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THE USE OF STIMULABLE BIOLUMINESCENCE FROM MARINE DINOFLAGELLATES AS A MEANS OF DETECTING TOXICITY IN THE MARINE ENVIRONMENT.

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ABSTRACT: Phytoplankton bioassays have been used as biological tools in assessing environmental impact from contaminants. Series of experiments were designed to measure the acute and sublethal effects of heavy metals (tributyltin, copper, and zinc) and storm drain effluent on the light output from marine bioluminescent dinoflagellates (Pyrocystis lunula in earlier experiments and Gonyaulax polyedra in later experiments). Cultured cells were exposed to various concentrations of a metal or storm drain effluent from hours up to 11 days. Measurable differences in light output have been observed in as little as 3 h when compared to control cells.

KEYWORDS: bioluminescence, toxicity testing, dinoflagellates, metals, storm drain effluent

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Phytoplankton bioassays have been used as biological tools in assessing environmental contamination. The use of these organisms in bioassays is justified by the ecological role they play as primary producers with respect to other trophic levels, and their inherent sensitivity to toxic chemicals (Salazar 1985; Walsh et al. 1985). Also, phytoplankton bioassays tend to be simple, rapid, and inexpensive when compared to more complicated and involved assays using fish and invertebrate species.

Historically, phytoplankton bioassays have involved the enumeration of phytoplankton cells to determine stress in algal populations when exposed to a single toxicant or chemical mixture (Lapota et al. 1989). These assays have been successful, but can be labor intensive. Therefore, other toxicological endpoints for the measurement of toxicity in phytoplankton are being investigated. In our laboratory, a bioassay has been developed which measures the light output from bioluminescent dinoflagellates for assessment of toxic effects when exposed to a single toxicant or mixture. Bioluminescence is a visible blue light produced either intermittently or continuously by numerous terrestrial and aquatic organisms. Many of the marine dinoflagellate species are able to produce bioluminescence as part of their daily physiological processes. Similarly, some marine bacteria also emit this light. Early observations indicated that the presence of various toxicants reduced the light intensity of bioluminescent bacterial cultures (Beijerinck 1889). The utility of bioluminescent bacteria as a test organism has since been investigated in testing air quality (Sie and Thanos 1966), herbicides (Tchan and Chiou 1977), and other toxic chemicals (Johnson et al. 1942). A bacterial bioluminescent assay (using freeze-dried cultures of a cold water bacterium) has been developed for the detection of toxic compounds (Bulich 1979).

Only recently has work been initiated using bioluminescent dinoflagellates as bioassay organisms. It was observed that exposure to toxic compounds quenched the bioluminescence from cultures of the dinoflagellate Pyrocystis lunula by as much as 40 percent following a 2-h exposure (Hannan et al. 1986). This species has also been used in acute assays to detect toxicity from drilling muds (Stiffey, pers. comm.). In our laboratory, successful use of this type of bioassay has provided data on the acute response and has demonstrated the chronic effects, from hours up to 11 days, on dinoflagellate cells of Pyrocystis lunula and Gonyaulax polyedra upon exposure to several metals and storm drain effluent. In this paper, the methods and preliminary results of these tests using stimulable bioluminescence as an endpoint for the detection of toxicity will be discussed.

MATERIALS AND METHODS

Materials

Materials used were as follows: reagent grade copper(II)sulfate pentahydrate and zinc sulfate heptahydrate (Aldrich Chemical Co.); tributyltin chloride (Aldrich Chemical Co.); American Society for Testing and Materials sea-salts (Lake Products Co.).

Culture and Bioassay Containers

Optical grade spectrophotometric cuvettes and polycarbonate flasks were used. All cuvettes and polycarbonate flasks were seawater aged for several days prior to first time use. Containers used in experiments were routinely soaked in RBS (critical cleaner) for 24 h and then soaked in 4N nitric acid for 4 h.

Bioassay Organisms

Two species of marine bioluminescent dinoflagellates were used in the assays. Pyrocystis lunula was obtained from the North East Pacific Culture collection at the University of British Columbia, Canada. The other species, Gonyalaux polyedra, was isolated from San Diego Bay. Both species were maintained in enriched seawater medium (ESM) according to American Society for Testing and Materials Standard Guide for Conducting Static 96-h Toxicity Tests with Microalgae (ASTM 1990). Tetrasodium ethylenediaminetetraacetic acid (EDTA), a chelator, was removed from preparation of the ESM during assays (Davey et al. 1970). Cultures were maintained in 250-mL polycarbonate flasks under a light regime of 12:12 h (light:dark) at approximately 100 fc from cool white bulbs. The cells' day-night cycle was reversed to accommodate daytime testing while the cells were in their night phase and most stimulable for light production. Cultures of P. lunula were maintained at a room temperature of 22°C prior to and during the assay; where as, G. polyedra was maintained at 18°C. Typically, P. lunula was cultured at approximately 3 to 4 x 10^3 cells/mL with medium changes at 1 month intervals. G. polyedra was cultured at twice this density with medium changes weekly due to their higher cell division rates.

