Oyster Hatchery Manual

Protocols for North Carolina Oyster Hatchery Operations

Compiled and edited by
Philip S. Kemp Jr.
Carteret Community College
Aquaculture Technology Program
Nelson Bullock
Jeff Militano
Tom Osborne
Dana Schmidt
Transitional Production Program

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Table of Contents

3) Strip spawning
4) Counting sperm on eggs
5) Counting oyster eggs
6) Hydrogen peroxide induced spawn
7) Serotonin induced spawn

Section D: Drain downs and Larviculture Protocols

1) Determining larvae size
2) Calibrating ocular micrometer
3) Determining sieve size
4) Alternative method measuring sizes
5) Counting Larval Oysters
6) Daily tank drain down
7) Cleaning larvae tanks
8) Restocking larvae
9) Larvae tank feeding
10) Larvae feeding chart
11) Harvesting eyed-larvae
12) Remote setting
13) Larvae tank record sheet

Section E: Examining Trouble Areas

1) Agar plates for algae culture
2) Fluid thioglycollate bacteria indicator test
3) Agar plates for aerobic bacteria
4) Serial dilutions for bacteria testing
5) Extracting dilutions
6) Interpreting results of bacteria cultures
7) Protocol for total aerobic bacteria and Vibrios
8) Chlorine treatment for ciliates in algae

Section F: Facilities and Operations Protocols

1) Large dock pump operation
2) Hydrotech 801-802 filter operation
3) Transfer pump operation
4) Sand filter operation
5) Using U-V filtration
6) Testing water quality
7) Maintaining a chlorine bath
8) Cleaning the Floor
9) Determining tank volumes
10) Making a kalwall useable
11) Cutting and gluing PVC pipe
12) Making a sieve
13) Making a counting slide
14) Using the MIC-D digital microscope

Miscellaneous:

1) How to write a protocol

Section A: Algae Culture Protocols

1) General Algae system setup
2) Algae room daily tasks
3) Sterile technique inoculating test tubes
4) Inoculating stock cultures
5) Inoculating aerated flasks
6) Inoculating non-aerated flasks
7) Inoculating starter culture flasks
8) Inoculating carboys
9) Inoculating Kalwalls
10) Cleaning and preparing carboys
11) Cleaning and preparing kalwalls
12) Cleaning and preparing kalwalls alternative method
13) Preparing media for small algae cultures
14) Adding nutrients to cultures
15) Harvest kalwall algae batch
16) Harvest semi-continuous algae culture
17) Restocking a kalwall
18) Steam sterilizer operation
19) Using the Yamato SM510 autoclave
20) Installing disk filter
21) Making cotton plugs
22) Making a three-stem apparatus
23) Making tube filters
24) Cleaning tube filters
25) Counting algae with a hemacytometer
26) Measuring algae concentrations through spectrophotometry
27) De-chlorination
28) Algae culture record sheet

General algae system setup:

An algal production system should have the following basic components:

- Algae culture vessels of various sizes and shelves for culturing and storage.
- Vessels include: test tubes, 125-ml flasks, 2000-ml flasks, 5-gallon carboys, 60-gal kalwalls
- Artificial illumination to optimize growth (cool white fluorescent)
- Sterilization equipment to maintain axenic media and facilitate sterile technique.
- Equipment includes: Autoclave, steam sterilizer, alcohol lamp, chlorine bath.
- Aeration system to mix cultures and deliver CO2 to speed up growth; dry air pump
- Filters for air supply to prevent contamination
- Filtered seawater supply free of particulates to make culture media
- Fertilizer and metasilicate nutrients for algal cultures
- Miscellaneous components include glass tubing, rubber stoppers, cotton gauze, sterile cotton, plastic tubing, heavy duty aluminum foil, alcohol, bleach, Ortho-tolidine (OTO), Sodium thiosulfate

Sequencing algae culture vessels:

1) Inviolate cultures (test tubes) house incoming cultures of algae species. Replicate them for backups. Maintain inviolate and axenic cultures in test tubes. Use them only in the event an algae culture becomes contaminated.
2) Stock cultures (125-ml flasks) are maintained axenically and used to inoculate starter cultures and to restart stock cultures.
3) Starter cultures (1000-ml or 2000-ml flasks) are used to inoculate carboys.
4) Carboys (5-gallons) are used to inoculate kalwalls and to feed small larvae cultures
5) Kalwalls (60-gallons) are used to culture feeding quantities of algae
6) Open tanks are used to culture algae in very large hatcheries.

General operating notes for culturing algae:

- ✓ Keep facilities as clean as possible; minimize traffic
- ✓ Separate sterile test tubes from working cultures; maintain a large number of backup cultures
- ✓ Work with smallest most sterile cultures first then with progressively larger cultures
- ✓ Add silicate before autoclaving and add fertilizer after autoclaving and cooling media
- ✓ Sterilize large culture media by chlorination, pasteurization, u/v filtration, ultra-filtration
- ✓ Use sterile technique when harvesting, inoculating, transferring or counting algae cultures
- ✓ Use heavy duty aluminum foil which can be reused several times
- ✓ Take advantage of natural lighting where possible
- ✓ Use condensation-free aeration system; replace airlines yearly

Algae Room Daily Tasks

Materials: Microscope and slides, autoclave/sterilizer, alcohol burner, anti-bacterial hand soap, bleach, clean rags, gloves

Note: Keep the algae lab as clean as possible. Wash hands with anti-bacterial soap before working with algae cultures. Dampen a clean rag with a mild chlorine bleach solution and keep it handy to wipe hands during the algae workup. Wear laboratory gloves, if desired, to minimize chlorine sensitivity.

Beginning of day:

- Make a daily overview of algae cultures to insure that aeration and lighting are operating properly and that there are no crashed cultures.
- Check air supply line for condensation buildup and purge if required.
- Correct aeration problems by checking for valve adjustments or clogged filters. Replace clogged air filters using sterile technique.
- Check the carbon dioxide injection for proper operation. Note the CO2 tank pressure.
- Replace any non-working light bulbs in fluorescent light fixtures.
- Check the chlorine bath for sufficient level of chlorination by dropping in one drop of OTO (should turn very yellow, not slightly yellow and not orange or red). Add 5 ml bleach if required and stir to raise residual chlorine level.
- Remove items from autoclave and place on cooling rack or storage shelves if cooled.
- Reload and start autoclave if there is a backlog of items to be sterilized.

Working day:

- Swirl stock cultures to resuspend algae and note any that are not growing properly (set aside bad cultures for disposal) be sure not to wet the cotton plugs.
- When working with algae cultures begin with the smallest cultures and continue to the larger ones. Algae cultures maintained in algae production operations are (from small to large): inviolate cultures, stock cultures, starter cultures, carboys and kalwalls.
- Transfer and replicate inviolate cultures to new test tubes once per month.
- Transfer and backup new stock cultures and new starter cultures once per week.
- Inoculate cultures that were autoclaved and prepared the previous day
- Dechlorinate media storage kalwalls and verify with OTO.
- Restart harvested kalwalls.
- Check cultures for contamination by protozoans or bacteria on a regular basis according to appropriate protocol; record in lab notebook.
- Discard infected algae cultures and troubleshoot source of contamination.
- Empty crashed or contaminated cultures and discard.
- Wash and clean empty kalwalls and carboys.

End of day:

- Refill algae kalwall cultures and media storage kalwalls with filtered seawater and bleach.
- Clean glassware and prepare new items for autoclaving; start autoclave.
- Place other items in chlorine bath; mop floor with antibacterial detergent before leaving.


NANO

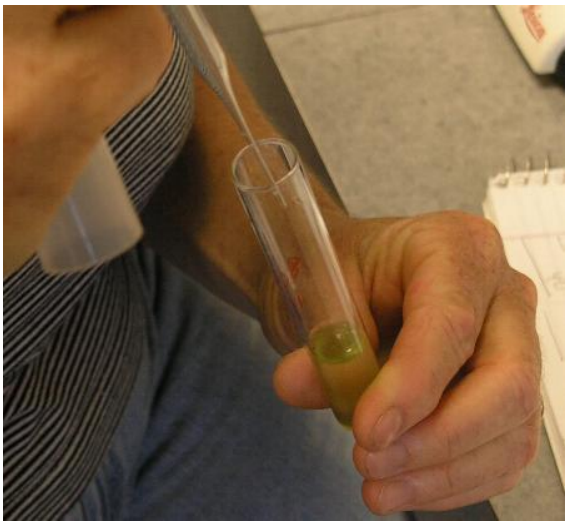

NVA
7/24
1000
900
700
600
500
400
300

Sterile technique and inoculating test tubes:

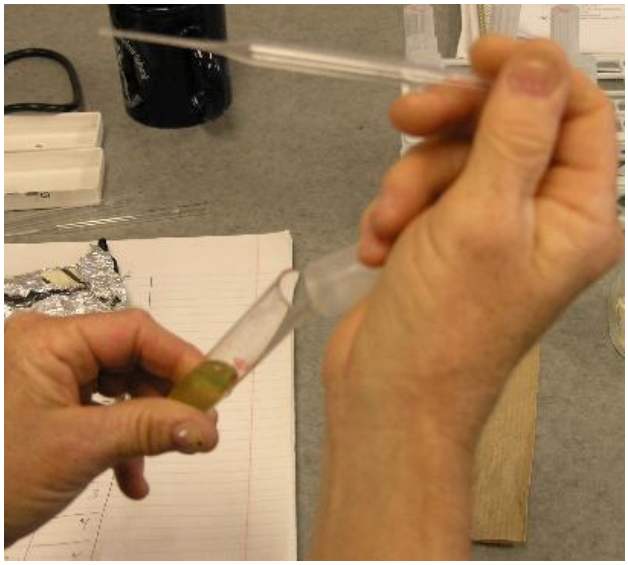
Sterile technique is an important skill in the oyster hatchery and is used in all aspects of algae culture. Test tubes are inviolate, which means that they are kept as a back up for cultured algae species and are only used in the event a species becomes contaminated.

Materials: Selected algae cultures for transfer, sterilized test tubes of media for inoculation, sterile pipettes, alcohol or Bunsen burner, igniter, chlorinated wiping cloth

1) Insure that you have an uncluttered working area and clean countertop by wiping with an alcohol swab or a chlorinated wiping cloth. Wash hands thoroughly.
2) Using the proper protocols insure that the mother culture is clean and not contaminated by protozoans or bacteria. For protozoans use sterile technique to obtain a drop of the culture for examination. Bacterial contamination must be determined in advance.
3) Select desired mother algae cultures to inoculate new daughter cultures.
4) Assemble test tubes with fertilized sterile media. Start 6-10 new test tubes from each inviolate starter culture. Label receiving test tubes with species, date, your initials and any special conditions of the culture.
5) Organize the workplace by placing cultures and sterile flasks or test tubes in such a position that you do not have to reach across the burner for cultures or inoculants.
6) Light an alcohol lamp or Bunsen burner. The burner is used to sterilize all transfers.
7) Hold the selected mother culture with the left hand and a sterile pipette with the thumb and first two fingers of the right hand (left-handed individuals switch hands).



8) Quickly flame the lip of the mother culture exposing all sides of the tube lip to the burner flame. Rotate your hand at the wrist to accomplish flaming the test tube lip; do not rotate the test tube by swiveling it in your fingers as you may drop it.
9)



10) Insert the sterile pipette to the mother culture and draw the desired amount for transfer. Use 0.5 to 1 ml of inoculant for test tubes with 6 ml of media. You may wish to vary the amount of inoculant according to the performance in your facility for each algae species. Use less inoculant to produce cultures that are stable for longer periods of time. Use more inoculant to produce cultures that are ready sooner for transfer to working stock cultures.
11) Re-flame the lip of the mother culture and re-insert the cotton plug; replace mother culture in test tube rack.
12) Without putting down the pipette immediately pick up the receiving container and repeat procedure to remove plug and flame the lip.
13) Be sure to hold both plug and pipette in such a way that they do not touch the counter or other objects.
14) Quickly flame the lip of the receiving tube per above description. Insert pipette and dispense contents to the new tube. Flame tube again and replace cotton plug.
15) This completes the inoculation procedure.
16) Replace new daughter culture in test tube rack.
17) Label tubes with species, date, your initials and any special conditions of the culture.
18) Cover test tube cultures and place in a lighted location.
19) Place used cotton plugs, pipettes and test tubes in receptacles for cleaning and reuse.

Inoculating stock cultures from test tubes

Materials: Selected mother cultures in test tubes, 125-ml flasks with 60 ml of sterilized seawater media, one flask for each mother culture to be transferred, alcohol burner or Bunsen burner and lighter, chlorinated wiping cloth

Inoculating the first set of stock cultures in flasks

- Gather required materials and wash hands thoroughly
- Clean the work area and wipe with chlorinated wiping cloth
- Light the burner
- Hold the flask in your left hand and pick up the mother culture test tube with your right hand. Using the pinky finger of your right hand remove the cotton plug from the flask and flame the lip of the flask. Using the pinky finger of the left hand remove the plug from the test tube and flame the lip.
- Quickly pour the contents of the test tube into the open flask without touching the lips of the two containers.
- Flame the lip of the flask and replace the cotton plug. This completes the inoculation procedure.
- Place the used test tube in a container for cleaning later. Place cotton plugs and aluminum foil in storage receptacles for reuse.
- Cover cotton plug and upper neck of flask with aluminum foil to extend shelf-life and reduce chance of contamination.
- Label flasks with species, date, your initials and any special conditions of the culture.
- Place newly inoculated cultures in lighted location

Replicating stock cultures

- Once several working stock cultures have been established they can be used to inoculate new working stock cultures instead of using test tubes. Follow the above procedure using a flask culture in place of a test tube.
- It is important to continue to perform routine tests on working flask cultures for bacterial and protozoan contamination so that only axenic cultures are used for inoculations.

Inoculating aerated flask cultures

Materials: Selected inoculant starter culture, Bunsen burner, alcohol spray bottle

1) Assemble materials
2) Wash hands
3) Spray the neck of carboy or flask to be harvested
4) Open clip on the harvest line
5) Place collection container under the outlet of the harvest line
6) Note for algal use harvests, a longer piece of tubing may be needed than the tube used as the harvest line which is already connected to the , this can be done by attaching a longer tube to the existing harvest line using an coupling. Remember to spray the portion of tubing that will be inserted into the harvest line with alcohol.
7) Close the gas outlet line clip
8) Algae culture should then flow out of the harvest line into the collection container
9) Open the gas outlet line
10) Close the harvest line when the proper amount of algae is collected
11) Spray aerated flask with alcohol
12) Label with algae species, date, your initials and any special conditions of the culture

Note: During harvest the harvest line may retain some drops of algae. To remove these spray the inside of the line with alcohol. This can be done either by removing the harvest line and replacing it, or spraying out the line. The dirty line can also be left on the three stem aeration unit and sprayed if caution is taken not to let alcohol flow into the culture vessel

Inoculating a non-aerated flask

Materials: clean 1000 mL flask with sterilized sea-water, isopropyl alcohol in a spray bottle, alcohol burner, smaller 500 mL flask that contains the inoculant, F/2 Fertilizer, both part A and B, larger autoclaved cotton stopper for the large flask.

Procedure for inoculating a non-aerated flask

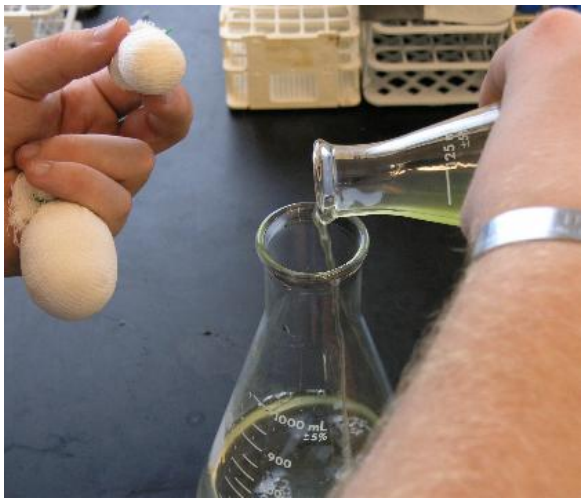
1) Assemble materials
2) Light the alcohol burner
3) Spray both hands with isopropyl alcohol
4) Remove the cap from the larger flask and sterilize the flask by heating the lip of the flask with the flame. NOTE: the flame should not actually touch the flask.
5) Add 5 drops of part a, and 5 drops of part b fertilizer.
6) Remove the cotton stopper from the 500 mL flask, and sterilize the rim of the flask.
7) Re-sterilize the lip of the 1000 mL flask, and pour the inoculants into the larger flask, without letting the flasks touch.
8) Re-sterilize the lip of the larger flask
9) Grab the larger cotton stopper from the autoclaved package and Quickly place it in the larger flask.

