Stimulable and Spontaneous Bioluminescence in the Marine Dinoflagellates, Pyrodinium bahamense, Gonyaulax polyedra, and Pyrocystis lunula

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ABSTRACT P. bahamense, G. polyedra, and P. lunula exhibit interspecies differences in stimulable and spontaneous bioluminescence. For each species the total number of photons that can be emitted upon mechanical stimulation is a constant, regardless of the time during scotophase at which stimulation occurs. Ratios of stimulable bioluminescence per organism during scotophase and photophase are as high as 950:1 for laboratory cultures and have been observed as high as 4000:1 for natural populations of P. bahamense. Spontaneous emission in darkness shows flashing as well as low-level continuous emission. Natural populations of P. bahamense, placed in darkness during natural photophase, exhibit a dual character to their stimulable bioluminescence. Mechanical stimulation techniques are described for rapid and reproducible stimulation of bioluminescence.

INTRODUCTION

In previous papers dealing with the natural rhythms of bioluminescence of the tropical marine dinoflagellate Pyrodinium bahamense (Seliger et al., 1962; Taylor et al., 1966; Seliger and McElroy, 1968), both the shapes of the daily stimulable bioluminescence curves and the night-to-day ratios of stimulable bioluminescence differed markedly from the results reported by Hastings and Sweeney (1957, 1958, 1959), Sweeney and Hastings (1957, 1958, 1960), and Sweeney et al. (1959) for laboratory cultures of a different species, Gonyaulax polyedra.
We therefore established unialgal laboratory cultures of \textit{P. bahamense} and \textit{G. polyedra}, as well as a third nonmotile species, \textit{Pyrocystis lunula}, and developed precise mechanical stimulation and light measurement techniques. We have made a comparative study of the bioluminescence of these three species under identical conditions of laboratory culture as well as of the bioluminescence of natural populations of \textit{P. bahamense}. The present paper describes our techniques and the results of these comparisons.

\textbf{MATERIALS AND METHODS}

\textbf{A. Dinoflagellate Cultures}

Table I lists the data relative to the origins and culture conditions for the three species of dinoflagellates investigated. Only actively growing, log-phase cultures were used in the experiments. Fernbach flasks containing 1500 ml of media were inoculated at 500/ml and grown into log phase without stirring. Concentrations were determined by microscopic counting after gentle stirring which did not injure the organisms. 3 ml of the culture solutions were pipetted gently into 16 mm O.D. test tubes during photophase and replaced in open wire racks in the growth chamber to insure recovery from even this mild disturbance. Tubes were then removed from the racks without jarring, for insertion into the bioluminescence assay geometries, at times depending upon the properties being investigated.

\textbf{B. Light Assay Techniques}

All relative measurements of bioluminescence were made with EMI 9558 or EMI 6097 phototubes with cathodes at negative high voltage. Dynode resistor load currents, depending on phototube voltage, were of the order of 1 ma. Instantaneous anode
currents never exceeded 0.01 ma and more usually were below 0.001 ma. Electrometer DC amplifiers (Seliger et al., 1962) were used throughout in conjunction with Sanborn Model 320 recorders.

At this point we shall introduce a terminology to describe the various parameters that were measured. All three species of dinoflagellates exhibit nocturnal stimulable bioluminescence. The measurements of natural populations in Oyster Bay, Jamaica, W. I., were made with a photometer unit containing an impeller pump which stimulated the organisms to emit light and in addition maintained a constant flow of fresh

organisms. Above some minimum pumping speed the observed mechanically stimulable intensity, MSI, was directly proportional to the concentration of dinoflagellates being pumped. In the laboratory measurements, a 3 ml volume of solution containing the organisms was either stirred or bubbled until no further bioluminescence was emitted. The total stimulable light, TSL, emitted by the sample was measured with the circuit shown in Fig. 1 a. The mechanically stimulable intensity, MSI, as a function of time was obtained with the differentiating circuit shown in Fig. 1 a. There is also a very low-intensity bioluminescence observed in darkness under conditions of no external stimulation. This spontaneous unstimulated intensity, SUSI, was measured with the circuit shown in Fig. 1 b. The phototube gain and feedback resistors were set so that the largest SUSI flashes could be recorded in recorder channel 1 without saturation, while recorder channel 2, at up to 100 times the sensitivity of channel 1 (at a level

![Figure 1](image-url)
where the phototube dark current was just discernible) recorded small flashes not
detected in channel 1, as well as the continuous emission. In this way we could obtain
SUSI flashing rates and pulse height distributions simultaneously with the continuous
low-level SUSI. We also use LD 12:12 to indicate a light-dark photoperiod of 12 hr
of light (photophase) followed by 12 hr of dark (scotophase). Times during these
phases will be referred to as D_6 or L_n. For example, D_6 means 6 hr into the dark
period. The properties of bioluminescence that were examined as well as the time
periods involved are summarized in Table II.

