section three

Techniques for identification and assessment of contaminants in aquatic ecosystems

chapter nineteen

Coral reproduction and recruitment as tools for studying the ecotoxicology of coral reef ecosystems

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Contents

Introduction

Aquatic toxicology is a discipline that deals with the effects of chemicals and potential pollutants on individual organisms, usually with the intent of applying such data to understanding effects on populations and community structure and function. Aquatic organisms have a number of different life-history stages through which they must successfully pass, in order to become part of a population, and hence, assays that deal with only one stage, such as the adult form, may miss critical effects and have limited predictive value.

Many benthic aquatic organisms in both the protostome and deuterostome phyla, including Porifera, Cnidaria, Annelida, Crustacea, Echinodermata, and Mollusca, spawn gametes (eggs and sperm) into the water column, with subsequent external fertilization and development into planktonic larval stages, which eventually settle on specific substrata prior to metamorphosis into the juvenile form. These organisms respond to

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chemical cues for synchronization of spawning events among conspecifics, for mediation of egg–sperm interactions and for metamorphic induction in response to particular substrata, aggregations of adults or preferred prey.[1] Since so many steps in the life cycles of marine organisms are chemically mediated, the lack of a measurable effect of a xenobiotic on adult organisms at a range of concentrations does not necessarily mean the chemical is safe from a population or community perspective if it interferes with chemical cues at earlier life-history stages. Additionally, 100% successful fertilization of eggs and development of larvae in a bioassay with a subsequent 0% recruitment rate has the same overall effect on the population as 100% mortality at the fertilization stage. Finally, the duration of the observation period following exposure of an early life-history stage of an organism is important, as initial survival does not necessarily mean growth, development and reproductive ability will be unaffected later on.

Scleractinian corals provide an excellent set of organisms for use in aquatic toxicologic studies in tropical marine environments. Many species are simultaneous hermaphrodites that release combined egg–sperm packets during highly predictable annual spawning events. The gametes are easy to collect and manipulate, the large numbers available lend themselves to experiments with large sample sizes and hence rigorous statistical testing, and the larvae that result from successful fertilizations often have specific cues necessary for settlement and metamorphosis, allowing for multiple stage testing. Additionally, specific genetic crosses can be controlled and as a result, the confounding effects of genetic variability among individuals can be controlled. Genetic lines can be reared for later exposure and determination of biomarker expression. Gametes can be collected from the same coral colonies over several years if care is taken in collecting and maintaining ''donor'' corals.

Materials required

Material	Source	Comments
Gravid corals; at least two different colonies of the same species	Local coral reef communities	See (Richmond and Hunter, 1990; Richmond, 1997) Reference 2, for timing and location. Collecting permits is usually necessary
Sea water system	Field station, aquarium, or laboratory; can be open, closed, or recirculating	Corals have specific requirements for water quality, light, pH, temperature, and water motion
Basins/containers large enough to hold individual colonies	Aquarium store, hardware store, department store	Containers should be conditioned prior to use; glass is preferable for some experiments; plastic basins should be soaked to remove plasticizers
Plankton netting (Nytex) 105-μm mesh	Aquatic Ecosystems catalog #M105 (www.aquaticeco.com)	The netting is used to make sieves to collect gamete bundles. Coral eggs typically range in size from ca. 180 to 360 μm. A mesh size of 105 μm works for most species

Material	Source	Comments
2-in. PVC pipe	Hardware stores	Good diameter for making sieves for separating egg–sperm clusters. Nytex is attached to end of PVC pipe with hot melt glue
Hot melt glue and glue gun	Hardware or department stores	Used to attach plankton netting to PVC pipe sections for sieves
250-ml specimen cups, beakers or equivalent	VWR, Scientific Products or most laboratory suppliers	Glassware is preferable over plastic
Pasteur pipettes and bulbs	VWR, Scientific Products, etc.	Both 5- and 9-in. pipettes are useful
1-l glass beakers, fleakers or jars	Most labware suppliers	1200-ml vessels allow for raising batches of 1000 larvae at a density of 1 larva/ml
Air pumps (aquarium type or system), lines and airstones	Any pet shop or store with pet department	Need gentle aeration to keep floating eggs from getting trapped in the surface tension of the water
Hemocytometer	VWR, Scientific Products, etc.	Necessary for determining sperm density if the separation technique is used

Procedures

Selection of coral species

Coral species are selected based on their reproductive behavior and timing, growth rate, growth form, sensitivity to environmental parameters, cultivation characteristics, and biogeographic distribution patterns. Our laboratory has been able to routinely raise larvae from 13 species of corals that represent a variety of growth forms, habitats, and range of sensitivities to stress. *Pocillopora damicornis*, a brooding species, is widely distributed across the Pacific Ocean and produces viable planula larvae monthly, throughout the year, in Micronesia and Hawaii.[3] These larvae contain a full complement of zooxanthellae upon release from the parent colony. As such, this coral is good for studies requiring competent larvae but not for experiments to test the effects of chemical compounds on fertilization, larval development, or acquisition of symbiotic zooxanthellae. *P. damicornis* is a non-perforate species, meaning tissue is relatively easy to remove from the carbonate exoskeleton, which is a helpful characteristic for assays requiring subsequent colony tissue analysis. This species also releases relatively low quantities of mucus and is easy to maintain and transplant.

