Project #: G-32-663  
Center #: 10/24-6-R8640-0A0  

Cost share #:  
Center shr #:  

Contract #: 5R-1113-NALX  
Prime #:  

Subprojects ?: N  
Main project #:  

Project unit:  
Project director(s):  

BIOLOGY  
SNELL T W  
BIOLOGY  

Unit code: 02.010.134  
(404)894-3700  

Sponsor/division names: ENVIRON PROTECTION AGENCY  
Sponsor/division codes: 129  
EPA ATL - GA  
002  

Award period: 950629 to 960629 (performance) 960629 (reports)  

Sponsor amount  New this change  Total to date  
Contract value  0.00  24,965.00  
Funded  0.00  24,965.00  

Cost sharing amount  0.00  

Does subcontracting plan apply ?: N  

Title: DEV OF A CORAL SURROGATE FOR CORAL REEF BIOASSAYS  

PROJECT ADMINISTRATION DATA  

OCA contact: Anita D. Rowland  894-4820  

Sponsor technical contact  
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U.S. ENVIRONMENTAL PROTECTION AGENCY  
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ATLANTA, GA 30365  

Security class (U,C,S,TS) : U  
Defense priority rating : NA  

Equipment title vests with: Sponsor  
NONE PROPOSED  

Administrative comments -  
PROCESSED 1.25.96 BUDGET REVISION  

Sponsor issuing office  
NANCY M. WATKINS EXT. 6822  (404)347-2374  

U.S. ENVIRONMENTAL PROTECTION AGENCY  
345 COURTLAND STREET, N.E.  
ATLANTA, GA 30365  

FAX NO. 404/347-2400  

ONR resident rep. is ACO (Y/N): N  
NA supplemental sheet  
GIT
NOTICE OF PROJECT CLOSEOUT

Closeout Notice Date 11/08/96

Project No. G-32-663 __________________________________________
Center No. 10/24-6-RB640-0A0________________________

Project Director SHELL T W__________________________
School/Lab BIOLOGY_________

Sponsor ENVIRON PROTECTION AGENCY/EPA ATL - GA________________________

Contract/Grant No. 5R-1113-NALX________________________
Contract Entity GTRC

Prime Contract No. __________________________

Title DEV OF A CORAL SURROGATE FOR CORAL REEF BIOASSAYS________________________

Effective Completion Date 960629 (Performance) 960629 (Reports)

Closeout Actions Required:

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<tr>
<th>Action</th>
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<tr>
<td>Final Invoice or Copy of Final Invoice</td>
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<td>Final Report of Inventions and/or Subcontracts</td>
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Comments__________________________________________________________________

Subproject Under Main Project No. ________________________________

Continues Project No. ________________________________

Distribution Required:

- Project Director Y
- Administrative Network Representative Y
- GTRI Accounting/Grants and Contracts Y
- Procurement/Supply Services Y
- Research Property Management Y
- Research Security Services N
- Reports Coordinator (OCA) Y
- GTRC Y
- Project File Y
- Other N
1. Title: Development of Coral Surrogates for Coral Reef Bioassays  
EPA contact: Dr. Roland Ferry, Coastal Management Section, Water Management Division, Region IV, Atlanta

2. RATIONALE FOR PROJECT

There is widespread agreement that the health of coral reef systems worldwide are in a state of decline. The exact causes of this apparent decline is not known due to the variety of symptoms including coral diseases, reduced coral growth, bleaching in corals and other photosynthetic mixotrophs, and impaired coral recruitment. Nutrients from point and nonpoint sources and sediments from coastal watersheds are suspected to be the primary anthropogenic agents of reef health decline.

At present, coral reef assessments are generally limited to evaluations of community structure and dynamics. These assessments are limited in that they cannot provide cause and effect information regarding community conditions, and by the time quantifiable community level changes occur, the damage to the reef has been done. Analytical techniques are needed that: 1) provide early warning of impending impacts to coral health, 2) are relatively fast (short duration), low cost, and that do not require a high level of technical sophistication, 3) quantify sub-lethal responses to pollutants and other environmental stressors, and 4) relate directly to coral biology and physiological responses. Our main objective in this work, therefore, was to develop methods for rapid toxicity assessment using coral surrogates.

3. RESULTS

Culture of an Experimental Animal

After evaluating several invertebrate models for use as coral surrogates in toxicity tests, we chose the mangrove anemone, Aiptasia pallida (Phylum Cnidaria, Class Anthozoa). It seemed the best candidate because of its ease of culture, year-round availability of test animals, clonal reproduction, and toxicant sensitivity apparently similar to stony corals. Anemones were originally obtained on live rock from the Florida Keys and have been in culture at Georgia Tech for over one year, reproducing continuously. Culture conditions were 35 ppt synthetic seawater (Instant Ocean), 25°C, with a flow rate of 0.5 L per minute in 20 liter tanks in a 2000 L recirculating system. Fluorescent light was provided at about 3000 lux, 14 h light, 10 h dark. Reproduction is by separation of cells from the pedal disk region of adults which develop into small polyps in 7-8 days. We took advantage of this clonal reproduction by collecting the tiny polyps and separating them into test wells for toxicity tests.