<table>
<thead>
<tr>
<th>Property of bioluminescence measured</th>
<th>Time period of measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute photon emission per organism</td>
<td>D_4 to D_8</td>
</tr>
<tr>
<td>Increase of MSI and TSL in dark</td>
<td>L_11 to D_4</td>
</tr>
<tr>
<td>Constancy of MSI and TSL during dark phase</td>
<td>D_1 to D_12</td>
</tr>
<tr>
<td>Decrease of MSI and TSL in light</td>
<td>D_6 to L_6</td>
</tr>
<tr>
<td>Entrained rhythm of TSL</td>
<td>D_6 to D_20*</td>
</tr>
<tr>
<td>Entrained rhythm of SUSI</td>
<td>D_6 through D_48*</td>
</tr>
<tr>
<td>Diphasic character of TSL</td>
<td>Placed in dark at L_4; MSI and TSL assayed subsequently</td>
</tr>
<tr>
<td>Dark recovery of TSL</td>
<td>Stimulated at D_6; MSI and TSL assayed subsequently at 5 min intervals</td>
</tr>
</tbody>
</table>

* D_20 means that the lights were not turned on at D_12 but that the organisms were continued in darkness for 8 more hr.
† D_48 means continued darkness for 36 hr past D_12.

C. Mechanical Stimulation of Organisms

The dinoflagellates were mechanically stimulated by both bubbling and stirring. The
sample test tube containing 3 ml of dinoflagellates in culture medium was fixed
vertically and reproducibly alongside the end-on phototube face. Stirring was
accomplished with the "U" portion of a straightened Giant Gem paper clip, fitted into
the rotor of an 1800 rpm motor. The U portion of the clip extended approximately
two-thirds of the way into the sample and rotation produced an extremely turbulent
solution. Bubbling was produced by means of a small air pump adjusted to a flow of
1.5 liters per min through a No. 15 cannula (0.137 mm i.d.), with the cannula tip
extending halfway down into the sample.

D. Spontaneous Unstimulated Intensity

Samples of SUSI were measured as a thin layer (6 mm) of solution in a flat-bottomed
quartz cylindrical cell (2.5 cm i.d. (inside diameter)) sitting directly on the flat face
(5 cm D) of a vertically mounted phototube. The cell was covered by a hemispherical
mirror to increase the efficiency of the light collection and also to retard evaporation
during the measuring period (see Table II). In this way the geometry was reasonably
constant for all organisms emitting within the volume.
RESULTS

All data are reported in absolute units of photons per second per organism for intensities (MSI, SUSI) or photons per organism for total light (TSL), based on an independent absolute calibration reported separately.\(^1\)

A. Experimental Precision

There are several sources of error in the determination of TSL per organism. There are (a) possible nonuniformities in organism concentrations in the culture flasks; (b) real differences in TSL per organism in any particular species, depending on the age and density of the culture, the previous light-dark history of the culture, the natural variability of the organisms themselves, and differences among different cultures of the same species; (c) changes over long periods of time, in either spectral sensitivity or amplification factors of the phototubes used for light measurements; (d) variations in the degree of turbulence produced by the mechanical stimulation method.

For (a) we have been able to demonstrate that with gentle stirring in the 1 liter culture flasks, successive withdrawals of 1 ml samples for microscopic counting could be counted with a coefficient of variation\(^2\) of 6%.

For (b) we have shown that for all three species TSL is constant from approximately \(D_1\) through \(D_{12}\) within the precision of a set of measurements made over a short time interval. The coefficient of variation of TSL for a series of 20 consecutive samples of \(G.\) polyedra was 11%.

We have followed individual cultures from the time of inoculation through log phase of growth into stationary phase, in some cases for as long as 27 days. Typical data for TSL per organism determined at \(D_4\) for all three species are shown in Table III. Since we have not yet studied the effects of intensity and the spectral quality of ambient light on the growth and bioluminescence of these organisms, we maintained our culture conditions constant so that at least the species comparisons could be made. We did verify, however, that a factor of two increase in ambient light intensity had no effect on the observed bioluminescence.

For (c) we used a hermetically sealed, radioactive light source, consisting of \(^1\)C homogeneously incorporated into a luminescent epoxy resin. With this constant intensity light source we have been able to maintain a continuing check on our phototube sensitivities.


\(^2\) Defined as the standard deviation divided by the mean in per cent.
For (d) we verified that changes of ±50% in the bubbling rate or the stirring rate did not affect the TSL readings during scotophase.

B. Photoperiod-Entrained Rhythms of Nocturnal TSL per Organism

1. LABORATORY CULTURES

None of the three species exhibits exactly the same kinetics of MSI. For example the MSI of *P. bahamense* during photophase and subsequent to the onset of scotophase is shown in Fig. 2. Mechanical stimulation of a population of *P. bahamense* results in a cumulative flash which has the same relative shape in scotophase as in photophase.