Several broadcast spawning acroporids (e.g., *Acropora humilis*, *A. tenuis*, *A. valida*, *A. danae*, *A. surculosa*, and *A. wardii*) and favids (*Goniastrea retiformis* and *Leptoria phrygia)* have proven to be useful in fertilization, development, symbiotic algal uptake, and recruitment bioassays. Species in these two groups, along with corals in the family Poritidae, are among the most commonly studied coral species. Since our research aim is to develop protocols that are widely applicable, we continue to add coral species that have appropriate biogeographic distribution patterns.

Collection of corals

Corals are collected from the field several days prior to predicted planulation or spawning. For the brooding species *P. damicornis*, colonies are collected 2–3 days after the new moon, as this species has been found to planulate within several days of the lunar first quarter, each month of the year, in Micronesia.[3] Timing is different in Hawaii and Australia, demonstrating the need for accessing data on the reproductive timing of local populations.

Spawning species are collected 2 days after the June–August full moons on Guam (Richmond and Hunter, 1990),[1] and in March–May in Palau. In all cases, corals are checked in the field for the presence of gametes each month leading up to spawning. Generally, if a coral is ripe, the eggs will be pigmented (pink, red, or orange) and are easily seen on the colony side of a broken branch or fragment (Figure 19.1). If no color is observed, the branch tip or fragment is brought back to the laboratory for examination under a compound microscope. For the acroporid and favid corals studied on Guam to date, eggs can be observed developing up to 6 months prior to spawning (January), appearing as white growths along the mesenteries. As spawning approaches, the eggs increase in size and eventually become pigmented. In the species that are simultaneous hermaphrodites (containing both eggs and sperm within the same colony and polyp), sperm begin to develop within a month or two of spawning, after eggs have already formed.

When ripe colonies are found, they are gently removed from the substratum with a hammer and chisel, and kept submerged until they are transferred to coolers filled with seawater and quickly transported to running seawater tables or aquaria. It is important to collect at least two colonies of each species, preferably some distance apart (to increase the probability of the two being genetically different) to allow for outcrossing in fertilization assays. Experiments have demonstrated self-fertilization rates for the simultaneously hermaphroditic corals are low or non-existent.[1,4]

Collection of gametes and larvae

The acroporid corals proven most appropriate for toxicology studies are all simultaneous hermaphrodites that release combined egg–sperm bundles[2] (Richmond and Hunter, 1990;

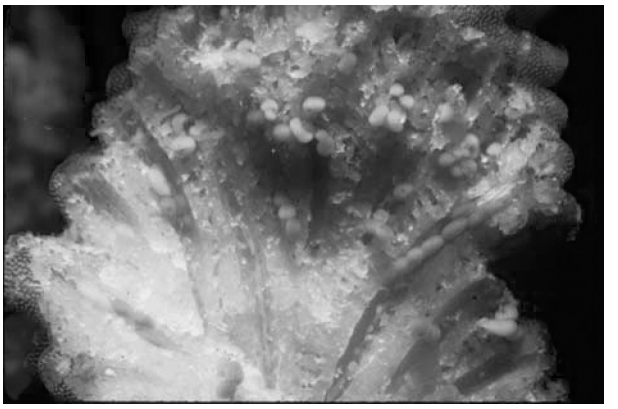

Figure 19.1 **(see color insert following page 464)** Cross-section of coral colony with pink eggs and white spermaries.

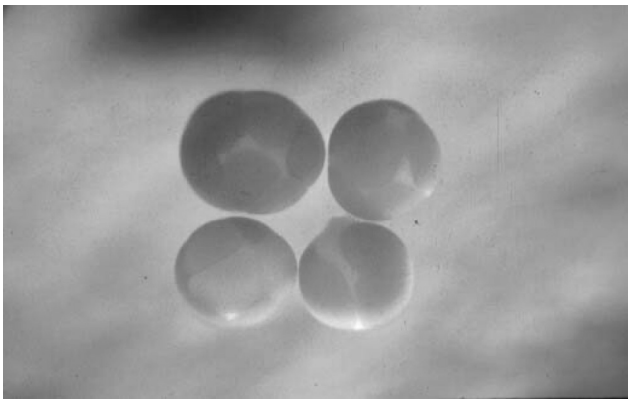

Figure 19.2 **(see color insert)** Combined egg–sperm clusters.

