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RICE UNIVERSITY, M.S., 1982

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AN AQUATIC BIOASSAY
UTILIZING THE LEMNACEAE (DUCKWEEDS)
IN A STATIC SYSTEM

by

GARY JAMES GRANT

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

MASTER OF SCIENCE

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APRIL, 1982
ABSTRACT

AN AQUATIC BIOASSAY
UTILIZING THE LEMNACEAE (DUCKWEEDS)
IN A STATIC SYSTEM

Gary James Grant

Growth optimized cultures of *Lemna minor* and *Spirodela polyrrhiza* were developed and used to assess phytotoxicity of two chemicals. Bioassay sensitivity was demonstrated by the dose response curve for Hydrothal-191, a chemical used primarily for the control of aquatic weeds. A photographic technique using a light-sensitive circuit was developed to automate quantitative determination of biomass. A computer program was developed for unbiased statistical comparisons of test and control groups.

The techniques developed are useful in determining the relative toxicity of new and existing chemicals, as well as providing data needed for environmental hazard assessment. Duckweeds grown under optimized conditions exhibited higher growth rates and were more sensitive to linear alkylbenzene sulfonate (L.A.S.) than has been previously reported in the literature. *L. minor* exhibited a significantly lower EC$_{50}$ than *S. polyrrhiza* for both Hydrothal-191 and L.A.S. while the two species were equally sensitive in response to ammonium carbamate.
ACKNOWLEDGMENTS

The development of a bioassay employing the Lemnaceae was suggested by Dr. C. H. Ward and carried out under his direction. The author will be forever indebted to him for his patience and guidance during the course of the project.

The author would like to express his gratitude to the late Dr. William Hillman who kindly supplied duckweed cultures for experimentation, Dr. Olin B. Cecil for his suggestions and inspiring thoughts, and Mrs. Elizabeth Earl Cecil for generously providing unlimited access to computer facilities at The Learning Place. A special appreciation is extended to Phyllis Grant for her endless emotional and technical support.
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Bacterial Bioassay</td>
<td>2</td>
</tr>
<tr>
<td>Fish Bioassay</td>
<td>4</td>
</tr>
<tr>
<td>Algal Bioassays</td>
<td>13</td>
</tr>
<tr>
<td>Aquatic Macrophyte Bioassay</td>
<td>29</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>38</td>
</tr>
<tr>
<td>Obtaining Axenic Cultures</td>
<td>38</td>
</tr>
<tr>
<td>Optimization of Growth</td>
<td>39</td>
</tr>
<tr>
<td>Biomass Measurements</td>
<td>52</td>
</tr>
<tr>
<td>Experimental Design</td>
<td>56</td>
</tr>
<tr>
<td>Growth Rate Determination and Data Analysis</td>
<td>61</td>
</tr>
<tr>
<td>RESULTS</td>
<td>63</td>
</tr>
<tr>
<td>DISCUSSION AND CONCLUSIONS</td>
<td>72</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>74</td>
</tr>
<tr>
<td>APPENDICES</td>
<td>76</td>
</tr>
<tr>
<td>Appendix A</td>
<td>76</td>
</tr>
<tr>
<td>Appendix B</td>
<td>85</td>
</tr>
<tr>
<td>Appendix C</td>
<td>87</td>
</tr>
<tr>
<td>Appendix D</td>
<td>89</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>No.</th>
<th>Table Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Biomass Monitoring</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>Comparison of Knop's, Hoagland's and Hutner's Media by Compound</td>
<td>41</td>
</tr>
<tr>
<td>3</td>
<td>Environmental Conditions</td>
<td>47</td>
</tr>
<tr>
<td>4</td>
<td>Growth Rates of Controls</td>
<td>47</td>
</tr>
<tr>
<td>5</td>
<td>L.A.S. Toxicity in Dynamic and Static Systems</td>
<td>69</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Typical BOD curve</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Typical fish toxicity curve</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Real-time toxicity curve</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>Batch growth curve</td>
<td>18</td>
</tr>
<tr>
<td>5</td>
<td>Computation of maximum specific growth rate</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>Typical algal growth curve</td>
<td>22</td>
</tr>
<tr>
<td>7</td>
<td>Determination of algistatic concentration</td>
<td>25</td>
</tr>
<tr>
<td>8</td>
<td>Algicide toxicity study, <em>Selenastrum</em></td>
<td>27</td>
</tr>
<tr>
<td>9</td>
<td>Growth rate vs illumination intensity</td>
<td>33</td>
</tr>
<tr>
<td>10</td>
<td>Growth rate vs media, <em>L. minor</em></td>
<td>44</td>
</tr>
<tr>
<td>11</td>
<td>Reproducibility of controls (<em>L. minor</em>)</td>
<td>49</td>
</tr>
<tr>
<td>12</td>
<td>Reproducibility of controls (<em>S. polyrrhiza</em>)</td>
<td>51</td>
</tr>
<tr>
<td>13</td>
<td>Photograph of duckweed culture</td>
<td>55</td>
</tr>
<tr>
<td>14</td>
<td>Optical measuring system</td>
<td>58</td>
</tr>
<tr>
<td>15</td>
<td>Frond number vs current (<em>r^2 = 0.96</em>)</td>
<td>60</td>
</tr>
<tr>
<td>16</td>
<td>Toxicity of Hydrothal-191 to two species of duckweed</td>
<td>65</td>
</tr>
<tr>
<td>17</td>
<td>Toxicity of ammonium carbamate to two species of duckweed</td>
<td>67</td>
</tr>
<tr>
<td>18</td>
<td>Toxicity of Linear Alkylbenzene Sulfonate (L.A.S.) to two species of duckweed</td>
<td>71</td>
</tr>
</tbody>
</table>
INTRODUCTION

Biological evaluation of the aquatic environment is essential to the assessment of water quality and is the only true measure of the effects of pollutants. The utility and demand for biological assessment and biomonitoring will receive greater attention in the future as a result of three recently enacted legislations:

1) the Toxic Substances Control Act (TSCA);

2) the Resource Recovery and Conservation Act (RCRA); and

3) the Clean Water Act.

The bioassay test performs a dual role in the enforcement of these laws as a technique to classify toxicants and as an assessment tool in monitoring toxic discharges. On-site dynamic bioassays using indigenous species will provide both a truer indication than laboratory tests of the impact of a discharge on a given receiving stream and a basis for determining the real level of abatement action required. The use of these data for the determination of discharge limitations is likely to avert unnecessary expenditures for wastewater treatment and prevent ecological disaster because of inadequate treatment. A few of the existing bioassay techniques will be presented in order to develop the concepts and current status of biomonitoring. One deficient methodology, aquatic macrophyte phytotoxicity testing, will be
discussed in detail.

There are numerous organisms that have been used or proposed for use in bioassay techniques. Few of these are currently routine; however, their use is sure to expand as legislation continues to show a concern for environmental protection. A few of the organisms that are classified as "tentative" include 1) phytoplankton, 2) zooplankton, 3) daphnia, 4) scleractinian coral, 5) marine annelids, 6) crustaceans, and 7) aquatic insects (1). The important concept is that no single organism can be used universally for the assessment of water quality. Rather, several species representative of biota in a given receiving water must be investigated. When evaluating biological effects of toxicants it is also important to be aware of the organism's position in the food web. DDT has provided a good example of bioconcentration of a chemical through the food web. Certainly the environmental impact is magnified when exposure of all possible organisms is considered.

Bacterial Bioassay

One of the simplest bioassays involves counting the number of indicator bacteria present in a water sample. Culture media are used which select for the coliform and streptococci groups. The number of fecal coliform, fecal streptococci, and total coliform organisms is usually expressed per per 100 ml of sample water (1). It is dangerous as well as
difficult to culture pathogenic bacteria in the laboratory such as those producing typhoid (Salmonella), cholera (Vibrio), or parasitic dysentery (Endamoeba histolytica). Instead, indicator organisms are cultured, realizing that when they are present there is a high probability that fecal contamination has occurred. By looking at various ratios of the indicator bacteria it can be determined if the source of the contamination is man or animals.

Biochemical Oxygen Demand (BOD) is also a bioassay which is routinely used to evaluate water quality. BOD is an empirical test used to determine the relative oxygen requirements for degradation of soluble organics in wastewaters, effluents, and polluted waters. Its primary application is in measuring waste loadings to treatment plants and in evaluating the efficiency of treatment (soluble organic removal). It is important to realize that BOD values cannot be legitimately compared unless the results have been obtained under identical test conditions. The extrapolation of test results to actual stream oxygen demands is highly questionable because the laboratory environment does not reproduce stream conditions such as temperature variation, photoperiod, biological population, water movement, and dissolved oxygen concentration (2). A synopsis of the BOD technique follows. As bacteria in the test water (either indigenous or added as seed) grow and consume oxygen, carbon
dioxide is released due to respiration. A quantity of potassium hydroxide is used to remove the carbon dioxide as it is evolved. A net decrease in pressure above the liquid in the test vessel occurs and is measured with a mercury manometer. Since the pressure drop is linearly related to oxygen consumption, the mercury column can be calibrated in mg/l BOD (2). Alternatively, the sample may be initially saturated with oxygen and the decreasing dissolved oxygen concentration can be monitored throughout the experiment (Fig. 1). A 5-day BOD is most often performed when this technique is utilized.

**Fish Bioassay**

The most popular bioassays in common use are presently those involving fish. Fish bioassay techniques are somewhat more advanced in development and consequently a number of terms have evolved. These include the following definitions (3,4,5,6):

1. **Lethal Concentration (LC)**—Indicates the concentration at which a specified percentage of test organisms are killed over a specified exposure period (e.g., 96-hr LC50).

2. **Effective Concentration (EC)**—Indicates the concentration that a specified effect other than lethality occurred (e.g., 96-hr EC50 to produce paralysis).
Fig. 1. Typical BOD curve (2).
3. Incipient Lethal Level (ILL)--Indicates the concentration at which 50% of the population can live for an indefinite time.

4. Safe Concentration (SC)--Indicates the maximum concentration of a toxicant that has no observable harmful effects after long-term exposure over one or more generations.

5. Maximum Allowable Toxicant Concentration (MATC)--Indicates the concentration of toxicant that may be present in a receiving water without causing significant harm to productivity or to other parameters.

