# Standard Operating Procedures
# Chen Laboratory Zebrafish Facility
## CCSR 3151

*Updated August 16, 2018*

# General Rules

1. Before and after handling fish, system water, or equipment that may come in contact with fish or system water (tanks, nets, etc.), hands must be washed with soap and water.

2. Water, tanks, and fish from the Quarantine system (*indicated with YELLOW labels*) must never be introduced to the Main system (*indicated with WHITE labels*).

3. Separate fish nets should be used for the Quarantine and Main systems. *Red fish nets* are used for the Quarantine system, and *blue fish nets* are reserved for the Main system. The nets should be placed in corresponding Virkon S disinfectant buckets when not in use.

4. Fish should only be exposed to system water as tap water contains chlorine or chloramines, which kills fish.

5. Watch for signs of overfeeding: fat fish, uneaten food on the bottom of the tank, clogged screens in baby tanks. The rule of thumb is to give the fish as much as they will eat in about 5 minutes.

6. Watch for signs of underfeeding: skinny fish, really hungry fish, slow growth of young fish.

7. Maintain a fish density of between 5-15 fish per liter.

8. Do not enter the fish room when the lights are out (between 10 PM and 8 AM).

# Fish Health

## Health Assessment:

1. Remove any dead fish or fish that display symptoms of disease, and record these data in the fish facility log. If pathology services are warranted, sick

fish should be kept alive and shipped to either the Stanford Dept. of Comparative Medicine staff or the Zebrafish International Resource Center's pathology services. Dead fish should be placed in a cooler at +4° C or fixed in paraformaldehyde (or Bouin's solution) for necropsy analysis.

2. Symptoms of nematode infection: skinny, arched body or a failure to mate or feed.

3. Symptoms of TB infection: raised scales, open lesions, skin ulcers, and wound spots.

4. For more extensive descriptions and illustrations of zebrafish symptoms, please refer to the illustrative chart in the zebrafish facility.

## Euthanization:

1. Adult fish that exhibit symptoms of illness or infection should be either provided to the Stanford Dept. of Comparative Medicine staff or the Zebrafish International Resource Center's pathology services.

2. Adult fish that require euthanization due to infertility, age, or experimental need should be anesthetized with lethal dose of Tricaine (200-300 mg/L) in system water or E3 solution containing 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$, buffered to a final pH of 7 using sodium bicarbonate.

3. Tricaine used for this purpose must be pharmaceutical grade (e.g. Western Chemical). Always wear nitrile gloves, lab coat, and safety glasses when handling Tricaine (work in fume hood when using Tricaine powder). Stock solutions can be frozen and stored for up to six months and must be labeled with an expiration date.

4. Fish should then be submerged in ice water (5 parts ice/1 part water, 0-4° C) for at least 20 minutes following cessation of opercular (i.e., gill) movement from Tricaine treatment.

# Water Quality Maintenance:

*The fish facility includes two independent, recirculating systems (Quarantine and Main systems), manufactured by Pentair Aquatic Habitats. Fish facility water is obtained directly from the CCSR building's deionized water system and stored in a 90-gal holding tank. A fifteen percent water exchange occurs daily for each recirculating system (Quarantine and Main systems) using an automatic timer. Water in the racks is passed through charcoal and 50 micron mechanical filters and irradiated with ultraviolet light in a continuous cycle. The aquaculture systems also have automatic pH/conductivity sensors that measure and dose at regular time intervals.*

## Daily Water Quality Testing:

1. At 10 AM, collect at least 30 mL of Q and M system water into two 50 mL Falcon tubes labeled accordingly. For both Quarantine and Main, fill 6 glass test tubes with 5 mL sample water for testing.

