

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 *P. bahamense* and *G. polyedra*, as well as a third nonmotile species, *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 *P. bahamense*. The present paper describes our techniques and the results of these comparisons.

MATERIALS AND METHODS

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

TABLE I
ORIGINS AND CULTURE CONDITIONS FOR DINOFLAGELLATES

Organism and habitat	Isolation	Culture conditions	Generation time	Standing crop
			days§	ml ⁻¹
<i>P. bahamense</i> Plate, armored dinoflagellate, shallow, well-lit bays of West Indies	B. M. Sweeney, 1966, Oyster Bay, Jamaica, West Indies	*, ‡	4.5	4500
<i>G. polyedra</i> Stein, armored dinoflagellate, cosmopolitan	B. M. Sweeney, 1952, Scripps pier, La Jolla, Calif.	*, ¶	3.7	13,000
<i>P. lunula</i> Schütt, unarmored dinoflagellate, cosmopolitan	E. Swift, 1965, South Atlantic, 730 miles east of Rio de Janeiro	*, ¶	4.3	5000

* Temperature 25°C; Sylvania high yield cool white fluorescent lamps, 500 ft-c; LD 12:12 cycle; no shaking.

‡ Growth medium, Sweeney and Hastings (1957).

¶ Growth medium, Swift and Taylor (1967).

§ Under our conditions. In Jamaica, in nature, generation times of 2.5 days have been observed
|| Approximately 21 days subsequent to inoculation at 500/ml.

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.

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 stimutable 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

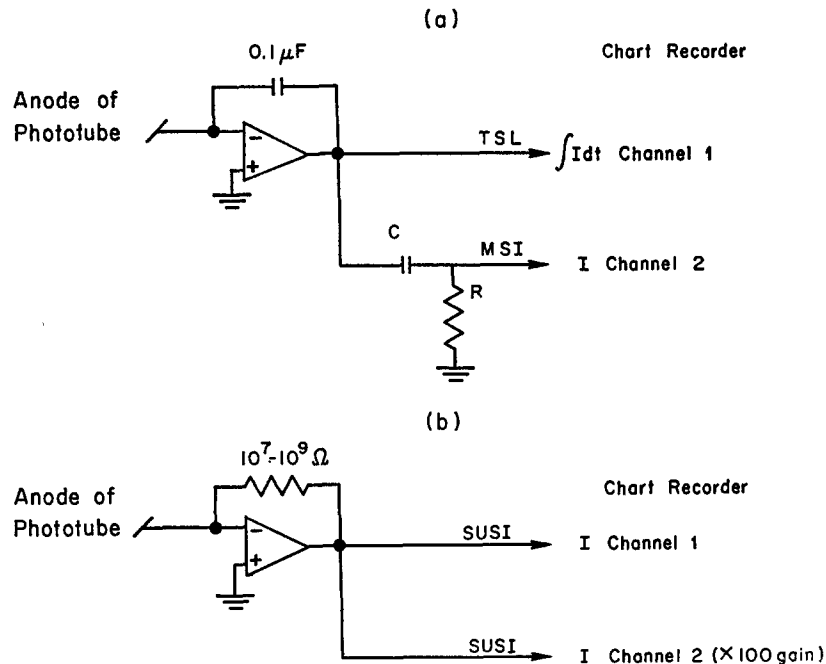


FIGURE 1. Schematic drawings of the feedback DC amplifiers used for measurement of dinoflagellate bioluminescence. (a) Integrating circuit with RC differentiating network at output of DC amplifier; (b) standard circuit for measuring light intensities.

organisms. Above some minimum pumping speed the observed *mechanically stimutable 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 stimutable light*, TSL, emitted by the sample was measured with the circuit shown in Fig. 1 a. The mechanically stimutable 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

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_n 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 II
PARAMETERS MEASURED AND TIME PERIODS OF MEASUREMENT

Property of bioluminescence measured	Time period of measurement
Absolute photon emission per organism	D_4 to D_8
Increase of MSI and TSL in dark	L_{11} to D_5
Constancy of MSI and TSL during dark phase	D_1 to D_{12}
Decrease of MSI and TSL in light	D_6 to L_6
Entrained rhythm of TSL	D_8 to D_{20}^*
Entrained rhythm of SUSI	D_0 through D_{48}^\dagger
Diphasic character of TSL	Placed in dark at L_6 ; MSI and TSL assayed subsequently
Dark recovery of TSL	Stimulated at D_6 ; MSI and TSL assayed subsequently at 5 min intervals

* 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 variation² of 6 %.

For (b) we have shown that for all three species TSL is constant from approximately D₁ through D₁₁ 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 D₄ 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 ¹⁴C 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.

¹ Seliger, H. H., W. H. Biggley, and E. Swift. 1969. Total stimutable light emission in the marine dinoflagellates, *Pyrodinium bahamense*, *Gonyaulax polyedra* and *Pyrocystis lunula*. *Photochem. Photobiol.* In press.

² Defined as the standard deviation divided by the mean in per cent.