Figure 19.3 **(see color insert)** Floating gamete bundles collected in sieves.

Figure 19.2). Upon release, these float to the surface and can be collected on sieves with a mesh size of 105 μm or, alternatively, collected individually with a Pasteur pipette and placed intact into a 250-ml container with UV-sterilized/filtered seawater (Figure 19.3).

Two different systems have been developed, depending upon the experiments that will be performed. In fertilization studies, or those testing the effects of water-soluble pollutants, the clusters are washed with 0.45 μm (Millipore) UV-sterilized/filtered seawater, with the sperm being collected as the wash. The eggs are re-suspended in filtered/sterilized seawater, and fertilized with sperm from another colony of the same species, at a sperm concentration of ca. 10^5 sperm/ml.[4] Outcrossing has been found to be necessary as self-fertilization rates are relatively low. Higher concentrations of sperm often result in polyspermy and non-viable embryos. Fertilized embryos are placed into 1- and 2-l glass or plastic culture flasks with aeration, to provide a degree of water motion. Densities of eggs and developing larvae should not exceed 1/ml of seawater. Water is changed every 12 h during the first 48 h by siphoning from the bottom of the vessel, and daily afterwards until the larvae are ready to be settled onto appropriate substrata. Antibiotics are not necessary and handling of larvae should be minimized.

In studies using larval development, recruitment, zooxanthellae acquisition, and survivorship, we have simplified the fertilization procedure, and simply added the appropriate number of gamete clusters from two different colonies of the same species to 1 l of filtered seawater to achieve the proper sperm density. For example, for most acroporids, 50–100 gamete clusters from each of two colonies yield a sperm density of ca. 10^5 sperm/ml, and yield between 800 and 1200 larvae, with over 90% fertilization success. For the favid corals, crossing 150–200 clusters from each of two colonies in 1 l of seawater has been effective. The ''cluster'' technique makes a number of experiments logistically feasible at high levels of replication, and hence statistical rigor.

The planula larvae of the brooding coral *P. damicornis* are approximately 1 mm in diameter. Adult colonies measuring 15–20 cm in diameter are placed into 3- to 9-l vessels that overflow into containers constructed from plastic beaker bases with walls of 80-μm Nytex plankton netting. The corals are kept under continuous flow conditions of 1 l/min, and the buoyant larvae, which float out of the bowls, are collected in the mesh of the cups. This species has been found to planulate at night, so collectors are checked each morning for the presence of larvae.

Bioassays

Fertilization bioassays address the first critical, chemically-mediated step in the reproductive process and can be performed in a variety of vessels and a using a range of concentrations based on the question being asked and the characteristics of the substance being tested. When gamete quantity is limited, 15-ml prewashed glass scintillation vials are used with ca. 100 eggs (50 each from two different colonies; 5–8 clusters from each, for a variety of acroporid and favid corals) per vial, in 10 ml of UV-sterilized/filtered seawater, with a sperm density of ca. 10^5 sperm/ml. With the expectation that outcrossing will occur when using the ''cluster'' technique, developing larvae will be of two different genotypes. If follow-up experiments are to be performed on the larvae that form from the fertilization assays, using 100 eggs collected from a single colony and fertilized with sperm separated from a conspecific colony will presumably yield larvae of a single genotypic cross, and may reduce variability in experimental results.

Experiments are scored by viewing samples under a dissecting microscope for the number of eggs fertilized, the number of embryos reaching the planula larval stage versus the number of eggs/embryos that are non-viable.[5] A watch glass or other concave dish makes counting easier. Careful and limited handling allows for the larvae that do form to be reared further for recruitment and algal acquisition assays.

Recruitment assays

Coral larvae from spawning species become competent to settle between 18 and 72 h following fertilization, depending on species and egg size.[1] The smaller eggs (from favids) develop cilia and become competent more quickly than the larger *Acropora* eggs. The brooded larvae of *P. damicornis* are fairly non-specific and will settle on a variety of substrata pre-conditioned with bacterial/diatomaceous films. The *Acropora* and *Goniastrea* larvae are more selective, and our previous experiments indicate that several species are highly specific, settling only on particular species of crustose coralline algae (*Hydrolithon reinboldii*).

