MOTE MARINE LABORATORY
1989-1990 RED TIDE PROGRAM

Second quarterly report
For the period January 1 - March 31, 1990

FLORIDA DNR CONTRACT NO. C-6074

Submitted to: Dr. Karen Steidinger, Chief
Florida Marine Research Institute
Florida Dept. of Natural Resources
100 Eighth Avenue SE
St. Petersburg, FL 33701-3093

Submitted by:
Dr. Richard H. Pierce
Ms. Karen de Borja
Dr. Armand J. deRosset
Ms. Patricia A. Hasbrouck
Mr. Michael S. Henry
Dr. Robert E. King
Mr. August R. Kotelowski
Mr. Melville R. Levi
Dr. Charles S. Nevin
Mr. L. Scott Proffitt
Mote Marine Laboratory
1600 Thompson Parkway
Sarasota, Florida 34236
(813) 388-4441

Dr. John D. Buck
Department of Marine Science
University of Connecticut
Noank, Connecticut

April 10, 1990

MTR No. 170b
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>TOPIC</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Bioaccumulation of Brevetoxins in Fish</td>
<td>1</td>
</tr>
<tr>
<td>II. Culture Maintenance and Ecological Studies</td>
<td>6</td>
</tr>
<tr>
<td>III. Red Tide Bloom Monitoring</td>
<td>7</td>
</tr>
<tr>
<td>IV. Bacteriological Studies</td>
<td>8</td>
</tr>
<tr>
<td>A. Baseline Bacteriological Studies: Gulf of Mexico</td>
<td>8</td>
</tr>
<tr>
<td>B. Marine Bacteria Associated with Growth Phases of Red Tide Cultures</td>
<td>9</td>
</tr>
<tr>
<td>V. Toxin Chemistry</td>
<td>18</td>
</tr>
<tr>
<td>VI. Recommendations</td>
<td>25</td>
</tr>
</tbody>
</table>
LIST OF TABLES AND FIGURES

<table>
<thead>
<tr>
<th>TOPIC</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1. Water Quality Parameters for <em>Harenula</em> Bioaccumulation Study IV</td>
<td>3</td>
</tr>
<tr>
<td>Table 2. <em>G. breve</em> Cell Counts and Toxin Concentration.</td>
<td>5</td>
</tr>
<tr>
<td>Table 3. Monthly combined production and use of Laboratory cultures of <em>G. breve</em>.</td>
<td>6</td>
</tr>
<tr>
<td>Table 4. <em>G. breve</em> Monitoring during a small bloom.</td>
<td>7</td>
</tr>
<tr>
<td>Table 5. Various genera recovered from Non-red tide Gulf waters.</td>
<td>8</td>
</tr>
<tr>
<td>Table 6. Accumulated population counts on <em>G. breve</em> and its associated bacteria.</td>
<td>12</td>
</tr>
<tr>
<td>Table 7. Brevetoxin Concentration in Red Tide Cultures.</td>
<td>14</td>
</tr>
<tr>
<td>Table 8. pH changes over time.</td>
<td>15</td>
</tr>
<tr>
<td>Table 9. Kinetics of Adsorption of Brevetoxins on Polygosil SiO₂/C₁₈ Run DT-42</td>
<td>19</td>
</tr>
<tr>
<td>Table 10. Table 10. Run DT-45 - Toxin Breakthrough Test.</td>
<td>20</td>
</tr>
<tr>
<td>Table 11. Run DT-45 - Mass Balances.</td>
<td>22</td>
</tr>
<tr>
<td>Table 12. Precision Study of Solid Extraction Analysis of Brevetoxin Solution with Polygosil SiO₂/C₁₈.</td>
<td>23</td>
</tr>
<tr>
<td>Table 13. Precision Study of Solid Extraction Analysis of Red Tide Culture with Polygosil SiO₂/C₁₈.</td>
<td>24</td>
</tr>
</tbody>
</table>

Figure 1. Brevetoxin Breakthrough over Polygosil Silica Bonded Octadecane | 21   |
I. BIOACCUMULATION OF BREVETOXINS IN FISH

Introduction
This series of experiments was undertaken to investigate the accumulation of brevetoxins in fish that are exposed to sublethal concentrations or red tide. The scaled sardine *Harengula jaguana* was chosen as the test organism because it is a local species of plankton-consuming fish.

The first set of experiments established the acute toxicity of red tide to the sardine; $LC_{50} = 6.9 \times 10^5$ cells/liter for lysed cells, and $7.4 \times 10^5$ cells/liter for exposure to whole cells (First Quarterly Report, January 5, 1990). The next set of studies focused on techniques of sublethal exposure and *Harengula* feeding on the red tide organism. These studies showed that brevetoxins were found in the viscera of exposed fish, but problems were encountered in maintaining consistent concentrations of viable red tide cells to which the fish were exposed over extended periods of time, requiring improved exposure techniques to obtain reproducible results.

The experiment described below utilized a new technique to acclimate the red tide culture to attain a stable population prior to the addition of fish. This was needed to provide sufficient numbers of viable cells for the fish to consume and to reduce the amount of extra-cellular toxins which are toxic to the fish. Extra-cellular toxins increased stress and mortality even when the number of viable cells was below toxic levels. In this experiment, the fish were exposed to sublethal concentrations of red tide cells for a 72-hour period in an attempt to simulate natural red tide bloom conditions. Following exposure, the fish viscera was extracted and analyzed for the presence of brevetoxins.

**Goals**
1) To establish stable, viable cultures of *G. breve* at sublethal concentration levels (less than $1 \times 10^6$ cells/liter) prior to exposure of *Harengula*. This is necessary to reduce extra-cellular toxins released from lysed cells which are readily toxic to the fish, resulting in toxin stress and premature mortality.
2) To maintain *Harengula* in exposure and control tanks for 72 hours with a minimum of mortality.
3) To successfully induce feeding behavior of *Harengula* when exposed to sublethal concentrations of red tide.
4) To expose a sufficient number of fish such that the viscera would contain measurable amounts of brevetoxins if they were bioconcentrated by the fish.

**Methods**

Three tanks were prepared, each with 36 liters of filtered (0.2μ) seawater; air stones were set at low aeration levels and allowed to equilibrate for 24 hours. Tank A was the fish control tank with no red tide, Tank B; fish exposure to red tide organisms, and Tank C; red tide control with no fish. A filtered seawater culture (FF-10) of *G. breve* in the log phase of its growth curve (concentration of 33.1 x 10^6 cells/l) was used in this experiment. This original culture was diluted three-fold and allowed to stabilize for 24 hours before addition to the appropriate tanks. Previous bioassays of this type (First Quarterly Report, January 5, 1990) had involved addition of small volumes (500 ml) of red tide culture in concentrations in the range of 3 x 10^6 cells/l. Due to the inability of this small volume of culture to establish a stable *G. breve* population in the tanks, and our knowledge that a critical volume of red tide culture is required to acclimate the new culture for initiation of cell growth, a larger volume of culture (2,500 ml) was added to establish a more stable *G. breve* population.

At 7 a.m. on 2/14/90, approximately 2.5 liters of the 11 x 10^6 cells/l culture were added to tanks B and C to create a concentration of 0.75 x 10^6 cells/l in each tank. At 4 and 10 hours after addition, cell counts were taken, and the culture was replenished as needed to maintain a 0.75 x 10^6 cells/l count in each tank. Salinity, temperature, pH, and dissolved oxygen (D.O.) were monitored twice daily in conjunction with cell counts (see Table 1). The two tanks containing red tide cells (B and C) were allowed to stabilize for 48 hours at which time cell counts indicated both tanks were maintaining at approximately 0.5 x 10^6 cells/l.

