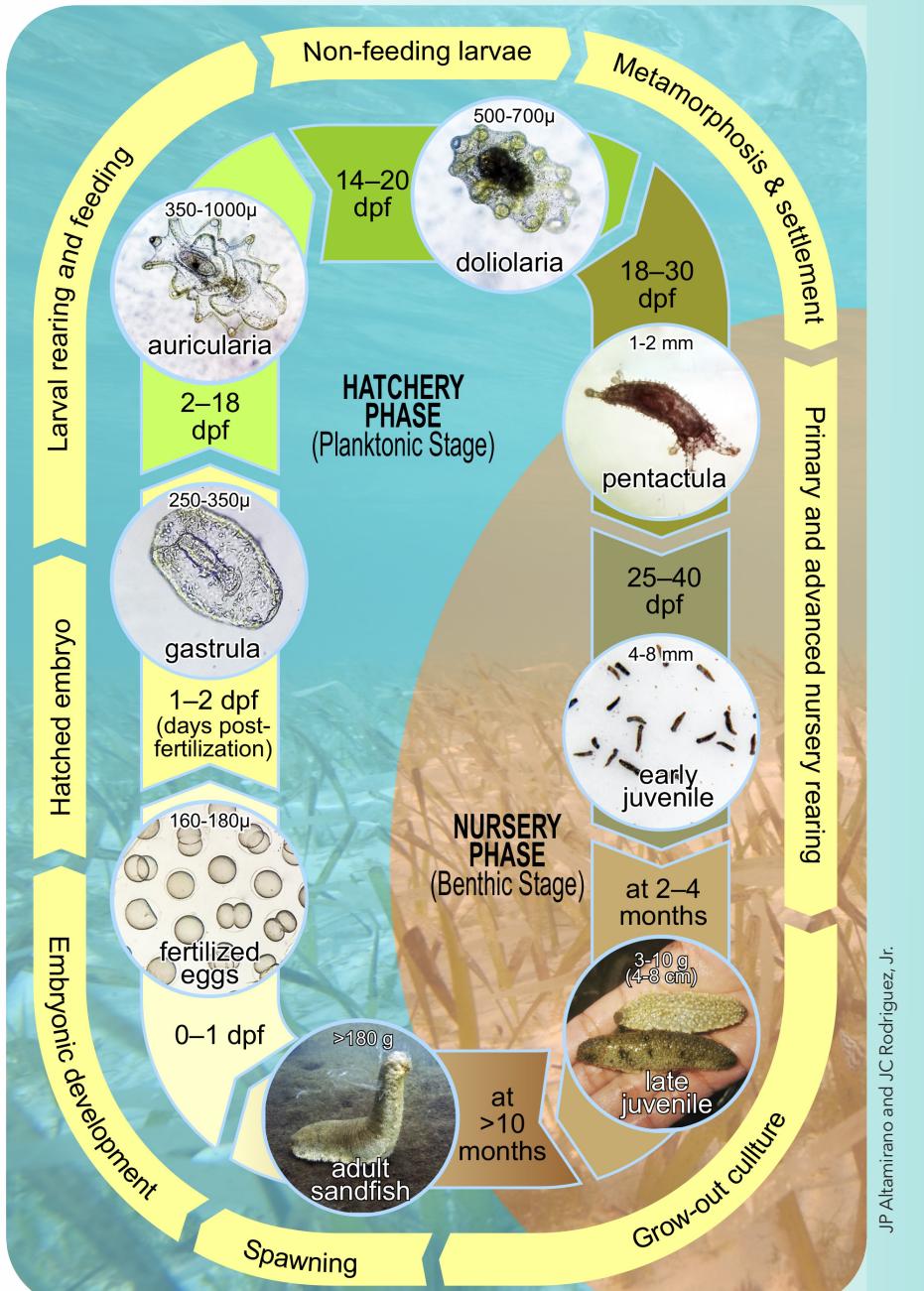


Production Phases and Life Cycle of SANDFISH *Holothuria scabra*



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AEM 48 Seed production of sandfish (*Holothuria scabra*) in Vietnam *Nguyen Dinh Quang Duy* (2010) An extension manual describing broodstock management, larval rearing, and management of nursery systems.

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SEA CUCUMBER Hatchery and Nursery Production



Southeast Asian Fisheries Development Center
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Why culture SEA CUCUMBER?

Sea cucumbers are highly valued marine commodities, with prices reaching up to US\$ 2,000 per kilo, when processed and dried into *trepang* or *beche-de-mer*. A great majority of traded sea cucumbers comes from wild harvest causing severe decline in stocks. Sea cucumber mariculture using hatchery-bred juveniles can offer an alternative income source especially for coastal communities, while protecting the remaining wild populations.



Expensive dried sea cucumbers for sale in Chinese markets

What is SANDFISH?

Sandfish is the common English name for one particular tropical species of sea cucumber called *Holothuria scabra*. It is one of the most threatened tropical species because of its high price and ease in collection. It is typically found in shallow intertidal sandy-muddy shores, commonly associated with seagrass beds and sand flats. Sandfish has one of the highest potential for aquaculture because hatchery production technology of this species is established. SEAFDEC/AQD is one of the leading institutions developing the production technologies for sandfish.



Sandfish *Holothuria scabra* in their natural habitat

The SEAFDEC/AQD Sea Cucumber Hatchery

The SEAFDEC/AQD maintains a small-scale sea cucumber hatchery facility for sandfish spawning, larval rearing and juvenile culture. Continuous life support systems like flow-through seawater and aeration are maintained. Natural microalgal food such as *Chaetoceros* sp. and *Navicula* sp. are produced within the facility as well. Various experimental research are being conducted here in order to enhance sandfish production.



SEAFDEC/AQD sea cucumber hatchery, established in 2010

How to breed SANDFISH?

Broodstock conditioning

Broodstock collected from the wild are conditioned in tanks with sandy-mud substrate and flow-through seawater. They are fed with a mixture of powdered *Sargassum*, formulated feed and *Navicula* sp. slurry. After spawning, they are returned to the field where they were collected for natural recovery.



Cleaning and preparation of sandfish broodstock for spawning induction

Spawning induction

A pre-defecated spawning group of 20-60 sexually mature sandfish are induced to spawn using non-lethal thermal and food stimulations. Males are expected to spawn first by releasing a steady stream of white milt with sperm through the gonopore – a small genital opening above the anterior or front end of the body. This may last up to 3 hours. Females spawn by releasing quick bursts of eggs after a characteristic bulging around the gonopore. Females typically perform 2-3 bursts at about 5 minutes interval. Although at SEAFDEC/AQD, we have recorded as much as 22 bursts from a single female within 1 hour.

Larval Rearing

Fertilized eggs are stocked in tanks filled with filtered and UV-treated seawater at 100-500 eggs/L at optimum temperature (26-30°C) and salinity (28-33 ppt). Auricularia stage larvae are fed daily with *Chaetoceros calcitrans*. Water exchange (20-50%) is done every two days while siphoning out wastes from the tank bottom. At Doliolaria stage, corrugated plastic sheets painted with *Spirulina* paste are added into the rearing tank to induce settlement. Metamorphosed Pentactula are fed with *Navicula* sp. slurry.



Larval development monitoring (left)



Preparation of settlement plates (right)

Nursery Rearing

Post-metamorphic or early juvenile sandfish (4-10 mm), at 30-45 days old, are transferred from larval tanks in the hatchery into floating hapa nets (1 m x 2 m x 1.2 m) in sea-based or tank-based nurseries. Nursery hapas are made of fine-meshed (>1 mm) net suspended with a floating PVC frame. In good sites, sandfish juveniles grow to 2-4 g within 1-2 months, depending on season and sea conditions. At this size, they are ready for advanced nursery rearing in pens or ponds.



Sandfish nursery pen in a protected cove at SEAFDEC/AQD's Igang Marine Station in Nueva Valencia, Guimaras, Philippines

SANDFISH

ATTRIBUTES

- Sandfish is an Echinoderm.
- There are more than 1000 species of sea cucumber known to exist.
- The sea cucumber *Holothuria scabra* has an elongated body with leathery skin whose color ranges from gray to black.
- They live in sandy-muddy substrate and feeds on organic matter which is mainly composed of mud or sand, bacteria and benthic algae.
- Males and females are not distinguishable unless they have spawned.
- At least 100 species are found in the Philippines, 25-30 species are commercially traded.
- They can be found throughout the shallow tropical waters of the Indo-Pacific region.



WHY VENTURE INTO SEA CUCUMBER FARMING?

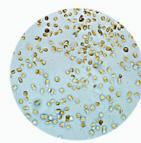
- Stock enhancement is one major reason of farming.
- Bech-de-mer , trepang or dried sea cucumbers are being exported to Asian markets due to its pharmaceutical, nutraceutical and culinary uses.
- It is being sold at price ranging from 1000 to 6000 pesos per kilogram.

THE HATCHERY

1

NATURAL FOOD | Phytoplankton Culture

Phytoplankton species commonly cultured:
Chaetoceros gracilis
Chaetoceros calcitrans
Isochrysis galbana
Navicula ramosissima



Mass culture of Microalgae

- Prepare 180 Liters UV filtered sea water in clean containers.
- Fertilize with: 200ml manusol, 200 ml Algafer and 200 ml sodium silicate.
- Pour 20L of inoculants into the fertilized sea water.
- Harvest cultured microalgae during its peak exponential growth when cells are of good quality which is 3 days after culturing.

The techniques and methods used at GMFDC Sea Cucumber hatchery are based on the protocols of University of the Philippines Marine Science Institute.

2

BROODSTOCKS | Collection and Selection

Sea cucumber broodstock may be bought from local dealers or collected from the wild. It should weigh 200 to 500 grams. Broodstock should be healthy, undamaged and with no visible skin lesion.



Management and Maintenance

- Sea cucumbers can be held in tanks, sea pens or ponds.
- The stocking density should be <500g / m².
- Blended shrimp starter feed is used at 1-1.5g/m³.
- Broodstock held in pens, ponds or tanks for several months are easier to spawn than the ones taken directly from the wild.

SPAWNING | Induction Method

Spawning is usually done 3 to 2 days before full moon or new moon of the month. The hatchery uses the combined effect of dry treatment (desiccation) and food shock technique using *Spirulina*.

Dry Treatment

- Broodstock are placed in a tank without water and left to dry for 45 minutes.

Spirulina Bath

- After dry treatment, the tank (or basin) is filled with sea water and broodstocks are immersed.
- Blended *Spirulina* powder (15 grams) is then added.
- Allow 45 minutes of bathing.

3

Spawning & Egg Collection

- Broodstocks are allowed to spawn in tanks.
- Spawning usually takes an hour or more.
- Sperms appear whitish while eggs appear in orange.
- Collect eggs separately in a large container and sperms are added to fertilize them.
- Study eggs under a microscope to determine if fertilization has already occurred.



4

LARVAL REARING

Sandfish transforms into different stages as it develops. Below is a table showing the different stages and the appropriate management techniques to be applied during a specific larval stage. Larval tanks with sand-filtered seawater and aeration system, and natural food are basic requirements in larval rearing.

No. of Days	Stage	Feeding	Water Management
0	Fertilized egg	Non-feeding stage	No water exchange.
2-5	Early Auricularia	After 2 days, larvae are fed with microalgae (Cgr-Cc) with concentration of 20,000 cells/ml.	50% water exchange is done every 2 days.
7-13	Late Auricularia	Microalgal feed (Cgr-Cc) concentration is increased to 40,000 cells/ml	50% water exchange is done every 2 days.
13-18	Doliolaria	Doliolaria larvae are non-feeding stage. However, feeding is maintained since larvae do not metamorphose evenly.	50% water exchange is done every 2 days. Tanks are provided with corrugated plastic plates smeared with <i>Spirulina</i> .
18-25	Pentactula	Pentactula larvae are fed with Cgr-Cc combined feed.	50% water exchange is done every 2 days.
25-30	Juvenile	Juveniles are transferred intanks with flow-through system where water is actively replenished.	

5

JUVENILE REARING

When juveniles reach 1-2mm (23 - 35 days after fertilization) they are transferred from larval tanks to floating hapa nets. Growth and survival of juveniles are very density-dependent and thus grazing area is an important consideration in nursery rearing.

Primary Nursery

- Juveniles are carefully harvested and detached from the larval tanks by siphoning them or individual picking.
- Stocking density: 1000 first stage juveniles per hapa net.
- Juveniles are regularly monitored.



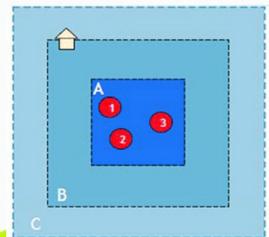
Secondary Nursery

- After a month in primary nursery, the juveniles are thinned-out and transferred in the secondary nursery hapa/ floating hapa.
- Stocking density: 500 second stage juveniles per hapa net.
- Floating hapas are regularly monitored and cleaned.
- From ocean nurseries/ floating hapa, juveniles (20 mm or >1g) are harvested after two months and transferred to sand-conditioned tanks in the hatchery.
- Muddy-sandy substrate collected from sites that supported wild sandfish population is used in sand conditioning.

GROW-OUT CULTURE

After sand-conditioning, juveniles are transferred for grow-out culture in a sea ranch. Sea cucumbers are allowed to grow for several months until they reach at least 300 g for harvest.

The ranch has the following zones and dimensions as suggested by the UP MSI:



6

- Zone A is 50 x 50 m with 3 100 sq. m. circular monitoring pens.
- Zone B is 1-hectare nursery area. This is for sea cucumber monitoring only.
- Zone C is a 5-hectare buffer area. No-take area for sea cucumbers.



This information is made for you by the



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**National Fisheries Research and Development Institute
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SEA CUCUMBER CULTURE

Hatchery Techniques and Protocols of the Seed Production
of the Sea Cucumber *Holothuria scabra*
at GMFDC, Guiuan Eastern Samar.

2017 ISSUE

Hatchery Manual for Sea Cucumber Aquaculture in the U.S. Affiliated Pacific Islands



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Masahiro Ito

Hatchery-Based Sea Cucumber Farming

It is common knowledge that aquaculture farms will result in employment opportunities for island communities and provide potential source for exports. A hatchery-based sea cucumber production is to make available for stock enhancement program and for aquaculture-based farming enterprises. The author (Masahiro Ito) is an independent sea cucumber hatchery consultant and a former director of aquaculture research and extension of COM. He has proprietary technology in possession which can significantly boost the sea cucumber juvenile production in the hatchery. He has agreed to write this manual to contribute to the benefit of the U.S.-Affiliated Pacific Islands and its future industry development. The main objective is to provide the advanced methodologies and to improve the hatchery technology for the holothurian sea cucumbers, particularly the sandfish (*Holothuria scabra*) in the U.S.-affiliated Pacific islands.

Status of World Sea Cucumber Trading

The sandfish sea cucumber business was once prosperous and has been a valuable source of income for decades in the tropical and subtropical coastal communities, but it was based on “boom and bust” business resulting over-fishing to the level of extinction of this high-valued species. Similar phenomenon on almost all sea cucumber fishery have occurred worldwide. Despite of these facts, a sustained demand for bêche-de-mer (processed sea cucumbers) from China and other Asian sea food markets has pushed up the price of this favored *holothurian* sea cucumber species. Most of the sandfish product which has been regarded as one of the most valuable tropical sea cucumber is traded and sold in the dried form in the Asian market mainly in Hong Kong where the products are distributed into mainland China. Dried sea cucumbers are brought from all over the world to be bought and sold in Hong Kong. Traders and wholesalers are located along Nam Pak Hong Street in the Sheung Wan area in the north-west of Hong Kong Island. Hong Kong and Guangzhou in Guangdong province, China, have been tightly connected since the birth of Hong Kong in the 19th century. Through this channel, most of the dried marine products imported into Hong Kong are re-exported to Guangdong, from where they are traded throughout China. Currently, retail prices of the sandfish in Hong Kong are from around US\$50 for the low quality with small sized products to US\$300 per kg for high quality with larger size and the highest quality sandfish fetches between US\$500 and US\$800 per kg. The “Australian” or “Australian-made” sandfish have always been regarded as the highest quality and price in Hong Kong wholesale and retail markets.

This hatchery manual includes the following topics; i.e. broodstock management and juvenile production work of the sea cucumber sandfish, notes on microalgae culture, complete larval development as well as descriptions of post-larvae and juveniles of the sandfish and the black teatfish (*Holothuria whitmaei*):

- 1) quarantine culture of the broodstock, recovering them from spawning stress and conditioning for spawning induction by using down-weller “Habitat Simulator” system;
- 2) microalgae culture of benthic diatoms and knowledge of heterotrophic algae (micro-organisms) for feeding the settled pentactula and early juvenile stages;
- 3) spawning induction methods with disinfection of spawners, fertilization, collection and incubation of eggs;
- 4) larvae rearing including specific knowledge of feeding capability of larval stages and combination of feed, calculating amount of larval feed mix, controlling algal cell suspension, adjusting feeding amount and rearing water volume, and knowledge of optimal larval development by expecting

- proportions of larval and post-larval stages between day-1 and day-11;
- 5) settlement techniques including preparations of settlement plates and tanks, maintenance of benthic diatom culture and water quality, nutrient media preparation and culture techniques of benthic diatoms and/or naturally occurring epiphytes, knowledge on the types of benthic diatoms (*Navicula* sp. & *Cocconeis* sp.) and symbiotic heterotrophic micro-organisms in the mangrove ecosystem for feeding pentactula and early juveniles;
 - 6) culture of pentactula and early juvenile stages in the settlement tank (the 1st phase nursery culture) from day-11 to day-56 or 8 weeks after spawning (approximately 2-month-old juveniles of 6 – 15 mm, 0.2 – 1g size), including calculation of feeding mix amount for the juvenile culture and preparation of feed mix;
 - 7) grow-out culture using the down-weller “habitat simulator” tanks from day-56 or 8 weeks (onset of the 2nd phase nursery culture) until 5-6 months old (approximately 20 – 50mm, 5 – 20g size).

Broodstock Management

Elsewhere, the sandfish broodstock are usually held either in FRP (fiber-reinforced plastic) raceways, concrete tanks or earthen ponds for spawning work (Figs. 1a-c). The COM’s hatchery in Pohnpei, Federated States of Micronesia, uses freshly caught sandfish broodstock from nearby its hatchery a day prior to the spawning induction work without doing any conditioning work.



Figure 1a. Habitat simulators.



Figure 1b. Concrete tanks.



Figure 1c. Earthen ponds.

The sandfish habitat is characterized by a seagrass bed of the tidal flat along the mangrove-covered shoreline from low-tide line to 10 – 20m deep in subtidal zone with soft muddy or sandy substrate. Seagrass bed is characterized by turtle-grass such as *Thalassia* spp. or by eel-grass such as *Zostera* spp. in the Indo-Pacific region (Fig. 2a-b). It is said that stocking density of the sandfish grow-out in a pond is one or two animals per square meter and the broodstock may be stocked at 3 – 5 per m⁻² in a tank for conditioning if they are provided good aeration, water flow (water exchange rate at 400% per day), ample feeding with periodic tank cleaning at least once a fortnightly or renewal of tank with fresh muddy sand substrate (Duy, 2011; Purcell et al., 2012).

A key technological innovation developed by the author is a land-based broodstock culture system with a “down-weller” or “habitat simulator” tank system. The system uses a combination of closed recirculating seawater and partial flow-through method, which enables a long-term holding and domestication of healthy broodstock for selective breeding programs rather than relying on wild-caught parents on each hatchery operation. The tank system holds 5 - 10 broodstock per m⁻² by providing with good air, water circulation (100% daily water exchange) and enabled to feed without periodic tank cleaning or renewal of

Sandfish Holding Tank for Broodstock & Juveniles

combined partial flow-through + closed re-circulating seawater

“Habitat Simulator”

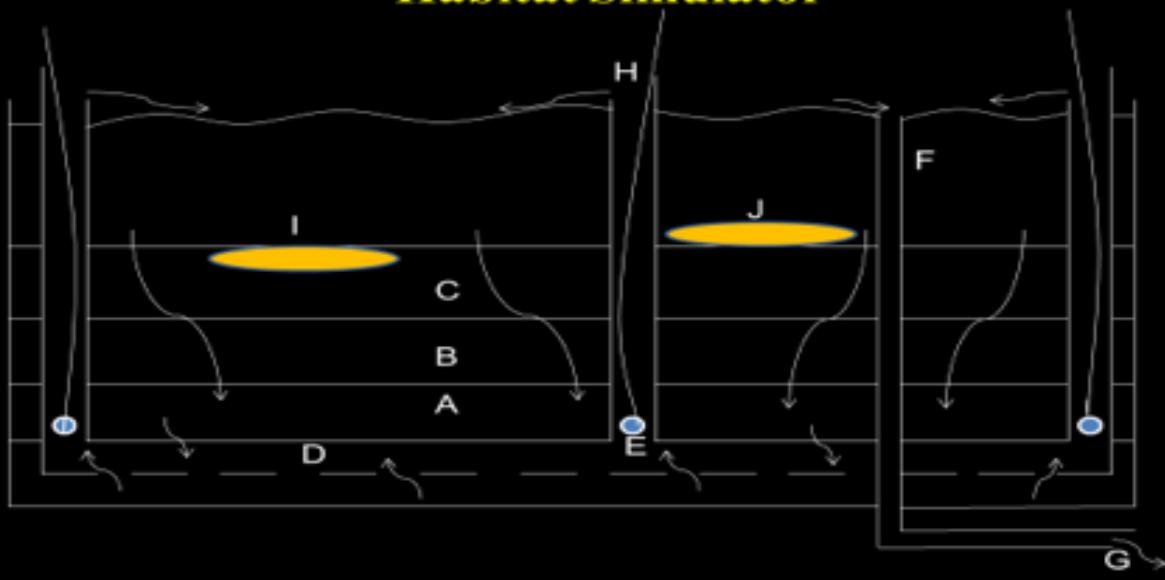


Figure 3. Diagram of down-weller “Habitat Simulator” tank. A: coral rock, B: coral gravel & sand, C: fine sand & silt, D: perforated pipe, E: air stone, F: overflow standpipe, G drain, H: airlift pipe, I & J: sandfish

tank. The down-weller was consisted of two or three layers of substrates to form a false-bottom structure; sand and mud, which are collected from tidal flat areas in the mangrove shore. Water re-circulates through the surface muddy and sand layer by air-lift pump which also maintains aerobic condition of the tank bottom and substrates (Fig. 3).

For quarantine purpose, an Australian private hatchery uses this tank system to prevent spreading potential disease among the wild-caught or domesticated broodstock (Fig. 4). Furthermore, this system has been used for recovering the spawners which had been injured or stressed during the transportation and/or spawning induction work. In Pohnpei of the Federated States of Micronesia, the COM built 2,500L rectangular tanks with down-weller system were made for broodstock recovering (Fig. 5) and juvenile grow-out (Fig. 6). Routine maintenance of the down-weller system for the broodstock is to: 1) avoid the macro-green algae (e.g. *Enteromorpha* spp.) over-grown on the tank surface; and 2) adjust aeration so as not to give strong air to drag too much seawater into the perforated piping system. To prevent green algae over-grown, use shade-screen to cut too much sunlight onto the tank. Continuously strong air-lift causes hardening the sandy substrates from strong downward water movement.



Figure 2a. Seagrass beds of turtlegrass (*Thalassira* species).



Figure 2b. Seagrass beds of eelgrass (*Zostera* species).



Figure 4. Down-wellers for commercial



Figure 5. Quarantine and recovery for broodstock in Pohnpei, Micronesia.



Figure 6. Down-wellers for juvenile grow-out in Pohnpei, Micronesia.



Figure 7a. Transporting in bags.



Figure 7b. Selecting broodstock for spawning.

1-1. Feeding broodstock

The broodstock in the “down-weller” tanks are fed daily with 1~2 % BW (body wet weight) per individual depending on the purposes; i.e. grow-out or quarantine at 1%, fattening or recovery at 1.5~2%. For practical reason, it is recommended to feed them weekly. The feed consists of dried alga Spirulina, fishmeal and seaweed of which ratio varies depending on the sizes and conditions of the animals; approximately 1 vs. 20 vs. 10. Mud/silt collected from tidal flat zone of the mangrove-covered shore is also included as an important food for both broodstock and juveniles. The amount of mud is equivalent to a total weight of other three foods. The amount of food should be adjusted by increasing or decreasing according to their average body weight. Therefore, the body weight needs to be measured at least monthly or bi-monthly. Feeding the broodstock in the Habitat Simulator can be done without a renewal

of sandy and/or muddy substrates because this down-weller system itself contains organic matters such as seaweed and detritus. Currently with no mortality has been recorded by using with a combination of dried seaweed, fishmeal and mangrove-silt.

1-2. Transporting broodstock

When transporting broodstock for a long distance (4 hours or more) from wild habitat to the hatchery, the animals should be packed individually in a plastic zip-bag in a polystyrene box or ice-chest (Fig. 7a-b). It is better using ambient seawater the same water collected at the habitat and better inserting ice-gel pack (s) in the box to keep temperature at lower than 25 °C during the transportation. If the animals are found eviscerated (vomited the gut/internal organ), they should be removed from the spawners and held in the recovery-fattening tank for at least six months period for the next spawning work.

2. Microalgae Culture Management

For microalgae culture of benthic diatoms refer to “Trainer's manual for hatchery-based pearl farming” (Ito, 2005), “Development of pearl aquaculture and expertise in Micronesia” (Ito, 2006) or “A hatchery operations manual for rearing sandfish, *Holothuria scabra*, in Tarawa, Republic of Kiribati.” (SPC, 2015). Detailed descriptions of the microalgae culture management and techniques had been written by the author during the COM Land Grant Program's pearl project in 2001-2013, which offered basic but practical knowledge on the microalgae culture in the tropical conditions.

2-1. Precaution for microalgae culture

- soak in freshwater and wash with detergent, brushing off dirt/wastes. Although it is not always necessary, hydrochloric acid (5 - 10% HCl solution) can be used for cleaning flasks by soaking when the dirt is difficult to clean off. Collect the used hydrochloric acid in a glass bottle for re-use.
- rinse with freshwater 5 - 10 repeats, completely wash off residue of detergent or chemicals.
- dry flasks upside down and avoid air-born dirt inside the flask.
- spray alcohol (isopropyl-alcohol or ethanol 75 % solution), rinse with distilled /filtered rainwater, and wait for dry upside down.
- put the lid on (aluminum foil) or place them in a dust-free cabinet for longer storage.
- rinse with filtered (0.2 µm or 1µm) seawater and, if available, UV-sterilized seawater before use.
- make sure washing your hand, particularly dirty finger nails and oily fingers, with soap and rinse off any residue of soap/chemicals, and then spray alcohol before commencing work.
- spray alcohol on the surface of culture flasks/containers/fittings/ working bench when entering the room.
- keep the floor and bench clean and dry and, if necessary, clean a floor with chlorinated freshwater.
- soak your feet in the chlorine bath before stepping into the room.
- periodically check and clean air filter/air outlet of air pump, air-conditioner and ventilator.

- always keep the room door/windows closed and avoid unnecessary entry into the room.

The hatchery staff tend forget general precautions for the microalgae culture work and how to properly operate the autoclave. Usually, hatchery operation elsewhere uses 121 °C for 45-60 minutes for larger flasks such as 3~5L high density culture, and small 100~250mL flasks for stock culture are sterilized for 10-15 minutes at 121°C. Periodical maintenance of autoclave is also necessary by changing or refreshing water in the chamber.

2-2. Culture methods for the sea cucumber hatchery

Sea cucumber hatchery work involves microalgae culture of planktonic and benthic diatoms. The author also uses mud/silt collected for the tidal flat zone of the mangrove shore for feeding the settled pentactula and early juvenile stages as well as broodstock. This kind of mud contains nutrient rich, particularly Omega-3 ($\omega 3$) fatty acids, derived from heterotrophic algae (micro-organisms).

Live microalgae are not required for feeding broodstock (adults) of the sea cucumbers. During the larval and post-larval rearing, however, live and/or dried microalgae are used toward settlement stage (pentactula stage) and after settlement to juveniles. The author simplified feeding methods for the larval rearing to reduce workload of culturing live microalgae. With higher survival rate at 30 - 40 % from day-1 to the settlement stage, the author has been using a single live planktonic diatom species of *Chaetoceros muelleri* together with dried form of microalga, *Spirulina* sp. For settlement phase and post-settlement rearing of juveniles, the author developed to use two kinds of live benthic diatoms (*Navicula* sp. and *Coconeis* sp.) by combining with dried *Spirulina* during the pentactula and early juvenile stages and during the juvenile stage by combining fishmeal, seaweed, *Spirulina* and tidal flat mud. Note that there are eight types of benthic diatoms and *N. ramosissima* (Type-A benthic diatom) and *C. scutellum* (Type-B benthic diatom) are commonly used at abalone hatchery for post-settlement juvenile culture in Japan (Kawamura, 1998). The author has been using two types as live epiphytes on the settlement substrates for the sea cucumbers, such as *N. jeffreyii* for type-A and *Coconeis* sp. for type-B. Master stock culture of these benthic diatoms can be purchased commercially such from Commonwealth Scientific Industrial Organization (CSIRO) in Australia or elsewhere. Culture media of these benthic diatoms or naturally occurring epiphytes are same as planktonic diatom such as *C. muelleri* with nutrient media strength varies from 1/100th to 1/10th. Starter high density (3L - 5L flasks) & mass culture (20L carboys - 100L polycarbonate tanks) are used for the above three diatom species. For these benthic diatom culture techniques and work plan, refer to Chapter 4 (Larval Rearing) and 5 (Settlement Techniques).

For specific knowledge of heterotrophic algae, refer to some of many publications such as “*Schizochytrium limacinum* sp. nov., a new thraustochytrid from a mangrove area in the west Pacific Ocean” (Honda et al., 1998), “Fatty acid composition and squalene content of the marine microalga *Schizochytrium mangrovei*” (Jiang et al., 2004), “Effects of dried algae *Schizochytrium* sp., a rich source of docosahexaenoic acid, on growth, fatty acid composition, and sensory quality of channel catfish *Ictalurus punctatus*” (Li et al., 2009), and “Heterotrophic cultivation of microalgae as a source of docosahexaenoic acid for aquaculture” (Taberna, 2008).



Figure 8a. Cold water treatment using ice cubes.



Figure 8b. Cold water treatment in algae room.



Figure 9a. 1ppm iodine bath.



Figure 9b. Disinfecting (1 min.)



Figure 9c. Rinse with freshwater.



Figure 10a. Thermal shock.



Figure 10b. Gently stirring.



Figure 10c. Siphoning droppings.



Figure 11a. Spirulina bath (12g/60L seawater)



Figure 11b. Monitoring water temp.



Figure 12a. Spawning.



Figure 13a. Collecting eggs.



Figure 13b. Sampling for counting eggs.



Figure 13c. Cutting broodstock.



Figure 13d. Stripping gonads.