Experimental Design

Dinoflagellate cells were exposed to various concentrations of tributyltin chloride (TBTCl), copper (II) sulfate (Cu_2SO_4), zinc sulfate ($ZnSO_4$) or storm drain

effluent. P. lunula was used in earlier experiments with TBTCl, storm drain effluent, and Cu_2SO_4; whereas, G. polyedra was tested in later experiments with Cu_2SO_4 and $ZnSO_4$. P. lunula was eliminated from further testing because it is an open ocean species and therefore may not be representative of species found in polluted bay and coastal waters. Consequently, G. polyedra was adopted for use in the assays because of its wide distribution in bay and coastal waters of most continents.

The emphasis of this study also shifted to the use of metals recommended by the Environmental Protection Agency for the standardization of bioassays. The range of metal concentrations tested was primarily determined based on the known sensitivities of other species.

A working solution of test article (test metal or storm drain effluent) or deionized water (for controls), seawater medium, and dinoflagellate cells was prepared for each experimental concentration and control. Aliquots of the working solution were delivered to cuvettes (test chambers) and light output measured. Assays ranged in duration from 3 to 11 days.

Three hundred cells per cuvette were used in all experiments. This number was determined from previous experiments in which light output vs. cell number in P. lunula was measured. The results indicated that as light output increased with cell number the coefficient of variation (C.V.) decreased (Fig. 1). A C.V. of approximately 10% was obtained using 300 cells; therefore, this cell number was adopted for use in the assay.

Determination of stock culture density

At the beginning of each assay, the stock culture density was determined by pipetting 1 mL of stock culture into 25 mL of medium (stock cultures were diluted for ease of enumeration). Subsamples of the dilute stock solution, in 1-mL aliquots, were pipetted into settling chambers which contained a 5% buffered formalin solution. The subsamples were enumerated in the settling chambers with an Olympus IMT-2 inverted microscope at 40x magnification. A total of 4 subsamples were enumerated and the mean calculated. The mean cell number was then multiplied by the dilution factor for determination of stock density.

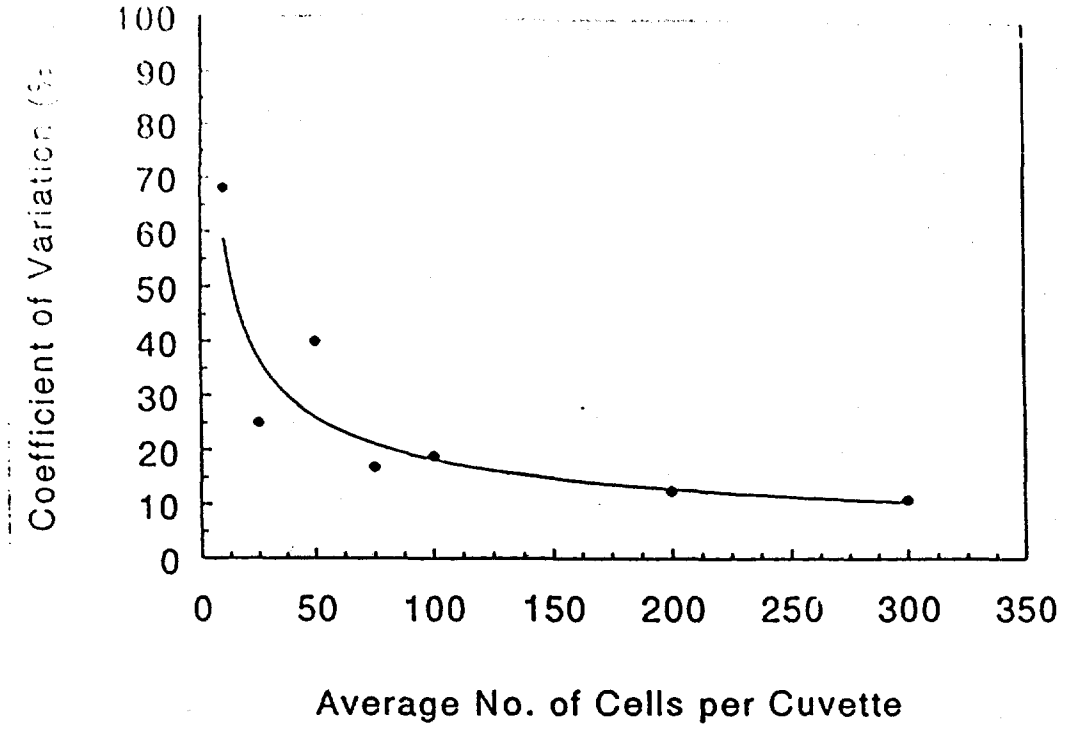

Fig. 1--Pyrocystis lunula. The coefficient of variation (%) of mean light output versus the number of cells stimulated per cuvette.

