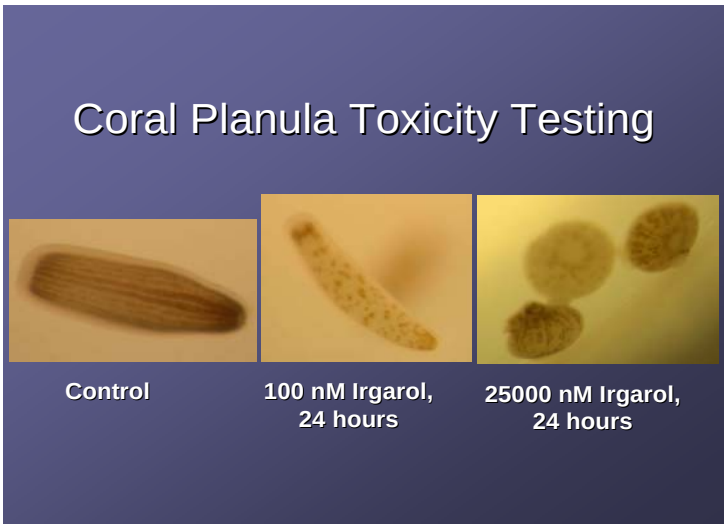
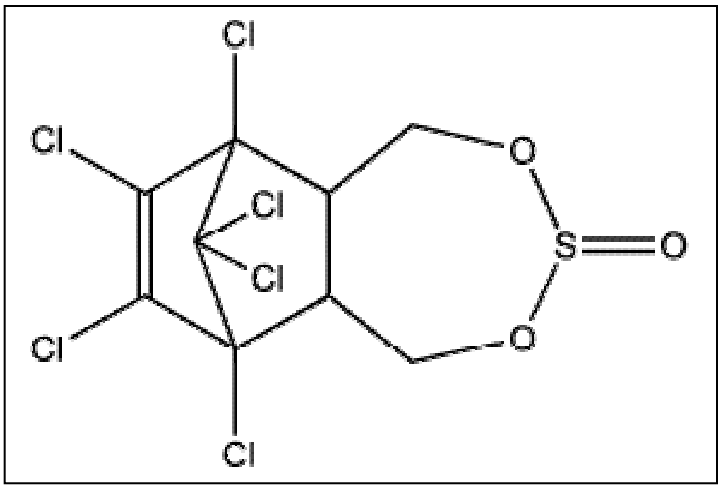
Laboratory Toxicity Testing for the Meso-American Reef





Chapter 1
Experimental Design and Analysis

The purpose of this document is to complement the World Wildlife Fund's *Contaminant Chemistry and Biomarker Diagnostic Site Assessment Protocol Manual for Assessing the Effectiveness of Agricultural Better Management Practices in the Mesoamerican Reef*. Once a contaminant or sets of contaminants are found in the local environment of a population or in a population that is exhibiting a stressed physiological condition, the next step is to evaluate the possible toxicological association between the contaminant(s) and the stressed condition. One way of doing this is to conduct laboratory-based toxicological experiments. Toxicological experiments generally have three objectives. The first purpose is to ascertain whether or not a contaminant results in an effect on a biological system. The second objective is to determine how much of an effect is present as a result of the concentration of the exposure. The third objective, though often forgotten or ignored in many ecotoxicological endeavors, is to understand the nature or mechanism of toxicity. It is fulfillment of this third objective that allows us to determine if populations in the field that are exposed to a known contaminant are producing a specific pathological effect. When a specific pathological profile is generated by a particular contaminant in the laboratory, and that same pathological profile is seen in a natural population exposed to that same contaminant, the investigator can generate a strong, convincing argument that the contaminant is acting as a toxicant, and is to a degree adversely affecting the population.

This document proposes three model species for laboratory studies. Only one of the species used in the Meso-American Reef monitoring and assessment program (*Porites asteroides*) is also used as a laboratory model. The species that is not used in the Meso-American Reef monitoring and assessment program is a sea urchin, *Tripneustes ventricosus*. Instead of using adults in the most of the toxicological experiments, early development stages of each species (e.g., embryo, blastula, planula) will be used. There are two principle reasons for using early stage development models. The first reason is one of cost and infrastructure. Rearing adult coral or sea urchins requires extensive culturing infrastructure as well as a more costly laboratory dosing design and structure. Since there are few labs in the Caribbean that have this capacity, it is more cost-effective to use juvenile stages. The second reason is that juvenile stages are usually much more sensitive to the toxic effects of a contaminant than the adult stage. The advantage here is that the experimental system requires less time and fewer infrastructures requirement (e.g., dosing chambers) to observe an effect. Juvenile-stage toxicity testing produces data that has importance to an ecological risk assessment – it directly addresses an aspect of the risk characterization for reproductive fitness and recruitment. In spite of these and other advantages, two caveats of juvenile-stage toxicity testing are (1) the uncertainty associated with the pathological profile and (2) differences in the toxic-responses between juvenile and adult forms.

Types of Toxicity Tests

There are three types of laboratory-toxicity tests that can be applied to each of the laboratory models: (1) chemical-exposure challenge, (2) sediment pore-water or sediment

challenge, and (3) Toxicity Identification Evaluation. Toxicity Identification Evaluation is a specific methodology that is used to help identify the cause or source of toxicity, and will not be covered in this document. A good resource for learning more about Toxicity Identification Evaluation is Norberg-King, TJ., et al (eds). 2005. Toxicity reduction and toxicity identification evaluations for effluents, ambient waters, and other aqueous media. Pensacola, FL: Society of Environmental Toxicology and Chemistry (SETAC).

Chemical-exposure challenge is the most straight-forward experimental design. This experiment design consists of exposing the laboratory-model species to a known concentration of a pure chemical compound or a known concentration of a mixture of chemicals. Some chemical compounds will require an organic solvent or a pH altered solvent to dissolve the solute. Such solvents are known as "carriers", and if the target chemical requires a carrier (e.g., ethanol, acetone, dimethyl sulfoxide), a "carrier" control must be included in your experimental design. This basically consists of the highest volume of solvent used to deliver a chemical used in the experiment. For example, if 20 microliters of acetone was used to deliver the chemical compound, a set of replicates should be exposed to 20 microliters of acetone.

Sediment can accumulate contaminants, which can reach concentrations detectable by many trace contaminant chemistry analyses, as well as result in toxic effects to benthic organisms. Contaminants in high concentrations in sediment may pose a significant ecological risk. Interstitial water of marine sediments (porewater; the liquid component of a sediment matrix) is the principle solvent in marine sediments, and may contain the majority of contaminants. Toxicity testing of marine sediments and porewater can be a powerful tool in measuring the impact a contaminant or mix of contaminants present in the environment. Sediment and porewater toxicity testing is usually conducted as one of the first-steps in an environmental assessment of determining whether an anthropogenic activity or event may be causing an impact on the local biota whose influences are not readily obvious. Sediment and porewater-toxicity testing can be used for different types of investigations in the Meso-American Reef system. Sediment and porewater toxicity testing can be used as an inexpensive screening method to justify trace-contaminant chemistry analysis. A toxic effect with the assay, with ecological data for that area indicating a degrading community or population provides significant evidence that a specific coral reef is being impacted. This information can also be used to justify a more sophisticated investigation to determine what exactly is impacting the coral reef, and the nature of the contributing effect produced by the stressor. A good resource for sediment and porewater toxicity testing methods can be found in Carr, R.S. and Nipper, M (eds) 2003. Porewater toxicity testing: Biological, chemical, and ecological considerations. Pensacola, FL, USA: Society of Environmental Toxicology and Chemistry (SETAC).