Selection of Toxicological Endpoints

The first experiments examined the suitability of several anemone endpoints for toxicity assessment including ingestion rate, enzyme activity, and behavior. Over the past several years, we have developed several sublethal assays based on the
measurement of fluorescent markers. Ingestion of fluorescently labeled beads has proven to be a useful endpoint in rotifers (Jukelka and Snell 1994) and daphnids and paramecia (Jukelka and Snell 1995). We tested anemone polyp uptake of beads suspended in seawater as well as in their food. We succeeded in feeding young (2-10 mm) anemones 2 μm fluorescently labeled beads. We could visualize beads in the gut, but the animals proved too thick to accurately quantify fluorescence. Another problem was that many beads stuck to the external surface of the anemones, confounding our ingestion measurements. Using another approach, we labeled *Artemia* by feeding them fluorescent beads, then fed the *Artemia* to anemones. We had some success with this approach, but the variance in ingestion rates between replicates was high. Detection of differences among toxicant treatments required samples sizes of 20 animals or more for each treatment, which was impractical with our current culture facility. For these reasons, we concluded that our techniques for quantification of anemone ingestion are probably not suitable as endpoints for rapid toxicity assessment.

The second set of experiments involved the examination of a variety of enzyme substrates that yield fluorescent products upon cleavage. We have investigated nine substrates and their usefulness in visualizing *in vivo* enzyme activity (Burbank and Snell 1994) in anemones (Table 1). The most consistent results were obtained with FDA, so we focused on esterases. Enzyme activity was reduced in response to toxicant exposure, but much variation remained unexplained by the regression (Figure 1). We tried several approaches like normalizing for the weight of anemones and attempting to use similar size (age) individuals, but the unexplained variance could not be reduced.

Table 1. Enzyme substrates investigated using anemones.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Target Enzyme</th>
<th>Source</th>
<th>Stock solution</th>
<th>Observations</th>
<th>qualitative fluorescence after 15 min</th>
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</thead>
<tbody>
<tr>
<td>FDA</td>
<td>esterases</td>
<td>Molecular Probes F7378 F119F-5025</td>
<td>1 mg/ml DMSO then 50:1 dilution</td>
<td>concentrated in body cavity, tentacle periphery</td>
<td>+++</td>
</tr>
<tr>
<td>cFDA</td>
<td>esterases</td>
<td>Molecular Probes C1361 lot #10B-5</td>
<td>1 mg/ml acetone</td>
<td>limited to body cavity</td>
<td>++</td>
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<tr>
<td>DCFDA</td>
<td>peroxidase, esterase</td>
<td>Molecular probes D-399 lot # 54111</td>
<td>1 mg/ml acetone</td>
<td>contained fluorescence, throughout</td>
<td>+</td>
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<tr>
<td>FDP</td>
<td>alkaline phosphatase</td>
<td>Molecular Probes F-2999 lot # 1552-1</td>
<td>1 mg/ml acetone</td>
<td>intense, in body cavity, possibly minor leaking</td>
<td>++++</td>
</tr>
<tr>
<td>PLA2</td>
<td>phospholipase</td>
<td>Molecular Probes N3786 lot # 4542-3</td>
<td>5 mg/ml DMSO</td>
<td>fluorescence leaking</td>
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<tr>
<td>EFEE</td>
<td>microsomal dealkylase</td>
<td>Molecular Probes F-425 lot #7A</td>
<td>1 mg/ml acetone</td>
<td>external fluorescence only</td>
<td>+</td>
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<tr>
<td>Enzyme</td>
<td>Type</td>
<td>Molecular Probes</td>
<td>Concentration</td>
<td>Solvent</td>
<td>Fluorescence</td>
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<tr>
<td>----------</td>
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<tr>
<td>FDGlu</td>
<td>glucosidase</td>
<td>Probes 1 mg/ml</td>
<td>no</td>
<td>acetone</td>
<td>no fluorescence</td>
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<tr>
<td>FDGal</td>
<td>galactosidase</td>
<td>F-1179 Probes</td>
<td>1 mg/ml</td>
<td>acetone</td>
<td>no fluorescence</td>
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<tr>
<td>FDGlcu</td>
<td>glucuronidase</td>
<td>F2915 Probes</td>
<td>1 mg/ml</td>
<td>DMSO</td>
<td>no fluorescence</td>
</tr>
</tbody>
</table>

FDA = fluorescein diacetate  
cFDA = 5-carboxyfluorescein diacetate  
DCFDA = 2,7-Dichloro-fluorescein diacetate  
FDP = fluorescein diphosphate, tetraammonium salt  
PLA2 = 2-[6-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine  
EFEE = Fluorescein monoethyl ether ethyl ester  
FDGlu = fluorescein di-β-D-glucopyranoside  
FDGal = fluorescein di-β-D-galactopyranoside  
FDGlcu = fluorescein di-β-D-glucuronide

Figure 1. Esterase activity vs. pentachlorophenol (PCP) concentration. X-axis: A - log mg PCP/L, B - mg PCP/L, Y-axis is % control fluorescence per mg dry weight of test anemone.