Exposure Technique

Calculations were made to determine the volume of stock culture to use in order to obtain a desired cell concentration of 100 cells/mL. The following equation was used to determine the volume of stock culture to add to the working solution:

$$V_s = C_f V_f / C_s \qquad (1)$$

Where,

V_s = volume of stock solution to add to final volume of working solution, mL,

C_f = concentration of cells desired in cuvettes, 100 cells/mL,

V_f = final volume of working solution desired, mL,

C_s = concentration of dinoflagellate stock solution, cells/mL.

Enough medium per flask was used to accommodate 3 mL per cuvette, 4 replicate cuvettes, and the number of test periods within an assay. Appropriate volumes of metal solutions or storm drain effluent were added to make up test concentrations. All metal stock solutions were prepared in deionized water. It was unnecessary to adjust the salinity of the stock metal solutions due to the small volume used (≤1 mL). In contrast, the salinity of the storm drain effluent was adjusted to 33 ‰ (parts per thousand) with ASTM salts prior to delivery.

The dinoflagellate stock solution was added to the working solution after the addition of test article or deionized water and gently mixed. The working solution plus dinoflagellate cells were delivered to cuvettes to a total volume of 3 mL at approximately 300 cells per cuvette. Cuvettes were maintained at temperature and light regime as previously described. Stimulable bioluminescence was measured at each test period (i.e., 3 or 4 h, 24 h, 48 h, 72 h, etc.) for all assays; however, the time of the test periods varied between some assays. Cells were kept in the dark for 3 or 4 h prior to testing. Dinoflagellate cells within cuvettes were discarded after each test period.

Chemical Analyses

Samples of the working solutions were frozen at the beginning of some of the assays to confirm initial metal concentrations. This was conducted to verify the accuracy of added metal concentrations. TBTCl was measured by purge and trap hydride derivatization followed by atomic absorption spectrophotometry (Stallard et al. 1989). The other metals were analyzed by standard EPA protocol (EPA Method 6010).

Instrumentation used for Measuring Bioluminescence

The laboratory plankton test chamber (LPTC) measures light output from either individual bioluminescent marine plankters or from cultures of bioluminescent plankton (Lapota and Losee 1984). Many of the dinoflagellate species require a mechanical stimulus to emit light. Consequently, the chamber lid of the LPTC was fitted with a direct drive stirring system for creating shear within the vials (Fig. 2).

The stainless steel shaft terminates into a 6mm teflon® propeller and, when seated into the cuvettes or vials, extends approximately 2/3 into the volume for complete mixing. A variable controller drives the stirrer at approximately 2500 r/min. This stirring rate was observed to stimulate maximum bioluminescence from dinoflagellates in our preliminary studies (Lapota, unpublished data).

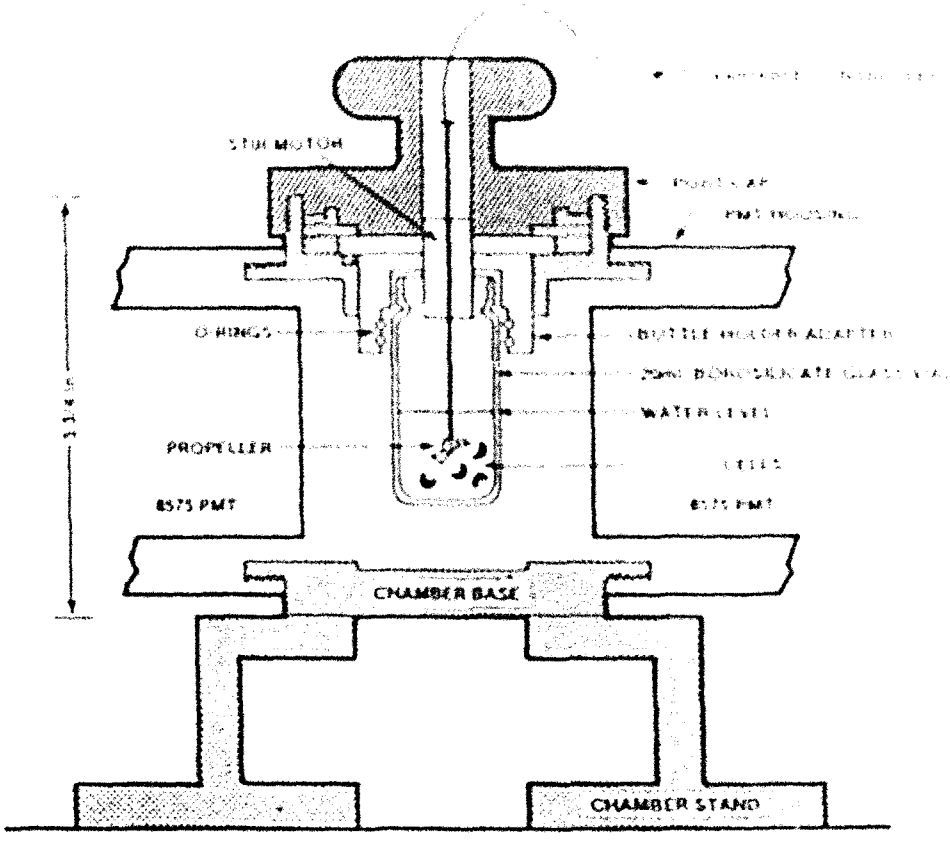

Fig. 2--Schematic view of the Laboratory Plankton Test Chamber. Dinoflagellate cells are maintained in glass vials or cuvettes filled with medium and stimulated to emit light upon mechanical stirring. The photomultiplier tubes (PMTs) view and integrate the light output from the cells.