The MSI flashes of *P. lunula* are essentially the same as those of *P. bahamense*. However, as the result of mechanical stimulation, *P. lunula* emits a low-level continuous glow, presumably due to cell damage, which persists after mechanical stimulation has stopped. A glow is also observed if a droplet of culture medium containing the organisms adheres to the side of the test tube and the

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**TABLE III**

EXAMPLES OF EXPERIMENTAL PRECISION FOR LABORATORY CULTURES THROUGHOUT LOG PHASE GROWTH

<table>
<thead>
<tr>
<th>Time after inoculation</th>
<th><em>P. bahamense</em></th>
<th><em>G. polyedra</em></th>
<th><em>P. lunula</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells in 3 ml</td>
<td>TSL/cell</td>
<td>Cells in 3 ml</td>
</tr>
<tr>
<td>days</td>
<td>photons × 10⁸</td>
<td></td>
<td>photons × 10⁸</td>
</tr>
<tr>
<td>4</td>
<td>860</td>
<td>3.30</td>
<td>2,810</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>2,040</td>
</tr>
<tr>
<td>6</td>
<td>1,090</td>
<td>2.72</td>
<td>4,020</td>
</tr>
<tr>
<td>7</td>
<td>1,350</td>
<td>3.19</td>
<td>6,000</td>
</tr>
<tr>
<td>8</td>
<td>1,000</td>
<td>3.63</td>
<td>9,000</td>
</tr>
<tr>
<td>11</td>
<td>2,440</td>
<td>3.92</td>
<td>10,350</td>
</tr>
<tr>
<td>12</td>
<td>2,840</td>
<td>3.00</td>
<td>16,620</td>
</tr>
<tr>
<td>13</td>
<td>3,390</td>
<td>3.55</td>
<td>17,840</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td>22,190</td>
</tr>
<tr>
<td>15</td>
<td>3,480</td>
<td>4.78</td>
<td>23,140</td>
</tr>
<tr>
<td>18</td>
<td>6,570</td>
<td>3.55</td>
<td>32,150</td>
</tr>
<tr>
<td>20</td>
<td>6,830</td>
<td>3.92</td>
<td>38,410</td>
</tr>
<tr>
<td>22</td>
<td>13,440</td>
<td>2.29</td>
<td>38,280</td>
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<tr>
<td>25</td>
<td>12,220</td>
<td>3.00</td>
<td>40,020</td>
</tr>
<tr>
<td>27</td>
<td>12,700</td>
<td>2.73</td>
<td>50,810</td>
</tr>
</tbody>
</table>

Average TSL<sub>cell</sub> : 3.35 × 10⁸
Coefficient of variation : 17%

Average TSL<sub>cell</sub> : 1.17 × 10⁹
Coefficient of variation : 8.2%

Average TSL<sub>cell</sub> : 38.9 × 10⁹
Coefficient of variation : 19%
solution begins to evaporate. For this reason, only the paper clip stirring technique, which evoked the maximum initial MSI and resulted in the least splashing of solution to the sides of the test tubes, was used for TSL measure-

![Graph](image.png)

**Figure 2.** Shapes of MSI flashes of *P. bahamense* populations during the transition from photophase to scotophase. The ordinates are in relative intensity units for each curve. The curves are displaced vertically from one another by distances corresponding to the time during scotophase at which MSI was measured. The three vertical sections were measured at relative electronic gains of 1, 1/10, and 1/100.

ments of *P. lunula*. During photophase the continuous glow emitted as the result of mechanical stimulation produced a much larger contribution to TSL than the MSI flash. We assumed that this was due to injury to the or-
ganisms. Therefore for all TSL measurements of *P. lumula* we arbitrarily integrated the light emission over the time period during which the flash decays to 10% of its peak value. During scotophase, the glow induced by mechanical stimulation was negligible compared with the MSI flash and therefore the time period over which TSL was measured was not as critical.

*G. polyedra* does not exhibit this stimulation-induced glow. However, during photophase there are two different types of mechanically stimulable bio-
luminescence (Fig. 3). There is a low-level continuous MSI component which can persist for several minutes. This component is extremely sensitive to the degree of turbulence produced by the stimulation technique and is much less intense for bubbling (even under our optimum conditions) than for stirring. Around \( D_0 + 10 \) min MSI flashes become apparent and the low-level continuous MSI decreases. Subsequent to \( D_0 + 30 \) min there is no observable low-level continuous MSI, and the MSI flashes of \( G. \) polyedra have the same relative shapes as those for \( P. \) bahamense and \( P. \) lunula. However, even in

scotophase, TSL of \( G. \) polyedra was more sensitive than that of the other two species to degree of turbulence, and TSL values obtained with paper clip stirring were consistently higher than those obtained with bubbling.