Toxin analyses were performed on the original undiluted culture (1-liter sample), and on 6-liter samples from tank B and C at 4, 24, 48, and 120 hours. Toxin extraction from culture was by solid phase extraction through 3 gram beds of silica-bonded octadecane at a flow rate of 3 liters per hour. The columns were then blown dry and back extracted with 15 ml methanol in three 5-ml fractions. These fractions were analyzed by our standard HPLC procedures in 85/15 methanol/water at a wavelength of 215 nm.
The sample volume was reduced in vacuo, and an aliquant injected for analysis of minor toxins. Fish viscera were processed by homogenization in 10 ml 0.15 m NaCl, frozen, and the moisture removed by lyophilization. The dry powder was then extracted with methanol (MeOH) to recover brevetoxins. Clean-up consisted of flash chromatography through a silica column, followed by elution through a silica/alumina column. The MeOH eluant was evaporated to 1 ml volume, filtered through 0.2μ and analyzed for brevetoxins by HPLC.

Table 1. Water Quality Parameters for Harenugula Bioaccumulation Study IV: A = fish control, B = fish exposed to red tide, C = red tide control.

<table>
<thead>
<tr>
<th>Date &amp; Time</th>
<th>Tank</th>
<th>Activity</th>
<th>S ‖ /oo</th>
<th>T°C</th>
<th>pH</th>
<th>D.O. mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>2/14/90</td>
<td>A</td>
<td>-</td>
<td>35</td>
<td>21.3</td>
<td>7.85</td>
<td>8.4</td>
</tr>
<tr>
<td>0700</td>
<td>B</td>
<td>+ 2.5 l red tide</td>
<td>35</td>
<td>21.8</td>
<td>8.00</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>+ 2.5 l red tide</td>
<td>35</td>
<td>21.5</td>
<td>8.10</td>
<td>8.3</td>
</tr>
<tr>
<td>2/15/90</td>
<td>A</td>
<td>-</td>
<td>35</td>
<td>21.5</td>
<td>7.95</td>
<td>8.5</td>
</tr>
<tr>
<td>0700</td>
<td>B</td>
<td>Acclimate cells</td>
<td>35</td>
<td>22.0</td>
<td>7.95</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>Acclimate cells</td>
<td>35</td>
<td>22.0</td>
<td>7.90</td>
<td>6.7</td>
</tr>
<tr>
<td>2/16/90</td>
<td>A</td>
<td>+ 15 fish</td>
<td>35</td>
<td>21.5</td>
<td>7.95</td>
<td>8.4</td>
</tr>
<tr>
<td>0700</td>
<td>B</td>
<td>+ 15 fish</td>
<td>35</td>
<td>22.0</td>
<td>8.00</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>-</td>
<td>35</td>
<td>21.8</td>
<td>8.00</td>
<td>8.3</td>
</tr>
<tr>
<td>2/17/90</td>
<td>A</td>
<td>-</td>
<td>35</td>
<td>21.5</td>
<td>7.55</td>
<td>7.2</td>
</tr>
<tr>
<td>0700</td>
<td>B</td>
<td>+ 1.35 l ³</td>
<td>36</td>
<td>22.0</td>
<td>7.65</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>+ 0.35 l ³</td>
<td>35</td>
<td>22.0</td>
<td>7.95</td>
<td>7.8</td>
</tr>
<tr>
<td>2/18/90</td>
<td>A</td>
<td>-</td>
<td>35</td>
<td>21.5</td>
<td>7.60</td>
<td>7.4</td>
</tr>
<tr>
<td>0700</td>
<td>B</td>
<td>+ 0.72 l ³</td>
<td>35</td>
<td>22.0</td>
<td>7.80</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>+ 0.56 l ³</td>
<td>35</td>
<td>22.0</td>
<td>7.95</td>
<td>7.7</td>
</tr>
<tr>
<td>2/19/90</td>
<td>A</td>
<td>-</td>
<td>35</td>
<td>22.0</td>
<td>7.55</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>-</td>
<td>35</td>
<td>22.0</td>
<td>7.65</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>-</td>
<td>35</td>
<td>22.0</td>
<td>7.85</td>
<td>8.0</td>
</tr>
</tbody>
</table>

¹ Dilute culture of red tide, 11 x 10⁵ cells/liter, added to produce ca. 750 x 10³ cells/liter in tanks.
Results and Discussion

1) Red Tide Culture Acclimation:

A major problem experienced with red tide bioassay and bioaccumulation has been the lysing of red tide cells after dilution of the original culture. Dilution is necessary to produce sublethal concentrations in the seawater tanks for exposure of the fish. Cell lysing not only produces a nonreproducible exposure situation, requiring continued additions of new culture, but also produces an unknown amount of extra-cellular toxins that are much more acutely toxic to the fish than are the toxins while they remain within the viable red tide cells.

To address this problem, a new technique was devised to produce an acclimated red tide culture at low cell counts prior to the addition of the fish. As described above in the methods section, the red tide exposure tanks were prepared by adding 2.5 liters of red tide culture to 32 liters of seawater followed at 24 hours by an additional 1.5 liters to provide a viable red tide culture at ca. 0.75 x 10^6 cells/liter by 48 hours acclimation. These results (Table 2) indicate that the addition of 10% of red tide culture to the seawater provided a stable red tide culture at sublethal cell concentrations. Cell concentrations throughout the 120 hour test are shown in Table 2, along with the total toxin concentration at select times. These data show stable cultures at the 32 hour period, with apparent culture growth occurring in the experimental tanks by 48 hours.

2. Fish Exposure to Red Tide Cells:

At 48 hours, fish were added to tanks A and B. Tank C remained as a red tide control to observe how well the cells would survive in the absence of fish. Within 24 hours of exposure to fish, the cell population in B was reduced 97%, while the red tide with out fish diminished by 57%. Red tide culture was added periodically to keep the cell count between 0.50 and 0.75 x 10^6 cells/liter. During the final 24 hour exposure, the cells in the fish tank were reduced by 92%, where as the red tide cell count in the tank with out fish was again reduced by 57%, indicating that the fish were reducing the number of viable cells either by consuming the cells or causing the cells to lyse.
Table 2. *G. breve* Cell Counts and Toxin Concentration.

<table>
<thead>
<tr>
<th>Date (hours)</th>
<th>Tank</th>
<th>Cell Count x10^3</th>
<th>Total Toxins ug/l</th>
<th>Activity ug/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>2/12</td>
<td>-</td>
<td>33,000</td>
<td>595</td>
<td>18.1</td>
</tr>
<tr>
<td>2/14 0700</td>
<td>-</td>
<td>11,000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2/14 0700+4</td>
<td>B</td>
<td>755</td>
<td>12.4</td>
<td>16.5</td>
</tr>
<tr>
<td>2/14 0700+4</td>
<td>C</td>
<td>930</td>
<td>14.9</td>
<td>16.0</td>
</tr>
<tr>
<td>2/14 10</td>
<td>B</td>
<td>349</td>
<td>-</td>
<td>Add 1.5 l</td>
</tr>
<tr>
<td>2/14 10</td>
<td>C</td>
<td>581</td>
<td>-</td>
<td>Add 0.9 l</td>
</tr>
<tr>
<td>2/15 24</td>
<td>B</td>
<td>523</td>
<td>9.9</td>
<td>18.8</td>
</tr>
<tr>
<td>2/15 24</td>
<td>C</td>
<td>581</td>
<td>6.7</td>
<td>11.4</td>
</tr>
<tr>
<td>2/16 32</td>
<td>B</td>
<td>785</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2/16 32</td>
<td>C</td>
<td>523</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2/16 48</td>
<td>B</td>
<td>901</td>
<td>9.4</td>
<td>10.6</td>
</tr>
<tr>
<td>2/16 48</td>
<td>C</td>
<td>872</td>
<td>7.4</td>
<td>8.4</td>
</tr>
<tr>
<td>2/16 55</td>
<td>B</td>
<td>785</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2/16 55</td>
<td>C</td>
<td>698</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2/17 72</td>
<td>B</td>
<td>29</td>
<td>-</td>
<td>Add 1.35 l</td>
</tr>
<tr>
<td>2/17 72</td>
<td>C</td>
<td>378</td>
<td>-</td>
<td>Add 0.35 l</td>
</tr>
<tr>
<td>2/17 82</td>
<td>B</td>
<td>494</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2/17 82</td>
<td>C</td>
<td>320</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2/18 96</td>
<td>B</td>
<td>116</td>
<td>-</td>
<td>Add 0.72 l</td>
</tr>
<tr>
<td>2/18 96</td>
<td>C</td>
<td>203</td>
<td>-</td>
<td>Add 0.56 l</td>
</tr>
<tr>
<td>2/19 106</td>
<td>B</td>
<td>439</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2/19 106</td>
<td>C</td>
<td>581</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2/19 120</td>
<td>B</td>
<td>58</td>
<td>0.8</td>
<td>13.9</td>
</tr>
<tr>
<td>2/19 120</td>
<td>C</td>
<td>320</td>
<td>6.4</td>
<td>19.7</td>
</tr>
</tbody>
</table>