Attached to the side of the chamber were horizontally mounted RCA 8575 photomultiplier tubes (PMTs) with an S-20 response. The PMT operates in the photon count mode with the output displayed on a multichannel analyzer as a time sequence of counts (1-s time channels) from the integrated light production (Fig. 3). The instantaneous intensity of the signal is proportional to the number of counts in each channel. The PMT signal is also diverted into a scaler/timer which integrates PMT counts for a 100-s measurement period (most of the light signal is produced within the first 50 s). The multichannel analyzer displayed the kinetics of the light output from the dinoflagellates while the scaler count provided the total number of PMT counts from the measurement period. Total number of PMT counts were recorded per cuvette.

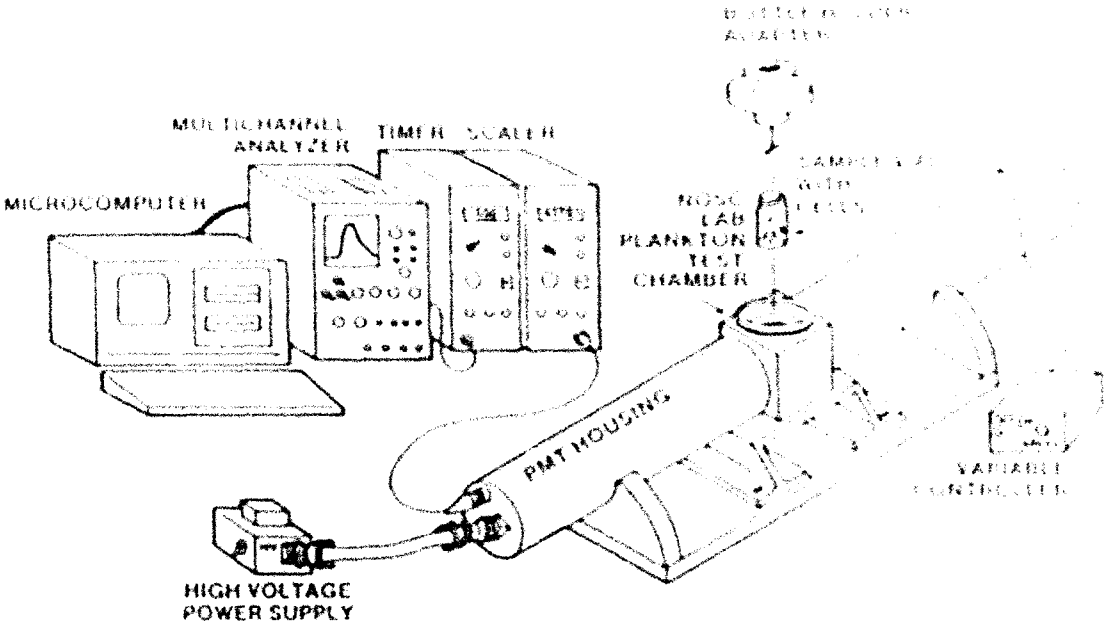

Fig. 3--Schematic of instrumentation used to measure bioluminescence from dinoflagellate cells. The light from the cells is measured by PMTs and the signal intensity is integrated by the scaler. The integrated light output from the cells can be displayed on the multichannel analyzer and recorded on a microcomputer.

Data Analyses

Mean light output (PMT counts) and the coefficient of variation were calculated for experimental and control groups. A statistical software package was used to perform Dunnett's test for determining the statistical significance between experimental and control means within an assay. Experimental means were graphically displayed as the percent change in light output of the control mean over time. Data were plotted with a cubic spline fit program to observe acute and chronic light reduction trends. An IC_{50} (a graphically estimated concentration that is likely to cause a 50% reduction or inhibition of a biological process, such as light output) was estimated for all assays according to the American Society for Testing and Materials Standard Guide for Conducting Static 96-h Toxicity Tests with Microalgae (ASTM 1990).

RESULTS

The trend of light reduction as a response to increasing dose level of test article was observed in all assays. The coefficient of variation of control groups was

10% or less in all experiments. This was in contrast to the coefficient of variation of experimental groups which ranged from less than 10 to as high as 80%. The high degree of variation seen in some of the experimental groups may have been correlated with fewer viable cells since light reduction was also reduced in these groups.

