Diseases in the hatchery are caused either by *opportunistic* pathogens or by *host-specific* pathogens. These two kinds of diseases require different management approaches. Disease causing agents which are of regulatory consequence and considered to be of major risk for geographic movement are referred to as "reportable" or "certifiable" diseases.

The diseases one finds most often in bivalve hatcheries are caused by bacteria that live in the marine environment, whether or not bivalve larvae are present, but that opportunistically take advantage of the high densities of larvae in the hatchery. Because of this, opportunistic diseases are often called *management* diseases, the commonest of which is *vibriosis*.

Based on the knowledge we have today, we generally consider most bacterial diseases of bivalve larvae to be management diseases. Viruses are host-specific pathogens, as are some parasitic diseases. Having made this distinction, however, it should be noted that certain bacterial and fungal pathogens of larvae are rather host specific, so that in reality there is not always a clear distinction between opportunistic pathogens and host-specific pathogens.

Opportunistic Diseases

Opportunistic diseases are caused by bacteria or other microorganisms that exist in the marine environment. Poor management practices can allow them to gain entrance or proliferate at any number of sites in a hatchery, and good management can control or eliminate them. The overall approach to managing these diseases is as follows:

1. Maintain pathogen-free algal stocks and expand cultures.
2. Maintain absence or low levels of pathogenic vibrios and other disease-causing microorganisms in the system (water column and surfaces) by proper water filtration, hygiene of system surfaces, and frequent water changes.
3. Isolate infected stocks and associated equipment at first sign of disease.
4. Discard infected stocks and sterilize equipment.
5. Identify source of contaminants, and modify and clean system.

BACTERIOLOGICAL SAMPLING

Disease-causing bacteria can enter the hatchery system at any of its several components. To determine whether and to what extent bacteria are present, then, one must sample bacterial growth throughout the hatchery. Samples may be taken from water, algal stocks, container surfaces, or larvae.

Bacteriological sampling can be done at several different levels of detail. It may be adequate simply to locate large numbers of likely problem-causing bacteria without actually counting bacteria. If, however, an actual count is needed, counting, while more detailed and time consuming, is possible in the production hatchery or can be done by an assisting laboratory.

Once bacterial "hotspots" have been located and corrective measures taken (as outlined above), the areas should be sampled again to determine the success of the measures.

The following procedures are directed toward the detection of *vibrios*, since *vibriosis* is the best-known bacterial disease, but they can be adapted to other bacteria. The steps outlined assume that the reader has some basic knowledge of bacteriological methods. Any hatchery worker who has successfully cultivated algae in order to feed bivalve larvae will be able to perform these procedures.

Note: Dispose of used bacteriological plates carefully (by sterilization, if this is practical) to avoid contaminating your system.

Supplies and Equipment

- Bacteriological growth medium (see below) used to make bacteriological culture plates.
- Refrigerator for storing prepared bacteriological growth medium and bacteriological plates.
- Range top, hot plate or gas burner for boiling bacteriological medium.
- Autoclave or pressure cooker for sterilizing bacteriological medium.
- Heatproof container (Glass beaker or flask) in which to mix and boil bacteriological medium.
- Household bleach diluted 1:20 with tap water.
- Sterile plastic inoculation loops (10µL volume).
- Sterile swabs.
- Sterile spreaders.
- Pipet (100 µL volume).
- Plastic petri dishes (100 mm x 15 mm).
- Weighing balance.
- Sterile 1.5 mL microcentrifuge capped tubes.
- Sterile seawater for dilutions.
- Sharpie for labeling plates.
- Parafilm.

Bacterial Growth Media

Thiosulfate citrate bile salt sucrose agar (TCBS) is a widely used medium for the *selective isolation* and purification of *vibrios* from a variety of sources. TCBS (Difco Laboratories) is used in determining the presence of *vibrios*, many species of which appear yellow and cause the agar medium to turn yellow. *Vibrios* that are able to use sucrose will form yellow colonies, while sucrose-negative strains will form green colonies. Green colonies are typically considered to be more pathogenic and therefore their presence is of greater concern. Other types of growth medium may be required for special purposes.

To prepare TCBS agar follow label directions. After bringing TCBS medium to a boil, allow the flask to cool to the touch before pouring plates. *Do not autoclave the medium!* While the agar is cooling, wipe down work surface areas with the 1:20 household bleach solution. Then carefully pour 15-20 mL of medium (enough liquid to cover the bottom of the dish with 0.5 inch of medium) into each 100 x 15 mm petri dish using sterile technique. Allow the medium to remain at room temperature for 24 hours or longer to solidify and to remove excess moisture. Package plates in sealed plastic bags and refrigerate upside down until use to prevent condensation.