3. Brevetoxin Analyses:

The concentration of brevetoxins in tank B and C is given along with the corresponding cell count in Table 2 as total toxin concentrations in ug/liter and as ug/10^6 cells. The concentration in ug/liter provides an indication of the amount of toxin to which the fish were exposed, and ug/10^6 cells gives an indication for the presence of excess extra-cellular toxin in addition to the amount expected to be inside the red tide cells that were present. These data indicate that the fish were consuming the red tide organisms rather than lysing the cells, since cell lysis would have released the toxins to the water.

Unfortunately, toxins were not recovered from fish viscera, not even from those spiked with standard PbTx-2 and PbTx-3. These results indicate a problem with the extraction and analysis procedure, which is currently undergoing a thorough evaluation.
II. CULTURE MAINTENANCE AND ECOLOGICAL STUDIES

Culture Propagation

The laboratory stock cultures of Gymnodinium breve suffered a severe setback when an accidental fire struck on March 10, 1990 causing a loss of power and thus lighting and temperature control in the main culture room. A total of 51 liters of culture died immediately following the fire, and the remaining 65.5 liters survived in the room with temporary lighting for an additional week before their demise. All 116.5 liters were run through solid phase extraction procedures and the toxins collected. Currently 48 liters of "back up" culture are located in the Trailer Culture room and are doing well. These carboys will be used as inoculate when the facilities can accommodate proper transfers. Normal culture activities over the last quarter are listed in Table 3 (fire damage is not included). Both the main culture room and the red tide lab suffered serious smoke damage and clean up continues through the first week of April. Some power has been restored in the area of the fire making the autoclave facilities now available; however, the Main Culture room will require painting after further clean up as well as recertification of the laminar flow hood.

Table 3. Monthly combined production and use of Laboratory cultures of G. breve.

<table>
<thead>
<tr>
<th>Month</th>
<th>Total Vol. of New Culture Inoculated (Liters)</th>
<th>Total Vol. of Culture Used for Testing (Liters)</th>
<th>Net Gain/Loss of Overall Culture Vol. (Liters)</th>
</tr>
</thead>
<tbody>
<tr>
<td>January</td>
<td>84</td>
<td>91.5</td>
<td>- 7.5</td>
</tr>
<tr>
<td>February</td>
<td>88</td>
<td>56</td>
<td>+ 32.0</td>
</tr>
<tr>
<td>March</td>
<td>12</td>
<td>20</td>
<td>- 8.0</td>
</tr>
</tbody>
</table>

Total Available Volume: 48.0 Liters
(March activities refer only to those nine days prior to the fire)
III. RED TIDE BLOOM MONITORING

On February 25, 1990, the Department of Natural Resources in St. Petersburg reported a red tide bloom of 14,000 cells/liter in New Pass. From 2-26-90 to 3-09-90 samples were taken off New Pass dock to monitor the bloom by changing cell concentrations. Cells were enumerated via quick scan of the three well slide or via the Sedgwick-Rafter slide in conjunction with a Whipple disc. Average temperature and salinity were 20.5°C and 34.1%o, respectively. Samples were also taken in several other places to track the extent of the bloom and the data collected is found in Table 4.

Cell levels did not remain concentrated for an extended period of time; thus, no fish kills were sighted and little to no respiratory effects were reported by individuals in the affected area. Monitoring of the pass continued until levels had assuredly dropped.

Table 4. *G. breve* Monitoring during a small bloom.

<table>
<thead>
<tr>
<th>Date</th>
<th>Site</th>
<th>Concentration (Cells/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-26-90</td>
<td>New Pass Dock</td>
<td>8,600</td>
</tr>
<tr>
<td></td>
<td>Mote Dock Bayside</td>
<td>2,000</td>
</tr>
<tr>
<td>2-27-90</td>
<td>Siesta Public Beach</td>
<td>No R.T.</td>
</tr>
<tr>
<td></td>
<td>Lido Public Beach</td>
<td>233,000</td>
</tr>
<tr>
<td></td>
<td>New Pass Dock</td>
<td>203,000</td>
</tr>
<tr>
<td>2-28-90</td>
<td>New Pass Dock</td>
<td>14,333</td>
</tr>
<tr>
<td>3-01-90</td>
<td>Captiva Island</td>
<td>No R.T.</td>
</tr>
<tr>
<td></td>
<td>New Pass Dock</td>
<td>8,670</td>
</tr>
<tr>
<td>3-02-90</td>
<td>New Pass Dock</td>
<td>3,000</td>
</tr>
<tr>
<td>3-03-90</td>
<td>New Pass Dock</td>
<td>5,000</td>
</tr>
<tr>
<td>3-04-90</td>
<td>New Pass Dock</td>
<td>3,000</td>
</tr>
<tr>
<td>3-05-90</td>
<td>New Pass Dock</td>
<td>3,000</td>
</tr>
<tr>
<td>3-06-90</td>
<td>New Pass Dock</td>
<td>2,667</td>
</tr>
<tr>
<td>3-07-90</td>
<td>New Pass Dock</td>
<td>No R.T.</td>
</tr>
<tr>
<td></td>
<td>Long Boat Key Gulf side</td>
<td>No R.T.</td>
</tr>
<tr>
<td></td>
<td>Long Boat Key Bay side</td>
<td>No R.T.</td>
</tr>
<tr>
<td>3-08-90</td>
<td>New Pass Dock</td>
<td>No R.T.</td>
</tr>
<tr>
<td>3-09-90</td>
<td>New Pass Dock</td>
<td>No R.T.</td>
</tr>
</tbody>
</table>
IV. BACTERIOLOGICAL STUDIES

A. Baseline Bacteriological studies: Gulf of Mexico

1) Qualitative

Forty of the sixty-five bacteria isolated from the Gulf water samples taken between 10-06-90 and 10-07-90 have been identified to genus. Some genera present have been found in our own *G. breve* cultures at Mote Marine Laboratory. Speciation of these genera will hopefully provide a differentiation between the two sets of bacteria. Those genera found in the Gulf samples are listed in Table 5.

Table 5. Various genera recovered from Non-red tide Gulf waters.

