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Appendix 1. Preparation of samples for diagnosis

A. Live samples

Obtain 3-5 or more diseased and moribund individuals, and an equal number of normal crabs from the pond and tank (Photo 1). Separately place crabs in boxes with moistened tissue paper for shelter during transport. Crabs can be transported even without water, but it is advisable to bring them to the laboratory early in the morning or late in the afternoon to avoid too much exposure to heat and prevent drying up.

B. Iced samples

Materials needed: ice packs, plastic bags, styrofoam box or other insulated containers

If delivery of live samples is not possible, samples can be sent iced or frozen (Photo 2). Obtain 3-5 or more diseased and moribund individuals, and an equal number of normal crabs from the pond and tank. Wrap individually in plastic bags to prevent water from damaging the tissues. Separate normal from diseased specimen. Place packed samples between layers of ice in a styrofoam box or other insulated containers. Specimens should reach the laboratory for analysis within 24 hours.

C. Fixed samples

Materials needed: Bottles with water-tight cap, fixatives (see Appendix 2), dissecting scissors, forceps, scalpels

If samples cannot be delivered to a laboratory within 24 hours, specimens should be fixed before submission. These specimens can be processed for histopathology only.

Put eggs and larvae in screw-cap containers. The number of specimens to be submitted should be based on the tabulated guides in page 8. For juveniles and adults, open the body (Photo 3) to allow the fixative to penetrate inner tissues. Place crab in a bottle or container. Then, add fixatives (Davidson’s fixative or 10% buffered formalin) at the ratio of 10 parts fixative to 1 part tissues (Photo 4). Containers should be sealed tightly to prevent spillage and evaporation of fixative.

D. Samples for diagnosis of associated viruses by PCR

Samples for diagnosis of associated viruses by means of polymerase chain reaction (PCR) test should be fixed in 95% ethanol. For large specimens, dissect the gills (Photo 5) and fix tissues in 95% ethanol. These samples should be submitted to a laboratory as soon as possible.
### Appendix 2. Fixatives and fixation procedures

**Formulae of Fixatives**

<table>
<thead>
<tr>
<th>A. Davidson’s Fixative</th>
<th>B. 10% Buffered Formalin</th>
</tr>
</thead>
<tbody>
<tr>
<td>For 1 Liter:</td>
<td>For 1 Liter:</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>Formalin</td>
</tr>
<tr>
<td>330 ml</td>
<td>100 ml</td>
</tr>
<tr>
<td>Formalin</td>
<td>Distilled water</td>
</tr>
<tr>
<td>220 ml</td>
<td>900 ml</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>Sodium phosphate,</td>
</tr>
<tr>
<td>115 ml</td>
<td>monobasic</td>
</tr>
<tr>
<td>Distilled water</td>
<td>Sodium phosphate,</td>
</tr>
<tr>
<td>335 ml</td>
<td>dibasic, anhydrous</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C. 95% Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>For 1 liter:</td>
</tr>
<tr>
<td>Absolute ethanol</td>
</tr>
<tr>
<td>950 ml</td>
</tr>
<tr>
<td>Distilled water</td>
</tr>
</tbody>
</table>

**Caution:**
- Store all fixatives in tightly capped containers and place unused fixatives in the refrigerator to prevent evaporation of water.
- Exercise caution when handling fixatives as they can be harmful when inhaled and toxic when ingested.
- Protect hands with gloves and use aspirators when dispensing them.
- Work in well ventilated rooms or under a fume hood to prevent lingering toxic fumes.

Web-based Resources

Appendix 3. Preparation of fresh mounts for microscopic examination

The simple techniques shown in Photos 1-6 allow microscopic examination of small live specimens like crab larvae and eggs, or small sections of gills, hepatopancreas, and other tissues.

Procedure:

1. Transfer small specimens like larva or eggs using a pipette
2. Cut-away or select samples to be examined
3. Place a drop of clean seawater on a slide
4. Place tissue sample on the slide with water
Cover with a glass coverslip if necessary

Place the slide on the microscope stage and adjust focus starting with the lowest power objectives before switching to higher magnifications.

Do not flood glass slides with too much water to avoid wetting the microscope stage and objectives. Seawater is corrosive.
Appendix 4. Techniques for isolation of microorganisms from various samples

A. Media for isolation of bacteria

Bacteria occurring at the sites of infection can be isolated using several types of culture media in plates (Photo 1). Dehydrated forms of media in powder or granular forms are commercially available from various suppliers.