Analysis of Data

Chemical-exposure toxicity tests or various sediment/porewater toxicity tests will be based on either a single exposure/effect design or a dose-response design. A single exposure/effect design is often used with sediment/porewater toxicity tests; for example, replicates of your laboratory model are exposed directly to a specific volume of extracted porewater. Hypothesis-testing methods (e.g., univariate methods: Student's t-Test or

Analysis of Variance) can determine whether porewater exposure induced a response. Dose-response design is the experimental design used as examples in the following chapters. One objective of this design is to determine whether a specific parameter changed in treatment-exposure populations from the reference, or whether there are differences among the treatment concentrations. Hypothesis testing methods are beyond the scope of this chapter, but any Statistics Primer textbook should be able to explain these concepts and constraints in applying them to a given situation.

Exploring the relationship between a treatment variable (i.e., concentration of a chemical compound) and an effect variable is an important goal for ecotoxicology. Understanding the relationship between a contaminant and causing a stress on a biological system provides essential information to resource management: (1) it allows the resource manager to estimate the risk of a biological resource in adversely responding to a specific pollutant or anthropogenic activity and (2) it provides information to a weight-of-evidence argument of whether or not a contaminant is having an impact on a biological entity. Modeling is the act of defining the relationship between an exposure treatment and a biological effect. In ecotoxicology, once a model has been established, it can be used to (1) predict biological effects to a concentration of the contaminant where actual data is lacking, (2) provide a level of confidence in the prediction, and (3) determine if there is a relationship between a pattern of effects and a pattern of exposures. The methodology for modeling described in this chapter is valid with only two variables. Methods for modeling three or more variables are beyond the scope of this chapter, but include methods such as Multiple Regression Analysis and Canonical Correlation Analysis.

No model is complete – or perfect. Toxicologists can conduct an experiment with a finite series of treatments. In the following chapters, it is suggested that the toxicologist begins with five different treatments and a reference. The concentration difference between treatments is arbitrary: it is up to the researcher to posit the treatment variables. Concentrations can be a doubling per treatment, or be based on a log scale. For example, a researcher exposes coral planula to five concentrations of a pesticide at 10 millimolar, 20 millimolar, 40 millimolar, 80 millimolar, and 160 millimolar. If the researcher finds the pesticide at a concentration of 50 micromolar in the environment, what is the probable effect of that concentration? To answer this without having to repeat the exposure experiment for that concentration, a relationship between concentration and effect are modeled. One method of modeling is regression analysis. Regression analysis is a way of examining a potential relationship

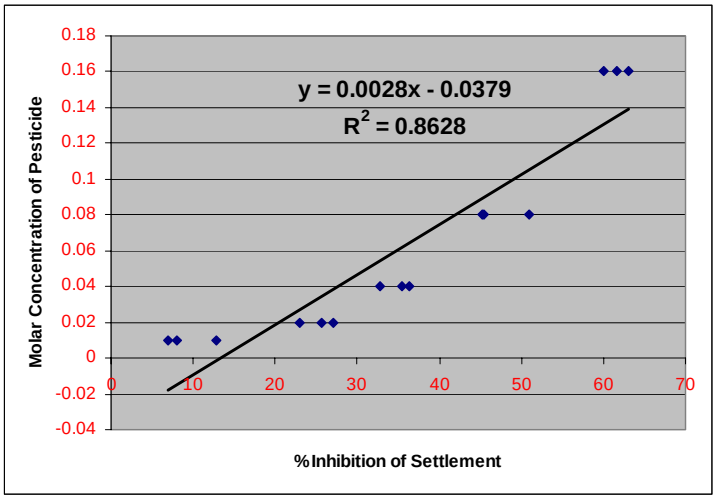


Figure 1.1 Linear regression of dose-response data

between the treatment variables and the effect variables. In this example, the effect variable would be inhibition of coral settlement. In regression analysis, the treatment variable is on one axis (usually the y-axis) of a graph, and the effect variable is on the other axis. Regression is the fitting of a line (or curve) to a set of data on a graph defined by the two variables. The easiest type of regression analysis is a linear regression analysis (Figure 1.1). The correlation coefficient (r) indicates the strength of the relationship between the treatment variable and the effect variable. The coefficient of determination (r^2) is the square of the correlation coefficient, a measure of the proportion of the variance in a set of data that is accounted for by the linear regression model; the closer to 1, the greater the explanatory power of the model.

As demonstrated in Figure 1.1, dose-response behavior is rarely linear over a wide range of concentration exposures, resulting in a lowered accuracy of estimating an effect based on a known concentration. This estimating of an effect when the concentration is known is called *interpolation*. It should be cautioned that interpolation of an effect is valid only within a regression line established by the data. It can not estimate the effect of a concentration outside of the regression line; such an estimation is called an extrapolation. One very common interpolation in ecotoxicology is the Effect Concentration 50 (EC_{50}) or the Lethal Concentration 50 (LC_{50}); this value allows you to compare the effect of one chemical on the laboratory model with the effect of another chemical on the same laboratory model. The EC_{50} and LC_{50} are most valuable as components in the risk characterization phase in modeling a risk assessment for a compound on a species, or even an ecosystem. A common method for modeling non-linear dose-response data is the Probit/Log Transforms and Regression. Probit/Log transformation is a transformation of the raw data into probability (Probit) and logarithmic (log) scales. The effect or response data are expressed as Probit values while concentrations of a chemical or stressor (e.g., temperature) are expressed on the log scale. Once data are transformed, it is subjected to a linear regression analysis, and a line and a correlation coefficient are determined. Doing Probit/Log transformations manually is cumbersome (daunting for large data sets), and is best done using specialized software and a computer. Commercial software that can do these transformations includes:

- ToxCalc and CETIS by Tidepool Scientific Software, Inc.
- JMP of SAS by SAS Institute, Inc.
- SigmaStat by SPSS, Inc.

Free software that can do these transformations is provided by the U.S. Environmental Protection Agency and can be accessed by the internet at the web address: http://www.epa.gov/nerleerd/stat2.htm.

Chapter 2
Conducting Experimental Toxicity Studies on Coral Larvae

This experimental procedure can be conducted in either gamete broadcast-spawning coral species, such as *Montastraea annularis*, or in "brooding" coral species, such as *Porites astreoides*.

Exposure Chamber - Exposures should be conducted in the appropriate dosing chamber, both in size and composition. Particular attention should be given to composition. Many organics will adhere to glass, plastic, and metal surfaces. The ion strength of seawater usually decreases the solubility of an organic xenobiotic and increases the rate at which an organic xenobiotic adheres to the side of the chamber. This, in turn, reduces the actual concentration of the substance to which the organism is exposed. If the adhesion coefficient is high between an organic xenobiotic and the surface material of the chamber, it can affect (usually increases) the solubility equilibrium of the organic xenobiotic. Pyrex or other type of laboratory glass can be used as a chamber for most metal-xenobiotic exposures (e.g., CdCl). Plastics of any kind should be avoided. Besides the high adhesion of organic xenobiotics to plastic surfaces, chemicals in the plastic matrix can leach into the exposure solution (e.g., pthalates), and create significant artifact. Although alternative materials can reliably be used with some xenobiotics, organic xenobiotics, with their high binding capacities require chambers with the lowest possible adhesion coefficient. The preferred material in this case is Teflon© , the perfluoroalkoxy (PFA) resin formulation. Polytetrafluoroethylene (PTFE)-Teflon can also be used, but care must be taken as this material degrades once the chamber has been in use over 250 working days.