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Bacteria recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/4 mile off Big Pass</td>
<td>1 <em>Photobacterium</em></td>
</tr>
<tr>
<td></td>
<td>2 <em>Alteromonas</em></td>
</tr>
<tr>
<td></td>
<td>1 <em>Aeromonas</em></td>
</tr>
<tr>
<td></td>
<td>1 <em>Alcaligines</em></td>
</tr>
<tr>
<td></td>
<td>1 <em>Agrobacterium</em></td>
</tr>
<tr>
<td>Sarasota County Artificial Reef</td>
<td>6 <em>Photobacterium</em></td>
</tr>
<tr>
<td>Yellow buoy near New Pass</td>
<td>2 <em>Vibrio</em></td>
</tr>
<tr>
<td></td>
<td>1 <em>Cytophaga</em></td>
</tr>
<tr>
<td>New Pass Bell (10-06-90)</td>
<td>4 <em>Photobacterium</em></td>
</tr>
<tr>
<td></td>
<td>4 <em>Vibrio</em></td>
</tr>
<tr>
<td>New Pass Bell (10-07-90)</td>
<td>2 <em>Xanthomonas</em></td>
</tr>
<tr>
<td></td>
<td>1 <em>Vibrio_ parahaemolyticus</em></td>
</tr>
<tr>
<td></td>
<td>1 <em>Vibrio</em></td>
</tr>
<tr>
<td></td>
<td>2 <em>Photobacterium</em></td>
</tr>
<tr>
<td></td>
<td>1 <em>Pseudomonas/ Alteromonas</em></td>
</tr>
<tr>
<td>2 miles out from New Pass</td>
<td>1 <em>Photobacterium</em></td>
</tr>
<tr>
<td></td>
<td>1 <em>Xanthomonas</em></td>
</tr>
<tr>
<td>2 miles out from Siesta Beach</td>
<td>1 <em>Chromobacterium</em></td>
</tr>
<tr>
<td></td>
<td>3 <em>Pseudomonas/ Alteromonas</em></td>
</tr>
<tr>
<td>Siesta Beach</td>
<td>1 <em>Aeromonas</em></td>
</tr>
<tr>
<td></td>
<td>2 <em>Vibrio</em></td>
</tr>
<tr>
<td></td>
<td>1 <em>Acinetobacter</em></td>
</tr>
</tbody>
</table>
B. Marine Bacteria Associated with Growth Phases of Red Tide Cultures

1) Quantitative

Ten carboys of 6-liter cultures of Gymnodinium breve were established by inoculation with cultures of similar age and cell concentration. Five carboys were of the artificial-based medium, NH15, three experimental with two as controls, and five were of the natural seawater-based medium, FF10, three experimental with two controls. The experimental carboys were subjected to cell counts (microscopic), pH monitoring, and heterotrophic bacteria enumeration (via spread plate) twice a week as well as toxin analysis every two weeks over a six-week period. The controls were subjected only to bacteria enumeration and cell counts but not toxin analysis over the same six-week period. Controls were established to verify that the removal of 1 liter from the experimental carboys for toxin analysis would not greatly affect the growth of either the bacteria or the dinoflagellate. Cell count samples were taken by removing approximately 10-15 ml with a syringe and sterile tubing. Bacteria samples were taken by aseptically removing approximately 2-3 ml and placing it in a sterile test tube. The cultures were mixed by placing the carboy at a 75 degree angle and gently rotating it. One liter samples were also removed, after mixing, for toxin analysis. The data collected on the counts is found in Table 6, toxin analysis in Table 7, and pH changes in Table 8.

2) Qualitative

In general those bacteria found within the cultures of FF10 media parallel the growth of the dinoflagellates in a more precise manner than seen in the original flask experiment (Red tide 3rd Quarterly Report ’88-89). Slight irregularities do exist within a few of the carboys but are probably due to sampling error or possibly an error in procedures of the spread plate method. The NH15 cultures also display general trends of increase in bacteria as the dinoflagellate increases. However, the bacteria, within the NH15, appear to reach a plateau and remain there over several sampling periods. There is no way of knowing if this trend was temporary or would have persisted due to the nature of the experiment’s demise (fire). Though the experiment was prematurely ended, the trends still indicate a relation between the bacteria’s growth and the growth of the dinoflagellates. This may indicate a purpose to the bacteria
being within the cultures and not just incidental contamination. Once again, a biochemical role has yet to be established, but experimentation with a labeled amino acid might prove useful in this endeavour.

Since the cultures were still well within growth phase as the toxins were being analyzed, one would expect to see a general increase in their concentration as the cell concentration increases. This was the case within the two sample runs that were analyzed. As expected BTx3 was found to increase only after a noticeable drop in BTx2 being a byproduct of the latter toxin. BTx3, BTx6/9, BTx2, BTx1, BTx5, and BTx7 were isolated from those carboys analyzed with BTx3, BTx6/9, BTx2, and BTx1 the most prevalent among the cultures. BTx5 showed up in minor quantities, and BTx7 was only isolated from the inoculate cultures which were four weeks old.

The additional parameter of pH monitoring was added to this investigation in an attempt to explain a phenomenon observed in the initial flask experiment. The Alteromonas/Pseudomonas bacterium recovered from the dinoflagellate cultures stimulated the production of CaCO₃ crystals when the bacterium was grown on Difco-Marine Agar. This was believed to be the result of increased pH in the medium as a result of proteolysis such that NH₄⁺ is released from amino acids and CaCO₃ is precipitated at increased pH levels. When the pH levels were read during the growth of the dinoflagellate, it was noted that the levels increased (alkaline) as the culture matured. FF10 cultures start around pH 7.5 and increase to around pH 7.8, whereas NH15 cultures started at around pH 8.0 and rose to as high as pH 8.12. The initial pH's noted are most likely due to the volume of media into which the culture was inoculated since they match or are near the normal pH ranges for sterile FF10 and NH15. The gradual increases, however, should be attributed to the culture growth within the media, as it is the only factor acting on the surroundings (note, culture in this instance refers to both the bacteria and dinoflagellates). As observed above, it is possible that this increase in pH might be created by the bacteria, thus providing a more suitable environment for G. breve. And if G. breve requires a higher pH to sufficiently survive, this factor might help explain why cultures grown in NH15 (pH = 8.0), an artificial medium with little to no nitrogen, do better than cultures grown in FF10 (pH = 7.5) (Red Tide Final Report '88-89).
Several important observations were made within this investigation. They include:

1) The parallel nature of the growth of the associated bacteria in the cultures with their *G. breve* counterpart.
2) The increased production of toxins with the increase of cell growth.
3) The alkaline nature of the cultures as growth occurs giving insight into one parameter of growth possibly provided by the bacteria which may be necessary for the survival of *G. breve*.

One final test of the ten carboy experiment should help to verify all three points. Furthermore, stock cultures can be monitored for pH as samples for cell counts are being taken to increasingly generate data on this occurrence. Speciation of both the bacteria in the cultures and in non-red tide waters will upgrade the baseline data obtained and collection of samples from an actual bloom would help to round out the information thus far.
Table 6. Accumulated population counts on *G. breve* and its associated bacteria.