Nutrient Agar with 1.5% salt

Preparation (for 1 Liter):

- Nutrient Broth: 8 grams
- Agar: 15 grams
- Sodium chloride: 15 grams
- Distilled water: 1000 ml

Weigh dry ingredients and put them in a heat resistant flask or bottle. Add distilled water. Cap lightly then sterilize by autoclaving at 121°C and at a pressure of 15 pounds per square inch (psi) for 15 minutes. After sterilization, allow medium to cool. Swirl bottle for thorough mixing before dispensing medium into sterile plates. Sea water may be used instead of distilled water. Do not add sodium chloride to the medium.

Thiosulfate citrate bile sucrose agar (TCBS)

Preparation (for 1 Liter):

- TCBS medium: 88 grams
- Distilled water: 1000 ml

Weigh dry powder and put in a heat resistant flask or bottle. Add distilled water. Cap bottle lightly, then sterilize by boiling in a water bath until TCBS agar powder is totally dissolved. After sterilization, allow medium to cool enough for easy dispensing into sterile plates.

Note: Since TCBS medium already contains sodium chloride, do not prepare this using seawater.

These media can be inoculated by spread plate or streak methods. The table below gives the interpretation of colonies on TCBS after 18-24 hour incubation. Bacterial colonies growing on this medium are in Photos 2 and 3.

<table>
<thead>
<tr>
<th>Appearance of Colonies</th>
<th>Microorganisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flat, 2-3 mm in diameter, yellow</td>
<td><em>Vibrio cholerae, Vibrio cholerae</em> El Tor</td>
</tr>
<tr>
<td>Small, blue-green center</td>
<td><em>Vibrio parahaemolyticus, V. harveyi</em></td>
</tr>
<tr>
<td>Large, yellow</td>
<td><em>Vibrio alginolyticus</em></td>
</tr>
<tr>
<td>Blue</td>
<td><em>Pseudomonas, Aeromonas, and others</em></td>
</tr>
<tr>
<td>Very small, translucent</td>
<td>Enterobacteriaceae and others</td>
</tr>
</tbody>
</table>
B. Isolation of fungi from crab eggs and larvae

Media:

Peptone Yeast-extraxta Glucose (PYG) Broth

1.25 g Bacto-Peptone
1.25 g Yeast Extract
3.0 g Glucose
1.0 liter sea water

Weigh dry ingredients and put them in a heat resistant flask or bottle. Add seawater. Cap lightly then sterilize by autoclaving at 121°C and at a pressure of 15 psi for 15 minutes. After sterilization, allow medium to cool. Add 500 ug/ml (final concentration) of Penicillin and Streptomycin to inhibit bacterial growth. Swirl bottle for thorough mixing before dispensing medium into sterile plates.

Note

To prepare PYG agar, add 15.0 g of agar/liter of PYG broth.

Isolation of fungi from eggs/larvae

1. Examine freshly sampled eggs or larvae
2. Under a dissecting microscope, separate fungi-infected eggs or larvae from normal ones
3. Isolate one egg or one larva and place in a test tube with 10 ml PYG broth with antibiotic
4. Observe for mycelial growth in the next 3 days
5. Remove hypha from the tube and rinse with sterile sea water
6. Place hypha in a petri plate with ~10 ml sterile sea water
7. Incubate at room temperature
8. After 18-24 hours, examine sporulation of fungi
9. If sporulation occurs, pippet out 0.1 ml aliquot and place 0.5 ml of sample on PYG agar plate with antibiotic
10. Spread the drops on the agar surface by shaking the plate
11. Incubate plate for more than 2-3 days to evidently see isolated hyphal growth of germinated spores

Propagate fungus on solid medium by cutting agar blocks that contain hyphae and placing them on new PYG agar plates.

References


Appendix 5. Chlorination of water and rearing paraphernalia

Chlorine is a widely used disinfectant to kill most bacteria, viruses and other microorganisms. It is added to water as hypochlorite powder (70% activity) or solutions (such as Chlorox, Purex, with 5% available chlorine).

Disinfection of rearing water using calcium hypochlorite (70% activity)

1. For chlorination with calcium hypochlorite powder, use Table 1 to determine the required amount of bleach powder for the volume of rearing water. For example, if the water volume is 0.5 ton or 500 liters and the desired chlorine concentration is 15 ppm, the amount of calcium hypochlorite needed is 10.7 g. Dissolve this amount first in a small volume of water (500 ml).
2. For chlorination with ordinary household bleach (Purex, Chlorox, etc. with 5% available chlorine), use Table 2 to determine the amount of bleach to be used for the volume of water.
3. Fill the tank with the desired volume of water then add the calcium hypochlorite solution.
4. Allow chlorinated water to stand for at least 12 hours and up to 24 hours. Check the residual chlorine level by means of portable kits available in the market. Neutralize remaining chlorine with equal amount of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$), before using the water.