Light Exposure – Light is an essential environmental factor for nominal coral symbiotic physiology, as well as an essential factor for the toxicity of many xenobiotics. For example, a primary mechanism of toxicity for methyl violagen (paraquat) is the inhibition of complete electron flow in the photosynthetic electron transport chain and the induction of a "Mehler Reaction"; the transfer of electrons to diatomic oxygen to produce superoxide, resulting in oxidative-stress toxicity. This toxic reaction is light dependent.

The quantity and quality of light is an essential exposure factor. Light quality refers to the spectrum composition of photons with specific wavelengths. For example, some incandescent light bulbs produce photons that have wavelengths that are dominated by wavelengths in the red and green spectrum (550 nm to 760 nm) and emit very little or no photons in the blue spectrum of light (e.g., 370 nm to 459 nm). Hence, the spectrum composition of light may impact the efficiency of photosynthesis, but also on the toxicity of photo-dependent toxicants (e.g., herbicides, fungicides). Natural light is the best source to use for an ecotoxicological laboratory experiment. If natural light cannot be used, a "solar-simulator" light source can be used, but is rather expensive and often cannot achieve sufficient quantity of light to conduct an experiment. If neither of these two light sources is accessible, artificial light from incandescent and fluorescent sources may be used. This is the least preferred option, since this usually requires an array of different incandescent and/or fluorescent light sources and the use of a spectro-radiometer to determine light composition of the source(s).

Quantity of light is as important as the quality of the light. The rate of photosynthesis is directly proportional to the quantity of light (assuming the light source has the appropriate quality of light). The same principle holds for the onset (rate) and extent of photo-toxicity that a xenobiotic can induce -the more light, the greater the toxicity. A photometer is an instrument that measures the intensity of light; it cannot measure the quality of light. If artificial light is used, both a spectroradiometer and a photometer should be used to measure quality and quantity of light. A photometer can be used for natural light.

Exposure Chamber Design and Chamber Configuration – The best design for an exposure experiment is a randomized configuration of unjoined dosing chambers on the laboratory bench. This is possible if the chambers are not physically joined with one another (**Figure 2.1**), such as using Pyrex or Teflon Petri dishes. Each chamber is a replicate of a single treatment. Many invertebrate larval toxicological methods use plastic cell culture micro-well plates, or Teflon micro-well plates (**Figure 2.2**). A randomized configuration, theoretically, would be the best configuration, but such a configuration may induce a "proximity" artifact. Micro-wells are relatively close together and as a result of volatilization of the xenobiotic, it is possible that a control replicate micro-well may be inadvertently contaminated by the target xenobiotic if it is located adjoining to a micro-well with a high concentration of the xenobiotic. To overcome this artifact, the highest concentration exposure wells should be on the right hand side of the micro-well plate, and the control and lowest concentrations should be on the left hand side of the micro-well plate. Movement of air can be generated (with a fan) that goes from the left-side of the micro-well plate to the right-side of the micro-well plate. Water-level and salinity should be monitored to ensure that the movement of air does not significantly increase evaporation of the exposure solutions, creating another artifact.

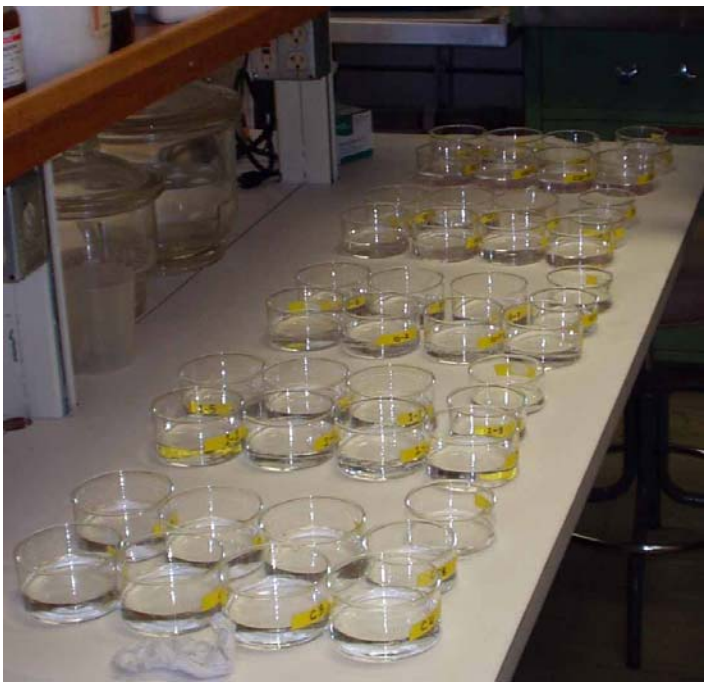


Figure 2.1 - Pyrex dishes can be used as dosing chambers. In this picture, there are five treatments with nine replicates; each chamber is a single replicate. Unjoined chambers should be randomly configured on the laboratory bench after the xenobiotic and the larvae have been added to the chambers.

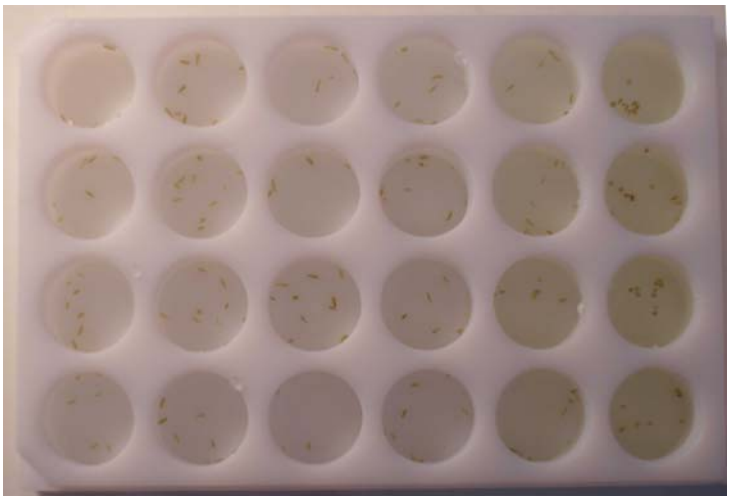


Figure 2.2 – Teflon micro-well plate. On the far left side of the plate, each of the wells in column 1 has 10 coral larvae and is the control treatment. The far-right of the plate is Column 6, which has 10 coral larvae per well and contains the highest concentration treatment of xenobiotic.

Example: The effects of light and dark environments on the LC50 value for KCN toxicity of coral planula

1. Around 5:00 pm (1800 hours), set planula traps for brooding coral species or initiate fertilization of gamete "broadcast" coral species.
2. The next day around 7:00 am (700 hours), collect planula traps.
3. Artificial seawater should be made with double distilled and activated carbon-filtered water. Sources for the salts should be made from "molecular-grade" chemicals.
4. From each trap, transfer and count the number of planula into a clean Petri dish or beaker that is filled with filtered artificial sea water.
5. Make up solutions of KCN to determine LC50 (24hour). Formula weight of KCN is 65.12. Make up a 100 mM stock solution by adding 0.16 grams of KCN to 1.0 mL of distilled water. Make sure the KCN crystals are completely dissolved in the water. Add the KCN solution to 24 mL of filtered seawater. This is now you 100 mM KCN stock solution.
    - 5 mM KCN - To make up a 20 mL solution of 5 mM KCN, take 1 mL of the 100 mM KCN stock solution, and add it to 19 mL of filtered seawater.
    - 4 mM KCN – To make up a 25 mL solution of 4 mM KCN, take 1 mL of the 100 mM KCN stock solution and add it to 24 mL of filtered seawater.
    - 3 mM KCN – To make up a 33 mL solution of 3 mM KCN, take 1 mL of the 100 mM KCN stock solution and add it to 32 mL of filtered seawater.
    - 2.5 mM KCN – To make up a 20 mL solution of 2.5 mM KCN, take 10 mL of the 5 mM KCN solution, and add it to 10 mL of filtered seawater.
    - 1 mM KCN – To make up a 10 mL solution of 1.0 mM KCN, take 2.5 mL of the 4 mM KCN solution, and add it to 7.5 mL of filtered seawater.
6. It is best to use planula from a single colony. To do this, you will need 240 planula from a single planula trap. Add 10 planula to each well using a plastic or glass pipette.
7. Remove as much excess seawater from each well, but leave just enough so that the planula are still floating in a drop of water.