Inoculating starter cultures

Starter cultures are maintained in static illuminated flasks and will keep for several months on the shelf in a temperature-controlled room. Keep starter cultures away from routine algae culture activities to reduce chance of contamination. Aerate starter cultures with the 3-stem apparatus if faster growth is required.

Materials: Selected stock cultures in 125-ml flasks, sterilized 1000 ml or 2000 ml flasks with fertilized sterile seawater media, alcohol lamp or Bunsen burner and lighter

- Assemble materials and wash hands with antibacterial soap.
- Light the burner
- Use sterile techniques described previously for inoculating stock cultures.
- Remove aluminum foil and cotton plug from mother culture, flame the rim of the flask and transfer to receiving flask. Flame the rim of the receiving flask and replace cotton plug.
- Cover cotton plug and upper neck of starter culture flask with aluminum foil to extend shelf life and reduce chance of contamination.
- This completes the inoculation procedure for starter cultures.
- Place used cotton plug and aluminum foil in receptacles for reuse. Place used flask in sink for cleaning.
- Label flasks with species, date, your initials and any special conditions of the culture.



Inoculating Carboys

Materials: Selected starter cultures (1000 or 2000 ml flasks), prepared 5-gallon carboy with sterilized seawater media, prepared and sterilized 3-stem apparatus for carboy aeration including air filter, alcohol spray bottle, dispensing bottles of F/2 algae fertilizer

- Assemble materials and wash hands thoroughly with antibacterial soap
- Spray outside of carboy neck with isopropyl alcohol and remove foil from carboy neck
- Remove aluminum foil and cotton plug from stock culture and pour contents into carboy
- Dispense 2.5 ml of F/2 Part A and 2.5 ml of Part B into carboy.
- Add 1/8-tsp of sodium metasilicate for diatom cultures
- Remove foil from the sterilized three stem apparatus and place apparatus in the carboy neck; secure the stopper in place. in carboy
- Connect airline to the longest stem of the three stemmed apparatus, which also contains the air filter. Secure the tube clips on the harvest and gas outlet tubes
- Place used cotton plug and aluminum foil in receptacles for reuse. Place used flask in sink for cleaning.
- Label carboy with species, date, your initials and any special conditions of the culture.

Inoculating Kalwalls

Materials: Spray bottle of isopropyl alcohol, dispensing bottle of chlorine bleach, dispensing bottle of sodium thiosulfate, dropper bottle of OTO chlorine indicator solution, dispensing bottles of F/2 algae fertilizer Parts A and B, container of sodium metasilicate and plastic teaspoon, stepstool

- One day prior to kalwall inoculation fill a 60-gallon kalwall with 50 gallons of filtered u/v sterilized seawater and chlorinate with 30 ml of chlorine bleach for 24 hours.
- Wash hands thoroughly with antibacterial soap before working with algae cultures.
- To inoculate, first dechlorinate kalwall by adding 30 ml of sodium thiosulfate. Turn on air for 20 seconds; turn off air. Test with 1 drop of OTO to insure there is no residual chlorine.
- Remove three-stem apparatus from carboy with algae culture for transfer; place a clean paper towel damped with isopropyl alcohol over the carboy opening.
- Carry the carboy over to the kalwall and with assistance stand on the stepstool and slowly pour the carboy into the kalwall. Try to minimize the amount of splashing.
- Using clean technique add 25 ml of algae fertilizer part A and 25 ml of part B to kalwall. For growing diatoms add 1/2 teaspoon (13 mg/l) of sodium metasilicate.
- Spray kalwall lid perimeter with isopropyl alcohol and replace in kalwall.
- Place used three-stem apparatus and carboy in sink for cleaning.
- Label kalwall with species, date, your initials and any special conditions of the culture.

Restarting kalwalls for semi-continuous production

- Harvest kalwall down to one-half volume.
- Turn off air supply.
- Use clean technique to refill with filtered seawater that has previously been sterilized.
- Acceptable methods of sterilizing seawater media for kalwall cultures are chlorination/dechlorination, pasteurization, ultra-filtration/u-v treatment
- Dispense 13 ml of F/2 Part A and 13 ml F/2 Part B fertilizer.
- If the culture is a species of diatom add 1/4 teaspoon of sodium metasilicate.
- Spray lid perimeter with isopropyl alcohol and reposition in kalwall.
- Turn air supply on.

Cleaning and preparing carboys

Materials: Carboy brush with long handle, dish detergent or alconox, bleach, water hose

- After harvesting or draining carboy, move it to outside cleaning station.
- Spray out carboy with water hose.
- Add 1 ml of dish detergent or alconox and 1 ml of bleach to bucket with 1 gallon of water.
- Add small amount of water to carboy
- Dip brush in stock cleaning solution and brush the bottom and sides of carboy.
- Empty carboy and thoroughly rinse to remove soap and bleach.

Preparing carboys with media

Materials: Clean empty 5-gallon carboy, dispenser bottle of bleach, filtered and U/V sterilized seawater, aluminum foil

- Fill carboy with 4.5 gallons (15 liters) of filtered u/v treated seawater
- Add 3 ml of bleach so that the bleach is mixed by the filling process
- Adjust salinity, if desired, while filling the carboy by adding fresh water
- Make a cap from a square of aluminum foil, and put it over the carboy opening
- Replace the carboy on a shelf for a minimum of 24 hours before use.
- Label with the date of chlorination.
- Before use be sure to dechlorinate the carboy, as per the dechlorination protocol.

Cleaning and preparing kalwalls for algae culture

Materials:

Dish detergent, dispensing bottle of bleach, freshwater hose, long-handled brush, bucket, kalwall cleaning stand

Cleaning kalwalls

- Drain kalwalls, remove lid and air hose and take outside.
- Place kalwall on the cleaning stand.
- Spray out kalwall with a freshwater hose.
- Dispense 1 ml dish detergent and 1 ml bleach to 1 gallon of water in a bucket
- Dip brush in cleaning mixture and scrub kalwall thoroughly; repeat with additional cleaning mixture.
- Rotate kalwall to clean all sides.
- Clean the kalwall lid with brush and cleaning mixture.
- Detach kalwall valve and brush thoroughly.
- Detach air valve and examine for buildup; brush thoroughly.
- Replace kalwall valve and air valve and place kalwall back inside on support platform.

Preparing kalwall media

- Fill kalwalls with 50 gallons of filtered/UV saltwater.
- Avoid touching the upper inside wall of the kalwall with hose or hands.
- Add 30 ml of bleach while filling the kalwall to insure complete mixing of the bleach
- Adjust salinity if desired by adding freshwater while filling the kalwall
- Put lid back on kalwall and wait 24 hours before dechlorination and inoculation.

Cleaning and preparing kalwalls for algae culture alternative method

Materials:

Dish detergent, brush, concrete floor

Cleaning kalwalls

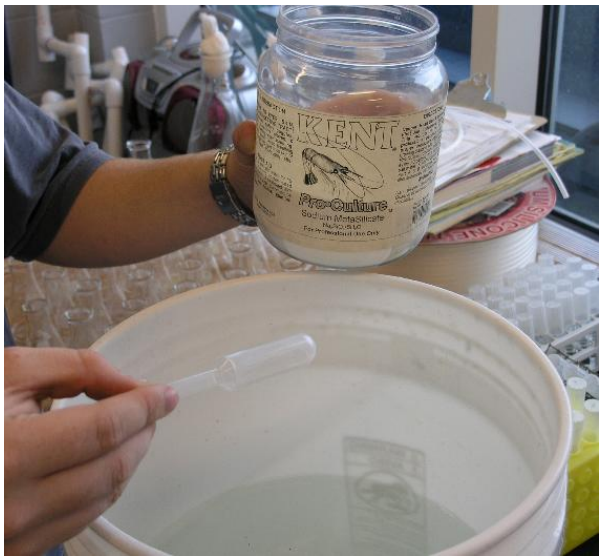
1) Place kalwall on level surface
2) Using a brush clean the kalwall thoroughly inside and out

Preparing media for small algae cultures

Materials: Clean 5-gallon bucket, filtered seawater, fresh water, dispensing bottles of algae F/2 fertilizer, sodium metasilicate, algae culture containers (test tubes, 125-ml and 1000-ml flasks)

Note: Always wash hands thoroughly before working with algae. Keep hands clean.

1) To prepare a quantity of stock solution algae growth media add 20 liters of filtered seawater to a clean 5-gallon bucket and test the salinity. Dilute seawater to 26 ppt salinity if required by stirring in fresh water. A salinity of 26 ppt is suitable for most saltwater algae cultures however higher or lower salinities may be used depending on the species cultured or the specific media being prepared.
2) Add 0.5 ml of fertilizer "Part A" and 0.5 ml of F/2 "Part B" per gallon (3.785 liters) of water. The resulting media is termed F/2 due to the strength of fertilizer used. Make stronger or weaker fertilized solutions for various algae species or culture regimes.
3) **Note:** Alternatively a solution of equal proportions of A & B can be pre-mixed in a clean dispensing container and used at a rate of 1 ml of this premixed fertilizer per gallon of water. Premixed fertilizer is not shelf stable and is only good for 1 week.
4) Add sodium metasilicate to the stock media solution at the rate of 13 mg/liter if it will be used for growing diatoms.
5) Fill culture flasks with prepared stock media at the following rates:
    a. 6 ml in test tubes
    b. 60 ml in 125-ml flasks
    c. 600 ml in 1000-ml flasks
6) Place cotton plugs or foam plugs snugly in the top of each container.
7) Cover the cotton plugs and the upper neck of the flasks with a small square piece of aluminum foil. This will reduce evaporation on the shelf and provide an additional level of long-term sterility.
8) Load flasks into the autoclave for sterilization.



Adding nutrients to ongrowing algae cultures

Materials:

Spray bottle with isopropyl alcohol
Container of sodium metasilicate and small plastic measuring spoons
Dispensing bottles with F/2 fertilizer either:

a) one bottle each for part A and part B
b) one bottle of pre-mixed fertilizer at ratio of 1:1 (part A: part B)

Note: Nutrients can be added to small cultures after inoculation however there is a small chance for contamination. To lessen the risk of contamination for small cultures sterilize media with nutrients. Sterilizing media with nutrients will destroy the vitamins and some minerals; however the cultures should grow at acceptable rates. Alternatively nutrients can be added using sterile techniques to minimize contamination. Larger cultures require the addition of nutrients when new seawater is added to semi-continuous cultures or when starting batch cultures. When using F/2 algae fertilizing media, the parts can be pre-mixed 1:1 and added at twice the recommended rate or they can be added separately. Pre-mixed fertilizer has a short shelf-life and is only good for one week; use pre-mixed fertilizer for small cultures to reduce chance of contamination.

- Wash hands with antibacterial soap before working with algae cultures
- Spray isopropyl alcohol around the area that will be handled when entering the culture to be fertilized.
- Using dispensing bottles and sterile technique add ½- ml each of part A and part B fertilizer per gallon of water; or add 1 mL of pre-mixed fertilizer per gallon of water.
- Using a small plastic measuring spoon add 13 mg/liter of sodium metasilicate to the culture

Nutrient addition rates for various media volumes

Media Volume (gallons)	Media Volume (approx liters)	F/2 Part A (ml)	F/2 Part B (ml)	F/2 A/B Pre-mix	Silicate (mg) (g)	Silicate (tsp)
0.25	1	3 drops	3 drops	6 drops	Trace	Trace
0.5	2	6 drops	6 drops	12 drops	Trace	Trace
1	4	0.5	0.5	1.0	50 mg	Pinch
5 (carboy)	19	3	3	6	250 mg	Pinch
10	38	5	5	10	0.5 g	1/8
20	76	10	10	20	1.0 g	1/4
25	95	13	13	25	1.0 g	1/4
30	114	15	15	30	1.5 g	1/3
40	150	20	20	40	2.0 g	1/2
50 (kalwall)	190	25	25	50	2.5 g	1/2

Harvest kalwall algae using batch method

Materials: Live algae, multiple species if required, harvester, container for harvest

Process:

1) Assemble tools and materials. Wash hands.
2) Remove harvester from chlorine bath. Attach the harvester to the kalwall.
3) Spray all contact areas with isopropyl alcohol.
4) Place harvester outlet into the algae transfer sump.
5) Place the hose from transfer sump into the container that is receiving the contents of the kalwall. Open valve and begin draining kalwall into the transfer sump.
6) Turn on the transfer pump located in the sump. Adjust the valve so that algae culture is not overflowing transfer sump.
7) Once the kalwall is empty close kalwall drain valve.
8) Using freshwater hose turn water into transfer sump, move feeding hose to drain and rinse out the algae transfer system.

Harvest kalwall algae using semi-continuous culture method

Materials: Live algae, multiple species if needed, harvester, container to hold algae for feeding or tanks with larvae

Process:

1.) Gather tools and Wash hands. Remove harvester from chlorine bath. Attach the harvester to the kalwall, being sure to spray all contact areas with isopropyl alcohol. Place harvester outlet into the algae transfer sump.
2.) Turn the valve, and begin draining kalwall into the transfer sump. Place the hose from transfer sump in the container that is receiving the contents of the kalwall.
3.) Turn on the transfer pump located in the sump. Adjust the valve so that algae culture is not overflowing transfer sump.
4.) Once the kalwall is drained to the half-full line close kalwall drain valve.
5) Refill the kalwall with sterilized media and add fertilizer

Restocking kalwalls

Materials: Alcohol spray bottle

1) Assemble materials and wash hands
2) Remove storage kalwall lid and test for Chlorine using OTO
3) If chlorine is present, use sodium thiosulfate to dechlorinate the water, this should be added in an amount equal to the amount of chlorine used to sterilize the medium
4) Spray the lid with alcohol and replace
5) Attach pump unit to the media storage kalwall
6) Check to insure that the hose is shut off at the outlet end
7) Open valve on storage kalwall and open the outlet end of hose. Allow to drain for 30 seconds, this will flush out any chlorinated water in the hose. Close hose
8) Open the kalwall to be filled, spray area to be handled with alcohol
9) Spray the fill hose end with alcohol, open the outlet end and place in the kalwall
10) Turn on pump and fill kalwall
11) Watch the storage tank to ensure it does not run out of medium as this will mean the pump will run dry, this is a convenient time t o have a partner to watch the storage tank while you watch the tank being filled
12) Turn off the pump when the desired level is reached in the kalwall being filled.
13) Fill the kalwall to within 4 inches of the top
14) Add fertilizer and silicone as needed, spray the exposed portion the lid
15) Remove the hose and replace the lid
16) Shut the valve on the storage kalwall
17) Using filtered water, UV is best, refill the storage kalwall and add 1 ml of chlorine per 4 liters added
18) Open the kalwall valve, open the hose outlet valve and allow to flow for 30 seconds
19) Shut the hose outlet valve, the water in the hose is now chlorinated water
20) Let the chlorinated water stand in both the hose and storage tank for 24 hours before dechlorinating and repeating the process.

Steam Sterilizer Operation

Note: Before using sterilizer remove the holding tray and insure that there is water in the Autoclave. Use only deionized, RO (reverse osmosis) or distilled water in the sterilizer. Fill sterilizer until the water level is ¼-inch below the bottom of the wire rack.

1) Preheat by turning the sterilizer on and note the red indicator light preheat cycle requires about 15 minutes. Turn the temperature knob until the pointer is positioned at the black mark (approximately at the two o'clock position on the dial).
2) While sterilizer is preheating, load holding tray with items to be sterilized including prepared media in flasks, test tubes or pipettes wrapped in aluminum foil. Mark all items with autoclave indicator tape. Insure items are securely balanced and not stacked on top of each other. Also insure that all items are autoclavable. If you are not certain, test a small amount of the material before running a full load.
3) Flip pressure exhaust valve on the lid to the vertical (open) position.
4) Place the lid on the sterilizer by inserting the flexible vent tube into the tube guard on the side of the holding tray and lowering the lid into place. Align lid with fastening brackets with a slight clockwise twist.
5) Position the clamps and tighten the wing nuts, two at a time until they are snug. Do not over tighten clamps. When clamps are snug, retighten with an additional ¼-turn by turning the two wing nuts diagonally across the lid from each other slowly and evenly.
6) Insure that the dial is set to 1100 watts and flip the pressure exhaust valve to the horizontal (closed) position.
7) Allow sterilizer to heat up and observe pressure gauge. When pressure reaches 250-degrees Fahrenheit (121 deg Celsius) or 15-psi start the timer for 30 minutes.
8) When the sterilizer has run for 30 minutes, turn off the switch and note that the indicator light turns off.
9) Allow sterilizer to cool until the pressure is near zero. Flip the pressure exhaust valve to vent excess pressure. Do not vent until pressure is below 5 psi.
10) Loosen the wings nuts slowly and flip all clamps off the lid. Turn the lid slightly counter clockwise to unlock lid and remove lid from pot. Position sterilizer lid to the side on the counter top. Be careful when placing the lid if it is hot.
11) Note that the autoclave indicator tape has been activated. Remove items from sterilizer with insulated gloves or hot pot holder. Place sterilized items in an appropriate location so they will not be confused with non-sterile items.