2. Use API test kits to monitor pH, nitrate, ammonia, nitrate, general hardness (GH), and carbonate hardness (KH). **All solutions should be shaken well before using, and when administering drops, the bottle should be held vertically to ensure consistent drop size. When matching the color of the sample to the provided color chart, hold sample against the white background of the card in a well-lit area.** The table on the following page provides details instructions and normal ranges for each test. Record all results in the daily log book.

| Test | **Instructions** | **Normal Range** | **If reading falls outside of normal range…** |
|---|---|---|---|
| **pH** | Add 3 drops and invert tube several times. Also record the sensor's pH reading for both Q and M in the log book. | 6.5-7.5 | If the pH is >7.4, use the High Range pH test kit instead. If the system's dosing equipment is not working properly, adjust manually with the acid/base buffer. If pH test doesn't match system reading, recalibrate probe. |

| Test | **Instructions** | **Normal Range** | **If reading falls outside of normal range…** |
|---|---|---|---|
| **Conductivity** | There is no manual conductivity test, but record the sensor reading for both Q and M | 300-500 uS | If the system's dosing equipment is not working properly, adjust manually with salt solution. |
| **Nitrite** | Add 5 drops and invert tube several times. Wait 5 minutes before reading. | 0-1 ppm | Increase water exchange to 20-30% to introduce fresh system water to dilute this contaminant |
| **Ammonia** | Add 8 drops from bottle #1, then add 8 drops from bottle #2, and invert tube several times. Wait 5 minutes before reading | 0-2 ppm | Increase water exchange to 20-30% to introduce fresh system water to dilute this contaminant |
| **Nitrate** | Add 10 drops from bottle #1, then cap tube and invert several times. Shake bottle #2 for 30 seconds (IMPORTANT!), then add 10 drops. Invert the tube for 1 min to mix. | 0-40 ppm | Increase water exchange to 20-30% to introduce fresh system water to dilute this contaminant |
| **General Hardness (GH)** | Record number of drops needed to change solution from orange to green. Convert this to units of ppm using the provided conversion chart | 1-3 drops (17.9-53.7 ppm) | |
| **Carbonate Hardness (KH)** | Record number of drops needed to change solution from blue to yellow. Convert this to units of ppm using the provided conversion chart | 1-2 drops (17.9-53.7 ppm) | |

# System Maintenance

## Pad Filters:

1. The square pad filters located near the sump should be switched out every 3 days for both Q and M systems, or sooner if the accumulated waste is inhibiting water flow.

## Mechanical Filters:

*The 50 micron mechanical filters for both Q and M systems should be changed once every 72-96 hours (3-4 days).*

Protocol for Quarantine System:

1. Stop the system using the touch screen interface.

2. Slowly screw the old filter loose and replace with a new 50 micron filter.

3. Turn the water flow back on and reset the 50 micron filter change alarm on the sensor.

Protocol for Main System:

1. Cut off water flow to the pH and conductivity sensors, and put the TGP sensor on bypass (take the TGP probe out).

2. Stop the system using the touch screen interface.

3. Cut off the main water valve to the fish tanks, and cut off any water flow to or from the mechanical filter.

4. Take a new 50 micron Main system mechanical filter and wash thoroughly with MilliQ water. Replace the old filter, open up valves to the filters, restart the system, and slowly open the water line to the fish tanks.

5. Turn back on the pH, conductivity, and TGP sensors (remember it takes 1 hour for the TGP probe to stabilize its reading outside the holder; after

1 hour the TGP probe can be return to the holder and taken out of the bypass), and reset the 50 micron filter alarm on the touchscreen.

## Carbon Filters:

*Carbon media (activated charcoal) is used to trap and filter out any carbon based impurities and chlorine. It is recommended that carbon media be changed every 2-3 weeks. Both systems are set up to be alarm every 262 hours (~ 11 days). To replace the carbon media:*

1. Pre-soak the carbon in the designated system bucket in RO water for at least 24 hpf.

2. Shut off the system and remove old carbon media.

3. Fill the appropriate container and rinse with RO water until the water runs clear.

4. Replace the carbon media and restart the system.

## Pentair's Required System Maintenance:

*Regular maintenance on the physical system is outlined by Pentair's guidelines and warranty agreement. Below is a table of the regular maintenances required and the frequency with which they should be completed. There is a yearly maintenance log on the fish room door that needs to be signed after the completion of each maintenance cycle.*