### Table 1. Guide for determining the amount of powder calcium hypochlorite to be added to get desired chlorine concentration for water disinfection

<table>
<thead>
<tr>
<th>Volume of Water</th>
<th>Amount (grams) of Calcium Hypochlorite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tons</td>
<td>Liters</td>
</tr>
<tr>
<td>------</td>
<td>--------</td>
</tr>
<tr>
<td>0.25</td>
<td>250</td>
</tr>
<tr>
<td>0.50</td>
<td>500</td>
</tr>
<tr>
<td>1</td>
<td>1,000</td>
</tr>
<tr>
<td>2</td>
<td>2,000</td>
</tr>
<tr>
<td>3</td>
<td>3,000</td>
</tr>
<tr>
<td>5</td>
<td>5,000</td>
</tr>
<tr>
<td>10</td>
<td>10,000</td>
</tr>
</tbody>
</table>

### Table 2. Guide for determining the amount of bleach solution (in milliliters) for water disinfection

<table>
<thead>
<tr>
<th>Volume of Water</th>
<th>Amount (ml) of Bleach Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tons</td>
<td>Liters</td>
</tr>
<tr>
<td>------</td>
<td>--------</td>
</tr>
<tr>
<td>0.25</td>
<td>250</td>
</tr>
<tr>
<td>0.50</td>
<td>500</td>
</tr>
<tr>
<td>1</td>
<td>1,000</td>
</tr>
<tr>
<td>2</td>
<td>2,000</td>
</tr>
<tr>
<td>3</td>
<td>3,000</td>
</tr>
<tr>
<td>5</td>
<td>5,000</td>
</tr>
<tr>
<td>10</td>
<td>10,000</td>
</tr>
</tbody>
</table>
Equipment and Materials

- Provide properly labeled materials like beakers, scoop nets, pails, etc. for exclusive use in individual tanks
- Materials like brushes, pails, scoop nets, water hoses, and glasswares that are used in different tanks may be disinfected between use in different tanks by dipping in 400 ppm chlorine and thoroughly rinsing with clean freshwater
- The same amount of chlorine can be used to disinfect contaminated rearing water and infected animals for disposal
- Disinfect tanks between rearing periods

References

Appendix 6. Disinfection and caring for berried crabs

1. Disinfect newly procured berried crabs in 150 ppm formalin bath for 30 minutes. See formalin solutions preparation guide below.

2. Place each berried crab in a 500-liter tank with aerated sea water.

3. Feed crabs with mussel meat, fish, marine worms, or squid at 10-15% of crab biomass daily. Remove uneaten feeds after 4 hours. Discontinue feeding on the day eggs become brown.

4. Siphon out detached eggs and excess food every day before water change. Change about 80% of the total water volume in the tank daily. Retain 20% of water in the tank to prevent egg desiccation.

5. Obtain a few eggs from the egg mass 2-3 times during the incubation period (9-14 days) to examine embryonic development and biofouling.

6. Apply 0.1 ppm Treflan (44% trifluralin) to water every other day to prevent fungal infection.

TABLE 1. Guide in the preparation of the formalin solutions

<table>
<thead>
<tr>
<th>Formalin concentration (ppm)</th>
<th>Volume of formalin solution (ml) per 10 liters</th>
<th>Volume of formalin solution (ml) per 100 liters</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>100</td>
<td>1.0</td>
<td>10.0</td>
</tr>
<tr>
<td>150</td>
<td>1.5</td>
<td>15.0</td>
</tr>
<tr>
<td>400</td>
<td>4.0</td>
<td>40.0</td>
</tr>
<tr>
<td>500</td>
<td>5.0</td>
<td>50.0</td>
</tr>
</tbody>
</table>

A 37-40% formalin solution should be considered as 100% stock. If a white precipitate forms, filter the formalin stock before use. Dilute the solution in sea water.