For (d) we verified that changes of $\pm 50\%$ 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

TABLE III
EXAMPLES OF EXPERIMENTAL PRECISION FOR LABORATORY CULTURES
THROUGHOUT LOG PHASE GROWTH

Time after inocula- tion	<i>P. bahamense</i>		<i>G. polyedra</i>		<i>P. lunula</i>	
	Cells in 3 ml	TSL/cell	Cells in 3 ml	TSL/cell	Cells in 3 ml	TSL/cell
<i>days</i>		<i>photons</i> $\times 10^{-8}$		<i>photons</i> $\times 10^{-8}$		<i>photons</i> $\times 10^{-8}$
4	860	3.30	2,810	1.26	1,520	20.2
5					2,040	45.6
6	1,090	2.72	4,020	1.24	2,040	51.0
7	1,350	3.19	6,000	1.21	2,520	39.2
8	1,000	3.63	9,000	1.05	2,910	46.5
11	2,440	3.92	10,350	1.20	5,570	34.9
12	2,840	3.00	16,620	1.01	4,910	47.7
13	3,390	3.55	17,840	1.20	5,520	40.4
14			22,190	1.10	6,920	36.5
15	3,480	4.78	23,140	1.15	7,400	35.6
18	6,570	3.55	32,150	1.14	9,270	35.6
20	6,830	3.92	38,410	1.03	9,220	36.5
22	13,440	2.29	38,280	1.27	9,960	37.1
25	12,220	3.00	40,020	1.34	12,010	38.1
27	12,700	2.73	50,810	1.19		
Average $\frac{\text{TSL}}{\text{cell}}$: 3.35×10^8		Average $\frac{\text{TSL}}{\text{cell}}$: 1.17×10^8		Average $\frac{\text{TSL}}{\text{cell}}$: 38.9×10^8		
Coefficient of variation: 17%		Coefficient of variation: 8.2%		Coefficient of variation: 19%		

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

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-

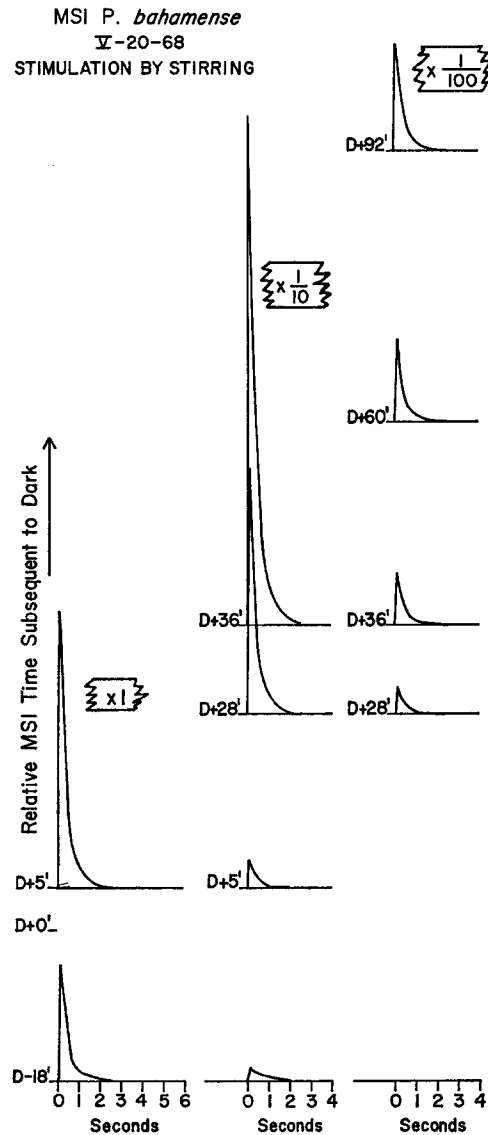


FIGURE 2. Shapes of MSI flashes of *P. bahamense* populations during the transition from photophase to scotophase. The ordinates are in relative intensity units for each curve. The curves are displaced vertically from one another by distances corresponding to the time during scotophase at which MSI was 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. lunula* 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

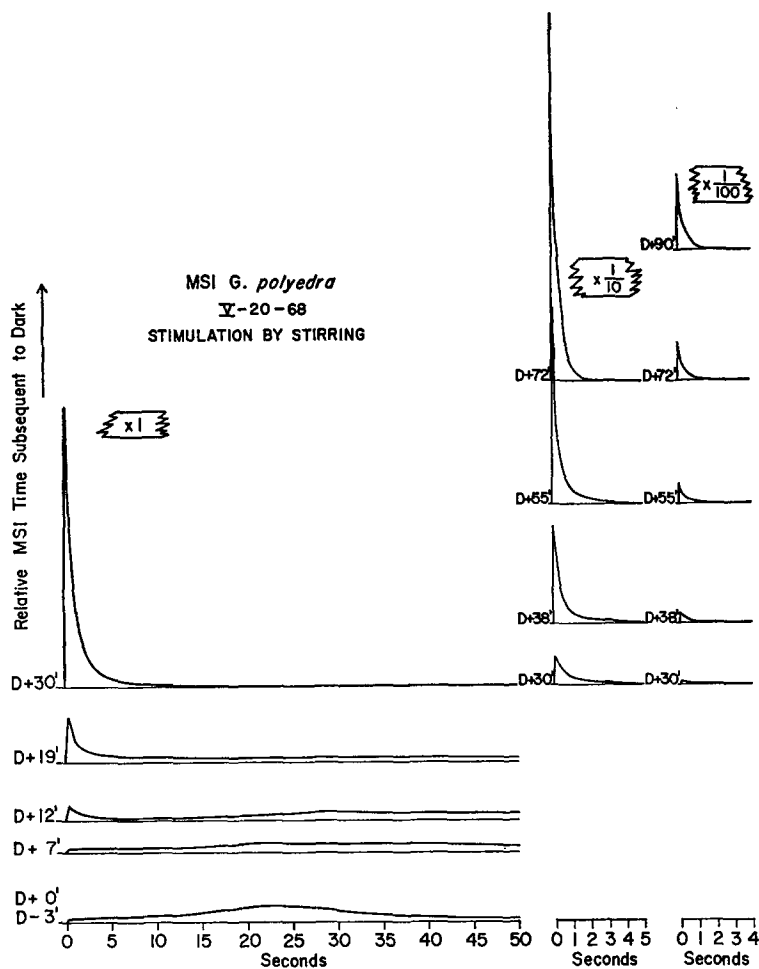


FIGURE 3. Shapes of MSI flashes of *G. polyedra* populations during the transition from photophase to scotophase. The ordinates are in relative intensity units for each curve. The curves are displaced vertically from one another by distances corresponding to the time during scotophase at which MSI was measured. The three vertical sections were measured at relative electronic gains of 1, 1/10, and 1/100.