Using the Yamato SM510 Sterilizer

- Prepare items for autoclaving.

- Wrap or cover with aluminum foil and tag with autoclave indicator tape.
- Open the front door of the autoclave and remove the book bottle.

- Attach drain hose to the valve on the front bottom of autoclave. Drain hose is stored on the left side of the autoclave.
- Open valve and drain out water; Close valve when water drains out and return coiled drain hose to hook on side of autoclave.
- Fill the book bottle with 3.5 liters of reverse osmosis or distilled water.
- Pour about 2.0 liters of water from book bottle into autoclave chamber. The water level should cover the heating coils and should be between the high- and low-water notches on the solid bottom plate. Do not cover the plate in the bottom of the sterilizer.

- Return the book bottle to its position in the front of the autoclave and insert hose with muffler.
- Insure that the strainer at the end of the hose is clean. Push bottle up on to the stopper.
- Close the front door.
- Load wire baskets with items to be sterilized. The autoclave will hold two baskets.

- Position items wrapped in tin foil so that water runs off them (flap turned towards the bottom of tank).
- Place baskets into the autoclave. Do not block exhaust port (hole at the top and back of the chamber).

- Close the top lid and turn clockwise 1/4-1/2 turn after contact until snug. Do not over tighten.

- Set the steam saucer cup with magnet on right side under the steam release vent.

- Turn the switch on (left side). Insure that the LED menu reads “sterilize and dry”, which is the second selection. Chose this selection while the display is blinking.
- Press “Enter” and insure that the temperature begins to rise.
- The autoclave is programmed to heat to 120 degrees Celsius and hold that temperature for 30 minutes; drying is set for 150 degrees Celsius for 30 minutes.
- The entire process takes about 1 hour 20 minutes and can be left to run the program, which will also shut down automatically.
- Refer to instruction manual for additional settings.

Installing Disk Filters

Materials: disk filters, 2 pieces rubber surgical tubing, isopropyl alcohol spray bottle

Steps:

1) Wash hands and spray an autoclaved disk filter with isopropyl alcohol on both connection ends.
2) Spray rubber tubing with isopropyl alcohol.
3) Attach surgical tubing to each end of disk filter.
4) Spray airlines with isopropyl alcohol.
5) Attach disk filter to airlines through the rubber surgical tubing.

Making cotton plugs

Materials: Cheese cloth or gauze, roll cotton or cotton balls, cotton string, test tubes and flasks of the sizes for which you are making plugs

1) Cut a piece of cheesecloth or gauze into a square whose sides are three times longer than the diameter of the flask or test tube for which you are making the stopper.

2) Make the "Okay" sign with your non-dominant hand and push the cloth into it.

3) Push pieces of cotton or cotton balls into the gauze until the resulting plug is packed and large enough that it fits snugly in the flask.

4) Place the plug into the flask and assure that the stopper is sufficiently large and fits tightly enough by lifting the flask by the plug.

5) Gather up the edges of the gauze and wind tightly with several wraps of cotton string at the point where the cotton filler ends. Finish with a knot or two half hitches to prevent unraveling. Trim gauze ends only slightly as the loose gauze is needed to grasp when performing sterile technique.

Making a three stem Algae Culture Apparatus

The purpose of this apparatus is to increase algal growth rates with improved gas exchange. One tube is used for air supply, another for venting and the third for harvesting algae. Harvesting is accomplished by closing the air vent and opening the harvest tube.

Materials: glass tubing, three-hole stopper, file or glass cutter, tubing which can be autoclaved

1) Assemble materials
2) Using a small triangular file, score the glass tubing at incremental lengths as follows: Cut three lengths of glass tube, a) two tubes which extend from the stopper to the bottom of the culture vessel plus 2 inches (one will be the aeration tube, the other will be the harvest tube, b) one tube that is 3 inches long, this will be the vent tube.
3) Using Vaseline as a lubricant and a rag to protect your hand in case of tube breakage, push the tubes through the stopper. End so that each tube shows a different length above the stopper; the 3-inch tube extends ½-inch above the stopper and the other tubes extend 1-inch and 1 ½-inch above the stopper.
4) The tube which ends closest to the stopper is the vent, the next longest is the harvest tube and the longest is the air supply tube. This will facilitate identification of the tubes.
5) Insert the finished stopper into the culture vessel to insure that the tubes are correctly fit.
6) Cut airline tubing into lengths: a) 3 inches for the air supply, b) 6 inches for the harvest line, c) 2 inches for the vent.
7) Place the flexible airline tubing over the ends of the respective glass tubing
8) The three-stem apparatus is finished.

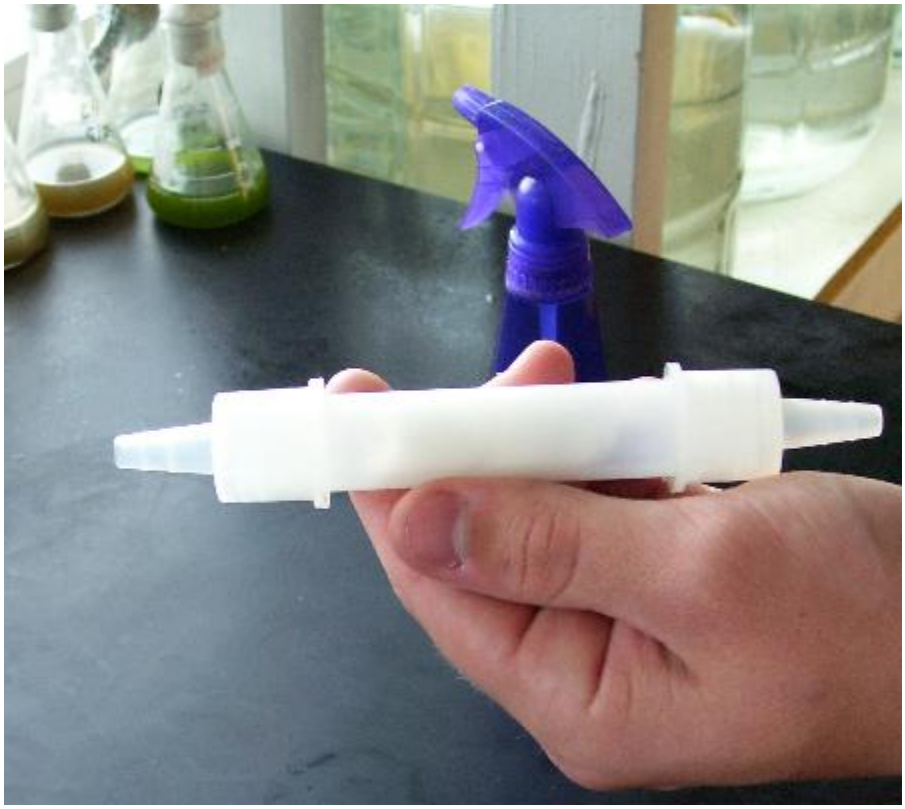
Note: when inserting glass tubing exercise care, hold tubing with gloves or a rag close the stopper to avoid break tube and harming yourself. Do not bend flexible airline tubing as it may seal together if bent in the autoclave.

Making Tube Filters

Materials: hard plastic tube between 3-5” long, two tapered end caps, airline tubing, isopropyl alcohol spray bottle, cotton balls, air source (with on/off valve)

Process of making tube filters:

1) Gather required tools and wash hands.
2) Spray all pieces that are needed with isopropyl alcohol, with the exception of the cotton balls.
3) Place two cotton balls in each end of the hard plastic tube, leaving the space in the middle of the tube empty.
4) Put the tube ends on the hard plastic tube.
5) Attach the required amount of airline hose to reach the sample you are going to aerate.
6) Attach one end of the airline hose to your air source, and turn the valve on.



Cleaning tube filters

Materials: Isopropyl Alcohol spray bottle, tube filter, cotton balls

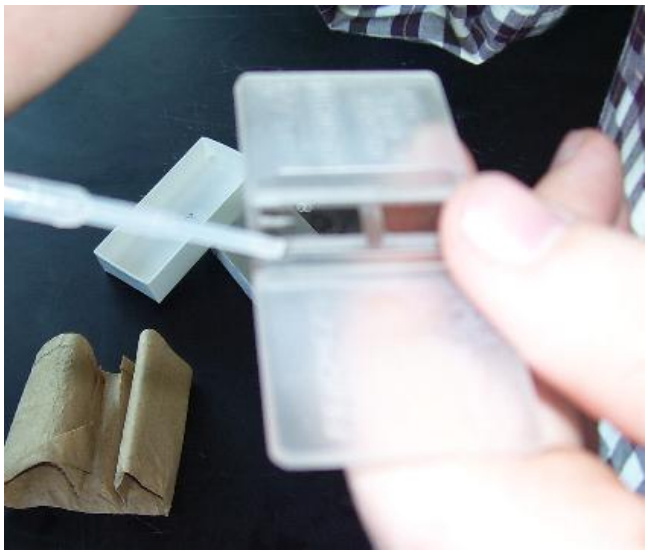
Cleaning tube filters:

1) Spray the entire area were the tube filter that needs to be cleaned is located
2) Disconnect the tube filter from the air source, as well as the airline hose.
3) Remove both of the tapered tube ends from the hard plastic tube.
4) Using a pencil or small rod, remove the cotton balls by pushing them through the tube and discard the used cotton balls.
5) Spray all parts with isopropyl alcohol
6) Add two new cotton balls to each end of the hard tube filter
7) Put the clean tapered tube ends back onto the hard plastic filter tube
8) Re-attach the airline hoses
9) Turn the air back on.

Counting algae with a hemacytometer

Materials: Sterile pipette, alcohol or Bunsen burner, tally counter, Lugol's solution and test tube, sample of algae obtained aseptically, compound microscope, hemacytometer and proper cover slip

- Locate the algae culture to be counted. Using sterile technique draw a 1-ml sample of the culture into the pipette (see protocol for aseptic technique). If you are counting motile species (or very small species) place twenty drops of algae culture in a test tube and add 2 drops of Lugol's solution. This will dilute the sample by $1/10^{th}$, which can be factored in later (multiply by the results by 1.1). Mix the sample well before counting.
- Place a clean cover slip on the hemacytometer.
- Gently place a drop of the sample from the pipette into each V-shaped channel of the hemacytometer (one on each side). The liquid will be drawn under the cover slip by capillary action. Wait five minutes for algae to settle.



- The hemacytometer grid consists of a 1-mm x 1-mm grid that is subdivided into twenty-five smaller boxes that each contains sixteen squares. As a result the entire grid has 25 boxes and 400 tiny squares (see diagram).
- Place the counting slide under the microscope at low power and find the grid. Increase magnification until the algae is clearly visible.
- For medium dense cultures randomly count all the algae cells in five of the boxes and multiply by five. Use a tally counter to assist in counting. Depending on the size of the algae and the density of the culture count enough boxes at random until you have counted at least 200-400 cells.
- For less dense count all the algae in the entire 1 sq. cm grid (25 squares) and the number may not reach 200.
- For denser cultures randomly count the algae in 25 small squares and multiply by 16.

- For extremely dense cultures dilute them by putting twenty drops of saltwater into a test tube and two drops of dense algae culture. Substitute two drops of Lugol's solution for two drops of saltwater if you are working with motile algae or if the cells are extremely small. Lugol's solution stains the cells and makes them easier to count. Mix thoroughly and count this diluted sample. Remember to factor in this 10:1 ratio when calculating the culture density (multiply results by 10).
- When counting cells that are directly on a line of the square border only count the cells on the lines that border the top and right sides of the square; do not count the cells that are on the lines of the left and bottom sides. This will insure an unbiased average count per square.

- Multiply the results appropriately to obtain a count for the entire grid. For example, if five boxes are counted multiply by five since there are 25 boxes; if the culture is very dense and you count only 20 small squares then multiply by twenty since there are 400 small squares.
- Repeat for the second grid on the hemacytometer. Average the two numbers.
- The cover slip floats on the liquid at a depth of exactly 0.1 mm resulting in a volume of 0.1 cubic millimeters. To obtain the culture density as number of cells per ml multiply the average count by 10,000 or (10^4) to determine the density. Remember to factor in any dilutions that were made in the counting process.
- Record results in the lab notebook.
- Rinse the hemacytometer with fresh water and wipe dry.
- Replace hemacytometer in protective case and return to storage drawer.

Measuring Algae Concentrations through Spectrophotometry

Programs developed for *Tetraselmis, T. isochrysis, C. isochrysis, Nannochloropsis,* and "Ply." (courtesy of Jordan Ridgeway)

When using the Aquamate, it is important to choose the appropriate wavelength. It is necessary to find the wavelength that is absorbed best and most regularly by the algae. The first step is to perform a "scanning" or measuring the absorbance of the sample at all wavelengths within a spectrum. The proper wavelength should be chosen from a regular and reproducible curve created by plotting absorbance vs. wavelength. By identifying and applying this wavelength, this assures that the measurements are more precise and are measuring algae as opposed to sediment or other foreign particles.

Scanning: Absorbance vs. Wavelength (fixed concentration)

The scanning range is 200-700 nm, within the visible spectrum. The visible spectrum ranges from approximately 260 nm to 720 nm. Most cultures peak at 670 - 705 nm.

Because absorbance is directly proportional to concentration when only the algae significantly absorbs the appropriate wavelength, a protocol can be made by establishing a linear fit of standard dilutions. After the appropriate wavelength is identified, concentrations of the starting sample are measured and serial dilutions prepared from that sample. Because the concentrations have been measured and then calculated for the serial dilutions, the absorbance of each dilution is plotted against its concentration. Therefore, the linear curve fit is a plot of concentration vs. absorbance at the appropriate wavelength.

Measure concentrations of starting samples with a hemacytometer using a minimum of 100 repeated samples of 0.0001 ml each. Calculate concentrations of serial dilutions.

The Linear Curve Fit: Absorbance vs. Concentration (fixed wavelength)

It is important to note the coefficient of the curve fit. The coefficient describes how linear the relationship is. The closer the coefficient is to 1.0, the more accurate the line because absorbance should be directly proportional to concentration.

Once this linear curve fit is created, the program is set. Now it is possible to measure a sample of unknown concentration and the Aquamate will compare the absorbance to the curve fit to give a concentration value.

Measuring concentration of Algae culture.

Note: When the Aquamate is on, the lid should be closed at all times except when inserting/removing cuvettes. This can damage the detector. Always make sure that the lid is completely closed when taking measurements. And insure that the Aquamate is turned off when not in use as the light bulb is expensive.

1) Obtain your sample
   a) It's important to properly stir the sample to get the proper concentration. This is especially important for non-motile species, but important for every culture.
   b) Only about 3 mL is needed.
   c) Sterile Collection Techniques should be used.
2) Prepare the cuvette
   a) Inspect the cuvette for scratches, smudges or other imperfections. Flat/clear sides are the only sides that should make a difference. If there are any imperfections that cannot be removed, then choose another cuvette. Kimwipes are useful because they don't leave lint on the surface.
   b) Transfer your sample to the cuvette. ~3 mL should be sufficient. Make Sure to stir the sample before and after transfer. This is easily done by squeezing the bulb of your pipette several times. Squeeze in the corners of the cuvette and sample holder.
   c) Wipe down the cuvette with a Kimwipe.
3) Prepare the Aquamate
   a) Turn it on by flipping the switch on the back and allow to boot. Usually takes a minute or two.
   b) Select "LIBRARY"
   c) Select the program for your species of algae.
   d) Zero the Aquamate: Prepare a clean cuvette with distilled water and press "ZERO."
   e) Measure your sample.
      i) Make sure that your sample is stirred.
      ii) Press "RUN."
      iii) The concentration and absorbance values should be displayed
      iv) Multiple samples can be measured.
4) Shutting down the Aquamate
   a) Press "HOME" and wait for menu to appear.
   b) Turn off the by flipping the switch.

De-chlorination

Materials: Sodium thiosulfate, OTO test solution, isopropyl alcohol

Process for de-chlorinating:

1) Spray the opening or lid of the item that needs to be de-chlorinated with isopropyl alcohol
2) Put 1 drop of OTO test solution in the chlorinated media.
3) If the water turns yellow, orange, or red, add 1 ml of sodium thiosulfate for every 1 ml of bleach that was added to water.
4) Allow a few minutes for the sodium thiosulfate to mix.
5) Recheck with the OTO test again to be sure it has been fully de-chlorinated.
6) If the OTO produces a yellow color add 10 ml sodium thiosulfate.
7) Repeat until the OTO produces no color change.

Algae Culture Record Sheet

Date	Note the conditions of individual cultures, growth rates, bacteria/protozoan tests, crashes.