Figure 14. Incubating eggs in 1,000L

3. Spawning Induction

Spawning induction work involves; conditioning and disinfection of spawners; inducing by stimulations or stressing such as exposing to the air, changing water temperature, water pressure and/or salinity, and chemical or food; fertilization and washing of eggs; and collection, sampling, counting and incubation of eggs (see Figs 8-14). When using a 2,500L tank for a small-scale juvenile production work, about 50 - 60 broodstock (spawners) are used for single larval run. Prior to spawning induction work before transferring from cold water treatment to spawning tank, all the spawners are disinfected by iodine, in which the animals were immersed in 1 ppm iodine bath (freshwater) for 1 minute. Spawning induction are usually done by: 1) stress by handling with exposure to the air, 2) thermal shock from cold (20-22 °C) to warm water (32-34 °C), 3) chemical stimulation by dried microalga Spirulina (20g/100L) in seawater for 30 minutes, 4) changing water pressure (decreasing/increasing water level), and/or changing salinity (decreasing salinity to about 30 ppt).

Collection of the spawned eggs are usually two-step approaches; 1) the first batch by scooping the spawned eggs by beakers and 2) the second batch by draining spawning induction tank. For a small-scale work, the former method is better to obtain cleaner with enough number of eggs. This also requires careful and continuous observation of female spawning posture. Noticeable change is observed in gonophore shape by swelling outwardly. Therefore, swift and timely scooping actions to collect eggs are required. If the latter method is used, collection of eggs should be commenced soon after several females spawned before the spawning tank becoming cloudy from too many sperms.

For incubating the fertilized eggs, stocking density should be less than 10 eggs per mL. Seawater is filtered to 1 µm by using filter-bags or cartridge filters, which does not necessarily required sterilization by in-line UV sterilizer unless virus infection or other disease has been reported from the surrounding environment. A combination of plankton screen (50 µm and 80 µm or 90 µm) is essential for collecting eggs.

3-1. Spawning procedures

The following describes timeline of spawning induction work which is based on a combination of 1) physical stress; 2) exposure to air, 3) thermal shock from cold to warm water, 4) chemical (dried algae Spirulina-bath) and/or 5) changing water pressure.

- Start preparing boiled seawater in deep pan (20-40L) using firewood (or use immersion heaters in the spawning tank) to maintain the induction tank water temperature at 33-34 °C.
- Cleaning off dirt from the body surface, measuring body weight (BW) and selecting spawners to be at least 200g, so the smaller ones should be returned to the broodstock holding tank. Before transfer to a cold water, quickly rinse with filtered seawater.
- Prepare cold seawater (1 µm filtered) beforehand, in the preceding day by placing it in the algae room. Transfer spawners to 100L cold treatment tank at about 20-22 °C and keep them for at least 2~3 hours, preferably for overnight.
- Transfer spawners to iodine (freshwater) bath at 1ppm of iodine (or 100ppm of Betadine®*) for 1minute (= 60 seconds). *Betadine® contains 1%W/V iodine. Therefore, 1g Betadine contains 0.01g iodine. To make 1ppm iodine solution (or 0.1g iodine per 100L), add 10g Betadine® per 100L to make 1ppm iodine (freshwater) bath.
- Start spawning induction work immediately after the iodine bath, rinse off iodine with filtered seawater and transfer to the spawning tank (2,500L raceway with approximately 1,000L water volume) at 33-34°C.
- Wait spawning (male and female) for at least an hour and keep cleaning droppings on the tank floor by siphoning. Use a plunger to stir gently spawning tank water and keep mixing the warm water.
- If the spawners dose not respond to the above thermal shock, transfer them to “Spirulina bath” for 30 minutes at 12g of Spirulina in 60L of filtered seawater. Spirulina is dissolved faster and better in freshwater (or rainwater), so prepare 12g Spirulina in about 500mL rainwater before making 60L seawater solution.
- Rinse off the Spirulina with filtered seawater and introduce them again to the spawning tank.
- Wait for the spawning. Males usually spawn before females release eggs.
- Observe spawning posture of female(s) and scoop the eggs with beakers when the female releases the eggs. If excess eggs are needed after confirming the females finished spawning, drain the spawning tank to collect remaining eggs inside the tank. Use a combination of 50 and 80 µm-pore size mesh screen to collect and wash the eggs. If no female responded after two hours, return all the spawners to broodstock holding tank.
- After washing/rinsing off sperms for 10-20 minutes, transfer the eggs into a 20L bucket to make 15L volume of 1 µm filtered seawater.
- Take at least two samples of 2mL volume while stirring the bucket by a plunger.
- Count the eggs under microscope with an aide of Rafter Counting Chamber and estimate total number of eggs obtained. While counting the eggs, check the fertilization by confirming the 1st polar body or more advance embryonic development such as 2-cell stage, 4-cell stage, and so on.
- Stock the eggs in incubator tanks, maximum stocking density of the eggs being 10 eggs per mL.

3-2. In vitro Fertilization (Gonad Stripping Method)

At present, a method using thermal shock with or without Spirulina bath treatment has been effective, but it has not always resulted in 100% success rate of the sea cucumber spawning induction work. Sooner or later, it is inevitable to develop an effective method for spawning both males and females. A Japanese group of scientists (Kato et al., 2009) found that neuronal peptides induced oocyte (ovum) maturation and gamete spawning of the Japanese sea cucumber *Apostichopus japonicus*. They extracted the neuronal peptides and so synthesized it chemically, which was effective to mature 150 µm diameter or larger eggs. They also experimented to inject this synthesized hormone into the body cavity of the sea cucumbers, resulting the male and female spawned 60 minutes and 80 minutes later, respectively. Unfortunately, they did not describe how far the eggs developed as embryos and whether their hatchery work went through to settlement as pentactula stage. Therefore, no information was available from their study for fertilization and hatching rates as well as survival rates during the larval and post-larval rearing works. They also stated that this chemical did not work effectively on immature ova and, thus, they concluded that the maturation mechanisms and process of ova/spermatozoa still needed further studies. Synthesizing and producing such a neuro-hormone commercially could be very expensive and won't be available for the Indo-Pacific region in foreseeable future. The important fact is that developing techniques of artificial maturation of oocytes (ova/spermatozoa) and activation of gametes (sperms) are the keys to success in obtaining the fertilized eggs. In this end, a gonad stripping method* could be an alternative to spawning induction work near future, either using synthesize neuronal hormone or other chemicals such as ammonia-seawater which has been used for commercial pearl oyster hatcheries in Japan, or just use of natural seawater.

**Note that the gonads are removed from the parent animal by cutting a small portion of the body and the gametes are obtained by stripping/squeezing the gonads (Fig. 13c-d). This is called "gonad stripping method".*

Although no one has been successful for in vitro fertilization of the sea cucumbers, the author thought that it was worthwhile for the hatchery technicians to understand principle and procedures of this method. During the hatchery training workshop in May 2015 at the Fijian Government's hatchery in Galoa, the author used filtered seawater (1 µm nominal pore) without using any other chemicals for the gonad stripping method for the sandfish (Ito, 2015). As a result, fertilized eggs subsequently underwent embryonic and larval development to the settlement as pentactula stage. Although the number of eggs and resultant pentactula were very small, several hundred, and low survival rate at less than 10 % to day 11, this method may be economical and could be the first step towards future improvements for a large-scale juvenile production on a regular basis.

4. Larval Rearing

Larval rearing of the sandfish sea cucumber requires knowledge of feeding capability of larval stages, suitable combination of food, calculating amount of feed mix, controlling algal cell (feed mix) suspension, adjusting feeding amount and rearing water volume, water quality control, and knowledge of optimal larval development in changing proportions of larval and post-larval stages; i.e. from hatching as auricularia stage to settlement as pentactula stage. Approximately 18-24 hours after spawning depending on water temperature, larval rearing work commences by draining the incubator to collect gastrula and/or auricularia larvae. For collecting larvae and post-larvae, a combination of 80 µm and 100 µm mesh

screen is to be used. Larval rearing tanks should be completely drained every other day, on days 1, 3, 5, 7, 9 and 11. The larval specimens need to be kept alive but immobilized/anesthetized by isopropyl alcohol for counting, measurements and microscopic photographs. For longer term preservation, use formalin (10% seawater formalin). Water temperature in the larval rearing could be better between 27-30 °C.

Hatchery facility should be maintained good conditions in terms of hygiene and efficiency for larval and post-larval rearing work:

- animals such as cats need to be kept away in- and out-side the hatchery, around the indoor and under-cover tanks and indoor storage areas;
- microalgae culture room should not be a sort of storage room with scattered lab supplies and equipment on the culture benches, dusty air-conditioners and air pumps without filter maintenances;
- air supply system needs to be functioning effectively, with sufficient air pressure in the under-cover areas as well as in the microalgae culture room;
- in-use or used tools should not be scattered on the floors, e.g. filter cartridges, filter-bags, hoses, pipes, buckets, air-stones, airlines, pipe-fittings, nets, plumbing machines; air leaks from many outlets with unnecessary accessories and fittings;
- sea water and freshwater supply piping are better to be simple and do not need unnecessary connections, diversions and outlets fittings;
- the hatchery staff understand general hygiene procedure before and during the hatchery operation.

4-1. Precautions before, during and after working at hatchery

For precautions of the onsite hatchery work, refer to “Trainer's manual for hatchery-based pearl farming” (Ito, 2005) or “A hatchery operations manual for rearing sandfish, *Holothuria scabra*, in Tarawa, Republic of Kiribati.” (SPC, 2015). The following were also described in those manuals.

- make sure your hands are clean. Wash your hand with soap, particularly dirty finger nails, before starting work.
- Soak your feet in “chlorine-bath” before entering in the larval and/or microalgae room.
- don't work with you own shoes. Always wear designated boots or work with barefoot.
- always rinse again the cleaned and dried equipment with filtered rainwater (1 µm) before use.
- for the used equipment/tools, wash first with chlorinated (public) water to wash out the waste/dirt.
- second-wash by using detergent and wash-off the dirt thoroughly with a soft sponge or brush.
- rinse with 1 µm-filtered rainwater and completely wash out residual soap/detergent.
- soak in chlorine-batch (a diluted Sodium Hypo-chloride, NaHClO) for overnight. Don't mix with soap or this may release Cl₂ (chloride gas).
- rinse completely with filtered rainwater (1 µm). Make sure “no residual chlorine”.



Figure 15. Seawater filters and UV sterilizer.



Figure 16a. Larval rearing (2,000L).



Figure 16b. Larval rearing (5,000L).



Figure 17. Sieves for collecting larvae.

- always hang and dry the equipment after being cleaned. Do not leave them on the floor or dirty bench.
- if necessary, use isopropyl-alcohol spray (75 % solution) and wait for it to dry. *Note that the use of methanol (methyl-alcohol) will become a health hazard in a small microalgae culture room.
- don't touch inside of the cleaned surface of equipment and tools such as bucket/ container/tank/tub/flask, etc.
- wash filter bags, cartridges and housings after every use. Wash out the dirt with pressurized freshwater, filtered rainwater (1µm), soak in chlorine-bath, rinse with filtered rainwater (1 µm) and dry them on a designated bench. Keep the filter bags, cartridges in sealable plastic bags each with alcohol-sprayed inside. For the filter housings, spray alcohol inside and store them upside-down on the bench.
- make sure always clean the floor; wash with freshwater (chlorinated town-water or rainwater). It is the best that the floor is a “dry” condition when you start working in the following morning.
- don't disturb animal (larvae/juveniles/broodstock) and minimize giving shock or stress to the animals. Avoid unnecessary entry to the microalgae culture room and larval rearing unit.

4-2. Preparation for the larval rearing

The seawater for the larval rearing should be filtered to 1µm with a bag filter or cartridge filter. In-line UV-sterilizer is not necessarily required (Fig 15). When the day-1 larvae exceeded 0.35 larvae per mL in a rearing tank, the stocking density should be adjusted to make acceptable number of larvae in each tank: e.g. between 0.5 - 0.7 million larvae in a 2,000L tank or 1.25 - 1.75 million larvae in a 5,000L tank (Figs. 16). Gentle aeration is given throughout the larval rearing and the tank must be protected by a lid (tank cover) from debris from the ceiling. If the stocking density is less than 0.25 larvae per mL, the rearing water volume must be adjusted (reduced) to maintain required range of algal cell suspension based on *C. muelleri* culture density and number of larvae in each day. A combination of sieves is usually 80/100 µm

throughout larval rearing and each sieve must be deep and wide enough (30 cm deep x 50 cm wide) to do sieving efficiently from a larger diameter drain pipe e.g. 25.4 cm (2 inch) pipe (Fig. 17).

Apart from tools and equipment, preparation of the live microalgae species (*C. muelleri*, *Navicula* sp. and *Cocconeis* sp.) need to be cultured at least two weeks before commencing spawning and larval run. For continuous culture and feeding the larvae, several subcultures should be made after commencing larval rearing, instead of starting from new stock culture (Figure 18). Larval rearing period with microalga *C. muelleri* feeding would finish within two weeks on day-14 after spawning. When a hatchery operation is planned a single spawning0larval run, therefore, it is not useful to start any new culture of *C. muelleri*. Generally, 7 days needed for a 2 - 5L high density starter cultures to be ready for starting 20 - 100L mass cultures, and these mass culture needs further 4 - 5 days to use for feeding the larvae. The author uses dried microalga Spirulina to mix with live diatom *C. muelleri* for larval and post-larval rearing. *Navicula* sp. and *Cocconeis* sp. are used for feeding settled pentactula and early juvenile stages up to two months old in settlement tanks. If live microalgae are used for sea cucumber hatchery, therefore, it is necessary to culture diatoms.



Figure 18. Microalgae (diatoms) for feeding larvae and post-larvae.

4-3. Feeding methods for larval rearing

Mixture of *C. muelleri* and Spirulina are given for feeding the larvae twice a day. Feeding is maintained by estimating algal cell suspension in each larval tank, starting from approximately 1,000 up to 20,000 cells per mL during the two-weeks larval rearing. Total cell suspension in each day is attained by combining these two feeds and expressed as the number of *C. muelleri* cells, where amount of Spirulina is computed and expressed as the number of *C. muelleri* cells. Feeding ratio of *C. muelleri* and Spirulina is approximately 80 % and 20 %, respectively. Feeding is divided into two; one in the morning between 8 and 10 AM, and the other in the late afternoon between 4 and 6 PM. The author developed daily feeding tables with simplified data-inputting methods, therefore, the hatchery staff are usually trained to use such feeding tables.

A hatchery protocol with daily work schedule for larval rearing was summarized and shown in Table 1. The hatchery staff needs to count the number of living larvae and post-larvae after tank draining every two days and *C. muelleri* to input daily culture density (million cells per mL). The feeding amount of both *C. muelleri* and Spirulina are obtained instantly in those feeding tables. All feeding amount were converted and expressed as *C. muelleri* cells. For the Spirulina, the hatchery staff simply measures the dry-weight according to the feeding table, prepare for AM or PM feeding amount, dissolve in the freshwater (about 500 mL) and wait for an hour before adding to the rearing tanks. It is advised the stocking density of larvae from onset of larval rearing on “day 1” to be between 0.25 - 0.35 larvae per mL.

Table 1. Hatchery protocols for juvenile production of the sandfish sea cucumber.

HATCHERY PROTOCOL (WORK SCHEDULE)	*larval & post-larval development based on the water temperature at 29 ± 1 °C
days of run (size.; µm)	*larval rearing tank (LR) = 1 x 5,000L
Algal cell suspension (as <i>C. muelleri</i> cells mL ⁻¹)	*settlement tank (ST) = 4 x 5,000L (bottom area = 5,000 m ²); settlement plates=50cm x 50cm x 800~1,000 plates per 5,000L tank *plates = corrugated plastic plates *juvenile down-weller tank (JHS) = 4 x 10,000L (bottom area = 10,000 m ²); sandy bottom covered with mangrove mud (dried mud sieved through 100 µm screen)
-3d before spawning	Start conditioning settlement tanks and plates; add benthic diatoms (i.e. <i>Navicula</i> sp. & <i>Coccconeis</i> sp. 200L each in 5,000L settlement tank) & nutrient (100% strength) to culture in the settlement tanks
0 (egg: 150-160)	Collecting (sieves 50 & 80 µm mesh screen), washing, counting & incubating eggs (up to 50 million eggs per 5,000L incubator)
1 (420 x 320)	Draining incubator & collecting gastrula & early auricularia (sieve 80 & 100 µm) approx. 20 hours after fertilization; sampling, counting & stocking to larval rearing tanks (1.5 million larvae in 5,000L tank); start feeding larvae with live microalga <i>Chaetoceros muelleri</i> and dried microalga <i>Spirulina</i> sp. as soon as stocking larvae.
900-1,500 cells/mL	
2 - 4,000-7,500 cells/mL	
3 - 7,000-12,000 cells/mL	Larval tank draining (80 & 100 µm) & tank change (100% Water Exchange); early auricularia (20%) + mid-auricularia (80%)
4 - 8,000-14,000 cells/mL	
5 (800-1000) - 8,500-15,000 cells/mL	Larval tank draining (80 & 100 µm) & tank change (100% WE); mid-auricularia (20%) + late-auricularia (8%)
6 - 9,000-17,000 cells/mL	
7 (800-1200 x50-80)	Larval tank draining (80 & 100 µm) & tank change (100% WE); late-auricularia + fully developed late auricularia; remove settlement plates to dry; drain settlement tanks & refill the settlement tanks with new benthic diatoms (200L each in 5,000L tank) & nutrient (1/20 th strength)
9,500-18,000 cells/mL	
8 - 10,000-20,000 cells/mL	Spray <i>Spirulina</i> on the settlement plates (<i>Spirulina</i> 30g per liter freshwater solution) & dry the plates
9 - 8,000-18,000 cells/mL	Larval tank draining (80 & 100 µm) & tank change (100% WE); late-auricularia + fully developed late auricularia + post-late auricularia (= metamorphosing to doliolaria) + doliolaria (~ 5%)
10 - 6,000-16,000 cells/mL	Return the plates to the settlement tanks
11 (400-1200 x 60-80)	Larval tank draining (80 & 100 µm), collect larvae & post-larvae & transfer them to the settlement tanks; late-auricularia + fully developed late auricularia + post-late auricularia + doliolaria + pentactula; *survival rate from day-1 = ave. 30% (approx. 0.5 million each in 5,000L settlement tanks)
4,000-13,000 cells/mL	
12 - 2,000-6,000 cells/mL	*feeding only with <i>C. muelleri</i> for the swimming stages (no need of using <i>Spirulina</i>)
13 - 500-2,000 cells/mL	*feeding only with <i>C. muelleri</i> for the swimming stages (no need of using <i>Spirulina</i>)
14 - 200-1,000 cells/mL	Finish using <i>C. muelleri</i> & <i>Spirulina</i> for feeding larvae; fully developed late auricularia + post-late auricularia + doliolaria + pentactula
15 (~ 1mm)	Start flow-through over 24 hrs. 100% WE; (inlet water = 1 µm filter & outlet water = 100 µm screen); post-larvae; Start feeding daily with 3 foods (fishmeal 5g + seaweed 5g + mud 12.5g) per 100,000 post-larvae; *Sieve mud, fishmeal & seaweed with 100 µm screen for feeding; *no need of giving <i>Spirulina</i> as the settlement plates still covered with <i>Spirulina</i>
21 (1 ~ 2mm)	flow-through 24 hrs.100% WE; (inlet = 1 µm filter & outlet = no screen); continue daily feeding at 0.25% of BW with 3 foods (fishmeal 10g + seaweed 10g + mud 25g) per 100,000 early juveniles; *sieve feed-mix with 100 µm screen for feeding
28 (1-mo) (2~5 mm, 0.01~0.1g)	flow-through 24 hrs.100% WE; start daily feeding at 0.25% of BW with 4 foods (<i>Spirulina</i> : fishmeal : seaweed : mud = 1 : 2 : 2 : 5); *start giving <i>Spirulina</i> ; *settlement success rate from day-11 to 1-mo = 20-50%
30	Start weekly feeding at 0.25% of BW with 4 foods (<i>Spirulina</i> , fishmeal, seaweed & mud)
Day 35	Continue weekly feeding
Day 42	Continue weekly feeding
Day 49	Start transferring juveniles (2-mo = ave. 1g BW, 10-20mm BL) from settlement tank (ST) to down-weller “habitat simulator” for juveniles (JHS);
Day 56 (2-mo = J2) (0.2~2g)	*increase proportion of fishmeal and seaweed (<i>Spirulina</i> , Fishmeal & Seaweed = 1 : 2.5 : 2.5); *stocking density of JHS = approx. 10,000~1,500 x J2 per m ² (= 10,000~15,000 xJ2 in a 10,000L JHS tank);

(Example 1)

If the rearing tank volume is 2,000 L (=2,000,000 mL),
C. muelleri culture density is 3.0 million cells/mL, and
today's total amount of *C. muelleri* required is 2,000 mL

“total *C. muelleri* cells in the rearing tank” =
“culture density of *C. muelleri* ” x “ today's required amount of *C. muelleri* ”
(= 3 million x 2,000 = 6.0 billion cells)

Therefore, today's “ *C. muelleri* cell suspension ” in the 2,000L tank
= 6 billion cells / 2,000,000 mL = 3,000 cells/mL

(Example 2)

If today's required total feeding amount of larva is 12,000 cells/larva/day,
number of larvae in 2000L tank is 500,000,
today's required algal cell suspension is 3,000 cell/mL, and
today's algal culture density is 3 million cells/mL

“required total algal cells in the 2,000 L rearing tank”
= 12,000 (cells/larva/day) x 500,000 (larvae) = 6 billion cells
*algal cell suspension in this 2000L tank = 6 billion cells / 2 million mL = 3,000 cells/mL

Therefore, today's “total amount of algae” = 6 billion cells / 3 million cells/mL = 2,000 mL

For feeding the larvae, the hatchery staff must take algal samples (= *C. muelleri*) and counts the culture density; input each counting results into the designated cell in the MS Excel spreadsheet (“HIROITO CONSULTING's Sandfish Feeding Tables”), of which table automatically calculates the daily (in the morning-AM and afternoon-PM) feeding amount of both *C. muelleri* and Spirulina. “estimated available food per larva as CM” is based on the past results of larval feeding experiments of the sandfish and other sea cucumber species by the author and others in Japan and elsewhere, which ranges from 3,000 to 60,000 cells/larva/day.

Note that a commercially available dried alga Spirulina contains certain amount of dried form of cells (e.g. 2.6 billion cells per 1 g). The author found about three different sizes (e.g. 10 µm x 15 - 50 µm) and shapes (e.g. rectangular). Average size of *C. muelleri* is about 10 x 5 µm. 1 g of Spirulina cells are approximately to 42.8 billion *C. muelleri* cells. Therefore, it is necessary to estimate approximate volumes of those different sizes of Spirulina cells and to convert to the number of cells to *C. muelleri*. This is because total feeding amount is expressed as the number of *C. muelleri* cells and the feeding ratio (based on the number of cells) of *C. muelleri* vs. Spirulina is 80 % vs. 20 %.

4-4. Example of two spawning-larval runs in September 2017 at COM's Nett Point Hatchery in Pohnpei, FSM.

During the work, 1.4 million eggs (trial 1) and 5.3 million eggs (trial 2) were stocked in the 1,000L incubators. Approximately 20 hours after spawning, each yielded 0.42 million larvae (trial 1) and 2.5 million larvae (trial 2). As the two 2,000L round tanks were needed each with approximately 600,000

larvae for the trial 2. The followings were the results of larval runs between day 1 and day 11:

- trial 1 at survival rate of 10.9 % with 63,750 swimming stages on day 11 including late auricularia, doliolaria and pentactula from 416,250 larvae on day 1;
- trial 2-1 with 67,500 from 620,625 at survival rate of 15.3%;
- trial 2-2 with 221,250 from 620,625 at survival rate of 35.6%

Average survival rate of the three larval runs was 20.6% from day 1 to day 11. Total 353,500 larvae and post-larvae were transferred to three 2,500L settlement tanks.

4-5. Larval Development

Detailed morphological descriptions of the complete larval and post-larval development for both sandfish (*H. scabra*) and black teatfish (*H. whitmaei*) were given in Appendix 1 with notes on larval growth and durations in Appendix 2. Comparisons of morphological features of larval and post-larval stages of these two holothurian sea cucumbers were also given in Appendix 3.

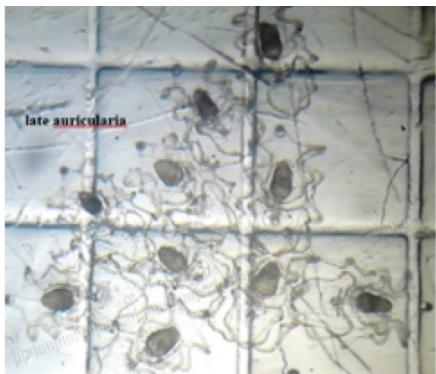


Figure 19. Late auricularia.



Figure 20. Post-late auricularia & doliolaria.

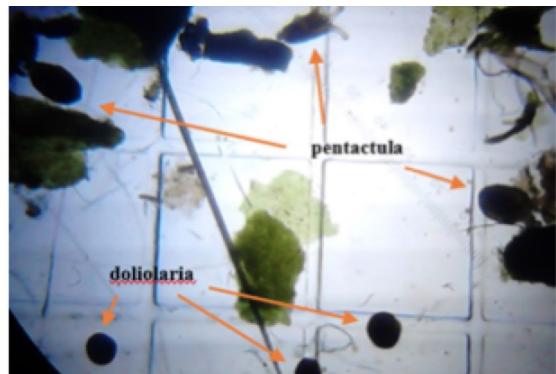


Figure 21. Doliolaria settling and pentactula settled.

5. Settlement Techniques

Settlement techniques involves conditioning of settlement plates and tanks, culturing benthic diatoms. The pentactula generally settles on the sandy sea grass bed such as *Talassira* spp. (for sandfish) in tidal flat zone, coralline gravels and rocks in the intertidal or littoral zone (e.g. black teatfish, greenfish). In the hatchery, the pentactula settle anywhere in the tank and so corrugated plastic plates are usually used as settlement substrates. Surface of settlement substrates such as plastic plates and inside the tank are conditioned with dried alga Spirulina and/or with epiphytes either naturally occurring or cultivated. The author uses two species of benthic live diatoms (*Navicula* sp. & *Cocconeis* sp.) as epiphytes on the settlement substrates. On day 11, larval rearing tank was drained and all swimming stages including fully developed late auricularia, metamorphosing post-late auricularia and doliolaria were collected onto a 100 µm screen (Figs. 19-20). A small percentage of pentactula up around 5% (Fig. 21) might have also settled on this day, so soft brushes were used to collect those settled individuals. Samples were taken for counting to estimate number of living individuals of all stages. Then, they were transferred to settlement tanks.

Table 2. Dimension of settlement plates used at COM's Hatchery in Pohnpei, FSM.

Settlement Plates (type)	Length (m)	Width (m)	Surface Area - both sides (square meters)
Large A (LA) =	0.65	0.44	0.57
Large B (LB) =	0.44	0.44	0.39
Medium (M) =	0.39	0.33	0.26

and a full strength of nutrient media (i.e. medium F2 in Australia and Fiji, OFCF/JICA's medium in Kiribati, MI medium and Kent's F2 media at COM in FSM) together with sodium meta-silicate 15 - 30 g were added to the 2,500L tank. About 10 days after the initial diatoms culture, the settlement plates were removed for drying and the tanks were drained to renew the culture water (1 µm filtered seawater) and to refill with new benthic diatoms (100L mas culture each) and 1/20th strength of nutrient media. After a couple of days for drying, the settlement plates were sprayed (or painted) with Spirulina (30g/L solution) and keep drying for a day or two (Figs. 22-23). On day 10, a day prior to transfer larvae and post-larvae to settlement tanks, return the Spirulina-sprayed plates in the settlement tanks. During the work at COM's hatchery in 2017, three different sizes of corrugated plates were used for settlement (refer to the table 2 of plate's dimensions). In a 2,500 L settlement tank, 300 - 400 plates of the type-M are mainly deployed. The plates were washed by detergent, disinfected by chlorine and dried for a couple of days before growing benthic diatoms on the plates. For larval rearing with a 2,000L tank, two of 2,500 L rectangular tanks (raceways) were used for settlement of doliolaria to pentactula, approximately up to 100,000 pentactula in each settlement tank (Fig. 24). On day 11, about 200,000 swimming stages, which consist of feeding and non-feeding stage of late auricularia, post-late auricularia and doliolaria, are expected in each 2,000L rearing tank out of 600,000 larvae on day 1.

5-2. Transferring swimming and settled stages to settlement tank.

Onset of the settlement (on day 11) is called “the 1st phase nursery culture”, which continues for about 2 - 3 months until the juveniles of 1- 2 g body weight (BW in wet weight) in average are transfer further to a down-weller “juvenile habitat simulator” tank or bag net “hapa” for a pre-growout or “the 2nd phase nursery culture”.

Living individuals on day 11 are usually consisted of the settled pentactula stage (10 - 15%), transitional doliolaria stage (20 - 25%), metamorphosing late auricularia = post-late auricularia stage (30 - 40%), fully-developed late auricularia (20 - 30%) and late auricularia stage (10 - 20%). It is better to transfer procedure when the proportion of settling or settled animals (50% < post + dolio + penta) occupy more than a half in the larval rearing tank. The larval rearing tank is drained to collect all the animals onto 80 µm and 100 µm sieve. Collected specimens should be poured over the plates in the settlement tanks.



Figure 22. Spraying Spirulina.



Figure 23. Drying plates after spray.

5-1. Conditioning settlement substrates.

Conditioning settlement plates and tanks should commence at least two weeks prior to settlement. To start culturing benthic diatoms *Cocconeis* sp. and *Navicula* sp. in the settlement tank, 2,500L tank for example, 100L each of mass-cultured those diatoms

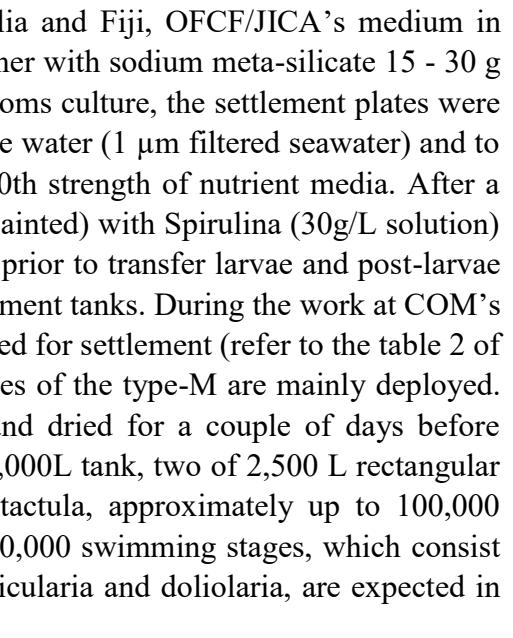


Figure 24. Settlement tank.

Toward day 11, all the settlement substrates (plates and tank surface) should be covered with light brown -colored benthic diatoms. If sunlight is too strong, the settlement tanks will be dominated by green alga (e.g. *Enteromorpha* spp.). In that case, the tanks should be covered by shade-cloths (75-80% shading rate). If pink- colored organisms (pathogenic bacteria *Pseudomonas* spp.) began visible after a week of benthic diatom culture in the settlement tank, those plates, tank surface and fittings such as air stones, air tubing, PVC pipes or ropes must be removed from the tank and disinfected by chlorine or discarded all or part of them to avoid further infection of the pathogenic bacteria. Such infection is always caused by inexperienced hatchery technician's careless setting of the settlement tank and plates, poor skill of culturing microalgae and larval rearing.