| Maintenance | System | Frequency |
|---|---|---|
| pH Sensor Calibration | BOTH | Every 2 weeks |
| Conductivity Calibration (Monthly) | BOTH | Monthly |
| Clean Air Manifold (2 weeks) | BOTH | Every 2 weeks |
| Level Sensor (monthly) | BOTH | Monthly |
| Lubricate O-rings (monthly) | MAIN | Monthly |
| TGP Calibration (2 weeks) | MAIN | Every 2 weeks |

| | | |
|---|---|---|
| Water Exchange Calibration (2 weeks) | BOTH | Every 2 weeks |
| Water Exchange Clean (monthly) | BOTH | Monthly |
| Pressure relief valve (monthly) | MAIN | Monthly |
| Pump Bolts (monthly) | BOTH | Monthly |
| Sensaphone Batteries (monthly) | -- | Monthly |
| Clean heater (monthly) | BOTH | Monthly |
| Piston Filters (6 months) | BOTH | Every 6 months |
| Clean Flow Sensor (3 months) | MAIN | Every 3 months |
| UV Bulb (yearly) | BOTH | Yearly |
| UV Sleeves (yearly) | BOTH | Yearly |
| Dosing Tubing (yearly) | BOTH | Yearly |

# Fish Feeding

## Brine Shrimp Production:

### Supplier:

We currently use Hatching Shell-Free Brine Shrimp Eggs E-Z Egg from Brine Shrimp Direct. We order 10 kg at each order which lasts ~ 10 wks.

Occasionally there are quality issues and the supplier notifies us of a delay. If there is a delay, we use capsulated brine shrimp. Below is the detailed protocol for preparing shrimp.

### Decapsulation:

*Decapsulation removes the chorion (outer shell) from Artemia cysts via bleach treatment. This eliminates the need to separate nauplii from their hatching shells before feeding and is especially useful when high quality Artemia cysts are unavailable.*

**Materials Needed:**

Bleach (100%): 8.7 L bleach (1 gal = 3.785 L) ~2.3 gal bleach or 3 x 3⁄4 gal bottles

Buffered salt solution: (25 ppt salt; 2.5% NaOH) and premake in a plastic gallon container: 100 g NaCl, 100 g NaOH, 4.25 L MilliQ water

Sodium Thiosulfate (1.0%): 60 g sodium thiosulfate of 94.8 g sodium thiosulfate pentahydrate, 6 L MilliQ water

Saturated Brine Solution: 2.4 kg NaCl (use a bag to weight this out), 8.0 L tap water (make this directly in the hatchery while dehydrating overnight)

**Protocol:**

<u>Cyst Hydration:</u>

1. Add 2 cans of unbleached Artemia cysts to 10 L of MilliQ water in the hatching cone specified for decapsulation (located near the Chen Lab weigh station).

2. Aerate for 1 hour at room temperature.

3. After 1 hour has passed, examine cysts under a microscope. At this the cysts should be completely spherical, not like a deflated basketball. If not, continue hydration for up to 2 hours and check every 15 minutes.

4. Filter cysts through a medium sized brine shrimp bag (1000-µm mesh) and rinse with tap WATER). This can be done with the water outlet in the chemical hood.

5. Add 4 L of Buffered Salt Solution to the cone and transfer rinsed cysts back to cone to aerate.

Decapsulation:

1. Turn aeration down and add 8.7 L chilled bleach (or whatever vol. will fit) to the cone with a funnel. Cysts should begin to change color, first turning from brown to gray and then from gray to orange. Check constantly, this color change will happen very quickly. Note that over-bleaching will prevent the cysts from hatching.

2. As soon as 90% of the cysts turn orange, stop the reaction by quickly transferring cysts into 100-µm mesh bag and rinsing with cool tap water.

Neutralization of residual chlorine:

1. Transfer mesh bag into a large container and pour 1% sodium thiosulfate. Soak cysts in sodium thiosulfate for 1 min, then rinse with MilliQ water.

Dehydration for long-term storage:

1. Transfer cysts to a normal hatchery cone with 8 L saturated brine solution and aerate for 18-24 hours (**overnight**).