Single periods of the photoperiod-entrained rhythms of nocturnal TSL per organism in absolute units of quanta for \( P. \) bahamense, \( G. \) polyedra, and \( P. \) lunula are shown in Figs. 4–6, respectively. It should be emphasized that the data of Figs. 4–6 are repeatable during every photoperiod over the entire culture life (inoculation through stationary phase) as implied by Table III. Because of the large scotophase to photophase variations, the TSL data are plotted on a logarithmic scale. The abscissae are in hours relative to the beginning of scotophase or photophase, which in laboratory cultures are step functions. The scotophase is shaded. In Fig. 4, the results of bubbling and

Figure 4. Photoperiod-entrained TSL per organism for \( P. \) bahamense in absolute units of photons over a complete photoperiod.
paper clip stirring for \( P. \) bahamense are plotted together since TSL was equally sensitive to either method of stimulation.

The paper clip stirring data for \( G. \) polyedra are plotted in Fig. 5a separately from the bubbling data which are shown in Fig. 5b. As we have described above, TSL during scotophase (D\( _1 \) to D\( _1n \)) is the result of a large MSI flash which decreases to zero intensity within approximately 5 sec (Fig. 3). Thus TSL is independent of the integration time. However, during photophase, the integration time determines the total light measured. In Fig. 5a values of TSL per organism during photophase are shown for integration times of 5, 30, and 90 sec. The large differences between bubbling and stirring TSL during photophase for \( G. \) polyedra are evident from Fig. 5b in which for the same culture, TSL per organism is plotted for bubbling periods of 120 sec. Even during scotophase, TSL per organism obtained by bubbling is somewhat lower (15%) than that obtained by stirring.

The corresponding data for stirring TSL per organism for \( P. \) lunula, shown in Fig. 6, agreed with checking experiments in which bubbling was used for stimulation, although as explained above, the bubbling data were more erratic because of splashing.

For ease of discussion, we shall list the following points: (a) The increases in TSL per organism subsequent to D\( _0 \) are extremely rapid and exponential, with doubling times of 5–8 min for all three species. (b) Maximum values of TSL per organism are attained within 60–90 min of initiation of scotophase. (c) From D\( _1 \) through D\( _1n \), TSL per organism is constant within the precision of the measurements. (d) There is a small decrease in TSL per organism between D\( _1n \) and D\( _2h \), just before the initiation of photophase. The effect is largest for \( G. \) polyedra. (e) Subsequent to photophase, there are rapid exponential decreases in TSL per organism. However, under our laboratory growth conditions, the decrease for \( P. \) bahamense (Fig. 4) is not nearly as rapid as for \( G. \) polyedra or \( P. \) lunula. The half-times for decrease of TSL per organism are 50 min for \( P. \) bahamense, 9 min for \( G. \) polyedra, and 4–5 min for \( P. \) lunula. (f) The maximum scotophase-to-photophase ratios of TSL per organism are approximately 200:1 for \( P. \) bahamense and \( G. \) polyedra. For \( P. \) lunula the ratio is much larger and depends on the integration time during photophase. If we arbitrarily integrate over the time during which the photophase flash decays to 10% of its peak value, the ratio is 950:1.

2. NATURAL POPULATIONS

We performed similar measurements of TSL per organism with natural populations of \( P. \) bahamense, freshly removed from Oyster Bay, Jamaica, W. I. Samples taken during natural photophase were separated into two sets of test tubes. Set A was placed in total darkness one-half hour before sunset. Set B was allowed to remain in natural photoperiod. TSL per organism was meas-
ured for both sets at intervals throughout the next 19 hr. We obtained the following results: (a) The immediate rate of increase in TSL per organism for set A (step function scotophase) was greater than for set B (natural scoto-

![Graph](image)

**Figure 5a**

Photoperiod-entrained TSL per organism for *G. polyedra* in absolute units of photons over a complete photoperiod. (a), TSL per organism as a result of paper clip stirring. During photophase and the transition time D₆ to D₇ light emission was integrated for 5, 30, and 90 sec as shown by the encircled numbers. The data during scotophase represented by the solid circles are independent of time of integration. (b), TSL per organism as a result of bubbling. During photophase and the transition time D₆ to D₇ light emission was integrated for 120 sec. During scotophase the data are independent of time of integration.

(b) From 1 hr past sunset throughout the scotophase, TSL per organism was identical for both sets. (c) Subsequent to dawn, set B (natural photoperiod) exhibited the same rapid decreases in TSL per organism as the MSI natural photoperiod data of Seliger and McElroy (1968). (d) Set A, kept in darkness through D₁₇, exhibited a very gradual decrease in TSL per organism beginning around D₁₁. (e) At D₁₇, corresponding to L₄ of the natural photo-
period, set A was removed from the dark and placed in ambient sunlight. The decrease in TSL per organism was precipitous, even more rapid than observed for set B under natural photoperiod.