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 stimutable 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

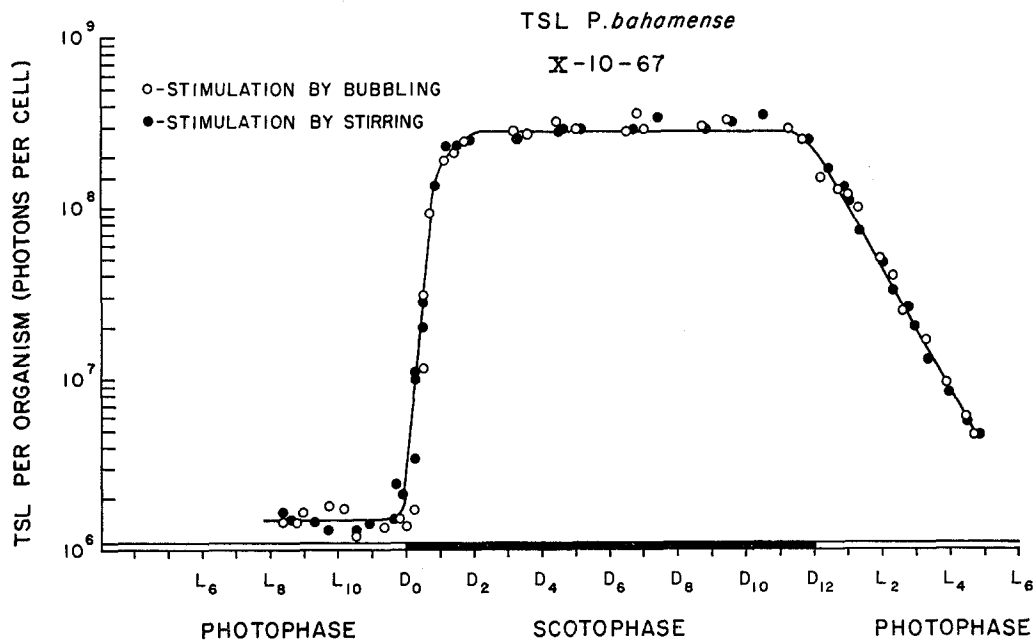


FIGURE 4. Photoperiod-entrained TSL per organism for *P. bahamense* in absolute units of photons over a complete photoperiod.

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

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. 5 *a* separately from the bubbling data which are shown in Fig. 5 *b*. As we have described above, TSL during scotophase (D_1 to D_{11}) 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. 5 *a* 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. 5 *b* 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_{11} , TSL per organism is constant within the precision of the measurements. (*d*) There is a small decrease in TSL per organism between D_{11} and D_{12} , 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-

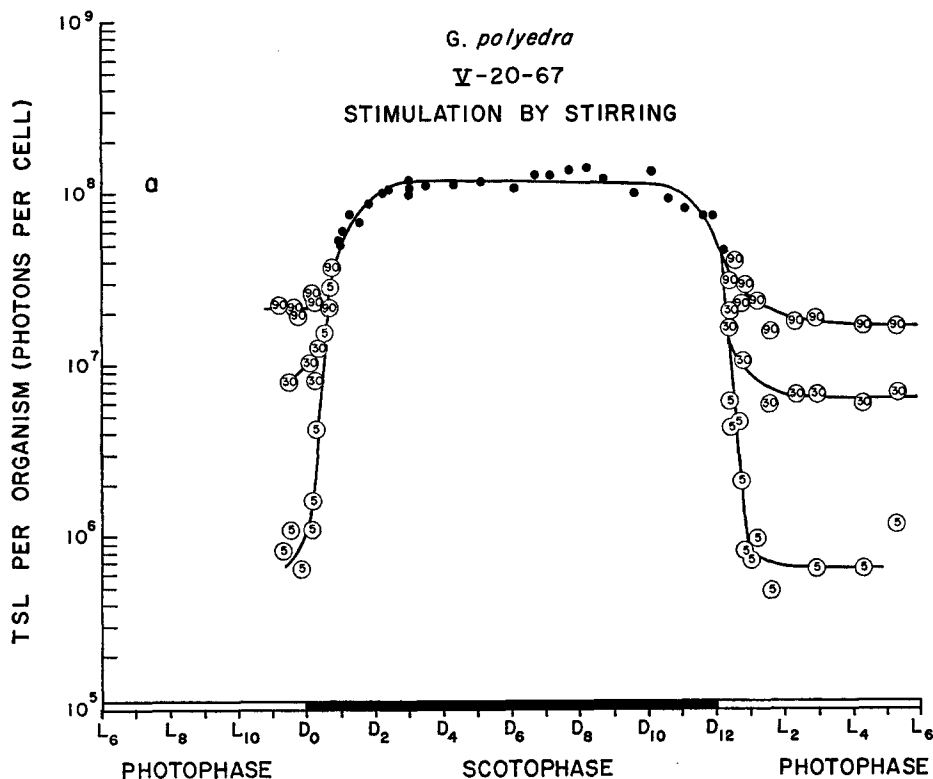


FIGURE 5 *a*

FIGURE 5. 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 D_0 to D_1 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 D_0 to D_1 light emission was integrated for 120 sec. During scotophase the data are independent of time of integration.

phase). (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 D_{17} , exhibited a very gradual decrease in TSL per organism beginning around D_{11} . (e) At D_{17} , corresponding to L_6 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.

