CHAPTER III

MATERIALS AND METHODS

Apparatus

Apparatus and equipment required for culturing and testing of potential toxicity of TBTO on the sea bass (*L. calcalifer*) are summarized in Appendix A.

Seawater

Diluted seawater used in the experiments was obtained by diluting natural seawater (from uncontaminated, uniform quality source) by dechlorinated tab water. Total residual chlorine was not allowed to be higher than 0.01 mg/L.

Cleaning test chambers and laboratory apparatus

1. New testing glassware were soaked overnight in a 10% HNO_3 acid solution and subsequently soaked overnight in sea water.
2. All test vessels, tanks, and other equipments that came in contact with test solutions or chemicals were washed with detergent and tap water after use to remove surface contaminants. The apparatus was then rinsed with 10% HNO_3 to remove scales, metals and bases following by acetone to removed organic components, if any. Finally, such equipment was rinsed thoroughly with distilled water.

Preparation of stock solution

Reagent-grade tributyltin oxide (TBTO; Fluka Chemical Ltd. > 96 % purity), was used in the experiments without futher purification. Stock solution was prepared by dissolved tributyltin oxide in acetone to a final concentration of 1000 mg/L, and

kept in a 4°C refrigirator until further required. The appropiate concentrations of TBTO were prepared from the stock solution by diluting with testing seawater (20 ppt).

Experimental animal

Twenty days old sea bass (*L. calcalifer*) , obtained from Prachub Khirikhan Coastal Aquaculture and Development Center, were placed into a 1000 L holding tank and slowly acclimatized in 20 ppt salinity water. The fish were fed twice daily during guarantine, holding, and acclimatization for signs of stress, physical damage, mortality, disease, and external parasites. Abnormal, injured, and dead individuals were discarded. The acclimation period was about 20 days until the fish seemed to be able to adapt to the laboratory condition.

Approximately 2-3 days prior to the experiment, appropriate numbers of equal-sized sea bass were transferred from a holding tank to an acclimation tank. The fish were maintained in the 20 ppt seawater at the ambient temperature for 48 h before placed in the test chambers.

Acute toxicity tests

1. Preliminary range-finding test

A toxicity range-finding test consisted of a down-scaled, abbreviated static acute test in which groups of fish were exposed to widely-spaced TBTO concentrations. Firstly, concentrations of 2.5, 5.0, 10.0, 20.0, 40.0 μg/L TBTO and a control were carried out. Subsequently, the finer concentrations of 1.0, 1.5, 2.0, 2.5, 5.0 μg/L and a control were employed, and finally 0.25, 0.50, 1.00, 1.50, 2.00 μg/L TBTO and a control were used, respectively. The fish were killed or affected from 0-100 % related to TBTO concentrations. The percent mortality of three preliminary range-finding tests are shown in Appendix B.

2. Definitive toxicity tests

Based on the results of the range-finding test described previously, the TBTO concentrations were selected between the highest concentrations that killed none or only a few test fish and the lowest concentration that killed almost or all sea bass. As a result, five different concentrations of TBTO and a control (at the highest acetone-solvent concentration of 1.4 mg/L) were chosen for a total of 18 treatments. The selected TBTO concentrations were 0, 0.6, 0.8, 1.0, 1.2, and 1.4 μg/L, respectively.

Test procedures

The static renewal tests were employed for all acute toxicity tests. The experiments were carried out in 14 L chambers containing 10 L of TBTO testing solution which were renewed every 24 h.

Ten sea bass (*L. calcalifer*) aged 45 days with an average of 0.2702 (± 0.0923) g in weight, and an average of 2.07 (± 0.24) cm in length were randomly allocated into each chamber provided with continuous aeration (rate were not exceed 100 bubbles/min.). The fish were not fed for 24 h before the beginning of the test and during the test which continued for 96 h. The number of dead fish in each test chamber were counted every 24 h from the beginning of the test until the end of experiment.