2. While keeping the aeration in, fill 1 L with resuspended cysts.

3. Store at 4 °C in saturated brine solution for up to 6 months.

## Feeding Adult Fish

Fish are fed twice a day on weekdays. On weekends, once a day is also suffice. Brine shrimp is the primary adult fish food. Add enough brine shrimp that can be consumed in 10 minutes.

Adult fish should also be fed supplemental foods (Zeigler Adult Zebrafish Diet) in the afternoon (5 PM). Shrimp and supplement are stored at 4 °C. Add enough food that can be consumed in 10 minutes. Please note that all supplemental food products that do not have a specified expiration date should be disposed of three months after opening. Always add a label to containers specifying the open date and expiration date.

On weekdays, feed the fish brine shrimp in the morning and afternoons (10 AM). *Do not feed fish in the mating tanks*—these will be fed upon their return to the Quarantine or Main system racks. On weekends, feed brine shrimp using the entire yield from the two hatcheries.

When feeding, avoid spilling shrimp or supplement on the tank lids, as this can promote bacterial growth. Use the Q and M squirt bottles for dispensing brine shrimp and the designated pipette feeder devices for dispensing supplement.

<u>Weekday AM:</u>

1. Remove the air input tube and lid from the left hatchery, clean it, and set it aside.

2. Drain the richest, orange portion of the culture into a brine shrimp net and wash thoroughly (2-3x) with system water. This is important to remove salt and debris in the culture.

3. Fill 1L flask with collected shrimp. Let settle so unhatched shrimp sink to the bottom.

4. Clean hatchery with brush and rinse with system water.

5. Fill hatchery with 12 L of system water. Put back the air tube and lid. Add 300 g salt and 2 falcon tubes (~70 mL) of shrimp.

6. Decant the shrimp from the 1L flask into the brine shrimp net. Try not to collect the brown-looking egg-sludge from the bottom and only collect the bright orange shrimp. Dump the sludge down the sink.

7. Return the shrimp to the 1L flask. Add water to 1L and let settle.

8. Drain again into brine shrimp net, rinse shrimp again, and return to 1L flask. Dilute total to 1L.

9. Spot a small aliquot of shrimp onto a depression slide and look at shrimp under microscope. Check for shrimp health, if <50% are alive do not feed the culture and feed supplement instead. Ideally there should be a limited amount of debris in the culture.

10. Portion the 1L of brine shrimp between the main and quarantine feeding bottles. Typically we use ~300 mL for quarantine depending on fish load.

Weekday PM:

1. At 5 PM, feed the adult fish the supplement. If there are larvae / young fry in the system, there will be a third hatchery labeled SNACK set up for them. Repeat the morning procedure using the SNACK hatchery.

Weekend:

1. All fish only get one feeding. Use both hatcheries to feed. On Sundays, check whether the SNACK hatchery needs to be set up. Feedings must be completed by 2 PM or else the shrimp will not hatch on time for Monday morning.

## Rotifer Production and Maintenance

Rotifers (*Brachionus plicatilis;* L-type rotifer) are maintained as a continuous culture at a density of ~ 20-100 rotifers per mL 15 ppt marine salt (Coral Life) solution in a 15 L conical hatching jar (CCH10; Aquatic Habitats) at 26.5 □C in the fish room.

Starting a new culture:

In event of a culture crash, fresh rotifers can be purchased from Reed Mariculture (Marine Rotifers [L-Type]; 1 million). Fresh rotifers can be kept in 4 □C for up to two days before beginning continuous culture.

. 1) Fill conical hatching jar with 15 L MilliQ water and 225 g marine salt.

. 2) While leaving rotifers in delivery bag, place rotifers in conical hatching jar to allow temperature equilibration for 30 minutes.

. 3) Cut open bag and release rotifers into hatching jar

. 4) Rotifers are then fed RotiGrow Plus (Reed Mariculture; stored at 4C) NOTE: We follow the Reed Mariculture recommendations for feeding based on volume we are harvesting.

. 5) One week after starting a new culture, the culture undergoes an entire water change and hatchery cleaning.