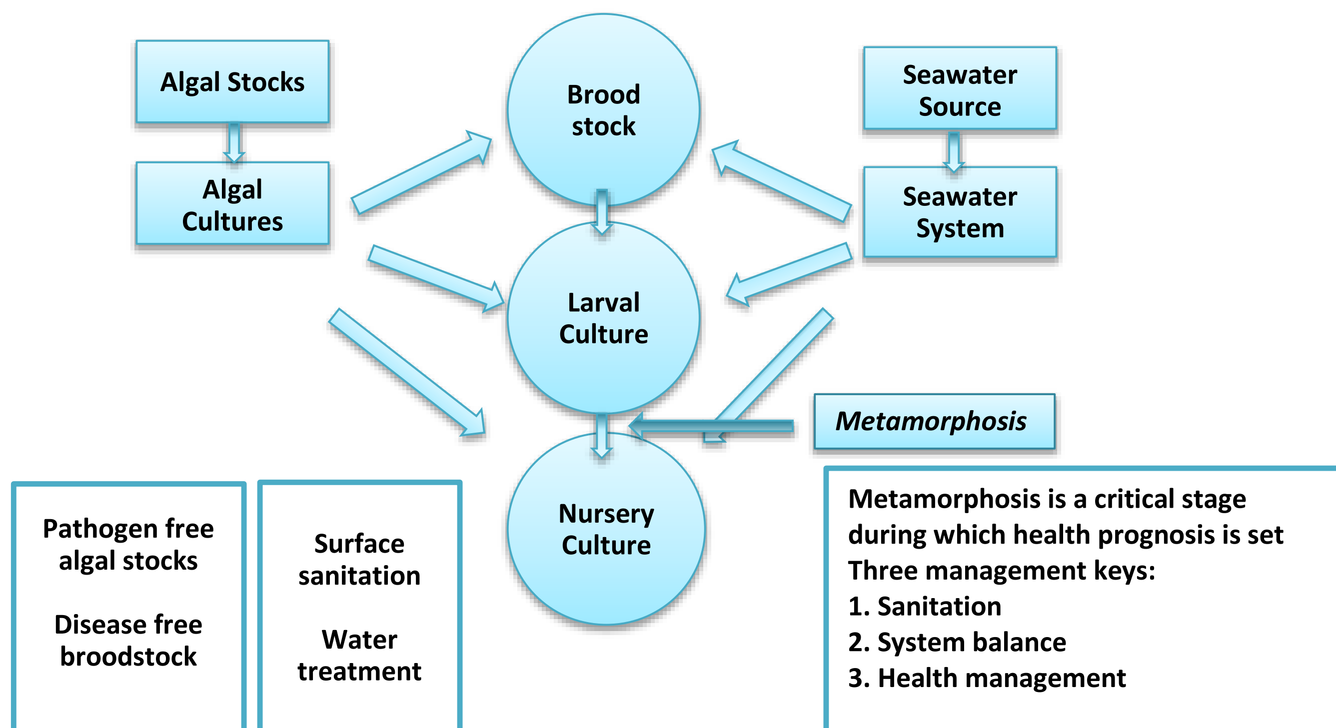
Another commonly used bacteriological growth medium is Marine Agar. Marine Agar (Difco Laboratories) is a *nonselective* medium on which vibrio and many environmental bacteria that require salt water will grow. It may be used if other bacteriological pathogens are suspected or if “complete” counts of bacteria in the hatchery system are being made.

To prepare Marine agar follow directions on label. After bringing the media to a boil, sterilize the medium, use an autoclave at 15 pounds of pressure for 15 minutes. When sterilization is complete, wipe down work surface areas with the 1:20 household bleach solution. Then carefully pour 15-20 mL of medium (enough liquid to coat the bottom of the dish with a 0.5 inch of medium) into each 100 x 15 mm petri dish using sterile technique. Allow the medium to remain at room temperature for 24 hours or longer to solidify and to remove excess moisture. Package plates in sealed plastic bags and refrigerate upside down until use to prevent condensation.

Taking the Samples

Bacteriological samples may be collected from a number of places: the seawater source, any intermediate seawater holding tanks, processed seawater as it is utilized, neutralizing solutions, algal stocks, expanded algal cultures, and larval and spat tanks. We will go through sample collection to determine the presence and relative abundance of bacteria for a qualitative analysis, not to develop actual quantitative results. A simple semi-quantitative presence or absence of vibrios on selective or non-selective mediums will enable you to monitor bacteriological levels in your own hatchery and allow you to determine the next diagnostic step, if needed.

Identify bacteriological problems and how to locate them by process of elimination and systematic sampling:



Schematic diagram of intensive hatchery and nursery production of molluscan shellfish with notes regarding health management. *Adapted from Elston & War (2003).*

Container Surface Samples

Using a sterile cotton swab, collect samples from a defined square (1-2 cm on a side). This must be an area which has been removed from the water but not dried. Either drain tanks down slightly or use standard sheets of fiberglass, glass, or similar material which can be removed easily from tanks for surface sampling. Dilute sample by placing the swab in a sterile microcentrifuge tube containing 1.0 mL sterile seawater or 2.0% sterile saline solution and vortexing or vigorously swirling. With a sharpie marker label and date the bottom of the TCBS agar plate. Streak the sample rinse from the swab onto a TCBS agar plate using a 0.01 mL (10µL) inoculating loop, this will give you a 1:1,000 dilution. Alternatively, remove a 100 µL sample with a sterile pipette and use a spreader to cover the agar with the sample. This will give you a 1:100 dilution. Allow plate to remain upright with the cover on to dry for a few minutes, seal plate with paraffin and then incubate plate upside down at room temperature. Examine in 24-48 hours.