For recruitment bioassays, chemical effects of potential pollutants are tested by exposing preferred substrata to the xenobiotic, rinsing the substrata, then placing them

into 250-ml beakers with clean, filtered seawater or by exposing larvae to the chemical and providing them with untreated substrata. A known quantity of larvae is added to each beaker (50–100 per replicate), and the substrata scored daily under a dissecting microscope for 5 days for larvae that have settled (come into contact with the substrata), metamorphosed (cemented to the surface and displaying evidence of calcification; Figure 19.4), remained swimming or died. Alternatively, larvae are exposed (generally for 12–24 h) and subsequently added to beakers with the appropriate untreated substrata. Our research has demonstrated that pollutants can interfere with metamorphic inducers associated with the substrata and/or with the inducer receptor of the larvae. It is important to address both possibilities. While fertilization and embryological development, stages in corals are particularly sensitive to water-soluble chemicals; lipophilic substances appear to have a greater effect on larval recruitment.

In assays designed to study the effects of chemicals on zooxanthellae uptake, competent larvae are placed into basins or aquaria containing cleaned coral rubble in 0.45 μm (Millipore) filtered seawater. Pieces are checked daily for the presence of settled larvae, and if present, fragments are transferred to separate containers for exposure to chemicals and the addition of zooxanthellae. Zooxanthellae for use in these assays can come from pieces of donor colonies of the same species placed in the container, from cultured lines, from zooxanthellae centrifuged from tissue preparations of conspecific colonies, or from zooxanthellae collected and filtered from ''induced expulsions.'' One known stress response of corals is the breakdown of the animal–algal symbiosis. A by-product of cyanide bioassays performed to determine the effects of cyanide fishing on reefs was the discovery that exposure of coral branches to cyanide at 0.1 g/l for 5 min resulted in free-swimming zooxanthellae evacuating the host cells and tissue within hours of exposure. Since zooxanthellae appear to have the same cyanide-resistant respiratory pathway found in other plants, clean zooxanthellae free of coral tissue residue can be acquired this way that have found to be functional and capable of colonizing coral recruits.

For bioassays using adult corals, colonies are cultivated from larvae to a size of approximately 5–10 cm in diameter. Depending on the question, corals are either

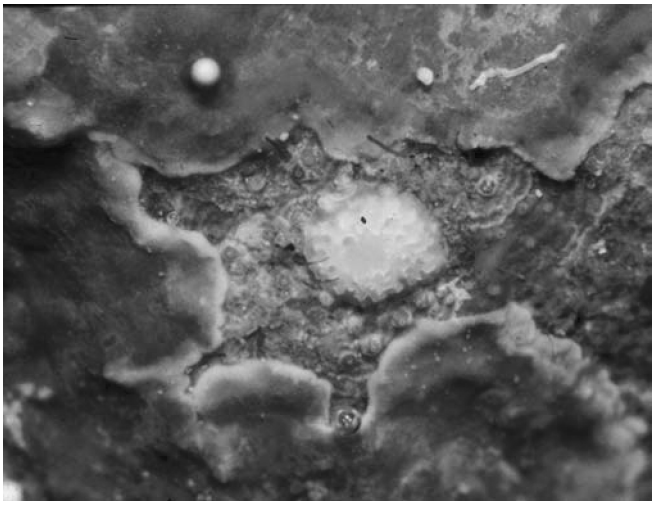

Figure 19.4 **(see color insert)** Coral larval recruit, settled and metamorphosed.

''pulsed'' and transferred to grow-out tanks with flowing seawater or maintained under test conditions in a closed system with air stones. We use separate containers for each coral, 10 corals per concentration (for replication, avoiding pseudoreplication) in a randomized block design. In pulse experiments, after exposure, tagged, treated and control corals are placed into a flowing seawater tank with a flushing rate of 21/min and observed for signs of bleaching, tissue loss, and death for a period of 30 days.

A great advantage of using corals is the ability to control genetic variability by using larvae from a single cohort. A single colony can be repeatedly ''harvested'' for gametes and larvae over a period of years with careful handling. For spawning species, once gametes are released and collected, colonies are tagged and transplanted back into the field, by cementing them in a marked area using a mixture of 7 parts cement to 1 part plaster of paris. Previous experiments have demonstrated that for several types of stress assays, genetic considerations are important, as variability among coral colonies across different genotypes was greater than the effect measured within a single genotype.

In conclusion, corals have proven to be valuable tools for ecotoxicological studies. Their reproductive behavior provides opportunities for studying effects of chemicals on cueing between conspecific colonies during spawning events, for studies of egg–sperm recognition, fertilization, embryological development, metamorphic induction, and acquisition of symbiotic zooxanthellae. The ability to rear corals of known genotypes in large numbers allows for statistically rigorous testing and for monitoring specific sites over time. Recent advances in the development of biomarkers of exposure in corals hold promise for determining the effects of pollutants at sublethal levels, and for measuring responses to mitigative measures. Such tools are proving useful to help resource managers address the effects of human activities on coral reef ecosystems, presently under threat world-wide.

Acknowledgments

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