The purpose of a short term fish bioassay is usually to answer one or more of the following questions (7):

Is the chemical safe?
How toxic is it?
Does it vary in toxicity?
Which fraction is most toxic?
Is available dilution sufficient to protect fish?
How effective are treatment methods in reducing toxicity?

Fish toxicity tests include both static and flow-through systems. The static test is more common and will be discussed to illustrate the technique. Results obtained from a static test are often analyzed and presented in a fashion
similar to that for flow-through systems. Many technical problems associated with the bioassay must first be confronted, such as selection of a test species, preparation of dilution water, feeding to ensure initial health of the test organisms, determination of adequate test water volume, acclimation of fish to test tanks, etc.

For each of the observation times a graph similar to Fig. 2 is constructed. The mortality observed in each test tank is plotted against the concentration in the tank. Concentration is plotted on a logarithmic scale and percent mortality is plotted on a probit scale. A line is then fitted so that vertical deviations are minimized. The LC$_{50}$ for that exposure time is then read from the graph. At each observation time the data are plotted and an LC$_{50}$ is determined. The result is a series of LC$_{50}$ values for increasing lengths of time. This series of LC$_{50}$ values is then used to construct a toxicity curve as illustrated in Fig. 3. This type of curve gives the experimenter an overall view of what is happening in the tests. For example, Fig. 3 (ammonia curve) shows a vertical asymptote after 300 minutes, indicating that acute mortality is over and the experiments might be ended earlier than expected. The important idea is that plotting such a toxicity curve as the experiment progresses may give the experimenter a number of clues about the action of the material being tested and will indicate whether it would be
Fig. 2. Typical fish toxicity curve: ppm F (●)(6).
Fig. 3. Real-time toxicity curve: ZnSO$_4$ as Zn, ppm (•), NH$_4$Cl as N, ppm (○)(6).
advantageous to continue the test beyond 96 hr. When the experiment has been completed, a more careful estimate should be made of the LC$_{50}$ for the longest time period used in the test. In routine tests this would usually be the 96-hr LC$_{50}$. If an asymptote has been reached in the toxicity curve (i.e., acute lethality has ceased) the final value should be called the incipient LC$_{50}$ or threshold lethal concentration (3,5,7).

As indicated, most fish studies report relative chemical toxicity in terms of LC$_{50}$. This value should not be used in setting allowable environmental concentrations without the use of a meaningful application factor or safety factor. The data can be extrapolated to obtain a threshold value; however, this is a mortality threshold. Unfortunately, few studies have investigated deleterious effects other than death.

**Algal Bioassays**

Algal bioassay tests have been developed for the determination of primary productivity in a receiving water. Test water and algae are placed in bottles and lowered to various levels within the lake or stream which provides in situ light quality as well as other environmental parameters. Incorporation of the $^{14}$C-bicarbonate carbon source by the algae in the flask is a measurement of carbon dioxide uptake, photosynthesis, and productivity.

Another bioassay test, the Algal Assay Procedure
Bottle Test (8), has been used in eutrophication research; variations are being adapted for toxicity testing. The test, as described by the EPA, is intended primarily for:

1) Assessment of a receiving water to determine its nutrient status and sensitivity to change.

2) Evaluation of materials and products to determine their potential effects on algal growth in receiving waters.

3) Assessment of effects on receiving waters of changes in waste treatment processes.

The test involves inoculating sterile test water with either a single species or a mixed culture of growing algae, usually species indigenous to the water being tested. Three sets of flasks are prepared: one set with only test water and inoculum; one with test water, inoculum, and a nitrogen spike, and one with a phosphorus spike. Biomass is then measured daily (Table 1) and specific growth rates calculated. The specific growth rate is calculated from the following equation:

\[ \mu = \frac{\ln(X_1/X_2)}{T_2-T_1} \text{ days}^{-1} \]

where \( X_2 \) = biomass at the end of the time interval

\( X_1 \) = biomass at the start of the time interval

\( T_2-T_1 \) = elapsed time in days

If biomass is determined indirectly (e.g., by optical den-
<table>
<thead>
<tr>
<th>Measurement*</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspended solids</td>
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</tr>
<tr>
<td>Absorbance (optical density)</td>
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<tr>
<td>Suspended carbon</td>
<td>mg/l</td>
</tr>
<tr>
<td>Cell counts</td>
<td>no./ml</td>
</tr>
<tr>
<td>Cell volume</td>
<td>μm³/ml</td>
</tr>
<tr>
<td>Chlorophyll fluorescence</td>
<td>units/cm</td>
</tr>
<tr>
<td>Extracted chlorophyll</td>
<td>μg/l</td>
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* U.S. Environmental Protection Agency (8)
sity, cell constituents, etc.) the specific growth rate may be computed directly from these determinations without conversion to biomass; however, the factor relating the indirect measurement to biomass must remain constant for the time period considered. The biomass is then plotted against time to obtain the algal growth curve (Fig. 4). The equation used to calculate the specific growth rate suggests that if the logarithmic portion of the growth curve is plotted on a semi-log scale the resulting line has a slope which equals the specific growth rate (Fig. 5). The algal growth curve for each experimental flask is plotted to determine which one achieves the highest maximum growth rate. If all three flasks are equal, then there is no nutrient limitation and nutrient spikes provided for no additional growth. If one of the spiked flasks shows increased growth rates, then the nutrient added is limiting in the test water and should be most carefully controlled as an influent because its addition allows for a faster rate of growth (Fig. 6).

Hall (9) attempted to develop a standard algal toxicity test. Algae were exposed to various toxicant concentrations in standard algal nutrient media for five days, and then washed and resuspended in pure media. The algae were then allowed to grow for nine more days as a recovery period to test if the algal cells were still viable. Hall outlined three important responses of algal cells to toxic substances:
Fig. 4. Batch growth curve (8).
Fig. 5. Computation of maximum specific growth rate (8).

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Cell Count (cells/ml)</th>
<th>( \mu_{\text{max}} = \frac{\ln(1.7 \times 10^5 / 5.8 \times 10^3)}{2} )</th>
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<tr>
<td>0</td>
<td>1.0 \times 10^3</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.1 \times 10^3</td>
<td>By inspection of the plot, the maximum growth rate occurred between days 2 and 4.</td>
</tr>
<tr>
<td>2</td>
<td>5.8 \times 10^3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.5 \times 10^4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.7 \times 10^5</td>
<td>= \frac{\ln(29.3)}{2}</td>
</tr>
<tr>
<td>5</td>
<td>3.3 \times 10^5</td>
<td>= 3.38/2</td>
</tr>
<tr>
<td>6</td>
<td>6.3 \times 10^5</td>
<td>= 1.69 days(^{-1})</td>
</tr>
<tr>
<td>8</td>
<td>2.3 \times 10^6</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>2.4 \times 10^6</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1.6 \times 10^6</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>2.4 \times 10^6</td>
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</tr>
</tbody>
</table>
Fig. 6. Typical algal growth curve: control (○), control + 0.05 mg l\(^{-1}\) N (★), control + 0.37 mg l\(^{-1}\) P (●) (8).
1) a reduction in growth rate, 2) an inhibition of cell division (algistatic response), and 3) cell death (algicidal response).

Algal biomass was determined periodically by cell counts and plotted semi-logarithmically versus time. Hall stressed that the concentration of interest is the algistatic concentration and defined it as that concentration that will inhibit growth, yet allow the organism to return to normal growth rate after removal of the chemical. Different concentrations of toxicants were prepared for algal cultures and the growth rates determined. The ratio of biomass at day 5 (end of toxicant exposure) to biomass at the time of inoculation was plotted against the log of toxicant concentration (Fig. 7). A ratio of one represents a 5-day algistatic response (no increase or decrease in cell number) while ratios above one were inhibitory responses and ratios below one were algicidal responses (Fig. 7). The 9-day recovery period was to ensure that no permanent effect on growth rate occurred. Hall tested his bioassay by exposing algae to various concentrations of a commercial swimming pool algicide (Fig. 8).

The ideas that Hall presented in his bioassay are important to consider; however, there are some aspects which are inconsistent with the goals of a bioassay technique. In the normal algal assay bottle test, biomass measurements are made at least daily. For many replicate flasks and many
Fig. 7. Determination of algistic concentration:
confidence interval (a), algistic concentration (b) (9).
Fig. 8. Algicide toxicity study, *Selenastrum*: AAP media (○); algicide concentrations: 0.01 mg l\(^{-1}\) (●), 0.40 mg l\(^{-1}\) (★), 0.5 mg l\(^{-1}\) (X), 2.50 mg l\(^{-1}\) (●) (9).
Relative Fluorescence units

DAYS

10.8 10.7 10.6 10.5 10.4 10.3 8 4 12 16 20 24
toxicant concentrations biomass determinations become rather
time consuming. Hall decided that by looking at the ratios
of cell numbers at day 0, 5, & 14, biomass measurements
were only necessary at the time of inoculation, day 5, and
at the end of the 9-day recovery period. This method saves
time, but there is no way to determine the nature of the
response between the time of inoculation and day 5. An
initial cell death might occur with subsequent adaptation
by the remaining algal cells resulting in regrowth of the
population. The environmental implications of this toxic-
cant would be different if interpreted by Hall's method
alone.

A common effect parameter in bioassay techniques is a
50% reduction in the growth rate of an organism. Hall ex-
cluded this parameter from his test because higher concen-
trations could be tolerated without permanent damage to the
organisms. This might be an acceptable endpoint for a com-
parative laboratory toxicity test; however, it is rather
misleading in the environmental setting.

If the growth rate of a primary producer such as algae
was to be held constant at zero, there would still be a net
decrease in the population with time due to predation. The
next higher species in the food web (the predator) would
also suffer a decline in growth rate due to a decreased prey
density.
This effect may easily cascade up the food web and have unpredictable effects upon the structure of the ecosystem in a receiving water. While the algistatic concentration might be useful in comparing the relative toxicity of chemicals as tested in the laboratory, it is not adequate in assessing environmental effects or defining effluent characteristics. Its only environmental application, although questionable, would be in a spill situation. Perhaps the more meaningful value for environmental concern would be the concentration which exerts a threshold effect upon growth rate.

