Chapter 3

Materials and Methods

3.1 Test animal

3.1.1 Prawns

Young and mature prawns were collected from a culture pond in Rangsit, Pathumthani province. Those living specimens were trasfered to the Marine Science Department, Faculty of Science, Chulalongkorn University, Bangkok. Each prawn was transferred to a 30 x 60 x 30 cm glass aquarium, containing about 30 litres of dechlorinated tap water. Aeration was supplied through an air-stone. About ten females were needed to maintain breeding stock at least two healthy mature male were required for mating. Artificial diet was fed 4 times, 08:00, 12:00, 16:00, 20:00, a day. To keep good quality of rearing water, exceed food and fecae on the bottom of each aquarium was drained everyday, about 10-20% of water was refilled. These aquaria were cleaned once a week.

3.1.2 Breeding of embryos and larvae

The newly-moulted ripe female was retained in the aquarium 2 to 3 hours for hardening of new shell. The old shell, fecae, and excess diet was all drained out and new water was refilled. After that period, a healthy mature male was introduced into the aquarium. After mating process succeeded, the male was retained until egg-laying took place in female before returning to its original aquarium. The berried prawn was served as embryo source, and eggs took about 16-17 days under this condition (28-29 °C in the glass aquarium) for incubation.

After hatching, the spawner was removed and transferred to a spare aquarium. Salinity acclimatization was performed by gradual adding of filterd brine (~100‰) prepared from salt using dipping system. A bottom cut-polyethylene bottle modified from a 1.25 or 2 litre-carbonated drink container with a small pore on top was applied. The device was hung above the larvae's aquarium,

brine was kept dipping until the salinity of about 14‰ was reached (12-14‰ of salinity have to be maintained; Ling, 1969b). The larvae were acclimated at the salinity for at least one day before transfering to a 200 litre-fiberglass tank, conataining 100 litres of rearing water. Aeration was supplied through air-stones to stir and oxygenate the rearing water. Evaporation loss was made up with freashwater to the original level. The water volume was gradually increased with the development of larvae. Larvae were fed with newly hatched *Artemia* naplii twice a day.

3.1.3 Selected testing stages

Early (1 day-old) and late (12 day-old) stages of *M. rosenbergii* embryos were tested for sensitivity trend to TBTO within 96 h of acute term. The tolerance range throughout the progressive development which figured out from the pretests was considered for setting appropriate concentration series in subacute test. For larvae, the resemble processes were also conducted in second to sixth stages. These instars consumed 24-72 h to become next stage, the larval tests were so limited to 48 h of exposure. In case of subacute term, 1 day-old larvae were treated with sublethal doses for 30 days comparing to control and solvent control.

3.2 Toxicity tests

TBTO toxicity tests for *M. rosenbergii* embryos and larvae were divided into 2 steps of action—acute toxicity tests to find out LC_{50}s, and subacute tests based on suborder concentration of average LC_{50}s. The experimental condition was designed to be a static with daily renewal tests. The study used decreasing TBTO concentrations at different exposure time information of Litnot *et al.* (1998) to make a decision of renewal tested water. Even though the flow-through system would offer the most consistent TBTO levels regimes over a exposure period, it requires more complicate apparatus and produces relatively large amount of wastewater. Besides, the declining concentration expected in this static protocol would reflect the daily fluctuation of TBT levels in natural condition which depend upon the local aquatic transportaion. However, according to the study of Litnot *et al.* (1998), daily water renewal manifest to provide sufficient bioavailable of TBT^+ and good quality of water for test organisms.

Stock solution I (1000 mg TBTO l^{-1}) was prepared by dissolving 0.25 g TBTO in 25 ml acetone. Dilution of stock solution I resulted in stock solution II (1000 µg TBTO l^{-1}) which served as working solution. Both stock solutions were preserved in refrigerator at 4 °C for a lifetime of one month). Each concentration of testing solution was made up from stock solution II with rearing water (14‰). All experiments were conducted in Physiology and Toxicology Laboratory, Marine Science Department, Faculty of Science, Chulalongkorn University. A black plastic sheet was installed on the window side to protect experimental units from direct sunlight. All experimental units were exposed to the room temperature (~28-29 °C).