After day 11, the larval rearing continues in a static water condition because there are still swimming and feeding stages of auricularia larvae (late auricularia, fully developed late auricularia and metamorphosing post-late auricularia). Therefore, it is necessary to provide microalga *C. muelleri* until around day 14. From day 15, the rearing water in the settlement tank should be switched from static to flow-through, continuously flowing in from filter bag and flowing out to overflow-drain pipe at 100 % water exchange rate over 24 hours. 100 µm screen is used to cover the drain outlet for minimize loss of swimming stages (Fig. 25).

5-3. Examples of juvenile productions using the settlement plates.

The measurements of the settlement tank (ST), settlement plates (LA, LB, M), sample plates showing surface areas (in square meters) and estimated number of juveniles were given in the following tables 3 and 4: 30 small sample plates, 0.1m x 0.1m corrugated plastic, were also made for estimating settlement on the tank surface sides and bottom. Total of 79,180 one-month-old juveniles (on day 28, J1W1) were estimated to settle in three tanks (Figs. 26-27).

6. Pentactula and Juvenile Rearing

Pentactula and the early juveniles settled on the plates or tank surface are given additional food daily with a mixture of Spirulina, fishmeal, seaweed and mud. The mixtures of these food are homogenized and passed through 100 µm mesh screen before feeding. The juveniles on the plates and tank surface are kept in the settlement tank for two or three months and then the juveniles are transferred to "the 2nd phase nursery culture" either in the down-wellers (or habitat simulators) for juveniles or hapas bag net. The 2nd nursery culture continues further two or three months before being transferred to grow-out farm/ ponds/ enclosure. In Australia, a large-scale private farming enterprise does not conduct "the 2nd phase nursery culture", except for selective breeding programs, but those grown juveniles of average 2 - 5 g BW from the settlement tanks are also transferred to ocean grow-out sites (Fig. 28-29).

Culture techniques of pentactula and early juvenile stages in the settlement tank (the 1st phase nursery culture) from day-11 to day-56 or 8 weeks after spawning (approximately 2-month-old juveniles of 10 – 20mm, average 1g size) include selection and collection of food, preparation of food (e.g. drying, sieving and storing) and calculation of amount of food based on the number and weight of juveniles. As the feeding begins with mixture of three food (fish meal, seaweed and mud), rearing method for the 1st nursery phase switches from a static water method to a flow-through method with 100 % water exchange rate over 24 hours. 1 µm filter bag is attached to the inlet of seawater and a 100 µm screen is attached to the drain outlet, the latter is for catching any swimming stages such as doliolaria and auricularia which

Table 3. Surface areas of settlement tank and plates used at COM's hatchery in September - October 2017 in Pohnpei, FSM.

Surface Area (square meters)	bottom	sides	up-down	total surface area
2,500L mark =	5.00	5.00	1.00	11.00
	Surface Area - both sides (square meters)	No. of plates used in Tank #1	No. of plates used in Tank #3	No. of plates used in Tank #2
plate-LA (0.65 x 0.44)	0.57	32	7	0
plate-LB (0.44 x 0.44)	0.39	0	142	0
plate-M (0.39 x0.33)	0.26	0	92	212
	Total surface areas of plates in ST#1-3	18.24	83.29	55.12
	Total surface area ratio (tank vs. plates)	1.66	7.57	5.01

Table 4. The estimated number of the early juveniles on day 28 (1-month after spawning) during the juvenile production work in September - October 2017 at COM's hatchery in Pohnpei, FSM.

	Tank #1	Tank #3	Tank #2
Results of Sampling on Day 28 (J1W1) plate-LA	498 juveniles on 6 out of 30 plates	906 juveniles on 4 out 15 plates	
Estimated number of juvenile on the plates	2,485	3,398	
Results of Sampling on Day 28 (J1W1) plate-LB		5,279 juveniles on 20 out of 215 plates	
Estimated number of juvenile on the plates		53,372	
Results of Sampling on Day 28 (J1W1) plate-M	161 on 2 out of 2 M (side) converted as 1/2 of LA (side)		355 juveniles on 33 out of 212 plates
Estimated number of juvenile on the plates	161		2,503
Results of Sampling on Day 28 (J1W1) sample plates (0.1mx0.1mx30plates)			68 juveniles on 30 out of 30 plates
total number of juveniles (on plates)	2,646	56,770	2,503
total number of juveniles (tank surface)	3,179	11,589	2,493
total number of juveniles (plates + tank)	5,825	68,359	4,996

Table 5. Examples of daily feeding amount after settlement (if total 200,000 settled) for the subsequent two weeks between day 15 and day 27.

ratio against 100,000 juveniles =	2.00	(If total 200,000 juveniles)		
	Spirulina (g)	Fishmeal (g)	Seaweed (g)	Mud (g)
d14	N/A	10.00	10.00	25.0
weekly	N/A	70.0	70.0	175.0
d21	N/A	20.0	20.0	50.0
weekly	N/A	140.0	140.0	350.0

Table 6. Feeding schedule for the sandfish juvenile grow-out for the COM's hatchery work in September – October 2017 in Pohnpei, FSM.

No. of Juveniles	*feeding amount changes from 0.25% - 0.25% - 0.25% - 0.5% - 0.5% - 1% of the average body weight in each month							
	daily amount of feed (g) = smaller size			Mud (g) = 3-feed total	daily amount of feed (g) = larger size			
26,458 (estimated number of pentactula on day 15) survival rate to 1M = 19%	Spirulina	Fishmeal	Seaweed		Spirulina	Fishmeal	Seaweed	
1-2M (0.01-0.5g) (ratio) 0.25% BW	1	2	2	3-feed total (g)	1	2	2	3-feed daily total (g)
*estimated number on day 28 in ST2 (2,500L tank)								
4,996	0.02	0.05	0.05	0.12	1.2	2.5	2.5	6.2
4,996	0.14	0.29	0.29	0.72	2.2	4.4	4.4	10.9
4,996	0.26	0.52	0.52	1.31	3.1	6.2	6.2	15.6
4,996	0.38	0.76	0.76	1.90	4.1	8.1	8.1	20.3
*2M transferred from ST#2 to JHS#3: 4 weeks total feed=	5.68	11.37	11.37	28.42	74.3	148.6	148.6	371.6
2-3M (0.2-2g) (ratio) 0.25% BW	1	2.5	2.5	3-feed daily total (g)	1	2.5	2.5	3-feed daily total (g)
5,000	0.4	1.0	1.0	2.5	4.2	10.4	10.4	25.0
5,000	0.8	2.1	2.1	5.0	5.7	14.3	14.3	34.4
5,000	1.3	3.1	3.1	7.5	7.3	18.2	18.2	43.8
5,000	1.7	4.2	4.2	10.0	8.9	22.1	22.1	53.1
survival rate to 3M = 100% 4 weeks total feed =	29.2	72.9	72.9	175.0	182.3	455.7	455.7	1225.0
3-4M (1-5g) (ratio) 0.25% BW	1	5	5	3-feed daily total (g)	1	5	5	3-feed daily total (g)
5,000	1.1	5.7	5.7	12.5	5.7	28.4	28.4	63
5,000	2.6	13.0	13.0	28.6	7.1	35.5	35.5	78
5,000	3.1	15.6	15.6	34.4	8.5	42.6	42.6	94
5,000	3.6	18.2	18.2	40.1	9.9	49.7	49.7	109
survival rate to 4M = 100% 4 weeks total feed =	73.6	367.9	367.9	809.4	218.8	1093.8	1093.8	2406.3
4-5M (2-10g) (ratio) 0.5% BW	1	10	10	3-feed daily total (g)	1	10	10	3-feed daily total (g)
5,000	2.4	23.8	23.8	50.0	11.9	119.0	119.0	250
5,000	3.3	32.7	32.7	68.8	14.9	148.8	148.8	313
5,000	4.2	41.7	41.7	87.5	17.9	178.6	178.6	375
5,000	5.1	50.6	50.6	106.3	20.8	208.3	208.3	438
survival rate 5M = 100% *4M transfer to farm/pond 4 weeks total feed =	104.2	1041.7	1041.7	2187.5	458.3	4583.3	4583.3	6926.0
5-6M (5-20g) (ratio) 0.5% BW	1	20	10	3-feed daily total (g)	1	20	10	3-feed daily total (g)
5,000	4.0	80.6	40.3	125.0	16.1	322.6	161.3	500
5,000	5.0	100.8	50.4	156.3	20.2	403.2	201.6	625
5,000	6.0	121.0	60.5	187.5	24.2	483.9	241.9	700
5,000	7.1	141.1	70.6	218.8	28.2	564.5	282.3	875
survival rate to 6M = 100% *5M transfer to farm/pond 4 weeks total feed =	155.2	3104.8	1552.4	4812.5	621.0	12419.4	6209.7	19250.0

are returned to the settlement tank. Depending on presence of the swimming stages i.e. late auricularia and post-late auricularia, feeding with *C. muelleri* ends on around day 14. The juvenile foods are prepared as follows:

- after collecting from the wild, seaweed (Fig.30) and mud (Fig. 31) are sundried (Fig. 32);
- mud is sieved through 200 or 250 µm screen (Fig 33) and the seaweed is chopped in fine pieces (Fig. 34);
- after weighing, seaweed and fish meal are dipped in 1 µm filtered seawater for a several hours or overnight (Fig. 35)
- Spirulina is dipped in freshwater (rainwater) for several hours or overnight;
- Use blender to make the softened-foods finer pieces;
- sieve through 100 µm before feeding the animals

The protein content is the most important element in feeding pentactula and juveniles. Low fat content (crude fat around 5%) is desirable to maintain better water quality during the larval rearing and the 1st phase nursery culture in the settlement tanks. The author uses dried alga Spirulina instead of Algamac® (*Algamac ProteinPlus®) because Spirulina has much higher protein with low fat contents and the latter has higher fat contents. The author's choice of fishmeal is for milkfish (herbivore fish) farming because of less crude fat contents (about 3 %) compared to other fishmeals (Fig. 36). The author also uses the seaweed Sargassum spp. and Gracilaria sp., which contains about 10% crude protein with other essential nutrients, for free feed from the wild supplementing Spirulina and fishmeal. Mud or silt can be collected from the tidal flat area of the mangrove shore. The author has been using to mix with other feed (i.e. seaweed, fishmeal and Spirulina) for the juvenile grow-out as well as broodstock conditioning. The mud



Figure 24. Settlement tank.



Figure 25. Screen-covered drain outlet.



Figure 26. Day 28 juvenile.



Fig 27. Day 28 settled juveniles.



Figure 28. 5 g size 3-mo juveniles



Figure 29. Juveniles 3-mo in ocean grow-out site.



Figure 30. Seaweed *Sargassum* sp.



Figure 31. Mud from mangrove tidal flat.



Figure 32. Drying seaweed and mud.



Figure 33. Dried and sieved mud.



Figure 34. Dried seaweed.



Figure 35. Preparation for feeding.



Figure 36. Fishmeal.



Figure 37. Down-weller “habitat simulators” for juvenile grow-out.

contains detritus with organic substances and symbiotic heterotrophic micro-organisms in the mangrove forest (i.e. *Schizochtrium* spp.) which are known to have high contents of poly-unsaturated fatty acid ($\omega 3$ fatty acid). This heterotrophic (propagating without using sunlight) organisms live in the mangrove shore. *Algamac® and Algamac ProteinPlus® are commercial products from Aquafauna BioMarine, USA.

From day 28 or one month after spawning to six-month-old juveniles, an example of feeding schedule was given in the following table 6: juveniles from 2-month-old (Settlement Tank #2 = ST2 with 4,996 J2W1) to 3-month-old in the 2,500L down-weller (Juvenile Habitat Simulator #3 = JHS3 with 5,000 J3W1) where the juveniles were produced from the spawning on September 8, 2017.

Juveniles attached on the plates are transferred on around day 56 (two-month-old after spawning) to the down-weller “habitat simulators for juveniles” (Figs. 37-38). Although the larger the animals, it is the easier for counting and estimating the standing crop, the juveniles were large enough (10 - 20 mm, 0.2 - 2 g) to estimate the number of individuals by a naked eye.

Between 2 - 3 months old (1 - 5 g) after spawning, down-wellers “habitat simulators for juveniles” are stocked with juveniles either being attached on or being detached from the settlement plates. Stocking density of each tank was approximately 1,500 juveniles per square meter. Those remaining juveniles in the settlement tanks need to be fed weekly but are to be transferred in earlier occasions to hapas or ocean grow-out sites. The juveniles in the habitat simulators will be fed weekly for subsequent two or three months before transferring to grow-out phase (farming) in the ocean sites, earthen ponds or restocking sites.



Figure 38. Estimating settled 2-mo juveniles by detaching from the plates.

7. Grow-out Culture

At around four or five months after spawning, the juveniles should reach to average 5 - 10 g. So, they are ready for farming. There is no published information available on the feeding techniques or feeding amount for both settled pentactula, early juveniles and juveniles up to six months after spawning. Also, there is no information about feeding techniques after six months old to harvesting size to export. The

Table 7. The results of counting of two-month-old juveniles attached on the settlement plates and estimate of those attached on the tank surface during the hatchery work in September - October 2017 at COM's hatchery in Pohnpei, FSM.

day 49 (Oct. 27-29, 2017)	Settlement Tank #1 (from LR2-1)	Settlement Tank #2 (from LR1)	Settlement Tank #2 (from LR2-2)
counted total number of juveniles (on plates)	2,154	2,975	17,067
estimated total number of juveniles (tank surface)	3,670	2,021	51,304
estimated total number of juveniles (plates + tank)	5,824	4,996	68,371

Table 8. Restocking JHS tanks with 2-mo juveniles.

2.5t Habitat Simulator Tank for Juveniles (with plates)	No. of Juveniles Counted
JHS#1 =	5,985
JHS#2 =	4,642
JHS#3 =	5,129
JHS#4 =	4,569
JHS#5 =	1,871
total =	22,196

Table 9. Number of 2-mo juveniles in the settlement tanks.

2.5t Settlement Tank after Transfer (without plate)	No. of Juveniles Estimated
ST#1 =	3,670
ST#2 =	2,021
ST#3 =	51,304
total =	56,995

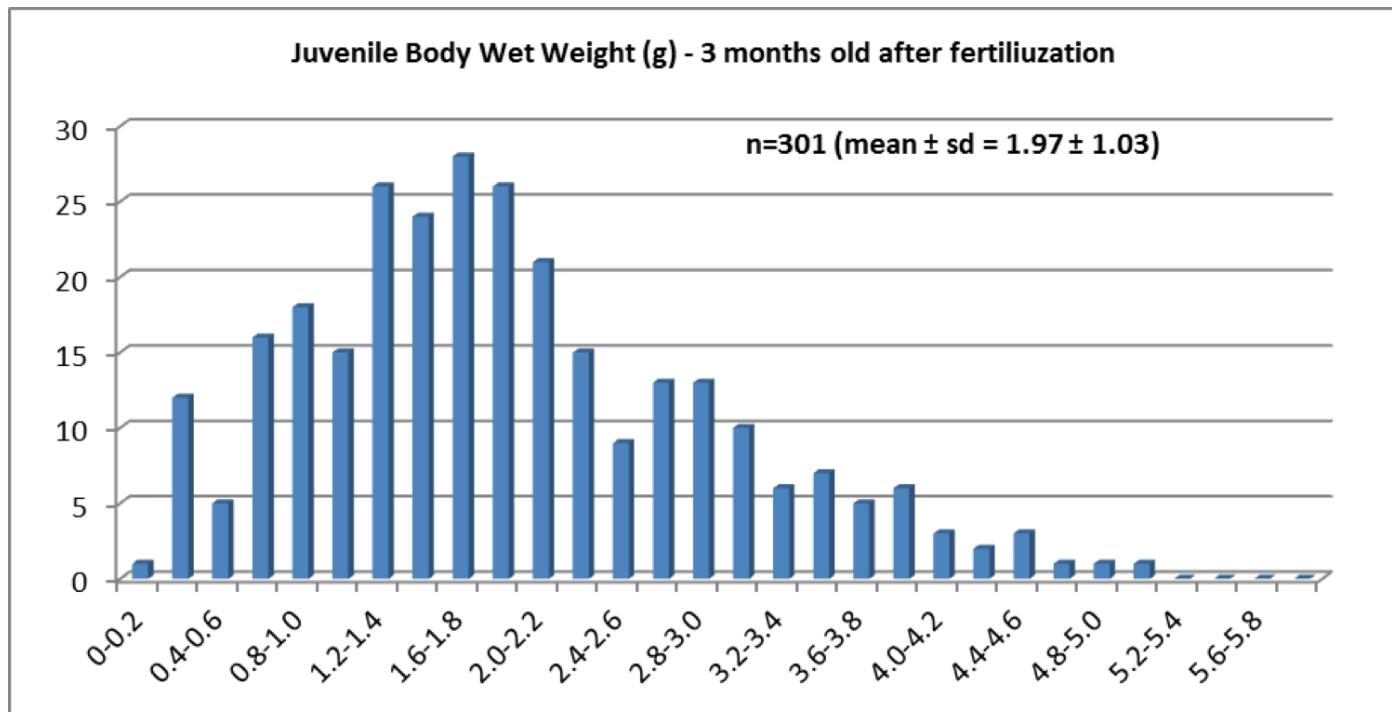
Table 10. Feeding table for 6-month-old juveniles in the downweller habitat simulator for juveniles.

	daily amount of feed (g) = smaller size			Mud (g) = 3-feed total	daily amount of feed (g) = larger size			Mud (g) = 3-feed total
	Spirulina	Fishmeal	Seaweed		Spirulina	Fishmeal	Seaweed	
6M (10-40g) (ratio) 1% BW	1	20	10	3-feed daily total (g)	1	20	10	3-feed daily total (g)
4,750	NA	306.5	153.2	475.0	NA	1225.8	612.9	1900
4,750	NA	536.3	268.1	831.3	NA	1379.0	689.5	2138
4,750	NA	766.1	383.1	1187.5	NA	1532.3	766.1	2375
4,750	NA	990.0	498.0	1543.0	NA	1685.5	842.7	2613
survival rate to 12M = 95% - 4 weeks total feed=	NA	18233.9	9116.9	28262.5	NA	40758.1	20379.0	63175.0

feeding table (Table 10) was developed by the author for private farming enterprise in Australia. Note that the feeding tables should be used only if actual or estimate of number of juveniles are not available. Feeding the juveniles and/or young adults are generally based on and calculated by the average body weight (wet weight). When they reach preferably to 10 - 20 g (average 5 - 10g) size after 3 - 5 months from spawning, they are ready for grow-out in the farm (ocean enclosures and/or ponds). Stocking density for the grow-out farming could be at 2 - 3 individuals per square meter (m²). In a hapa method elsewhere, 1- 2 g size about 2 or 3-month-old juveniles are stocked at 200 individuals per m². On the other hand in a down-weller juvenile habitat simulator (e.g. 10,000L in Australia) with tank floor area of approximately 10 m², initial stocking density is about 1,000 juveniles per m²). It is recommended to reduce the stocking density to at least half or preferably to 1/4 on around 5-month-old.

The following pages describe how to estimate feeding amount for the juveniles of 3-month-old. Also, explaining how accurate these feeding tables and how to use these tables developed by the author. For rearing early juveniles, it is recommended to feed the animals with 1/4 of adult grow-out amount = (1% body weight) x ¼ = 0.25% daily, and then increase the amount gradually to 0.5% and later around 5-month-old to 1%. The hatchery and farmhands must monitor the animals daily or at least weekly, such as measuring body weight by sampling monthly to adjust (increase) amount of weekly feeding amount. The following is an example of 300 juveniles of 3-month-old collected from a hapa elsewhere and measured body weight (mean ± sd = 1.97 ± 1.03 g). The histogram of these 301 (say 300) showed that they were smaller and closer to minimum weight group (1g group), indicating that they had not been fed well.

The following is how to estimate the weekly feeding amount of the 300 juveniles of 3-month-old, when they are required daily with 1% of body weight of food. Also, how-to estimate the feeding amount by using the juvenile feeding table when actual body-weight measurements were not done onsite.



(EXAMPLE) Histogram of the 3-month-old juvenile body weight obtained from hapa in a pond.

"How-to-Compute" weekly feeding amount (1% Body Weight) for 3-month-old "300" juveniles from hapa.

$$1.97g \times 1\% \text{ BW per day} = 1.97g \times 0.01 = 0.0197g \text{ per day per juvenile}$$

$$0.0197 \times 300 \text{ juveniles per day} = 5.91g \text{ per day} = 5.91 \times 7\text{days} = 41.37g \text{ per week in total}$$

*Spirulina vs. Fishmeal vs. Seaweed = 1 : 5 : 5 for the 3-month-old juveniles
therefore,

$$\text{Spirulina} = 41.37 \times 1/(1+5+5) = 3.76g \text{ per week (0.54g per day)}$$

$$\text{Fishmeal} = 41.37 \times 5/(1+5+5) = 18.8g \text{ per week (2.69g per day)}$$

$$\text{Seaweed} = 41.37 \times 5/(1+5+5) = 18.8g \text{ per week (2.69g per day)}$$

* If number of juvenile is 300, then the feeding amount is 300/10,000 of the figures given in the following Feeding Table.

$$\text{daily amount of 3-feed total} = 25 \times 300/10,000 = 0.75g \text{ when given 0.25\% BW}$$

If given 1% BW,

$$\text{daily amount} = 0.75g \times 1/0.25 = 3g \text{ and weekly amount} = 3g \times 7\text{days} = 21g$$

$$\text{Spirulina} = 21 \times 1/(1+5+5) = 1.91g$$

$$\text{Fishmeal} = 21 \times 5/(1+5+5) = 9.55g$$

$$\text{Seaweed} = 21 \times 5/(1+5+5) = 9.55g$$

However, the amount is based on the Smaller Group (1g).

If the juvenile's average body wet weight is 1.97g (= 2g), the feeding amount should be increased and then, re-calculated for the "300" juveniles.

i.e. Each 1 g increment is $(875-1,75)/4 = 1,75$ per week

$$1\text{g weight group} = 175; 2\text{g weight group} = 175 + 175 = 3,50g; 3\text{g weight group} = 350 + 175 = 525g$$

Feeding Table for the 10,000 Juveniles (3 M): Smaller Group (1g) and Larger Group (5g)								
	Smaller Group (1g)			Larger Group (5g)				
10,000 3M juveniles	Spirulina	Fishmeal	Seaweed		Spirulina	Fishmeal	Seaweed	
3-4M (ratio) 0,25%BW	1	5	5	3-feed daily total (g)	1	5	5	3-feed daily total (g)
wk-1 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
wk-2 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
wk-3 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
wk-4 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
Weekly Total (g)	15.9	79.5	79.5	175.0	79.5	397.7	397.7	875.0

4g weight group = $525 + 175 = 700$ g; 5g weight group = $700 + 175 = 875$ g

therefore,

weekly amount of the 2g weight group could be 350g per "10,000" juveniles

weekly amount of the 2g weight group of the "300" juveniles = $350 \text{g} \times 300/10,000 = 10.5 \text{g}$ when given 0.25% BW

therefore,

Spirulina = $10.5 \times 1/11 = 0.95$ g

Fishmeal = $10.5 \times 5/11 = 4.77$ g

Seaweed = $10.5 \times 5/11 = 4.77$ g

If given 1% BW, weekly feeding amount of 2g weight group = $10.5 \times 4 = 42.0$ g

therefore,

Spirulina = $0.95 \times 1/0.25 = 3.8$ g

Fishmeal = $4.77 \times 1/0.25 = 19.1$ g

Seaweed = $4.77 \times 1/0.25 = 19.1$ g

The estimated weekly feeding amount from BW measurements ($1.97 \text{g} \pm 1.03 \text{g}$) was total $41.37 \text{g} =$ Spirulina 3.76g + Fishmeal 18.8g + Seaweed 18.8g

Thus,

the above "Feeding Table" is very accurate ($+0.04 \text{g} < > +0.67 \text{g}$), almost the same feeding amount obtained from actual BW measurements.

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Hatchery Manual for Sea Cucumber Aquaculture in the U.S. Affiliated Pacific Islands



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Masahiro Ito

Hatchery-Based Sea Cucumber Farming

It is common knowledge that aquaculture farms will result in employment opportunities for island communities and provide potential source for exports. A hatchery-based sea cucumber production is to make available for stock enhancement program and for aquaculture-based farming enterprises. The author (Masahiro Ito) is an independent sea cucumber hatchery consultant and a former director of aquaculture research and extension of COM. He has proprietary technology in possession which can significantly boost the sea cucumber juvenile production in the hatchery. He has agreed to write this manual to contribute to the benefit of the U.S.-Affiliated Pacific Islands and its future industry development. The main objective is to provide the advanced methodologies and to improve the hatchery technology for the holothurian sea cucumbers, particularly the sandfish (*Holothuria scabra*) in the U.S.-affiliated Pacific islands.

Status of World Sea Cucumber Trading

The sandfish sea cucumber business was once prosperous and has been a valuable source of income for decades in the tropical and subtropical coastal communities, but it was based on “boom and bust” business resulting over-fishing to the level of extinction of this high-valued species. Similar phenomenon on almost all sea cucumber fishery have occurred worldwide. Despite of these facts, a sustained demand for bêche-de-mer (processed sea cucumbers) from China and other Asian sea food markets has pushed up the price of this favored *holothurian* sea cucumber species. Most of the sandfish product which has been regarded as one of the most valuable tropical sea cucumber is traded and sold in the dried form in the Asian market mainly in Hong Kong where the products are distributed into mainland China. Dried sea cucumbers are brought from all over the world to be bought and sold in Hong Kong. Traders and wholesalers are located along Nam Pak Hong Street in the Sheung Wan area in the north-west of Hong Kong Island. Hong Kong and Guangzhou in Guangdong province, China, have been tightly connected since the birth of Hong Kong in the 19th century. Through this channel, most of the dried marine products imported into Hong Kong are re-exported to Guangdong, from where they are traded throughout China. Currently, retail prices of the sandfish in Hong Kong are from around US\$50 for the low quality with small sized products to US\$300 per kg for high quality with larger size and the highest quality sandfish fetches between US\$500 and US\$800 per kg. The “Australian” or “Australian-made” sandfish have always been regarded as the highest quality and price in Hong Kong wholesale and retail markets.

This hatchery manual includes the following topics; i.e. broodstock management and juvenile production work of the sea cucumber sandfish, notes on microalgae culture, complete larval development as well as descriptions of post-larvae and juveniles of the sandfish and the black teatfish (*Holothuria whitmaei*):

- 1) quarantine culture of the broodstock, recovering them from spawning stress and conditioning for spawning induction by using down-weller “Habitat Simulator” system;
- 2) microalgae culture of benthic diatoms and knowledge of heterotrophic algae (micro-organisms) for feeding the settled pentactula and early juvenile stages;
- 3) spawning induction methods with disinfection of spawners, fertilization, collection and incubation of eggs;
- 4) larvae rearing including specific knowledge of feeding capability of larval stages and combination of feed, calculating amount of larval feed mix, controlling algal cell suspension, adjusting feeding amount and rearing water volume, and knowledge of optimal larval development by expecting

- proportions of larval and post-larval stages between day-1 and day-11;
- 5) settlement techniques including preparations of settlement plates and tanks, maintenance of benthic diatom culture and water quality, nutrient media preparation and culture techniques of benthic diatoms and/or naturally occurring epiphytes, knowledge on the types of benthic diatoms (*Navicula* sp. & *Cocconeis* sp.) and symbiotic heterotrophic micro-organisms in the mangrove ecosystem for feeding pentactula and early juveniles;
 - 6) culture of pentactula and early juvenile stages in the settlement tank (the 1st phase nursery culture) from day-11 to day-56 or 8 weeks after spawning (approximately 2-month-old juveniles of 6 – 15 mm, 0.2 – 1g size), including calculation of feeding mix amount for the juvenile culture and preparation of feed mix;
 - 7) grow-out culture using the down-weller “habitat simulator” tanks from day-56 or 8 weeks (onset of the 2nd phase nursery culture) until 5-6 months old (approximately 20 – 50mm, 5 – 20g size).

Broodstock Management

Elsewhere, the sandfish broodstock are usually held either in FRP (fiber-reinforced plastic) raceways, concrete tanks or earthen ponds for spawning work (Figs. 1a-c). The COM’s hatchery in Pohnpei, Federated States of Micronesia, uses freshly caught sandfish broodstock from nearby its hatchery a day prior to the spawning induction work without doing any conditioning work.



Figure 1a. Habitat simulators.



Figure 1b. Concrete tanks.



Figure 1c. Earthen ponds.

The sandfish habitat is characterized by a seagrass bed of the tidal flat along the mangrove-covered shoreline from low-tide line to 10 – 20m deep in subtidal zone with soft muddy or sandy substrate. Seagrass bed is characterized by turtle-grass such as *Thalassia* spp. or by eel-grass such as *Zostera* spp. in the Indo-Pacific region (Fig. 2a-b). It is said that stocking density of the sandfish grow-out in a pond is one or two animals per square meter and the broodstock may be stocked at 3 – 5 per m⁻² in a tank for conditioning if they are provided good aeration, water flow (water exchange rate at 400% per day), ample feeding with periodic tank cleaning at least once a fortnightly or renewal of tank with fresh muddy sand substrate (Duy, 2011; Purcell et al., 2012).

A key technological innovation developed by the author is a land-based broodstock culture system with a “down-weller” or “habitat simulator” tank system. The system uses a combination of closed recirculating seawater and partial flow-through method, which enables a long-term holding and domestication of healthy broodstock for selective breeding programs rather than relying on wild-caught parents on each hatchery operation. The tank system holds 5 - 10 broodstock per m⁻² by providing with good air, water circulation (100% daily water exchange) and enabled to feed without periodic tank cleaning or renewal of

Sandfish Holding Tank for Broodstock & Juveniles

combined partial flow-through + closed re-circulating seawater

“Habitat Simulator”

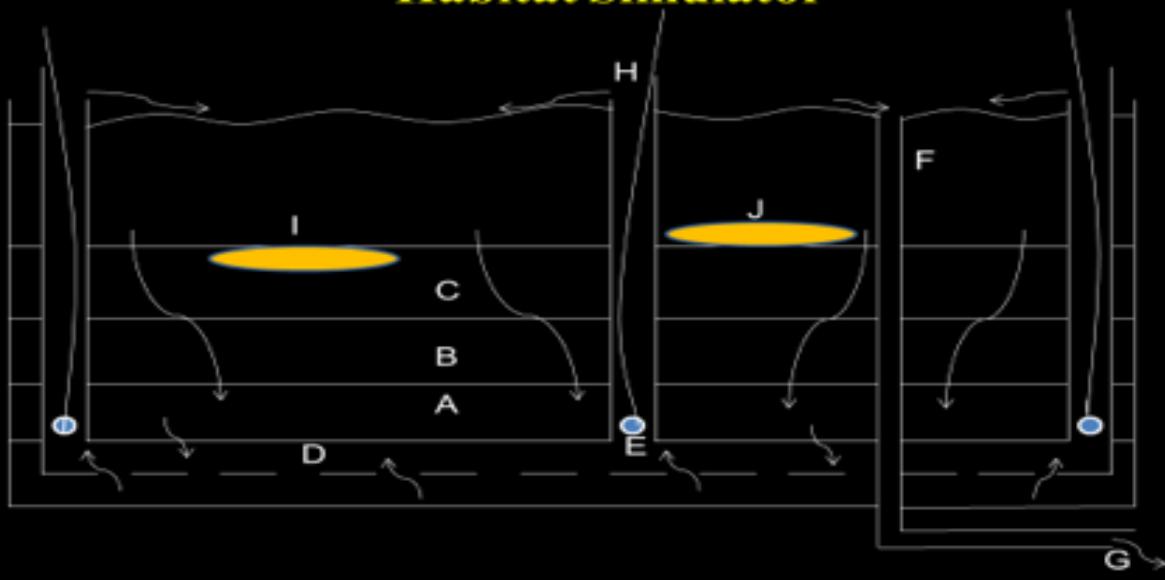


Figure 3. Diagram of down-weller “Habitat Simulator” tank. A: coral rock, B: coral gravel & sand, C: fine sand & silt, D: perforated pipe, E: air stone, F: overflow standpipe, G drain, H: airlift pipe, I & J: sandfish

tank. The down-weller was consisted of two or three layers of substrates to form a false-bottom structure; sand and mud, which are collected from tidal flat areas in the mangrove shore. Water re-circulates through the surface muddy and sand layer by air-lift pump which also maintains aerobic condition of the tank bottom and substrates (Fig. 3).