Section B: Broodstock Protocols

1) Broodstock system setup
2) How to start a Biofilter
3) Temperature maintenance on broodstock
4) Cleaning incoming oyster broodstock
5) Feeding instant algae
6) Feeding live algae
7) Maintenance Cleaning of Broodstock
8) Cleaning broodstock system
9) Broodstock record sheet

Broodstock System Setup

A broodstock room should have the following basic components

1) Aeration and water for the holding system
2) Large volume system for hold the broodstock and conditioning them
3) A temperature control to keep the system cold
4) Filtered water
5) Floor drain for cleaning the system
6) A portable sump for feeding and pump to feed from this sump to the system

System operation:

1) Shallow, 4-6 inch, raceway to hold oysters in mesh trays
2) Distinct groups of oysters should be housed in marked trays to distinguish broodstock
3) Preferably separate different stocks to discrete holding systems
4) The system should have a large sump to facilitate volume
5) Temperature should be set to a mild temperature (18-20 degrees C) to prevent accidental spawning
6) The pump for feeding is used to create a steady supply of food; the ideal food concentration is 100,000 cells/ml. This can be provided with either live algae or instant algae. When feeding add algae to the system directly and then set the algal sump to a speed which replaces the amount being consumed

Operating Advice:

1) Clean and quarantine all oysters to prevent disease transfer.
2) Clean the system and oysters weekly

How to start a biofilter

Materials: source of filtered sea water, bio media such as bio-balls, bio-barrels, bio-films, bio-sponge, ammonium chloride

1) Fill the tank with filtered sea water
2) Add bio-media for bacterial substrate
3) Calculate the volume of the system
4) Add ammonium chloride to bring the ammonia level to 4 mg/liter
5) Monitor the TAN, nitrite and nitrate levels of the system for two weeks
6) When nitrite and nitrate are at detectable levels and the ammonia level is reduced below 0.5 ppm the Biofilter is active
7) To speed the process put in bio-media from an active recirculation system.

Temperature Maintenance for brood stock oysters

Materials: brood stock conditioning system, brood stock oysters, heater/chiller, thermometer

Process for temperature maintenance for brood stock oysters:

1) Check the temperature in the brood stock system. The temperature should be around 18-20 C. Oyster brood stock develop eggs and sperm at constant temperatures and with provided algae. Warmer temperatures increase the rate of gamete development and cooler temperatures require longer development times.
2) Add in a heater or chiller to the system if necessary to lower or raise the temperature to the desired level.
3) The temperature should be checked daily and chilling systems maintained to insure there aren't any sudden large changes, which can trigger spawning at undesired times.

Cleaning incoming oyster broodstock

Materials: hard bristle brush, paint chipper, gloves, 5-gallon bucket, quarantine container, bleach.

Process:

1) Remove any hard fouling (worms, barnacles, muscles, other oysters, etc.) on oysters with paint chipper while wearing gloves.
2) Take a hard bristle brush and scrub oysters to remove any soft fouling (sea grass, mud, bryozoans, etc.) and rinse in a 5 gallon bucket filled with freshwater.
3) Fill the quarantine container with freshwater and 5ml of bleach.
4) Place the oysters in the quarantine container for 15 minutes.
5) If there are boring sponges present, place into direct sunlight for a few hours.
6) Rinse out the quarantine container and fill with saltwater.
7) Change the salinity so that it is between the salinity of the broodstock system and the water the oysters came from.
8) Add a small amount of instant or live algae and place the oysters in the container overnight.
9) Add the oysters to the broodstock system.
10) Watch new incoming broodstock for presence of spawning OR
11) Isolate incoming broodstock in separate containers until temperature is lowered and stabilized at 18-20 deg-C for several hours to insure no inadvertent spawning, then transfer new oysters to conditioning system

Feeding algae paste to broodstock oysters

Materials: algae paste, 1-liter beaker, peristaltic pump apparatus, 5-gallon bucket

Process for bulk feeding:

1) Read label found on bottle to determine density of cells in algae paste.
2) Calculate the volume of broodstock holding system including all tanks and trays.
3) Calculate the volume of instant algae in milliliters required to bring the cell count of the broodstock system to a desirable level (see chart).
4) Dispense the required amount of algae paste into a 1-liter beaker, add seawater and mix thoroughly.
5) Turn off the system circulation pump and distribute the algae mixture across the top of all broodstock trays. Insure that airstones are delivering air to tanks.
6) Re-fill 1-liter beaker with water from the system and wash out container back into the system until the container is clean.
7) After one to two hours have elapsed turn on the system circulation pump.

Process for distributed feeding:

a) Follow steps 1-4 above.
b) Fill 5-gallon bucket with filtered seawater
c) Add 1-liter of re-suspended algae paste mixture
d) Put an air stone in the bucket
e) Turn on peristaltic pump apparatus with suction in the bucket of algae mixture and the discharge directed to the system circulation pump suction
f) Calculate the required amount of algae mixture, compare that with the length of time for feeding and divide by the flow rate of the peristaltic pump. Adjust the pumping delivery time with an automatic controller.

Feeding live algae to brood stock

Materials: Live algae, multiple species if needed, harvester, container to hold algae for feeding or tanks with larvae

Process:

1) Follow the protocol for harvesting algae from kalwall.
2) After drawing the required amount of live algae move the bucket to the broodstock feeding container or larvae tank and pour contents in slowly to avoid splashing. Distribute the algae over all broodstock trays.
3) Repeat steps 1-3 for multiple species of algae if possible.
4) Refill algae kalwalls with sterilized seawater media. Follow the protocol for refilling and inoculating kalwalls.
5) Alternatively, for a timed release of algae, place the bucket of algae over the broodstock tank and start an airline siphon from the bucket to the broodstock tank. The algae will be dispensed over a longer period of time rather than pulsed into the tank.
6) If the conditioning system is outfitted with a protein skimmer it may be necessary to turn off the circulation pump and feed algae, then restart the circulation after broodstock have completed feeding.

Maintenance Cleaning of Broodstock:

Materials: water hose

1) Remove tray of oysters and place above the drain.
2) Spray them down with fresh water
3) Flip them over and spray again
4) Spray tray down as well and
5) Turn the oyster over so the lip is on top
6) Do this for all the trays in the system.

Optional:

7) The pump for the system is turned off
8) The drain lines of the system are routed to the room drain and one of the trays is drained.
9) This tray is then cleaned with brush and fresh water
10) The fresh water is then drained
11) Return the drains to the sump and turn to pump back on
12) Return the oysters to the system

Cleaning the Brood Stock System

Materials: 3-5ft. section of drain hose, small scrub brush, siphon hose, freshwater hose

Steps:

1) Unplug submersible pump.
2) Connect drain hose from raceway to a piece of hose long enough to reach the floor drain.
3) Disconnect standpipe and allow all water to drain out of raceways.
4) Remove trays containing oysters and spray with freshwater.
5) Scrub sides of raceways with a scrub brush.
6) Wash out raceways with freshwater hose.
7) Siphon out water in both sump tanks.
8) Replace standpipes in raceways.
9) Fill system with filter/U.V. saltwater.
10) Turn on submersible pump.
11) Check salinity and adjust if needed.

Broodstock Record Sheet

Date	Temp	Salinity	Feed		Water Quality/System/Broodstock Condition Notes
			Qty	Type	

Section C: Conduct Spawning Protocols

1) Spawning with thermal fluctuation
2) Identifying oyster spawns
3) Strip spawning
4) Counting sperm on eggs
5) Counting oyster eggs
6) Hydrogen peroxide induced spawn
7) Serotonin induced spawn

Spawning with thermal fluctuation

Materials: shallow tray (wood, fiberglass or plastic) with drain and overflow standpipe, 1000-watt submersible heater with thermostat, 30-gallon plastic drum of filtered/U.V. seawater, 5-gallon bucket, floating thermometers

Note: Black color for spawning tray allows easier observation of spawning activity

Spawning with thermal fluctuation

1) Clean spawning tray with brush and warm soapy water. Rinse thoroughly.
2) Fill drum with filtered and U/V treated seawater
3) Place submersible heater in drum, set thermostat for 28^{o} C. Heat water.
4) When heated, use 5-gallon bucket and fill tray with 28^{o} C water.
5) Place broodstock oysters from conditioning system into spawning tray.
6) Place thermometers in tray to monitor water temperature. Observe for spawning activity.
7) If spawning does not occur add ice to reduce temperature in spawning tray to 20^{o} C. Wait 30 minutes. Drain tray and repeat steps 4 and 6.
8) Continue thermal fluctuation for 6 hours.
9) If no spawning is observed replace broodstock in conditioning system.

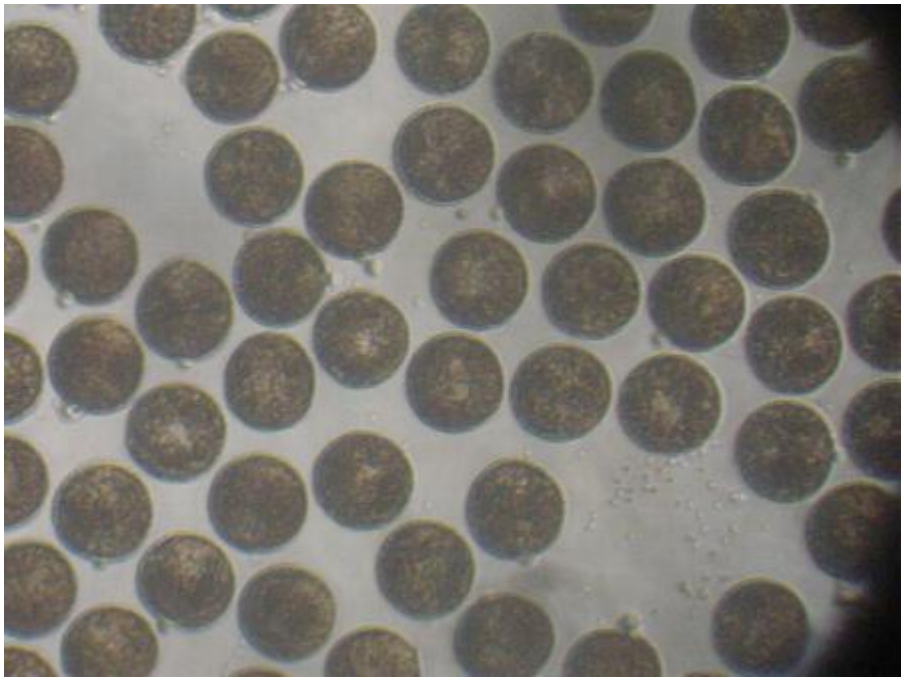
How to identify oyster spawn

Spawning is the broadcast of gametes, egg and sperm into the water column. You can observe a white or cream-colored cloudy stream exuding from the adult oysters. With experience one can tell the difference between male and female gametes without using the microscope. Males tend to have a spawn which is much less dense and whiter and rises to the surface. Females have a more yellow concentrated spawn which tends to sink. Strip spawning requires the identification of oyster sex before stripping the gametes.

Materials: water with gametes, pipette, microscope, cover slip

Identifying eggs and sperm:

1) Using a pipette take a sample of water from the area where spawn is observed.
2) Transfer sample to a microscope slide and cover with a glass cover slip.
3) Observe under low power, focus, and increase magnification to medium power.
4) Oyster eggs appear as rounded, oval, tear-drop or pear-shaped objects.
5) Sperm appear as tiny spots unless higher magnification is used. If sperm are motile they will appear to move around in the sample.



Strip spawning oysters

Materials: oyster knives, gloves, plastic buckets and pans, 20-micron and 100-micron sieves, microscope and slides, 5-liter pitcher, scalpels, bleach, filtered seawater, clean dry cloth rags.

1) Set up workspace on table with plenty of room to spread out.
2) Assemble materials. One pan with 1 ml bleach / liter of water and one with fresh rinse water.
3) Select broodstock oysters.
4) Open oysters by twisting oyster knife at hinge end to sever the ligament and pop oysters open. Slide knife under top shell to sever the muscle from the shell. Remove shell to clear tissue and then replace to avoid desiccation.
5) Clean knife and gloves in chlorinated water between each oyster to avoid cross contamination or fertilization. Dip in chlorinated pan then rinse water. Wipe with dry cloth.
6) Once all oysters have been opened proceed to the next step.
7) Using the tip of a scalpel collect a tiny amount of gonad from each oyster and transfer to microscope slide by barely touching it to the slide. Examine with microscope. Multiple samples can be put on a single slide but remember to keep them in order.
8) Identify the sex of each oyster and move males and females to separate areas.
9) Strip females first. Hold the oyster with the hinge end pointing downward. Slice the gonad and carefully scrape the tissue downward into a shallow container.

10) Commingle eggs from all females unless this is a paired-spawn.
11) Using the scalpels tease the female gonad tissue into very small pieces.
12) Transfer gonad tissue to a 5-liter graduated pitcher. Add 3-liters of filtered seawater. Using a plunger swirl rapidly and vigorously to dislodge eggs from the connective tissue.

13) Fill pitcher with water then decant through a 100-micron sieve and collect eggs on a 20-micron sieve leaving large chunks of tissue in pitcher. Thoroughly wash eggs on the sieve with filtered seawater.
14) Transfer eggs (backwash them with water from a pitcher or squirt bottle) from the sieve into a bucket of water with 10 liters of water and a slow air stone.
15) Hydrate eggs for 15 minutes.
16) While eggs are hydrating, strip male oysters into a pan, macerate tissue with 4 liters of clean seawater and decant through a 20-micron sieve leaving chunks behind. Collect the sperm suspension that passes through the sieve.

17) Add 100 milliliters of sperm suspension to the bucket with eggs and agitate with plunger.
18) Examine eggs with microscope for presence of sperm.
19) If no sperm are visible on the eggs add more sperm suspension.
20) Repeat until there are 2-3 visible sperm surrounding each egg.
21) When eggs show signs of dividing transfer them to the hatching tank

Counting sperm on oyster eggs

Materials: Microscope, sperm infused oyster eggs, microscope slide, cover slip, pipette

Process of looking for sperm:

1) Load a sample of the oyster eggs and sperm suspension onto the microscope slide using a pipette.
2) Find an oyster egg on the slide under the microscope, and look closely for the tiny sperm (transparent dots) that attach to the outer surface of the egg.
3) Count the number of sperm transparent dots that are surrounding the eggs.
4) A desirable sperm-to-egg ratio is roughly 3 visible sperm per egg.
5) If no sperm are present, or if there are fewer than 3 visible sperm per egg, add more sperm suspension to the eggs.
6) If there are far too many sperm per egg immediately pour the egg/sperm suspension through a 20-micron sieve and wash with filtered seawater.
7) Return the collected eggs to a clean bucket and fill with filtered seawater.
8) Re-examine the eggs and count sperm.

Counting oyster eggs

Materials: Microscope, Sedgwick-Rafter or home-made counting slide (see protocol for making a counting slide), 5-gallon graduated harvest bucket with collected oyster eggs, three clean 1-ml pipettes, plunger, and tally counter

Procedure for counting eggs:

1) Assemble materials
2) Place collected oyster eggs in a clean graduated harvest bucket
3) Add filtered seawater to the bucket and bring level up to 20-liters
4) Stir with the plunger by slowly raising and lowering plunger until thoroughly mixed and eggs have been brought up off of the bottom of the bucket.
5) Take three 1-ml samples using the pipettes. Obtain the samples while gently mixing with the plunger.
6) After all three samples have been taken discontinue stirring the bucket. Load the samples onto a Sedgwick-Rafter counting slide, which contains a grid to aid counting.
7) Using the tally counter and low power magnification, scan the slide from side-to-side in a "lawnmower" fashion and count the number of oyster eggs in each sample. Average the counts for the three samples and enter in the following formula:
   Average number of eggs counted X 20,000 milliliters = total number of eggs
8) Record the number of eggs and the date spawned on the larvae tank record sheet.

Note: the goal is to have approximately 50-200 eggs per milliliter to count, if the number far exceeds this value, then retake the samples using 0.25-0.5 milliliters and dilute by adding water to reach the 1-ml sample size. Remember to factor in the dilution when calculating the number of eggs.

Induced Spawning Using Hydrogen Peroxide

From Research Paper
Title: **"**Hydrogen Peroxide Induces Spawning in Mollusks, with Activation of Prostaglandin Endoperoxide Synthetase"
Authors: Daniel E. Morse, Helen Duncan, Neal Hooker, Aileen Morse
Institute: Marine Science Institute and Department of Biological Sciences, University of California, Santa Barbara
Date: 1976

Materials: filtered seawater, small containers, Tris buffer, hydrogen peroxide

Procedure:

(from research paper) Add 6 grams of Tris to 1 liter of seawater in a graduated beaker and stir until solids go into solution. The addition of Tris is needed to raise the pH to 9.1 and to buffer the pH drop from addition of hydrogen peroxide. Check pH with calibrated instrument to ensure pH is 9.1. Add 28 ml of 3% hydrogen peroxide (standard household hydrogen peroxide) to the seawater solution. Stir gently for 30 seconds. Pour solution into 4 liters of filtered water in a spawning container and add mollusks. Spawning should start to occur within 4 hours (end citation).