8. Add concentration of KCN to each well as indicated in **Figure 2.3**.
9. Place one microwell plate in a darkened room or a drawer, so that it receives no light. Place the other microwell plate under a neutral density filter net that reduces the peak ambient light by 50%. Maximal light levels should be between 800 and 1200 micromoles of photons per meter per second.
10. Examine planula using a dissecting microscope at Time 0, 4 hours after initial exposure, 12 hours after initial exposure, and 24 hours after initial exposure.

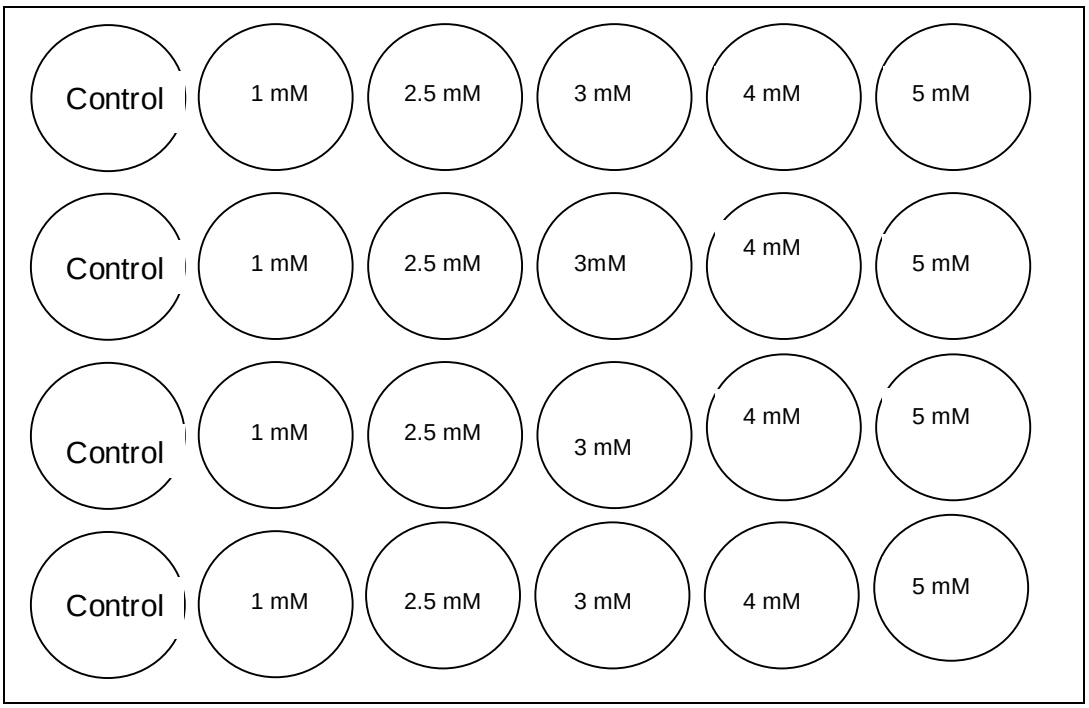
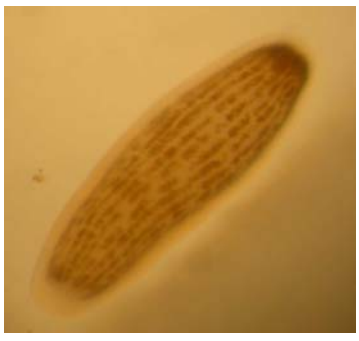


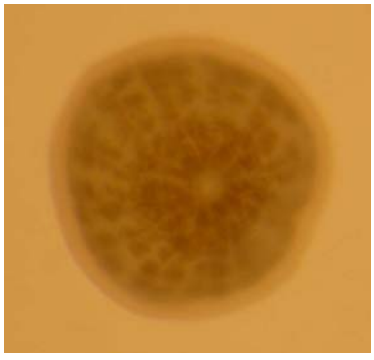
Figure 2.3

11. Depending on the species, planula will most often have three major morphologies: elongated, squat, and disk (**Figure 2.4**). Planula begin first elongated, then develop a squat morphology, and finally a disk morphology.



Elongated Planula

Squat Planula



Disk Planula

Figure 2.4

12. Record the number of planula alive and dead, and the morphological types for each of the ten planula in each of the wells.
13. If the lowest concentrations used in this experiment causes more than 50% mortality, repeat the experiment using a lower concentration series.

Effects

Exposure to a toxicant can result in a number of different effects. In planulae that contain zooxanthallae, loss of zooxanthallae (bleaching) is an important end-point to measure. Loss of zooxanthallae can be a qualitative measure (visual; **Figure 2.5**) or a quantitative measure (zooxanthallae counts via histology, chlorophyll *a* content).

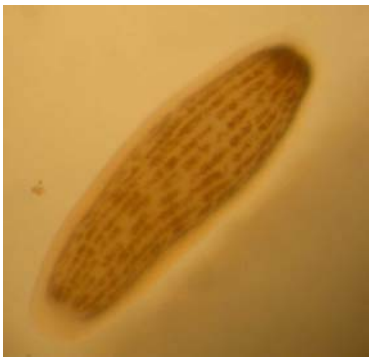


Figure 2.5A – Healthy Planula

Figure 2.5b – Bleached Planula after being exposed to an herbicide

Deformities may arise which can also be used as end-points of effect. It is important to distinguish between deformities and actual death.

Figure 2.6 – Planula exposed to a higher concentration of an herbicide for the same length time as the planula in

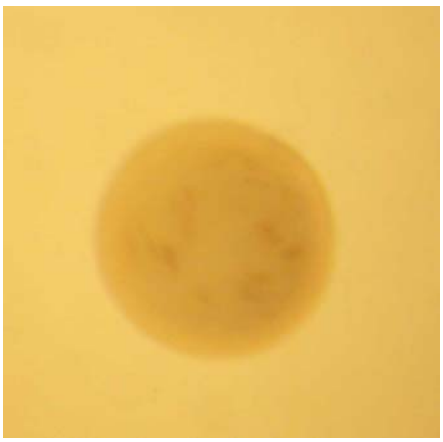


Figure 2.7 - Planula exposed to a plastic UV stabilizer. The elongated morphology has become spherical with an associated loss of its zooxanthallae

Figure 2.8 – Planula exposed to a fungicide developed two "mouths."

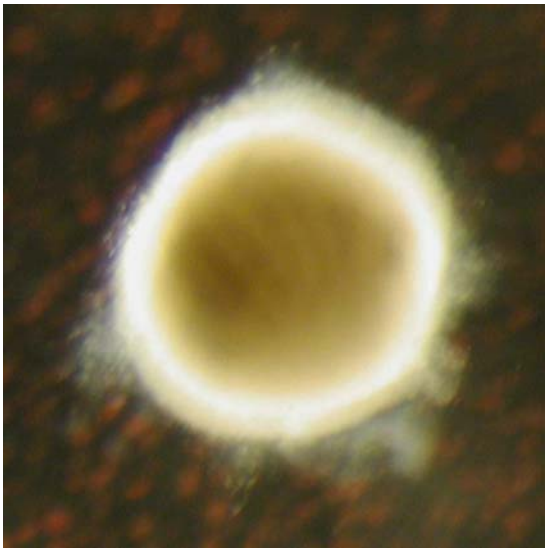


Figure 2.9 – Planula exposed to a metal oxide that destroyed the ectodermal layer within 30 minutes of exposure. Planula is dead.