For quarantine purpose, an Australian private hatchery uses this tank system to prevent spreading potential disease among the wild-caught or domesticated broodstock (Fig. 4). Furthermore, this system has been used for recovering the spawners which had been injured or stressed during the transportation and/or spawning induction work. In Pohnpei of the Federated States of Micronesia, the COM built 2,500L rectangular tanks with down-weller system were made for broodstock recovering (Fig. 5) and juvenile grow-out (Fig. 6). Routine maintenance of the down-weller system for the broodstock is to: 1) avoid the macro-green algae (e.g. *Enteromorpha* spp.) over-grown on the tank surface; and 2) adjust aeration so as not to give strong air to drag too much seawater into the perforated piping system. To prevent green algae over-grown, use shade-screen to cut too much sunlight onto the tank. Continuously strong air-lift causes hardening the sandy substrates from strong downward water movement.



Figure 2a. Seagrass beds of turtlegrass (*Thalassira* species).



Figure 2b. Seagrass beds of eelgrass (*Zostera* species).



Figure 4. Down-wellers for commercial



Figure 5. Quarantine and recovery for broodstock in Pohnpei, Micronesia.



Figure 6. Down-wellers for juvenile grow-out in Pohnpei, Micronesia.



Figure 7a. Transporting in bags.



Figure 7b. Selecting broodstock for spawning.

1-1. Feeding broodstock

The broodstock in the “down-weller” tanks are fed daily with 1~2 % BW (body wet weight) per individual depending on the purposes; i.e. grow-out or quarantine at 1%, fattening or recovery at 1.5~2%. For practical reason, it is recommended to feed them weekly. The feed consists of dried alga Spirulina, fishmeal and seaweed of which ratio varies depending on the sizes and conditions of the animals; approximately 1 vs. 20 vs. 10. Mud/silt collected from tidal flat zone of the mangrove-covered shore is also included as an important food for both broodstock and juveniles. The amount of mud is equivalent to a total weight of other three foods. The amount of food should be adjusted by increasing or decreasing according to their average body weight. Therefore, the body weight needs to be measured at least monthly or bi-monthly. Feeding the broodstock in the Habitat Simulator can be done without a renewal

of sandy and/or muddy substrates because this down-weller system itself contains organic matters such as seaweed and detritus. Currently with no mortality has been recorded by using with a combination of dried seaweed, fishmeal and mangrove-silt.

1-2. Transporting broodstock

When transporting broodstock for a long distance (4 hours or more) from wild habitat to the hatchery, the animals should be packed individually in a plastic zip-bag in a polystyrene box or ice-chest (Fig. 7a-b). It is better using ambient seawater the same water collected at the habitat and better inserting ice-gel pack (s) in the box to keep temperature at lower than 25 °C during the transportation. If the animals are found eviscerated (vomited the gut/internal organ), they should be removed from the spawners and held in the recovery-fattening tank for at least six months period for the next spawning work.

2. Microalgae Culture Management

For microalgae culture of benthic diatoms refer to “Trainer's manual for hatchery-based pearl farming” (Ito, 2005), “Development of pearl aquaculture and expertise in Micronesia” (Ito, 2006) or “A hatchery operations manual for rearing sandfish, *Holothuria scabra*, in Tarawa, Republic of Kiribati.” (SPC, 2015). Detailed descriptions of the microalgae culture management and techniques had been written by the author during the COM Land Grant Program's pearl project in 2001-2013, which offered basic but practical knowledge on the microalgae culture in the tropical conditions.

2-1. Precaution for microalgae culture

- soak in freshwater and wash with detergent, brushing off dirt/wastes. Although it is not always necessary, hydrochloric acid (5 - 10% HCl solution) can be used for cleaning flasks by soaking when the dirt is difficult to clean off. Collect the used hydrochloric acid in a glass bottle for re-use.
- rinse with freshwater 5 - 10 repeats, completely wash off residue of detergent or chemicals.
- dry flasks upside down and avoid air-born dirt inside the flask.
- spray alcohol (isopropyl-alcohol or ethanol 75 % solution), rinse with distilled /filtered rainwater, and wait for dry upside down.
- put the lid on (aluminum foil) or place them in a dust-free cabinet for longer storage.
- rinse with filtered (0.2 µm or 1µm) seawater and, if available, UV-sterilized seawater before use.
- make sure washing your hand, particularly dirty finger nails and oily fingers, with soap and rinse off any residue of soap/chemicals, and then spray alcohol before commencing work.
- spray alcohol on the surface of culture flasks/containers/fittings/ working bench when entering the room.
- keep the floor and bench clean and dry and, if necessary, clean a floor with chlorinated freshwater.
- soak your feet in the chlorine bath before stepping into the room.
- periodically check and clean air filter/air outlet of air pump, air-conditioner and ventilator.

- always keep the room door/windows closed and avoid unnecessary entry into the room.

The hatchery staff tend forget general precautions for the microalgae culture work and how to properly operate the autoclave. Usually, hatchery operation elsewhere uses 121 °C for 45-60 minutes for larger flasks such as 3~5L high density culture, and small 100~250mL flasks for stock culture are sterilized for 10-15 minutes at 121°C. Periodical maintenance of autoclave is also necessary by changing or refreshing water in the chamber.

2-2. Culture methods for the sea cucumber hatchery

Sea cucumber hatchery work involves microalgae culture of planktonic and benthic diatoms. The author also uses mud/silt collected for the tidal flat zone of the mangrove shore for feeding the settled pentactula and early juvenile stages as well as broodstock. This kind of mud contains nutrient rich, particularly Omega-3 ($\omega 3$) fatty acids, derived from heterotrophic algae (micro-organisms).

Live microalgae are not required for feeding broodstock (adults) of the sea cucumbers. During the larval and post-larval rearing, however, live and/or dried microalgae are used toward settlement stage (pentactula stage) and after settlement to juveniles. The author simplified feeding methods for the larval rearing to reduce workload of culturing live microalgae. With higher survival rate at 30 - 40 % from day-1 to the settlement stage, the author has been using a single live planktonic diatom species of *Chaetoceros muelleri* together with dried form of microalga, *Spirulina* sp. For settlement phase and post-settlement rearing of juveniles, the author developed to use two kinds of live benthic diatoms (*Navicula* sp. and *Coconeis* sp.) by combining with dried *Spirulina* during the pentactula and early juvenile stages and during the juvenile stage by combining fishmeal, seaweed, *Spirulina* and tidal flat mud. Note that there are eight types of benthic diatoms and *N. ramosissima* (Type-A benthic diatom) and *C. scutellum* (Type-B benthic diatom) are commonly used at abalone hatchery for post-settlement juvenile culture in Japan (Kawamura, 1998). The author has been using two types as live epiphytes on the settlement substrates for the sea cucumbers, such as *N. jeffreyii* for type-A and *Coconeis* sp. for type-B. Master stock culture of these benthic diatoms can be purchased commercially such from Commonwealth Scientific Industrial Organization (CSIRO) in Australia or elsewhere. Culture media of these benthic diatoms or naturally occurring epiphytes are same as planktonic diatom such as *C. muelleri* with nutrient media strength varies from 1/100th to 1/10th. Starter high density (3L - 5L flasks) & mass culture (20L carboys - 100L polycarbonate tanks) are used for the above three diatom species. For these benthic diatom culture techniques and work plan, refer to Chapter 4 (Larval Rearing) and 5 (Settlement Techniques).

For specific knowledge of heterotrophic algae, refer to some of many publications such as “*Schizochytrium limacinum* sp. nov., a new thraustochytrid from a mangrove area in the west Pacific Ocean” (Honda et al., 1998), “Fatty acid composition and squalene content of the marine microalga *Schizochytrium mangrovei*” (Jiang et al., 2004), “Effects of dried algae *Schizochytrium* sp., a rich source of docosahexaenoic acid, on growth, fatty acid composition, and sensory quality of channel catfish *Ictalurus punctatus*” (Li et al., 2009), and “Heterotrophic cultivation of microalgae as a source of docosahexaenoic acid for aquaculture” (Taberna, 2008).



Figure 8a. Cold water treatment using ice cubes.



Figure 8b. Cold water treatment in algae room.



Figure 9a. 1ppm iodine bath.



Figure 9b. Disinfecting (1 min.)



Figure 9c. Rinse with freshwater.



Figure 10a. Thermal shock.



Figure 10b. Gently stirring.



Figure 10c. Siphoning droppings.



Figure 11a. Spirulina bath (12g/60L seawater)



Figure 11b. Monitoring water temp.



Figure 12a. Spawning.



Figure 13a. Collecting eggs.



Figure 13b. Sampling for counting eggs.



Figure 13c. Cutting broodstock.



Figure 13d. Stripping gonads.



Figure 14. Incubating eggs in 1,000L

3. Spawning Induction

Spawning induction work involves; conditioning and disinfection of spawners; inducing by stimulations or stressing such as exposing to the air, changing water temperature, water pressure and/or salinity, and chemical or food; fertilization and washing of eggs; and collection, sampling, counting and incubation of eggs (see Figs 8-14). When using a 2,500L tank for a small-scale juvenile production work, about 50 - 60 broodstock (spawners) are used for single larval run. Prior to spawning induction work before transferring from cold water treatment to spawning tank, all the spawners are disinfected by iodine, in which the animals were immersed in 1 ppm iodine bath (freshwater) for 1 minute. Spawning induction are usually done by: 1) stress by handling with exposure to the air, 2) thermal shock from cold (20-22 °C) to warm water (32-34 °C), 3) chemical stimulation by dried microalga Spirulina (20g/100L) in seawater for 30 minutes, 4) changing water pressure (decreasing/increasing water level), and/or changing salinity (decreasing salinity to about 30 ppt).

Collection of the spawned eggs are usually two-step approaches; 1) the first batch by scooping the spawned eggs by beakers and 2) the second batch by draining spawning induction tank. For a small-scale work, the former method is better to obtain cleaner with enough number of eggs. This also requires careful and continuous observation of female spawning posture. Noticeable change is observed in gonophore shape by swelling outwardly. Therefore, swift and timely scooping actions to collect eggs are required. If the latter method is used, collection of eggs should be commenced soon after several females spawned before the spawning tank becoming cloudy from too many sperms.

For incubating the fertilized eggs, stocking density should be less than 10 eggs per mL. Seawater is filtered to 1 µm by using filter-bags or cartridge filters, which does not necessarily required sterilization by in-line UV sterilizer unless virus infection or other disease has been reported from the surrounding environment. A combination of plankton screen (50 µm and 80 µm or 90 µm) is essential for collecting eggs.

3-1. Spawning procedures

The following describes timeline of spawning induction work which is based on a combination of 1) physical stress; 2) exposure to air, 3) thermal shock from cold to warm water, 4) chemical (dried algae Spirulina-bath) and/or 5) changing water pressure.

- Start preparing boiled seawater in deep pan (20-40L) using firewood (or use immersion heaters in the spawning tank) to maintain the induction tank water temperature at 33-34 °C.
- Cleaning off dirt from the body surface, measuring body weight (BW) and selecting spawners to be at least 200g, so the smaller ones should be returned to the broodstock holding tank. Before transfer to a cold water, quickly rinse with filtered seawater.
- Prepare cold seawater (1 µm filtered) beforehand, in the preceding day by placing it in the algae room. Transfer spawners to 100L cold treatment tank at about 20-22 °C and keep them for at least 2~3 hours, preferably for overnight.
- Transfer spawners to iodine (freshwater) bath at 1ppm of iodine (or 100ppm of Betadine®*) for 1minute (= 60 seconds). *Betadine® contains 1%W/V iodine. Therefore, 1g Betadine contains 0.01g iodine. To make 1ppm iodine solution (or 0.1g iodine per 100L), add 10g Betadine® per 100L to make 1ppm iodine (freshwater) bath.
- Start spawning induction work immediately after the iodine bath, rinse off iodine with filtered seawater and transfer to the spawning tank (2,500L raceway with approximately 1,000L water volume) at 33-34°C.
- Wait spawning (male and female) for at least an hour and keep cleaning droppings on the tank floor by siphoning. Use a plunger to stir gently spawning tank water and keep mixing the warm water.
- If the spawners dose not respond to the above thermal shock, transfer them to “Spirulina bath” for 30 minutes at 12g of Spirulina in 60L of filtered seawater. Spirulina is dissolved faster and better in freshwater (or rainwater), so prepare 12g Spirulina in about 500mL rainwater before making 60L seawater solution.
- Rinse off the Spirulina with filtered seawater and introduce them again to the spawning tank.
- Wait for the spawning. Males usually spawn before females release eggs.
- Observe spawning posture of female(s) and scoop the eggs with beakers when the female releases the eggs. If excess eggs are needed after confirming the females finished spawning, drain the spawning tank to collect remaining eggs inside the tank. Use a combination of 50 and 80 µm-pore size mesh screen to collect and wash the eggs. If no female responded after two hours, return all the spawners to broodstock holding tank.
- After washing/rinsing off sperms for 10-20 minutes, transfer the eggs into a 20L bucket to make 15L volume of 1 µm filtered seawater.
- Take at least two samples of 2mL volume while stirring the bucket by a plunger.
- Count the eggs under microscope with an aide of Rafter Counting Chamber and estimate total number of eggs obtained. While counting the eggs, check the fertilization by confirming the 1st polar body or more advance embryonic development such as 2-cell stage, 4-cell stage, and so on.
- Stock the eggs in incubator tanks, maximum stocking density of the eggs being 10 eggs per mL.

3-2. In vitro Fertilization (Gonad Stripping Method)

At present, a method using thermal shock with or without Spirulina bath treatment has been effective, but it has not always resulted in 100% success rate of the sea cucumber spawning induction work. Sooner or later, it is inevitable to develop an effective method for spawning both males and females. A Japanese group of scientists (Kato et al., 2009) found that neuronal peptides induced oocyte (ovum) maturation and gamete spawning of the Japanese sea cucumber *Apostichopus japonicus*. They extracted the neuronal peptides and so synthesized it chemically, which was effective to mature 150 µm diameter or larger eggs. They also experimented to inject this synthesized hormone into the body cavity of the sea cucumbers, resulting the male and female spawned 60 minutes and 80 minutes later, respectively. Unfortunately, they did not describe how far the eggs developed as embryos and whether their hatchery work went through to settlement as pentactula stage. Therefore, no information was available from their study for fertilization and hatching rates as well as survival rates during the larval and post-larval rearing works. They also stated that this chemical did not work effectively on immature ova and, thus, they concluded that the maturation mechanisms and process of ova/spermatozoa still needed further studies. Synthesizing and producing such a neuro-hormone commercially could be very expensive and won't be available for the Indo-Pacific region in foreseeable future. The important fact is that developing techniques of artificial maturation of oocytes (ova/spermatozoa) and activation of gametes (sperms) are the keys to success in obtaining the fertilized eggs. In this end, a gonad stripping method* could be an alternative to spawning induction work near future, either using synthesize neuronal hormone or other chemicals such as ammonia-seawater which has been used for commercial pearl oyster hatcheries in Japan, or just use of natural seawater.

**Note that the gonads are removed from the parent animal by cutting a small portion of the body and the gametes are obtained by stripping/squeezing the gonads (Fig. 13c-d). This is called "gonad stripping method".*

Although no one has been successful for in vitro fertilization of the sea cucumbers, the author thought that it was worthwhile for the hatchery technicians to understand principle and procedures of this method. During the hatchery training workshop in May 2015 at the Fijian Government's hatchery in Galoa, the author used filtered seawater (1 µm nominal pore) without using any other chemicals for the gonad stripping method for the sandfish (Ito, 2015). As a result, fertilized eggs subsequently underwent embryonic and larval development to the settlement as pentactula stage. Although the number of eggs and resultant pentactula were very small, several hundred, and low survival rate at less than 10 % to day 11, this method may be economical and could be the first step towards future improvements for a large-scale juvenile production on a regular basis.

4. Larval Rearing

Larval rearing of the sandfish sea cucumber requires knowledge of feeding capability of larval stages, suitable combination of food, calculating amount of feed mix, controlling algal cell (feed mix) suspension, adjusting feeding amount and rearing water volume, water quality control, and knowledge of optimal larval development in changing proportions of larval and post-larval stages; i.e. from hatching as auricularia stage to settlement as pentactula stage. Approximately 18-24 hours after spawning depending on water temperature, larval rearing work commences by draining the incubator to collect gastrula and/or auricularia larvae. For collecting larvae and post-larvae, a combination of 80 µm and 100 µm mesh

screen is to be used. Larval rearing tanks should be completely drained every other day, on days 1, 3, 5, 7, 9 and 11. The larval specimens need to be kept alive but immobilized/anesthetized by isopropyl alcohol for counting, measurements and microscopic photographs. For longer term preservation, use formalin (10% seawater formalin). Water temperature in the larval rearing could be better between 27-30 °C.

Hatchery facility should be maintained good conditions in terms of hygiene and efficiency for larval and post-larval rearing work:

- animals such as cats need to be kept away in- and out-side the hatchery, around the indoor and under-cover tanks and indoor storage areas;
- microalgae culture room should not be a sort of storage room with scattered lab supplies and equipment on the culture benches, dusty air-conditioners and air pumps without filter maintenances;
- air supply system needs to be functioning effectively, with sufficient air pressure in the under-cover areas as well as in the microalgae culture room;
- in-use or used tools should not be scattered on the floors, e.g. filter cartridges, filter-bags, hoses, pipes, buckets, air-stones, airlines, pipe-fittings, nets, plumbing machines; air leaks from many outlets with unnecessary accessories and fittings;
- sea water and freshwater supply piping are better to be simple and do not need unnecessary connections, diversions and outlets fittings;
- the hatchery staff understand general hygiene procedure before and during the hatchery operation.

4-1. Precautions before, during and after working at hatchery

For precautions of the onsite hatchery work, refer to “Trainer's manual for hatchery-based pearl farming” (Ito, 2005) or “A hatchery operations manual for rearing sandfish, *Holothuria scabra*, in Tarawa, Republic of Kiribati.” (SPC, 2015). The following were also described in those manuals.

- make sure your hands are clean. Wash your hand with soap, particularly dirty finger nails, before starting work.
- Soak your feet in “chlorine-bath” before entering in the larval and/or microalgae room.
- don't work with you own shoes. Always wear designated boots or work with barefoot.
- always rinse again the cleaned and dried equipment with filtered rainwater (1 µm) before use.
- for the used equipment/tools, wash first with chlorinated (public) water to wash out the waste/dirt.
- second-wash by using detergent and wash-off the dirt thoroughly with a soft sponge or brush.
- rinse with 1 µm-filtered rainwater and completely wash out residual soap/detergent.
- soak in chlorine-batch (a diluted Sodium Hypo-chloride, NaHClO) for overnight. Don't mix with soap or this may release Cl₂ (chloride gas).
- rinse completely with filtered rainwater (1 µm). Make sure “no residual chlorine”.



Figure 15. Seawater filters and UV sterilizer.



Figure 16a. Larval rearing (2,000L).



Figure 16b. Larval rearing (5,000L).



Figure 17. Sieves for collecting larvae.

- always hang and dry the equipment after being cleaned. Do not leave them on the floor or dirty bench.
- if necessary, use isopropyl-alcohol spray (75 % solution) and wait for it to dry. *Note that the use of methanol (methyl-alcohol) will become a health hazard in a small microalgae culture room.
- don't touch inside of the cleaned surface of equipment and tools such as bucket/ container/tank/tub/flask, etc.
- wash filter bags, cartridges and housings after every use. Wash out the dirt with pressurized freshwater, filtered rainwater (1 μ m), soak in chlorine-bath, rinse with filtered rainwater (1 μ m) and dry them on a designated bench. Keep the filter bags, cartridges in sealable plastic bags each with alcohol-sprayed inside. For the filter housings, spray alcohol inside and store them upside-down on the bench.
- make sure always clean the floor; wash with freshwater (chlorinated town-water or rainwater). It is the best that the floor is a “dry” condition when you start working in the following morning.
- don't disturb animal (larvae/juveniles/broodstock) and minimize giving shock or stress to the animals. Avoid unnecessary entry to the microalgae culture room and larval rearing unit.

4-2. Preparation for the larval rearing

The seawater for the larval rearing should be filtered to 1 μ m with a bag filter or cartridge filter. In-line UV-sterilizer is not necessarily required (Fig 15). When the day-1 larvae exceeded 0.35 larvae per mL in a rearing tank, the stocking density should be adjusted to make acceptable number of larvae in each tank: e.g. between 0.5 - 0.7 million larvae in a 2,000L tank or 1.25 - 1.75 million larvae in a 5,000L tank (Figs. 16). Gentle aeration is given throughout the larval rearing and the tank must be protected by a lid (tank cover) from debris from the ceiling. If the stocking density is less than 0.25 larvae per mL, the rearing water volume must be adjusted (reduced) to maintain required range of algal cell suspension based on *C. muelleri* culture density and number of larvae in each day. A combination of sieves is usually 80/100 μ m

throughout larval rearing and each sieve must be deep and wide enough (30 cm deep x 50 cm wide) to do sieving efficiently from a larger diameter drain pipe e.g. 25.4 cm (2 inch) pipe (Fig. 17).

Apart from tools and equipment, preparation of the live microalgae species (*C. muelleri*, *Navicula* sp. and *Cocconeis* sp.) need to be cultured at least two weeks before commencing spawning and larval run. For continuous culture and feeding the larvae, several subcultures should be made after commencing larval rearing, instead of starting from new stock culture (Figure 18). Larval rearing period with microalga *C. muelleri* feeding would finish within two weeks on day-14 after spawning. When a hatchery operation is planned a single spawning0larval run, therefore, it is not useful to start any new culture of *C. muelleri*. Generally, 7 days needed for a 2 - 5L high density starter cultures to be ready for starting 20 - 100L mass cultures, and these mass culture needs further 4 - 5 days to use for feeding the larvae. The author uses dried microalga Spirulina to mix with live diatom *C. muelleri* for larval and post-larval rearing. *Navicula* sp. and *Cocconeis* sp. are used for feeding settled pentactula and early juvenile stages up to two months old in settlement tanks. If live microalgae are used for sea cucumber hatchery, therefore, it is necessary to culture diatoms.



Figure 18. Microalgae (diatoms) for feeding larvae and post-larvae.

4-3. Feeding methods for larval rearing

Mixture of *C. muelleri* and Spirulina are given for feeding the larvae twice a day. Feeding is maintained by estimating algal cell suspension in each larval tank, starting from approximately 1,000 up to 20,000 cells per mL during the two-weeks larval rearing. Total cell suspension in each day is attained by combining these two feeds and expressed as the number of *C. muelleri* cells, where amount of Spirulina is computed and expressed as the number of *C. muelleri* cells. Feeding ratio of *C. muelleri* and Spirulina is approximately 80 % and 20 %, respectively. Feeding is divided into two; one in the morning between 8 and 10 AM, and the other in the late afternoon between 4 and 6 PM. The author developed daily feeding tables with simplified data-inputting methods, therefore, the hatchery staff are usually trained to use such feeding tables.

A hatchery protocol with daily work schedule for larval rearing was summarized and shown in Table 1. The hatchery staff needs to count the number of living larvae and post-larvae after tank draining every two days and *C. muelleri* to input daily culture density (million cells per mL). The feeding amount of both *C. muelleri* and Spirulina are obtained instantly in those feeding tables. All feeding amount were converted and expressed as *C. muelleri* cells. For the Spirulina, the hatchery staff simply measures the dry-weight according to the feeding table, prepare for AM or PM feeding amount, dissolve in the freshwater (about 500 mL) and wait for an hour before adding to the rearing tanks. It is advised the stocking density of larvae from onset of larval rearing on “day 1” to be between 0.25 - 0.35 larvae per mL.

Table 1. Hatchery protocols for juvenile production of the sandfish sea cucumber.

HATCHERY PROTOCOL (WORK SCHEDULE)	*larval & post-larval development based on the water temperature at 29 ± 1 °C
days of run (size.; µm)	*larval rearing tank (LR) = 1 x 5,000L
Algal cell suspension (as <i>C. muelleri</i> cells mL ⁻¹)	*settlement tank (ST) = 4 x 5,000L (bottom area = 5,000 m ²); settlement plates=50cm x 50cm x 800~1,000 plates per 5,000L tank *plates = corrugated plastic plates *juvenile down-weller tank (JHS) = 4 x 10,000L (bottom area = 10,000 m ²); sandy bottom covered with mangrove mud (dried mud sieved through 100 µm screen)
-3d before spawning	Start conditioning settlement tanks and plates; add benthic diatoms (i.e. <i>Navicula</i> sp. & <i>Coccconeis</i> sp. 200L each in 5,000L settlement tank) & nutrient (100% strength) to culture in the settlement tanks
0 (egg: 150-160)	Collecting (sieves 50 & 80 µm mesh screen), washing, counting & incubating eggs (up to 50 million eggs per 5,000L incubator)
1 (420 x 320)	Draining incubator & collecting gastrula & early auricularia (sieve 80 & 100 µm) approx. 20 hours after fertilization; sampling, counting & stocking to larval rearing tanks (1.5 million larvae in 5,000L tank); start feeding larvae with live microalga <i>Chaetoceros muelleri</i> and dried microalga <i>Spirulina</i> sp. as soon as stocking larvae.
900-1,500 cells/mL	
2 - 4,000-7,500 cells/mL	
3 - 7,000-12,000 cells/mL	Larval tank draining (80 & 100 µm) & tank change (100% Water Exchange); early auricularia (20%) + mid-auricularia (80%)
4 - 8,000-14,000 cells/mL	
5 (800-1000) - 8,500-15,000 cells/mL	Larval tank draining (80 & 100 µm) & tank change (100% WE); mid-auricularia (20%) + late-auricularia (8%)
6 - 9,000-17,000 cells/mL	
7 (800-1200 x50-80)	Larval tank draining (80 & 100 µm) & tank change (100% WE); late-auricularia + fully developed late auricularia; remove settlement plates to dry; drain settlement tanks & refill the settlement tanks with new benthic diatoms (200L each in 5,000L tank) & nutrient (1/20 th strength)
9,500-18,000 cells/mL	
8 - 10,000-20,000 cells/mL	Spray <i>Spirulina</i> on the settlement plates (<i>Spirulina</i> 30g per liter freshwater solution) & dry the plates
9 - 8,000-18,000 cells/mL	Larval tank draining (80 & 100 µm) & tank change (100% WE); late-auricularia + fully developed late auricularia + post-late auricularia (= metamorphosing to doliolaria) + doliolaria (~ 5%)
10 - 6,000-16,000 cells/mL	Return the plates to the settlement tanks
11 (400-1200 x 60-80)	Larval tank draining (80 & 100 µm), collect larvae & post-larvae & transfer them to the settlement tanks; late-auricularia + fully developed late auricularia + post-late auricularia + doliolaria + pentactula; *survival rate from day-1 = ave. 30% (approx. 0.5 million each in 5,000L settlement tanks)
4,000-13,000 cells/mL	
12 - 2,000-6,000 cells/mL	*feeding only with <i>C. muelleri</i> for the swimming stages (no need of using <i>Spirulina</i>)
13 - 500-2,000 cells/mL	*feeding only with <i>C. muelleri</i> for the swimming stages (no need of using <i>Spirulina</i>)
14 - 200-1,000 cells/mL	Finish using <i>C. muelleri</i> & <i>Spirulina</i> for feeding larvae; fully developed late auricularia + post-late auricularia + doliolaria + pentactula
15 (~ 1mm)	Start flow-through over 24 hrs. 100% WE; (inlet water = 1 µm filter & outlet water = 100 µm screen); post-larvae; Start feeding daily with 3 foods (fishmeal 5g + seaweed 5g + mud 12.5g) per 100,000 post-larvae; *Sieve mud, fishmeal & seaweed with 100 µm screen for feeding; *no need of giving <i>Spirulina</i> as the settlement plates still covered with <i>Spirulina</i>
21 (1 ~ 2mm)	flow-through 24 hrs.100% WE; (inlet = 1 µm filter & outlet = no screen); continue daily feeding at 0.25% of BW with 3 foods (fishmeal 10g + seaweed 10g + mud 25g) per 100,000 early juveniles; *sieve feed-mix with 100 µm screen for feeding
28 (1-mo) (2~5 mm, 0.01~0.1g)	flow-through 24 hrs.100% WE; start daily feeding at 0.25% of BW with 4 foods (<i>Spirulina</i> : fishmeal : seaweed : mud = 1 : 2 : 2 : 5); *start giving <i>Spirulina</i> ; *settlement success rate from day-11 to 1-mo = 20-50%
30	Start weekly feeding at 0.25% of BW with 4 foods (<i>Spirulina</i> , fishmeal, seaweed & mud)
Day 35	Continue weekly feeding
Day 42	Continue weekly feeding
Day 49	Start transferring juveniles (2-mo = ave. 1g BW, 10-20mm BL) from settlement tank (ST) to down-weller “habitat simulator” for juveniles (JHS);
Day 56 (2-mo = J2) (0.2~2g)	*increase proportion of fishmeal and seaweed (<i>Spirulina</i> , Fishmeal & Seaweed = 1 : 2.5 : 2.5); *stocking density of JHS = approx. 10,000~1,500 x J2 per m ² (= 10,000~15,000 xJ2 in a 10,000L JHS tank);

(Example 1)

If the rearing tank volume is 2,000 L (=2,000,000 mL),
C. muelleri culture density is 3.0 million cells/mL, and
today's total amount of *C. muelleri* required is 2,000 mL

“total *C. muelleri* cells in the rearing tank” =
“culture density of *C. muelleri* ” x “ today's required amount of *C. muelleri* ”
(= 3 million x 2,000 = 6.0 billion cells)

Therefore, today's “ *C. muelleri* cell suspension ” in the 2,000L tank
= 6 billion cells / 2,000,000 mL = 3,000 cells/mL

(Example 2)

If today's required total feeding amount of larva is 12,000 cells/larva/day,
number of larvae in 2000L tank is 500,000,
today's required algal cell suspension is 3,000 cell/mL, and
today's algal culture density is 3 million cells/mL

“required total algal cells in the 2,000 L rearing tank”
= 12,000 (cells/larva/day) x 500,000 (larvae) = 6 billion cells
*algal cell suspension in this 2000L tank = 6 billion cells / 2 million mL = 3,000 cells/mL

Therefore, today's “total amount of algae” = 6 billion cells / 3 million cells/mL = 2,000 mL

For feeding the larvae, the hatchery staff must take algal samples (= *C. muelleri*) and counts the culture density; input each counting results into the designated cell in the MS Excel spreadsheet (“HIROITO CONSULTING's Sandfish Feeding Tables”), of which table automatically calculates the daily (in the morning-AM and afternoon-PM) feeding amount of both *C. muelleri* and Spirulina. “estimated available food per larva as CM” is based on the past results of larval feeding experiments of the sandfish and other sea cucumber species by the author and others in Japan and elsewhere, which ranges from 3,000 to 60,000 cells/larva/day.