This solution has been proven to induce spawning in abalone and certain other mollusks including *Donax variabilis*.

Protocol for serotonin-induced spawning

Serotonin is a molluscan reproductive hormone, which can be administered directly to ripe adult shellfish to induce spawning. This protocol is written (courtesy of Dan Speiser) for use with bay scallop (*Argopecten irradians*) but can also be used with other species.

Materials: serotonin, deionized water, 1-ml syringe with 18-gauge needle

1) Make 5 mM (millimolar) serotonin solution. Use serotonin hydrochloride and DI (deionized) water. Mix 1.06 grams of serotonin for every liter of water (106 mg per 100 ml). Keep the solution in the refrigerator once it is mixed.
2) Select ripe broodstock.
3) Inject scallops with serotonin. Remove scallops from water and wait until they gape. Then keep the valves open with your fingers. Do not force or pry the scallops open, as this can damage their valve hinge and strain or tear the adductor muscle. Use a syringe to inject about 1cc of solution into the adductor muscle. Alternatively the injection can be made into the gonad.
4) Observe for spawning activity. Place each animal in their own dish containing filtered sea water. It will take several minutes for spawning to begin. For most reliable results insure that scallops have ripe gonads with mature gametes.
5) Being hermaphrodites, bay scallops usually spawn the male gametes first. Then after a short hiatus of 20-30 minutes they begin spawning eggs.

Section D: Drain downs and Larviculture Protocols

1) Determining larvae size
2) Calibrating ocular micrometer
3) Determining sieve size
4) Alternative method measuring sizes
5) Counting Larval Oysters
6) Daily tank drain down
7) Cleaning larvae tanks
8) Restocking larvae
9) Larvae tank feeding
10) Larvae feeding chart
11) Harvesting eyed-larvae
12) Remote setting
13) Larvae tank record sheet

Determining larvae size

Materials: Microscope with graduated eye piece reticule (ocular micrometer), 35-micron cup sieve, pipette, microscope slide and cover slip, conversion table below

Note: Locate the tank records and determine the sieve size used for the last drain-down. Sieve sizes are chosen to allow for growth of larvae in culture. Following a normal two-day schedule for drain-downs allows time for oyster larvae to grow to the next sieve size. However, some batches grow faster or slower than others. Before increasing to the next larger sieve size, follow the protocol below for determining the size of larvae.

1) Swish the 35-micron cup sieve through the larvae tank by pulling it across the water surface to collect larvae. Use pipette to transfer larvae from cup to slide and place cover slip on the sample.
2) Observe objects being measured (larvae) under low magnification first and compare their size with the ocular micrometer scale. Increase to the highest magnification that the object being viewed is not larger than the scale of the ocular micrometer.
3) Using the table below measure length and width of the smallest and largest larvae.
4) Record the larval measurements on the tank record sheet.

Table of calibrations for ocular micrometer reticule in CCC microscope:

Objective	Color	Eyepiece	Total magnification	Ocular Micrometer Scale (microns / unit)
4X	Blue	10X	40X	25
10X	Green	10X	100X	10
40X	Yellow	10X	400X	2.5
100X	White	10X	1000X	1.0

For example: Under 10X magnification a larvae that measures 10 units long and 6 units wide is 100 microns long and 60 microns wide.

Note: Each microscope may have some variation from the above scale so calibrate the ocular micrometer for each microscope using a stage micrometer (see protocol). Label accordingly on the side of each microscope.

Calibrating ocular micrometer:
Place a stage micrometer on the microscope stage.
Observe theough low power (4X)
Simultaneously rotate the ocular micrometer and move the stage micrometer until the scales line up.

Calibrating the ocular micrometer

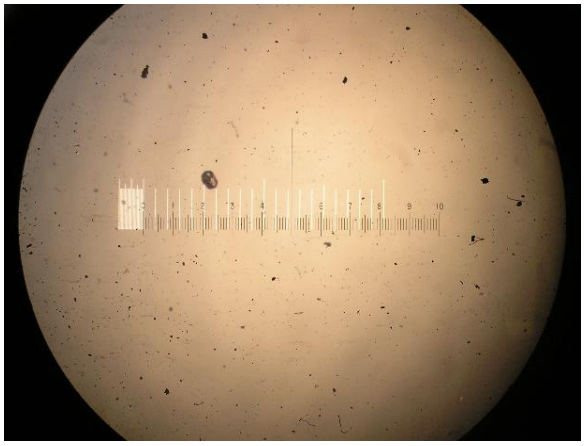
Materials: stage micrometer, microscope with ocular micrometer (graduated eyepiece reticule)

Procedure:

1) Place the stage micrometer on the microscope stage.
2) Observe under low power (4X).
3) Simultaneously rotate the ocular micrometer and manipulate the stage micrometer with the mechanical stage controls until the scales line up, side-by-side.
4) Adjust the positions until the zeros of both scales are perfectly aligned.
5) Observe the farthest measurement on the stage micrometer that perfectly aligns with a tick on the ocular micrometer. Record the measurement.
6) Determine the calibration for that magnification using the formula:

Stage micrometer measurement / Number of ocular micrometer units = microns/unit

7) Repeat procedure with each successively higher magnification objective.
8) Record the calibration on a sticky label and attach to the microscope body.



Typical calibration of ocular micrometer reticule:

Objective	Color	Eyepiece	Total magnification	Ocular Micrometer Scale (microns / unit)
4X	Blue	10X	40X	25
10X	Green	10X	100X	10
40X	Yellow	10X	400X	2.5
100X	White	10X	1000X	1.0

Note: Total magnification is found by multiplying the eyepiece magnification by the objective magnification to yield total magnification

Determining sieve size to use

Materials: Microscope with ocular micrometer reticule in eyepiece, 35-micron cup sieve, pipette, microscope slide and cover slip, and protocol for determining larvae size.

Note: Sieves are sized according to the square mesh measurement. The critical dimension for determining sieve size to use is the diagonal measurement, which determines what passes through the sieve. Using the Pythagorean Theorem the diagonal measurement can be determined, which is approximately 1.4 times the square mesh measurement.

Procedure:

1) Determine the size of larvae using the appropriate protocol.
2) Using the chart below, select the proper sieve size by matching the smallest dimension of the larvae with the diagonal measurement of the sieve:

Diagonal measurements and larvae sizes for various sieve sizes (rounded values):

Sieve size	Diagonal mesh measurement	Smallest larvae to sieve
20	28	30
35	50	60
55	78	80
64	91	100
75	106	110
100	141	150
125	177	180
150	212	220
180	255	260
200	283	290
225	318	320
250	352	350

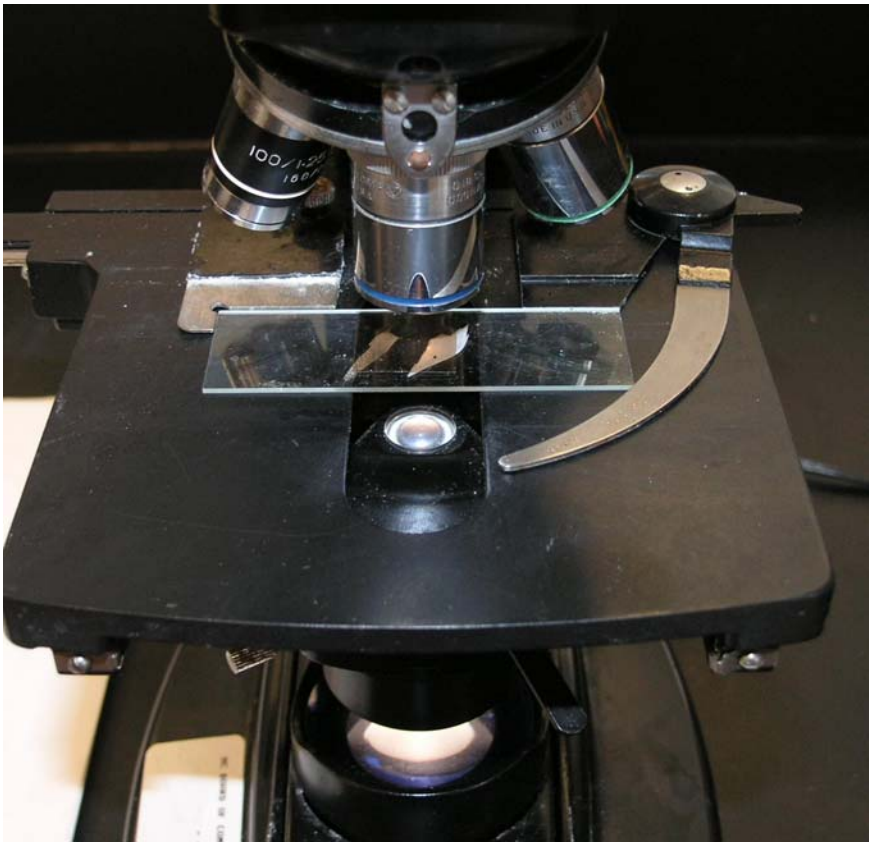
Alternative method for measuring sizes (if ocular micrometer is not available)

Materials: small (2-3 millimeter) squares of various sizes of sieve material, microscope, slides and cover slip

Procedure:

1) Place a sample of larvae on a microscope slide.
2) Using forceps place a small square of Nitex nylon mesh material into the sample containing larvae. Estimate the closest mesh size for larvae size based on your experience.
3) Place cover slip over both sample and sieve material.
4) Observe through microscope on low power first; increase magnification as desired.
5) Compare sizes of larvae and sieve material squares to estimate the size of larvae and to determine the most appropriate sieve size to retain the larvae scheduled for drain down.

Note: this method can also be used to roughly calibrate an ocular micrometer if a stage micrometer is not available. You may wish to make a permanent measuring slide by gluing several small squares of various size Nitex mesh to a microscope slide and gluing on a cover slip with clear hot glue or silicone caulk.



Counting Larval Oysters

Materials: Microscope, Sedgwick-Rafter counting slide or home-made counting slide, 5-gallon graduated harvest bucket with larvae from tank drain-down, three 1-ml clean pipettes, plunger, tally counter

Procedure:

1) Assemble materials
2) Using filtered seawater bring the graduated harvest bucket volume up to 20 liters.
3) Stir the harvest bucket with the plunger by slowly raising and lowering plunger until thoroughly mixed and larvae have been brought up off of the bottom of the bucket.
4) Take three 1-ml samples using the pipettes, and obtain the samples while mixing.
5) After all three samples have been taken; load them onto the Sedgwick-Rafter or home-made counting slide.
6) Count each sample. Average the three counts. Determine the number of larvae by using the following formula:

Average number of larvae counted X 20,000 milliliters = total number of larvae

7) Record the number of larvae on the tank record sheet

Note: try to have approximately 50-200 larvae per milliliter to count, if the number far exceeds this value, retake the samples using 0.25-0.5 milliliters and dilute by adding water to reach the 1-ml sample size. Remember to factor in the dilution when calculating the number of eggs.

Daily larvae tank drain-down

Drain downs are broken into 3 major steps (see appropriate protocols for each):

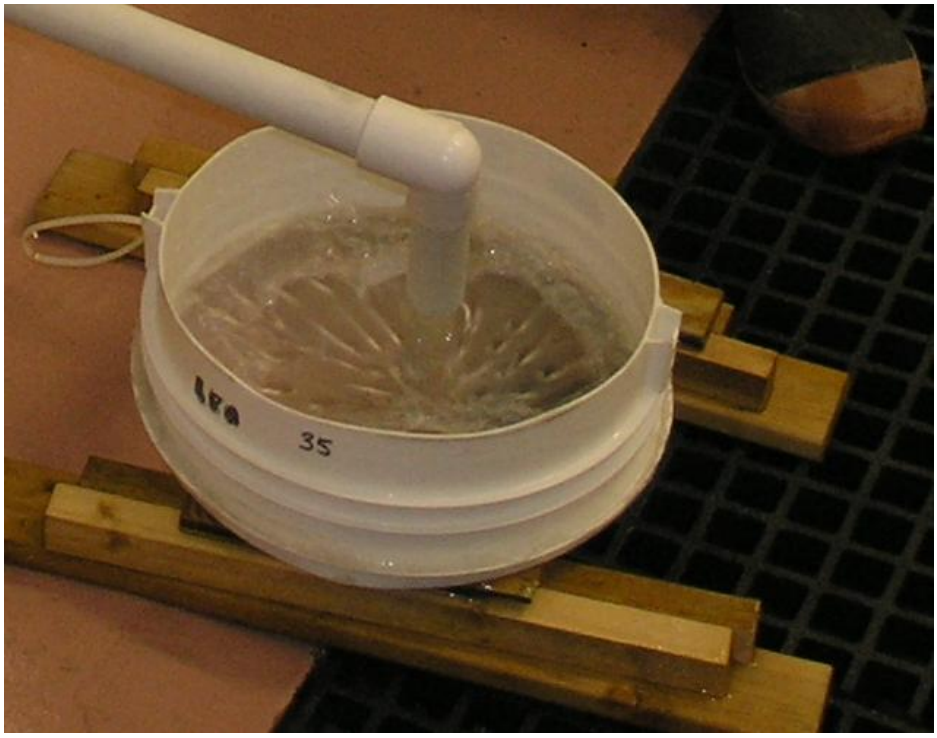
1) Determining the sieve size needed.
2) Draining down the tank and harvesting larvae.
3) Restocking the larvae.

Materials: sieves of the proper size for larvae to be drained (see appropriate protocol)

Note: Locate the tank records and determine the sieve size used for the last drain-down. Sieve sizes are chosen to allow for growth of larvae in culture. Following a normal two-day schedule for drain-downs allows time for oyster larvae to grow to the next sieve size. However, some batches grow faster or slower than others. Before increasing to the next larger sieve size, follow the protocol below for determining the size of larvae.

Draining larvae tanks:

1) Remove two of the appropriate sized sieves from the chlorine bath and rinse in fresh water.
2) Clean and prepare a 20-liter graduated harvest bucket by adding 10 liters of filtered water.
3) Adjust water temperature in the bucket to approximately that of the larvae cultures. Prepare saltwater spray hose and spray bottles by filling with filtered seawater.
4) Place the sieve under the drain and open valve to begin draining larvae tank. Insure that the waste water is directed to the drain.



5) Submerge sieve in an overflow pan if desired or elevate sieve with wooden blocks to allow better drainage.
6) Attend draining and observe sieve for over flowing especially when using smaller sieve sizes.

7) When the sieve begins to fill up and flow rate through it slows down, the sieve can be sharply smacked with a short piece of PVC pipe and it will “unclog”. As clogging becomes more frequent (usually only a problem for smaller sieve sizes) exchange with the alternate sieve.
8) Wash the larvae on the sieve using a gentle stream of filtered sea water from the supply hose or use a spray bottle. While washing them direct the larvae toward one side of the sieve.
9) Move the sieve over the graduated harvest bucket and invert. Backwash the larvae from the sieve into the harvest bucket. Put an air stone into the harvest bucket to keep collected larvae suspended and aerated.
10) Repeat steps 5-7 until the tank is completely drained.
11) Unless larvae are close to setting do not wash contents from the tank bottom into the sieve. The bottom sediments contain accumulated waste and dead larvae, which can be discarded.
12) After harvesting larvae, count them and enter on tank record sheet.
13) When a new tank is ready follow the protocol for restocking larvae.

Note: Larvae in early stages appear pink in color and collect in one area of the sieve, algal particles and other debris appear brown or green and stay suspended in the sieve while larvae generally sink.

Cleaning larvae tanks

Materials: Scrub brush, 5-gallon bucket, Alconox soap, bleach, fresh water hose

Procedure:

1) Larvae tanks must be cleaned while they are still wet. Within ten minutes of drain-down, rinse the inside of the tank with fresh water to remove loose particles off and drain them off.
2) Prepare a cleaning solution bucket with 4 gallons of fresh water, 5 ml bleach and 1 tsp soap.
3) Scrub the tank thoroughly with the cleaning solution.
4) Rinse the cleaning solution from the tank with a fresh water sprayer hose.
5) Tank may be refilled immediately or allowed to dry and refilled as needed.

Restocking Larvae

Materials: Tank to receive the larvae filled with filtered seawater, harvest bucket of larvae to be stocked, gentle spraying filtered seawater hose, over-sized sieve

Note: larvae may be restocked into a tank that has not been filled yet and the tank can continue to be filled if the temperature of incoming filtered water is close to that of the larvae tank.
Note also: the density of larvae in tanks varies with the stage of development (see chart below).

(from Hadley, N., A Manual for the Culture of the Hard Clam, *Mercenaria*, in South Carolina.)