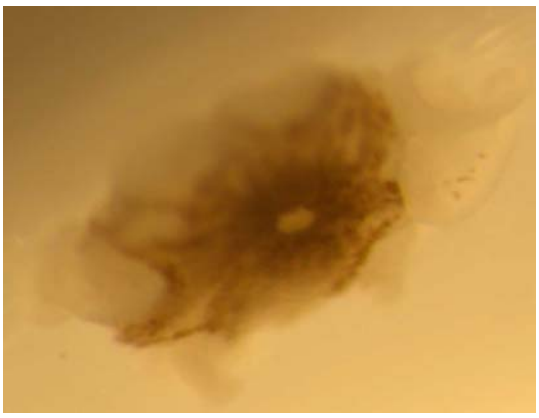


Figure 2.10 – Planula exposed to a high concentration of fungicide; it is dead.

Chapter 3
Conducting Experimental Toxicity Studies on Adult Coral

To conduct toxicity studies on adult coral, the method is divided into two phases: Phase 1 is coral culturing and Phase 2 is the actual exposure experimental design. For Phase 1, corals must be brought into the laboratory and acclimated to laboratory conditions. Corals should be cultured under natural light conditions which are controlled by shading with a neutral density filter (e.g., black net). Peak light levels should be about 2/5 lower than peak natural irradiance during the day (e.g., 800-1,200 micromoles of photons per meter per second). Water flow over the coral is another important parameter to consider: flow over the coral should be at least 0.05 liters per second. There is a significant difference between keeping corals as a hobby aquarist and culturing corals for physiological and toxicological experimentation. Though many hobbyists and aquariums may raise corals that look healthy, the goal of the physiologist/toxicologist is to use specimens that are in a physiologically "normal" condition that is observed under natural environmental conditions. Hobbyists and professional aquarists can raise a number of tropical corals in an azooxanthallic state (lacking zooxanthallae); this is an altered state and not one that reflects the desired physiological condition for experimentation under realistic environmental parameters. This does not mean that one should ignore the knowledge and experience of hobbyist or commercial aquarists; coral cultivation is an art and a science, and successful efforts to cultivate coral within the laboratory can be tremendously facilitated by their knowledge.

Corals should be cultured on glass slides or Teflon stubs at least two months prior to initiating exposure experiments. Once every two to three days, the slides or stubs should be cleaned with either a camel-hair or plastic-hair paint brush to remove algae that has settled on the slides/stubs. Coral nubbins can be grown in relatively high density (**Figure3.1**).

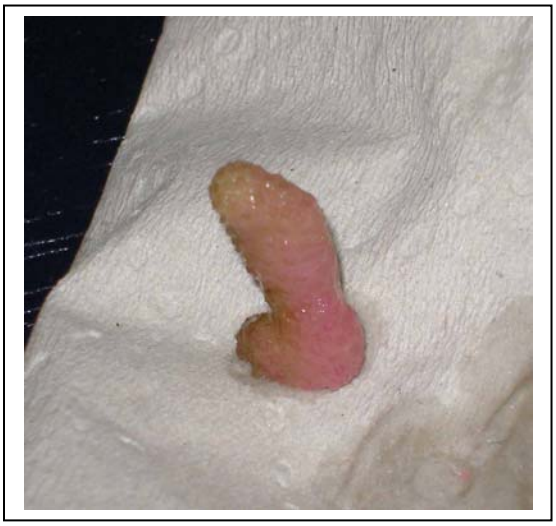
Figure 3.1 – Coral nubbins can be cultured in high density on platforms that can allow the corals to be easily transferred to dosing chambers. Oftentimes, it may be advantageous to dose more than a single species of coral, reducing the amount of waste generated and the time required to conduct experiments. Notice the red coralline algae.

Phase I: Creating coral nubbins – In this example, *Stylophora pistillata* nubbins are created and set on glass microscope slides.

Step 1 - Cut corals to a length of 1.5 cm using a pair of wire cutters that have been cleaned of oil and grease with a laboratory detergent (e.g., Alconox).

Step 2a – Remove excess seawater from the coral nubbin with a clean, lint-free paper towel. Do not use toilet tissue. Lint or paper particulates can adhere to the coral, and be caught in the glue matrix.

Step 2b – Resulting coral nubbin.



Step 3a – Use superglue (cyanoacrylate) for gluing the coral nubbin to the glass slide. Do NOT use an epoxy or silicon-based putty.

Step 3b – Put 150 microliters of glue onto the slide as a single drop.

Step 4 – Place one end of the coral nubbin into the glue droplet.

Step 5 – Hold the coral nubbin in place on the superglue droplet for no longer than one minute.

Step 6 – The coral nubbin should be free standing when released. Place the coral slide in seawater after the nubbin has been on the glass slide for two more minutes.

After a period of two months, most coral species cultivated under "optimal" environmental conditions should have skeleton and new tissue deposited over the adhesion zone and onto the glass slide or Teflon stub. It is recommended that coral nubbins should not be used for toxicity studies until new deposition of skeleton/tissue is observed. Deposition of new skeleton/tissue is an indication that not only has the nubbin recovered from the lesion stress resulting from its creation, but that it is in a physiological condition indicative of growth (**Figure 3.2**).

Figure 3.2

Phase II: Exposure to xenobiotic - The same issues concerning light exposure, exposure-chamber composition, temperature and exposure-chamber configuration for coral planula should also be considered for adult coral. The exposure system for adult coral is more complicated than the exposure system for larval exposures. Several additional issues must be considered.

- Concentration of oxygen in the dosing solution is important. Aeration can be accomplished by using a PFA-Teflon 5 mm diameter tube that comes directly out of an aquarium air flow generator and is perforated with small diameter holes on the end of the tube that is placed in the chamber. Do not use an airstone.
- If conducting a LC50 (Lethal Concentration 50; the concentration at which 50% of the population dies) determination, exposure times should be carried out for at least 96 hours.
- Flow-through system vs. recirculating/static exposure systems – Most ecotoxicologists will recommend that a flow-through system be used; a system where water from a reservoir that contains the xenobiotic is passed through the dosing chamber and into waste container. The benefits of the flow-through system is that the organism is always being exposed to fresh xenobiotic and that wastes generated by the organism in the dosing chamber are carried away. The major drawback of this system for corals is the amount of waste water generated. Hundreds of liters of waste per day can be generated by a single dosing chamber. Recirculating/static exposure systems produce a fraction of the waste generated with a flow-through system. The drawbacks with this system are (1) the xenobiotic can undergo metabolic or photolytic degradation producing an artifact of either too low concentration in regards to the defined exposure concentration and (2) metabolic wastes generated by the organism can reach concentrations that cause an artifactual "stressed" condition unrelated to your intended test agent. Water changes, probably twice daily, are usually needed to prevent these artifacts.
- Waterflow in recirculating/static exposure systems is an important factor for adult coral exposure experiments. Lack of waterflow over coral nubbins can induce a stress artifact. Use of a plastic aquarium flow-jet is inappropriate, since it is made of plastic. A Teflon pump or flow-jet can be custom manufactured to fit the exposure chamber used. Alternatively, each chamber can be set on a magnetic stir plate, and a Teflon-coated magnetic stir bar can be used to generate flow.
- Coral nubbins should be transferred from cultivation tanks to the dosing chambers at least 3-4 days before beginning the exposure experiment. Transfer of the corals out of the water, into air, and then into water again is equivalent to an ischemic/reperfusion shock. This can alter the tolerance levels of an organism to a xenobiotic, especially if that xenobiotic's mechanism of toxicity includes oxidative stress. Allowing the nubbins 3-4 days of recovery before initiating the experiment will allow for the cellular physiology of the nubbins to return to normal.