Tributyltin Chloride

The TBTCl assay was set up to observe light reduction effects on P. lunula cells for a period of 11 days with measurement periods selected every 48 h. The four experimental exposures of TBTCl tested were 0.05 (nominal), 1.6, 4.2, and 12.8 ug/L (actual). A reduction in light output from cells exposed to 1.6, 4.2, and 12.8 ug/L TBTCl was observed (Fig. 4). The IC_{50} decreased from 8.5 ug/L at 120 h to 3.0 ug/L at 264 h. Cells exposed to concentrations of 1.6 to 12.8 ug/L continued to exhibit a statistically significant (p=0.05) decline in light output from 120 to 264 h. A final reduction in light output of approximately 72, 90, and 99% was observed for the 1.6, 4.2, and 12.8 ug/L TBTCl exposed cells, respectively.

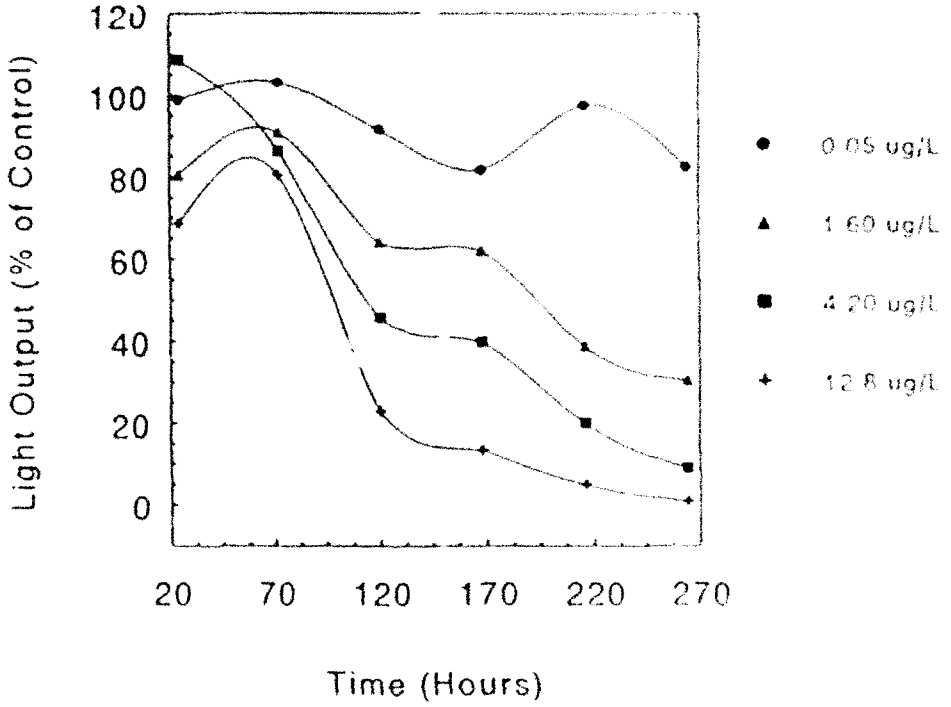

Fig. 4--P. lunula. Response of cells exposed to ug/L levels of TBTCl. Data are plotted as the % of light output of the control group versus time per experimental concentration.

Storm Drain Effluent

The objective of the storm drain effluent assay was to quantify the toxic effects of environmental chemical mixtures. The sample, collected in San Diego Bay, was known to contain heavy metals (such as copper at 18 mg/L and zinc at 50 mg/L), petroleum hydrocarbons, suspended solids, and trace organics (Gadbois, pers. comm.). Analyses of the results of the 4-day assay indicated that the 3 lowest levels of storm drain effluent, for the most part, had little effect on light reduction in G. polyedra (Fig. 5). However, the cells exposed to 6.25% effluent and greater exhibited a statistically significant (p=0.05) reduction in light output in as little as 3 h. A 3-h IC_{50} was calculated at 4.3% effluent and changed little by day 4 (4%).