. 6) Keep water tinted green with RotiGrow Plus. Feed Rotifers 2x a day with RotiGrow Plus. NEVER ALLOW CULTURE TO APPEAR CLEAR OR ROTIFERS WILL STARVE!

Daily maintenance of continuous culture:

To maintain fresh rotifer production, at least 25% culture volume must be replaced with fresh 15 ppt marine salt solution when density increases. Rotifer feeding with RotiGrow Plus is required once in the morning and once in the afternoon.

. 1) Determine rotifer density by pipetting 1 ml culture into a glass vial and counting the number of rotifers present. (If this number is <75, it may be best not to perform a water change that day)

. 2) If there are larval fish to raise, see "**Feeding Baby Fish"**.

. 3) Drain 25% of rotifer culture into the sink and refill to 15 L with fresh 15 ppt marine salt
solution (60 g marine salt in 4 L MilliQ water)

. 4) Feed rotifers 1 ml Rotigrow Plus. NOTE: On weekends, a single feeding of 2 ml

RotiGrow Plus is sufficient. However, water changes are mandatory unless otherwise instructed.

. 5) Every week, the rotifer culture undergoes an entire water change and hatchery cleaning.
In short, collect all rotifers in compound net (see below) and replace entire 15 L of 15 ppt water.

## Feeding Baby Fish

Zebrafish larvae are raised until 5 dpf in a 28.5 □C incubator. At 5 dpf, larvae are fed rotifers until 10 - 12 dpf (5 - 7 days of rotifer feeding) and then are introduced into the adult system and fed brine shrimp until adulthood. Rotifers are cultured at 15 ppt salinity; however, they are co- cultured with zebrafish larvae at 5 ppt salinity. It is not the rotifers themselves that provide nutrition to the larvae but rather the food the rotifers eat. Starved rotifers will do no good for raising baby fish!

. 1) Transfer 5 dpf larvae into approximately 200 ml volume of 5 ppt water (~ 30-40 larvae per 3 L tank).

. 2) After checking rotifer density, drain 10 - 25% (depending on density) of rotifer culture into a compound collection net composed of a 150 μm mesh (N2150A; Aquatic Habitats) within a 25 μm bag (N1025; Aquatic Habitats). Large debris are filtered out through the 150 μm mesh and the rotifers are retained within the 25 μm bag. (See *Daily maintenance of continuous culture* for instructions of how to maintain culture.)

. 3) Resuspend collected rotifers in 5 ppt water

. 4) Feed enough rotifers to the zebrafish larvae to create a “blizzard” of rotifers (increases
chance that larvae will find and consume the rotifers)

. 5) Add enough RotiGrow Plus to the co-culture (larvae + rotifers) to tint the water green.

. 6) The next morning, replace co-culture water with fresh 200 ml 5 ppt water and re-dose
with freshly collected rotifers.

. 7) Repeat until the larvae are are 10-12 dpf and introduce larvae into the adult system.

. 8) Once larvae are 45+ dpf, move larvae into larger tanks to provide space for growth

# Fish Husbandry

## Setting up Crosses

1. Set up mating crosses about one hour after the second feeding.

2. Assemble Aquatic Ecosystems breeding tanks with plastic partitions and fill with system
water.

3. Place 1-5 female fish in one compartment and an equal number of male fish in the other.

4. Keep the segregated fish in the climate- and light-controlled fish room to allow mating
the next morning.

5. Remove the plastic partitions to allow the fish to mate as soon as possible after the light
comes on the next morning (8 AM).

6. Check for eggs every 30 minutes or so and collect them in separate Petri dishes.

7. Wash eggs extensively with egg water, using a mesh sieve to remove debris.

8. Place eggs in new Petri dishes, approximately 50 embryos/10-cm dish with about 30 mL
of egg water.

9. Incubate eggs at the external 28.5 °C incubator to ensure properly staging. Remove dead
or contaminated eggs regularly to ensure maximal survival rates.

## Bleaching Embryos for Main System Aquaculture

Embryos to be transferred to the Main System *must* be bleached prior to their introduction. Procedures for this process can be found in Nüsslein-Volhard's Zebrafish book, which is located in the laboratory.