Aqueous Samples

With a sharpie marking pen, draw a solid line down the middle of the outside of the bottom of a TCBS plate and label one half 10^{-1} and the other half 10^{0} along with the sample name and date. Label an empty sterile microcentrifuge tube 10^{0} and add approximately 1 mL of any aqueous solution sample (e.g. water, algal culture). Make a 1:1,000 dilution by transferring a sterile inoculating loop sample (10 µL) to 1 mL of sterile seawater in a microcentrifuge tube labeled 10^{-1}. Gently swirl the inoculating loop in the dilution tube, close the cap and flick the bottom of the tube several times to mix. Using the inoculating loop, streak a sample of the 10^{-1} dilution onto the labeled 10^{-1} half of the plate and then streak a sample of the 10^{0} dilution onto the labeled 10^{0} half of the plate. This will give you a 1:100 dilution. *It is important to streak from the most dilute sample to the least dilute sample if using the same inoculating loop.*

Alternatively, simply label the bottom of the plate 1:10 and remove a 100 µL sample with a sterile pipette and use a spreader to cover the agar with the sample. This will give you a 1:10 dilution. Allow plate to remain upright with the cover on to dry for a few minutes, seal plate with paraffin and then incubate plate upside down at room temperature. Examine in 24-48 hours.

Algal Stock Culture Samples

With a sharpie marker label and date the bottom of the TCBS or Marine agar plate. Using asceptic techniques, remove a sample from an algal stock culture using a sterile 0.01 mL (10µL) inoculating loop. Using a loop, streak the sample onto a labeled TCBS and/or Marine Agar plate. This will give you a 1:100 dilution. Alternatively, using a 0.100 mL pipet (100µL) transfer the culture to a labeled TCBS or Marine Agar plate, and use a spreader to cover the agar with the sample. This will give you a 1:10 dilution. Allow plate to remain upright with the cover on to dry for a few minutes, seal plate with paraffin and then incubate plate upside down at room temperature. Examine in 24-48 hours.

Interpretation

After the 24 or 48 hour incubation period it is time to examine the plates. Count each individual colony and multiply by the dilution factor. For example if you count 20 total colonies and you diluted your sample 1:10 your actual count is 20x10=200 colony forming units (CFU) per ml. Make separate counts and determinations of the number of yellow and green colonies on TCBS plates.

It is not uncommon to have some level of bacteria in your hatchery. So, what numbers can you expect to see under normal conditions? Naturally we do not want to see anything in our incoming water or in our algae cultures, however we expect to find some bacteria in tanks.

<u>Incoming water</u>: The amount of general bacteria in your incoming water will depend on the source of that water and type of pretreatment. Well water will typically have a much lower level of environmental bacteria (and unlikely to have any pathogenic bacteria) compared to water pumped in from a body of water such as the Indian River Lagoon. Ideally you **do not want any *Vibrio* in your system water**. Monitoring your incoming water at specific points will alert you to the need to clean reservoirs, hoses, and replace or clean filters and UV bulbs.

Algae cultures: It is not uncommon to find bacterial growth (as seen on Marine agar plates) in algae cultures, particularly as the cultures are expanded. However **any level of *vibrio* is unacceptable in algae cultures** that are being fed to larval bivalves.

Tank culture water: It is not uncommon to have counts as high as 1,000,000 CFU/mL of total bacteria (Marine agar plates). Anything over that number is unacceptable. Vibrio counts (yellow colonies) can be as high as 10,000 CFU/mL in healthy larval tanks. Vibrio counts (blue-green colonies) can be as high as 1,000 CFU/mL. Keep in mind that these are **maximum** numbers that have been reported in tanks of healthy larvae. Add a stressful situation such as high ammonia, poor quality algae, etc. and your larvae may easily succumb to vibriosis. Ideally yellow colony counts should be below 1,000 CFU/mL and blue-green colony counts 0.

Tanks & equipment: Bacteria adhere to surfaces. No level of *Vibrio* is acceptable on tank surfaces or equipment used to drain down larvae, including buckets, sieves & beakers. So chlorinate, rinse and scrub, scrub, scrub. Then test surfaces to see how well you scrubbed! The larvae themselves will harbor some bacteria that will be transferred to the tank culture water. Don't make it worse!

Some references:

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TCBS plates with sucrose utilizing isolates (yellow) and non-sucrose utilizing isolates (green):