**Aquatic Macrophyte Bioassay**

Algae are satisfactory organisms for determining the nutrient status of waters, relative toxicity of chemicals in the laboratory, and in examining toxicity in the water column. Fish are useful for comparing the toxicity of chemicals in the laboratory and can be useful for in-stream monitoring. Fish are also valuable for looking at chemicals both in the water column and in the sediments, depending upon the feeding habits of the particular species tested. Evaluations of phytotoxicity currently do not involve aquatic vascular plants. Not only is a bioassay for an aquatic macrophyte lacking, but none of the available bioassays are designed to measure chemicals which tend to partition at the air/water interface, such as surfactants. Macrophytes
contribute significantly to the productivity of some aquatic ecosystems. Floating macrophytes, such as members of the family Lemnaceae (the duckweeds) appear to be organisms well suited for this type of examination of aquatic settings. Some of the attributes of the Lemnaceae which suggest their suitability as experimental organisms include the following (10):

1. Wide distribution
2. Easily cultured in the laboratory
3. Easily measured biomass
4. Reasonable growth rates
5. Manageable size
6. Relative structural simplicity

The Lemnaceae are small floating plants which are found at or near the water surface. The four genera in the family (from largest to smallest) are Spirodela, Lemna, Wolffiella, and Wolffia (11). The four species which are globally distributed are L. minor, L. trisulca, S. polyrrhiza, and S. oligorhiza; however, L. trisulca is somewhat limited to slightly warmer areas (10).

The entire family is in need of modern taxonomic treatment because many of the diagnostic characters include such things as seed ribbing or fruit shape, which are seldom seen since flowering is relatively rare. Most of the studies in the literature have been involved with attempts to induce
flowering in the laboratory with limited success and no reproducible results. The main difference distinguishing the genera is root number; Spirodela has two or more roots, Lemna has one, and Wolffiella and Wolffia have no roots.

When developing a bioassay one should optimize environmental conditions so that growth may proceed at a maximum rate. The primary parameters affecting the growth of the Lemnaceae are nutrients and their availability, light, temperature, and pH. The Lemnaceae respond as typical higher plants with respect to nutrient requirements and grow well on totally inorganic media (10). Hutner's medium, the most commonly used medium in the literature, appears to be the best for maintaining vigorous growth over prolonged periods (12). Hoagland's medium also has been used extensively in studies of the Lemnaceae.

Light is typically considered a growth-limiting parameter for organisms which photosynthesize. To ensure maximum growth it is important to maintain cultures above the light saturation level but intensities which are deleterious. Many different light saturation values are found in the literature, varying from 25 to 117 \( \mu E \text{ m}^{-2} \text{ sec}^{-1} \) (150 to 700 ft-c). A recent study by Bishop (13) has shown a light saturation value of 117 \( \mu E \text{ m}^{-2} \text{ sec}^{-1} \) (700 ft-c)(Fig. 9). Based on Bishop's data, growth experiments should be conducted at intensities above 117 \( \mu E \text{ m}^{-2} \text{ sec}^{-1} \). Literature values for temperature
Fig. 9. Growth rate vs illumination intensity, L. minor (13).
optima seem to agree that there is little effect upon growth in the 20-30°C range (13).

Several studies of the pH requirements of the Lemnaceae failed to optimize all other environmental parameters and the results are difficult to compare. Experiments conducted by McLay (7) on cultures maintained on pH-adjusted medium that was changed every two days found normal growth to occur for *Lemna* and *Spirodela* within the pH range of 4-10; optimal growth occurs at pH 6.2.

Reproduction in the Lemnaceae can be either sexual or vegetative, but since flowering is so rare, the vegetative process is predominant. A few species have been seen to flower in the laboratory, but only with a complex variation in temperature and photoperiod (14). This is an advantage in an experimental organism because genetic variation is reduced with vegetative reproduction allowing one particular clone to be maintained to ensure uniformity in all experiments. New "daughter" fronds (or leaf structures) are produced from two vegetative primordia (pockets) on either side of the "mother" frond, near the node (place of root attachment). Daughter fronds usually stay attached until they form daughter fronds of their own and are most often seen in colonies of three. Species of *Wolffia* and *Wolffiella* have only one vegetative pocket. Daughter fronds can be produced alternately from the right and left sides and the one
that is first to produce a daughter remains constant for a clone and determines its "handedness" (10).

Root length in *Spirodela* and *Lemna* varies greatly from species to species and depends upon environmental conditions. Functional importance of the root is difficult to evaluate since nutrients are taken up through the lower frond surface and normal growth is seen whether root elongation is or is not present (10).

During periods of adverse environmental conditions the Lemnaceae form turions. Fronds under normal conditions have large intercellular spaces which contain air and provide buoyancy in the aquatic environment. Turions are dense structures with little air in the intercellular spaces and show increased starch supplies. These structures remain dormant until conditions improve (10).

For any bioassay, an adequate measurement of biomass is necessary. The most reliable measure of biomass in the duckweeds appears to be frond number (10). However, frond size will vary slightly, depending upon maturation stage. Since the Lemnaceae grow only in a planer form on the water surface, a surface coverage measure may prove to be a more accurate parameter to estimate biomass. Any type of weight measurement might prove misleading since it is more largely indicative of starch content rather than other growth features. Growth rates are calculated as suggested
for algal growth experiments.

When investigating a growth response to chemicals in test water it is important to avoid simultaneous metabolism by the test organism and contaminant microorganisms. Other bacterial or algal species may also be parasitic and alter growth rate due to their interactions. The most common method cited in the literature for obtaining an axenic culture of duckweed has been to submerge the plant in 0.5% sodium hypochlorite for 30 seconds. The plant is then placed in medium supplemented with 0.2% dextrose. If the cultures become turbid they are considered contaminated. Clear cultures are considered axenic (15). This method is discussed below in Materials and Methods.

After the proposed test organism has been evaluated, it is appropriate to determine which parameters should be monitored. The toxicity and evaluation procedures used with algal bioassays appear to be readily applicable to the Lemnaceae. When using the Lemnaceae to evaluate the relative toxicity of a chemical, it would be most valuable to look at the concentration which reduces the growth rate by 50% (EC50), while a threshold effect on growth rate would be most reasonable when testing natural waters or effluents for allowable concentrations. The proposed bioassay technique should first be tested with a chemical which is expected to produce a toxic effect. For duckweed, such a chemical would be a com-
mercial aquatic herbicide such as endothall.

The role of bioassay techniques has become more important with the recent increased concern for environmental impacts from introduced chemicals. For example, Section 311 of the 1972 Federal Water Pollution Control Act Amendments uses the fish LC$_{50}$ concentration to categorize hazardous chemicals as follows:

<table>
<thead>
<tr>
<th>Category</th>
<th>Toxicity Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>LC$_{50}$ less than 0.1 mg/l</td>
</tr>
<tr>
<td>A</td>
<td>LC$_{50}$ of 0.1-1.0 mg/l</td>
</tr>
<tr>
<td>B</td>
<td>LC$_{50}$ of 1.0-10 mg/l</td>
</tr>
<tr>
<td>C</td>
<td>LC$_{50}$ of 10-100 mg/l</td>
</tr>
<tr>
<td>D</td>
<td>LC$_{50}$ of 100-500 mg/l</td>
</tr>
</tbody>
</table>

Bioassay techniques for vertebrates (fish) and for algal species have been developed and are currently in use. However, there is no bioassay using a representative of the aquatic macrophytes, or a bioassay which selectively examines chemicals with the greatest tendency to partition at the air/water interface. The research reported in this thesis indicates that species of the Lemnaceae would be excellent organisms to meet both of these needs.
MATERIALS AND METHODS

Obtaining Axenic Cultures

The procedure described by Hillman to obtain axenic cultures was attempted without success (10). Several fronds of \textit{L. minor} and \textit{S. polyrrhiza} (obtained from William Hillman) were submerged in sodium hypochlorite for 30 seconds, rinsed with sterile deionized water, and placed in sterile medium. The medium remained clear for several days and would have been deemed axenic by Hillman's criteria. However, a sample of medium was plated on microbial culture medium and contaminant microorganisms were detected. It was assumed that these remaining microorganisms had not been destroyed by the treatment because they were not in a rapid growth phase. Subsequently, this assumption was tested.

Cultures of the duckweeds were then prepared which would allow for improved microbial growth. \textit{L. minor} and \textit{S. polyrrhiza} cultures were prepared in unsterile Hutner's medium that was supplemented with 0.2\% dextrose and 600 mg/l pancreatic digest of casein. After approximately 48 hr a visibly decreased clarity of the medium indicated the presence of microbial flora. This suggested that contaminant microbes were in a rapid growth phase and that spore forms were reduced in numbers. The fronds were then rinsed for 5 minutes in sterile Hutner's medium and subsequently submerged in a 0.5\% solution of sodium hypochlorite for time periods
varying from 5 to 10 minutes. The fronds were next placed in 33% sterile Hutner's medium supplemented with 0.5% dextrose and 600 mg/l pancreatic digest of casein. The cultures were monitored for several days. Flasks containing fronds exposed for 5 to 7 minutes were visibly contaminated with organisms. The fronds with longer exposure times were very chlorotic in appearance, but were maintained in a controlled environment chamber at 25°C with a fluorescent light intensity of 151 μE m⁻² sec⁻¹ (900 ft-c). After about 10 days some of the fronds in the 8 and 9 minute exposure groups exhibited small green areas in the center of the fronds, indicating that a portion of the meristematic tissue had survived and was continuing to grow. After about 10 more days a substantial population of fronds had developed and the medium remained clear. Aliquots of the medium were plated again with no microbes detected. Stock cultures were maintained on the supplemented medium while fronds used for testing purposes were acclimated on sterile culture medium for 10 days without the addition of carbohydrates or organic nutrients. Before inoculating test flasks, medium from the acclimating flasks was plated on bacteriological agar to ensure that no contamination had occurred.