Chapter 4
Conducting Experimental Toxicity Studies on Sea Urchin Embryos

Sea urchin embryology is one of the best studied animal development systems. Sea urchin embryos have been used as models of toxicity since 1892[1], and have been internationally recognized as ecotoxicological models since the early 1970s[2]. The availability of sea urchin species on Meso-American coral reefs, the abundance of sea urchin ecotoxicological literature, the ease of cultivation and handling, and the low costs associated with conducting toxicity experiments using sea urchins, makes this animal an attractive model for conducting basic toxicity studies and Toxicity Identification Evaluations.

Sea urchin embryo toxicity tests are so common that there is an American Society for Testing and Materials (ASTM) standard guide to conducting sea urchin embryo toxicity tests. This document should be obtained before conducting any of the larval assays in this manual, especially since the ASTM Guide provides a detailed overview of conducting static toxicity tests. This documents is entitled *Standard Guide for Conducting Static Acute Toxicity Tests with Echinoid Embryos (Reapproved in 2004; modified 2006) Designation E:1563-1598* and can be obtained from ASTM International, 100 Barr Harbor Drive, PO Box C700, West Conshohocken, PA 19428-2959, United States. Another good protocol to reference for conducting sea urchin embryo toxicity tests is *Ghirardini A.N. et al., (2005) Sperm cell and embryo toxicity tests using the sea urchin Paracentrotus lividus. In Techniques in Aquatic Toxicology, Vol. 2. G.K. Ostrander (ed). CRC Press. Boca Raton. Pp147-168*.

Tripneustes ventricosus, *Diadema antillarum*, and *Echinometra viridis* are three species of sea urchin, commonly found in Meso-American reefs, which have been used in the past as model species for sea urchin embryo toxicity assays. *Tripneustes* and *Echinometra* are preferable species over *Diadema*, whose punctures can be quite painful.

Adult sea urchins can be reared in the laboratory, or preferably, collected (with the appropriate permits) from a reference site that is in good "health." The life history for the species used as a model must be known, especially its reproductive timing. Some species spawn during the winter months, while other species can spawn once a month depending on the availability of food and exposure to favorable environmental conditions. The ratio of sperm and egg for optimal fertilization must also be determined before toxicity tests can be performed. This should be determined in each laboratory regardless of the ratios identified by other laboratories that use sea urchins taken from other reference sites, in order to account for possible genotypic and ecotypic differences.

[1]Theel, H. (1892) On the development of *Echinocyamus pusillus* (O. F. Muller). Nova Acta R. Soc. Scient. upsal. Ser. III, 15 (6): 1-57.

[2]Kobayashi, N. (1971) "Fertilized Sea Urchin Eggs as an Indicatory Material for Marine Pollution Bioassay, Preliminary Experiments." *Marine Biology Laboratory*: 379–406

Materials for sea urchin embryo toxicity testing include the following:

- Sea Salts, Sigma-Aldrich, catalog #S9883

- Distilled water
- Activated carbon filter
- Salinity refractometer
- Dosing chambers
- Analytical balance
- Potassium chloride, reagent grade
- Glacial acetic acid
- 5 mL syringe and small gauge needle
- Pasteur pipettes (glass)
- Neubauer hemacytometer counting chamber
- Glass microscope slides
- 500 mL and 1000 mL Pyrex beakers
- pH meter
- Dissecting microscope with 10x and 40x magnification
- Inverted microscope with 10x, 40x, and 100x magnification
- 20-100 microliter and 100-1000 microliter pipettes
- 15 mL polypropylene Falcon tubes
- 10% formalin buffered in artificial sea water

Gamete ratio for fertilization

1. Double distilled water that has been filtered through an activated carbon filter can be mixed with the sea salts to create a saline solution of 37 parts per thousand, or a salinity concentration that is equivalent to that of the environment from which the sea urchins are collected. Use the salinity refractometer to determine the salinity of your solution, and remember to make sure that the refractometer is calibrated.
2. Sea urchins should be collected from the reference site the day of this test. Sea urchins can be collected in buckets or coolers with seawater. Care should be taken not to jostle or shake the containers with the sea urchins, since physical agitation will induce the sea urchins to spawn.
3. Spawning can be induced by injecting about 1 mL of 1 M KCl into the coelom (Figure 4.1).
4. If the urchin spawns, place the sea urchin on the mouth of a 500 mL Pyrex beaker filled with 150 mL of saltwater with the peristome (the

Figure 4.1. Injection of KCl into the coelom through the peristome.

membrane surrounding the mouth) facing down, so that the spawn fluid drips into the beaker (Figure 4.2). Allow to spawn for 20 minutes.

5. Spawn enough sea urchins so that you have at least one beaker of eggs and one beaker of sperm

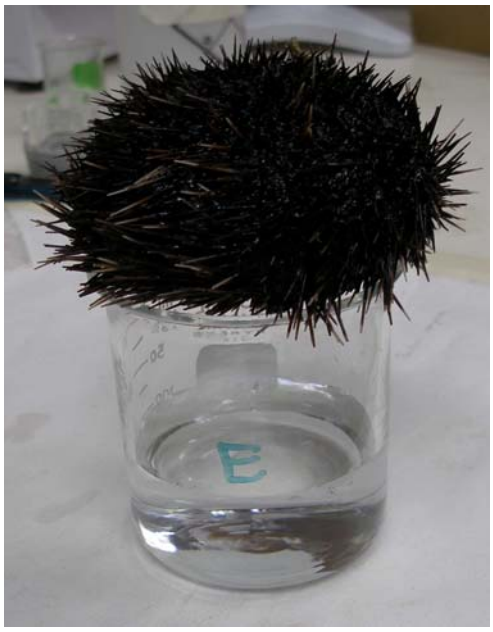


Figure 4.2. Collection of gamete from a forced-spawned sea urchin.

6. Within an hour after spawning, mix the sperm/salt water suspension to homogeneity. In a 15 mL Falcon tube, add 1 mL of glacial acetic acid to 8.9 mL of double distilled, carbon-filtered water. Take 0.1 mL of the sperm suspension and add it to the 8.9 mL diluted acetic acid solution. Cap the tube, and mix by gently inverting the tube 10 times. Allow the tube to set on the lab bench for 10 minutes.
7. After the 10 minute incubation, mix the contents of the Falcon tube again by gentle inversion (10 times) and then add 100 µL of the sperm/acetic acid suspension to each of the two counting mounts on the Neubauer hemacytometer counting chamber. Protocols on using a hemacytometer can be found at the following two websites:

- http://www.animal.ufl.edu/hansen/protocols/hemacytometer.htm
- http://www.protocol-online.org/prot/Cell_Biology/Cell_Culture/General_Procedures/Cell_Counting/index.html

8. In using a Neubauer hemacytometer, sperm density can be determined by the following formula:

[(dilution factor)(# of sperm counted)(hemacytometric conversion factor)/(# of squares)] mm^3/mL = [sperm/mL]

which is converted to

[(100)(# of sperm counted)(4000)/400)] x 1000 = [sperm/mL]

9. Egg density can be determined by placing 100 µL of the egg suspension on a microscope slide. Count the total number of cells in the 100 µL drop. If there are too many to count, dilute the egg suspension by half with artificial sea water. Add 100 µl of egg suspension and place the suspension on a microscope slide. Count total number of cells in the 100 µL drop. Do this until you can count about 50 eggs in 100 µL of egg suspension.