Note that a commercially available dried alga Spirulina contains certain amount of dried form of cells (e.g. 2.6 billion cells per 1 g). The author found about three different sizes (e.g. 10 µm x 15 - 50 µm) and shapes (e.g. rectangular). Average size of *C. muelleri* is about 10 x 5 µm. 1 g of Spirulina cells are approximately to 42.8 billion *C. muelleri* cells. Therefore, it is necessary to estimate approximate volumes of those different sizes of Spirulina cells and to convert to the number of cells to *C. muelleri*. This is because total feeding amount is expressed as the number of *C. muelleri* cells and the feeding ratio (based on the number of cells) of *C. muelleri* vs. Spirulina is 80 % vs. 20 %.

4-4. Example of two spawning-larval runs in September 2017 at COM's Nett Point Hatchery in Pohnpei, FSM.

During the work, 1.4 million eggs (trial 1) and 5.3 million eggs (trial 2) were stocked in the 1,000L incubators. Approximately 20 hours after spawning, each yielded 0.42 million larvae (trial 1) and 2.5 million larvae (trial 2). As the two 2,000L round tanks were needed each with approximately 600,000

larvae for the trial 2. The followings were the results of larval runs between day 1 and day 11:

- trial 1 at survival rate of 10.9 % with 63,750 swimming stages on day 11 including late auricularia, doliolaria and pentactula from 416,250 larvae on day 1;
- trial 2-1 with 67,500 from 620,625 at survival rate of 15.3%;
- trial 2-2 with 221,250 from 620,625 at survival rate of 35.6%

Average survival rate of the three larval runs was 20.6% from day 1 to day 11. Total 353,500 larvae and post-larvae were transferred to three 2,500L settlement tanks.

4-5. Larval Development

Detailed morphological descriptions of the complete larval and post-larval development for both sandfish (*H. scabra*) and black teatfish (*H. whitmaei*) were given in Appendix 1 with notes on larval growth and durations in Appendix 2. Comparisons of morphological features of larval and post-larval stages of these two holothurian sea cucumbers were also given in Appendix 3.

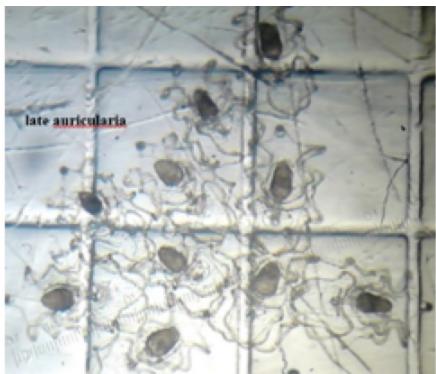


Figure 19. Late auricularia.



Figure 20. Post-late auricularia & doliolaria.

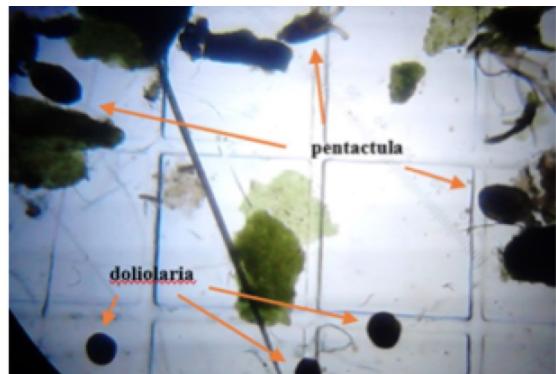


Figure 21. Doliolaria settling and pentactula settled.

5. Settlement Techniques

Settlement techniques involves conditioning of settlement plates and tanks, culturing benthic diatoms. The pentactula generally settles on the sandy sea grass bed such as *Talassira* spp. (for sandfish) in tidal flat zone, coralline gravels and rocks in the intertidal or littoral zone (e.g. black teatfish, greenfish). In the hatchery, the pentactula settle anywhere in the tank and so corrugated plastic plates are usually used as settlement substrates. Surface of settlement substrates such as plastic plates and inside the tank are conditioned with dried alga Spirulina and/or with epiphytes either naturally occurring or cultivated. The author uses two species of benthic live diatoms (*Navicula* sp. & *Cocconeis* sp.) as epiphytes on the settlement substrates. On day 11, larval rearing tank was drained and all swimming stages including fully developed late auricularia, metamorphosing post-late auricularia and doliolaria were collected onto a 100 µm screen (Figs. 19-20). A small percentage of pentactula up around 5% (Fig. 21) might have also settled on this day, so soft brushes were used to collect those settled individuals. Samples were taken for counting to estimate number of living individuals of all stages. Then, they were transferred to settlement tanks.

Table 2. Dimension of settlement plates used at COM's Hatchery in Pohnpei, FSM.

Settlement Plates (type)	Length (m)	Width (m)	Surface Area - both sides (square meters)
Large A (LA) =	0.65	0.44	0.57
Large B (LB) =	0.44	0.44	0.39
Medium (M) =	0.39	0.33	0.26

and a full strength of nutrient media (i.e. medium F2 in Australia and Fiji, OFCF/JICA's medium in Kiribati, MI medium and Kent's F2 media at COM in FSM) together with sodium meta-silicate 15 - 30 g were added to the 2,500L tank. About 10 days after the initial diatoms culture, the settlement plates were removed for drying and the tanks were drained to renew the culture water (1 µm filtered seawater) and to refill with new benthic diatoms (100L mas culture each) and 1/20th strength of nutrient media. After a couple of days for drying, the settlement plates were sprayed (or painted) with Spirulina (30g/L solution) and keep drying for a day or two (Figs. 22-23). On day 10, a day prior to transfer larvae and post-larvae to settlement tanks, return the Spirulina-sprayed plates in the settlement tanks. During the work at COM's hatchery in 2017, three different sizes of corrugated plates were used for settlement (refer to the table 2 of plate's dimensions). In a 2,500 L settlement tank, 300 - 400 plates of the type-M are mainly deployed. The plates were washed by detergent, disinfected by chlorine and dried for a couple of days before growing benthic diatoms on the plates. For larval rearing with a 2,000L tank, two of 2,500 L rectangular tanks (raceways) were used for settlement of doliolaria to pentactula, approximately up to 100,000 pentactula in each settlement tank (Fig. 24). On day 11, about 200,000 swimming stages, which consist of feeding and non-feeding stage of late auricularia, post-late auricularia and doliolaria, are expected in each 2,000L rearing tank out of 600,000 larvae on day 1.

5-2. Transferring swimming and settled stages to settlement tank.

Onset of the settlement (on day 11) is called “the 1st phase nursery culture”, which continues for about 2 - 3 months until the juveniles of 1- 2 g body weight (BW in wet weight) in average are transfer further to a down-weller “juvenile habitat simulator” tank or bag net “hapa” for a pre-growout or “the 2nd phase nursery culture”.

Living individuals on day 11 are usually consisted of the settled pentactula stage (10 - 15%), transitional doliolaria stage (20 - 25%), metamorphosing late auricularia = post-late auricularia stage (30 - 40%), fully-developed late auricularia (20 - 30%) and late auricularia stage (10 - 20%). It is better to transfer procedure when the proportion of settling or settled animals ($50\% < \text{post} + \text{dolio} + \text{penta}$) occupy more than a half in the larval rearing tank. The larval rearing tank is drained to collect all the animals onto 80 µm and 100 µm sieve. Collected specimens should be poured over the plates in the settlement tanks.



Figure 22. Spraying Spirulina.



Figure 23. Drying plates after spray.

5-1. Conditioning settlement substrates.

Conditioning settlement plates and tanks should commence at least two weeks prior to settlement. To start culturing benthic diatoms *Cocconeis* sp. and *Navicula* sp. in the settlement tank, 2,500L tank for example, 100L each of mass-cultured those diatoms



Figure 24. Settlement tank.

Toward day 11, all the settlement substrates (plates and tank surface) should be covered with light brown -colored benthic diatoms. If sunlight is too strong, the settlement tanks will be dominated by green alga (e.g. *Enteromorpha* spp.). In that case, the tanks should be covered by shade-cloths (75-80% shading rate). If pink- colored organisms (pathogenic bacteria *Pseudomonas* spp.) began visible after a week of benthic diatom culture in the settlement tank, those plates, tank surface and fittings such as air stones, air tubing, PVC pipes or ropes must be removed from the tank and disinfected by chlorine or discarded all or part of them to avoid further infection of the pathogenic bacteria. Such infection is always caused by inexperienced hatchery technician's careless setting of the settlement tank and plates, poor skill of culturing microalgae and larval rearing.

After day 11, the larval rearing continues in a static water condition because there are still swimming and feeding stages of auricularia larvae (late auricularia, fully developed late auricularia and metamorphosing post-late auricularia). Therefore, it is necessary to provide microalga *C. muelleri* until around day 14. From day 15, the rearing water in the settlement tank should be switched from static to flow-through, continuously flowing in from filter bag and flowing out to overflow-drain pipe at 100 % water exchange rate over 24 hours. 100 µm screen is used to cover the drain outlet for minimize loss of swimming stages (Fig. 25).

5-3. Examples of juvenile productions using the settlement plates.

The measurements of the settlement tank (ST), settlement plates (LA, LB, M), sample plates showing surface areas (in square meters) and estimated number of juveniles were given in the following tables 3 and 4: 30 small sample plates, 0.1m x 0.1m corrugated plastic, were also made for estimating settlement on the tank surface sides and bottom. Total of 79,180 one-month-old juveniles (on day 28, J1W1) were estimated to settle in three tanks (Figs. 26-27).

6. Pentactula and Juvenile Rearing

Pentactula and the early juveniles settled on the plates or tank surface are given additional food daily with a mixture of Spirulina, fishmeal, seaweed and mud. The mixtures of these food are homogenized and passed through 100 µm mesh screen before feeding. The juveniles on the plates and tank surface are kept in the settlement tank for two or three months and then the juveniles are transferred to "the 2nd phase nursery culture" either in the down-wellers (or habitat simulators) for juveniles or hapas bag net. The 2nd nursery culture continues further two or three months before being transferred to grow-out farm/ ponds/ enclosure. In Australia, a large-scale private farming enterprise does not conduct "the 2nd phase nursery culture", except for selective breeding programs, but those grown juveniles of average 2 - 5 g BW from the settlement tanks are also transferred to ocean grow-out sites (Fig. 28-29).

Culture techniques of pentactula and early juvenile stages in the settlement tank (the 1st phase nursery culture) from day-11 to day-56 or 8 weeks after spawning (approximately 2-month-old juveniles of 10 – 20mm, average 1g size) include selection and collection of food, preparation of food (e.g. drying, sieving and storing) and calculation of amount of food based on the number and weight of juveniles. As the feeding begins with mixture of three food (fish meal, seaweed and mud), rearing method for the 1st nursery phase switches from a static water method to a flow-through method with 100 % water exchange rate over 24 hours. 1 µm filter bag is attached to the inlet of seawater and a 100 µm screen is attached to the drain outlet, the latter is for catching any swimming stages such as doliolaria and auricularia which

Table 3. Surface areas of settlement tank and plates used at COM's hatchery in September - October 2017 in Pohnpei, FSM.

Surface Area (square meters)	bottom	sides	up-down	total surface area
2,500L mark =	5.00	5.00	1.00	11.00
	Surface Area - both sides (square meters)	No. of plates used in Tank #1	No. of plates used in Tank #3	No. of plates used in Tank #2
plate-LA (0.65 x 0.44)	0.57	32	7	0
plate-LB (0.44 x 0.44)	0.39	0	142	0
plate-M (0.39 x0.33)	0.26	0	92	212
	Total surface areas of plates in ST#1-3	18.24	83.29	55.12
	Total surface area ratio (tank vs. plates)	1.66	7.57	5.01

Table 4. The estimated number of the early juveniles on day 28 (1-month after spawning) during the juvenile production work in September - October 2017 at COM's hatchery in Pohnpei, FSM.

	Tank #1	Tank #3	Tank #2
Results of Sampling on Day 28 (J1W1) plate-LA	498 juveniles on 6 out of 30 plates	906 juveniles on 4 out 15 plates	
Estimated number of juvenile on the plates	2,485	3,398	
Results of Sampling on Day 28 (J1W1) plate-LB		5,279 juveniles on 20 out of 215 plates	
Estimated number of juvenile on the plates		53,372	
Results of Sampling on Day 28 (J1W1) plate-M	161 on 2 out of 2 M (side) converted as 1/2 of LA (side)		355 juveniles on 33 out of 212 plates
Estimated number of juvenile on the plates	161		2,503
Results of Sampling on Day 28 (J1W1) sample plates (0.1mx0.1mx30plates)			68 juveniles on 30 out of 30 plates
total number of juveniles (on plates)	2,646	56,770	2,503
total number of juveniles (tank surface)	3,179	11,589	2,493
total number of juveniles (plates + tank)	5,825	68,359	4,996

Table 5. Examples of daily feeding amount after settlement (if total 200,000 settled) for the subsequent two weeks between day 15 and day 27.

ratio against 100,000 juveniles =	2.00	(If total 200,000 juveniles)		
	Spirulina (g)	Fishmeal (g)	Seaweed (g)	Mud (g)
d14	N/A	10.00	10.00	25.0
weekly	N/A	70.0	70.0	175.0
d21	N/A	20.0	20.0	50.0
weekly	N/A	140.0	140.0	350.0

Table 6. Feeding schedule for the sandfish juvenile grow-out for the COM's hatchery work in September – October 2017 in Pohnpei, FSM.

No. of Juveniles	*feeding amount changes from 0.25% - 0.25% - 0.25% - 0.5% - 0.5% - 1% of the average body weight in each month							
	daily amount of feed (g) = smaller size			Mud (g) = 3-feed total	daily amount of feed (g) = larger size			
26,458 (estimated number of pentactula on day 15) survival rate to 1M = 19%	Spirulina	Fishmeal	Seaweed		Spirulina	Fishmeal	Seaweed	
1-2M (0.01-0.5g) (ratio) 0.25% BW	1	2	2	3-feed total (g)	1	2	2	3-feed daily total (g)
*estimated number on day 28 in ST2 (2,500L tank)								
4,996	0.02	0.05	0.05	0.12	1.2	2.5	2.5	6.2
4,996	0.14	0.29	0.29	0.72	2.2	4.4	4.4	10.9
4,996	0.26	0.52	0.52	1.31	3.1	6.2	6.2	15.6
4,996	0.38	0.76	0.76	1.90	4.1	8.1	8.1	20.3
*2M transferred from ST#2 to JHS#3: 4 weeks total feed=	5.68	11.37	11.37	28.42	74.3	148.6	148.6	371.6
2-3M (0.2-2g) (ratio) 0.25% BW	1	2.5	2.5	3-feed daily total (g)	1	2.5	2.5	3-feed daily total (g)
5,000	0.4	1.0	1.0	2.5	4.2	10.4	10.4	25.0
5,000	0.8	2.1	2.1	5.0	5.7	14.3	14.3	34.4
5,000	1.3	3.1	3.1	7.5	7.3	18.2	18.2	43.8
5,000	1.7	4.2	4.2	10.0	8.9	22.1	22.1	53.1
survival rate to 3M = 100% 4 weeks total feed =	29.2	72.9	72.9	175.0	182.3	455.7	455.7	1225.0
3-4M (1-5g) (ratio) 0.25% BW	1	5	5	3-feed daily total (g)	1	5	5	3-feed daily total (g)
5,000	1.1	5.7	5.7	12.5	5.7	28.4	28.4	63
5,000	2.6	13.0	13.0	28.6	7.1	35.5	35.5	78
5,000	3.1	15.6	15.6	34.4	8.5	42.6	42.6	94
5,000	3.6	18.2	18.2	40.1	9.9	49.7	49.7	109
survival rate to 4M = 100% 4 weeks total feed =	73.6	367.9	367.9	809.4	218.8	1093.8	1093.8	2406.3
4-5M (2-10g) (ratio) 0.5% BW	1	10	10	3-feed daily total (g)	1	10	10	3-feed daily total (g)
5,000	2.4	23.8	23.8	50.0	11.9	119.0	119.0	250
5,000	3.3	32.7	32.7	68.8	14.9	148.8	148.8	313
5,000	4.2	41.7	41.7	87.5	17.9	178.6	178.6	375
5,000	5.1	50.6	50.6	106.3	20.8	208.3	208.3	438
survival rate 5M = 100% *4M transfer to farm/pond 4 weeks total feed =	104.2	1041.7	1041.7	2187.5	458.3	4583.3	4583.3	6926.0
5-6M (5-20g) (ratio) 0.5% BW	1	20	10	3-feed daily total (g)	1	20	10	3-feed daily total (g)
5,000	4.0	80.6	40.3	125.0	16.1	322.6	161.3	500
5,000	5.0	100.8	50.4	156.3	20.2	403.2	201.6	625
5,000	6.0	121.0	60.5	187.5	24.2	483.9	241.9	700
5,000	7.1	141.1	70.6	218.8	28.2	564.5	282.3	875
survival rate to 6M = 100% *5M transfer to farm/pond 4 weeks total feed =	155.2	3104.8	1552.4	4812.5	621.0	12419.4	6209.7	19250.0

are returned to the settlement tank. Depending on presence of the swimming stages i.e. late auricularia and post-late auricularia, feeding with *C. muelleri* ends on around day 14. The juvenile foods are prepared as follows:

- after collecting from the wild, seaweed (Fig.30) and mud (Fig. 31) are sundried (Fig. 32);
- mud is sieved through 200 or 250 µm screen (Fig 33) and the seaweed is chopped in fine pieces (Fig. 34);
- after weighing, seaweed and fish meal are dipped in 1 µm filtered seawater for a several hours or overnight (Fig. 35)
- Spirulina is dipped in freshwater (rainwater) for several hours or overnight;
- Use blender to make the softened-foods finer pieces;
- sieve through 100 µm before feeding the animals

The protein content is the most important element in feeding pentactula and juveniles. Low fat content (crude fat around 5%) is desirable to maintain better water quality during the larval rearing and the 1st phase nursery culture in the settlement tanks. The author uses dried alga Spirulina instead of Algamac® (*Algamac ProteinPlus®) because Spirulina has much higher protein with low fat contents and the latter has higher fat contents. The author's choice of fishmeal is for milkfish (herbivore fish) farming because of less crude fat contents (about 3 %) compared to other fishmeals (Fig. 36). The author also uses the seaweed Sargassum spp. and Gracilaria sp., which contains about 10% crude protein with other essential nutrients, for free feed from the wild supplementing Spirulina and fishmeal. Mud or silt can be collected from the tidal flat area of the mangrove shore. The author has been using to mix with other feed (i.e. seaweed, fishmeal and Spirulina) for the juvenile grow-out as well as broodstock conditioning. The mud



Figure 24. Settlement tank.



Figure 25. Screen-covered drain outlet.



Figure 26. Day 28 juvenile.



Fig 27. Day 28 settled juveniles.



Figure 28. 5 g size 3-mo juveniles



Figure 29. Juveniles 3-mo in ocean grow-out site.



Figure 30. Seaweed *Sargassum* sp.



Figure 31. Mud from mangrove tidal flat.



Figure 32. Drying seaweed and mud.



Figure 33. Dried and sieved mud.



Figure 34. Dried seaweed.



Figure 35. Preparation for feeding.



Figure 36. Fishmeal.



Figure 37. Down-weller “habitat simulators” for juvenile grow-out.

contains detritus with organic substances and symbiotic heterotrophic micro-organisms in the mangrove forest (i.e. *Schizochtrium* spp.) which are known to have high contents of poly-unsaturated fatty acid ($\omega 3$ fatty acid). This heterotrophic (propagating without using sunlight) organisms live in the mangrove shore. *Algamac® and Algamac ProteinPlus® are commercial products from Aquafauna BioMarine, USA.

From day 28 or one month after spawning to six-month-old juveniles, an example of feeding schedule was given in the following table 6: juveniles from 2-month-old (Settlement Tank #2 = ST2 with 4,996 J2W1) to 3-month-old in the 2,500L down-weller (Juvenile Habitat Simulator #3 = JHS3 with 5,000 J3W1) where the juveniles were produced from the spawning on September 8, 2017.

Juveniles attached on the plates are transferred on around day 56 (two-month-old after spawning) to the down-weller “habitat simulators for juveniles” (Figs. 37-38). Although the larger the animals, it is the easier for counting and estimating the standing crop, the juveniles were large enough (10 - 20 mm, 0.2 - 2g) to estimate the number of individuals by a naked eye.

Between 2 - 3 months old (1 - 5 g) after spawning, down-wellers “habitat simulators for juveniles” are stocked with juveniles either being attached on or being detached from the settlement plates. Stocking density of each tank was approximately 1,500 juveniles per square meter. Those remaining juveniles in the settlement tanks need to be fed weekly but are to be transferred in earlier occasions to hapas or ocean grow-out sites. The juveniles in the habitat simulators will be fed weekly for subsequent two or three months before transferring to grow-out phase (farming) in the ocean sites, earthen ponds or restocking sites.



Figure 38. Estimating settled 2-mo juveniles by detaching from the plates.

7. Grow-out Culture

At around four or five months after spawning, the juveniles should reach to average 5 - 10 g. So, they are ready for farming. There is no published information available on the feeding techniques or feeding amount for both settled pentactula, early juveniles and juveniles up to six months after spawning. Also, there is no information about feeding techniques after six months old to harvesting size to export. The

Table 7. The results of counting of two-month-old juveniles attached on the settlement plates and estimate of those attached on the tank surface during the hatchery work in September - October 2017 at COM's hatchery in Pohnpei, FSM.

day 49 (Oct. 27-29, 2017)	Settlement Tank #1 (from LR2-1)	Settlement Tank #2 (from LR1)	Settlement Tank #2 (from LR2-2)
counted total number of juveniles (on plates)	2,154	2,975	17,067
estimated total number of juveniles (tank surface)	3,670	2,021	51,304
estimated total number of juveniles (plates + tank)	5,824	4,996	68,371

Table 8. Restocking JHS tanks with 2-mo juveniles.

2.5t Habitat Simulator Tank for Juveniles (with plates)	No. of Juveniles Counted
JHS#1 =	5,985
JHS#2 =	4,642
JHS#3 =	5,129
JHS#4 =	4,569
JHS#5 =	1,871
total =	22,196

Table 9. Number of 2-mo juveniles in the settlement tanks.

2.5t Settlement Tank after Transfer (without plate)	No. of Juveniles Estimated
ST#1 =	3,670
ST#2 =	2,021
ST#3 =	51,304
total =	56,995

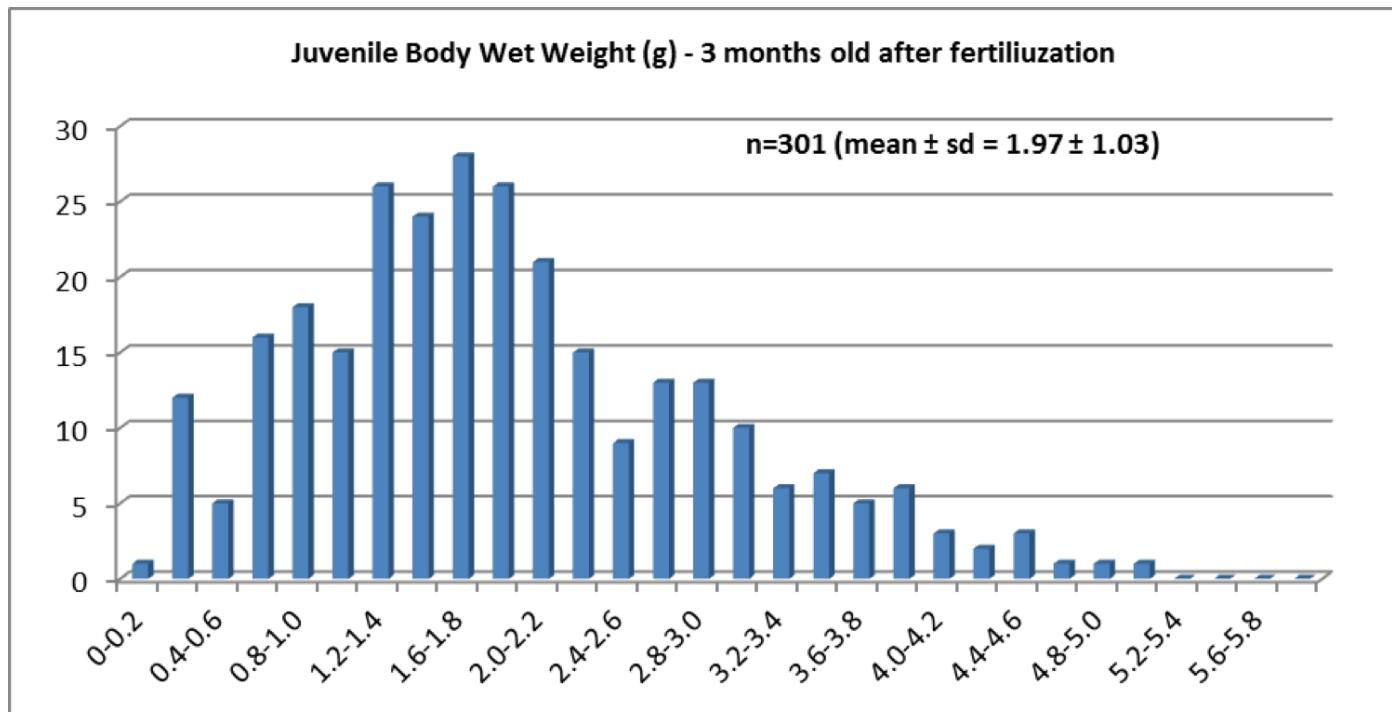
Table 10. Feeding table for 6-month-old juveniles in the downweller habitat simulator for juveniles.

	daily amount of feed (g) = smaller size				daily amount of feed (g) = larger size			
	Spirulina	Fishmeal	Seaweed	Mud (g) = 3-feed total	Spirulina	Fishmeal	Seaweed	Mud (g) = 3-feed total
6M (10-40g) (ratio) 1% BW	1	20	10	3-feed daily total (g)	1	20	10	3-feed daily total (g)
4,750	NA	306.5	153.2	475.0	NA	1225.8	612.9	1900
4,750	NA	536.3	268.1	831.3	NA	1379.0	689.5	2138
4,750	NA	766.1	383.1	1187.5	NA	1532.3	766.1	2375
4,750	NA	990.0	498.0	1543.0	NA	1685.5	842.7	2613
survival rate to 12M = 95% - 4 weeks total feed=	NA	18233.9	9116.9	28262.5	NA	40758.1	20379.0	63175.0

feeding table (Table 10) was developed by the author for private farming enterprise in Australia. Note that the feeding tables should be used only if actual or estimate of number of juveniles are not available. Feeding the juveniles and/or young adults are generally based on and calculated by the average body weight (wet weight). When they reach preferably to 10 - 20 g (average 5 - 10g) size after 3 - 5 months from spawning, they are ready for grow-out in the farm (ocean enclosures and/or ponds). Stocking density for the grow-out farming could be at 2 - 3 individuals per square meter (m²). In a hapa method elsewhere, 1- 2 g size about 2 or 3-month-old juveniles are stocked at 200 individuals per m². On the other hand in a down-weller juvenile habitat simulator (e.g. 10,000L in Australia) with tank floor area of approximately 10 m², initial stocking density is about 1,000 juveniles per m²). It is recommended to reduce the stocking density to at least half or preferably to 1/4 on around 5-month-old.

The following pages describe how to estimate feeding amount for the juveniles of 3-month-old. Also, explaining how accurate these feeding tables and how to use these tables developed by the author. For rearing early juveniles, it is recommended to feed the animals with 1/4 of adult grow-out amount = (1% body weight) x ¼ = 0.25% daily, and then increase the amount gradually to 0.5% and later around 5-month-old to 1%. The hatchery and farmhands must monitor the animals daily or at least weekly, such as measuring body weight by sampling monthly to adjust (increase) amount of weekly feeding amount. The following is an example of 300 juveniles of 3-month-old collected from a hapa elsewhere and measured body weight (mean ± sd = 1.97 ± 1.03 g). The histogram of these 301 (say 300) showed that they were smaller and closer to minimum weight group (1g group), indicating that they had not been fed well.

The following is how to estimate the weekly feeding amount of the 300 juveniles of 3-month-old, when they are required daily with 1% of body weight of food. Also, how-to estimate the feeding amount by using the juvenile feeding table when actual body-weight measurements were not done onsite.



(EXAMPLE) Histogram of the 3-month-old juvenile body weight obtained from hapa in a pond.

"How-to-Compute" weekly feeding amount (1% Body Weight) for 3-month-old "300" juveniles from hapa.

$$1.97g \times 1\% \text{ BW per day} = 1.97g \times 0.01 = 0.0197g \text{ per day per juvenile}$$

$$0.0197 \times 300 \text{ juveniles per day} = 5.91g \text{ per day} = 5.91 \times 7\text{days} = 41.37g \text{ per week in total}$$

*Spirulina vs. Fishmeal vs. Seaweed = 1 : 5 : 5 for the 3-month-old juveniles
therefore,

$$\text{Spirulina} = 41.37 \times 1/(1+5+5) = 3.76g \text{ per week (0.54g per day)}$$

$$\text{Fishmeal} = 41.37 \times 5/(1+5+5) = 18.8g \text{ per week (2.69g per day)}$$

$$\text{Seaweed} = 41.37 \times 5/(1+5+5) = 18.8g \text{ per week (2.69g per day)}$$

* If number of juvenile is 300, then the feeding amount is 300/10,000 of the figures given in the following Feeding Table.

$$\text{daily amount of 3-feed total} = 25 \times 300/10,000 = 0.75g \text{ when given 0.25\% BW}$$

If given 1% BW,

$$\text{daily amount} = 0.75g \times 1/0.25 = 3g \text{ and weekly amount} = 3g \times 7\text{days} = 21g$$

$$\text{Spirulina} = 21 \times 1/(1+5+5) = 1.91g$$

$$\text{Fishmeal} = 21 \times 5/(1+5+5) = 9.55g$$

$$\text{Seaweed} = 21 \times 5/(1+5+5) = 9.55g$$

However, the amount is based on the Smaller Group (1g).