Light quality and photo period was maintained at the laboratory illumination and 12L : 12 D, respectively. The salinity of seawater containing TBTO was 20 ppt.

The criteria for death was usually a lack of movement and of reaction to gentle prodding. During all tests, general observations of erratic swimming, loss of reflex, discoloration, changes in behavior, excessive mucus production, hyperventilation, opaque eyes, curved spine, and cannibalism were quantified and reported.

Dissolved oxygen, temperature, pH and conductivity were measured at the initiation of the tests and every 24 h thereafter. Hardness and alkalinity were measured periodically from reservoir water. (Methods and instruments were summarized in Appendix A)

Data analysis for acute toxicity tests

Probit analysis was used to estimate LC50 and the associated 95% confidence interval. The analysis consisted of adjusting the data for mortality in the control, and using a maximum likelihood technique to estimate the parameters of the underlying log tolerance distribution. Mortality data from the replicated chambers were combined before LC50 determinations were carried out.

Sublethal toxicity test

Experimental procedures

The static renewal tests were employed for all sublethal toxicity tests. The experiments were carried out in 12 glass aquaria, 30×60×30 cm, containing 30 L of an appropriate concentration of TBTO solution. Three different concentrations of TBTO (0.03, 0.05, 0.10 μg/L) and control, were employed with triplicate aquaria. The TBTO test solutions were replaced completely every 72 h.

Juvenile sea bass aged 60 days were randomly allocated into aquaria described above, with continuous aeration, until each aquaria contained 15 fish (or a total of 45 fish for each treatment). The fish were fed twice daily with adult brine shrimp (*Artemia* sp.) except on the measuring day.

The light quality was maintained at the laboratory illumination. The photo period and salinity were at 12L : 12D and 20 ppt, respectively.

The following parameters were measured and recorded in all test aquaria :

- Temperature (C°) was measured every day.
- DO, pH, and conductivity were measured every 3 days.
- Hardness and alkalinity were measured periodically from reservoir water. (methods and instruments were summarized in Appendix A)

The tests were terminated after 8 weeks. Oxygen consumption and growth of the sea bass were also recorded.

Oxygen consumption tests

1. Oxygen consumption at short-term experiments

Six experimental treatments included four different concentrations of TBTO (0.03, 0.05, 0.10, and 1.0 μg/L) and two controls (seawater and acetone) were carried out to measure their effects on oxygen consumption of the sea bass using a Gilson differential respirometer. The fish were placed individually in the active flasks filled with 40 mL newly prepared seawater of experimental salinity (20ppt) and dosed with the respective test concentrations of TBTO. After acclimated in solutions for 1 hour, oxygen consumption of such a fish was determined.

2. Oxygen consumption in continuous TBTO-exposure for 8 weeks

Fish in experimental treatments (control ,0.03 , 0.05 and 0.10 μg/L TBTO-expose) were ramdomly selected from test aquaria to measure the oxygen consumption. The fish were individually placed in the active flasks filled with 40 mL newly prepared seawater of experimental salinity and dosed with the respective test concentrations of TBTO. The oxygen consumption from each treatment were determined at the start of the experiment (Week 0) and every other weeks for 8-week period.

Oxygen consumption was expressed as specific oxygen consumption rate (milligrams of oxygen per grams wet weight per hour, $mgO_2/g/h$).

The test procedures employed for a Gilson differential respirometer, and calculation were illustrated in Appendix C.

Growth tests

The standard body length and weight (wet weight) in experimental treatments (control ,0.03 , 0.05 and 0.10 μg/L TBTO-exposed) were individually measured. These two parameters were determined at the start of the experiment (week 0) and every other weeks for 8-week period. The length and the weight were calculated expressed as the relative growth rate. The formula used to calculate growth parameters were described in Appendix C.

Data analysis for sublethal toxicity tests

One-way analysis of variance ($P \leq 0.05$) was applied to all oxygen consumption and growth data to determine the TBTO effects. Duncan's New Multiple Range Test was used to compare the means. Regression was used to determine the relationship between length and weight variables.