10. Add sperm to eggs in the following ratios: 5,000:1 (sperm to egg), 10,000:1, 20,000:1, and 30,000:1. The egg concentration should be about 100 eggs per 100 µL.
11. After 30 minutes, count the number of fertilized vs. unfertilized eggs there are in a 100 µL fertilization suspension.
12. Plot out the results, with the sperm:egg ratio on the x-axis and the *percentage of fertilization* on the y-axis. Determine by regression analysis the optimal sperm:egg ratio. Optimal ratio is defined by the highest incidence of successful fertilization.

A fertilized egg (embryo) has a "halo," or fertilization membrane, that is readily visible (marked by the double black arrow; Figure 3). Eggs that are not fertilized do not have the fertilization halo.

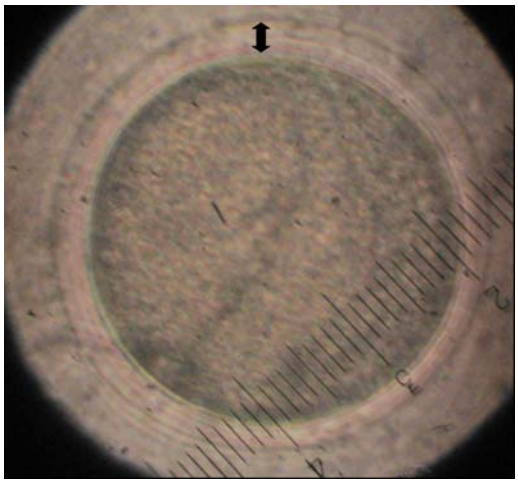


Figure 4.3. Sea urchin egg recently fertilized. The fertilization halo is easily recognizable. The double-black arrow indicates the width of the halo.

Procedure for Embryo Toxicity Test

1. Prepare the test solutions, and then fill the appropriately labeled testing chambers with the test solution. There should be at least three replicates per test treatment, including for the sea water control and a carrier solvent control if a carrier solvent was used. When calculating the test solution concentration, you should factor into your calculation that you will be adding 100 µL of zygote solution to the final test volume.
2. Zygote production. Fertilize the eggs with sperm at the appropriate concentration. The final concentration of zygotes should be 30 zygotes per 100 uL.
3. Allow twenty minutes to pass after adding the sperm to the eggs.
4. The final test volume in each test chamber should not exceed 15 zygotes per milliliter. It is recommended that you use a 2 mL test chamber, so that each chamber should hold about 30 zygotes.
5. It is acceptable to have more or less than 30 zygotes per chamber; provided the minimum number of zygotes is 20 and the maximum is 40.
6. Add zygotes to the test chambers.

7. Examine each test chamber for the number of fertilized zygotes per total number of sea urchin egg/zygotes of each chamber. This is a quality control step to ensure a baseline for actual zygotes in each test chamber.
8. The exposure period can persist up to 48 hours. Embryos should be examined with a microscope every 12 hours, tabulating the number of healthy looking developing embryos versus the number of those that are deformed. If possible, photo-document each replicate at each time point. A wide-field (low magnification; 4x or 10x) image should be taken, and a higher-magnified (40x or 100x) image should also be taken.
9. At the end of the exposure, development can be stopped by adding 400 µL of 10% formalin/seawater to the dosing vessel. This will preserve the embryos for at least 2-4 days to allow for more detailed observations of individual embryos or larval forms in each test chamber.
10. Using a microscope, count the number of deformed embryos and describe and photo-document the dominant deformation morphology.

- The end-point for the sea urchin embryo toxicity test is not lethality, but a deformation of morphology effect. Hence, one determines with this test an Effect Concentration (EC) for a specific contaminant or matrix.

- Oftentimes, a reference toxicant can be used as a quality control. Copper chloride is often used as a reference toxicant. A serial concentration from 10 to 150 µg L^{-1} will usually produce a toxic effect at various stages of development, depending on the concentration of copper. A good reference for this is Lee, D.R. (1980) Reference toxicants in quality control of aquatic bioassays. In Aquatic Invertebrate Bioassays, Buikema and Cairns (eds). ASTM STP 715: Philadelphia, American Society for Testing and Materials. Pp 188-199.

- A number of different "types" of deformities may arise at any developmental state. Figure 1 in *Standard Guide for Conducting Static Acute Toxicity Tests with Echinoid Embryos (Reapproved in 2004; modified 2006) Designation E:1563-1598* provides an excellent illustration of correct developmental morphologies as well as examples of typical deformities that may arise. This is by no means a complete list, and ultimately, deformities will be defined and recognized based on the investigator's *power of observation*. Any deformity that does arise as a result of experimental exposure should be adequately described and photo-documented. Different toxicants that produced similar deformities at the same stage of development may have the same or similar modes of toxicity.

Normal stages of development for Tripneustes species, from egg to plutei:

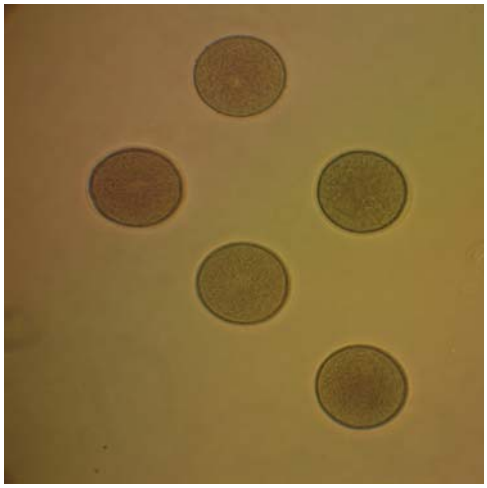


Fig. 4.4 Unfertilized eggs

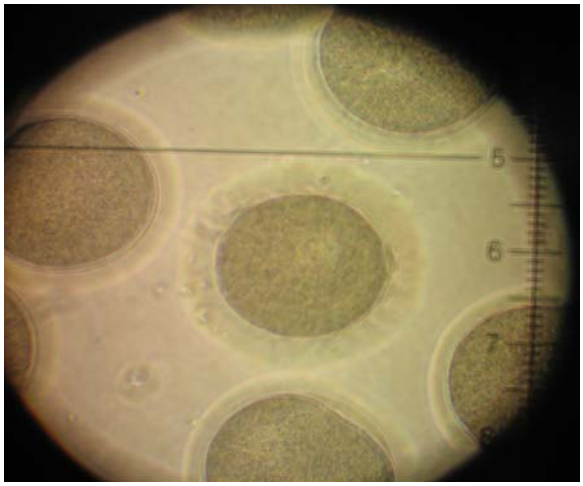


Fig. 4.5 Single-cell zygote.
Notice the Fertilization Halo.