**Optimization of Growth**

While Hutner's medium was reported most extensively in the literature for culturing duckweeds, other media were also
examined. *L. minor* cultures were placed in Knop's, Hoagland's, and 33% Hutner's media (Table 2). After several days of growth, no increase in frond numbers was seen with Knop's medium, while Hoagland's and Hutner's provided for equally rapid growth (Fig. 10). It was noted, however, that the fronds in Hoagland's medium had rather elongated roots which may indicate nutrient deficiencies (10). An inoculum of two fronds of *L. minor* consistently covered the entire surface (78.5 cm²) of the Corning storage jars used to culture the organisms after a period of 10 days. In order to optimize the nutrient concentration, *L. minor* was grown in 50%, 33% and 25% Hutner's medium. Frond counts were made daily for 10 days. Growth rate in the 25% Hutner's medium was significantly less than either the 33% or 50% concentrations. The 33% and 50% concentrations showed equal growth rates during the 10-day growth period.

Numerous attempts to determine an optimum pH for duckweed culture are found in the literature (7). However, few investigators used the same organism or the same nutrient medium nor did they attempt to ensure an axenic culture of the duckweeds. In order to better assess the optimum pH range, sterile 33% Hutner's medium was adjusted to various initial pH values by the addition of 0.1M KOH or 0.1M HCl. Samples of medium from each flask were aseptically taken every other day for pH determination and frond counts were made daily for 10 days.
### TABLE 2

**COMPOSITION OF KNOP'S, HOAGLAND'S, AND HUTNER'S MEDIA BY COMPOUND**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Knop's</th>
<th>Hoagland's</th>
<th>Hutner's (33%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄•7H₂O</td>
<td>250.0</td>
<td>242.738</td>
<td>104.167</td>
</tr>
<tr>
<td>EDTA</td>
<td>100.0</td>
<td>12.851</td>
<td>104.167</td>
</tr>
<tr>
<td>KOH</td>
<td>62.0</td>
<td>8.715</td>
<td>83.333</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>--</td>
<td>--</td>
<td>83.333</td>
</tr>
<tr>
<td>Ca(NO₃)₂•4H₂O</td>
<td>--</td>
<td>581.388</td>
<td>73.750</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>--</td>
<td>--</td>
<td>41.667</td>
</tr>
<tr>
<td>ZnSO₄•7H₂O</td>
<td>17.64</td>
<td>0.109</td>
<td>13.729</td>
</tr>
<tr>
<td>Na₂MoO₄•2H₂O</td>
<td>--</td>
<td>--</td>
<td>5.25</td>
</tr>
<tr>
<td>FeSO₄•7H₂O</td>
<td>10.0</td>
<td>12.26</td>
<td>5.188</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>22.8</td>
<td>1.403</td>
<td>2.958</td>
</tr>
<tr>
<td>CuSO₄•5H₂O</td>
<td>3.14</td>
<td>0.038</td>
<td>0.821</td>
</tr>
<tr>
<td>KNO₃</td>
<td>250.0</td>
<td>248.892</td>
<td>--</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>250.0</td>
<td>67.011</td>
<td>--</td>
</tr>
<tr>
<td>NaCl</td>
<td>--</td>
<td>28.804</td>
<td>--</td>
</tr>
<tr>
<td>MnSO₄•H₂O</td>
<td>--</td>
<td>0.757</td>
<td>--</td>
</tr>
<tr>
<td>H₂MoO₄(85%)</td>
<td>1.848</td>
<td>0.010</td>
<td>--</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>16.8</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>MnCl₂•4H₂O</td>
<td>2.88</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Co(NO₃)₂•6H₂O</td>
<td>0.98</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Element</td>
<td>Knop's</td>
<td>Hoagland's</td>
<td>Hutner's (33%)</td>
</tr>
<tr>
<td>---------</td>
<td>----------</td>
<td>------------</td>
<td>----------------</td>
</tr>
<tr>
<td>K</td>
<td>211.723</td>
<td>120.672</td>
<td>68.070</td>
</tr>
<tr>
<td>N</td>
<td>34.728</td>
<td>103.447</td>
<td>18.518</td>
</tr>
<tr>
<td>S</td>
<td>36.292</td>
<td>33.154</td>
<td>16.734</td>
</tr>
<tr>
<td>P</td>
<td>56.900</td>
<td>15.252</td>
<td>14.819</td>
</tr>
<tr>
<td>Ca</td>
<td>6.067</td>
<td>98.674</td>
<td>12.517</td>
</tr>
<tr>
<td>Mg</td>
<td>24.659</td>
<td>23.943</td>
<td>10.275</td>
</tr>
<tr>
<td>Zn</td>
<td>4.514</td>
<td>0.028</td>
<td>5.055</td>
</tr>
<tr>
<td>Mo</td>
<td>0.931</td>
<td>0.005</td>
<td>2.082</td>
</tr>
<tr>
<td>Fe</td>
<td>2.009</td>
<td>2.463</td>
<td>1.042</td>
</tr>
<tr>
<td>B</td>
<td>3.986</td>
<td>0.245</td>
<td>0.517</td>
</tr>
<tr>
<td>Na</td>
<td>--</td>
<td>11.331</td>
<td>0.499</td>
</tr>
<tr>
<td>Cu</td>
<td>0.799</td>
<td>0.010</td>
<td>0.209</td>
</tr>
<tr>
<td>Cl</td>
<td>11.765</td>
<td>17.473</td>
<td>--</td>
</tr>
<tr>
<td>Mn</td>
<td>0.799</td>
<td>0.246</td>
<td>--</td>
</tr>
<tr>
<td>Co</td>
<td>0.198</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
Fig. 10. Growth rate vs media, L. minor:

50% Hutner's medium  (Td = 2.36 days) (●)
33% Hutner's medium  (Td = 1.30 days) (●)
50% Hoagland's medium (Td = 1.92 days) (●)
Growth rates were difficult to compare due to erratic growth. It was noted that the medium of each flask increased to near pH 8.5 at which time growth was greatly reduced and a white precipitate was seen at the bottom of the flasks. In order to adequately compare and optimize growth rates, the control of pH was investigated. Cultures of L. minor were placed in flasks with 33% Hutner's medium adjusted to various values from pH 5.0 to 7.0. The fronds were then aseptically transferred to fresh, pH adjusted medium every other day for 10 days. Growth rates were not significantly different within the pH range tested. Changing the culture medium every other day would be time-consuming and introduce a possible source of culture contamination for large-scale experimentation.

Initial attempts at preparing a buffered solution of Hutner's medium failed when copious precipitation occurred upon autoclaving. If a pH 6.5, 1.0M phosphate buffer (KH₂PO₄/NaOH) was autoclaved separately and added to sterile Hutner's medium aseptically at room temperature, then no precipitation occurred. Various volumes of the 1.0M buffer were added to 1.5 liter volumes of 33% sterile Hutner's medium. Flasks were then inoculated with L. minor and monitored for 10 days. No difference was detected in growth rates when 20 ml, 25 ml, or 30 ml of the 1.0M buffer was added to 1.5 liter volumes of the 33% Hutner's medium (0.013M, 0.016M, and 0.020M buffer solutions). In all cases growth rates were not significantly
different when compared to cultures maintained at pH 6.5. For routine experiments, 0.020M phosphate buffered solutions of 33% Hutner's medium were used.

In order to further optimize growth rates of the Lemnaceae, the effects of light intensity and temperature were investigated. Zurzycki (17,18) found that light intensities of greater than 200 $\mu$E m$^{-1}$ sec$^{-1}$ (1200 ft-c) produced peculiar plastid abnormalities in *L. trisulca*, and intensities above 840 $\mu$E m$^{-2}$ sec$^{-1}$ (5000 ft-c) inactivated the photosynthetic mechanism. Studies utilizing *L. minor* and *S. polyrrhiza* have shown a decrease in growth rates below an intensity of 115 $\mu$E m$^{-2}$ sec$^{-1}$ (700 ft-c) (19). Bishop and Perry (13) also found light saturation to occur in *L. minor* at 150 $\mu$E m$^{-2}$ sec$^{-1}$ (900 ft-c) (Fig. 9). Landolt (19) found a temperature optimum for the Lemnaceae at about 25°C, with relatively rapid growth being maintained between 20°C and 30°C. Wilkinson (20) found an optimum temperature for growth of *L. minor* at 25°C and a significant increase in growth rate between 18.5 and 92.3 $\mu$E m$^{-2}$ sec$^{-1}$ (110 and 550 ft-c) illumination. All experimental and stock cultures were maintained at a temperature of 25°C under a continuous illumination (fluorescent) of 151 $\mu$E m$^{-2}$ sec$^{-1}$ (900 ft-c) to ensure growth optimized conditions (Table 3). Growth rates obtained with these conditions were comparable among replicates and reproducible (Table 4, Figs. 11, 12).
### TABLE 3

**ENVIRONMENTAL CONDITIONS**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth Medium</td>
<td>33% Hutner's Medium</td>
</tr>
<tr>
<td>pH</td>
<td>Buffered to pH 6.5</td>
</tr>
<tr>
<td></td>
<td>(0.015M phosphate buffer)</td>
</tr>
<tr>
<td>Light Intensity</td>
<td>151 μE m$^{-2}$ sec$^{-1}$ (900 ft-c)</td>
</tr>
<tr>
<td></td>
<td>Fluorescent</td>
</tr>
<tr>
<td>Temperature</td>
<td>25 ± 1°C</td>
</tr>
</tbody>
</table>

### TABLE 4

**GROWTH RATES OF CONTROLS***

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth Rate ($\mu \times 10^{-3}$)</th>
<th>Doubling Time ($T_d$)</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. minor</td>
<td>8.766 ± 0.20 hr$^{-1}$</td>
<td>1.43 ± 0.03 days</td>
<td>0.997</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. polyrrhiza</td>
<td>5.603 ± 0.14 hr$^{-1}$</td>
<td>2.24 ± 0.6 days</td>
<td>0.983</td>
</tr>
</tbody>
</table>

* Determined by the slope of a least squares linear regression of the growth curves (Figs. 11, 12)
Fig. 11. Reproducibility of controls, *L. minor*:

April 10, 1980 (Td = 1.44 days)(*)
April 24, 1980 (Td = 1.62 days)(●)
May 13, 1980   (Td = 1.35 days)(○)
Fig. 12. Reproducibility of controls, *S. polyrrhiza:

April 4, 1980  (Td = 2.05 days) (★)
May 23, 1980   (Td = 1.96 days) (●)
June 13, 1980  (Td = 2.17 days) (○)
Biomass Measurements

A bioassay procedure was developed to assess the toxicity of various chemicals to *L. minor* and *S. polyrrhiza* which allowed biomass to be measured over time without contaminating or removing organisms from the experimental flask. Hillman (10) reviewed the various measurements appropriate for determining growth rates and concluded that an increase in frond number was not only the most reliable but also the easiest method. Concentrated solutions of toxicants were prepared in Hutner's medium and filter sterilized using a 0.45 μm filter in a Millipore filtration system. Proper volumes of the concentrated toxicant were added to 1.5 liter volumes of 33% strength Hutner's medium to yield test media with desired toxicant concentrations. One 150 ml of toxicant medium was aseptically placed in each 100 mm diameter storage jar (Corning Glass). These jars were excellent for maintaining axenic cultures and the flat glass top provided good frond visibility when the flask was placed on a microbial colony counter and illuminated from below. Each test flask was initially inoculated with two or three fronds of either *L. minor* or *S. polyrrhiza* and placed in an environmental chamber (New Brunswick Scientific).