If the juvenile's average body wet weight is 1.97g (= 2g), the feeding amount should be increased and then, re-calculated for the "300" juveniles.

i.e. Each 1 g increment is $(875-1,75)/4 = 1,75$ per week

$$1\text{g weight group} = 175; 2\text{g weight group} = 175 + 175 = 3,50g; 3\text{g weight group} = 350 + 175 = 525g$$

Feeding Table for the 10,000 Juveniles (3 M): Smaller Group (1g) and Larger Group (5g)								
	Smaller Group (1g)			Larger Group (5g)				
10,000 3M juveniles	Spirulina	Fishmeal	Seaweed		Spirulina	Fishmeal	Seaweed	
3-4M (ratio) 0,25%BW	1	5	5	3-feed daily total (g)	1	5	5	3-feed daily total (g)
wk-1 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
wk-2 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
wk-3 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
wk-4 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
Weekly Total (g)	15.9	79.5	79.5	175.0	79.5	397.7	397.7	875.0

4g weight group = $525 + 175 = 700$ g; 5g weight group = $700 + 175 = 875$ g

therefore,

weekly amount of the 2g weight group could be 350g per "10,000" juveniles

weekly amount of the 2g weight group of the "300" juveniles = $350\text{g} \times 300/10,000 = 10.5\text{g}$ when given 0.25% BW

therefore,

Spirulina = $10.5 \times 1/11 = 0.95$ g

Fishmeal = $10.5 \times 5/11 = 4.77$ g

Seaweed = $10.5 \times 5/11 = 4.77$ g

If given 1% BW, weekly feeding amount of 2g weight group = $10.5 \times 4 = 42.0$ g

therefore,

Spirulina = $0.95 \times 1/0.25 = 3.8$ g

Fishmeal = $4.77 \times 1/0.25 = 19.1$ g

Seaweed = $4.77 \times 1/0.25 = 19.1$ g

The estimated weekly feeding amount from BW measurements ($1.97\text{g} \pm 1.03\text{g}$) was total $41.37\text{g} =$ Spirulina 3.76g + Fishmeal 18.8g + Seaweed 18.8g

Thus,

the above "Feeding Table" is very accurate ($+0.04\text{g} < > +0.67\text{g}$), almost the same feeding amount obtained from actual BW measurements.

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Culture of Sea Cucumbers in Korea: A Guide to Korean Methods and the Local Sea Cucumber in the Northeast U.S.

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CULTURE OF SEA CUCUMBERS IN KOREA: A guide to Korean methods and the local sea cucumber in the Northeast U.S.

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In an effort to develop suitable culture techniques for sea cucumber (*Cucumaria frondosa*) in the Northeast, this guide reviews the current knowledge of *C. frondosa* biology and reports on techniques for the hatchery culture of the Japanese sea cucumber *Apostichopus japonicus* learned during a research exchange between the United States (NOAA Sea Grant) and South Korea (National Fisheries Research and Development Institute). The final portion of the guide discusses the potential adoption of the culture techniques for *A. japonicus* for use with *C. frondosa*.

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INTRODUCTION

Sea cucumbers are a valuable fishery around the globe. The United Nations Food and Agriculture Organization (FAO) estimated the global production from both aquaculture and capture fisheries to be 153,183 metric tonnes in 2011 (FAO 2013a). Of that production, roughly 85% is the Japanese sea cucumber *Apostichopus japonicus* (1). Sea cucumbers are developed into a variety of products including dried muscle, fermented guts, and dried gonads (FAO 2013b). Wild stocks have been heavily fished in many areas around the world. As a result of overfishing, sea cucumber culture techniques have been developed for variety of species. Hatchery production has centered in the Pacific Rim and is used both as a source of juveniles for various culture activities and for stock enhancement and restoration efforts. Currently hatchery production exists for approximately a dozen different tropical and temperate species (Purcell et al. 2012, Table 1).

Integrated Multi-trophic Aquaculture (IMTA) efforts in northeastern North America have focused on various combinations of fish, shellfish, and algae. Sea cucumbers have been proposed as one potential species that could be added as a consumer of benthic deposits. Within the Gulf of Maine, the predominate sea cucumber species, *Cucumaria frondosa* (2), has been fished commercially in Maine since 1990. Landings peaked in 2004 with a harvest of just over 10 million pounds (ME DMR 2013). The value of the fishery in Maine fluctuated between \$66,000 and \$562,000 from 1994 until the peak in 2004. The price

per pound has more than tripled since the peak harvest in 2004. The economic value for the Maine fishery is based on the boat price paid to fishermen, and does not represent the significant increase in value that occurs with processing into various final consumer products.

Sea cucumbers from the Maine fishery traditionally have gone to the Asian food market, however the potential for various nutraceuticals may represent a higher value use. For example, trials are underway to examine the potential for Frondoside A, a compound extracted from *C. frondosa*, to treat cancer (Attoub et al. 2013). At least one company in the region is processing sea cucumbers for the extraction of various nutraceuticals.

A recent review of the potential for culture of *C. frondosa* in the Northeast (Nelson et al. 2012a) highlighted advantages and challenges for commercial culture of this species, in particular under IMTA. Strengths include an existing market for the product with good potential for increased nutraceutical use, a well-understood reproductive biology (especially for populations in the St. Lawrence River area), and evidence that *C. frondosa* would be a suitable species for integration in IMTA farms as a benthic filter feeder. Culture of this species faces several challenges. These include a relatively low market price, even when the fishery was at its peak, due to the thinner muscle wall, a long grow-out period, and a lack of published techniques for culturing this species.

Apostichopus japonicus



Michael Pietrak

Cucumaria frondosa



Hamel & Mercier (SEVE)

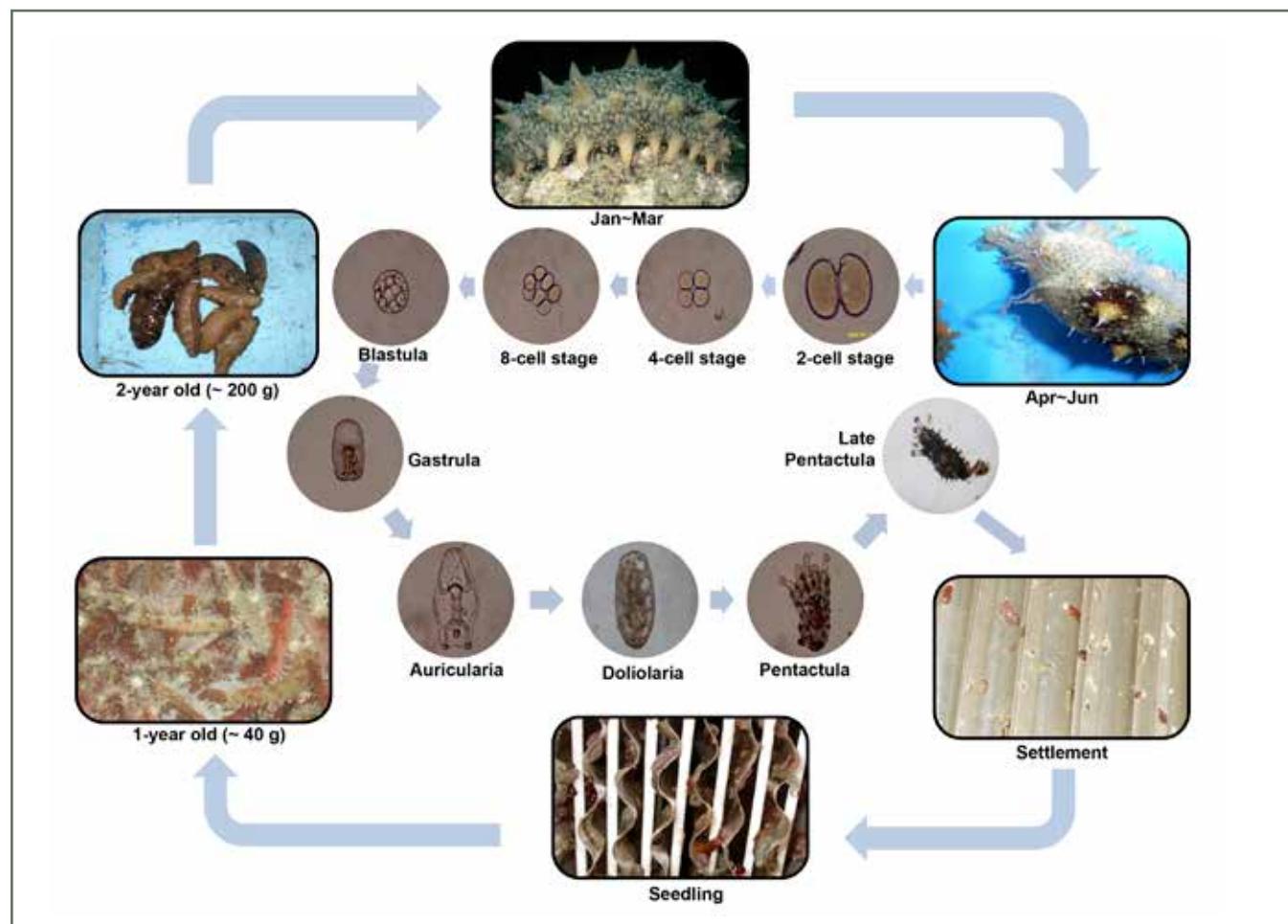
Table 1: Global hatchery production of sea cucumber juveniles. Modified from Table 1 in Purcell et al. 2012.

Country	Species cultured	Annual production of 1 g juveniles	Use of juveniles	Start year to end year
Australia (Northern Territory)	<i>Holothuria scabra</i>	62,000+	Sea ranching; pond farming	2004-ongoing
Australia (Queensland)	<i>H. scabra</i>	500,000	Sea ranching	2003-2009
Australia (Queensland)	<i>H. lessoni</i>	330,000	Sea ranching	2004-2009
Australia (Queensland)	<i>H. scabra</i>	1,000	Experimental	2004-2007
Canada	<i>Parastichopus californicus</i>	n/a	Pond farming	2009-ongoing
China	<i>Apostichopus japonicus</i>	>6 billion	Sea ranching; pond farming	1990-ongoing
Ecuador	<i>Isostichopus fuscus</i>	n/a	Experimental	2002-2008
Fiji	<i>H. scabra</i>	500	Experimental	2008-2010
FSM (Pohnpei)	<i>H. scabra</i>	10,000	Experimental	2009-ongoing
FSM (Yap)	<i>Actinopyga sp.</i>	n/a	Stock enhancement	2007
India (Tuticorn)	<i>H. scabra</i>	3000	Experimental	1988-2006
India (Tuticorn)	<i>H. spinifera</i>	n/a	Experimental	2001-2006
Iran	<i>H. scabra</i>	n/a	Experimental	2011
Japan	<i>A. japonicus</i>	>3 million	Stock enhancement	1977-ongoing
Kiribati	<i>H. fuscogilva</i>	500-8,000	Stock enhancement	1997-2009
Madagascar	<i>H. scabra</i>	200,000	Sea farming (pens)	2007-ongoing
Maldives	<i>H. scabra</i>	5 million	Sea ranching	1997-ongoing
Mexico	<i>I. fuscus</i>	300,000	Pond farming	2008-ongoing
New Caledonia	<i>H. scabra</i>	18,000	Experimental	2000-2006
New Caledonia	<i>H. scabra</i>	450,000+	Sea ranching; pond farming	2011-ongoing
New Zealand	<i>Australostichopus mollis</i>	n/a	Experimental	2007-ongoing
Palau	<i>Actinopyga mauritiana</i>	500,000	Stock enhancement	2009-2011
Palau	<i>Actinopyga miliaris</i>	50,000	Stock enhancement	2009-2011
Philippines (Bolinao)	<i>H. scabra</i>	32,000	Sea ranching	2001-ongoing
Philippines (Mindanao)	<i>H. scabra</i>	15,000	Sea ranching; pond farming	2009-ongoing
Philippines (Bolinao)	<i>Stichopus horrens</i>	500	Experimental	2009-ongoing
Philippines (Dagupan)	<i>H. scabra</i>	20,000	Experimental	2009-2011
Philippines (Iloilo)	<i>H. scabra</i>	11,000	Experimental	2010-ongoing
Saudia Arabia	<i>H. scabra</i>	n/a	Sea ranching	n/a
Solomon Islands	<i>H. scabra</i>	n/a	Experimental	1996-2000
USA (Alaska)	<i>P. californicus</i>	n/a	Experimental	2010-ongoing
Vietnam	<i>H. scabra</i>	200,000+	Pond farming	2001-ongoing

LIFE HISTORY AND BIOLOGY OF CUCUMARIA FRONDOSA

Mature adult sea cucumbers “broadcast spawn” their gametes. Fertilized embryos develop into pentacula, the five-tentacled larval stage in the life history of echinoderms. After a period of time in the plankton, the larvae settle to the sea floor and metamorphose into juveniles. *C. frondosa* average 20 cm in length, although they are capable of growing up to about 50 cm, and live in temperatures between -1.8 °C and 8 °C. Levin and Gudimova (2000) provide a good description of *C. frondosa*’s morphology.

Adult animals reach sexual maturity in about five years and around 80-100 mm in length. Adult animals display sexual dimorphism and can be easily distinguished when the feeding tentacles are extended. Females display a gonopore approximately 4 mm in diameter and a papilla approximately 7 mm in length with a single genital pore. Males possess a larger gonopore approximately 8-10 mm in diameter surrounded by between five and 22 papillae (Hamel and Mecier 1996).



Life history of sea cucumbers and milestones for their culture in Korea. Figure from NFRDI.

Spawning typically occurs in the St. Lawrence around mid-June (Hamel and Mercier 1996), while populations near Mount Desert Island in Maine spawned between the end of March and the end of May (Ross 2011). Day length is likely an important cue to start the process of producing gametes (Hamel and Mercier 1996, 1999), however cues in the mucus appear to be more important in synchronizing the spawning of animals in a given locality (Hamel and Mercier 1999). Following fertilization, the embryos develop into pentacula larvae. According to Hamel and Mercier (1996) this took nine days at 12 °C and 26 psu (practical salinity units; Table 2). Ross (2011) does not report embryo development timing beyond the 64-cell stage, which could take up to 48 hours. Ross did report that in one sampling season “elongated embryos” were found in the early plankton tows, and that lab ob-

servations showed that this stage was reached after four to seven days. Larvae prefer to settle on rock or gravel substrate after approximately 48 days after fertilization (Hamel and Mercier 1996).

Few have looked at growth rates, but Hamel and Mercier (1996) reported that the mean growth rate in the wild was 2 mm a month, while the maximal growth rate during the spring was 5-6 mm per month. Studies have also shown that the rate an individual *C. frondosa* inserts its tentacle into its mouth is related to the feeding rate of the animal (Holtz and MacDonald 2009). It has also been shown that *C. frondosa* are capable of feeding on high organic content materials, such as feces and fish feeds, from IMTA salmon farms (Nelson et al. 2012a). The absorption rate increased with increasing organic content, suggesting that *C. frondosa* might be cultured on salmon-mussel-algae IMTA farms.

Table 2: The time and size evolution of selected developmental stages of *Cucumaria frondosa* up to young sea cucumbers, under natural environmental conditions reproduced in the laboratory. Table modified from Hamel and Mercier 1996.

Stage of development	Time	Size (mm)
Fertilized oocytes	0 min	0.90±0.2
64-cell	24.0±2.0 h	1.40±0.5
Blastula	48.0±3.6 h	1.35±0.3
Hatching	62.0±8.0 h	1.30±0.4
Young gastrula	72.0±8.5 h	1.30±0.3
Late gastrula	4.3±0.5 d	1.40±0.5
Elongation	5.5±0.5 d	1.60±0.5
Vitellaria (uniformly ciliated)	8.0±1.0 d	1.55±0.3
Young pentactula (5 tentacles)	11.0±1.5 d	1.30±0.3
Appearance of the anal pore	13.0±1.0 d	1.35±0.4
Late pentactula (with pair of ambulacral podia)	17.0±1.4 d	1.40±0.5
Settlement (cilia loss)	46.0±2.0 d	1.40±0.4
Young sea cucumber with 2 pairs of ambulacral podia	3.0±0.2 months	1.70±0.5
Young sea cucumber with 5 pairs of ambulacral podia	4.0±0.2 months	1.90±0.5

Note: The temperature recorded throughout the development varied from 0 °C to 13 °C as reported in Figure 1 of Hamel and Mercier 1996. A new stage was considered attained when 50-60% of the embryos reached it.

KOREAN HATCHERY CULTURE TECHNIQUES

Hatchery culture systems

Culture facilities

In March 2013, a NOAA Sea Grant-sponsored delegation from the United States visited three different sea cucumber hatcheries near Gangneung in Gangwon Province, South Korea.

One of the facilities was a private hatchery that used discharged cooling water from a nearby coal-fired power plant as a heat source. This water was mixed approximately 50-50 with local seawater and sand filtered prior to use in the facility.

The second hatchery visited was a provincial government hatchery that reared various species for stock enhancement. A main seawater line supplied storage tanks for the entire facility. Incoming water was filtered and heated as required in the individual species culture buildings. Neither the private nor the provincial facility sterilized incoming water.

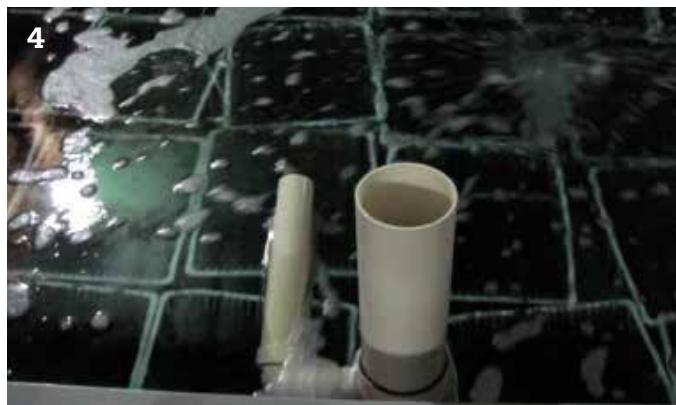
The final hatchery visited was an NFRDI research hatchery.

Culture tanks

All three hatcheries used similar rectangular culture tanks approximately five meters long and three meters wide, with a water depth of about one meter (**3**). A water inlet was located at the far side of the tanks; the bottom of each tank sloped slightly toward a drain at one end (nearest the walkway) to facilitate regular cleaning.

An internal standpipe of four-inch PVC acts as an emergency drain (**4**). The private hatchery attached a two- to four-inch "T" just below the water level in the tank with an unglued two-inch elbow that actually set the water level by having the pipe angled up, shown here. To partially drain the tank for the twice-daily water changes, the pipe is turned down, submerging it, draining the tank in a limited fashion.

A similar drain was installed between the tanks so that when moving the animals to the adjacent tank in order to clean the bottom, some of the already heated water could be saved. It is important to take the surface water, which is relatively free of sediments, and not the bottom water. At all locations the culture tanks were operated as static systems with partial water replacement twice a day, although the provincial hatchery had access to flowing seawater and recirculation systems.



Culture equipment

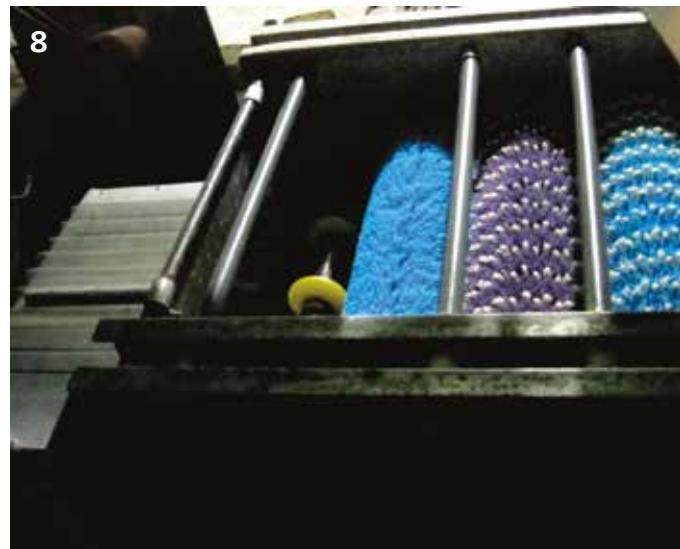
Very little equipment is required for spawning and rearing sea cucumbers in the hatchery. In addition to the culture tanks described above, it would be useful to have some smaller tanks for conditioning broodstock. The size and style of the tanks is not critical, but conditioning requires an ability to slowly raise the water temperature to 15 °C. Nets of the appropriate mesh size (200 µm for *A. japonicus* or 800 µm for *C. frondosa*) are required to transfer the fertilized eggs to the culture tanks described above, which are used for the entire culture cycle in the hatchery. Access to a dissecting microscope in order to view the development of

the larvae would be advantageous, however it is likely that an experienced operator will know the larval stage based on the degree-days since fertilization.

The most important pieces of equipment are the settling plates and the juvenile cucumber shelters (**5**). The shelters consist of an open plastic crate designed to hold file folders (**6**). Settling plates made from clear corrugated PVC sheets are cut to dimensions to fit inside the crate (**7**). In one of the hatcheries, the settling plates were treated with a vitamin mix prior to the first use, but this practice was not consistent at all of the hatcheries visited.



There is a need to clean the settling plates on a regular basis by hand washing/scraping or, with very dirty plates, an acid wash. One hatchery had a commercial plate washing machine that consisted of internal rollers that feed the individual panels and rotating brushes that scrub them clean (**8**).



Korean sea cucumber culture process

Broodstock conditioning and spawning

Adult *A. japonicus* are collected from the wild approximately 60 days in advance of the spawning season and placed into conditioning tanks at ambient seawater temperatures (9). The temperature is increased 1 °C per day until it reaches 15-18 °C. The animals are held at 15 °C for 800 degree days, at which point they should be ready to spawn. They can be fed a commercial diet during this time, which typically consists of powdered algae such as *Spirulina* spp. or *Dunaliella* spp. Feeding must be stopped the day before spawning to avoid contamination by sea cucumber feces.



Spawning is initiated through temperature shock: conditioned animals are moved from the holding tank directly into water that is 3-5 °C warmer. After release of both sperm and eggs, fertilized eggs can be removed with a net and placed into rearing tanks that have appropriately sized mesh covering the drains. The tanks should be set up as static systems. A sample of eggs can be taken and inspected for fertilization rates.

Larval culture

Fertilized eggs are collected from the spawning tanks and placed into the culture tanks at a density of 0.25 individuals mL⁻¹ (Kang et al. 2012). No specific density was provided, but the NFRDI culture tank shown here holds approximately one million larvae (10). Larvae are fed a diet of microalgae. For the first two days a 50-50 mix of *Isochrysis galban* and *Pavlova lutherii* is recommended. After day two they can be fed *Cheotoceros calcitrans*. Samples of larvae should be taken daily in order to monitor development. After approximately seven to eight days, larvae should be close to the pentacula stage and settlement. At this point shelters with clean settling plates can be placed in the larvae rearing tanks.



Upon settlement the new juveniles are transitioned to a feed mixture of mud or bentonite, yeast, and powdered algae (11).

Predation of larvae by zooplankton was reported to be a problem in at least one facility visited. This can likely be managed through good water filtration.

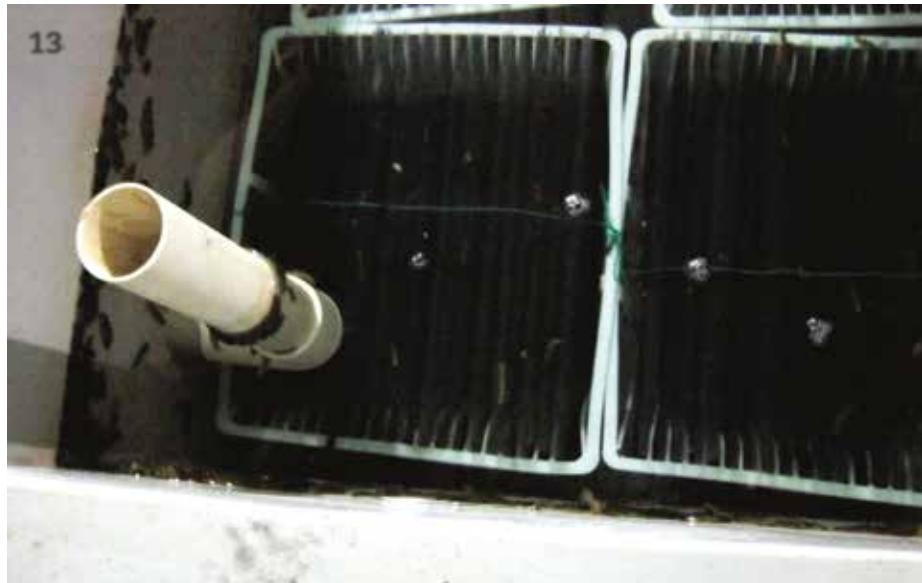


Juvenile culture

Juveniles are cultured on the settling plates for about a year until they are transferred to cage systems for grow-out at sea. Maintaining good husbandry practices in the hatchery is critical for preventing disease, which can be a significant issue. All of the systems visited maintained static water in the culture tanks, with twice-daily partial water changes at feeding times. In one facility, the water was changed before feeding in the morning and after feeding in the afternoon. The water temperature is maintained around 15 °C. Conservation of water heat was given as the reason for maintaining static systems. It is possible to use a flow-through system, as was observed at the NFRDI research lab, although animals were maintained under ambient water temperatures. It should be noted that the juvenile sea cucumbers are mobile and will move within and between the shelter and throughout the tank. Appropriate screening must be maintained on drains when conducting water changes or if using a flow-through system.

To maintain good water quality it is important to clean accumulated feed and feces from the bottom of the tanks approximately every five days. This is accomplished by moving the shelters to an adjacent tank that is empty of animals (**12**).

Surface water can be moved over to reduce the energy needed to heat the water. The image here shows a drain in the wall between adjacent tanks (**13**). Tilting the standpipe down allows clean, warm surface water from one tank to partially fill the adjacent tank where animals are moved while the old tank is cleaned. Mesh bags or screens should be placed on drains to capture loose animals in the tank being drained. Once drained, the tanks can be scrubbed clean and readied for the next transfer of animals. In addition to cleaning the tanks on a regular basis, the settling plates must also be changed out and cleaned about once every three months.



CULTURE POTENTIAL FOR CUCUMARIA FRONDOSA IN THE NORTHEAST

Cucumaria frondosa appears to be a potential candidate for culture in the northeastern United States. In particular, *C. frondosa* will likely make a good addition to IMTA sites being developed in the region, given its ability to feed on particulate organic wastes. Nelson et al. (2012b) reviewed the potential for culturing *C. frondosa* in the Northwest Atlantic and found it to be a potentially viable candidate species. Several major hurdles are immediately apparent to the culture of this organism. First, the current market for the wild caught *C. frondosa* does not appear to be valuable enough to make aquaculture economically viable. This situation is likely to change as higher-value uses of various extracts and nutraceuticals are further developed for both humans and animals. Second, a better understanding of some aspects of the basic biology is needed, in particular growth rates under various conditions and feeding levels. Finally, culture techniques will need to be adapted and optimized for this species.

A good starting point may be provided by the techniques observed in Korea, such as use of temperature shock to induce spawning. Nelson et al. (2012b) point out that hatchery production of juvenile sea cucumbers will likely be required as fishing for mature broodstock will compete with the wild fishery and juveniles are very difficult to find and collect in the wild. The long planktonic larval stage seen in *C. frondosa* compared to species raised in Korea may also pose additional problems. Hatchery producers will need to evaluate costs and feasibility of using the same tanks for juvenile culture and larval production. The long larval phase will also pose issues associated with water quality and treatment. Using static systems for the duration of the larval phase will likely not be possible, as a regular exchange of water will be needed to maintain water

quality. Prevention of larvae loss or damage will be an issue to contend with. Larval settlement onto the plate culture system used in Korea should be tried, however the spacing of the plates may need to be adjusted. The ability to grow sea cucumbers on a vertical surface will greatly increase space efficiency. As *C. frondosa* remains a planktonic feeder all of its life, their culture should not experience the transition to deposit feeders that was hinted at as a bottleneck in production in Korea, and might permit for the use of continuous flow culture tanks that will help to improve water quality and remove uneaten feed. If neutral to slightly negatively buoyant diets can be developed, flow through the tank should allow feed to be carried to all animals.

It will be necessary to develop grow-out techniques that can incorporate sea cucumbers onto IMTA sites. While the Korean design of a net pen cage that has a false bottom to separate the fish from the sea cucumbers might be feasible, other ideas should be considered. It may be more practical to culture sea cucumbers in submerged trays or cages similar to those used for shellfish culture. Most importantly, husbandry techniques will need to be developed that allow for the grow-out to occur in two and a half years or less. It would not be advisable to culture sea cucumbers for much longer than the grow-out period of the other organisms on the site, so that the entire site may be fallowed before starting the next crop of animals.

The development and optimization of culture techniques for *C. frondosa* is likely to take five to 10 years of research and grow-out experience. Because current medical trials may appear to be successful or other economic factors could change in a manner that will improve economics of sea cucumber culture, work on culture techniques should begin immediately.

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Pacific White Shrimp, *Litopenaeus vannamei*, Hatchery Industry in China

by

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Pacific white shrimp *L. vannamei*



A brief history of *L. vannamei* in China

1988

- *L. vannamei* was first introduced into China by Professor Zhang Weiquan of the Institute of Oceanology of Chinese Academy of Sciences from University of Texas Port Aransas Marine Science Laboratory. The postlarvae were provided by Texas A&M University. Nineteen postlarvae survived upon arriving in China.

1989

- First succeeded in maturation and spawning in captivity (up to zoea stage)

1992

- Succeeded in the production of postlarvae

1994

- Succeeded in the mass production of postlarvae

A brief history of *L. vannamei* in China (cont.)