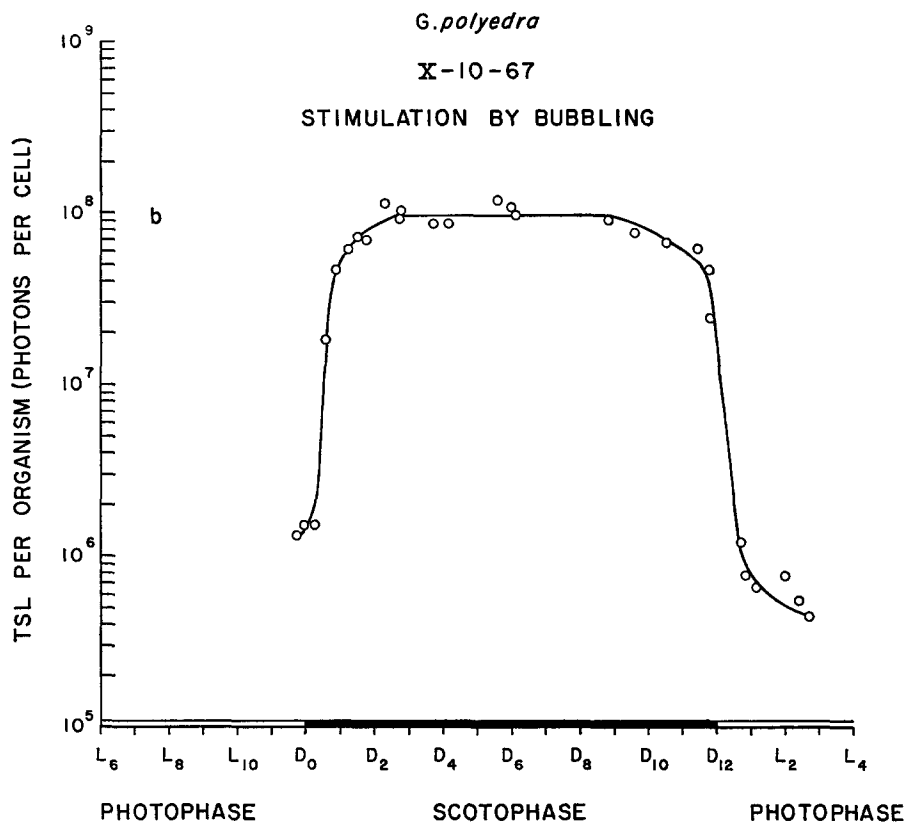


FIGURE 5b

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. baha-*

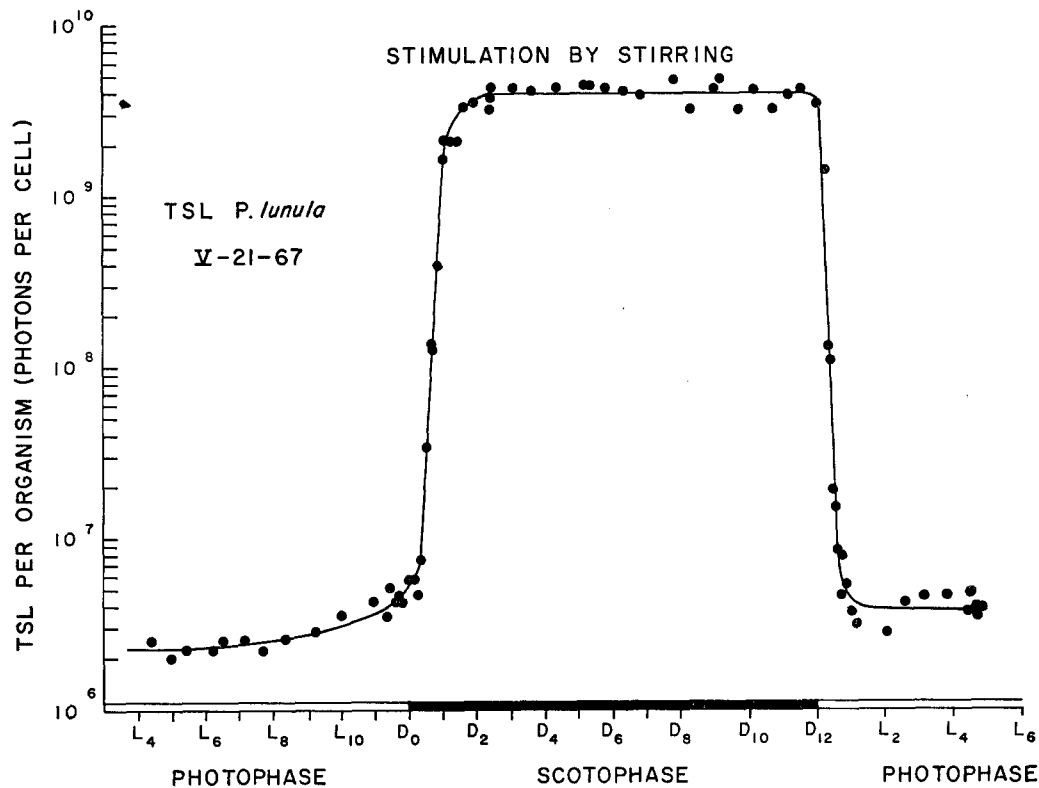


FIGURE 6. Photoperiod-entrained TSL per organism for *P. lunula* in absolute units of photons over a complete photoperiod.

mense in continued darkness there are: an increase in TSL during the interval D_0 to D_{12} , a decrease in TSL during D_{12} to D_{24} , an increase in TSL during D_{24} to D_{36} , and a decrease in TSL during D_{36} to D_{48} , 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*.