A frond count was made daily for each experimental flask using the microbial colony counter. A semi-automated technique for counting fronds was also developed which not
only made counting easier, but also provided a permanent record of the data. Each flask was momentarily removed from the environmental chamber, placed on a photocopy stand, and illuminated with four photographic floodlights. Kodalith (Eastman Kodak) copy film was exposed with a bellows-type camera at an f-stop setting of 5.6 for 1/8 second. Kodalith developer (Eastman Kodak) was used to develop the film utilizing a 3 minute developing cycle followed by a 30 second rinse in Kodak indicator stop bath. The film was then fixed for 5 minutes in Kodak Fixer, washed for 5 minutes in cool tap water, rinsed for 30 seconds in Photo-flo solution (Eastman Kodak) and allowed to air dry. Good latitude in exposure and developing times was noted since the film has fine grain size and very high contrast (Fig. 13). Since the fronds grow only in two dimensions on the surface of the medium, frond numbers could be easily counted using a microfilm reader to look at the outlines of the fronds.

Determining frond counts with the photographic technique and microfilm reader was easier than counting with the microbial colony counter. However, the process was still quite time-consuming when large numbers of fronds were to be counted on a daily basis. Several light detectors were investigated for possible use in quantifying a beam of incandescent light passing through the film. Most existing detectors were found to be either highly wavelength dependent,
Fig. 13. Photograph of duckweed culture.
not sensitive enough to detect significant frond differences, or too sensitive to not be affected by film grain size and distribution. A prototype device was then constructed which passed light through the film and focused the exit beam onto the surface of a phototransistor (Texas Instruments type TIL-81). The detector circuit was designed to utilize the device in a phototransistor mode with current flow from collector to emitter measured to quantify incident light (Fig. 14). Growth rates determined from a semi-log plot of incubation time vs current correlated well with growth rates determined with actual frond counts (Fig. 15).

**Experimental Design**

The toxicity of three chemicals including Hydrothal-191 (Pennwalt Corp.), linear alkybenzene sulfonate (L.A.S.) and ammonium carbamate was determined using this bioassay technique. Hydrothal-191 is a commercial preparation of a mono-(N,N-dimethylalkylamine) salt of endothall which has been widely used to control the growth of aquatic weeds, particularly hydriide (Hydrilla verticillata). Hydrothal-191 was chosen for testing since it would be expected to exert a toxic effect upon aquatic macrophytes such as the Lemnaceae. L.A.S. was chosen as a toxicant because of its presence in commercial detergents, and thus its opportunity to enter the aquatic environment in significant quantities. L.A.S. was also used as a test chemical by Bishop and Perry (13) in a
Fig. 14. Optical measuring system.
Bi-convex lens from
Kodak Carrousel slide projector

5 cm 13.7 cm 62.0 cm 11.0 cm
Film

High-intensity Film-mounting Stage Phototransistor
light Source
(detector)

100 ohms

5 v Detector Circuit Ammeter
Fig. 15. Frond number vs current ($r^2 = 0.96$).
dynamic system utilizing *L. minor*. Ammonium carbamate was tested because of its use in the manufacture of pesticides and presence as a by-product. These chemicals were selected to evaluate the potential of the duckweed bioassay in routine toxicity testing and hazard assessment.

For each chemical, tests were conducted with both *L. minor* and *S. polyrrhiza*. A range-finding test was first conducted using toxicant concentrations that were incrementally increased by factors of ten. At least three replicate flasks were prepared for each chemical concentration and each of the two species of duckweed. Growth rates of control flasks (buffered Hutner's medium, without toxicant) were compared to experimental flasks to approximate the toxicant concentration at which growth rates began to be affected. A second evaluation was then conducted using toxicant concentrations in a narrower range and a dose-response curve constructed comparing growth rate (as a percentage of controls) to increasing toxicant concentrations. In order to make comparisons among toxicants and among results using other bioassays, the EC$_{50}$ (that concentration which reduced growth rates to 50% of controls) was extrapolated from the toxicity curve.

**Growth Rate Determination and Data Analysis**

The photographic frond measuring technique was not developed in time to evaluate growth rates in the toxicant
study. However, frond counts were made periodically for 10 days, at which time the water surface was about 90% covered with fronds. A semi-log plot was then made of frond number vs time and growth rate was calculated from the slope of the linear portion of this plot. Growth rate was then converted to the more concise form of doubling time using the following equation:

\[ T_d = \frac{\ln(3.322 \times \mu \times 24)}{-1} \text{ days} \]

where \( T_d = \) doubling time (days)
\( \mu = \) growth rate (hours\(^{-1}\))

Comparison of various treatment groups was accomplished through a three-step statistical approach. A least squares regression analysis was first used to determine the slope (growth rate) of the best linear relationship with at least three replicates for each treatment group being evaluated. An analysis of variance (ANOVA) technique was then performed on all concentrations to determine if the growth rates (slopes) were statistically different. Dunnett's test was subsequently used to determine which groups (concentrations) were significantly different (alpha = 0.05) when compared to a designated control group (21). Data analysis was performed with a computer program written in Basic for a TRS-80 (Tandy Corp.) microcomputer system (Appendix A). A dose-response curve was constructed by plotting toxicant concentration vs growth rate percentage of control and the EC\(_{50}\) was determined.
RESULTS

The purpose of conducting the bioassay with specific toxicants was to demonstrate the utility of the bioassay as well as to obtain some toxicity data. The initial concentrations of Hydrothal-191 used in range-finding tests were 0.1, 1.0, 10.0, and 100.0 ppm (Appendix B). Since initial results indicated a rapid onset of toxicity in the range of 1.0 to 10.0 ppm, concentrations in a narrower range were then utilized for a more precise assessment of toxicity (Appendix B). The EC₅₀ for Hydrothal-191 was computed from the dose-response curve as 1.4 ppm for L. minor and 2.1 ppm for S. polyrrhiza (Fig. 16). The toxicity curve indicates a "no-effect" region for both species and a rapid onset of toxicity.

Significantly higher concentrations of ammonium carbamate were necessary to produce a toxic effect on both L. minor and S. polyrrhiza. A range-finding test in the range of 0.1 to 100.0 ppm (Appendix C) produced toxic effects for both species between 100 and 1000 ppm. A bioassay with additional concentrations in this range showed an EC₅₀ of 205 ppm ammonium carbamate for L. minor and 196 ppm for S. polyrrhiza (Fig. 17).

L.A.S. was also found to exert a toxic effect on both L. minor and S. polyrrhiza. A range-finding test produced a dose-response curve indicating toxic effects in the range of 1.0 to 10.0 ppm L.A.S. (Appendix D). A bioassay testing
Fig. 16. Toxicity of Hydrothal-191 to two species of duckweed: \textit{L. minor} (EC$_{50}$ = 1.4 ppm) (●), \textit{S. polyrrhiza} (EC$_{50}$ = 2.1 ppm) (X)
Fig. 17. Toxicity of ammonium carbamate to two species of duckweed: \textit{L. minor} (EC$_{50}$ = 205 ppm)(●), \textit{S. polyrrhiza} (EC$_{50}$ = 196 ppm)(■).
concentrations between 0.1 and 10.0 ppm L.A.S. was conducted and the dose-response curve indicated an EC$_{50}$ of 2.0 ppm L.A.S. for *L. minor* and 4.1 ppm for *S. polyrrhiza* (Fig. 18). L.A.S. was also used as a test chemical by Bishop and Perry (13) in a dynamic system using *L. minor*. They found an EC$_{50}$ of 2.7 ppm using 1% Hutner's medium and a light intensity of 75 μE m$^{-2}$ sec$^{-1}$ (450 ft-c) (Table 5).
<table>
<thead>
<tr>
<th>Bioassay System</th>
<th>L.A.S. EC&lt;sub&gt;50&lt;/sub&gt; (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bishop's (12) dynamic system</td>
<td>2.7</td>
</tr>
<tr>
<td>Grant's static system</td>
<td>2.0</td>
</tr>
</tbody>
</table>
Fig. 18. Toxicity of Linear Alkylbenzene Sulfonate (L.A.S.) to two species of duckweed: L. minor (EC$_{50}$ = 2.0 ppm)(●), S. polyrrhiza (EC$_{50}$ = 4.1 ppm)(★).
DISCUSSION AND CONCLUSIONS

The bioassay technique presented uses growth optimized cultures of *L. minor* and *S. polyrrhiza*. The dose-response curve of Hydrothal-191 demonstrates the sensitivity of this bioassay. For routine use the feasibility of automated biomass determination was demonstrated with a photographic technique using a light-sensitive circuit for quantifying results. A statistical interpretation has been demonstrated which allows comparison of test and control groups in an unbiased manner. This statistical method utilizes linear regression analysis, analysis of variance, and Dunnett's test, and has been incorporated into a computer program.