3. COMPARISONS OF TSL PER ORGANISM

Circadian rhythms of TSL have been reported for *G. polyedra* (Hastings and Sweeney, 1957), for *P. lunula* (Swift, 1967), and for a heterogeneous natural population (Kelly and Katona, 1966). These rhythms were observed under constant external conditions of illumination, either continuous dim light or continued darkness, as differentiated from the photoperiod-entrained rhythms of TSL. In captured natural samples of *P. bahamense* kept in continued darkness we have observed higher values of TSL at times corresponding to "night" and lower values of TSL at times corresponding to "day." In continued darkness the magnitudes of the TSL values were damped completely after 72 hr (see also Fig. 1 of Kelly and Katona, 1966). Microscopic observation
showed that the damping of the TSL values was due to death of the organisms in continued darkness. Laboratory cultures of all three species grown into log phase under LD 12:12 photoperiod will be essentially killed after 72 hr of extended darkness. Laboratory cultures of *P. bahamense* are the most sensitive to extended darkness; they are killed within 48 hr. With this limitation placed on the observations we have verified that for laboratory cultures of *P. bahamense* in continued darkness there are: an increase in TSL during the interval D₉ to D₁₂, a decrease in TSL during D₁₂ to D₂₄, an increase in TSL during D₂₄ to D₄₆, and a decrease in TSL during D₄₆ to D₄₈, corresponding to a highly damped oscillation. For *G. polyedra* and *P. lunula* the damping is less extreme and permits the observations of TSL periodicity for an additional 24 hr. In all cases the damping is due to death of the organism. Although, as one of the reviewers has pointed out, these observations of periodicity do not extend over an appreciable length of time, we feel that this suggests the presence of an endogenous circadian rhythm of TSL in *P. bahamense*.

**Figure 6.** Photoperiod-entrained TSL per organism for *P. lunula* in absolute units of photons over a complete photoperiod.
We further observed for all three species that during the time interval D₁₂ to D₁₈ the dinoflagellates did not die and the rates of decrease of TSL per organism for cultures in extended darkness were markedly different than the rates of decrease of TSL per organism for cultures in normal LD 12:12 photo-period (for these latter organisms the comparable time interval was L₀ to L₈). The comparisons of these two rates for all three species are shown in Fig. 7.

**Figure 7.** a, decrease in TSL per organism for laboratory cultures of *P. lunula* subsequent to initiation of photophase, compared with TSL per organism in continued darkness. Open triangles, photophase; solid triangles, continued darkness. b, decrease in TSL per organism for laboratory cultures of *G. polyedra* subsequent to initiation of photophase, compared with TSL per organism in continued darkness. Open triangles, photophase, 30 sec paper clip stirring; open circles, photophase, 120 sec bubbling; solid triangles, paper clip stirring in continued darkness; solid circles, bubbling in continued darkness. c, decrease in TSL per organism for laboratory cultures of *P. bahamense* subsequent to initiation of photophase, compared with TSL per organism in continued darkness. +, Normal photophase, 500 ft−; open triangles, photophase, 2000 ft−; solid triangles, continued darkness.
Due to the fact that the organisms remain viable at least through $D_{18}$, the data of Fig. 7 present a valid comparison of the effect of light on the reduction of TSL. The half-times for both scotophase- and photophase-induced changes in TSL per organism for all three species under the different experimental conditions are summarized in Table IV.

We set up a separate culture of *P. bahamense* in natural Baltimore photoperiod (2500 ft-c at noon) in an effort to approach the spectral quality and intensity of ambient sunlight in Jamaica. As shown in Fig. 8, even under these conditions natural photophase TSL for *P. bahamense* remained essentially unchanged from that measured for fluorescent light photophase. We have, how-

<table>
<thead>
<tr>
<th>Species</th>
<th>Laboratory photoperiod</th>
<th>Natural photoperiod</th>
<th>Extended darkness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Increase in TSL</td>
<td>Decrease in TSL</td>
<td>Increase in TSL</td>
</tr>
<tr>
<td></td>
<td>subsequent to step-function</td>
<td>subsequent to step-function</td>
<td>during</td>
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</tr>
<tr>
<td></td>
<td>min</td>
<td>min</td>
<td>min</td>
</tr>
<tr>
<td><em>P. bahamense</em></td>
<td>5-8</td>
<td>40-50</td>
<td>6-10</td>
</tr>
<tr>
<td><em>G. polyedra</em></td>
<td>7</td>
<td>6-9</td>
<td>-</td>
</tr>
<tr>
<td><em>P. lunula</em></td>
<td>5</td>
<td>4-5</td>
<td>-</td>
</tr>
</tbody>
</table>

* Laboratory culture grown in natural photoperiod (Baltimore sunlight, 2500 ft-c, noon).
† Laboratory culture grown in fluorescent light LD 12:12 cycle (500 ft-c).

ever, been able to produce an extremely rapid decrease in TSL per organism for a laboratory culture of *P. bahamense* if at $L_9$ the culture is placed for only 10-15 min at 17 cm from a 1000 w high pressure mercury arc (5000 ft-c).