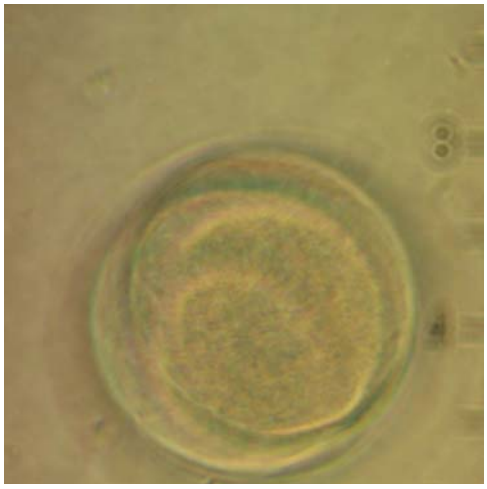


Fig. 4.6 Beginning of cell division

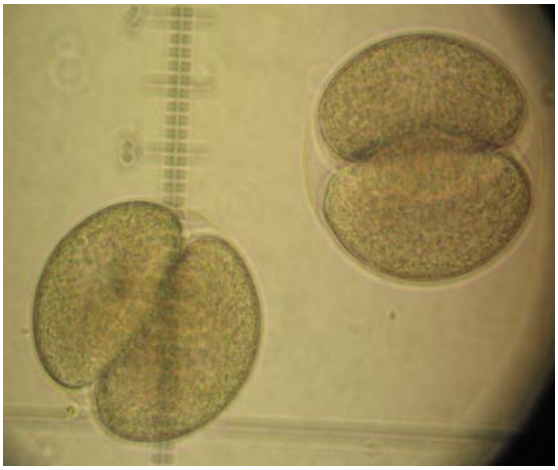


Fig. 4.7 Two-cell stage

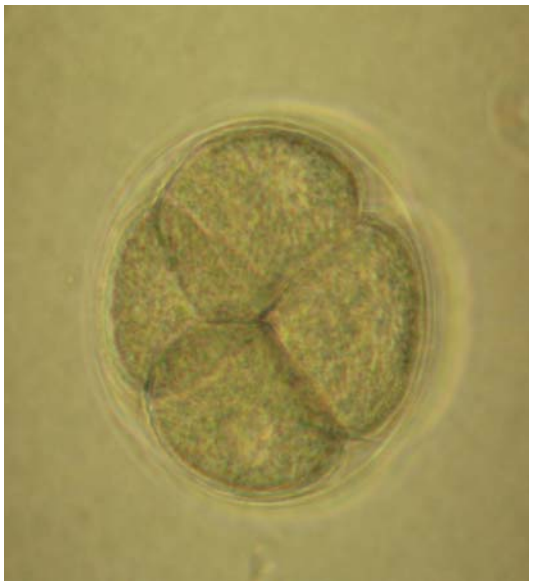


Fig. 4.7 Four cell stage

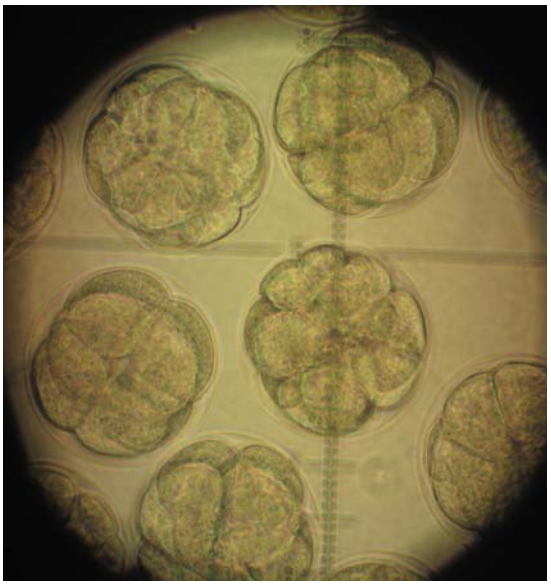


Fig. 4.8 Eight and sixteen cell stage

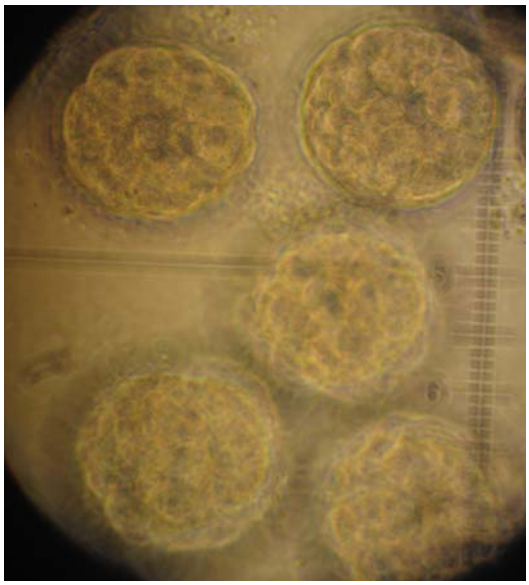


Fig. 4.9 32-cell stage

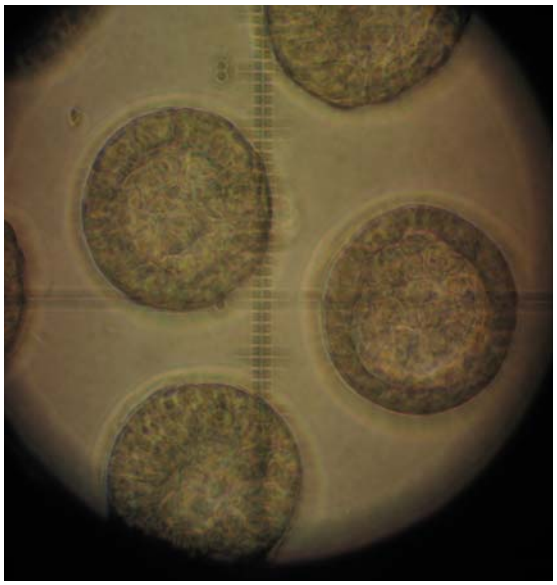


Fig. 4.10 Blastula

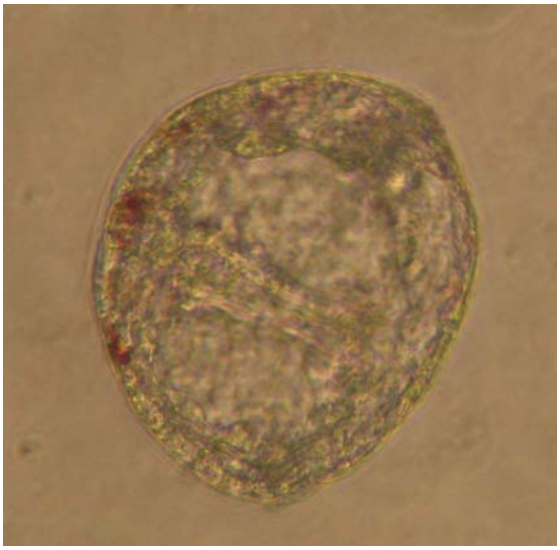


Fig. 4.11 Gastrula

Fig. 4.12 Prizm

Fig. 4.13 Early stage pluteus

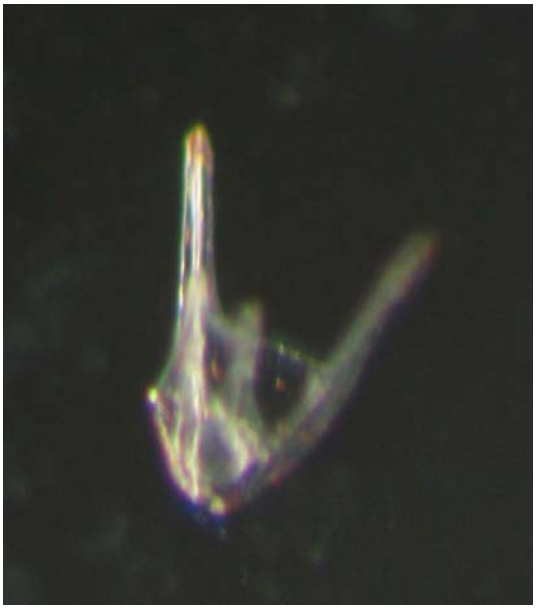


Fig. 4.14 Late stage pluteus