The duckweed bioassay that has been presented is feasible for use in routine toxicity testing. Results obtained from these tests are useful in evaluating relative toxicity of new and existing chemicals and provide useful data in environmental hazard assessment. The environmental conditions which have been defined for this bioassay have shown higher rates of growth and greater sensitivity to a given toxicant (L.A.S.) than was previously reported by Bishop and Perry (13). Interestingly, *L. minor* exhibits a significantly lower EC<sub>50</sub> than *S. polyrrhiza* for Hydrothal-191 and L.A.S. (33% and 51% lower, respectively) while the two species are essentially equivalent in their response to ammonium carbamate. Additional comparative data needs to be obtained in order to
completely characterize the differences between the two species. A wide data base should be established using chemicals with known hazard potentials in order to carefully define results obtained with this bioassay relative to existing toxicity data. Concurrently, a dynamic system should be developed using similar environmental conditions defined by this static system that would enable the comparison of static and dynamic systems for the development of hazard assessment.
REFERENCES


APPENDIX A

BASIC COMPUTER PROGRAM

10  '**********************************************************************
20  '******   GARY GRANT       ******
30  '******   NOVEMBER 1980   ******
40  '**********************************************************************
50  '
60  '
70  'PROGRAM DEVELOPMENT WAS MADE POSSIBLE BY
75  '**********************************************************************
80  '******   THE LEARNING PLACE   ******
90  '******   DALLAS, TEXAS 75240   ******
95  '
100 '**********************************************************************
110 '*** LINEAR REGRESSION PROGRAM ***
120 '**********************************************************************
130  '
140 CLS:INPUT "ENTER NAME OF EXPERIMENT";TX$
150  GJ=LEN(TX$)
160  GJ=(82-GJ)/2
170  LPRINTTAB(GJ);TX$
180  FOR I=1 TO 5:LPRINT CHR$(138):NEXT I
190 '**********************************************************************
200 '*** VARIABLES & DEFINITIONS ***
210 '**********************************************************************
220  '
230  ' K=# OF TREATMENT GROUPS
240  ' NN=# OF REGRESSIONS ENTERED
250  ' N= # OF DATA POINTS IN CURRENT REGRESSION
260  ' SX,SY=SUMMATION OF X'S & Y'S FOR CURRENT REGRESSION
270  ' B= SLOPE OF CURRENT REGRESSION
280  ' A= Y-INTERCEPT OF CURRENT REGRESSION
290  ' V1= SUMMATION OF X[2]'S FOR CURRENT REGRESSION
300  ' V2= SUMMATION OF Y[2]'S FOR CURRENT REGRESSION
310  ' V3= TOTAL SUM OF SQUARES
320  ' V4= REGRESSION SUM OF SQUARES
330  ' V5= RESIDUAL SUM OF SQUARES
340  ' V6= X-BAR (AVERAGE X)
350  ' V7= Y-BAR (AVE. Y)
360  ' V8= SUMMATION OF X*Y
370  ' V9= SUMMATION OF SCRIPT X[2] ; (X(I)-X(BAR))
380  ' W1= SUMMATION OF SCRIPT X*Y
390  ' W2= SUMMATION OF SCRIPT Y[2
400  '
410  FG=0
420  CLEAR 250
DIM KK(29): 'ARRAY TO STORE # OF REGRESSIONS (REPLICATES)
' IN EACH TREATMENT GROUP.
DIM VA(20,10): 'ARRAY TO STORE CALCULATIONS FROM EACH RUN
DIM X2(20,10)
DIM X3(20,10): 'SS-residual
DIM X4(20,10): 'DF-residual
DIM XD(20): 'SAVED X's
KK(K)=NN
K=K+1: NN=0
CLS:PRINT @ 75,"ENTER NAME OF TREATMENT GROUP(CONC., etc.)";
INPUT T$(K): IF W$="N" THEN 560
FOR I=1 TO 4:LPRINT CHR$(138):NEXT I
FOR I=1 TO 80: LPRINT ";":NEXT I:LPRINT CHR$(138)
IF K>1 THEN 1540
' BEGIN DATA ENTRY
NN=NN+1
N=0:B=0:A=0:RR=0
SX=0:SY=0:VL=0:V2=0:V3=0:V4=0:V5=0:V6=0:V7=0
V8=0:V9=0:WL=0:W2=0
CLS
L=LEN(T$(K))
L=64-L
L=(L/2)+64
PRINT @ L,T$(K)
PRINT @ 202,"";
PRINT USING "ENTER DATA FOR REPLICATE NO. #":;NN
PRINT:PRINT:PRINT:PRINT
IF FG=1 THEN X=XD(N+1):GOTO 770
PRINT "ENTER X ('E' FOR END OF DATA)
PRINT ""( 'D' TO DELETE LAST POINT)";:INPUT W$
IF W$="E" THEN 900
IF W$="D" THEN 1620
X=VAL(W$)
XD(N+1)=X
IF XD(N+1)=-1 THEN 900
PRINT "ENTER Y"
Y=VAL(Y$): IF Y$="E" THEN 900
IF Y=0 THEN GOTO 780
Y=LOG(Y)
N=N+1
SX=SX+X
SY=SY+Y
VL=VL+(X*Y)
V2=V2+(Y*Y)
V8=V8+(X*Y)
PRINT:GOTO 700
'******************************************************************************
' *** END OF DATA - SUMMARIZE CURRENT REGRESSION ***
'******************************************************************************
940 XD(N+1)=-1:FG=0
950 V6= SX/N
960 V7=SY/N
970 V9= V1-((SX*SX)/N)
980 W1= V9-((SX*SY)/N)
990 W2= V2-((SY*SY)/N)
1000 B= W1/V9
1010 A= V7-(B*V5)
1020 V3=W2
1030 V4= ((W1*W1)/V9)
1040 V5= V3-V4
1050 IF V3=0 THEN RR=1: GOTO 1070
1060 RR=V4/V3
1070 '
1080 '**********************************************************************
1090 '*** STORE RESULTS IN ARRAY ***
1100 '**********************************************************************
1110 'KK(K)=# OF REPLICATES IN GROUP K
1120 'T$(K)=NAME OF K-th GROUP
1130 'VA(K,NN)=SLOPE FOR NN-th REPLICATE IN GROUP K
1140 VA(K,NN)=B
1150 X2(K,NN)=V9:'STOES SUMMATION OF SCRIPT X*X
1160 X3(K,NN)=V5
1170 X4(K,NN)=N-2
1180 '
1190 '**********************************************************************
1200 '*** PRINT OUT RESULTS OF CURRENT REGRESSION ***
1210 '**********************************************************************
1220 '
1230 L=LEN(T$(K))
1240 L=64-(L+6)
1250 L=L/2
1260 CLS
1270 PRINT @ 197,"N=  ";N
1280 PRINT @ 261,"SLOPE=  ";B
1290 PRINT @ 325,"Y-INT.=  ";A
1300 PRINT @ 389,"R-squared=":RR
1310 PRINT:PRINT "HARDCOPY OF REGRESSION RESULTS (Y/N)"
1320 WS=INKEY$:IF W$="" THEN 1320
1330 IF WS="N" THEN 1480
1340 LPRINTTAB(10) "GROUP :";T$(K)
1350 LPRINTTAB(10) "REPLICATE #:";NN
1360 LPRINTTAB(10) "**************"
1370 LPRINT CHR$(138)
1380 LPRINTTAB(10) "N=  ";N
1390 LPRINTTAB(10) "SLOPE=  ";B
1400 LPRINTTAB(10) "Y-INT.=  ";A
1410 LPRINTTAB(10) "R-squared=":RR
1420 FOR JJ=1 TO 50
1430 LPRINT "*";
1440 NEXT JJ
1450 LPRINT CHR$(138):LPRINT CHR$(138):LPRINT CHR$(138)
1460 PRINT @ 725,"HIT ANY KEY TO CONTINUE"
1470 WS=INKEY$: IF WS="" THEN 1470
1480 CLS: PRINT @ 138,"1.) ENTER ANOTHER REPPLICATE,SAME TREATMENT GROUP"
1490 PRINT @ 202,"2.) ENTER NEXT TREATMENT GROUP"
1500 PRINT @ 266,"3.) END OF DATA- PERFORM ANALYSIS OF VARIANCE"
1510 PRINT @ 330,"4.) ABORT PROGRAM"
1520 PRINT @ 798,"SELECT OPTION:";INPUT W
1530 ON W GOTO 1540 ,1580 ,1690 ,4130
1540 CLS
1550 PRINT @ 138,"SAME X'S (Y/N):"; WS=INKEY$: IF WS="" THEN 1550
1560 IF WS="Y" THEN FG=1
1570 GOTO 580
1580 CLS
1590 PRINT @ 138,"SAME X'S (Y/N):"; WS=INKEY$: IF WS="" THEN 1590
1600 IF WS="Y" THEN FG=1
1610 GOTO 500
1620 
1630 '**** DELETE LAST DATA POINT ****
1640 
1650 N=N-1
1660 PRINT:PRINT "LAST POINT DELETED"
1670 PRINT
1680 GOTO 710
1690 '**************************************************************************
1700 '*** A.N.O.V.A. SECTION ***
1710 '**************************************************************************