A third set of experiments focused on the analysis of anemone behavior using image analysis. We chose to examine anemone tentacle spread because observations of toxicant stressed animals indicated that drooping tentacles was one of the first and most consistent stress responses. We developed a method for measuring tentacle spread using image analysis with the program NIH Image. Images were captured with a video camera and digitized with a Macintosh computer. The maximum width of the tentacles was traced on screen and the distance recorded. The tentacle spread of each animal was measured under a stereomicroscope at 5X magnification, then they were exposed to either PCP or mercury for 0.5 to 4 hours in 5 ml seawater at 25°C in the dark. Tentacle spread of each animal was re-measured after toxicant exposure and the percent reduction from the initial distance was calculated.

Tentacle spread is an ecologically relevant measurement because it estimates the food gathering ability of an anemone. In unstressed conditions, anemones spread their
tentacles widely to capture as many prey as possible. As individuals become stressed, their tentacles droop, decreasing the surface area available for food capture. As stress intensifies, anemones eventually completely withdraw their tentacles.

Two types of experiments were performed. The first was a time series to determine the best exposure time for detecting a response. We wanted the best compromise between short exposure for rapid results and sufficient time for clear toxic effects to be manifested. The reaction to 3 mg PCP/L was rapid. After 1 hour exposure, a significantly smaller tentacle spread was observed as compared to the initial spread (Figure 2A). This reduction in tentacle spread gradually increased, reaching its maximum of 65% after 4 hours. We chose a two hour PCP exposure since this was sufficient to observe a significant toxic effect and short enough to allow several tests to be performed in a typical workday.

To investigate the range of PCP concentrations causing adverse effects, we conducted a concentration-response experiment. A two hour exposure to PCP concentrations up to 1.4 mg/L had no significant effect on tentacle spread (Figure 2B). At 3 mg PCP/L, tentacle spread was reduced by 40% as compared to the initial condition, yielding a NOEC of 1.4 and a LOEC of 3 mg/L. The regression equation is $y = -13.3 \ln x - 18.9$, with a $r^2 = 0.71$. From this regression, an EC50 of 10.4 and EC20 of 1.1 mg PCP/L was calculated.

![Figure 2. The effect of PCP on anemone tentacle spread. A - the x-axis is hours exposure to PCP and the y-axis is percent reduction in tentacle spread from the initial condition. Vertical bars indicate one standard error. B - The effect of PCP concentration on anemone tentacle spread.](image)

Exposure to 0.5 mg Hg/L caused a similar reduction in anemone tentacle spread (Figure 3A). Mercury toxicity was apparent after only 0.5 hours exposure when a 40% reduction in tentacle spread was recorded as compared to 0% for controls. Tentacle
spread declined further after 1 hour exposure, then leveled off at about a 65% reduction from initial width through 3.5 hours. Clearly, mercury is a fast acting toxicant causing anemone tentacles to droop from their normally erect, outstretched position.

A two hour exposure to mercury concentrations up to 0.05 mg/L had no significant effect on tentacle spread (Figure 3B). At 0.15 mg Hg/L, tentacle spread was reduced by 65% as compared to the initial condition, yielding a NOEC of 0.05 and a LOEC of 0.15 mg/L. The regression equation is $y = -12.7 \ln x - 78.4$, with a $r^2 = 0.86$. From this regression, an EC50 of 0.11 and EC20 of 0.10 mg Hg/L was calculated.

![Figure 3](image)

Figure 3. The effect of Hg on anemone tentacle spread. A- the x-axis is hours exposure to PCP and the y-axis is percent reduction in tentacle spread from the initial condition. Vertical bars indicate one standard error. B - The effects of mercury concentration on anemone tentacle spread.

4. CONCLUSIONS

1) The anemone Aiptasia pallida ingested fluorescently labelled 2 μm beads, but ingestion is not likely to be a useful endpoint for rapid toxicity assessment.

2) Four out of nine enzyme substrates investigated yielded moderately strong in vivo enzyme activity. Only esterase activity was strong enough for quantification. Variability between individuals was large due to differences in size, age, and other unknown factors. A substantially larger number of replicates was needed to get consistent results. Providing large numbers of test animals was not feasible with the current culture system.

3) Measurement of tentacle spread was the most promising endpoint for rapid toxicity assessment using anemones. Tentacle spread was quantified using image analysis
and decreased dose-dependently with increasing toxicant exposure. Significant reduction in tentacle spread was observed after 60 minutes exposure to 3 mg PCP/L and after 30 minutes of exposure to 0.5 mg Hg/L. The NOECs for two hour exposures were 1.4 mg PCP/L and 0.05 mg Hg/L.

4) These results suggest that for animals like anemones and corals, molecular biomarkers may provide the best means of rapid toxicity assessment. Future work should focus on developing molecular probes that are sensitive indicators and that can be related to specific physiological responses to stress.

5. REFERENCES