We further observed for all three species that during the time interval D_{12} to D_{18} 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 photoperiod (for these latter organisms the comparable time interval was L_0 to L_6). The comparisons of these two rates for all three species are shown in Fig. 7.

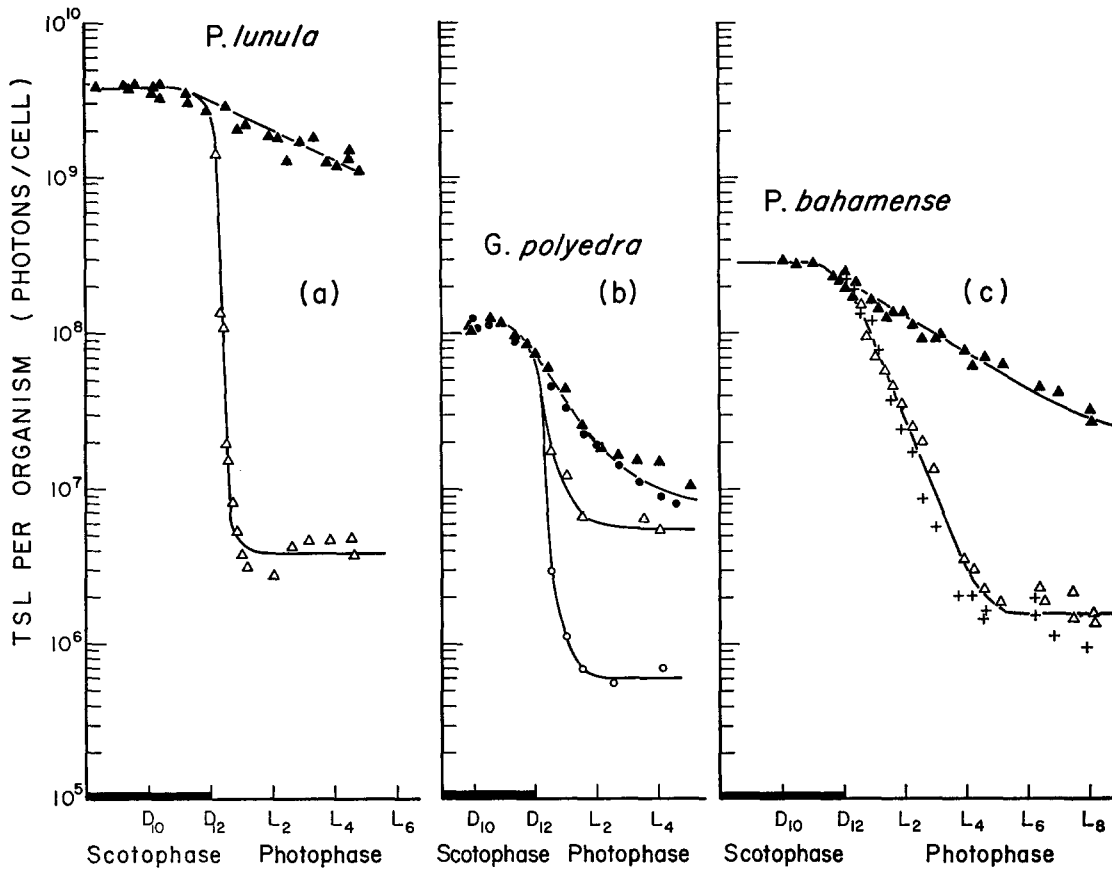


FIGURE 7. *a*, decrease in TSL per organism for laboratory cultures of *P. lunula* subsequent to initiation of photophase, compared with TSL per organism in continued darkness. Open triangles, photophase; solid triangles, continued darkness. *b*, decrease in TSL per organism for laboratory cultures of *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 IV
HALF-TIMES FOR SCOTOPHASE- AND
PHOTOPHASE-INDUCED CHANGES IN TSL

Species	Laboratory photoperiod		Natural photoperiod			Extended darkness
	Increase in TSL subsequent to step-function laboratory scotophase	Decrease in TSL subsequent to step-function laboratory photophase	Increase in TSL during natural Jamaica scotophase	Decrease in TSL subsequent to natural Jamaica photophase	Decrease in TSL subsequent to natural Baltimore photophase	Decrease in TSL subsequent to D_{12} in continued darkness
	<i>min</i>	<i>min</i>	<i>min</i>	<i>min</i>	<i>min</i>	<i>min</i>
<i>P. bahamense</i>	5-8	40-50	6-10	10-20	54*	160‡ 330*
<i>G. polyedra</i>	7	6-9	—	—	—	70‡
<i>P. lunula</i>	5	4-5	—	—	—	180‡

* 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_0 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 stimutable 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. 10 a) both methods of stimulation give essentially the same MSI flash