1720 
1730 FOR I=1 TO 10: LPRINT CHR$(138):NEXT I
1740 KK(K)=NN
1750 
1760 'PRINT OUT OF DATA
1770 L=LEN(TX$)
1780 L=(84-L)/2
1790 LPRINTTAB(L),TX$
1800 LPRINT CHR$(138):LPRINTTAB(22) "ANOVA FOR GROWTH RATE (SLOPES) COMPARISON"
1810 FOR I=1 TO K
1820 LPRINT CHR$(138):FOR M=1 TO 80
1830 LPRINT "**";
1840 NEXT M
1850 LPRINT CHR$(138)
1860 LPRINT "GROUP:";T$(I)
1870 LPRINT CHR$(138)
1880 LPRINT " SLOPES:"
1890 FOR J=1 TO KK(I) STEP 3
1900 IF KK(I)+1-J=2 THEN 1950
1910 IF KK(I)+1-J=1 THEN 1970
1920 LPRINTTAB(7);VA(I,J);TAB(7);VA(I,J+1);TAB(7);VA(I,J+2)
1930 NEXT J
1940 GOTO 1990
1950 LPRINTTAB(7);VA(I,J);TAB(7);VA(I,J+1)
1960 GOTO 1930
1970 LPRINTTAB(7);VA(I,J)
1980 GOTO 1930
1990 NEXT I
2000 LPRINT CHR$(138):FOR M=1 TO 80
2010 LPRINT "*" ;
2020 NEXT M
2030 FOR I=1 TO K
2040 A1=A1+KK(I)
2050 FOR J=1 TO KK(I)
2060 A2=A2+VA(I,J)
2070 A3=A3+(VA(I,J)*VA(I,J))
2080 A4=A4+VA(I,J)
2090 NEXT J
2100 A5=A5+(A4*A4/KK(I))
2110 A4=0
2120 NEXT I
2130 A6=A1-1
2140 A7=K-1
2150 A8=A1-K
2160 A9=(A2*A2)/A1
2170 B1=A3-A9
2180 B2=A5-A9
2190 B3=B1-B2
2200 B4=B2/A7
2210 B5=B3/A8
2220 F=B4/B5
2230 GOSUB 3730 : ' TO GET F-critical (F-.05(2),DF-gps.,DF-err.)
2240 GOSUB 3830
2250 IF F<FC THEN 2330 : ' SLOPES ARE EQUAL
2260 CLS:LPRINT CHR$(138);LPRINT CHR$(138);PRINT:PRINT:PRINT
2270 PRINTTAB(10);
2280 PRINT USING "F= ##### F(.05(2),##,##,##,##,##). =
#####";F;A7;A8;FC
2290 PRINTTAB(10) "H : GROWTH RATES ARE ALL EQUAL >>** REJECTED
**<<"
2300 PRINTTAB(20) " ** GROWTH RATES ARE NOT ALL EQUAL **"
2310 LPRINT CHR$(138)
2320 GOTO 2430
2330 ' SLOPES ARE ALL EQUAL
2340 CLS:PRINT:PRINT:LPRINT CHR$(138):LPRINT CHR$(138)
2350 PRINTTAB(10);
2360 PRINT USING "F= ##### F(.05(2),##,##,##,##)
#####";F;A7;A8;FC
2370 PRINT:PRINTTAB(10) "H : THE GROWTH RATES ARE ALL EQUAL ***
ACCEPTED ***
2380 LPRINTTAB(10) "F= ";F
2390 LPRINTTAB(10) "F(.05(2),";A7;"",";A8;")=";FC
2400 LPRINT CHR$(138):LPRINTTAB(10) "Ho: GROWTH RATES ARE ALL
EQUAL  **** ACCEPTED ****"
2410 LPRINT CHR$(138)
2420 GOTO 4130
2430 PRINT @ 852,"HIT ANY KEY TO CONTINUE"
2440 WS=INKEY$: IF W$="" THEN 2440
2450 CE
2460 PRINT:PRINTTAB(10) "1.) PERFORM DUNNETT'S TEST ON DATA"
2470 PRINTTAB(10) "2.) END PROGRAM"
2480 INPUT W
2490 ON W GOTO 2510 ,4130
2500 GOTO 2480
2510 
2520 '...........................................
2530 '*** DUNNETT'S TEST ***
2540 '...........................................
2550 GG=1
2560 'C1=SS-residual- CONTROL GROUP
2570 'C2=SS-residual- K-th GROUP
2580 'C3=DF-residual- CONTROL GROUP
2590 'C4=DF-residual- K-th GROUP
2600 'C5=S-y.x-squared, pooled
2610 ',
2620 FOR I=1 TO KK(1)
2630 C1=C1+X3(I,1)
2640 C3=C3+X4(I,1)
2650 C5=C5+X2(I,1)
2660 NEXT I
2670 FOR M=1 TO K
2680 SM=0
2690 FOR N=1 TO KK(M)
2700 SM=SM+VA(M,N)
2710 NEXT N
2720 AV(M)=SM/KK(M)
2730 NEXT M
2740 'AV(M) CONTAINS X-bar FOR EACH GROUP
2750 ',
2760 '...........................................
2770 ' RANK ORDERING ARRAY AV(K) *
2780 '...........................................
2790 ',
2800 Q1=AV(1)
2810 FOR II= 1 TO (K-1)
2820 FOR JJ= (II+1) TO K
2830 IF AV(II)<= AV(JJ) THEN 2870
2840 SA=AV(II); VS=TS(II); RR= KK(II)
2850 AV(II)=AV(JJ); TS(II)=TS(JJ); KK(II)=KK(JJ)
2860 AV(JJ)=SA; TS(JJ)=VS; KK(JJ)=RR
2870 NEXT JJ
2880 NEXT II
2890 '*** SEARCH FOR NEW RANK OF CONTROL AVERAGE
2900 '  
2910 FOR II= 1 TO K  
2920 IF Q1=AV(II) THEN Q1=II:GOTO 2940  
2930 NEXT II  
2940 ' Q1 = ORDERED RANK OF CONTROL GROUP **  
2950 LPRINTTAB(31) "**************************"  
2960 LPRINTTAB(31) "* DUNNETT'S TEST *"  
2970 LPRINTTAB(31) "**************************"  
2980 '  
2990 FOR LL=1 TO 5  
3000 LPRINT CHR$(138)  
3010 NEXT LL  
3020 LPRINT "GROUP MEANS :"  
3030 FOR J=1 TO K  
3040 LPRINTTAB(14),AV(J),J,TAB(5),T$(J)  
3050 LPRINT CHR$(138)  
3060 NEXT J  
3070 LPRINT CHR$(138):LPRINT CHR$(138)  
3080 LPRINTTAB(30);  
3090 FOR J=1 TO 20  
3100 LPRINT ";";  
3110 NEXT J  
3120 LPRINT CHR$(138)  
3130 LPRINTTAB(5) "Ho : growth rate (CONTROL) = growth rate  
3140 LPRINT CHR$(138):LPRINT CHR$(138)  
3150 FOR I=1 TO 82:LPRINT ";";NEXT I  
3160 FOR J=1 TO K  
3170 IF J=Q1 THEN 3360  
3180 LPRINT "COMPARISON : CONTROL vs. #";J  
3190 DL=AV(Q1)-AV(J)  
3200 SE=SQRT((1/KK(Q1))+(1/KK(J)))  
3210 SE =SQR(SE)  
3220 QP=DL/SE  
3230 QP=ABS(QP)  
3240 PP=J-Q1  
3250 PP=ABS(PP)+1  
3260 GOSUB 3620  
3270 LPRINT CHR$(138)  
3280 LPRINTTAB(5) "difference =";DL  
3290 LPRINTTAB(5) "SE =";SE  
3300 LPRINTTAB(5) "ABS(q) =";QP  
3310 LPRINTTAB(5) "p =";PP  
3320 LPRINTTAB(5) "ABS(q) =";QP  
3330 LPRINTTAB(5) "q .05(2),";AZ;",";PP;" =";QC  
3340 IF QP>QC THEN GOSUB 3380 : ' REJECT Ho  
3350 IF QP<QC THEN GOSUB 3500 : ' ACCEPT Ho  
3360 NEXT J  
3370 END  
3380 ' DUNNETT'S TEST -- REJECT Ho  
3390 '
3400 LPRINT CHR$(138)
3410 LPRINTTAB(15) "**************************"
3420 LPRINTTAB(15) "* REJECT H0 *"
3430 LPRINTTAB(15) "**************************"
3440 LPRINT CHR$(138)
3450 FOR LL=1 TO 80
3460 LPRINT "*";
3470 NEXT LL
3480 LPRINT CHR$(138);LPRINT CHR$(138)
3490 RETURN
3500 ' DUNNETT'S TEST -- ACCEPT H0
3510 '
3520 LPRINT CHR$(138)
3530 LPRINTTAB(15) "**************************"
3540 LPRINTTAB(15) "* ACCEPT H0 *"
3550 LPRINTTAB(15) "**************************"
3560 LPRINT CHR$(138)
3570 FOR LL=1 TO 80
3580 LPRINT "*";
3590 NEXT LL
3600 LPRINT CHR$(138); LPRINT CHR$(138)
3610 RETURN
3620 ' SUBROUTINE TO GET q'
3630 '
3640 AZ=0
3650 FOR II=1 TO K
3660 AZ=AZ+KK(II)
3670 NEXT II
3680 AZ=AZ-K
3690 CLS
3700 PRINT:PRINT
3710 PRINT "ENTER q'.05(2),";AZ",";PP" ; "";INPUT QC
3720 RETURN
3730 '****************************
3740 '*** F-critical TABLE ***
3750 '****************************
3760 '
3770 CLS:PRINT @ 138,"n-1 =",A7
3780 PRINT @ 202,"n-2 =",A8
3790 PRINT:PRINT
3800 PRINT "ENTER F-(.05(2),n-1,n-2)"; INPUT FC
3810 RETURN
3820 GOTO 4130
3830 '****************************
3840 '*** PRINTOUT OF ANOVA SUMMARY ***
3850 '****************************
3860 '
3870 LPRINT CHR$(138);LPRINT CHR$(138);LPRINT CHR$(138)
3880 LPRINTTAB(31),"**************************"
3890 LPRINTTAB(31),"* ANOVA SUMMARY *"
3900 LPRINTTAB(31),"**************************"
3910 LPRINT CHR$(138)
3920 LPRINT "SOURCE OF"
3930 LPRINT "VARIATION       SS   DF"
3940 FOR LL=1 TO 80
3950 LPRINT "-";
3960 NEXT LL
3970 LPRINT CHR$(138)
3980 LPRINT "TOTAL ",BL,"   ",A6
3990 LPRINT "GROUPS ",B2,"   ",A7,B4
4000 LPRINT "ERROR ",B3,"   ",A8,B5
4010 LPRINT CHR$(138)
4020 FOR LL=1 TO 80
4030 LPRINT "-";
4040 NEXT LL
4050 LPRINT CHR$(138)
4060 LPRINTTAB(5) "F =",F
4070 LPRINTTAB(5) "F.05(2),k-1,N-k =";FC
4080 LPRINTTAB(6) "k-1 =A7"
4090 LPRINTTAB(6) "N-k =A8"
4100 IF F>FC THEN LPRINTTAB(10) "*** REJECT Ho *** GROWTH RATES ARE NOT ALL EQUAL";GOTO 4120
4110 LPRINTTAB(10) "*** ACCEPT Ho *** GROWTH RATES ARE ALL EQUAL"
4120 RETURN
4130 END
APPENDIX B