<table>
<thead>
<tr>
<th>Carboy</th>
<th>Day</th>
<th><em>G. breve</em></th>
<th>Bacteria/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>0</td>
<td>10.3 +/- 1.2</td>
<td>580,000</td>
</tr>
<tr>
<td>(FF10)</td>
<td>4</td>
<td>19.9 +/- 2.1</td>
<td>2,580,000</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>21.5 +/- 1.4</td>
<td>7,100,000</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>19.3 +/- 1.3</td>
<td>1,470,000</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>20.2 +/- 2.4</td>
<td>4,660,000</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>23.5 +/- 1.6</td>
<td>5,500,000</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>22.4 +/- 2.3</td>
<td>7,100,000</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>22.6 +/- 2.8</td>
<td>8,800,000</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>22.5 +/- 0.6</td>
<td>7,200,000</td>
</tr>
<tr>
<td>C-2</td>
<td>0</td>
<td>11.0 +/- 1.6</td>
<td>1,350,000</td>
</tr>
<tr>
<td>(FF10)</td>
<td>4</td>
<td>19.0 +/- 2.4</td>
<td>2,980,000</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>17.8 +/- 1.5</td>
<td>8,400,000</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>11.9 +/- 1.0</td>
<td>3,700,000</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>21.3 +/- 2.9</td>
<td>2,720,000</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>22.3 +/- 1.3</td>
<td>10,400,000</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>20.7 +/- 2.3</td>
<td>8,700,000</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>23.0 +/- 2.0</td>
<td>5,100,000</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>19.4 +/- 0.4</td>
<td></td>
</tr>
<tr>
<td>C-3</td>
<td>0</td>
<td>7.1 +/- 0.5</td>
<td>420,000</td>
</tr>
<tr>
<td>(NH15)</td>
<td>4</td>
<td>11.5 +/- 1.8</td>
<td>1,340,000</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>12.8 +/- 1.9</td>
<td>1,240,000</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>18.0 +/- 2.5</td>
<td>670,000</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>18.1 +/- 0.4</td>
<td>900,000</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>25.0 +/- 2.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>19.7 +/- 1.2</td>
<td>900,000</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>22.6 +/- 1.1</td>
<td>1,210,000</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>26.4 +/- 2.6</td>
<td>1,070,000</td>
</tr>
<tr>
<td>C-4</td>
<td>0</td>
<td>10.2 +/- 1.0</td>
<td>440,000</td>
</tr>
<tr>
<td>(NH15)</td>
<td>4</td>
<td>11.5 +/- 1.8</td>
<td>750,000</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>14.3 +/- 0.5</td>
<td>780,000</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>12.8 +/- 1.9</td>
<td>330,000</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>16.6 +/- 1.3</td>
<td>670,000</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>19.3 +/- 2.1</td>
<td>280,000</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>19.4 +/- 0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>22.8 +/- 2.3</td>
<td>540,000</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>23.4 +/- 1.7</td>
<td>1,010,000</td>
</tr>
</tbody>
</table>
| E-1  | 0   | 11.5 +/- 1.0
|      | (FF10) |   |
|      | 4   | 17.8 +/- 0.65  | 1,030,000 |
|      | 7   | 18.6 +/- 1.1   | 5,700,000 |
|      | 11  | 15.3 +/- 2.7   | 2,260,000 |
|      | 14  | * 21.3 +/- 2.5 | 5,300,000 |
|      | 18  | 23.6 +/- 1.4   | 4,700,000 |
|      | 21  | 22.3 +/- 0.7   | 660,000   |
|      | 25  | 20.6 +/- 2.1   | 11,900,000|
|      | 28  | * 17.0 +/- 1.3 | 9,400,000 |

| E-2  | 0   | 9.9 +/- 1.1    | 640,000   |
|      | (FF10) |   |
|      | 4   | 20.9 +/- 2.1   | 2,110,000 |
|      | 7   | 20.8 +/- 3.5   | 6,500,000 |
|      | 11  | 13.6 +/- 0.75  | 2,940,000 |
|      | 14  | * 20.3 +/- 0.96| 3,700,000 |
|      | 18  | 23.8 +/- 2.2   | 9,500,000 |
|      | 21  | 21.4 +/- 2.2   | 1,910,000 |
|      | 25  | 25.5 +/- 2.2   | 6,600,000 |
|      | 28  | * 22.3 +/- 2.3 | 5,200,000 |

| E-3  | 0   | 11.3 +/- 1.1   | 630,000   |
|      | (FF10) |   |
|      | 4   | 21.8 +/- 3.0   | 2,730,000 |
|      | 7   | 21.9 +/- 2.8   | 4,200,000 |
|      | 11  | 14.5 +/- 1.9   | 2,780,000 |
|      | 14  | * 17.0 +/- 1.5 | 920,000   |
|      | 18  | 26.8 +/- 2.6   | 8,200,000 |
|      | 21  | 20.6 +/- 1.9   | 2,390,000 |
|      | 25  | 23.1 +/- 2.6   | 3,300,000 |
|      | 28  | * 21.6 +/- 1.3 | 8,000,000 |

| E-4  | 0   | 11.0 +/- 1.4   | 264,000   |
|      | (NH15) |   |
|      | 4   | 13.6 +/- 2.2   | 330,000   |
|      | 7   | 15.1 +/- 1.5   | 810,000   |
|      | 11  | 13.1 +/- 1.5   | 500,000   |
|      | 14  | * 19.3 +/- 1.7 | 490,000   |
|      | 18  | 20.1 +/- 2.2   |           |
|      | 21  | 19.9 +/- 0.8   |             |
|      | 25  | 21.9 +/- 2.1   |             |
|      | 28  | * 23.3 +/- 2.2 |             |

<p>| E-5  | 0   | 8.7 +/- 1.5    | 241,000   |
|      | (NH15) |   |
|      | 4   | 12.0 +/- 1.5   | 1,620,000 |
|      | 7   | 12.3 +/- 0.64  | 540,000   |
|      | 11  | 13.8 +/- 2.5   | 730,000   |
|      | 14  | * 18.5 +/- 1.9 | 980,000   |
|      | 18  | 25.6 +/- 2.0   | 1,010,000 |
|      | 21  | 21.7 +/- 1.1   | 1,020,000 |
|      | 25  | 28.8 +/- 2.1   | 940,000   |
|      | 28  | * 23.5 +/- 2.5 | 1,480,000 |</p>
<table>
<thead>
<tr>
<th>Carboy</th>
<th>Date</th>
<th>Toxin ug/mill. cells</th>
<th>BTx  -3</th>
<th>-6/9</th>
<th>-2</th>
<th>-7</th>
<th>-5</th>
<th>-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-6</td>
<td>0</td>
<td>8.4 +/- 0.9</td>
<td>226,000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>13.0 +/- 2.3</td>
<td>380,000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>13.5 +/- 1.1</td>
<td>680,000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>16.6 +/- 2.7</td>
<td>580,000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14 *</td>
<td>17.8 +/- 1.6</td>
<td>520,000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>23.0 +/- 2.2</td>
<td>1,350,000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>22.3 +/- 1.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>27.4 +/- 2.0</td>
<td>990,000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>28 *</td>
<td>24.2 +/- 2.3</td>
<td>810,000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7. Brevetoxin Concentration in Red Tide Cultures.
Table 8. pH changes over time.