C. Kinetics of MSI in Photophase and Scotophase

The shapes of MSI during the transition from photophase to scotophase have already been described. The question now arises as to what happens to MSI if the lights are not turned on subsequent to $D_{12}$? Does the organism exhibit a gradually reduced "scotophase" MSI or are the kinetics of MSI those of "photophase"? Figs. 9 and 10 are direct tracings of curves from the chart of the two-channel recorder of Fig. 1 a, for *P. bahamense* and *G. polyedra*, respectively, for representative times during scotophase, photophase, and for the organisms continued in the dark past $D_{12}$. The electronic gains for measurements during these different time periods were quite different from one another and the curves represent only the shapes of the MSI flashes and the
integrated light intensities. Both *P. bahamense* and *P. lunula* exhibit the same kinetics of stimulable bioluminescence, independent of phase of photoperiod. In the case of *G. polyedra*, we have presented side-by-side comparisons of stirring and bubbling data, the latter taken at the same electronic gain with a fresh sample within 2 min of the stirring measurement. During scotophase (Fig. 10a) both methods of stimulation give essentially the same MSI flash kinetics, although the bubbling results are always slightly lower than those for stirring. The double peak in the MSI flash is a stirring artifact. It is during photophase (Fig. 10b) that the marked difference in stimulability arises, giving rise to the concept of two different types of mechanically stimulable bioluminescence.

In all three cases, during extended scotophase or continued darkness, the kinetics of stimulable bioluminescence are essentially those of the normal scotophase.
D. Spontaneous Unstimulated Intensity (SUSI)

Hastings and Sweeney (1959) reported an endogenous circadian rhythm of SUSI for *G. polyedra* in continued darkness and in constant dim light. With our present equipment, we have been able to look at some of the details of this SUSI. For *P. bahamense*, *G. polyedra*, and *P. lunula*, SUSI consists, to different degrees, of a low-level continuous emission which is proportional to cell concentration, individual small flashes from single cells, and occasional transient glows lasting for several seconds.

In Section B 3 we have referred to the rapid death of the dinoflagellates under conditions of extended darkness. This applies as well to measurements of SUSI in extended darkness.

*G. polyedra*, as expected, exhibits an endogenous rhythm of SUSI in com-
plete darkness. Any periodicity in SUSI for *P. bahamense* or *P. lunula* is apparently so highly damped that it is not observable except for the initial scotophase time interval. Comparable data for SUSI for *G. polyedra*, *P. bahamense*, and *P. lunula* are shown in Fig. 11 a, b, and c. SUSI is plotted as the number of photons per organism per second, averaged over a 5 min period, measured at 15 min intervals from D₀ through D₄₈ (36 hr of darkness past D₄₈).

SUSI for *G. polyedra* (Fig. 11 b) is predominantly a glow and can be divided into three parts: (a) a low-level constant portion from D₉ to D₁₀ where the average rate of light emission per organism is 280 photons per second; (b) a narrow peak at D₁₁ with a half-width of 100 min and a maximum intensity per organism of 1500 photons per second; (c) a subsequent decrease to intensities lower than (a) from D₁₈ through D₂₈ at which time (a) and (b) are repeated.
E. Dark Recovery of Stimulable Bioluminescence

During scotophase all three species, after having been stimulated to exhaustion, can recover part of their initial bioluminescent capacity within a short time. This process can be repeated several times, although each recovery results in progressively smaller values of TSL; this is especially true for *P. lunula*, presumably due to cell damage during stimulation. Recovery data are plotted for all three species in Fig. 12, as per cent of initial TSL as a function of time subsequent to stimulation. The recovery is exponential with half-

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**Figure 11.** SUSI in photons per second per organism beginning at scotophase, D₀, and continued in darkness through Dₙ. a, *P. bahamense*. Open circles, total SUSI, flashes plus glow; heavy line, glow only. b, *G. polyedra*. Open triangles, flashes only; heavy line, glow only. c, *P. lunula*. Open circles, total SUSI, flashes plus glow; heavy line, glow only.
times of 18, 20, and 30 min for *P. bahamense*, *G. polyedra*, and *P. lunula*, respectively.

**F. Spectral Intensity Effects and Phase Setting**

A natural population was removed from Oyster Bay in Jamaica during natural photophase and placed in complete and continuous darkness. For the data of

![Graph](image)

**Figure 12.** Recovery of stimulable bioluminescence for *G. polyedra*, *P. bahamense*, and *P. lunula*. Time is measured subsequent to complete stimulation by the technique used to measure recovery.

Fig. 13 (open circles), this was done at 13:12, approximately the middle of natural photophase, although the time is not critical. There was an immediate rapid increase in TSL reaching a value greater by a factor of approximately 40 within an hour, at which time a plateau was reached. The plateau was maintained until approximately the time of day at which the *in situ* *P. bahamense* began their nocturnal increase in TSL per organism. At this time the
Figure 13. Relative TSL per organism for natural populations of
P. mahaffyana. The results are plotted over the same time period, in
the in situ TSL (△) and in the continuous dark (X). For
comparison, the in situ TSL (△) is normalized to the TSL (X).