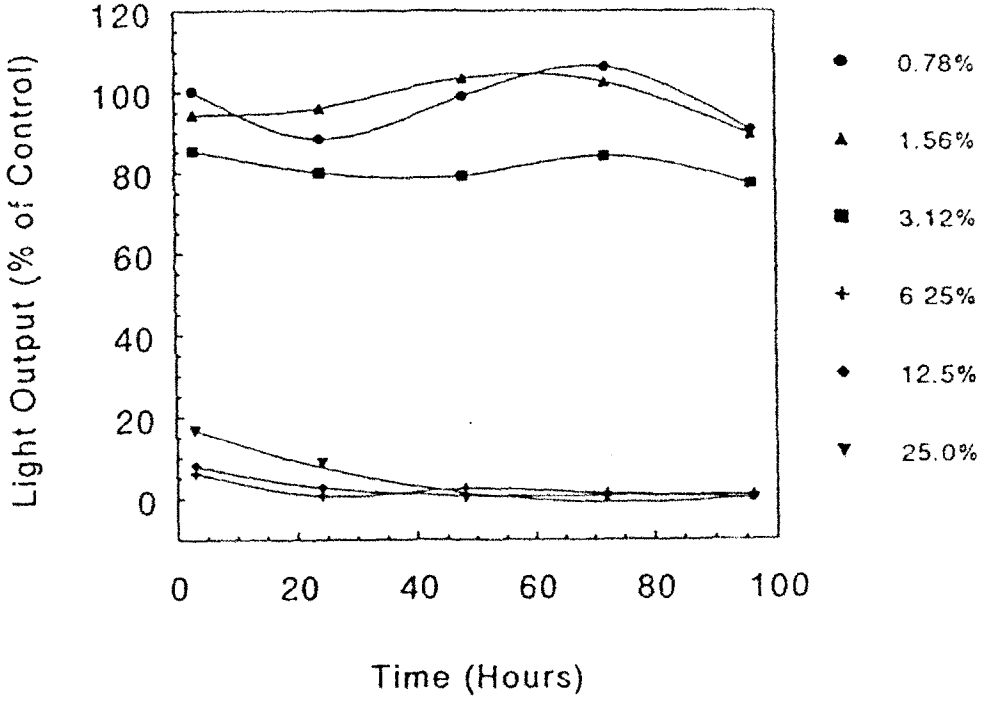

Fig. 5--P. lunula. Response of cells exposed to % levels of storm drain effluent. Data are plotted as the % of light output of the control group versus time per experimental level.

Copper

Cells of P. lunula were exposed to 6 concentrations of copper sulfate (Cu_2SO_4) which ranged from 1 to 20 mg/L for a period of 4 days. Measured levels of copper were within +/- 10% of nominal values in samples tested. Sampling was

initiated 3 h following setup of the assay and continued each day 24 h apart. The cells at the lowest concentration experienced no reduction in light output; however, statistically significant ($p=0.05$) effects in light reduction at concentrations of 2.5 to 20 mg/L, in reference to control values, were measured (Fig. 6). Less than 10% of the light measured in control cells was measured at concentrations from 5 to 20 mg/L Cu_2SO_4. A 48-h IC_{50} was estimated at 3.2 mg/L while a 96-h IC_{50} was estimated at 2.1 mg/L.

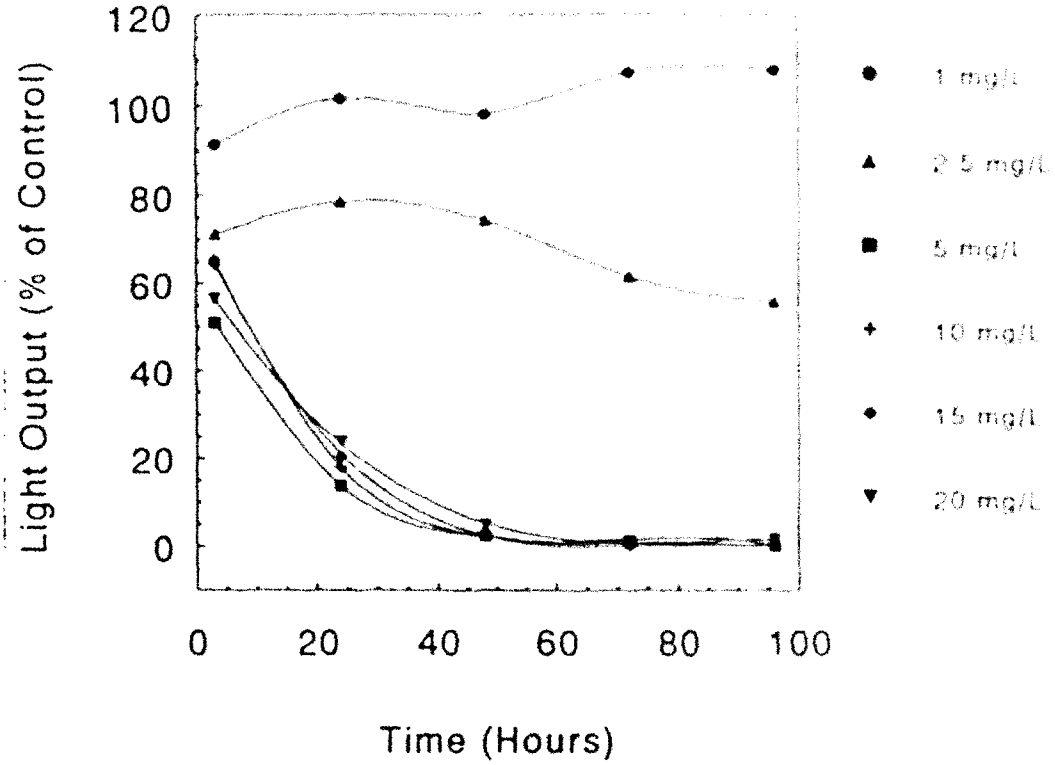

Fig. 6--P. lunula. Response of cells exposed to mg/L concentrations of Cu_2SO_4. Data are plotted as the % of light output of the control group versus time per experimental concentration.

