown to the carboy.
7. Aseptically place a sterile bubbling tube in the carboy. Large sterile forceps will be needed to push the stopper down into the neck of the carboy. Add a sterile inline filter to the top of the bubbling tube, being sure to get the inlet side up.
8. Remove carboy from hood, add a small piece of tygon tubing appropriate sized to fit tube from air and CO₂ source and connect bubbling tube to an air source and bubble air through carboy for at least 1 hour.
9. Put carboy back in hood and using aseptic technique add inoculum and replace bubbling tube.
10. Place carboy on light rack and connect bubbling tube to CO₂ source and allow to grow until desired density it reached.

Concentrated Media Tubes for use in carboys

1. Autoclave full rack of 25mm test tubes with caps (caps should be loose and a piece of autoclave tape over cap and tube with date on tape).
2. When cool, place in clean hood and using aseptic technique add 15mLs sterile F/2 concentrate + vitamins to each tube.
3. Add 15mLs of Essential Nutrients 1 (EN1).
4. Add 7.5mLs of Essential Nutrients 2 (EN2).
5. If F/2 + vitamins + essential nutrients + silicate is desired, using aseptic technique add an additional 10mLs sterile Silicate concentrate to each tube. Recap tightly and invert several times to mix well.
6. Label rack with contents of tubes and date prepared. Make sure label indicates all ingredients in the tubes. You can use one of the large blue test tube racks and have several types of tubes stored in one rack as long as the tubes are separated by a blank row and the rack is well labeled.
7. Store rack in refrigerator in the dark to help maintain vitamins.

There will be precipitate in the tubes with silicate so be sure to mix well before using.

Carboy Bubbling Tube Preparation

1. Bubbling tubes can be made by cutting a piece of glass tubing 23 inches long. The tubing we currently have is 48 inches and both ends are smooth. Measure from each end of long tube and mark 23 inches with a sharpie. Cut out the middle to make 2 tubes approximately 23 inches long and use the smooth tip for the up end of the bubbling tube and the rough cut end for the down end.
2. Add a 3 inch Styrofoam stopper in which a hole has been made to accommodate the glass tube.
3. Add a short piece of thick walled tygon tubing to the top of the tube. This is used to attach the sterile inline filter for the air source.
4. Make up 6 tubes.
5. Using the self-sealing large autoclave bags place 3 tubes in one bag stopper end down and together to one side of bag. Then place the other 3 tubes stopper end down in the second bag, again keep ends together in same area of bag.
6. Turn one bag over so the blue plastic side of one bag lines up with the paper side of the other bag. Push the down ends of tubes into the opposite bag making sure the three down tubes from one bag are in opposite corners of the bags.
7. Slide bags together so self-sealing strips overlap the opposite bag.
8. Carefully (slowly/gradually) remove self-sealing strip and seal it against the opposite bag.
9. Turn the bags over and so the same to the other side.
10. Add a small strip of autoclave tape to the edges of the seals.
11. Date bag and autoclave.
12. Store in cabinet until needed being sure to place new bags in back and oldest bags in front of cabinet so oldest stock is used first.

Adding Stopper/Tube Assembly to each carboy

ALL OF THE FOLLOWING SHOULD BE DONE ASEPTICALLY IN THE HOOD

1. Wipe around carboy cap and upper neck with alcohol. Remove cap on carboy and rest on top of carboy.
2. Carefully open one end of the sterile tube pack. Remove one stopper/tube assembly from pack (touch only on the top portion of the tube assembly to keep sterile and avoid touching within 1-2 inches of the stopper so this area remains sterile should the depth of the tube need to be adjusted once it is placed in the carboy) and without touching the top of the carboy lower the tube into the carboy until the stopper sits on the carboy top.
3. Using the sterile long forceps push the stopper into the carboy. From the top using a gentle twisting motion being careful not to touch sides of stopper or the insides around the carboy top push the stopper down into the carboy neck. Just do the best you can as this is difficult to do without touching something.
4. Now adjust the glass tube in the stopper so it is just off the bottom of the carboy somewhere around the side edge with the stopper well down in the carboy neck.
5. Attach a sterile in-line filter to the piece of Tygon tubing on the end of the bubbling tube. Be sure the inlet side is up and do not touch the inner edges of the Tygon tubing when inserting the filter.
6. Make sure the Tygon tubing is snugly attached to the glass bubbling tube. Autoclaving tends to expand the tubing.
7. Place a short piece of Tygon tubing on the inlet side of the in-line filter to attach the aeration line to.
8. Repeat the procedure until the bubbling tubes are used at one end of the pack.
9. Be very careful not to touch the glass tubes from the other bubbling tubes.
10. Fold over the end of the pack and tape it closed, making sure the glass ends of the remaining tubes are protected.
11. Invert the pack and open the other end of the pack and remove the tubes as needed and place in carboys as described above

Addition of inoculum

Inoculum should be added aseptically by pouring 1000 mL of heavy suspended cells into a sterile 1000mL beaker and then pouring the inoculum into a carboy. This should be done in the hood to maintain sterility. Avoid dribbling the inoculum down the sides of the carboy or beaker as much as possible when pouring from the beaker. The hood is limited with respect to space that is why I like to put the stopper assemblies all in the carboys first and then inoculate. Otherwise you have packets of autoclaved tubes, uninoculated carboys, and a carboy containing inoculum in the hood at the same time. A full carboy of heavy inoculum will inoculate 16 carboys.

Carboy Culture Maintenance

Carboy cultures should be stirred vigorously twice per day to resuspend and settled algae. CO₂ flow rates should be checked periodically to make sure CO₂ tank is not empty.

Adjusting air/CO₂ flow and lighting

Place inoculated carboys somewhere where they will receive adequate light and aeration. Adjust the rate of aeration so that bubbling is moderate to vigorous. Light should be reduced until the cells have a chance to acclimate to the new growing conditions. Light can be increased after a day or so. Sometimes cultures will bleach out if they are given too much light too soon and is often a function of inoculum density.

If CO₂ is needed for faster growth, it can be added at this time. Make sure all gas bottles are chained securely to the wall or other secure structure.