Published July 1, 1969
population confined in the dark exhibited a second rapid and exponential increase in TSL per organism, as though scotophase had just begun. For comparison the relative MSI data for the in situ bay population, obtained over the same time interval with the underwater photometers described by Seliger and McElroy (1968), are shown as the solid triangles. The nocturnal increase in TSL per organism for the sample confined in the dark remains in phase with the natural photoperiod and thus is independent of the time during photophase at which the dinoflagellates are artificially placed in the dark.

The initial dark-induced rise is immediate. The duration of the initial plateau is dependent upon the time during natural photoperiod when the organisms are placed in the dark. In nature, the two effects cannot be separated.

The same experiments repeated with laboratory cultures of *P. bahamense*, placed in darkness at L₆ and at L₉ (Fig. 14), showed a gradual rise to a maximum value of TSL per organism at D₁.
DISCUSSION

Under carefully controlled conditions of organism growth and with precisely defined stimulation and bioluminescence assay techniques, it has been possible to describe and compare several characteristics of the bioluminescence of three species of dinoflagellates. These characteristics such as the changes with time of the MSI flash, the TSL per organism, and SUSI are indicative of the physiological state of the organisms and should provide a sensitive assay technique for observing the effects of physical and chemical perturbants on the organisms.

Although in nature it has been our experience that dinoflagellates are seldom maximally stimulated mechanically to emit all of their potential luminescence, we have been able to demonstrate that they can be maximally stimulated without preventing further growth of the culture. This potential for stimulable luminescence, although different for different species, is a constant throughout essentially the entire scotophase (D₁ through D₁₁), in agreement with the data of Seliger and McElroy (1968) for natural populations of P. bahamense. The data presented are not intended primarily to demonstrate the existence of photoperiod-entrained and endogenous rhythms of bioluminescence; for the most part these have already been established by many previous workers. Rather we have examined the detailed shapes of the bioluminescent emissions from three species over their photoperiods as well as in extended darkness. For the photoperiod-entrained rhythm there are extremely rapid increases and decreases in TSL per organism (Table IV), so that for a complete photoperiod the stimulable bioluminescence has essentially a square-wave shape (Figs. 4-6). These shapes are completely different from the sine wave shapes reported by previous workers. In extended darkness, however, the endogenous rhythm of TSL does exhibit a sine wave character as described by Hastings and Sweeney (1957), Kelly and Katona (1966), and Swift (1967). However, since extended darkness for even 12–24 hr results in permanent injury to the dinoflagellates, we have limited our comparisons to TSL to the first 6 hr (D₁₂ through D₁₈) of extended darkness. From the data of Fig. 7 and the last column of Table IV, it may be seen that the rates of decrease of endogenous TSL are different for the three species, over and above the fact that these rates are each significantly lower than their respective photoinhibited rates of decrease during photophase. For G. polyedra and P. lumula our laboratory photophase intensities are sufficient to achieve a maximum photoinhibition, as evidenced by the rate of decrease of TSL. For the tropical P. bahamense a much higher light intensity is required. There are two aspects of the photoinhibition during photophase that must be examined: (a) the rate of decrease of TSL should have a spectral intensity dependence; (b) from the data of Fig. 13 and the fact that, for P. bahamense, the \( \frac{\text{MSI (scotophase)}}{\text{MSI (photophase)}} \)
ratios in nature, in Jamaica sunlight, are higher than those observed for our laboratory cultures indicates that the 
Scotophase TSL Photophase TSL
ratios as well should exhibit a spectral intensity dependence.

We have been able to achieve a rate of decrease in TSL per P. bahamense as fast as that for P. lunula by placing the P. bahamense culture close to a high intensity 1000 w mercury arc for as little as 10 min at L0. However, we have also observed that transitory morphological changes in pigment distributions occur when dinoflagellates are subjected to light intensities higher than those occurring during their normal photophase. For this reason all our measurements with the exception of the Hg arc illumination have been performed on cultures inoculated and grown up into log phase under the same spectral intensity and photoperiod as were used in the experiments.

Since of the three species, P. bahamense is the only representative of shallow tropical waters, it is not unreasonable to believe that the light intensities of our laboratory environmental chambers and even Baltimore photophase are not sufficient to effect the rapid TSL decrease observed in Jamaica sunlight. In laboratory culture, photophase is 500 ft-c. In Jamaica, sunlight illumination is as high as 8800 ft-c.

Nothing is known at present of the mechanism by which mechanical stimulation triggers light emission.

As in the case of the marine bacteria, there does not appear to be any selective advantage to the bioluminescence of dinoflagellates. In nature non-luminous species are often found together with luminous species (Nordli, 1957).

The significant differences in stimulability during photophase between P. bahamense and G. polyedra, which are responsible for the bubbling vs. stirring differences shown in Fig. 5, are demonstrated in Figs. 2 and 3 as well as in Figs. 9 b and 10 b. There are also differences in degree of recovery of potential for stimulable bioluminescence among all three species (Fig. 12).