RAW DATA

Hydrothal-191 Toxicity Tests

Toxicity of Hydrothal-191 to *L. minor*

<table>
<thead>
<tr>
<th>Hydrothal-191 Concentration (ppm)</th>
<th>Growth Rate $\mu$ (hr$^{-1}$)</th>
<th>Td (days)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0216598 ± 0.001499</td>
<td>0.58 ± 0.04</td>
<td>100</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0209965 ± 0.001754</td>
<td>0.60 ± 0.05</td>
<td>96.9</td>
</tr>
<tr>
<td>0.3</td>
<td>0.019726 ± 0.001500</td>
<td>0.64 ± 0.04</td>
<td>91.1</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0204939 ± 0.001500</td>
<td>0.61 ± 0.08</td>
<td>94.6</td>
</tr>
<tr>
<td>0.7</td>
<td>0.0192673 ± 0.000722</td>
<td>0.65 ± 0.03</td>
<td>89.0</td>
</tr>
<tr>
<td>1.0*</td>
<td>0.014698 ± 0.0013131</td>
<td>0.85 ± 0.08</td>
<td>67.9</td>
</tr>
<tr>
<td>2.0*</td>
<td>0.004112 ± 0.0016867</td>
<td>3.05 ± 1.5</td>
<td>19.0</td>
</tr>
<tr>
<td>3.0</td>
<td>--</td>
<td>--</td>
<td>0</td>
</tr>
<tr>
<td>10.0</td>
<td>--</td>
<td>--</td>
<td>0</td>
</tr>
</tbody>
</table>

* Significant difference detected at alpha = 0.05
  EC$_{50}$ = 1.4 ppm
APPENDIX B (cont'd)

Toxicity of Hydrothal-191 to *S. polyrrhiza*

<table>
<thead>
<tr>
<th>Hydrothal-191 Concentration (ppm)</th>
<th>Growth Rate ( \mu ) (hr(^{-1}))</th>
<th>Td (days)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0124483 ± 0.0017819</td>
<td>1.01 ± 0.15</td>
<td>100</td>
</tr>
<tr>
<td>0.1</td>
<td>0.015076 ± 0.0011181</td>
<td>0.83 ± 0.07</td>
<td>82.6</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0132441 ± 0.0009945</td>
<td>0.95 ± 0.07</td>
<td>100.1</td>
</tr>
<tr>
<td>0.7</td>
<td>0.0135936 ± 0.0014844</td>
<td>0.92 ± 0.08</td>
<td>100.1</td>
</tr>
<tr>
<td>1.0</td>
<td>0.0105068 ± 0.001641</td>
<td>1.19 ± 0.19</td>
<td>84.4</td>
</tr>
<tr>
<td>2.0*</td>
<td>0.0066645 ± 0.001027</td>
<td>1.88 ± 0.30</td>
<td>53.5</td>
</tr>
<tr>
<td>5.0</td>
<td>--</td>
<td>--</td>
<td>0</td>
</tr>
</tbody>
</table>

* Significant difference detected at alpha = 0.05

EC\(_{50}\) = 2.1 ppm
APPENDIX C

RAW DATA

Ammonium Carbamate Toxicity Tests

Toxicity of Ammonium Carbamate to L. minor

<table>
<thead>
<tr>
<th>Ammonium Carbamate Concentration (ppm)</th>
<th>Growth Rate μ (hr⁻¹)</th>
<th>Td (days)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.019614 ± 0.0012404</td>
<td>0.64 ± 0.04</td>
<td>100.0</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0200513 ± 0.0011975</td>
<td>0.63 ± 0.04</td>
<td>102.2</td>
</tr>
<tr>
<td>1.0</td>
<td>0.0207745 ± 0.0004138</td>
<td>0.60 ± 0.01</td>
<td>105.9</td>
</tr>
<tr>
<td>5.0</td>
<td>0.0204351 ± 0.0016467</td>
<td>0.61 ± 0.02</td>
<td>104.2</td>
</tr>
<tr>
<td>10.0</td>
<td>0.0197316 ± 0.0010346</td>
<td>0.64 ± 0.02</td>
<td>100.6</td>
</tr>
<tr>
<td>50.0</td>
<td>0.197116 ± 0.002091</td>
<td>0.64 ± 0.03</td>
<td>100.5</td>
</tr>
<tr>
<td>100.0</td>
<td>0.01684 ± 0.0001024</td>
<td>0.74 ± 0.01</td>
<td>85.9</td>
</tr>
<tr>
<td>75.0</td>
<td>0.0182873 ± 0.0007259</td>
<td>0.69 ± 0.02</td>
<td>93.2</td>
</tr>
<tr>
<td>150.0</td>
<td>0.0187176 ± 0.0077693</td>
<td>0.67 ± 0.01</td>
<td>91.0</td>
</tr>
<tr>
<td>125.0</td>
<td>0.0193829 ± 0.0007174</td>
<td>0.65 ± 0.02</td>
<td>93.8</td>
</tr>
<tr>
<td>175.0</td>
<td>0.0147541 ± 0.0015376</td>
<td>0.85 ± 0.04</td>
<td>71.4</td>
</tr>
<tr>
<td>250.0*</td>
<td>0.0037182 ± 0.0048097</td>
<td>3.37 ± 0.95</td>
<td>18.0</td>
</tr>
</tbody>
</table>

* Significant difference at alpha = 0.05
EC₅₀ = 205 ppm
APPENDIX C (cont'd)

Toxicity of Ammonium Carbamate to *S. polyrrhiza*

<table>
<thead>
<tr>
<th>Ammonium Carbamate Concentration (ppm)</th>
<th>Growth Rate $\mu$ (hr$^{-1}$)</th>
<th>$T_d$ (days)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0240956 ± 0.001726</td>
<td>0.89 ± 0.05</td>
<td>100.0</td>
</tr>
<tr>
<td>50.0</td>
<td>0.0141151 ± 0.0012457</td>
<td>0.89 ± 0.04</td>
<td>100.1</td>
</tr>
<tr>
<td>100.0</td>
<td>0.0126885 ± 0.000719</td>
<td>0.99 ± 0.03</td>
<td>90.0</td>
</tr>
<tr>
<td>125.0</td>
<td>0.0128655 ± 0.0015166</td>
<td>0.97 ± 0.05</td>
<td>91.3</td>
</tr>
<tr>
<td>150.0*</td>
<td>0.0100446 ± 0.0012019</td>
<td>1.25 ± 0.07</td>
<td>71.3</td>
</tr>
<tr>
<td>175.0*</td>
<td>0.008118 ± 0.0016971</td>
<td>1.55 ± 0.14</td>
<td>57.6</td>
</tr>
<tr>
<td>Control-2</td>
<td>0.0163411 ± 0.0007917</td>
<td>0.77 ± 0.02</td>
<td>100.0</td>
</tr>
<tr>
<td>75.0</td>
<td>0.0164541 ± 0.0010708</td>
<td>0.76 ± 0.02</td>
<td>99.3</td>
</tr>
<tr>
<td>250.0*</td>
<td>0.005067 ± 0.000803</td>
<td>2.48 ± 0.17</td>
<td>31.0</td>
</tr>
</tbody>
</table>

* Significant difference at alpha = 0.05

EC$_{50}$ = 196 ppm
APPENDIX D

RAW DATA

Linear Alkylbenzene Sulfonate Toxicity Tests

Toxicity of L.A.S. to L. minor

<table>
<thead>
<tr>
<th>L.A.S. Concentration (ppm)</th>
<th>Growth Rate μ (hr⁻¹)</th>
<th>Td (days)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0223026 ± 0.001394</td>
<td>0.56 ± 0.04</td>
<td>100.0</td>
</tr>
<tr>
<td>0.1</td>
<td>0.021009 ± 0.001050</td>
<td>0.60 ± 0.03</td>
<td>94.2</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0207637 ± 0.001050</td>
<td>0.06 ± 0.05</td>
<td>93.1</td>
</tr>
<tr>
<td>1.0</td>
<td>0.0196262 ± 0.001227</td>
<td>0.64 ± 0.04</td>
<td>88.0</td>
</tr>
<tr>
<td>2.0*</td>
<td>0.0117534 ± 0.001098</td>
<td>1.07 ± 0.10</td>
<td>52.7</td>
</tr>
<tr>
<td>3.0*</td>
<td>0.0027432 ± 0.000552</td>
<td>4.57 ± 0.92</td>
<td>12.3</td>
</tr>
<tr>
<td>5.0*</td>
<td>--</td>
<td>--</td>
<td>0</td>
</tr>
<tr>
<td>10.0*</td>
<td>--</td>
<td>--</td>
<td>0</td>
</tr>
</tbody>
</table>

* Significant difference at alpha = 0.05
EC₅₀ = 2.0 ppm
APPENDIX D (cont'd)

Toxicity of L.A.S. to *S. polyrrhiza*

<table>
<thead>
<tr>
<th>L.A.S. Concentration (ppm)</th>
<th>Growth Rate $\mu$ (hr$^{-1}$)</th>
<th>$T_d$ (days)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0114024 ± 0.000622</td>
<td>1.10 ± 0.06</td>
<td>100.0</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0112542 ± 0.000507</td>
<td>1.11 ± 0.05</td>
<td>98.7</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0110831 ± 0.000392</td>
<td>1.13 ± 0.04</td>
<td>97.2</td>
</tr>
<tr>
<td>1.0</td>
<td>0.0108779 ± 0.000662</td>
<td>1.15 ± 0.07</td>
<td>95.4</td>
</tr>
<tr>
<td>2.0</td>
<td>0.0089282 ± 0.000701</td>
<td>1.40 ± 0.11</td>
<td>78.3</td>
</tr>
<tr>
<td>3.0*</td>
<td>0.0073203 ± 0.000642</td>
<td>1.71 ± 0.15</td>
<td>64.2</td>
</tr>
<tr>
<td>5.0*</td>
<td>0.003968 ± 0.000779</td>
<td>3.16 ± 0.62</td>
<td>34.8</td>
</tr>
<tr>
<td>10.0*</td>
<td>--</td>
<td>--</td>
<td>0</td>
</tr>
<tr>
<td>100.0*</td>
<td>--</td>
<td>--</td>
<td>0</td>
</tr>
</tbody>
</table>

* Significant difference at alpha = 0.05
EC$_{50}$ = 4.1 ppm