Embryos should be bleached between 10 and 28 hpf. After 28 hpf, the chorion is partially deraded and the bleach can damage the embryo. Since bleaching interferes with hatching, either pronase must be added or embryos must be manually dechorinated 12 hours after bleaching.

1. Sterilize working area with 70% ethanol.

2. Set up 5 washing solutions in the following order: Bleaching Solution, E3, Bleaching Solution, E3, E3

3. Tranfer embryos to a clean tea strainer and incubate them in each bath (5 minutes each). Be sure that embryos remain submerged during treatment.

4. Wash embryos into a new Petris dish with E3 medium supplemented with methylene blue. No more than 75 embryos / dish.

## Tank Labeling

All aquaculture tanks containing zebrafish larvae and adult fish should be clearly labeled using removable tape. Wildtype fish and transgenic fish should be segregated (except for crosses) and should be housed in separate aquaculture tanks. Tank labels should include information on the fish line (wildtype, transgenic, or mutant strain) and the date of birth. Please note that the use of transgenic fish is restricted by the California Department of Fish and Game. No transgenic fish should ever leave the lab without proper authorization.

## Tank Sanitization

Aquaculture and mating tanks should be sanitized between uses, particularly in preparation for Main System fish. The tanks should be soaked for 24 hours in a

0.6% solution of sodium hypochlorite and soaked for at least one hour in a 0.3% solution of sodium thiosulfate (to quench any residual sodium hypochlorite). The tanks should then be transferred to the Chen Lab dishwasher located in CCSR 3141 and washed using the normal cycle on sterilization mode. Sterilized aquaculture tanks should be stored in the designated laboratory shelves. Sterilized mating tanks should be stored on the carts located in the fish facility itself. Sodium hypochlorite and sodium thiosulfate baths are changed monthly.

### Fish Net Sanitization

Fish nets should be sanitized between each use, and care should be taken to segregate nets used with Main System versus Quarantine System fish. The nets should be soaked for at least 30 minutes in Virkon S disinfectant solution (1 tablet/500 mL) and then washed with copious amounts of MilliQ water prior to use. This step is especially important since trace amounts of disinfectant can kill the fish. The Virkon S cleaning solutions should be changed weekly.

# Sentinel Health Program

The sentinel program is designed to detect and monitor the presence of fish pathogens in the main system of the fish facility. Sentinel fish are sampled once a year for diagnostic testing through VSC.

For sentinel testing, 6 fish are chosen from the SUMP for testing – half will be sent for histopathology and half to PCR detection of pathogens. If there are no fish in the SUMP, the oldest lines are chosen as these are the most susceptible to disease. Fish are fixed according to ZIRC's histopathology guidelines. Records are kept in the fish facility shared Dropbox folder.

The fish will be submitted to VSC every year in October.

# Emergency Preparedness

In the event that either the main or quarantine system water flow shuts off, the Sensaphone monitoring system will alarm and call the following phone numbers in this order until someone answers: the lab phone, James Chen, Tracfone and Robert Pearce. If the sensaphone alarms, proceed immediately into the fish facility and turn off the alarm. Assess the situation and turn the water flow back on if it is safe to do so.

Following a disaster event: immediate response by the animal staff is to notify the appropriate first responders. First Responders include: outside Emergency Response personnel (fire, police, rescue squad), Facilities Management personnel (James Chen, and Robert Pearce), and the VSC on-call staff. Animal Program personnel should be trained to always follow instructions given by First Responders, the Officer in Charge, or other emergency response personnel.

Assessment of the impact to the Animal Program is one of the first steps. Animal Program personnel may be denied entry or allowed only limited entry to an area that is deemed to be unsafe or compromised. The three critical areas to assess are: water supply, facility structure, utilities, and equipment; personnel; and research animal health. Efforts should be coordinated with VSC to gain access to an alternative water supply - i.e. water truck and an air cooling system. Adversely affected animals will be euthanized immediately. Once program and facility operations have returned to normal, a critical evaluation of the event cause, program response, and recovery process should be performed.