In a similar experiment, G. polyedra was exposed to 6 concentrations of Cu_2SO_4; however, the concentrations ranged from 0.10 to 20 mg/L and the duration of the assay was 72 h. Light reduction was statistically significant ($p=0.05$) at Cu_2SO_4 levels of 0.10 mg/L and greater throughout the assay. Almost complete light reduction was measured 4 h after assay setup at concentrations of 1 to 20 mg/L. Cells exposed to 0.10 mg/L produced 30% of the control light output at 4 h, and continued to decay to approximately 14% of control values at 72 h. (Fig. 7).

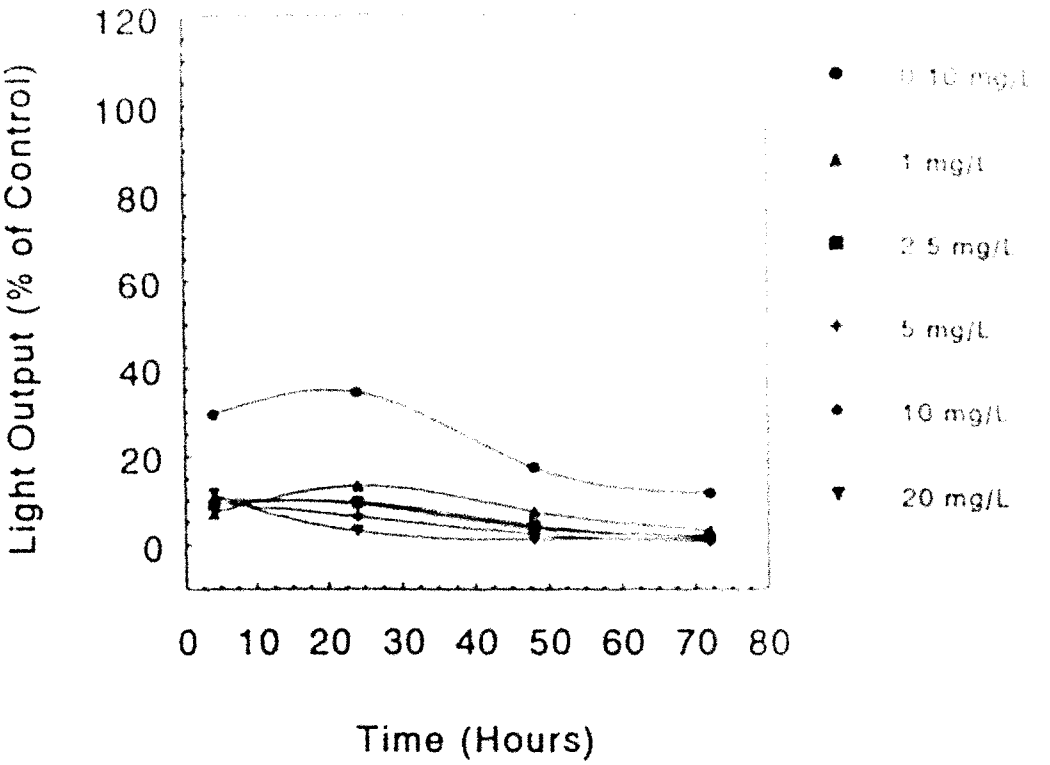

Fig. 7--G. polyedra. Response of cells exposed to mg/L concentrations of Cu_2SO_4. Data are plotted as the % of light output of the control group versus time per experimental concentration.

To further describe the sensitivity of G. polyedra to copper and determine a threshold value, several assays were ran in the 0.010 - 0.10 mg/L range. Cells exposed to 0.10 mg/L Cu_2SO_4 exhibited diminished light output (30% of the control's light output) at 24 h following initial setup. No effect on light output was measured when cells were exposed to 0.010 mg/L. We observed less than 50% of control light output at 50 mg/L Cu_2SO_4. An IC_{50} value was calculated and was observed to decrease with time during the 3-day exposure (Table 1).

Zinc

The zinc sulfate ($ZnSO_4$) assay was conducted using G. polyedra. A range of 0.10 mg/L to 10 mg/L was chosen to represent levels which might enter bays from storm drain effluents. The actual levels of zinc were within +/- 10% of nominal values in samples tested. A statistically

significant ($p=0.05$) decrease in light output was measured at 5 and 10 mg/L. No reduction of light output was observed at 0.10 mg/L and 1 mg/L $ZnSO_4$ (Fig. 8). 3-h and 48-h IC_{50} values of 7 mg/L and 1.7 mg/L were calculated, respectively.

Table 1--IC_{50} trends for G. polyedra exposed to Copper over time.