<table>
<thead>
<tr>
<th>Carboy</th>
<th>Date</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>2-09-90</td>
<td>7.48</td>
</tr>
<tr>
<td>(FF10)</td>
<td>2-13-90</td>
<td>7.77</td>
</tr>
<tr>
<td></td>
<td>2-16-90</td>
<td>7.88</td>
</tr>
<tr>
<td></td>
<td>2-20-90</td>
<td>7.78</td>
</tr>
<tr>
<td></td>
<td>2-23-90</td>
<td>7.82</td>
</tr>
<tr>
<td></td>
<td>2-27-90</td>
<td>7.82</td>
</tr>
<tr>
<td></td>
<td>3-02-90</td>
<td>7.80</td>
</tr>
<tr>
<td></td>
<td>3-06-90</td>
<td>7.80</td>
</tr>
<tr>
<td></td>
<td>3-09-90</td>
<td>7.81</td>
</tr>
<tr>
<td>C-2</td>
<td>2-09-90</td>
<td>7.53</td>
</tr>
<tr>
<td>(FF10)</td>
<td>2-13-90</td>
<td>7.74</td>
</tr>
<tr>
<td></td>
<td>2-16-90</td>
<td>7.81</td>
</tr>
<tr>
<td></td>
<td>2-20-90</td>
<td>7.78</td>
</tr>
<tr>
<td></td>
<td>2-23-90</td>
<td>7.82</td>
</tr>
<tr>
<td></td>
<td>2-27-90</td>
<td>7.81</td>
</tr>
<tr>
<td></td>
<td>3-02-90</td>
<td>7.80</td>
</tr>
<tr>
<td></td>
<td>3-06-90</td>
<td>7.80</td>
</tr>
<tr>
<td></td>
<td>3-09-90</td>
<td>7.76</td>
</tr>
<tr>
<td>C-3</td>
<td>2-09-90</td>
<td>8.01</td>
</tr>
<tr>
<td>(NH15)</td>
<td>2-13-90</td>
<td>7.97</td>
</tr>
<tr>
<td></td>
<td>2-16-90</td>
<td>8.03</td>
</tr>
<tr>
<td></td>
<td>2-20-90</td>
<td>8.03</td>
</tr>
<tr>
<td></td>
<td>2-23-90</td>
<td>8.09</td>
</tr>
<tr>
<td></td>
<td>2-27-90</td>
<td>8.08</td>
</tr>
<tr>
<td></td>
<td>3-02-90</td>
<td>8.06</td>
</tr>
<tr>
<td></td>
<td>3-06-90</td>
<td>8.07</td>
</tr>
<tr>
<td></td>
<td>3-09-90</td>
<td>8.05</td>
</tr>
<tr>
<td>C-4</td>
<td>2-09-90</td>
<td>8.00</td>
</tr>
<tr>
<td>(NH15)</td>
<td>2-13-90</td>
<td>8.01</td>
</tr>
<tr>
<td></td>
<td>2-16-90</td>
<td>8.07</td>
</tr>
<tr>
<td></td>
<td>2-20-90</td>
<td>8.04</td>
</tr>
<tr>
<td></td>
<td>2-23-90</td>
<td>8.11</td>
</tr>
<tr>
<td></td>
<td>2-27-90</td>
<td>8.08</td>
</tr>
<tr>
<td></td>
<td>3-02-90</td>
<td>8.07</td>
</tr>
<tr>
<td></td>
<td>3-06-90</td>
<td>8.06</td>
</tr>
<tr>
<td></td>
<td>3-09-90</td>
<td>8.04</td>
</tr>
<tr>
<td>Date</td>
<td>Value</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>2-09-90</td>
<td>7.46</td>
<td></td>
</tr>
<tr>
<td>2-13-90</td>
<td>7.55</td>
<td></td>
</tr>
<tr>
<td>2-16-90</td>
<td>7.60</td>
<td></td>
</tr>
<tr>
<td>2-20-90</td>
<td>7.61</td>
<td></td>
</tr>
<tr>
<td>2-23-90</td>
<td>7.68</td>
<td></td>
</tr>
<tr>
<td>2-27-90</td>
<td>7.69</td>
<td></td>
</tr>
<tr>
<td>3-02-90</td>
<td>7.67</td>
<td></td>
</tr>
<tr>
<td>3-06-90</td>
<td>7.62</td>
<td></td>
</tr>
<tr>
<td>3-09-90</td>
<td>7.59</td>
<td></td>
</tr>
<tr>
<td>2-09-90</td>
<td>7.50</td>
<td></td>
</tr>
<tr>
<td>2-13-90</td>
<td>7.75</td>
<td></td>
</tr>
<tr>
<td>2-16-90</td>
<td>7.80</td>
<td></td>
</tr>
<tr>
<td>2-20-90</td>
<td>7.73</td>
<td></td>
</tr>
<tr>
<td>2-23-90</td>
<td>7.80</td>
<td></td>
</tr>
<tr>
<td>2-27-90</td>
<td>7.77</td>
<td></td>
</tr>
<tr>
<td>3-02-90</td>
<td>7.77</td>
<td></td>
</tr>
<tr>
<td>3-06-90</td>
<td>7.76</td>
<td></td>
</tr>
<tr>
<td>3-09-90</td>
<td>7.74</td>
<td></td>
</tr>
<tr>
<td>2-09-90</td>
<td>7.56</td>
<td></td>
</tr>
<tr>
<td>2-13-90</td>
<td>7.68</td>
<td></td>
</tr>
<tr>
<td>2-16-90</td>
<td>7.73</td>
<td></td>
</tr>
<tr>
<td>2-20-90</td>
<td>7.72</td>
<td></td>
</tr>
<tr>
<td>2-23-90</td>
<td>7.80</td>
<td></td>
</tr>
<tr>
<td>2-27-90</td>
<td>7.78</td>
<td></td>
</tr>
<tr>
<td>3-02-90</td>
<td>7.77</td>
<td></td>
</tr>
<tr>
<td>3-06-90</td>
<td>7.75</td>
<td></td>
</tr>
<tr>
<td>3-09-90</td>
<td>7.73</td>
<td></td>
</tr>
<tr>
<td>2-09-90</td>
<td>8.01</td>
<td></td>
</tr>
<tr>
<td>2-13-90</td>
<td>8.01</td>
<td></td>
</tr>
<tr>
<td>2-16-90</td>
<td>8.07</td>
<td></td>
</tr>
<tr>
<td>2-20-90</td>
<td>8.04</td>
<td></td>
</tr>
<tr>
<td>2-23-90</td>
<td>8.11</td>
<td></td>
</tr>
<tr>
<td>2-27-90</td>
<td>8.08</td>
<td></td>
</tr>
<tr>
<td>3-02-90</td>
<td>8.06</td>
<td></td>
</tr>
<tr>
<td>3-06-90</td>
<td>8.05</td>
<td></td>
</tr>
<tr>
<td>3-09-90</td>
<td>8.05</td>
<td></td>
</tr>
<tr>
<td>2-09-90</td>
<td>8.01</td>
<td></td>
</tr>
<tr>
<td>2-13-90</td>
<td>8.03</td>
<td></td>
</tr>
<tr>
<td>2-16-90</td>
<td>8.08</td>
<td></td>
</tr>
<tr>
<td>2-20-90</td>
<td>8.04</td>
<td></td>
</tr>
<tr>
<td>2-23-90</td>
<td>8.11</td>
<td></td>
</tr>
<tr>
<td>2-27-90</td>
<td>8.08</td>
<td></td>
</tr>
<tr>
<td>3-02-90</td>
<td>8.06</td>
<td></td>
</tr>
<tr>
<td>3-06-90</td>
<td>8.05</td>
<td></td>
</tr>
<tr>
<td>3-09-90</td>
<td>8.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>E-6</td>
<td>2-09-90</td>
<td>8.02</td>
</tr>
<tr>
<td>(NH15)</td>
<td>2-13-90</td>
<td>8.02</td>
</tr>
<tr>
<td></td>
<td>2-16-90</td>
<td>8.08</td>
</tr>
<tr>
<td></td>
<td>2-20-90</td>
<td>8.04</td>
</tr>
<tr>
<td></td>
<td>2-23-90</td>
<td>8.12</td>
</tr>
<tr>
<td></td>
<td>2-27-90</td>
<td>8.08</td>
</tr>
<tr>
<td></td>
<td>3-02-90</td>
<td>8.06</td>
</tr>
<tr>
<td></td>
<td>3-06-90</td>
<td>8.06</td>
</tr>
<tr>
<td></td>
<td>3-09-90</td>
<td>8.06</td>
</tr>
</tbody>
</table>
Evaluation of Silica-bonded octadecane (Si0d.C.1S) Adsorbent for Analysis of Brevetoxins.

A silica-bonded octadecane adsorbent, labeled Polygosil 60-63200, supplied by Keystone Scientific Co., had been evaluated for the solid extraction of brevetoxins from red tide cultures.

The material is 12% carbon with a particle size of 60-200 Angstroms; bulk density is 0.65 g/ml, and the void volume, measured by methanol intrusion into a dry column, is 39%. It is not N-capped.

The program of evaluation comprised 1) test for kinetics and equilibria of adsorption of brevetoxins from aqueous (culture medium) solution; 2) estimate of the toxin capacity of the adsorbent by means of a breakthrough test; and 3) determination of the precision of solid extraction by running a sixfold replicate analysis.

The brevetoxin solutions were prepared by filtering red tide cultures through 3μ, or finer, filters.

The program was supplemented by an estimation of precision of analysis of a red tide culture. This study considers, in addition to the effectiveness of the adsorbent, variations in culture sampling and filtration.

Adsorption Equilibrium and Kinetics

An acceptable adsorbent for brevetoxin analysis must have a high partition coefficient for the toxins in favor of the solid phase versus culture medium (or seawater). The toxins must be adsorbed rapidly enough so that the required flow rates are not unreasonably slow, or the bed depths unreasonably large.