3.2.1 Acute toxicity tests

Embryo toxicity tests

On the range finding test, 3 day-old embryos were treated with nine concentrations between 0.5 to 128 µg TBTO l^{-1} in arithmetic series. Ten embryos were tested in an experimental unit using 100-ml glass jar containing about 90 ml of test solution for 96 h. Three replicates were prepared for each concentration. The media was stirred by bubbles supplied through a 4 mm-diameter Tygon tube for activating the embryos and aerating the water. The experimental condition was static with daily water renewal. During this process, each unit was gently poured whole content through a small mosquito larvae-net. The unit was refilled and embryos were immediately turned to the container.

In second trial, a new concentration series was therefore set up at 25, 50, 100, 200, and 400 µ g TBTO l^{-1} and 3 day-old larvae were tested. For third trial, 10 d-old embryos were treated at concentrations 200, 250, 300, 350 and 400 µg TBTO l^{-1} for 96 h.

Because of uncertainty in response of the embryos to TBTO, the new range finding test were revised in forth trial with 500, 1,000, and 5,000 µg TBTO l^{-1}. Finally, the actual definitive test was run at 250, 350, 500, 700 and 1000 µg l^{-1} in 24-well tissue culture plates containing about 1.5 ml of TBTO solution. Aeration was allowed by diffusion.

Larval toxicity tests

Based on LC_{50}s of pre-postlarval stage for zebra shrimp *Penaeus japonicus*, 0.7 μg TBTO l^{-1} of 48 h LC_{50} for mysis 1 and 5.3 μg TBTO l^{-1} of 24 h LC_{50} for mysis 3 (Lignot *et al.*, 1998), TBTO in the same series were applied as core of concentration for *M. rosenbergii* larvae. Both minimum and maximum ends were expanded three steps in geometric series with a factor of two. Subsequently, the range was reset with the same factor and resulting in a whole one series. In addition, a new maximum value was added to cover the 100% mortality. At the end, the concentrations for first trial were 0.1, 0.2, 0.4, 0.8, 1.5, 3, 6, 12, 24, 48 and 96 μg l^{-1} and forth stage larvae were tested.

In second range finding test, five concentrations; 3, 6, 12, 24 and 48 μg l^{-1} were conducted to meet appropriate lethal concentration range. The test was performed in 1^{st}, 2^{nd}, and 6^{th}-stage larvae for 48 h. The actual range was set in geometric series with a factor of 1.5 for five concentration values. The round-up values were 4, 6. 9, 14 and 20 μg l^{-1} and were used for actual acute toxicity test.

The condition was static with daily renewal test, aeration was supplied into each experimental units through a 4-mm diameter of Tygon tube at the rate of 2 bubbles per second or 0.66 $cm^3 s^{-1}$. During exposure, larvae were fed *Artemia* sp. once a day to avoid cannibalism except in the first stage. About one drop of concentrated nauplii of newly hatched *Artemia* were added into each experimental unit at the beginning of the tests and after solution renewal. The experiments were conducted in 100 ml glass jars containing about 90 ml of test solution, ten larvae were tested in each experimental unit. Three replicates were set for each concentrations. At each endpoint of 24 h the larvae were checked for mortality. The criteria for vital consideration were: (i) colour—opaque flesh of necrosis indicated lethality took place by few hours, pale salmon-pink indicated degraded flesh; (ii) movement—transparent larvae which lying on the bottom of the container, can not move after touch with dropper tip and can not swim to the surface after bubbling. Although the inert larva did not response to the stimulation and seem to be a dead one, its epipodite may still be functioned when observed microscopically. However, all of those were considered as dead larvae and were discarded. For embryonic effects of TBTO, the same manner procedures were performed.

3.2.2 Subacute toxicity tests

Embryo toxicity tests

To facilitate the recognition of embryo developmental rate in subacute toxicity test, the embyonic development observation was carried out. The outcome was added to the previous study of Ling (1969a) in order to supplement existing data on staging of *M. rosenbergii* embryos.