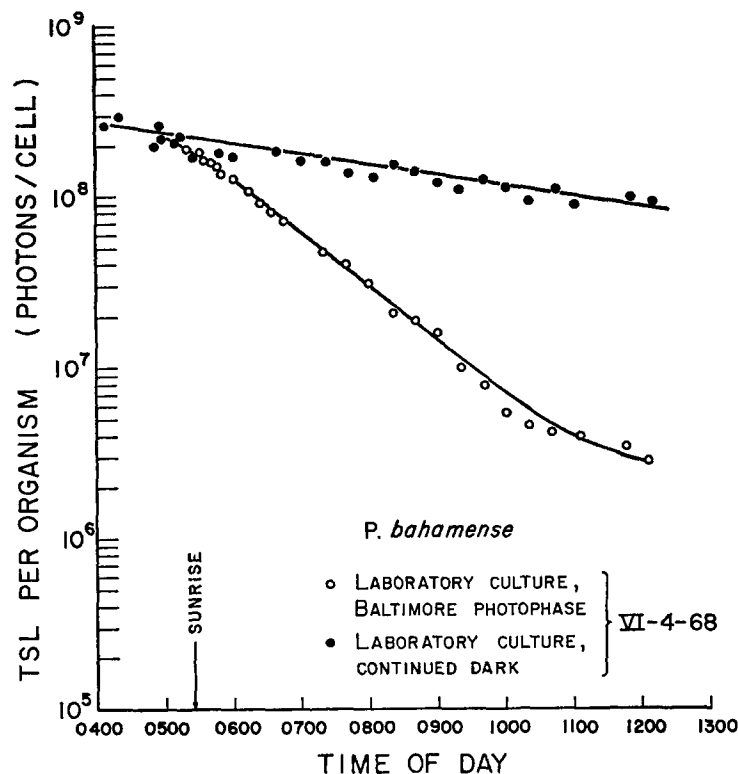


FIGURE 8. TSL per organism for laboratory cultures of *P. bahamense* during natural photophase compared with organisms remaining in constant darkness. Open circles, organisms in natural photophase in Baltimore (sunny day); solid circles, organisms kept in continuing darkness.

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. 10 b) that the marked difference in stimulability arises, giving rise to the concept of two different types of mechanically stimutable bioluminescence.

In all three cases, during extended scotophase or continued darkness, the kinetics of stimutable 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

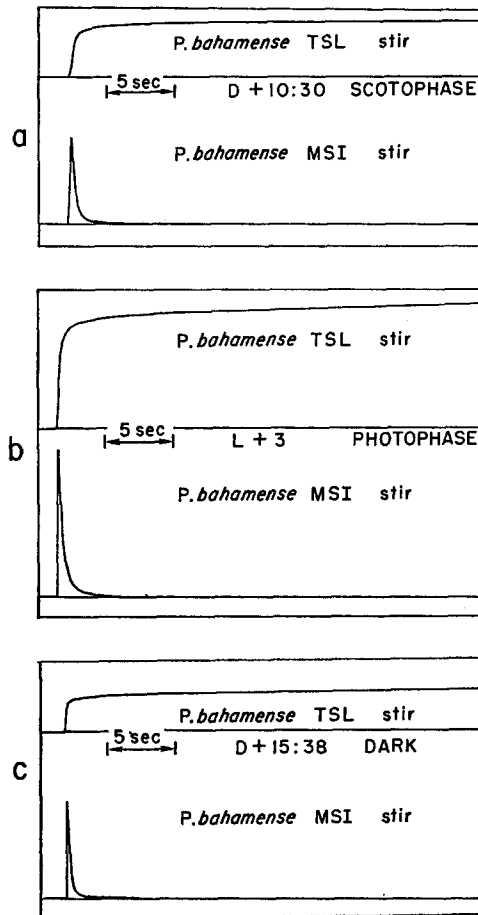


FIGURE 9. Direct tracings of chart records of TSL and MSI for *P. bahamense* for representative times during a), scotophase, b), photophase, and c), continuing darkness past D_{12} . The electronic gains for a, b, and c were different and the curves show only relative shapes.

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

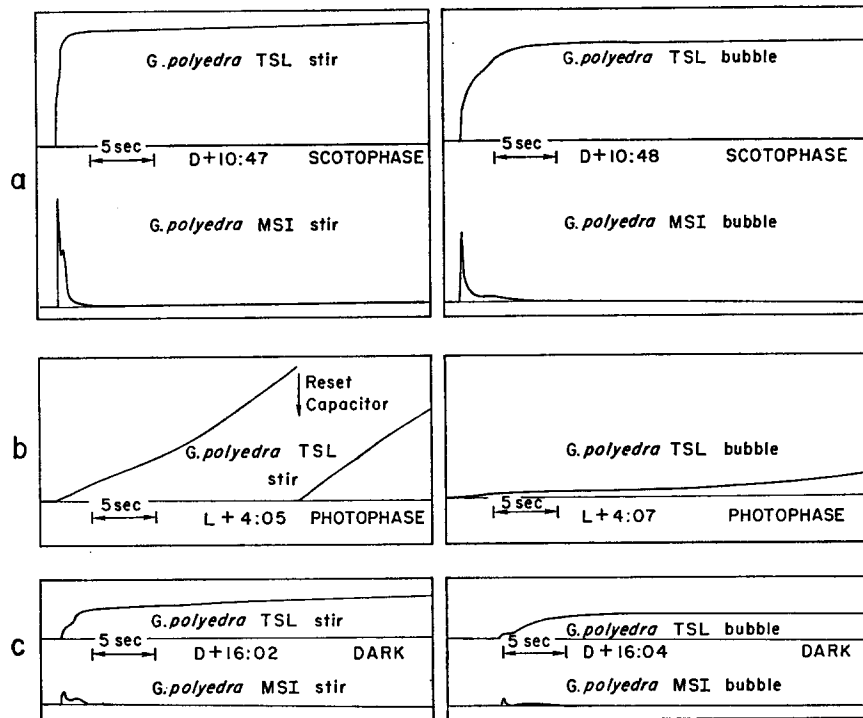


FIGURE 10. Direct tracings of chart records of TSL and MSI for *G. polyedra* for representative times during *a*, scotophase, *b*, photophase, and *c*, continuing darkness past D_{12} . The side-by-side stirring and bubbling curves can be compared directly. The electronic gains for *a*, *b*, and *c* were different and the curves show only relative shapes. In *b*, MSI was so small that it is not shown.

of photons per organism per second, averaged over a 5 min period, measured at 15 min intervals from D_0 through D_{48} (36 hr of darkness past D_{12}).