Time (h)	IC_{50} values (mg/L)
4	0.085
24	0.070
48	0.065
72	0.060

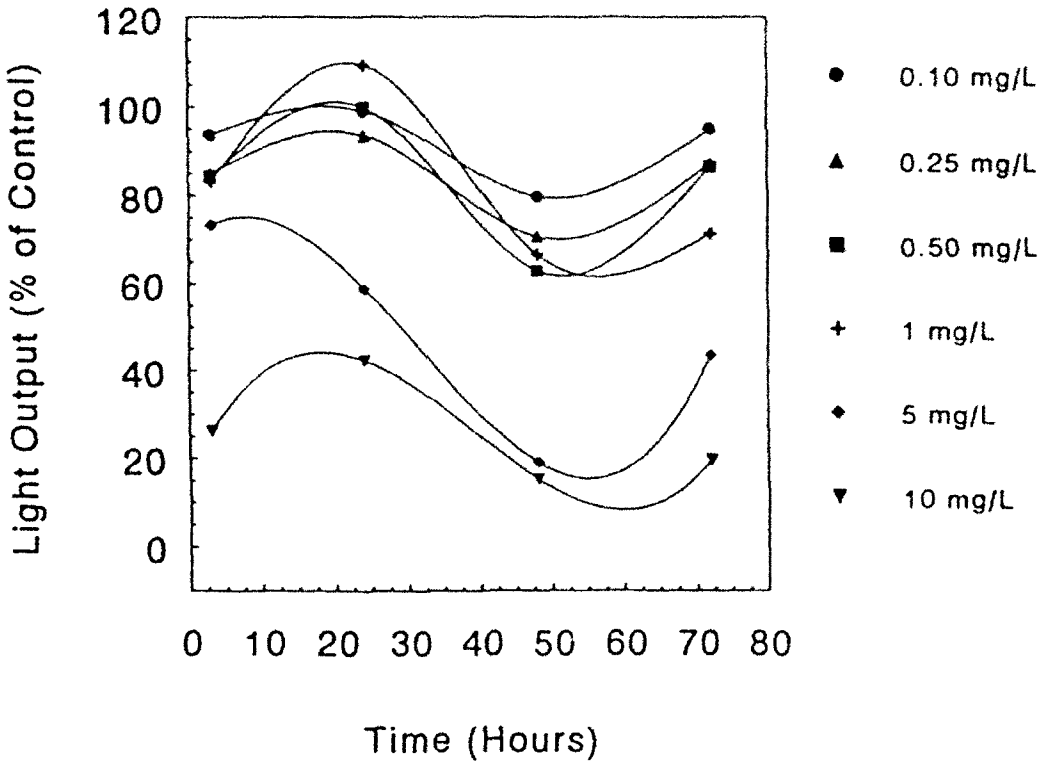

Fig. 8--G. polyedra. Response of cells exposed to mg/L levels of $ZnSO_4$. Data are plotted as the % of light output of the control group versus time per experimental concentration.

CONCLUSION

The results of these preliminary assays indicate the utility of using bioluminescent dinoflagellates as bioassay organisms. Light output seems to be inversely related to the toxicity of the test article. The results of these assays indicate that these organisms may be as, or more, sensitive than many of the traditional bioassay organisms.

The sensitivity of the organisms used in this assay has compared favorably with other "standard" bioassay organisms. The 0.060 - 0.080 mg/L IC_{50} values for copper in G. polyedra are lower than the 96-h EC_{50} value (effective concentration that inhibits growth) reported for Daphnia sp. (0.430 mg/L), the 1-h IC_{50} value for a Vibrio bacterial bioluminescence test (0.620 mg/L), and the 96-h LC_{50} value for fathead minnows (3.3 mg/L). In other studies, the EC_{50} values of zinc reported for Daphnia sp., trout, and fathead minnows (8.9, 26, and 169 mg/L, respectively) were higher than the 3-h IC_{50} for zinc in this study (7 mg/L)(Lankford and Eckenfelder 1990). Additionally, our calculated 4-d IC_{50} value of 4% for the storm drain effluent compared favorably to the IC_{50} value of 3% determined with Microtox®, a bacterial bioluminescent assay (Gadbois, pers. comm.).

We believe the techniques developed for this assay are a valuable addition to any program trying to assess toxicity to marine organisms from contaminated waters and possibly sediments. The entire procedure is quick to set up and sampling can be limited to 1.5 h a day for periods from a few days up to 2 weeks. The immediate advantages of using this assay would be to assess sublethal acute and/or chronic effects from exposures to metals, antifoulant paints, storm drain discharges, sediments, and possibly leachates from landfills adjacent to bays and estuaries. This assay could be used as part of a larger toxicity assessment encompassing other marine species which represent different taxa and levels of biological organization.

Few algal species are employed as assay organisms. Typically, the species that have been chosen are for convenience and not necessarily on their sensitivity to contaminants. There needs to be an expanded effort on screening algal species for determining which species may be more sensitive and/or applicable for use in a bioassay. Because of the complex and unknown behavior of multiple contaminants in the aquatic environment, the effects upon phytoplankton (dinoflagellates, diatoms, algae) cannot be predicted solely from their chemical compositions.

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