1994 – 1999

- Small scale growout production

1999

- Real start and acceptance of *L. vannamei* farming after the collapse of *Penaeus monodon* farming (mainly due to the epidemic white spot virus disease)

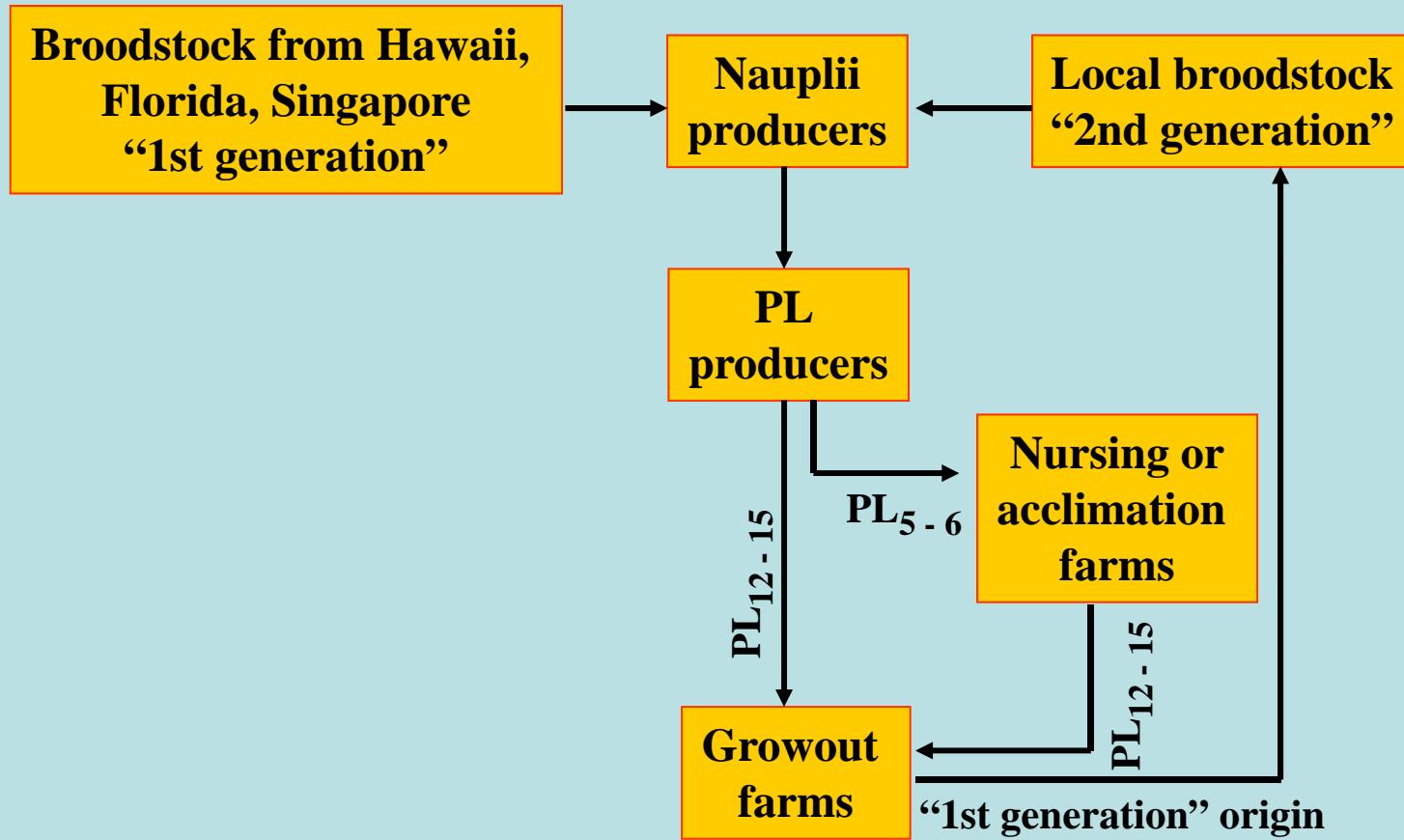
2001

- Farming area for *L. vannamei* started to expand quickly.

2007

- Annual production of shrimp reached a record-high of 1.28 million metric tons among which the majority were *L. vannamei*.

L. vannamei hatchery industry in China: diversification



L. vannamei hatcheries in China

Estimated number of hatcheries in China

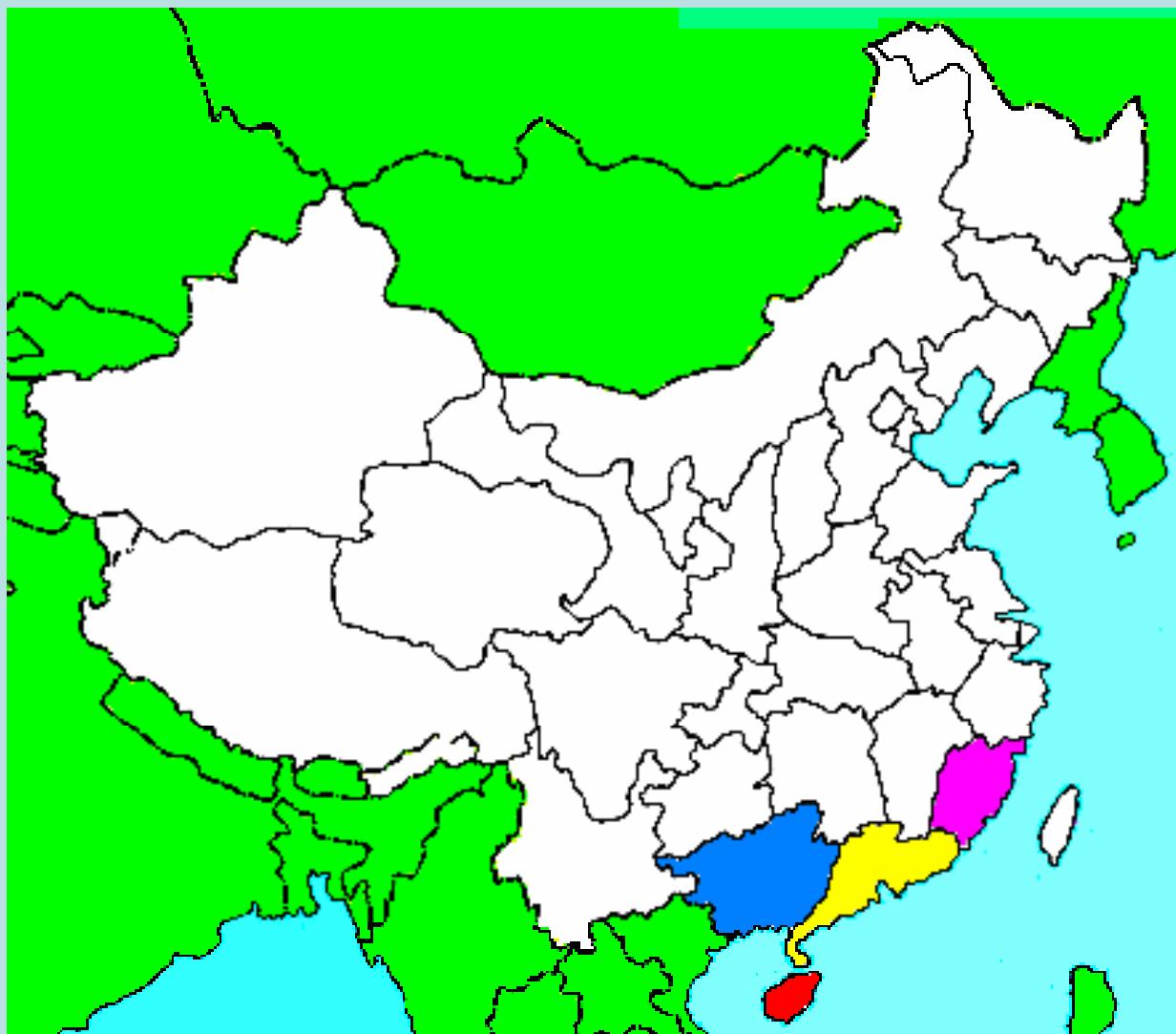
Between 2,600 and 2,700 established and 1,500 to 1,600 in production and at least 90% of them are situated in the provinces of Guangdong, Guangxi, Fujian and Hainan.

Sizes of hatcheries

From 500 m³ to 2,000 m³ larvae-rearing area per hatchery and production from 50 millions to 500 millions of postlarvae each year per hatchery.

Estimated total requirement of postlarvae

Between 300 billions and 400 billions each year to satisfy the growout production of 800,000 to 1 million metric tons of shrimp.



- Fujian
- Hainan
- Guangxi
- Guangdong

Major *L. vannamei* PL producing areas in China



**Wenchang and Qionghai, the major *L. vannamei*
PL producing areas in Hainan**



Fangcheng, Beihai and Dongxing, the major *L. vannamei* PL producing areas in Guangxi



**Longhai, Zhangpu, Xiaman and Xiapu, the major
L. vannamei PL producing areas in Fujian**



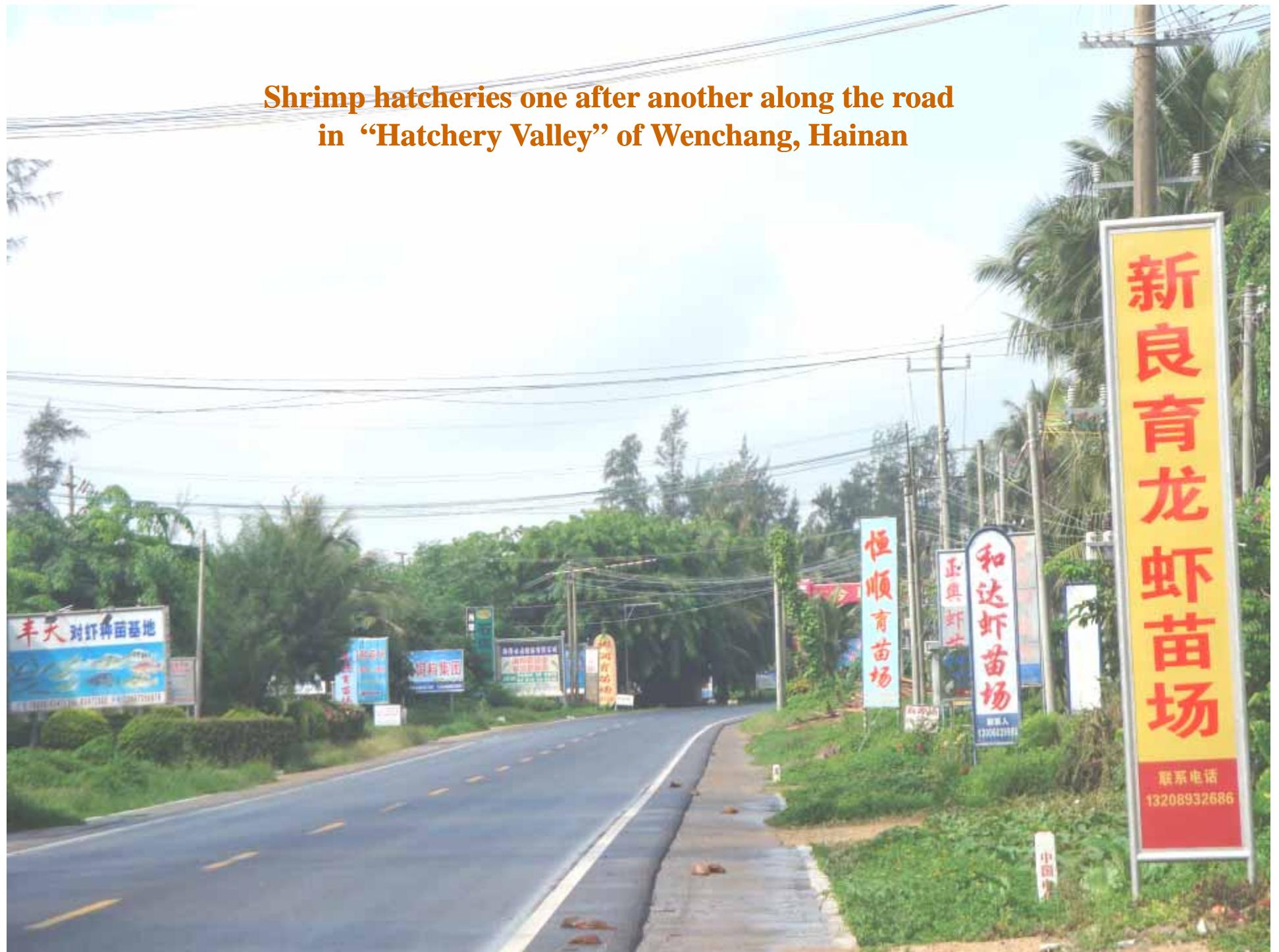
Zhanjiang (Donghai Island), the major *L. vannamei* PL producing area in Guangdong



**Shrimp hatcheries one after another along the road
in “Hatchery Valley” of Wenchang, Hainan**



Shrimp hatcheries one after another along the road
in “Hatchery Valley” of Wenchang, Hainan



L. vannamei hatcheries: water treatment

Filtration

- Sand filter (most commonly used)
- Cartridge filter
- Bag filter (most commonly used)

Removal of organic matters

- Foam fractionator (protein skimmer)
- Activated carbon filter (most commonly used)

Sterilization

- Ozonation
- UV sterilization
- Chlorination (most commonly used)
- Formalin (becoming popular)

L. vannamei hatcheries: water treatment

Adjustment of alkalinity

It is a common practice to bring up the alkalinity of seawater to a minimum of 130 ppm. The effect on the survival is significant.

Application of EDTA

Constant application of EDTA at 10 - 20 ppm is generally required. Heavy metals often exist in the seawater source.



**The water treatment system in Evergreen's shrimp hatchery
(Sand filters, protein skimmers and ozonators)**

***L. vannamei* hatcheries: broodstock**

Source of broodstock

- 1. Imported, so-called “1st generation” SPF broodstock**
- 2. Locally-raised, so-called “2nd generation” broodstock**

Size of broodstock

Males: > 40 g each

Females: > 45 g each

Age of broodstock

> 8 months and < 12 months

Cost of broodstock

- 1. Imported: >US\$ 40 each male or female**
- 2. Locally-raised: US\$ 2 - 3 each male or female**

L. vannamei hatcheries : broodstock

Density of broodstock

10 – 15 shrimp/m², males and females separated

Induced maturation

- Unilaterally eyestalk ablation on female
- Highly nutritive feed, mainly squid, polychaete worms, oyster and calf liver (formulated feeds, not popular)

Reproductive performance of broodstock

Expectation: 200,000 to 300,000 nauplii per spawn and a minimum of 14 spawns within 5 months after eyestalk ablation, totaling a minimum of 3 millions of nauplii per mother shrimp (imported broodstock)

L. vannamei hatcheries: nauplii to postlarvae

Capacity of larvae-rearing tank

10 - 20 m³ per tank

Initial density of nauplii

150 – 200 per liter of water

Survival rates

Naupliar stage: 0 – 90%

Zoea stage: 0 – 70% (most critical stage)

Mysis stage: 50 – 90%

PL stage: 80 – 90%

Overall from nauplii to PL_{12 - 15}: 0 - 50%

Production of PL

Up to 100,000 PL_{12 - 15} per m³ of water

L. vannamei hatcheries: nauplii to postlarvae

Feeding

Zoea stage:

- Live algae (mainly *Skeletonema sp.* and *Chaetoceros sp.*)
- Algal powder
- Formulated feed (in microencapsulated, flake or microbound form)
- Newly-hatched Artemia nauplii (cold or heat-shocked, starting from Zoea II or Zoea III stage)

Mysis stage:

- Formulated feed
- Live newly-hatched Artemia nauplii

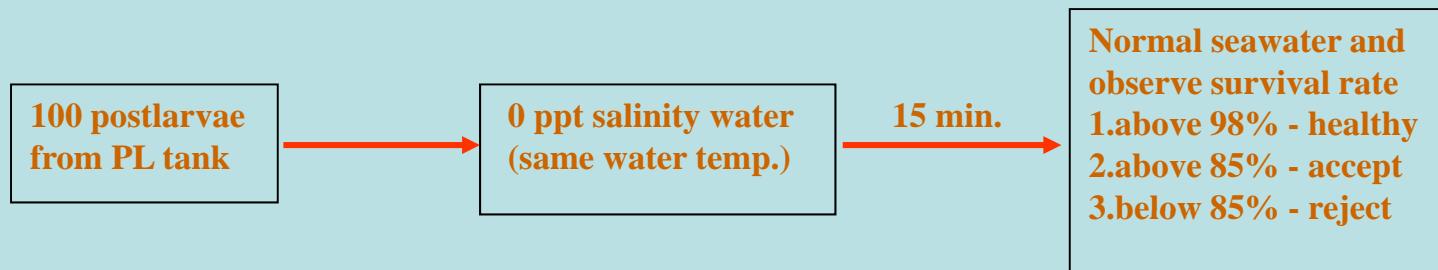
PL stage:

- Formulated feed
- Live newly-hatched Artemia nauplii

L. vannamei hatcheries: quality assurance of PL

Before harvesting PL, the following steps are taken to ensure quality of PL:

1. PCR check for viral infection
2. Microscopic observation on abnormality
3. Stress test (salinity shock)



* Postlarvae have to be PL₁₀ or older for 0 ppt salinity shock test.

L. vannamei hatcheries: costs of larvae-rearing

Nauplii (1st generation)

- Selling price: RMB 10 – 15 (US\$1.5 – 2.2) per 10,000 nauplii
- Cost per 10,000 PL_{12 - 15} based on 30% survival: RMB 33 – 50 (US\$ 4.8 – 7.3)

Feeding cost

RMB 27 - 30 (US\$ 4.0 – 4.4) per 10,000 PL_{12 - 15}

Other cost

RMB 20 - 25 (US\$ 2.9 – 3.7) per 10,000 PL_{12 - 15}

Total cost

RMB 80 - 100 (US\$ 11.7 – 14.6) per 10,000 PL_{12 - 15}

Selling price

RMB 120 - 160 (US\$ 17.6 – 23.4) per 10,000 PL_{12 - 15}

* Note: One US dollar is equivalent to RMB 6.83

L. vannamei hatcheries: costs of larvae-rearing

Nauplii (2nd generation)

- Selling price: RMB 1 - 2 (US\$ 0.15 – 0.30) per 10,000 nauplii
- Cost per 10,000 PL_{12 - 15} based on 30% survival: RMB 3.3 – 6.7 (US\$ 0.48 – 0.98)

Feeding cost

RMB 17 - 20 (US\$ 2.49 – 2.93) per 10,000 PL_{12 - 15}

Other cost

RMB 10 - 15 (US\$ 1.46 – 2.20) per 10,000 PL_{12 - 15}

Total cost

RMB 30 - 40 (US\$ 4.39 – 5.86) per 10,000 PL_{12 - 15}

Selling price

RMB 60 - 90 (US\$ 8.78 – 11.71) per 10,000 PL_{12 - 15}

* Note: One US dollar is equivalent to RMB 6.83

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Constraints

1. Inadequate supply of quality SPF broodstock

While the demand for “1st generation” PL is on the rise, some imported broodstocks perform poorly (low maturity rate, low fertilization rate, poor quality nauplii). Quality SPF broodstocks are in short supply and expensive.

2. Inferior quality of locally-raised broodstocks

Locally-raised broodstocks are not properly selected. The reproductive performance is poor and the growth of the offspring is slow with much size variation.

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Problems

- 1. Zoea II syndrome often occurs, resulting in low survival.**
- 2. Unknown causes of empty gut during mysis stage.**
- 3. The use of formalin in water treatment, which has become popular, may impose some side effects on shrimp health and possibly cause environmental hazards.**
- 4. The use of antibiotics still exists, though the incidents have been greatly reduced.**

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Problems

5. The use of probiotics, prebiotics, and immunoenhancers becomes popular. However, the effect is inconsistent. The commercially available health products are unreliable in qualities.
6. The overall survival rate from nauplii to PL is too low (< 25%). The technique of larvae-rearing needs to be further improved.
7. The contamination of pathogens especially protozoan parasites in mass culture of algae.

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Problems

8. The contamination of pathogens in live feed for broodstock.
9. The majority of the hatcheries operate without bio-security setup. The importance of the bio-security has been overlooked. Potential consequence is the viral infection in the hatchery. Many incidents of the viral infection in the hatchery have been diagnosed.

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Perspectives

1. The extension of the duration of the hatchery operation

Due to the establishment of the enclosure ponds in Northern Guangdong, Fujian and Zhejiang provinces, especially Yangtze River delta and Pearl River delta areas, demands for PL are now almost all year round. The hatcheries can thus operate on a non-stop basis. The operational costs are therefore significantly reduced.

2. Awareness of the importance of PL quality

The demand for good quality “1st generation” PL is expected to increase. The hatcheries, which produce low quality PL, will be phased out and those, especially major producers with well-established brand names will prevail.

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Perspectives

3. Stock improvement and selective breeding

China has to expedite its research and development on the stock improvement and selective breeding of *L. vannamei* in order to solve the existing problem of inadequate supply of quality broodstock .

4. The abuse or misuse of antibiotics

Effective measures has to be tightened on the control of illegal use of antibiotics and other prophylactic chemicals.

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Perspectives

5. Zoea II syndrome

The causes of Zoea II syndrome need to be identified and the problems resolved so that the overall successful rate of PL production can be ensured.

6. The use of formalin in water treatment

Though it is the most effective method in sterilization, research is urgently required to define the advantages and disadvantages of using formalin in water treatment.

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Perspectives

7. The use of probiotics, prebiotics and immunoenhancers

More research is required to justify the use of probiotics, prebiotics and immunoenhancers in larvae-rearing. The correct way of using those health products (eg. dosage and time of application) is yet to be defined. The governmental regulations on the production and sales of those products are anticipated.

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Perspectives

8. The use of formulated feed for broodstock

Partial replacement of live feed with formulated feed for broodstock needs to be encouraged and promoted.

9. Contamination in mass culture of algae

Method for solving the problem of the contamination of pathogens in mass culture of algae needs to be developed.

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Perspectives

10. Contamination in live feed for broodstock

Potential pathogenic contamination of live feed, such as polychaete worms and oyster, deserves more attention.

11. The establishment of the bio-security systems

The importance and significance of the bio-security systems for the shrimp hatchery industry needs to be highlighted.

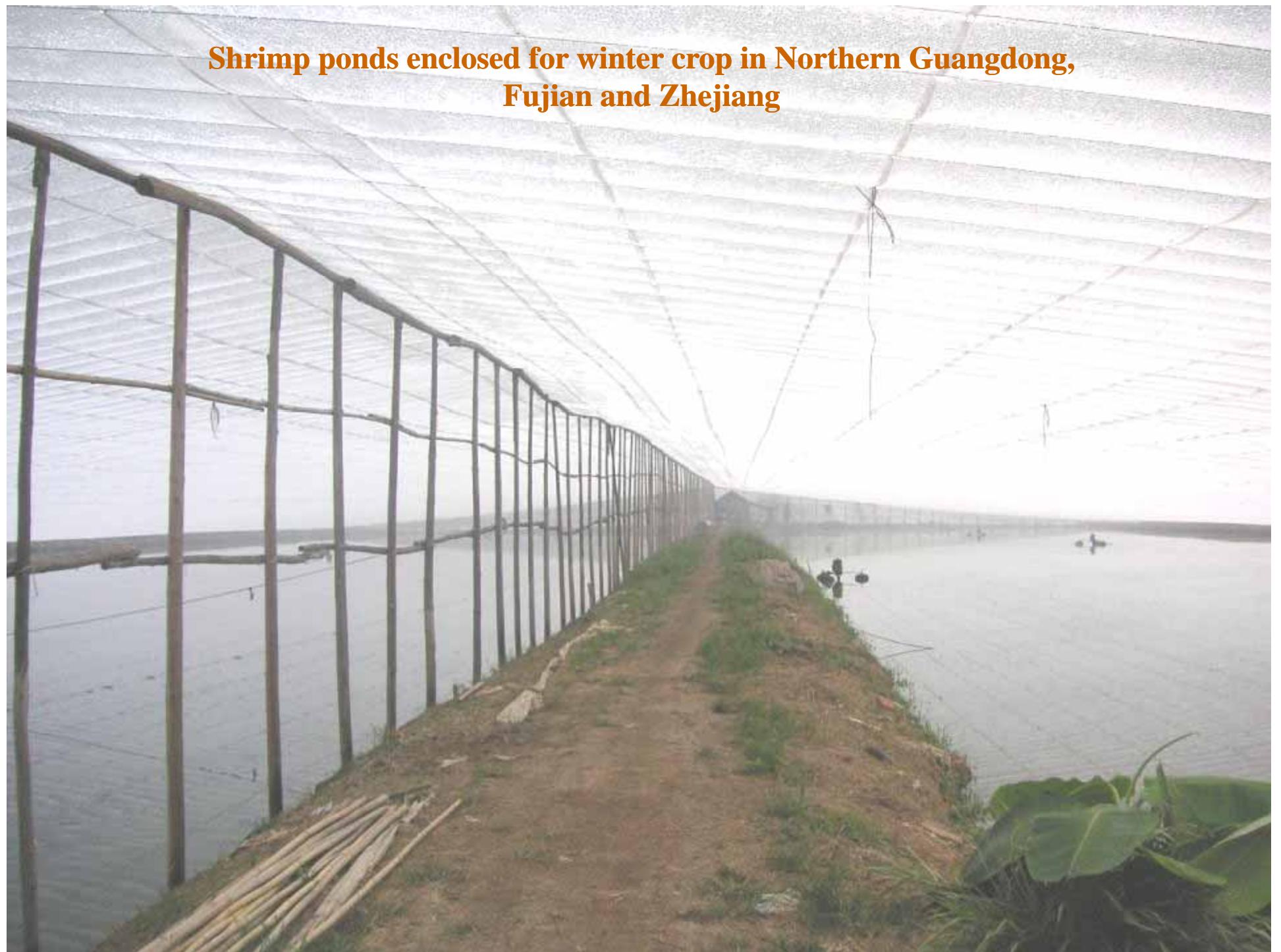
Typical *L. vannamei* intensive farm (Evergreen Group)



**Shrimp pond enclosed for winter crop in Northern Guangdong,
Fujian and Zhejiang**



**Shrimp ponds enclosed for winter crop in Northern Guangdong,
Fujian and Zhejiang**



**Shrimp pond enclosed for winter crop in
Northern Guangdong, Fujian and Zhejiang**



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**Shrimp pond enclosed for winter crop in Northern Guangdong,
Fujian and Zhejiang**



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**Shrimp pond enclosed for winter crop in Northern Guangdong,
Fujian and Zhejiang**



10. 23. 2007



Larvae-rearing room (Evergreen Group)



Typical *L. vannamei* larvae-rearing tanks in China



2004 9 11

Broodstock room (Evergreen Group)

L. vannamei spawners



Fully matured female *L. vannamei*



**Tight bio-security:
three steps in sterilization
(Evergreen hatchery)**



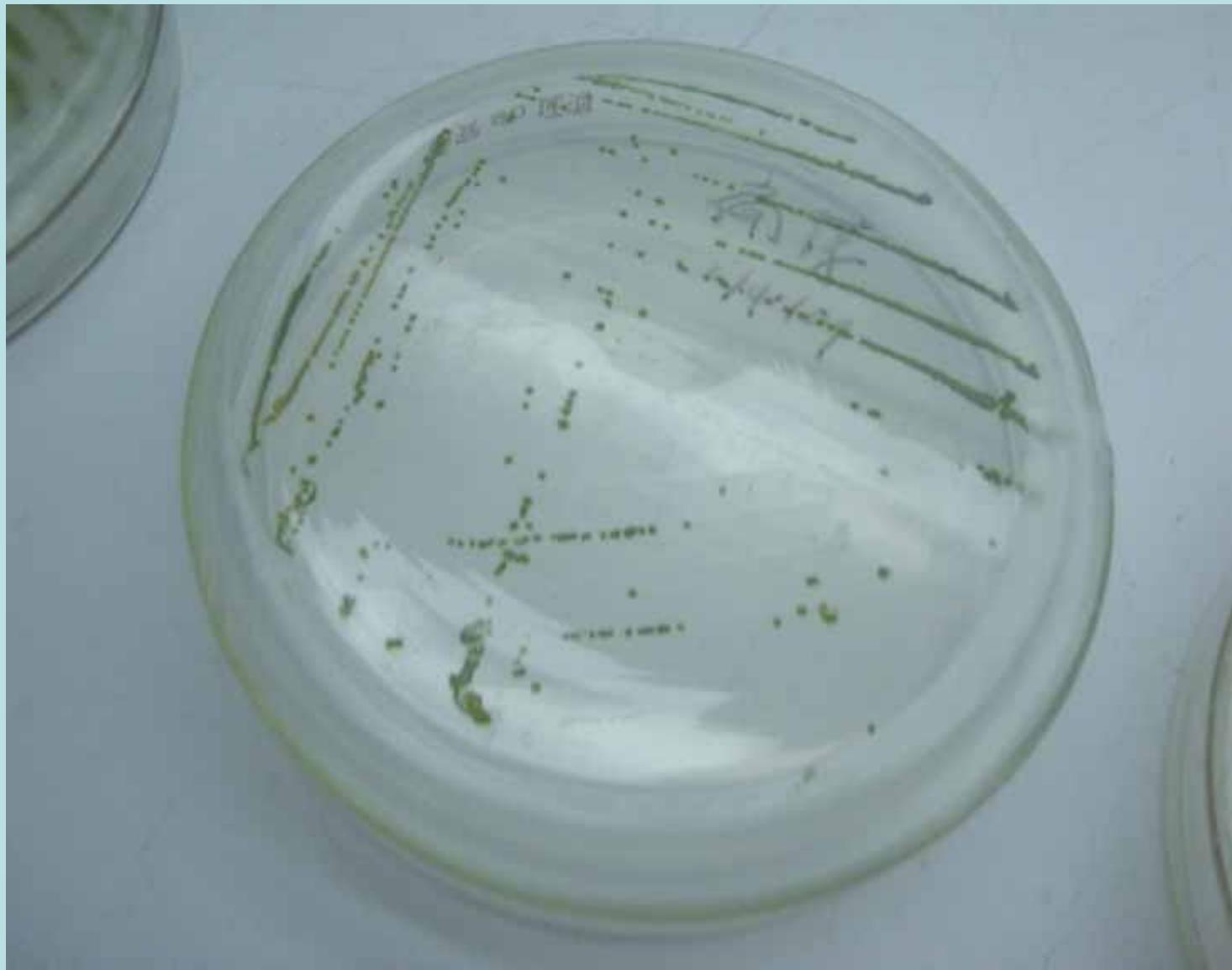
Vehicle washing basin at the gate



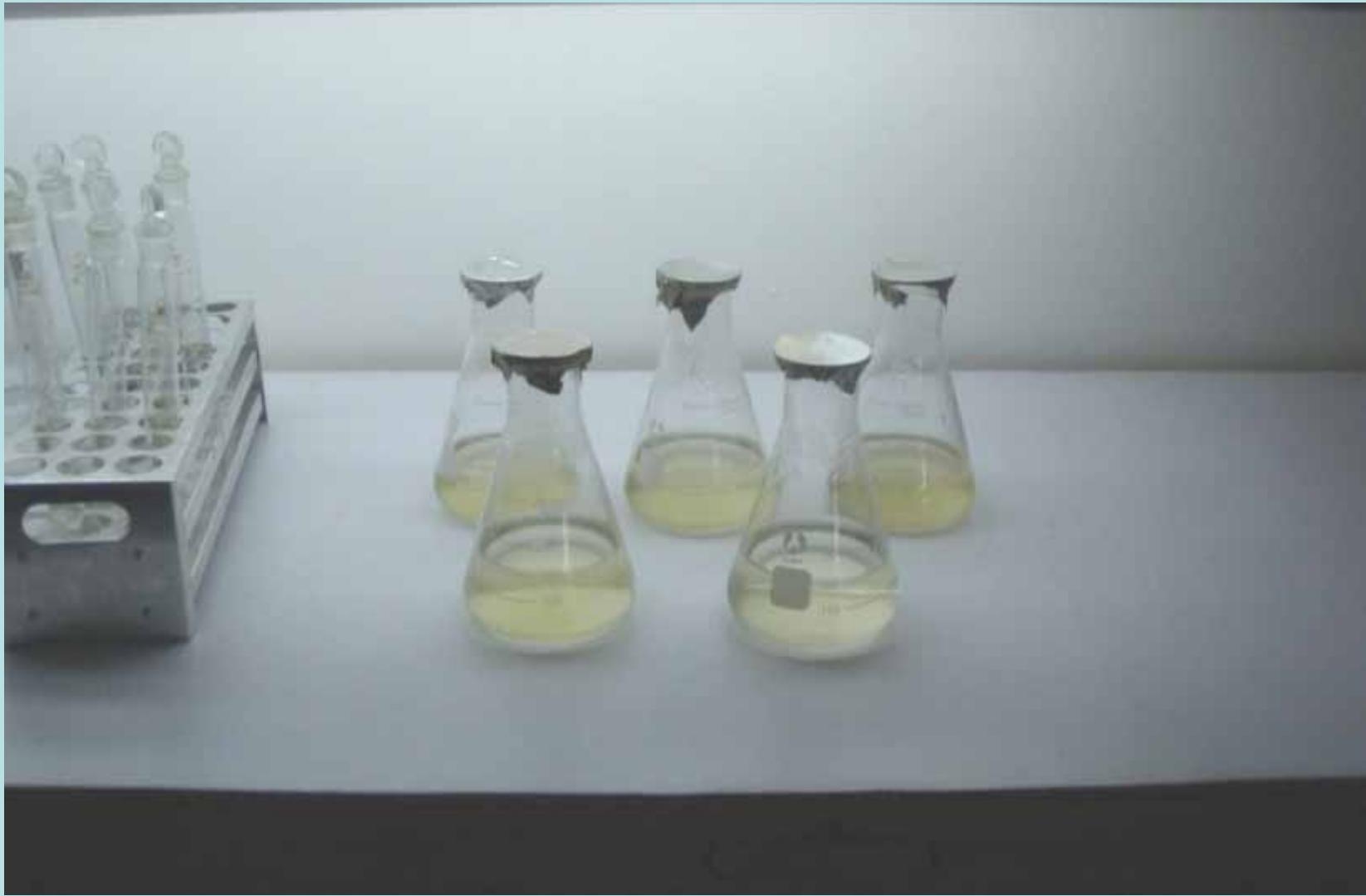
**Foot and hand sterilization before
entering larvae-rearing complex**



**Foot and hand sterilization before
entering larvae-rearing room**



Algal inocula in solid medium



Algal inocula in test tubes and flasks



Algal stock (*left:Chlorella sp.*, *right:Chaetoceros sp.*)



Algal stock (*Chaetoceros* sp.)