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 D_0 to D_{10} where the average rate of light emission per organism is 280 photons per second; (*b*) a narrow peak at D_{11} 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 D_{18} through D_{28} 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

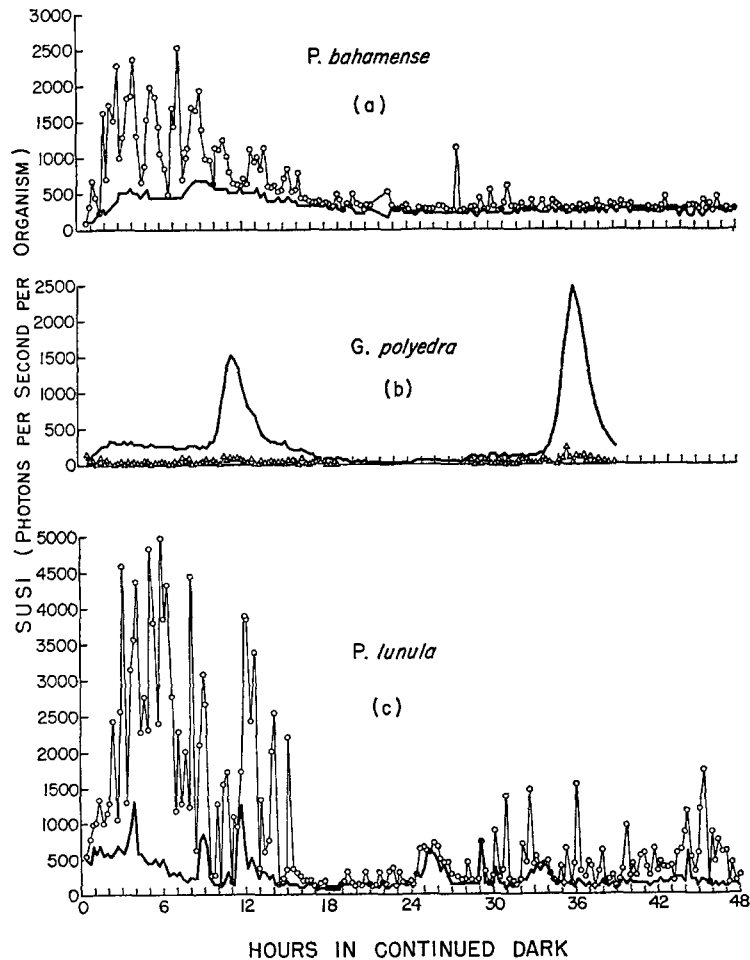


FIGURE 11. SUSI in photons per second per organism beginning at scotophase, D_0 , and continued in darkness through D_{48} . a, *P. bahamense*. Open circles, total SUSI, flashes plus glow; heavy line, glow only. b, *G. polyedra*. Open triangles, flashes only; heavy line, glow only. c, *P. lunula*. Open circles, total SUSI, flashes plus glow; heavy line, glow only.

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-

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

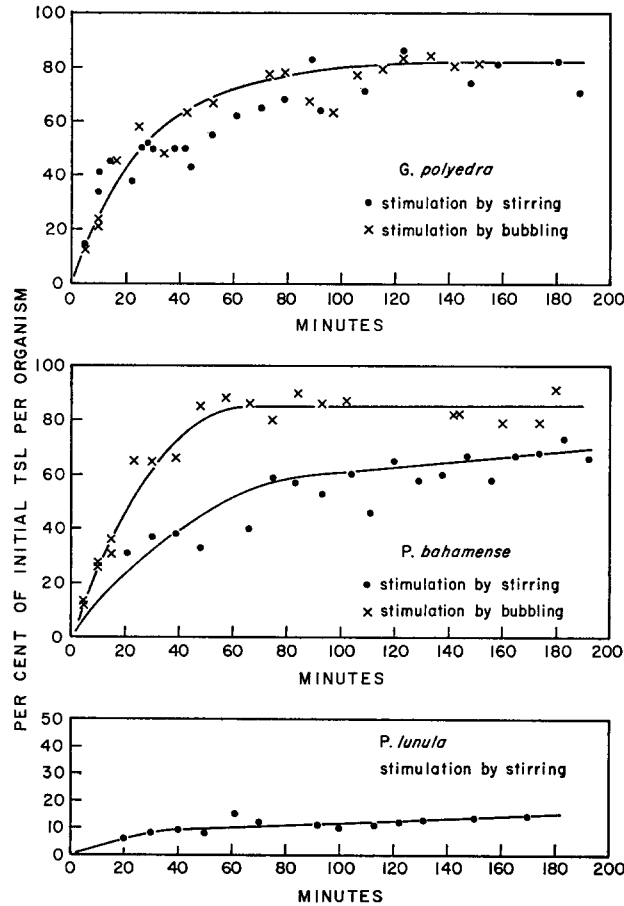


FIGURE 12. Recovery of stimulative bioluminescence for *G. polyedra*, *P. bahamense*, and *P. lunula*. Time is measured subsequent to complete stimulation by the technique used to measure recovery.