References


Appendix 7. Larval stages of mud crabs

- **Zoea 1: Day 0 - Day 4**
- **Zoea 2: Day 4 - Day 7**
- **Zoea 3: Day 7 - Day 10**
- **Zoea 4: Day 10 - Day 13**
- **Zoea 5: Day 13 - Day 17**
- **Megalopa: Day 17 - Day 23**
## Appendix 8. Scoring of extent of infestation or fouling

<table>
<thead>
<tr>
<th>Numerical Score</th>
<th>Microscopic findings*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No fouling organisms</td>
</tr>
<tr>
<td>0.5</td>
<td>A few scattered fouling organisms</td>
</tr>
<tr>
<td>1</td>
<td>Fouling organisms present, but not abundant (i.e., not covering or affecting more than 10 to 25% of the total area examined)</td>
</tr>
<tr>
<td>2</td>
<td>Fouling organisms common and affecting or covering at least 25% of the total area examined.</td>
</tr>
<tr>
<td>3</td>
<td>At least 50% of the affected part covered.</td>
</tr>
<tr>
<td>4</td>
<td>All or nearly all (75 to 100%) of the affected surfaces covered</td>
</tr>
</tbody>
</table>

*To be determined by microscopic examination (at 50-100×) of the lamellae of a gill or appendages in unstained wet mounts or in histological section

---


<table>
<thead>
<tr>
<th></th>
<th>Salinity (ppt)</th>
<th>Temperature (°C)</th>
<th>Dissolved Oxygen (ppm)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eggs</td>
<td>30-35</td>
<td>27-30</td>
<td>&gt; 5.0</td>
<td>7.0-8.5</td>
</tr>
<tr>
<td>Zoea</td>
<td>24 - 32</td>
<td>26-30</td>
<td>&gt; 5.0</td>
<td>7.5-8.5</td>
</tr>
<tr>
<td>Megalopae</td>
<td>20-25</td>
<td>26-30</td>
<td>&gt; 5.0</td>
<td>6.5-9.0</td>
</tr>
<tr>
<td>Grow-out</td>
<td>16-32*</td>
<td>18-30</td>
<td>&gt; 5.0</td>
<td>6.5-9.0</td>
</tr>
<tr>
<td>Broodstock</td>
<td>30-35</td>
<td>27-29.5</td>
<td>&gt; 5.0</td>
<td>6.5-9.0</td>
</tr>
</tbody>
</table>

* 16-32 ppt = *S. serrata*; 16-24 ppt = *S. olivacea* and *S. tranquebarica*

References


Hoang DD. 1999. Preliminary studies on rearing the larvae of mud crab (*Scylla paramamosain*) in south Vietnam, pp. 147-152. In: Keenan CP, Blackshaw A. (eds), Mud Crab Aquaculture and Biology. Proceedings of an international scientific forum held in Darwin, Australia, 21-22 April 1997. ACIAR Proceedings No. 78, ACIAR, Canberra, Australia


Appendix 10. External anatomy of a mud crab

Appendix 11. Procedures for sample collection of soils for analysis

Soils are analyzed to determine its physical and chemical properties which are important in the culture and production of various species. It may be done before and during pond operation.

Following are the procedures for soil sampling to determine pH, lime requirement, organic matter, available phosphorous, available sulfur (sulfate -sulfur) and iron.

Soil Collection From Fish Ponds

Proper collection and preparation of soil samples intended for analysis are extremely important. Correct interpretation of the tests can be made only when the samples are truly representative of the soil conditions in the field. Sampling is easy when the soil is moist. However, samples may also be taken when soil is dry or is naturally wet as in paddy fields.

Materials

Soil sampler = Core sampler or sampler made of bamboo or PVC pipes
Pail and plastic bags for collection and mixing

Procedure

1. Divide the fishpond into lots, as shown below:

   ![Diagram of fishpond lots]

2. Collect core samples up to 1.5 feet deep in representative areas with uniform slope, texture and depth
3. Brush away any stone, rubbish, decayed wood or trash before taking soil sample. In collecting a composite sample, each of the lots in the pond should be represented. Take similar samples in 9 or more points of each lot. Avoid taking directly from fertilized band sector or portion
4. Mix all ten samples to obtain about 10 kg of composite sample
5. Place in containers and label properly to include information like location, area, surface or subsoil, etc.

* Based on guidelines provided by the Centralized Analytical Laboratory, SEAFDEC Aquaculture Department
Preparation of Soil Sample

1. Spread out the soil in thin layer on a labeled strong board or polyethylene film in a room protected from sunlight, dust and wind
2. Break the soil occasionally to hasten the drying process. It takes 4-7 days to dry samples
3. Pulverize the soil using a ball mill or wooden mallet
4. Sieve the ground soil sample through a 2 mm sieve. Crush the clods that do not pass and re-sieve
5. Store the soil sample in properly labeled plastic bags or glass jars prior to analysis
6. Submit samples to an analytical laboratory nearest your farm site