To test acceptability, three, 3-ml (0.5 g) beds of adsorbent, packed in 8 mm i.d. x 15 cm transfer pipettes between glass wool pads, were connected in tandem. Brevetoxin solution was prepared by filtering 11 liters of culture (carboys GG and JJ, 1/24/90) through 15 cm GF/D (3μ) glass fiber circles. Prefiltration serves to lyse the cells, thereby releasing the toxins to the water for adsorption onto the adsorbent, and the filters remove cellular debris and pigments as well. One liter of this solution was drawn through the assembly by means of a vacuum bottle at a rate of 1 liter/hour. The beds were separately water washed, blown superficially dry, and back extracted with 5 ml methanol.
The methanol solutions were analyzed for brevetoxins by HPLC. Results are given in Table 9.

Table 9. Kinetics of Adsorption of Brevetoxins on Polygosil SiO₂/C₁₈
Run DT-42

<table>
<thead>
<tr>
<th>Bed No.</th>
<th>BTx-3</th>
<th>BTx-9</th>
<th>BTx-2</th>
<th>BTx-7</th>
<th>BTx-1</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>62.8</td>
<td>52.4</td>
<td>323</td>
<td>14.9</td>
<td>84.0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10.0</td>
<td>1.3</td>
<td>35</td>
<td>0</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>72.8</td>
<td>53.7</td>
<td>360</td>
<td>14.9</td>
<td>85.5</td>
<td>586.9</td>
</tr>
</tbody>
</table>

Percent Recovery

<table>
<thead>
<tr>
<th></th>
<th>BTx-3</th>
<th>BTx-9</th>
<th>BTx-2</th>
<th>BTx-7</th>
<th>BTx-1</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>86.2</td>
<td>97.6</td>
<td>89.7</td>
<td>100</td>
<td>98.2</td>
<td></td>
</tr>
<tr>
<td>1 + 2</td>
<td>100</td>
<td>100</td>
<td>99.4</td>
<td>100</td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

Under conditions of the test, residence time of toxin solution in each bed was one second. Five toxins were substantially all adsorbed in two seconds. There is no problem with the equilibria or kinetics of the adsorption for toxin recovery from seawater.

**Breakthrough test**

To estimate the useful capacity of the adsorbent for brevetoxins, an extended adsorption run, DT-47, was made to develop a toxin breakthrough curve. The test bed was a 3 ml (0.5 g) sample of adsorbent contained in a 8 mm i.d. transfer pipette. In tandem downstream from this test bed was a 6 ml (1 g) bed of adsorbent. The latter was removed and replaced periodically. It provided a periodic analysis of toxins in the effluent from the test bed.

Toxin solution was prepared by filtering 10 l culture (Carboy MS-2, 2/6/90) through 15 cm GF/F (0.7 µ) glass fiber circles. The filtrate was stored overnight in cooled brown bottles. Toxin content, determined the following day in the course of the breakthrough test, was as follows: BTx-3, 80 µg/l; BTx-9, 76 µg/l; BTx-2, 276 µg/l; BTx-5, 5 µg/l; BTx-1, 77 µg/l; 514 µg/l total.

The solution was pumped through the test bed and analytical beds for 14 hours, at a rate of 0.5 l/hr. This corresponded to a residence time of 2 seconds.
in the test bed. The analytical beds were changed hourly. They were water washed and back extracted with 5 ml methanol for HPLC analysis.

Table 10 summarizes the total toxin concentration in the effluent as a function of the total volume (l) of solution and weight (mg) of toxins fed. Ratio of this concentration to the feed concentration (C/Co, where Co=514 μg/l) is plotted in Figure 1 as the breakthrough curve.

Table 10. Run DT-45 - Toxin Breakthrough Test.

<table>
<thead>
<tr>
<th>Spl #</th>
<th>Volume (l)</th>
<th>Toxins in Effluent (μg)</th>
<th>Total Toxins Fed (mg)</th>
<th>Total Effluent C (mg)</th>
<th>C/Co</th>
</tr>
</thead>
<tbody>
<tr>
<td>OT-45</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.71</td>
<td>1.1</td>
<td>0.36</td>
<td>1.5</td>
<td>0.003</td>
</tr>
<tr>
<td>2</td>
<td>0.55</td>
<td>1.4</td>
<td>0.64</td>
<td>2.5</td>
<td>0.004</td>
</tr>
<tr>
<td>3</td>
<td>0.58</td>
<td>3.5</td>
<td>0.94</td>
<td>6.0</td>
<td>0.011</td>
</tr>
<tr>
<td>4</td>
<td>0.55</td>
<td>5.7</td>
<td>1.22</td>
<td>10.3</td>
<td>0.020</td>
</tr>
<tr>
<td>5</td>
<td>0.57</td>
<td>22.4</td>
<td>1.52</td>
<td>39.2</td>
<td>0.020</td>
</tr>
<tr>
<td>6</td>
<td>0.61</td>
<td>31.8</td>
<td>1.83</td>
<td>52.1</td>
<td>0.101</td>
</tr>
<tr>
<td>7</td>
<td>0.55</td>
<td>35.9</td>
<td>2.11</td>
<td>65.2</td>
<td>0.126</td>
</tr>
<tr>
<td>8</td>
<td>0.48</td>
<td>22.6</td>
<td>2.36</td>
<td>47.0</td>
<td>0.091</td>
</tr>
<tr>
<td>9</td>
<td>0.48</td>
<td>24.4</td>
<td>2.61</td>
<td>50.8</td>
<td>0.098</td>
</tr>
<tr>
<td>10</td>
<td>0.45</td>
<td>36.4</td>
<td>2.84</td>
<td>80.8</td>
<td>0.157</td>
</tr>
<tr>
<td>11</td>
<td>0.52</td>
<td>6.05</td>
<td>3.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.42</td>
<td>56.9</td>
<td>3.32</td>
<td>135.4</td>
<td>0.263</td>
</tr>
<tr>
<td>13</td>
<td>0.48</td>
<td>65.5</td>
<td>3.57</td>
<td>136.4</td>
<td>0.265</td>
</tr>
<tr>
<td>14</td>
<td>0.60</td>
<td>74.6</td>
<td>3.88</td>
<td>124.3</td>
<td>0.241</td>
</tr>
</tbody>
</table>

After passage of 2.2 mg toxins, effluent concentration was 10% of feed composition. A short extrapolation indicates that effluent concentration reached 50% of feed at 5.0 mg. The useful capacity of the adsorbent for analytical purposes can be based on the 10% breakthrough, and amounts to nearly 0.5 wt.%. The useful capacity for preparative applications can be based on the 50% breakthrough, and amounts to 1 wt. %.

The test bed, at the conclusion of the experiment, was water washed and back extracted with methanol for HPLC analysis. Sample DT47-11 was lost. Its toxin content was estimated from values for samples DT47-12 preceding and following. Table 11 summarizes mass balances for five individual brevetoxins and the total toxins. Overall recovery was 90%. 

20
Figure 1. Breakthrough Curve for Brevetoxin through Silica-bonded Octadecane: Ratio of effluent toxin concentration (C) to feed toxin concentration (Co) plotted against total toxins (mg) in the feed solution.
Table 11. Run DT-45 - Mass Balances.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ot45-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toxins recovered in effluent</td>
<td>1</td>
<td>1.1</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>22.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5.2</td>
<td>1.3</td>
<td>24.7</td>
<td>0.6</td>
<td></td>
<td>22.4</td>
</tr>
<tr>
<td>7</td>
<td>5.4</td>
<td>0.9</td>
<td>26.0</td>
<td>0.4</td>
<td>3.2</td>
<td>35.9</td>
</tr>
<tr>
<td>8</td>
<td>3.6</td>
<td>0.7</td>
<td>15.8</td>
<td>0.5</td>
<td>2.0</td>
<td>22.6</td>
</tr>
<tr>
<td>9</td>
<td>4.0</td>
<td>1.8</td>
<td>16.2</td>
<td>0.4</td>
<td>2.0</td>
<td>24.4</td>
</tr>
<tr>
<td>10</td>
<td>5.2</td>
<td>1.2</td>
<td>26.2</td>
<td>0.6</td>
<td>3.2</td>
<td>36.4</td>
</tr>
<tr>
<td>11</td>
<td>6.2</td>
<td>1.4</td>
<td>32.5</td>
<td>0.8</td>
<td>5.8</td>
<td>46.7</td>
</tr>
<tr>
<td>12</td>
<td>7.3</td>
<td>1.7</td>
<td>38.7</td>
<td>0.9</td>
<td>8.3</td>
<td>56.9</td>
</tr>
<tr>
<td>13</td>
<td>7.9</td>
<td>2.1</td>
<td>44.0</td>
<td>0.9</td>
<td>10.6</td>
<td>65.5</td>
</tr>
<tr>
<td>14</td>
<td>7.8</td>
<td>2.3</td>
<td>52.3</td>
<td>0.9</td>
<td>11.3</td>
<td>74.6</td>
</tr>
</tbody>
</table>