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

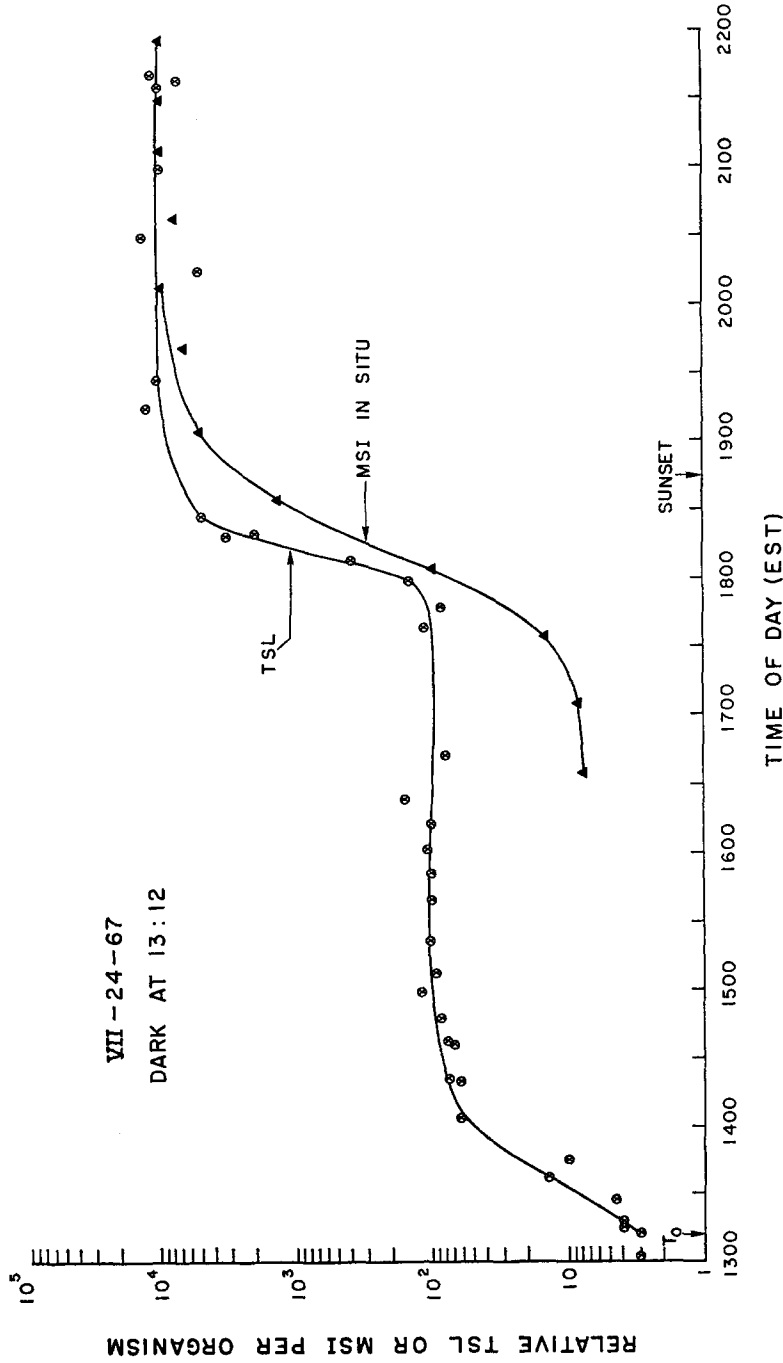


FIGURE 13. Relative TSL per organism for natural populations of *P. bahamense* removed from Oyster Bay in the middle of natural photophase and placed immediately in continued darkness (X). For comparison we have plotted, over exactly the same time period, the *in situ* MSI per organism, normalized to scotophase TSL (▲).

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

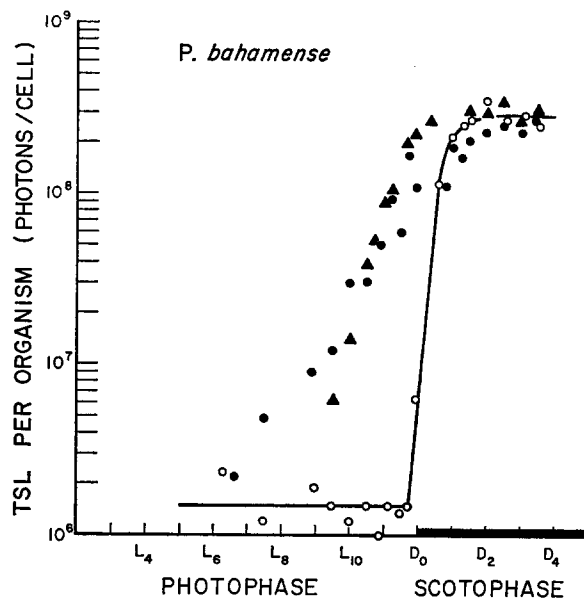


FIGURE 14. TSL per organism for laboratory cultures of *P. bahamense* as a function of photoperiod time. The cultures were removed from the fluorescent light photophase and placed immediately in continued darkness. For comparison, we have also plotted the normal photoperiod-entrained rise in TSL per organism. Solid circles, cultures removed from photophase at L_6 ; solid triangles, cultures removed from photophase at L_9 ; open circles, control removed from photophase at L_{12} .

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 L_6 and at L_9 (Fig. 14), showed a gradual rise to a maximum value of TSL per organism at D_1 .

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 stimutable luminescence, although different for different species, is a constant throughout essentially the entire scotophase (D_1 through D_{11}), 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 stimutable 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 (D_{12} through D_{18}) 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. lunula* 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 $\frac{\text{Scotophase TSL}}{\text{Photophase TSL}}$ 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 D₀ 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 D₃₆ 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 1.3×10^6 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 ≥ 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 L_{12} 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 stimutable bioluminescence (Seliger et al., 1961) as well as a second species which does.⁴

⁴ Seliger, H. H., and W. G. Fastie. Unpublished data.

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.

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