Algal stock (*Chaetoceros sp.* and *Chlorella sp.*)



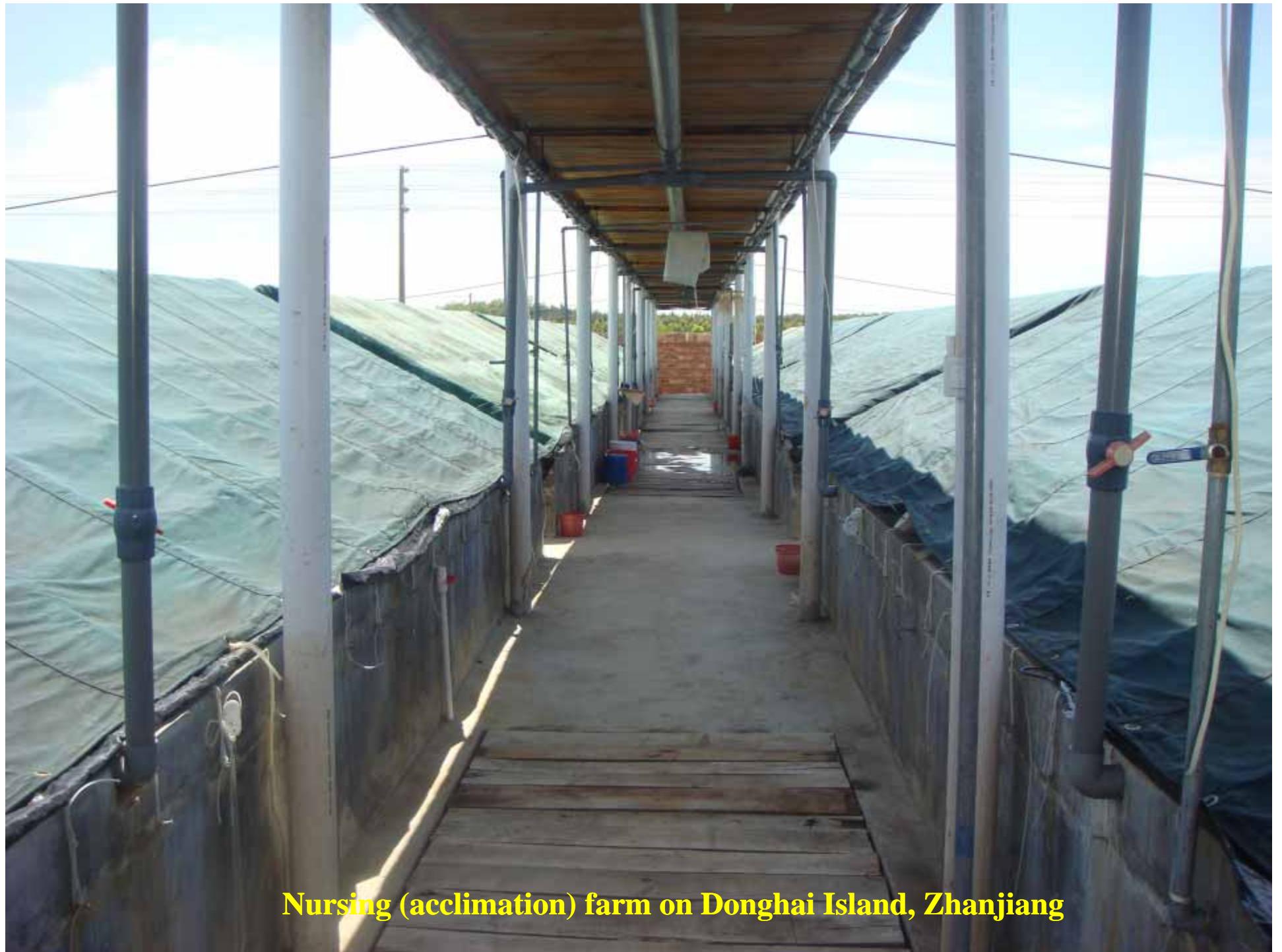
Mass culture of algae in outdoor concrete tanks



***L. vannamei* postlarvae**



L. vannamei postlarvae



Nursing (acclimation) farm on Donghai Island, Zhanjiang

Nursing (acclimation) tanks equipped with heaters





Thank you
For
Your Time and Patience

Evergreen Aquaculture Research Center on Donghai Island



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Using alternative low-cost artificial sea salt mixtures for intensive, indoor shrimp (*Litopenaeus vannamei*) production

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ABSTRACT

Inland marine shrimp production is increasing in popularity as recirculating aquaculture systems (RAS) can provide a fresh, high-value product year-round to nearby consumer markets. However, artificial sea salt mixtures are required for inland production, which can be a substantial portion of the production costs. The purpose of this experiment was to compare marine shrimp (*L. vannamei*) production and water quality dynamics in culture systems with various mixtures of a least cost salt (LCS) formulation and a commercial, complete sea salt (CSS) formulation. Previous research using this same LCS mixture found decreased shrimp production using 100% LCS compared to ratios with 25% CSS inclusion and higher. To further investigate the decreased shrimp performance in 100% LCS, this experiment used six salt different ratios of LCS and CSS between 100% LCS and 75% LCS:25% CSS. The treatments used in this study were 100% LCS, 97.5% LCS, 95% LCS, 90% LCS, 80% LCS, and 75% LCS. The results showed that use of the LCS formulation significantly lowered the cost of salt kg^{-1} shrimp produced. The LCS formulation cost \$8.83 USD to make 1 m^3 of water at 15 salinity, compared to \$12.89 using the 75%/25% LCS/CSS mixture. Salt mixtures had a significant impact on DO, pH, salinity, and turbidity; however, these differences did not seem to impact shrimp performance, as no significant differences were found in shrimp average weight, biomass, survival, FCR, or growth rate. The results from this study indicate that using the LCS formulation reduces artificial sea salt cost significantly while having no significant impacts on shrimp production.

1. Introduction

Due to high demand and exhausted natural fisheries stock, the aquaculture sector has grown rapidly, with aquaculture products currently representing more than 50% percent of seafood production for human consumption (FAO, 2020). Although most aquaculture is pond based, indoor aquaculture is growing in popularity, especially in inland areas where fresh seafood products are often difficult to acquire. Such indoor operations primarily utilize recirculating aquaculture systems (RAS). RAS are contained production systems that provide substantial environmental control, including solids filtration, biofiltration, temperature control, and other control mechanisms based largely on animal needs and climate considerations (Timmons and Ebeling, 2010). RAS also require much less space than pond-based aquaculture through higher production densities, allowing them to be situated in a variety of building types (Martins et al., 2010).

Due to the costs of RAS, high-value species are being investigated for their suitability in RAS production (Bunting and Shpigel, 2009). One

such species is the Pacific white shrimp (*Litopenaeus vannamei*), which can be marketed as a high-quality, fresh product in some niche markets (Moss and Leung, 2006; Van Wyk, 2006; Timmons and Ebeling, 2010). One limiting factor in RAS marine shrimp production is the need for salt to create marine or brackish water (Zohar et al., 2005). Most inland areas do not have direct access to saltwater; therefore, shrimp production operations must use an artificial marine salt mix (Whetstone et al., 2002; Watson and Hill, 2006). Shrimp can be grown at varying salinities from 30 g L^{-1} to 10 g L^{-1} , perhaps even lower in some cases, but require minimum levels of individual salt anions and cations, including Na, Mg, K, Ca, SO_4 , CO_3 , and Cl, and require specific ratios between pairs of ions like Na, Mg, K, and Ca (Saoud et al., 2003; Roy et al., 2007, 2010; Khanjani et al., 2020). Most commercial salt mixes attempt to replicate the mix of elements found in natural seawater, including trace minerals such as Fe, I, Zn, and Mn. Some of these trace elements have been shown to play a role in physiological functions when included in the shrimp diet, but the amounts of trace elements needed to meet animal requirements in water are not well documented (Ali, 2000; Lin et al.,

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2013). These commercial marine salt mixes can represent a significant portion of production costs for inland shrimp producers (Shinji et al., 2019; Maier, 2020). Reducing such costs is critical for the profitability of shrimp farms as the intensive indoor shrimp industry grows (Badiola et al., 2012; Quagrainie, 2015; Zhou and Hanson, 2017). Simplifying these salt mixes down to only essential elements may reduce costs for producers by reducing the number of ingredients and facilitating in-house made salt mixes.

A previous experiment found that there were no significant differences in shrimp production when using five salt mixtures: containing 100% commercial sea salt (CSS), 75% CSS and 25% of a least cost salt mix (LCS), 50/50% CSS/LCS, 25/75% CSS/LCS, and 100% LCS (Tierney et al., 2021). Although there were no significant differences in that study, shrimp survival in the 100% LCS treatment was low at 57%, whereas the other treatments averaged 70% survival. The LCS formula used in Tierney et al. (2021) was based on that published by Parmenter et al. (2009), who explored using the mixture in a low shrimp stocking density, outdoor setting which showed shrimp survival above 80%. The same LCS was also used by Galkanda-Arachchige et al. (2020) where it was found to be suitable for shrimp nursery settings, however the effectiveness of LCS in an intensive grow-out setting is still unclear. The purpose of the current experiment was to further examine salt mixtures in the range of 75% LCS to 100% LCS due to the low survival found in Tierney et al. (2021) and definitively determine if a LCS can result in adequate shrimp production, maintain acceptable water quality levels, and reduce salt cost in intensive, indoor shrimp aquaculture systems.

2. Materials and Methods

2.1. Experimental design and operation

This experiment took place in the Kentucky State University Sustainable Aquaculture Development Lab (SADL). The SADL is a 174 m² insulated and climate-controlled building used for indoor aquaculture research. Five treatments were developed for this experiment, each with 4 replicated tanks for a total of 20 tanks. Each treatment used different combinations of two salt mixes to reach the target salinity. The two salts were a commercial sea salt mixture, Crystal Sea Marine Mix (CSS) (Marine Enterprises International, Baltimore, MD, USA); and a least-cost salt mixture (LCS) made from sodium chloride (NaCl), magnesium sulfate (MgSO₄), magnesium chloride (MgCl₂), calcium chloride (CaCl₂), potassium chloride (KCl), and sodium bicarbonate (NaHCO₃) (Table 1). The LCS formula is based on a formula from Parmenter et al. (2009) and used by Tierney et al. (2021); all ingredients were mixed prior to being added to the tank. The CSS mix is commonly used in indoor shrimp farms in the U.S. It is a proprietary product and, according to the manufacturer, is an artificially formulated complete sea salt that duplicates the major, minor, and trace elements found in natural sea water (<https://www.meisalt.com/Crystal-Sea-Marinemix>). The individual treatments in the experiment were 75/25% LCS/CSS, 80/20% LCS/CSS, 90/10% LCS/CSS, 95/5% LCS/CSS, 97.5/2.5% LCS/CSS, and 100% LCS. These percentages of LCS were chosen to determine if higher percentages of LCS inclusion will result in decreased shrimp performance shown

Table 1

The formulation of the least cost salt (LCS) mixture at 15 salinity to make 1 m⁻³ of artificial seawater (Tierney et al., 2021).

Salt Formulation	
Ingredient Weight (g)	
NaCl	11,310
MgSO ₄	1830
MgCl ₂	855
CaCl ₂	376
KCl	240
NaHCO ₃	90

in Tierney et al. (2021). The specific brands and purity of each ingredient of the LCS are listed in Table 2.

All experimental tanks had a bottom area of 1 m² and had a volume of 1 m³. All tanks were HY systems and were equipped with an 18 L biofilter and an 18 L settling chamber for solids control, based on the design described by Ray et al. (2010). The biofilters were moving bed biological reactors (MBBR), and each contained 6 L of biological media (Curler Advance X-1, Aquaculture Systems Technologies, LLC. New Orleans, LA, USA.). At the start of the study, 3 L of biomedia from established shrimp production tanks was added to each MBBR to help ensure bacterial colonization in the filter. Water was pumped to the settling chamber from each tank using a 20 L min⁻¹ electric water pump. Water from the settling chamber was gravity fed into the MBBR, after which the water returned to the grow-out tank. Each tank was heated with a 1000-watt submersible electric heater, three 15 cm long ceramic air diffusers provided aeration to each shrimp tank, and one such diffuser supplied air to the biofilter.

2.2. Water quality

Salinity in all systems was kept at 15 throughout the experiment. Any water loss due to evaporation was replaced with dechlorinated municipal water. When pH fell below 7.8, this was adjusted with additions of 35 g of sodium bicarbonate. Temperature, dissolved oxygen (DO), pH, and salinity were all measured twice daily at approximately 08:00 and 16:00 h using a YSI Professional Plus Multi-Meter (YSI Incorporated, Yellow Springs, OH, USA). Total ammonia nitrogen (TAN), nitrite, and turbidity were measured once weekly during this study. Turbidity was measured using a Hach 2100Q Portable Turbidimeter; TAN and nitrite were measured using Hach methods 8155 and 8507 using a Hach DR6000 spectrophotometer (Hach Company, Loveland, CA, USA).

2.3. Shrimp husbandry

The shrimp used in this study were purchased from American Mariculture, Inc. (St. James City, FL, USA). Upon arrival, the shrimp were raised in two 3.4 m³ nursery tanks for 37 days before being stocked into the experimental tanks. The salinity in the nursery tanks was started at 30 and was lowered to 15 over the course of the nursery period. The shrimp were fed 6 different rations throughout the nursery. Four crumble diets with progressively larger sizes were fed through the first 27 days of the nursery (Raceway Plus 0, 1, 2, 3; Ziegler Brother, Inc., Gardners, PA, USA). Each of these diets contained 50% protein, 15% lipid, 1% fiber, 10% moisture, and 7.5% ash, according to the manufacturer. At 27 days the shrimp diet was transitioned to a 1.5-mm pelleted feed (Ziegler PL Raceway 40–9, 40% protein, 9% lipid, 3% fiber, 10% moisture, and 13% ash), and at 36 days transitioned to a 2.4-mm pellet (Ziegler Hyper-intensive Shrimp 35, 35% protein, 7% fat, 2%

Table 2

Components of the LCS mixture used in this study. Table shows the specific compound used, brand, and purity. Data collected from: Cargill, Incorporated, Minneapolis, MN, United States; Giles Chemical, Waynesville, NC, United States; Nedmag B.V., Groningen, Veendam, The Netherlands; Cal-Chlor Corporation, Lafayette, LA, United States; The Mosaic Company, Tampa, FL, United States; Solvay S.A., Brussels, Belgium.

Salt	Full Compound	Brand	Purity	Grade
NaCl	NaCl	Cargill	> 99%	Food-Grade
MgSO ₄	MgSO ₄ ·7 H ₂ O	Giles Chemical	> 99%	Technical-Grade
MgCl ₂	MgCl ₂ ·7 H ₂ O	Nedmag B.V.	> 99%	Feed-Grade
CaCl ₂	CaCl ₂	Cal-Chlor Corporation	> 94%	Industrial-Grade
KCl	KCl	The Mosaic Company	> 98%	Industrial-Grade
NaHCO ₃	NaHCO ₃	Solvay	> 99%	Food-Grade

fiber, 12% moisture, and 15% ash), which was also provided throughout the experiment. The shrimp were fed on 24-hour automatic belt feeders for the majority of the nursery stage to ensure continuous feed availability.

The post-nursery shrimp were stocked into the experimental tanks at 262 shrimp m⁻³ at an average individual shrimp weight of 2.9 g shrimp⁻¹. Throughout the experiment, the shrimp were fed 3 times daily at 08:00, 12:00, and 16:00 h. The amount of feed provided daily was calculated using an estimated FCR and weekly growth rate, along with water quality readings and periodic checks for uneaten feed at the bottom of the tanks. The study lasted 86 days, and at harvest, all shrimp were weighed and counted. The data gathered from the harvest was used to calculate average weight per shrimp, growth rates, FCR, survival, and total harvest weight m⁻³. Growth rate was calculated by dividing the average total weight gain per shrimp by the number of weeks in the study and FCR was calculated by dividing the total amount of feed added to each tank by the total shrimp weight harvested from the corresponding tank.

2.4. Salt cost

The cost of salt in USD for all treatments was generated by calculating the total cost of each salt mix to reach 15 g L⁻¹ salinity and the percent of each salt used in each treatment. In addition, the cost of salt m⁻³ and the shrimp production m⁻³ in each treatment were combined to calculate the cost of salt kg⁻¹ of shrimp.

2.5. Data management and analysis

All statistical analyses were performed using SigmaPlot 13.0 (Systat Software Inc., San Jose, CA, USA). All data was tested using Shapiro-Wilk and Brown-Forsythe tests to check normality and variance. All harvest data from shrimp, elemental analyses, and production costs were analyzed using a one-way ANOVA. Water quality parameters measured weekly were analyzed using a one-way repeated measures ANOVA. All results were considered significant at an α -value of < 0.05 , and if a significant difference was detected, a Tukey's HSD Test was applied.

3. Results

There were no significant differences in temperature, TAN, nitrite, TSS, or VSS ($p > 0.05$, Table 3). Significant differences were detected in DO, pH, salinity, and turbidity between treatments ($p < 0.05$). The DO concentration tended to increase as CSS concentration decreased, with 100% LCS and 97.5% LCS treatments having significantly higher dissolved oxygen levels than the other four treatments. The 100% LCS treatment had significantly lower overall pH than the 90% and 80% LCS treatments. Turbidity was significantly higher in the 100% LCS treatment compared to all other treatments except the 90% LCS treatment.

There were no significant differences detected between treatments in all tested shrimp production metrics, including average weight shrimp⁻¹, growth rate week⁻¹, FCR, kg of shrimp m⁻³, and survival ($p > 0.05$, Table 4). All average shrimp weights were between 20.7 g, and 22.2 g and average growth rates were 1.4–1.6 g week⁻¹, FCRs ranged from 1.4 to 1.6:1, shrimp production ranged from 4.3 to 4.7 kg m⁻³, and survival averaged 81% across all treatments with a range of 76.7–84.3%.

The cost of salt m⁻³ at 15 salinity was different between all treatments, and cost decreased as LCS percentage increased (Table 5). The cost of salt kg⁻¹ of shrimp produced was found to be lowest on average in the 97.5% LCS tanks, and significantly lower than the 80% and 75% LCS treatments (Table 6).

4. Discussion

Total ammonia nitrogen and nitrite levels were both maintained

Table 3

Mean \pm SD water quality values over the course of the study between each treatment (treatments are based on the amount of least cost salt (LCS) included; the remainder of the salt mixture was a commercial sea salt). Different superscript letters in a row denote significant differences between treatments ($p < 0.05$).

	Treatment					
	100% LCS	97.5% LCS	95% LCS	90% LCS	80% LCS	75% LCS
Temperature	27.3 \pm 0.1	27.3 \pm 0.1	27.3 \pm 0.1	27.3 \pm 0.1	27.3 \pm 0.1	27.3 \pm 0.1
Dissolved Oxygen (mg L ⁻¹)	6.5 \pm 0.0 ^a	6.5 \pm 0.0 ^a	6.4 \pm 0.0 ^c	6.4 \pm 0.0 ^b	6.4 \pm 0.1 ^{cd}	6.4 \pm 0.1 ^{bd}
pH	7.9 \pm 0.0 ^a	7.9 \pm 0.0 ^{ab}	7.9 \pm 0.0 ^{ab}	7.9 \pm 0.0 ^b	7.9 \pm 0.0 ^b	7.9 \pm 0.0 ^{ab}
Salinity	15.0 \pm 0.0 ^b	15.1 \pm 0.0 ^b	14.8 \pm 0.1 ^a	15.2 \pm 0.0 ^d	15.0 \pm 0.0 ^b	15.0 \pm 0.0 ^b
Ammonia (mg TAN L ⁻¹)	0.6 \pm 0.2	0.7 \pm 0.1	0.8 \pm 0.2	0.6 \pm 0.2	0.7 \pm 0.2	0.7 \pm 0.1
Nitrite (mg NO ₂ -N L ⁻¹)	0.9 \pm 0.2	0.8 \pm 0.2	0.9 \pm 0.2	0.9 \pm 0.2	0.9 \pm 0.2	0.8 \pm 0.2
TSS (mg L ⁻¹)	112.7 \pm 6.0	108.1 \pm 5.8	105.2 \pm 5.8	112.5 \pm 7.3	111.7 \pm 7.0	105.4 \pm 4.5
VSS (mg L ⁻¹)	77.1 \pm 5.5	71.9 \pm 3.2	70.0 \pm 4.5	72.9 \pm 6.1	67.5 \pm 4.9	67.7 \pm 2.8
Turbidity (NTU)	26.4 \pm 2.7 ^a	19.9 \pm 2.6 ^{bc}	19.2 \pm 3.4 ^{ab}	22.7 \pm 3.4 ^{ab}	20.2 \pm 3.2 ^{bc}	17.7 \pm 1.8 ^c

Table 4

Mean \pm SD shrimp production metrics at harvest between each treatment (treatments are based on the amount of least cost salt (LCS) included; the remainder of the salt mixture was a commercial sea salt). There were no significant differences between treatments with regard to these metrics.

	Treatment					
	100% LCS	97.5% LCS	95% LCS	90% LCS	80% LCS	75% LCS
Average Weight (g)	21.1 \pm 0.4	20.7 \pm 0.3	21.9 \pm 0.7	21.7 \pm 0.5	22.2 \pm 0.6	21.9 \pm 0.4
Growth rate (g/week)	1.5 \pm 0.0	1.4 \pm 0.0	1.5 \pm 0.1	1.5 \pm 0.0	1.6 \pm 0.1	1.5 \pm 0.0
FCR	1.6 \pm 0.1	1.5 \pm 0.0	1.4 \pm 0.1	1.5 \pm 0.1	1.4 \pm 0.1	1.4 \pm 0.1
Biomass (kg m ⁻³)	4.3 \pm 0.4	4.6 \pm 0.2	4.6 \pm 0.2	4.5 \pm 0.2	4.6 \pm 0.2	4.7 \pm 0.2
Survival (%)	77.5 \pm 6.6	84.3 \pm 3.2	81.1 \pm 3.9	79.2 \pm 3.8	76.7 \pm 5.8	82.3 \pm 2.0

Table 5

The cost of each ingredient used to make the least cost salt (LCS) mixture.

Ingredient	\$USD kg ⁻¹ Salt	\$USD m ⁻³ Water
NaCl	0.418	5.35
MgSO ₄	0.946	1.95
MgCl ₂	0.704	0.67
CaCl ₂	0.77	0.59
KCl	0.858	0.22
NaHCO ₃	0.726	0.06
Total	4.422	8.84

within acceptable ranges for shrimp production throughout this experiment (Alcaraz et al., 1999; Valencia-Castañeda et al., 2018). Although there were significant differences between treatments in DO levels, pH, salinity, and turbidity, these minute differences likely had little effect on the overall performance of the shrimp and were all within acceptable ranges (Zhang et al., 2006). The reason these subtle differences were detected as significant is because of the sensitivity of the repeated measures ANOVA. This test is useful when the same measurements are made repeatedly; however, consistent but minor differences over time

Table 6

Salt mixture cost m^{-3} of water, and kg^{-1} of shrimp produced. Treatments are based on the amount of least cost salt (LCS) included; the remainder of the salt mixture was a commercial sea salt. Different superscript letters in a column denote significant differences between treatments ($p < 0.05$).

Salt Mixture Costs		
Treatment	m^{-3} water	Kg^{-1} Shrimp
100% LCS	8.83	2.11 ^{ab}
97.5% LCS	9.24	2.03 ^a
95% LCS	9.64	2.09 ^{ab}
90% LCS	10.45	2.34 ^{ab}
80% LCS	12.08	2.63 ^b
75% LCS	12.89	2.76 ^b

often register as significant. There were no significant differences found between treatments in temperature, TSS, or VSS. Importantly, pH levels were maintained when using the LCS, even though a single source of alkalinity is used in the mixture (sodium bicarbonate). A complete sea salt mixture would likely include multiple buffers, such as calcium, potassium, and magnesium carbonate compounds.

The lack of significant differences in shrimp production between treatments has important implications for shrimp producers. The increased concentration of LCS used in production appears to have no detrimental impact on shrimp performance. Overall average survival was just above 80%, average FCR was 1.5 across all treatments, and the average growth rate was 1.5 g week⁻¹, all comparable to or exceeding recent shrimp studies using reduced cost salt mixtures and commercial mixtures at similar salinities (Tierney et al., 2021; Galkanda-Arachchige et al., 2020; Pinto et al., 2020). Shrimp in this study reached an average of 21.6 g individually at 86 days, a size that is within the range preferred by consumers in North America, Europe, and other regions (Wirth, 2014; Tabarestani et al., 2017). This production time scale falls within a competitive harvest schedule and the shrimp size is at the highest of the range recommended by Zhou and Hanson (2017) in their economic model, suggesting that the results of this study are commercially relevant.

These results further demonstrate the utility of this reduced-cost salt mix across several stages of shrimp growth, as the study by Galkanda-Arachchige et al. (2020) used an identical low-cost mixture and found equal performance of post-larval and juvenile shrimp between both low-cost and commercial salt mixes. Contrary to this study, Pinto et al. (2020) found severely decreased shrimp performance at 75% and 100% inclusion of low-cost salt, despite the low-cost salt mixtures having similar ingredients and levels of critical ions compared to the same commercial salt mix. There are several differences in the low-cost salt mixtures; this study used both MgCl and MgSO₄ for magnesium inclusion, while the mixture in Pinto et al. (2020) used exclusively MgSO₄. To maintain alkalinity, this study included NaHCO₃ in the low-cost salt mix and added small amounts of the same as needed, while Pinto et al. (2020) did not include NaHCO₃ but added Ca(OH)₂ as needed to raise pH. Pinto et al. (2020) concluded that the lack of trace minerals in the low-cost mixtures was likely the cause of decreased performance; however, this study and several others have reported adequate shrimp production without the inclusion of trace minerals (Parmenter et al., 2009; Galkanda-Arachchige et al., 2020). Another possible cause for the differences between this study and that of Pinto et al. (2020) is the sources of salts. Some salts can contain high levels of impurities, which may result in inadequate concentrations of target minerals or possibly toxic effects on shrimp. The LCS and CSS used in this study were identical to those used in Tierney et al. (2021) which found no significant difference in shrimp production between the CSS and the LCS. The results of this study found use of the LCS resulted in high survival, exceeding the results in Tierney et al. (2021) who noted shrimp jumping out of the tanks which may have resulted in the discrepancy in shrimp survival.

The similar shrimp performance between treatments, regardless of LCS concentration, influences the economics of shrimp production operations. The cost difference in salt between the 75% LCS and 100% LCS was just over \$4 USD m^{-3} , which could lead to significant cost savings for shrimp producers, especially those operating at large scale. The lower salt cost also reduced the cost of production kg^{-3} of shrimp by \$0.75 USD, which represents a 15% decrease in production cost over the CSS formulation. Although many shrimp production methods, including biofloc and hybrid systems, are designed to greatly reduce water use, some water is still discharged from the systems during normal operation (Avnimelech, 2015). The loss of this water (3–10% per production cycle) is usually replaced with newly mixed salt water, increasing salt use and production costs long term, which would be partially negated by replacement with the LCS mixture. The economics of pond-based shrimp farming are well studied; however, the feasibility of high intensity, indoor shrimp production is still unclear due to substantial upfront costs and variable system designs and production strategies (Moss and Leung, 2006; Badiola et al., 2012; Zhou and Hanson, 2017). Any reduction in production cost may have significant impacts on this relatively new shrimp production style.

The salts that were used to make the LCS formulation in this study were each purchased in 23 kg bags that were shipped several hundred kilometers. However, in a commercial setting it is more likely that farmers would purchase these in bulk quantities and from local vendors if possible. This commercial-scale strategy would likely reduce the cost of the mixture even further. In fact, Maier (2020) points out that scale is one of the biggest factors influencing the profitability of indoor shrimp farming. He goes on to note that using the same LCS formulation tested in this study can significantly improve profit potential for farmers.

5. Conclusion

The study shows the feasibility and cost savings of a low cost, easily made salt mixture in high-intensity indoor shrimp production. The use of the LCS mixture should be considered by shrimp producers due to the significant decrease in production costs, similar shrimp performance, and water quality compared to a commercial marine salt mixture. Further research should examine the long-term water quality dynamics associated with the LCS formula and how mineral concentrations may change over time.

Ethical statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

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CRediT authorship contribution statement

Leo Fleckenstein: Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. **Thomas Tierney:** Formal analysis, Investigation, Writing – review & editing. **Jill Fisk:** Formal analysis, Investigation, Writing – review & editing. **Andrew Ray:** Conceptualization, Funding acquisition, Methodology, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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