Stage of development	Stocking density
Zygotes	20-30 / milliliter
Early veliger	5-10 / milliliter
Late veliger	1-2 / milliliter

Restocking larvae:

1) Move the container of larvae to the destination tank.
2) Select a sieve that is barely large enough to allow all larvae to pass through. This is intended to prevent unwanted debris from entering the new tank.
3) Position the sieve over the receiving tank.
4) Gently pour the contents of the larvae harvest bucket through the sieve and into the tank.
5) Rinse the bucket with the filtered seawater hose and pour through the sieve.
6) Add algae to the tank to feed the larvae.
7) Replace sieves in chlorine bath
8) Record date, number and size of larvae and other information on tank record sheet.

Larvae tank feeding

Materials: Live algae, tank with larvae, clean 5-gallon bucket

Note: Larvae can be brought through larval phase to competency on a diet of Isochrysis. However, it is thought that a diet, which includes multiple species, gives better nutrition and produces healthier larvae. Algal foods include single-celled green algae, golden/brown algae, and diatoms. The most important nutritional quality of algae is the amount of lipid they contain.

Principles of feeding larval shellfish:

a) Provide the proper size of food for the size of larvae being fed.
b) Determine the amount of algae required for the larvae and an extra amount for background.
c) Calculate the amount of algae to harvest and feed.
d) Monitor the algae density to determine frequency of future feed additions and to maintain the required density of algae in the larvae tank.
e) Periodically examine larvae with microscope to verify that the gut is full.

Feeding at calculated rates:

1) Determine larvae size / stage.
2) Determine proper algae species to feed (see protocol for larvae feeding chart).
3) From chart, determine the algae cell density required.
4) Determine the density of the algae cultures to be harvested.
5) Determine larvae tank volume in milliliters.
6) Calculate the amount of algae culture to harvest and feed by using the formula:

Algal feeding density X Tank volume / algae culture cell density = volume of algae to feed

6) Harvest the calculated volume of algae and deliver to the larvae tank.
7) Monitor algal densities in larval cultures to determine additional and new feeding rates.

Example: Ten-day oyster veligers require an algae feeding density of Isochrysis of 30,000 cells/ml (from table). A kalwall culture of T-iso is determined to have a density of 3 million cells/ml. Calculate the amount of algae to feed a 900-gallon larvae tank.
30,000 X (900 gallons X 3785 milliliters/gallon) / 3,000,000 cells/ml = 34 liters of algae to feed

Feeding by color:

1) Feeding algae to larvae cultures causes the water to take on a certain color depending on the species of algae and the amount fed
2) After enough repetitions hatchery personnel develop experience with their systems, which can allow them to feed algae to produce a certain tint to the larvae tank water. This tint is maintained through additional feedings to maintain adequate feed levels in larvae cultures.
3) For the CCC hatchery the color desired is sufficient tint to the water such that the tank bottom is barely able to be seen. Actively feeding larvae cause the water to slowly clear by removing the algae.
4) The advantage of feeding by color is that it does not require the extra time to perform counts of algae cell densities in all larvae cultures.

Larvae feeding chart

Age	Stage	Approx size (microns)	Algae species to feed	Algae density (cells/ml)	Larvae density (larvae/ml)
Spawn	eggs	50-70	none	none	8
24 hours	trochophore	50-70	Nanno, Iso	10,000	6
2-day	D-stage	60-80	Nanno, Iso	20,000	5
4-day	veliger	90-110	Nanno, Iso	25,000	4
6-day	veliger	120-140	Iso	25,000	4
8-day	veliger	130-150	Iso	30,000	3
10-day	veliger	140-160	Iso	30,000	3
12-day	veliger	150-170	Iso, Pav	40,000	2
14-day	veliger	180-200	Iso, Pav	40,000	2
16-day	veliger	220-240	Iso, Pav, Chaet	40,000	2
18-day	veliger	240-260	Iso, Pav, Chaet, Thal	50,000	1.5
20-day	veliger	280-300	Iso, Pav, Chaet, Thal, Tet	50,000	1.5
22-day	pediveliger	300-320	Iso, Pav, Chaet, Thal, Tet	60,000	1
24-day	pediveliger	300-360	Iso, Pav, Chaet, Thal, Tet	80,000	1

Key to algae species in chart:

Nanno	Nannochloropsis
Iso	Isochrysis galbana (clones: T-iso, C-iso)
Pav	Pavlova lutheri
Chaet	Chaetoceros spp. (C. neogracilis, C. calcitrans)
Thal	Thallassiosira spp. (T. pseudonana clone 3H, T. weisflogii)
Tet	Tetraselmis spp. (T. suecia, clone Ply 329, clone Plat-P)

Harvesting and storing eyed-larvae

Materials: Tank with eyed pediveliger larvae ready for harvest, 250-micron sieve, 125-micron sieve, coffee filter, Ziploc bag, permanent marker, refractometer, several 200-500 ml containers, Imhoff cone apparatus

Note: a 225-micron sieve can be used to harvest larvae but larger sieves retain healthier larvae, which will have greater percentage setting and survival in remote setting systems.

Process for harvesting and storing larvae:

1) Check to be sure the larvae are ready for harvest. Majority should have a foot, and almost all should have eyespots.
2) Stack sieves with the 250-micron sieve on top backed with the 125-micron sieve.
3) Place sieves under the drain and turn on the valve to drain the tank.
4) Harvest larvae on sieves.
5) Check the back-up 125-micron sieve to see whether many larvae are passing through the 250-micron sieve. If few larvae are passing through, remove the 125-micron sieve and continue using only the 250-micron sieve.
6) Gently wash the larvae from the larger mesh sieve into the Imhoff cone with a small amount of water.
7) When the larvae sink to the bottom of the cone estimate the number of larvae. Twenty milliliters equal approximately one million larvae.
8) Drain the larvae from the Imhoff cone onto a coffee filter by opening the valve at the bottom. Drain off only the larvae; do not drain water as this will cause excessive water accumulation on the filter paper.
9) Place the harvested larvae and moist coffee filters in a Ziploc bag and into the refrigerator for short term storage (up to one week).
10) Label each bag with the number of larvae, parent stock if known, salinity and temperature of the water from which they were harvested.
11) Examine smaller larvae with microscope that collected on the 125-micron sieve. Replace smaller larvae in new tank if they do not have foot and eyespot. Provide fresh algae and repeat procedure in one to two days.

Remote setting oyster larvae

Materials: Ziploc bag of eyed larvae, clean buckets, filtered tempered seawater, dissecting microscope, petri dish, refractometer

Note: Maintain regular communication with hatchery for the week prior to delivery of eyed-larvae to coordinate salinity and other pertinent information.

Prior to receiving larvae

1) Put cleaned cultch in shell bags, crates or other containers and load into tanks.
2) Fill tanks with filtered water.
3) Operate blower for about 2 days to decompose organic matter on cultch.
4) Flush water through tanks and fill with clean filtered water.
5) Notify the hatchery of salinity and temperature of water in setting tanks.

Receiving larvae

1) Open box and record shipping container temperature immediately upon arrival. .
2) Place larvae in shaded area and slowly warm them to ambient temperature.
3) Adjust salinity and temperature in a bucket of water to the hatchery conditions.
4) Place eyed-larvae into the bucket and observe for movement and swimming.
5) Alternatively examine larvae in a petri dish with the dissecting microscope.
6) Adjust salinity and temperature in bucket to that of the remote setting tanks.
7) When most larvae are swimming distribute them evenly around the setting tank.

Remote setting system operation

1) Insure air blower is operational daily and that there is even distribution of air.
2) Record temperature, dissolved oxygen and salinity in setting tanks.
3) Use small sieve and pipette to sample for presence of swimming larvae.
4) Larvae may be observed with microscope, dissecting scope or magnifying glass.

Record keeping

1) Use the remote setting data sheet to record information for each batch or setting tank.
2) Record the temperature and salinity from the hatchery.
3) Record the temperature and salinity of the ambient water.
4) Record the temperature and salinity in the setting tanks.
5) Record presence / absence of swimming larvae daily.
6) Record average number of set oysters per shell immediately post set.
7) Record average number of spat per shell at set intervals post deployment.


WEST BAY

Shellfish Larvae Record Sheet

Spawn date: ______________________ Species/Source: ________________________________

Date	Temp	Salinity	Sieve	Larval	Larvae	Feeding		Other Notes and Comments
	C	ppt	Size	Count	Size	Qty	Type	

Section E: Examining Trouble Areas Protocols

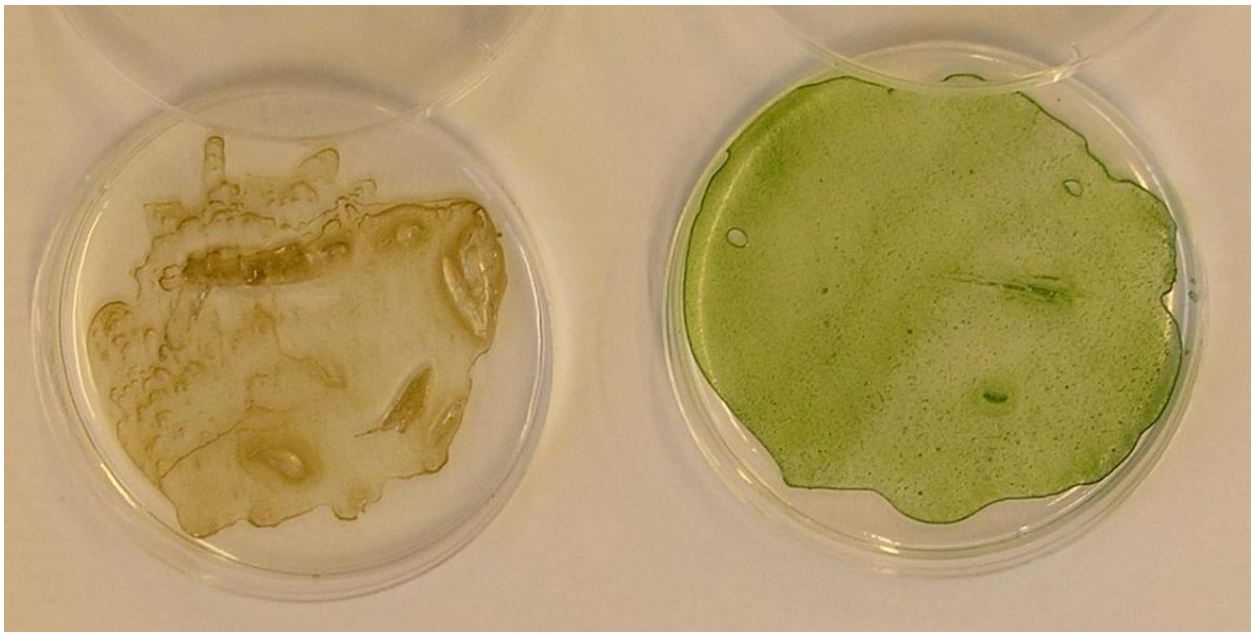
1) Agar plates for algae culture
2) Fluid thioglycollate bacteria indicator test
3) Agar plates for aerobic bacteria
4) Serial dilutions for bacteria testing
5) Extracting dilutions
6) Interpreting results of bacteria cultures
7) Protocol for total aerobic bacteria and Vibrios
8) Chlorine treatment for ciliates in algae

Agar plates for culturing marine bacteria and algae

Materials: 9 grams of Difco Bacto-agar, 1 liter of filtered seawater, microwave-safe glass pitcher with cover, scale, plastic spoon, 40-50 disposable petri dishes, F/2 algae fertilizer, metasilicate

Procedure:

1) Assemble materials. Wash hands with antibacterial soap and warm water.
2) Weigh 9 grams of Difco Bacto-agar to a weigh boat. Weigh 20 mg of sodium metasilicate.
3) Fill pitcher to 1-liter with filtered seawater. If diatoms will be cultured add metasilicate.
4) Place pitcher with cover in microwave; place plastic spoon on top of cover.
5) Microwave for 5 minutes on high setting or until water begins to boil.
6) Add agar to water and stir until dissolved.
7) Replace cover on pitcher and put spoon on top of cover; replace pitcher in microwave.
8) Microwave for 2 minutes on high setting. Stir while hot.
9) Repeat step 7-8 twice more and watch for boiling. Turn off microwave if contents begin to boil over the top of the pitcher. Reduce time to one minute or 30 seconds if foaming occurs. Microwave contents for a total of 6 minutes.
10) Allow pitcher of agar to cool in microwave. Remove when warm to the touch.
11) Lift cover carefully. Add ¼-ml (5 drops) of F/2 fertilizer. Stir with sterilized plastic spoon.
12) Line up petri dishes on the edge of a table. Pour approximately 20-25 ml agar into each plate.
13) Allow agar to cool and gel before using.
14) Using sterile technique transfer 0.5 ml of algae culture to the surface of agar plate. Spread across the agar surface with sterile steel triangle or glass rod “hockey stick”. Replace cover.



Fluid thioglycollate media (FTGM) test

Materials: Test tubes with autoclavable screw caps, 1-liter flask with 500 milliliters of filtered seawater, 14.5 grams of thioglycollate media (FTGM), scale, hot plate, 1-liter beaker, gloves, autoclave, 1-ml sterile pipette

Note: FTGM is used as a qualitative test to detect the presence of bacteria in algae culture media.

Procedure:

1) Assemble materials. Wash hands with antibacterial soap and warm water.
2) Weigh 14.5 grams of FTGM media
3) Mix the FTGM with 500 ml of filtered seawater
4) Place on a hot plate and boil until the mixture turns from a brown color to a clear yellow
5) Pour 10 ml of the mixture into test tubes filling until they are about half-full
6) Secure the lids to the test tubes and autoclave for 30 minutes at 121 degrees Celsius.
7) When cool, remove from the autoclave and place in storage on shelf.

Testing algae cultures for bacteria contamination

1) Using sterile technique and individual sterile Pasteur pipette, draw 0.25 ml from each algae culture to be tested.
2) Immediately place the sample of algae culture media in an individual test tube of FTGM and discard pipette. Use individual pipette for each sample. Label test tubes appropriately.
3) Place test tubes on a shelf in the dark or cover to prevent light from reaching the tubes.
4) Incubate for 24 hours and observe tubes. Look for a cloudy appearance in the tubes which indicates that bacteria are present in the sampled culture. Continue incubation for 24 hours more and use for comparison purposes.
5) This is a qualitative test and does not indicate the extent of contamination or species of bacteria. However, intuition can be used to compare cloudiness to give a relative comparison between and among tested cultures.

Agar plates for aerobic bacteria

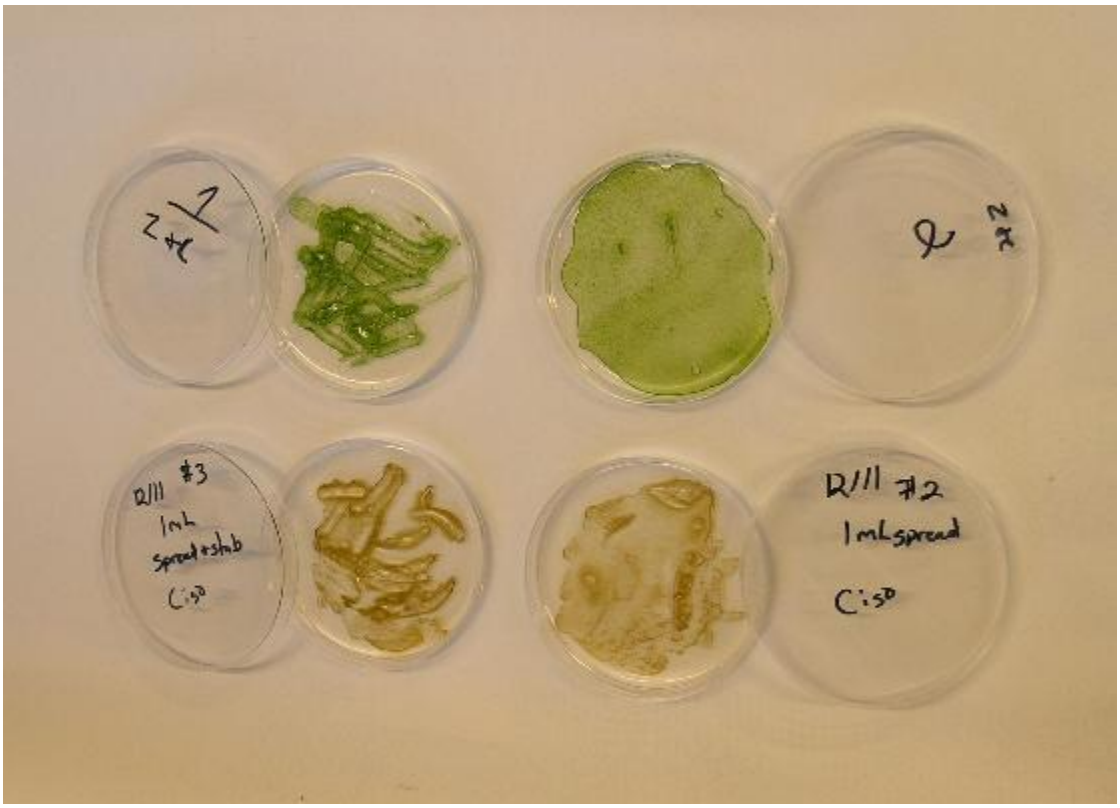
Materials: disposable petri dish plates 52-mm in diameter, 4.5 grams of Difco Bacto-Agar, 5 grams of Bacto-Peptone liquid media, 500 ml filtered seawater in a 1-liter flask, scale, hot plate

Note: 500 ml of agar will make 20-25 petri dish plates. Make agar according to needs.