All three species show an increase in SUSI subsequent to initiation of scotophase. P. bahamense and P. lunula show large fluctuations in SUSI over D9 to D12 (mainly due to erratic flashing) and then decrease to low levels. Of the three species, only G. polyedra exhibits well-defined peaks in both SUSI “glow” and cell division (Sweeney and Hastings, 1958) as functions of photoperiod. The former occurs at D11 and has a half-width of only 100 min, while the latter occurs at L1 with an even smaller half-width of 70 min.3 For all three species the Dc glow, represented by the heavy lines, is much less erratic than the flashes. For G. polyedra as contrasted with P. bahamense and P. lunula the contribution of flashes to SUSI was so small that in Fig. 11 b it is plotted separately. The second peak in SUSI glow around D34 is the expression of the circadian rhythm.

3 Unpublished data.
The distinction between glow or DC level and a large number of tiny flashes depends upon the signal-to-noise ratio of the phototube detector and the frequency response of the associated electronics. In our case the phototube dark noise was equivalent to an average dinoflagellate emission of $1.3 \times 10^4$ photons per sec. The input RC time constant of the DC amplifier was 0.01 sec. We arbitrarily set our “recognition” of flashes to those which, during any 0.01 sec time interval, emit a number of photons equal to the average number of “noise” photons; i.e., bioluminescent flashes emitting 1300 photons within 0.01 sec. Since the mean lifetime of the decay of dinoflagellate flashes is 0.05 sec, this corresponds to flashes containing $\geq 6500$ photons. Thus under ideal conditions SUSI flashes emitting approximately $10^{-5}$ of the scotophase MSI per organism for *P. bahamense* and *G. polyedra* and $10^{-6}$ of the scotophase MSI per organism for *P. lunula* could be detected.

We have not yet examined the effects of light intensity, temperature, nutrient levels, etc. on SUSI for these species and so we cannot say with certainty that neither *P. bahamense* nor *P. lunula* can be induced to synchrony. However, *G. polyedra* would appear to be an ideal choice with which to examine the correlation of SUSI with reproduction.

The data of Fig. 13 show an immediate increase in TSL by a factor of approximately 40 which can be considered as a reversal of photoinhibition. Then at a time corresponding to the natural scotophase, there is a further increase in TSL by a factor of 100. It is this former reversal of photoinhibition that can explain the increases and decreases in MSI in situ observed by Backus et al. (1965) during the course of a solar eclipse. It is apparent that the increases in TSL per organism shown in Figs. 13 and 14 are under photoperiodic control and that the arbitrary initiation of a dark interval during photophase, except for eliminating a relatively small photoinhibition, does not alter the phase of the expression of TSL. On the other hand, if photophase is continued past L14 there is no observable increase in TSL.

Seliger and McElroy (1968) have demonstrated that concentrations of natural populations of *P. bahamense* in Oyster Bay, Jamaica, W. I., can be assayed by measurement of scotophase MSI and by inference, TSL. The present data confirm this for laboratory cultures of *G. polyedra* and *P. lunula* as well. However, since photophase MSI is strongly dependent on ambient light intensity, it is no longer directly related to organism concentration. Thus large-scale mapping of *P. bahamense* concentrations in tropical bays by MSI can be made only during the night.

Although unfortunately we have not isolated and identified the species responsible, there is at least one bioluminescent plankton in Chesapeake Bay which does not exhibit a photoperiod-entrained diurnal rhythm in stimulable bioluminescence (Seliger et al., 1961) as well as a second species which does.  

Kelly and Katona (1966) have reported a photoinhibition of bioluminescence which is rapidly reversed by placing the organisms in darkness during photophase. Their technique for measuring bioluminescence was quite different from our own and their relative changes were several orders of magnitude smaller. It is therefore difficult to compare their results directly with ours.

The techniques and types of measurements described in this paper should permit a much more quantitative assay for the effects of photoinhibition and chemical agents on the expression of the rhythms of bioluminescent potential than has been available heretofore. In addition we would like to emphasize the importance of the comparative study of different species, both in laboratory culture and in natural populations, in view of the interspecies differences described in this paper.

We would like to thank Mrs. C. Eisner for her invaluable assistance in the maintenance of our laboratory cultures and in the experimental measurements. We also thank Mr. W. Esaias for his assistance in several of the light inhibition measurements.

This work was supported by the Division of Biology and Medicine of the Atomic Energy Commission, Contract AT (30-1) 2802. The experiments in Jamaica, West Indies were supported under Atomic Energy Commission Contract AT (30-1) 3480 and the Office of Naval Research, Contract NR-104-848, Nonr 4010 (16).

Contribution No. (560) of the McCollum-Pratt Institute, The Johns Hopkins University and Contribution No. (137) of the Chesapeake Bay Institute, The Johns Hopkins University.

This paper is based in part on material presented at the Fifth International Congress on Photobiology, Hanover, New Hampshire in August, 1968.

Received for publication 20 August 1968.

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