According to the 96 h LC_{50}s for early and late stage embryos, the subacute doses were set up from suborder mean of the 96 h LC_{50}s. The concentration series were: 3.5, 7, 14, 28 and 57 μg l^{-1}, about 120 embryos were tested at each concentration of TBTO in 24-well tissue culture plates containing about 1.5 ml of test solution in each well. The test was planned to run for 1 day-old embryos in 15-20 days of exposure, upon the hatching process. The newly fertilized eggs were separated individually from mother prawn using a soft brush, and retained in the wells with clean water for acclimaion, after 24 h the embryos were stereoscopically checked for survival, dead ones or unfertilized eggs were discarded before the test. Each embryo was checked for vitality, every 24 h the dull and/or edemous one was discarded. The experiment for that well was terminated and excluded from calculation. The water renewal was routinely done after that using a dropper, freshly-prepared solution was then immediatly refilled in the well with another dropper.

Larval toxicity tests

On pretests, sublethal concentration series were set for inhibition of larval development test. The larvae were exposed to the concentrations of 0.1, 0.2, 0.4, 0.8, and 1.6 μg TBTO l^{-1} for 48 h. Water renewal was performed in the same procedures of acute toxicity tests. At the endpoint the larvae were transferred from experimental units by small net, killed in 4% formaldehyde, identified and counted under microscope. The ratio of next stage larva(e) to total larvae was considered as developmental rate. The larvae with stunted growth and failed to reach the next stage were considered an affected ones. Third, forth, and fifth stage larvae were selected to try out.

For actual test, two concentrations plus control and solvent control was provided for subacute test using one-tenth of mean 48 h-LC_{50}s for the higher one and one-twenty for the other. Fifteen

larvae were tested in glass jar containing 1000 ml of test solution. About 50% of test solution was drained including fecal pellete and undesirable materials every 24 h using Tygon tube linked with a 20 ml plastic syringe and valves for controlling suction pressure. Five to fifteen larvae were randomly sampled to determine stage microscopically. Staging was based on pictures and descriptions in the larval development of *M. Rosenbergii* rearing in laboratory as described in Uno and Kwon chin soo (1969).

3.2.3 Data collection

Embryonic development

Pictures of untreated embryos newly detached from mother prawn were daily taken with a Cannon photomicroscope at 40 x until hatching.

Embryo toxicity tests

During acute toxicity tests, all embryos were carefully observed microscopically every 24 h of exposure until the end of 96 h. Mortality was recorded and dead embryos were discarded.

For subacute tests, during the sixth to seventh day development, embryos were steroscopically checked for the appearance of optic vesicles. Between seventh and eighth days, investigation for heart beating. Daily observation using steroscope was avoid on out of the ranges to prevent inducing thermal stress on embryos. Dead embryos were discarded during daily water renewal, parasitic or fungal infected ones were removed and excluded from calculation. At the tenth day the embryos of all experimental groups were evaluated for their vitality using a stereoscope. At hatching day, all larvae were carefully observed stereoscopically for malformations and recorded. Images of malformed hatchlings were acquired by a camera linked to a steroscope.

Larval toxicity tests

Lethality was checked and recorded every 24 h for acute toxicity tests. Stage composition was observed stereoscopically everyday, mean stage was calculated for growth rate.

3.2.4 Statistical analysis

For lethal toxicity tests, the relationship between the TBTO concentration and numbers of deads, at the end point, were calculated for LC_{50}s and its confidence intervals by probit method (Finney, 1971) using the EPA Probit Analysis Program version 1.5 (US Envrionmental Protection Agency).

As regards subacute larval toxicity tests, larval developmental rate as mean age in days ($\overline{A}_d$) was calculated from daily staging data of sampled larvae using the following equation:

$$\overline{A}_d = \frac{\sum_{i=1}^{n} f_i x_i}{n}$$

where f_i = numbers of sampled larvae in stage i; x_i = mean age in days of stage i which according to ceiteria described by Uno and Kwon chinn soo (1969): 0 day-1st stage, 2 days-2nd stage, 4 days-3rd stage, 7 days-4th stage, 10 days-5th stage, 14 days-6th stage, 17 days-7th stage, 20 days-8th stage, 24 days-9th stage, 28 days-10th stage, 31 days-11th stage, and 36 days for newly-transformed postlarval stage; n = total numbers of observations.

The $\overline{A}_d$ values from three replicates was then averaged to estimate the represented developmental rate on the sampling date.

The difference of developmental rate among treatments was determined by one-way ANOVA follows by multiple mean comparison using Duncan's new multiple range test.