Procedure:

1) Assemble materials. Wash hands with antibacterial soap and warm water.
2) Weigh 4.5 grams of Bacto-Agar and 5 grams of Bacto-Peptone liquid media. Place into the 1-liter flask containing 500 ml filtered seawater.
3) Swirl before heating to dissolve the powdered media. Place on hot plate and bring to a boil; boil for 2 minutes.
4) Place petri dishes side-by-side in a line on the edge of a table.
5) Carefully lift the lid of each dish with one hand and pour about 20-25 milliliters of the heated mixture into each petri dish with the other hand. Do not place the lid down on the table. Replace the lid and continue to the next dish. Wear insulated glove when handling hot flask.
6) Place petri dishes aside and allow the agar to cool and harden.
7) Aerobic agar plates are ready to use when the agar gels.

Note: Use the same protocol to make TCBS agar plates with the following exceptions: use 44.5 grams of TCBS media (instead of Bacto-agar and Bacto-peptone) and deionized water (instead of filtered saltwater).



Making serial dilutions for bacterial testing

Materials: Samples of water or algae culture to be tested, sterile test tubes each containing 9 ml of sterile filtered seawater, sterile pipettes, alcohol burner

Note: Water and algae culture samples are diluted to make counting easier. The dilutions are made to aid in counting.

Procedure:

1) Assemble materials. Wash hands with antibacterial soap and warm water.
2) Each sample will have 3 dilutions plus replicates if desired. Tubes with 9.0 ml of sterilized filtered seawater can be prepared ahead of time and stored on the shelf. Select the required number of test tubes containing 9-ml of sterile filtered seawater. Place in test tube rack.
3) Use one sterile pipette for each transfer. Select the number of required sterile pipettes and place in the work area.
4) Dilutions are made as follows:
    a. Dilution A (1:10): Transfer 1.0 ml of **raw sample** to test tube A. Mix well.
    b. Dilution B (1:100): Transfer 1.0 ml from tube A to tube B. Mix well.
    c. Dilution C (1:1000): Transfer 1.0 ml from tube B to tube C. Mix well.
5) Seal the dilutions and continue to extraction

Extracting serial dilutions

Note: For aerobic bacteria care should be taken to avoid incidental and cross-contamination For *Vibrio* specific media, TCBS, cross contamination should be avoided as much as possible.

Materials: Vacuum pump, 1-liter filtration flask, vacuum filter, 0.45-micron filter papers, 47-mm in diameter, forceps, pipette, test tubes, sterile seawater, alcohol burner, isopropyl alcohol

Procedure:

1) Assemble the filtration apparatus with 1-liter filter flask, vacuum hose and vacuum pump
2) Turn on the vacuum pump.
3) Put filter paper on the vacuum, using forceps. Pay attention to avoid cross-contamination.
4) To prevent the forceps from becoming contaminated handle only the outer edge of the filter both when loading and removing it. The outer edge is dry after the sample is loaded onto it and is not contaminated by the process.
5) Flame sterilize the forceps between filters by dipping in isopropyl alcohol and flaming. This will allow you to use the same forceps for the entire process. When not using the forceps place them on a dry rack.
6) Shake the sample. Using sterile technique draw and place a 5-ml aliquot of the first dilution on the gridded filter paper; load the sample toward the center of the filter paper.
7) Take care to avoid putting the sample dilutions on the edges of the vacuum dish. Use an individual sterile pipette for each dilution and sample.
8) Remove filter while the pump is running by picking up the filter with forceps at the dry edge.
9) Put the paper on an appropriate media petri dish and replace the dish cover.
10) Repeat steps 3-9 for each sample.
11) Turn off the vacuum pump.
12) Label all petri dish plates appropriately.
13) Incubate 18-24 hours at a temperature of 35º Celsius.

Interpreting results of bacteria cultures

Materials: petri dish cultures from incubator, dissecting scope

Procedure:

1) Remove petri dishes from incubator.
2) Place dish under low power dissecting scope.
3) Count the number of colonies on each plate.
4) Obtain the expansion factor for each dilution from the table below and calculate the number of bacteria according to the following formula:

Number of colonies counted X Expansion factor = Total number of bacteria per milliliter

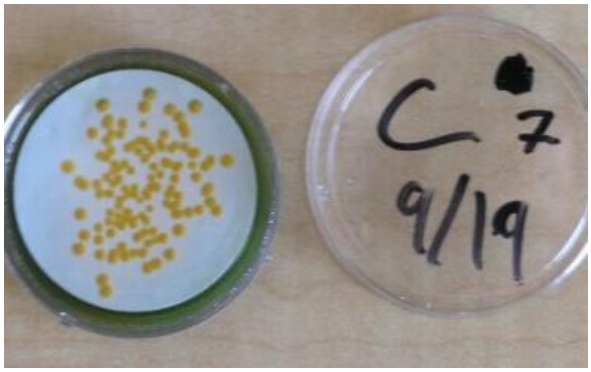
Dilution	Tube designation	Expansion factor
Full strength	Raw sample	0.2
1:10	A	2.0
1:100	B	20.0
1:1000	C	200.0

Note: To aid in understanding the calculation remember that each sample used 5-ml of media for each extraction.

For example: If a count of the sample plate yields 500 colonies, using the formula above, multiply 500 X 0.2 = 100 bacteria per milliliter of sample. When selecting a plate to use for counting look for one with around 200 or fewer colonies. In this example the 1:10 dilution should yield a count of approximately 50 bacteria colonies. Using the formula multiply 50 X 2.0 = 100 bacteria per milliliter.

For *Vibrio* testing the TCBS agar manufacturer provides this table to identify species:

Appearance of Colonies	Microorganisms
Flat, 2-3 mm in diameter, yellow	Vibrio cholerae, Vibrio cholerae type El Tor
Small, blue-green centre	Vibrio parahaemolyticus
Large, yellow	Vibrio alginolyticus
Blue	Pseudomonas, Aeromonas and others
Very small, translucent	Enterobacteriaceae and others



Quantifying Total Aerobic Bacteria and *Vibrios* in Oyster Larvae, Algal Samples, or Water

(Protocol courtesy of Dr. Jim Oliver, UNC-Charlotte)

Materials: gloves, prepared plates of MSWYE agar* or similar (e.g. ZoBell 2216 agar), TCBS agar, "hockey stick" or triangle spreaders, beaker of 95% ethyl alcohol, dilution tubes containing 9 ml filtered sterile seawater or artificial salt water (ASW)**, 1-ml sterile pipettes, 1-ml pipette bulbs, flame source such as alcohol lamp, candle, or Bunsen burner.

- Set up workspace on table with plenty of room to spread out.
- Assemble above materials.
- Select oyster larval, algal, or water samples to be analyzed.

Samples of Larvae:

1) Concentrate larvae by filtering a sample of them onto an appropriate filter, wash 2-3 times with filtered sterile seawater or artificial seawater (ASW), and then scrape larvae off into homogenizer tube containing 1 ml of sterile ASW.
2) Homogenize the entire larval sample to kill larvae and release gut bacteria. This is sample 1.
3) Make dilutions of the homogenized larvae by placing 0.1 ml of homogenized tissue into 0.9 ml of sterile seawater or ASW and mixing thoroughly. This is sample 2.
4) Repeat dilution into a fresh tube with 0.9 ml sterile seawater or ASW. This is sample 3.
5) From each tube (samples 1-3), remove 0.1 ml (using a sterile pipet and pipet aid) and place onto the center of an agar plate (TCBS agar for Vibrios, MSWYE agar for total marine bacteria).
6) Label with the larval sample ID, the sample number (1-3), and the date.
7) Remove bent metal or glass rod spreader from alcohol and shake off excess. Pass momentarily through flame. Allow the alcohol to burn off. (Do not heat spreader in flame).
8) Using the sterilized spreader, spread a 0.1 ml sample completely around the agar surface.
9) Place the spreader back into the alcohol, and leave until next sample is placed on medium.
10) Allow liquid on plates to absorb into the medium, then invert the plates and incubate at room temperature overnight.
11) Of the various plates, select one that has 20-200 colonies (this is considered the optimum dilution; the other plates should have many fewer or a far greater number of colonies). Count every bacterial colony that appears on that plate***.
12) After counting the "optimum" plate, multiply the total colony number by the "dilution" factor indicated on the table below.

Samples of Algae:

1) Place algae sample into homogenizer tube containing 1 ml of sterile ASW.
2) Homogenize the sample to release the bacteria from the algal surfaces. This is sample 1.
3) Make dilutions of the homogenized algae by placing 0.1 ml of homogenized algae into 0.9 ml of sterile seawater or ASW and mixing thoroughly. This is sample 2.
4) Repeat dilution into a fresh tube with 0.9 ml sterile seawater or ASW. This is sample 3.
5) From each tube (samples 1-3), remove 0.1 ml (using a sterile pipet and pipet aid) and place onto the center of agar plate (TCBS for Vibrios, MSWYE for total marine bacteria).
6) Label with the larval sample ID, the sample number (1-3), and the date.

7) Remove bent metal or glass rod spreader from alcohol and shake off excess. Pass momentarily through flame. Allow the alcohol to burn off. (Do not heat spreader in flame).
8) Using the sterilized spreader, spread a 0.1 ml sample completely around the agar surface.
9) Place the spreader back into the alcohol, and leave until next sample is placed on medium.
10) Allow liquid on plates to absorb into the medium, then invert the plates and incubate at room temperature overnight.
11) Of the various plates, select one that has 20-200 colonies (this is considered the optimum dilution; the other plates should have many fewer or greater number of colonies). Count every bacterial colony that appears on that plate***.
12) After counting the "optimum" plate, multiply the total colony number by the "dilution" factor indicated on the table below.

Samples of Water:

1) Place water sample into a sterile tube. This is sample 1.
2) Make dilutions of the water by placing 0.1 ml of the sample into a tube with 0.9 ml (or 1 ml into 9 ml) of sterile seawater or ASW and shaking thoroughly. This is sample 2.
3) Repeat dilution into a fresh tube with 0.9 ml sterile seawater or ASW. This is sample 3.
4) From each tube (samples 1-3), remove 0.1 ml (using a sterile pipet and pipet aid) and place onto the center of agar plate (TCBS for Vibrios, MSWYE for total marine bacteria).
5) Label with the larval sample ID, the sample number (1-3), and the date.
6) Remove bent metal or glass rod spreader from alcohol and shake off excess. Pass momentarily through flame. Allow the alcohol to burn off. (Do not heat spreader in flame).
7) Using the sterilized spreader, spread a 0.1 ml sample completely around the agar surface.
8) Place the spreader back into the alcohol, and leave until next sample is placed on medium.
9) Allow liquid on plates to absorb into the medium, then invert the plates and incubate at room temperature overnight.
10) Of the various plates, select one that has 20-200 colonies (this is considered the optimum dilution; the other plates should have many fewer or greater number of colonies). Count every bacterial colony that appears on that plate***.
11) After counting the "optimum" plate, multiply the total colony number by the "dilution" factor indicated on the table below.

Sample Number	Dilution factor
1	10
2	100
3	1000

For example: If plate number 2 had 48 colonies, multiply 48 times 100, to give a final number of 4,800 total bacteria present on the larvae sampled.

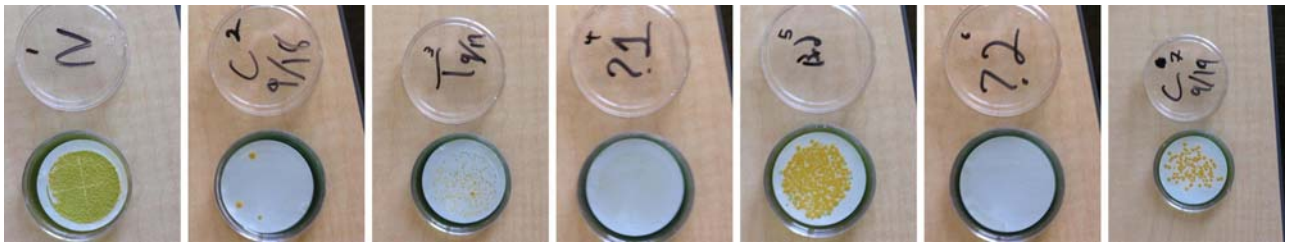
Note: Using this method, the smallest number of bacteria you can detect is 100 (from a single colony on plate number 1). If no colonies appear on plate #1, record the result as "less than 10" because it is not possible to detect zero colonies.
Note also: The number of bacteria you can detect will depend on the original number of larvae sampled (obviously, if you start with ten times as many larvae, you will get ten times more bacteria). So it is important to always start with approximately the same

number of larvae. Learn this through experience with your operation.

*MSWYE: 1 liter of "3-salts solution", 1g Proteose Peptone (Difco), 1g Yeast extract (Difco), 15 g agar. pH to 7.2-7.4, autoclave, and pour plates when agar has cooled to ~50°C. After plates solidify, invert, and incubate overnight at 37°C or on bench top for 2-3 days (this allows excess moisture to evaporate so that added larval/algal/water sample absorbs into the medium). "3-salts solution": 23.4 g NaCl, 0.75 g KCl, 7 g $MgSO_4 \cdot 7H_2O$.

**If sample to be tested is from significantly lower salinity (e.g. 15‰), dilute filtered seawater or ASW 1:1 with distilled water.

***Colonies on TCBS: sucrose + are yellow, sucrose – are green; some colonies may be black due to H_2S production.



Chlorine treatment for ciliates in algae cultures

(Courtesy of Ryan Murashige, NCSU)

Materials: chlorine bleach, contaminated algae cultures, scale, sodium thiosulfate, OTO

Procedure:

1) Calculate the volume of contaminated algae culture in liters
2) Add 50 mg chlorine bleach per liter of contaminated algae culture
3) Allow to stand for 45 minutes
4) Dechlorinate with 1 mg sodium thiosulfate per mg of chlorine bleach added
5) Check for residual chlorination with 1 drop of OTO (ortho-tolidine) solution. Insure that there is no color change with the addition of OTO.

Alternate procedure:

1) Calculate the volume of contaminated algae culture in liters
2) Add 10-15 ppm of chlorine bleach
3) Allow to stand for 2-4 hours
4) Dechlorinate with 0.1-0.15 mg/l sodium thiosulfate per ppm of chlorine bleach added
5) Check for residual chlorination with 1 drop of OTO (ortho-tolidine) solution. Insure that there is no color change with the addition of OTO.

Second alternate procedure:

1) Calculate the volume of contaminated algae culture in liters
2) Add acid to lower pH to 5.5-6.0
3) Allow to stand for 30 minutes.
4) Neutralize acid back to pH of 8.2

Section F: Facilities and Operations Protocols

1) Large dock pump operation
2) Hydrotech 801-802 filter operation
3) Transfer pump operation
4) Sand filter operation
5) Using U-V filtration
6) Testing water quality
7) Maintaining a chlorine bath
8) Cleaning the Floor
9) Determining tank volumes
10) Making a kalwall useable
11) Cutting and gluing PVC pipe
12) Making a sieve
13) Making a counting slide
14) Using the MIC-D digital microscope

Large dock pump operation

VFD Operation

- VFD should be set in auto mode when in operation, should press **"auto"** and VFD screen should say **"auto".**
- VFD screen should correspond with level sensor screen (blue box) percent value.
    - Press **"%"** on level sensor screen box, value shown will be percent full in 802 reservoir.
    - The value shown should correspond to bottom percent on large dock pump VFD screen.

Starting Large Dock Pump

-Step 1: Press **"auto"** on corresponding large dock pump VFD.
-Step 2: Listen for large volume of water flowing into 802 Hydrotech filter. If a lot of water is flowing then you are complete. If no water, continue to Step 3.
-Step 3: After 45 seconds with no water flowing press "Off" on the VFD.
-Step 4: At dock pump house, open 2" union valve and let water flow out until it stops. While valve is open fill pipe with fresh water until topped off. Close valve and repeat Step 1-2.

Switching Supply Lines Going Into 802 Filter

Note: During this process, it is important to always have a water supply pipe open for water to flow. Always open a valve before you close another. With an open valve the handle is parallel (matches direction) to the pipe. On a closed valve the handle is perpendicular to the pipe.