Toxins in effluent | 52.6 | 13.4 | 310.5 | 6.0 | 46.4 | 428.9 |
Toxins in adsorbent | 454.0 | 480.0 | 1543.0 | 10.0 | 567.0 | 3054.0 |
Toxins in recovered | 506.6 | 493.4 | 1853.5 | 16.0 | 613.4 | 3482.9 |
Toxins fed (7.55 l) | 604.0 | 573.0 | 2083.0 | 38.0 | 581.0 | 3879.0 |
Recovery on feed, % | 83.8 | 86.1 | 88.9 | 42.1 | 105.5 | 89.7 |

Precision of Analysis by Solid Extraction

A sixfold replicate analysis of a brevetoxin solution was made to determine the probable error of the analytical procedure. The solution was prepared by filtering red tide culture carboys M and N (1/29/90) through 15 cm GG/D (3x) glass fiber circles. Six 1.5 ml adsorbent beds were packed in 8 mm i.d. transfer pipettes between pads of glass wool. The beds were washed with methanol. Methanol was replaced gradually with water.

Brevetoxin solution was drawn through the beds into calibrated 1 liter vacuum bottles. A severe pressure drop built up. Passage of 1 liter solution required 2 hours or more under 28" Hg vacuum. The beds were water washed, blown superficially dry and back extracted with 5 ml methanol for HPLC analysis.

Results are summarized in Table 12. Four toxins were measured. The means and standard deviations for each toxin were calculated. The standard deviation was consistently close to 8% of the mean.
Table 12. Precision Study of Solid Extraction Analysis of Brevetoxin Solution with Polygosil SiO₂/C₁₈

<table>
<thead>
<tr>
<th>Replicate</th>
<th>DT43-</th>
<th>µg/liter of Brevetoxin</th>
<th>BTx-9</th>
<th>BTx-2</th>
<th>BTx-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>96</td>
<td>59</td>
<td>514</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>77</td>
<td>49</td>
<td>449</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>86</td>
<td>53</td>
<td>480</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>91</td>
<td>49</td>
<td>444</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>80</td>
<td>50</td>
<td>457</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>84</td>
<td>47</td>
<td>452</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>86</td>
<td>51</td>
<td>466</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>0₀,₁/mean</td>
<td>0.08</td>
<td>0.08</td>
<td>0.06</td>
<td>0.09</td>
<td></td>
</tr>
</tbody>
</table>

This study was repeated for two reasons. First, the flow rates were only half of the standard 1 l/hr for a 1.5 ml bed. Second, the study did not include possible variations in sampling red tide culture from a carboy, and filtering.

For the second precision test, a red tide culture, Carboy B (2/14/90), containing 6.5 l culture was divided in six parts using a 3 mm i.d. x 50 cm pyrex dip tube connected by a 1/8" i.d. tygon tubing to a 1 l vacuum bottle. The dip tube was moved around the carboy while a 1.05 l sample was drawn. Exactly 1 l of sample was measured in a graduate for subsequent filtration. The remainder provided a sample for cell count. The first three 1 l samples were filtered through a 15 cm GF/D (0.7µ) glass fiber circle. This was supported by a 15 cm GF/D (3µ) circle. The filter cakes were replaced before processing the last three samples. Filtrates and washings were returned to 1 liter bottles which served as chargers. In this test 9 mm i.d. x 10 cm chromatography tubes supplied by Spectrum Medical Industries replaced the transfer burettes. These tubes have end fittings to accommodate a transfer line at the top, and a teflon needle valve at the bottom. The latter helped control flow at 1 l/hr.

Results are summarized in Table 13. The variance in the first three samples was so much higher than in the last three that the two sets have been treated separately. The data suggest that in the first set, the filters were insufficiently washed after Sample DT47-1, and that toxins from this sample were included in the following two. The average toxin concentrations in each set were reasonably comparable. The standard deviations in the second set, expressed as
percentage of the means, were comparable to those in the preceding precision study, DT-43.

Table 13. Precision Study of Solid Extraction Analysis of Red Tide Culture with Polygosil SiO₂/C₁₈.

<table>
<thead>
<tr>
<th>Replicate No.</th>
<th>Cells/l x 10⁶</th>
<th>μg/liter of Brevetoxin</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BTx-3</td>
<td>BTx-9</td>
</tr>
<tr>
<td>1</td>
<td>34.6</td>
<td>147</td>
<td>74</td>
</tr>
<tr>
<td>2</td>
<td>38.9</td>
<td>209</td>
<td>161</td>
</tr>
<tr>
<td>3</td>
<td>32.9</td>
<td>175</td>
<td>153</td>
</tr>
<tr>
<td>mean</td>
<td></td>
<td>177</td>
<td>129</td>
</tr>
<tr>
<td>0.ₙ₋₁/mean</td>
<td></td>
<td>33</td>
<td>30</td>
</tr>
<tr>
<td>0.ₙ₋₂/mean</td>
<td></td>
<td>0.18</td>
<td>0.32</td>
</tr>
<tr>
<td>4</td>
<td>73.8</td>
<td>258</td>
<td>95</td>
</tr>
<tr>
<td>5</td>
<td>29.9</td>
<td>229</td>
<td>99</td>
</tr>
<tr>
<td>6</td>
<td>28.9</td>
<td>247</td>
<td>121</td>
</tr>
<tr>
<td>mean</td>
<td></td>
<td>244</td>
<td>105</td>
</tr>
<tr>
<td>0.ₙ₋₁/mean</td>
<td></td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>0.ₙ₋₂/mean</td>
<td></td>
<td>0.06</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Cell counts varied much more than would be expected from sampling a stirred culture. It is likely that the use of narrow bore tubing and high flow rates destroyed many cells.

Future work

The analytical procedure for brevetoxins in red tide cultures is fairly well established. At least two points need further work.

1) The procedure for washing filter cakes should be standardized. Filter cakes from 1 l culture samples will be washed with standardized volumes of water, and the amount of toxin in each successive washing determined.

2) A procedure for reducing the volume of the 5 ml methanol back extracts should be standardized. These extracts invariably contain some water remaining superficially on the adsorbent after it has been blown. This water inhibits evaporation of dissolved toxins as it increases in concentration during the evaporation. One method found useful in preparative applications is to partition the methanol back extract between saturated NaCl and dichloromethane. The toxins have been recovered in good yield from the organic phase. The latter is easy
to reduce in the rotary drier. Another proposal is to dry the methanol back extract over sodium sulfate prior to evaporation. Both will be tried.

VI. RECOMMENDATIONS

1. Major emphasis to be placed on recovery of *G. breve* cultures following renovation of fire-damaged culture room.
2. Modify and verify procedure for extraction and identification of brevetoxins in fish and clam tissue.
3. Isolate and purify brevetoxins from crude extract to prepare primary toxin standards.
4. Repeat studies of brevetoxin bioaccumulation in fish exposed to sublethal concentrations of red tide organisms.
5. Continue base-line bacteriology survey in preparation for studies during a red tide bloom.
6. Seek additional sources of funds to support field studies of brevetoxin bioaccumulation in fish and shellfish exposed during a natural red tide bloom.