-Step 1: While pump is going open valve to allow water to flow out supple line that you want to be hooked up. At this time you are allowing water to flow through both pipes.
-Step 2: Let water run out of the line you want to hook up for 2 minutes at max flow or until water doesn't smell like rotten eggs. Turn valve to stop water flowing through the pipe you want to hook up.
-Step 3: Open valves to allow for water to flow through out supply pipe that is located east of the concrete ponds. When water is flowing out of this pipe, turn both water supplies to 802 Hydrotech Filter off.
-Step 4: Take existing water pipe supply off 802 Hydrotech Filter and install the other supply pipe.
-Step 5: Turn valves to allow water to flow through supply pipe that is now connected to the 802 Hydrotech Filter.
-Step 6: Turn off valves that go to outside supply pipe (East of concrete ponds). Check to insure that water is going into filter.

Operating notes for 801 and 802 Hydrotech filters

Note: Before operating filters insure water is flowing through them and freshwater is on.

Resetting Filter

-Step 1: Check and write down problems with filter. Open corresponding filter box and note all lights that are on the ABB controller.
-Step 2: Turn outside knob on upper right corner to straight up and down position. All operation should stop, make note of anything that does not.
-Step 3: Go to main filter house breaker box and turn corresponding breaker off. Leave off until all lights inside of corresponding filter control box are off. Turn breaker on and turn knob outside of filter control box to automatic (away from you). Filter operation should return to normal.

High Pressure Pump Replacement

-Step 1: Turn corresponding filter control knob outside of control box to off position. If it does not stop turn corresponding filter breaker in main panel box inside filter house off.
-Step 2: Turn freshwater valve to pump off. Unscrew unions and take pump out. Leave freshwater valve off.
-Step 3: Outside on filter, disconnect red high pressure hose from filter, solenoid, and PVS pipe. Connect one end to outside freshwater supply and other end to filter. Reconnect solenoid and turn valve on the outside freshwater supply on. Return filter to automatic mode by turning knob outside of filter control box to automatic (turn top away from you) and if required turn breaker on.

Small transfer pumps (from Hydrotech filters 801 and 802)

Starting Pump

-Step 1: Make sure water is flowing into filtration system. Also make sure corresponding reservoir tank is filled.

-Step 2: At the corresponding VFD, turn knob on upper right outer side to automatic (turn top away from you). Feel and look at back of pump to see or feel if fan is rotating.

-Step 3: Check inside of building to see if water is running. If not go to end of main grate and open valve to corresponding pump. (Bottom 2" pipe is 802 Pump and Middle pipe or top 2" pipe is 801) Allow water to flow until flow rate increases then shut off valve. If water still is not flowing then repeat step 2 and 3.

Switching suction to concrete ponds

-Step 1: Turn pump knob on outside of box in upper right corner to off (straight up and down).

-Step 2: Down in basin inside filter house, turn corresponding pumps valve to reservoir off and turn pond valve on.

-Step 3: Outside at corresponding concrete pond, close submerged and open top valve. Top off pipe with water. Close top valve and open bottom valve.

-Step 4: At the corresponding VFD, turn knob on upper right outer side to automatic (turn top away from you). Feel and look at back of pump to see or feel if fan is rotating.

-Step 5: Check inside of building to see if water is running. If not go to end of main grate and open valve to corresponding pump. (Bottom 2" pipe is 802 Pump and Middle pipe or top 2" pipe is 801) Allow water to flow until flow rate increases then shut off valve. If water still is not flowing then repeat step 3-5.

Sand filter operation

- All three sand filters filled with 8 cubic feet of lightweight media from Aquatic Eco.

Backwashing filters (running parallel or series)

Note: Filters need to be backwashed every morning. Backwash filters one at a time

- Step 1: Turn 801 filter pump VFD down to **18.0 Hz** (connect wires)
- Step 2: Turn multiport valve on one filter to **"Backwash"**, let backwash for at least **10 minutes** or until clear, turn multiport valve to **"Rinse"** for **1 minute**. Return multiport valve to **"Filter"**.
- Step 3: Repeat Step 2 for other two filters one at a time.
- Step 4: After filters have backwashed and rinsed, and all multiport valves are back to filter, return VFD to original state (Automatic on PSI sensor)

Operating filters in parallel

- Step 1: Turn 801 filter pump VFD down to **18.0 Hz** (connect wires)
- Step 2: **Open** valves **1, 2, 4, and 6**
- Step 3: **Close** valves **3 and 5**
- Step 4: Return 801 filter pump VFD to original state

Operating filters in series

- Step 1: Turn 801 filter pump VFD down to **18.0 Hz** (connect wires)
- Step 2: **Open** valves **3 and 5**
- Step 3: **Close** valves **1, 2, 4, and 6**
- Step 4: Return 801 filter pump VFD to original state

Cleaning sand filters:

1) Turn off the water flow to the filter
2) Turn the valve to backwash
3) Turn the water back on for 30 minutes
4) Turn the water off and switch valve to rinse; turn water on for 2 minutes
5) Turn the water off, return valve to filter position and turn water back on.
6) If filters are in series turn the filter valve to recirc and repeat on other filters
7) Once all filters have been cleaned turn the valve to filter and turn water on again.

Periodically:

1) Remove the top and break apart the clumps with a powerful water spray.
2) Check to see if this accomplished by reaching in and feeling with the hand
3) Once this is done replace the top and clean as above.

Sand filter operation

Using UV Filtration

Materials: U-V filter, saltwater source

Process for using U-V Filtration

1.) Make sure the UV is connected to the water source, and there is a water hose on the harvest end.
2.) Turn on the water, and let it run for 5 minutes
3.) Plug the UV in and check to make sure the UV lights are on
4.) Let the water run with the UV on for 1 minute before using
5.) When finished, unplug the UV unit, and turn the water off.

Testing water quality

Materials: Saltwater liquid test kit, 50-ml beaker with water to be tested, refractometer, thermometer, dissolved oxygen meter

Ammonia:

1) Fill a clean test tube from the kit with 5 ml of the saltwater to be tested.
2) Add 8 drops of ammonia test solution #1 to test tube.
3) Add 8 drops of ammonia test solution #2 to test tube.
4) Attach cap and mix by inverting the test tube several times.
5) Allow the test tube to sit for 5 minutes.
6) Compare color of test tube to the saltwater ammonia color card found in the kit.

Nitrite:

1) Fill a clean test tube from the kit with 5mL of the saltwater to be tested.
2) Add 5 drops of nitrite test solution to test tube.
3) Attach cap and mix by inverting the test tube several times.
4) Allow the test tube to sit for 5 minutes.
5) Compare color of test tube to the saltwater nitrite color card found in the kit.

Nitrate:

1) Fill a clean test tube from the kit with 5 ml of the saltwater to be tested.
2) Add 10 drops of nitrate test solution #1 to test tube.
3) Attach cap and mix by inverting the test tube several times.
4) Mix nitrate test solution #2 for 30 seconds.
5) Take cap off test tube and add 10 drops of nitrate test solution #2 to test tube.
6) Attach cap and mix by inverting test tube several times for 1 minute.
7) Allow the test tube to sit for 5 minutes.
8) Compare color of test tube to the saltwater nitrate color card found in the kit.

pH:

1) Fill a clean test tube for the kit with 5 ml of the saltwater to be tested.
2) Add 5 drops of high range pH indicator solution to test tube.
3) Attach cap and mix by inverting the test tube several times.
4) Compare color of test tube to the saltwater pH color card found in the kit.

Test salinity:

1) Use a pipette to place 2 drops of water on the refractometer daylight plate prism.
2) Close lid on refractometer.
3) Point refractometer towards a light source, and using the eyepiece read the salinity.
4) Find the temperature of the water by placing the thermometer in the water for 30-60 seconds and read the results in degrees Celsius.
5) Measure dissolved oxygen in water using a dissolved oxygen meter.
6) Take probe out of holder, and place in the system you are testing.
7) Turn meter on, and wait until readings slow down or remain constant.
8) Test for dissolved oxygen at different sections of the system and at different depths.

Maintaining a chlorine bath

Materials: Bleach, container of fresh water, OTO

Process for maintaining a chlorine bath:

1.) Set up a container of fresh water, where the bath will be located
2.) Add 5 milliliters of bleach to the container.
3.) Check the bath daily with OTO. This indicates whether or not chlorine is present. If it isn't present, the OTO will cause no color change. Add 5 milliliters of bleach to the bath.
4.) Change the water once a week in the container.

Note: Do not leave equipment in the bath for over 24 hours as the chlorine can degrade plastics and other compounds.

Cleaning the Floor

Materials: Broom, chlorinated water (1 ml/gallon), 1-liter beaker, squeegee

Steps:

1. Pick up any trash found on floor.
2. Sweep floors to remove any dust or dirt.
3. Using the 1-liter beaker, pour generous amounts of chlorinated water across floor.
4. Using the broom sweep the chlorinated water around the floor
5. Using the squeegee push the residual chlorinated water to the drain located in the floor.
6. Make sure there is no water on the floor.

Measuring the volume of round and rectangular tanks

Materials: tape measure, calculator, tank of unknown volume

Procedure for round tanks:

1) Measure the diameter of the tank in centimeters (100 centimeters / meter)
2) Measure the depth of water in centimeters.
3) Calculate the volume of water using the following formula:

(Diameter / 2) X (Diameter / 2) X pi X depth = volume in cubic centimeters (milliliters)

For example: a round tank measures 80 cm dia. and 240 cm deep. Apply the above formula:
(80 cm / 2) X (80 cm / 2) X 3.14 X 240 cm = 1,206,371 milliliters, which is 1206 liters

Procedure for rectangular tanks:

1) Measure the sides of the tank and the depth of the water in centimeters.
2) Multiply these three numbers with the following formula to yield volume in cubic centimeters or milliliters:

Length (cm) X Width (cm) X Depth (cm) = Volume (cubic centimeters or milliliters)

For example: a tank measures 120 cm wide by 100 cm long and the water is 75 cm deep.
120 cm X 100 cm X 75 cm = 900,000 milliliters, which is 900 liters

Making a kalwall useable

Materials: Kalwall with conical bottom and 2-inch threaded female fitting, drill, drill bit sized appropriately, tap sized for the valve, 2-inch true union ball valve, 2-inch 90-degree PVC street elbow, thread tape, aeration valve

Procedure:

1) Remove kalwall from packaging
2) Lids for kalwalls come in a number of different sizes, the kalwall will be marked with a number, these are sometimes hard to interpret. Find a lid with the corresponding number
3) Drill and tap a hole in the back of the 2-inch elbow
4) Using thread tape screw the aeration valve in this hole
5) Put the 2-inch street elbow together with the ball and union valve. Insert a 2-inch diameter threaded close nipple. Wrap with thread tape and screw into the valve. Insure that the valve is oriented properly for flow.
6) Screw this assembly into the conical bottom of the kalwall. Take care to use three layers of thread tape to prevent leaking. A pipe or broom handle can be inserted into the assembly to make screwing it in easier

Cutting and gluing PVC pipe

Materials: PVC pipe and fittings, hand-held PVC cutting shears, hack saw, old rags, PVC cement and primer

Procedure for cutting PVC pipe:

1) For small diameter pipe use hand-held shears to cut pipe to desired length
2) For larger diameters (greater than 1 ½-inch diameter) use hack saw
3) Using an old rag, wipe the rough debris from pipe after sawing

Procedure for gluing PVC pipe:

1) Brush PVC primer onto the ends of the pipe and on the surfaces of the fitting to be glued
2) Brush a thin layer of PVC cement onto the end of the pipe and inside the fitting to be glued
3) Firmly push the pipe into the fitting until fully seated; twist ¼-turn and hold until glue sets
4) Allow 5-10 minutes to cure before using with water under pressure.

Making a sieve

Materials: sieve mesh, 5-gallon bucket with lid, sabre saw, hot glue gun and glue sticks

Procedure:

1) Select the proper mesh size for making the sieve
2) Saw completely around the bucket; maintaining the saw at a point 6-inches from the lid
3) Cut the center out of the lid leaving a 1-inch ring of lid, which can be snapped on the bucket
4) Cut a 13-inch square of mesh from the corner of the stock material so as to minimize waste
5) Place the mesh over the lip of the bucket
6) Simultaneously snap the lid onto the lip of the bucket and seizing the mesh onto the lid
7) Before completing the lid attachment begin pulling outward on the mesh to tighten it
8) Pull the mesh tightly across the open end of the bucket and complete attaching the lid.
9) Using the hot glue gun (or silicone caulk) fill the circular gap on the inside of the bucket next to the place where the mesh and lid meet.
10) The sieve is ready for use




Making a counting slide

Materials: microscope slide, silicone caulk, hot glue gun, round toothpicks, sharpie pen

1) Using a permanent marker draw a series of parallel lines lengthwise on a microscope slide approximately 3 millimeters apart.
2) Turn the microscope slide over so that the unmarked side is facing up.
3) Using a mini hot glue gun with a fine tip run a very thin bead of glue along each line and then run a bead of glue along the ends attaching all glue lines together.
4) Alternatively use silicone caulk instead of hot glue. Use a toothpick to spread the silicone caulk in thin linear beads along the lines. After the glue/caulk dries use isopropyl alcohol to remove the permanent marker lines.
5) The resulting slide has a series of parallel canals that are wide enough so that they can be viewed in a single pass of the microscope on low power.
6) Distribute a 1-ml sample along all of the canals and count objects.
7) The advantage of this type of slide is that the larvae do not need to be killed to count them, whereas using a Sedgewick-Rafter counting slide with swimming larvae requires that the larvae be killed in order to obtain and accurate count.

Using the MIC-D digital microscope

1) After installing program on computer, Click on Mic-D icon to start the program

2) Dialogue pop-up asks if you want to delete pictures taken in last use. Answer “No”

3) Dialogue pop-up installs camera. [For future reference, camera # 6880]

4) Use Windows Explorer or My Computer to create a new folder to store your pictures.

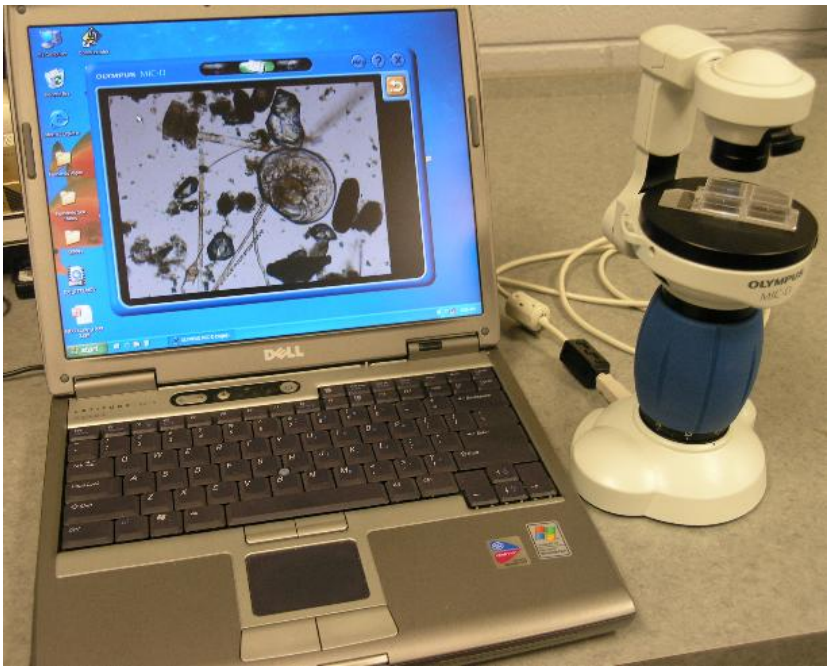
5) When the MIC-D program opens, find the picture storage folder on the right side. Select the folder that you created to save your pictures.

6) Load slide or well slide and place on stage. Rotate the light to the overhead position. Turn light switch on. Rotate body of scope to increase or decrease magnification. Select the best magnification for viewing and change the indicator in thumb print window. Focus with knob on side of stage. For best results begin with low magnification and increase.

7) To take a picture, focus subject and click the camera icon on right side. After taking pictures you can click on library (it is the middle top button) and view your pictures. Click on lower folder button to find your folder and click on each picture and bottom button to save them.

8) To take a motion picture click on the movie camera icon on right side of thumb print window. MIC-D will also take time lapse motion pictures.

9) Read the help file for additional information.



Section M: Miscellaneous Protocols

1) How to write a protocol

How to write a protocol:

Paragraph of introduction if necessary

Materials: enter materials here, use bold green underline for heading

Procedure: list steps to complete the task being described, use bold teal underline for heading

1) Set numbering for left alignment at 0 points
2) Tab space after 18 points
3) Indent at 18 points
4) List steps to complete the task being described

Note: any important points here, use bold orange underline for heading

Examples: are given to help understanding of the protocol, use bold plum underline for heading

Include charts or tables: to facilitate comprehension and increase usefulness.

Include pictures of the task, either following or in text, which help the reader's understanding.