Bioluminescence of Marine Dinoflagellates

I. *An underwater photometer for day and night measurements*

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ABSTRACT Portable light-baffled underwater photometers have been designed for the measurement of dinoflagellate bioluminescence by day and night. Maximal light emission is obtained by mechanical stimulation in a defined volume. The pump which stimulates the dinoflagellates also constantly replenishes the sample volume so that continuous measurements are possible. Evidence for both diurnal variation and vertical migration is presented. Using luminous bacteria for calibration a single dinoflagellate has been found to emit of the order of 10^{10} light quanta per flash. The technique suggests that large scale mapping of bioluminescence is feasible.

INTRODUCTION

Much of the "phosphorescence" of the sea is due to the light emission of dinoflagellates. These photosynthetic marine protozoa have a world-wide distribution and are of great importance to the food economy of the oceans. In warm waters and in tide-protected bays growth can be so intense that the water becomes colored because of the high concentration of organisms. Unfortunately, very little is known about the physiology and ecology of these organisms and the reason why blooms of dinoflagellates develop in open waters still remains a mystery. Haxo and Sweeney (1) have developed methods for growing one of these organisms in pure culture and Hastings (2) has studied in detail the rhythmic diurnal luminescence of these laboratory-grown cultures.

It is the purpose of this paper to describe photometric equipment for the quantitative measurement of maximal light emission by these organisms in their natural habitat and to present some of the observations which have been made in Chesapeake Bay, Maryland; Great Harbor, Woods Hole, Massachusetts; and in the luminous bay at Oyster Bay, Jamaica, British West

Indies. The portable, light-baffled equipment permits measurements to be made from a small boat during a 24 hour period without interference from ambient sun- or moonlight. Both horizontal and vertical light intensity mappings can be made with high precision. With a calibrated unit, the observation of single flashes concurrently with integrated light intensities permits direct conversion of observed light intensities to organism concentration. The techniques described could be of great importance for large scale mapping of oceans in order to provide valuable information about the distribution of this basic food supply.

The observations which are presented indicate large diurnal variations in light intensities as well as extensive vertical migration, both of which appear to be intimately related to such factors as previous photosynthetic activity, water temperature and salinity, and organism density. There are also large species differences with respect to these factors.

EQUIPMENT AND TECHNIQUES

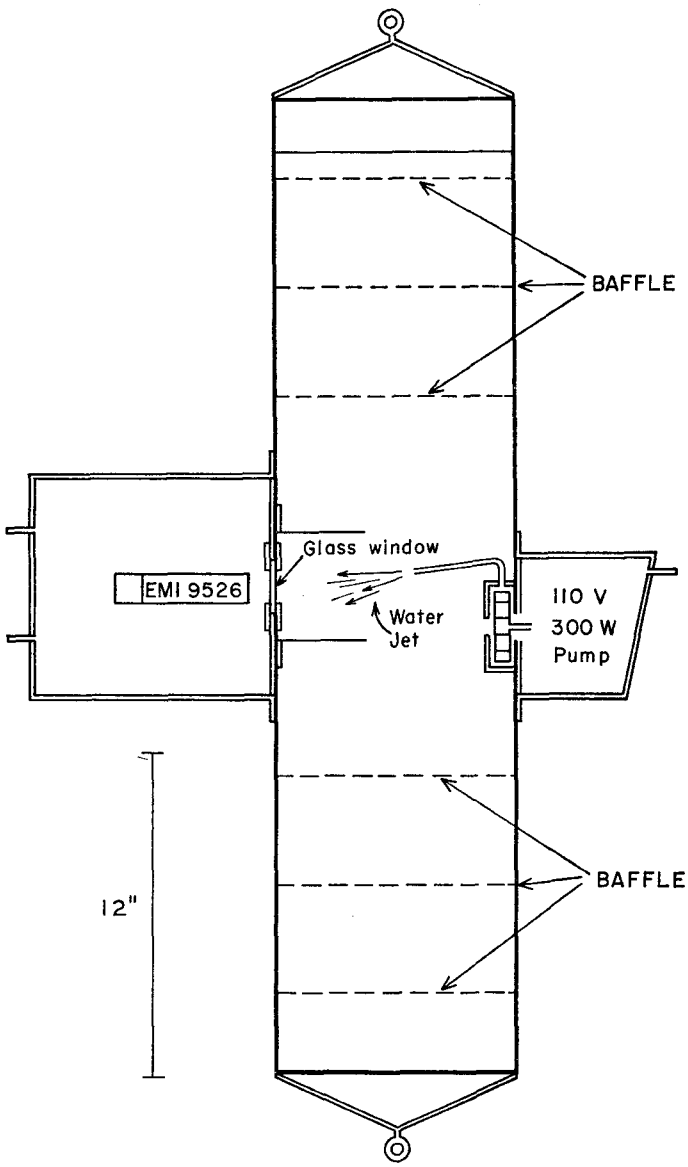
Photometric equipment for the measurement of underwater marine bioluminescence has been described by Clark *et al.* (3), by Backus (4) at the Woods Hole Oceanographic Institute, and in a previous note (5). The present equipment represents an improvement in design over that referred to in our previous communication although the concept of measurement of "stimulated bioluminescence" has remained the same.

From casual visual observations it is evident that freshly collected dinoflagellates will emit no light if they remain undisturbed. For this reason, therefore, we felt that it was essential in the design of the equipment to provide for maximal stimulation. In this way we could be sure of obtaining the maximum capacity for light emission. By depending upon "spontaneous" emission it is not possible to evaluate a number of factors known to be important for light emission. Organisms growing in the warm waters of the Caribbean, for example, appear to have a much lower threshold for stimulated light emission than do those growing in Chesapeake Bay or at Woods Hole. It is not yet known whether light emission from a single cell is an all or none phenomenon or is dependent upon the degree of stimulation up to a maximum of stimulation. In addition, we know that the light flashes are due to a large number of enzyme (luciferase)-catalyzed chemiluminescent oxidations in the individual cells and consequently the light intensity will depend upon the temperature, pH, and ionic environment. The total light emitted by any one cell will be dependent upon the substrate (luciferin) and enzyme (luciferase) concentrations and consequently the previous history of the cell will be important. This is particularly true for the photosynthetic dinoflagellates where one might expect large variations in light intensity not only from day to day but also depending upon the depth and transmission of the water in which the organisms grow. Since the individual cells can emit, even on maximal stimulation, varying amounts of light it is in principle better to measure the total light from a particular distribution rather than the individual flashes. The former is more directly related to the total substrate available.

We have therefore designed the electronic equipment to measure DC phototube current rather than relying on AC pulse-counting and its attendant pulse height discrimination. In addition, we have employed maximal mechanical (rather than electrical or chemical) stimulation and a light-baffled sample cell which can be used for both horizontal and vertical mapping. The Chesapeake Bay luminescence was used to make the initial tests and the "phosphorescent bay" at Oyster Bay, Jamaica, was later studied since it is one of the few places in the world where there exists a stable dinoflagellate culture with practically no tidal mixing. Thus, we can assume that intensity measurements over a period of night and day refer to the same population and are not greatly modified by tidal mixing.

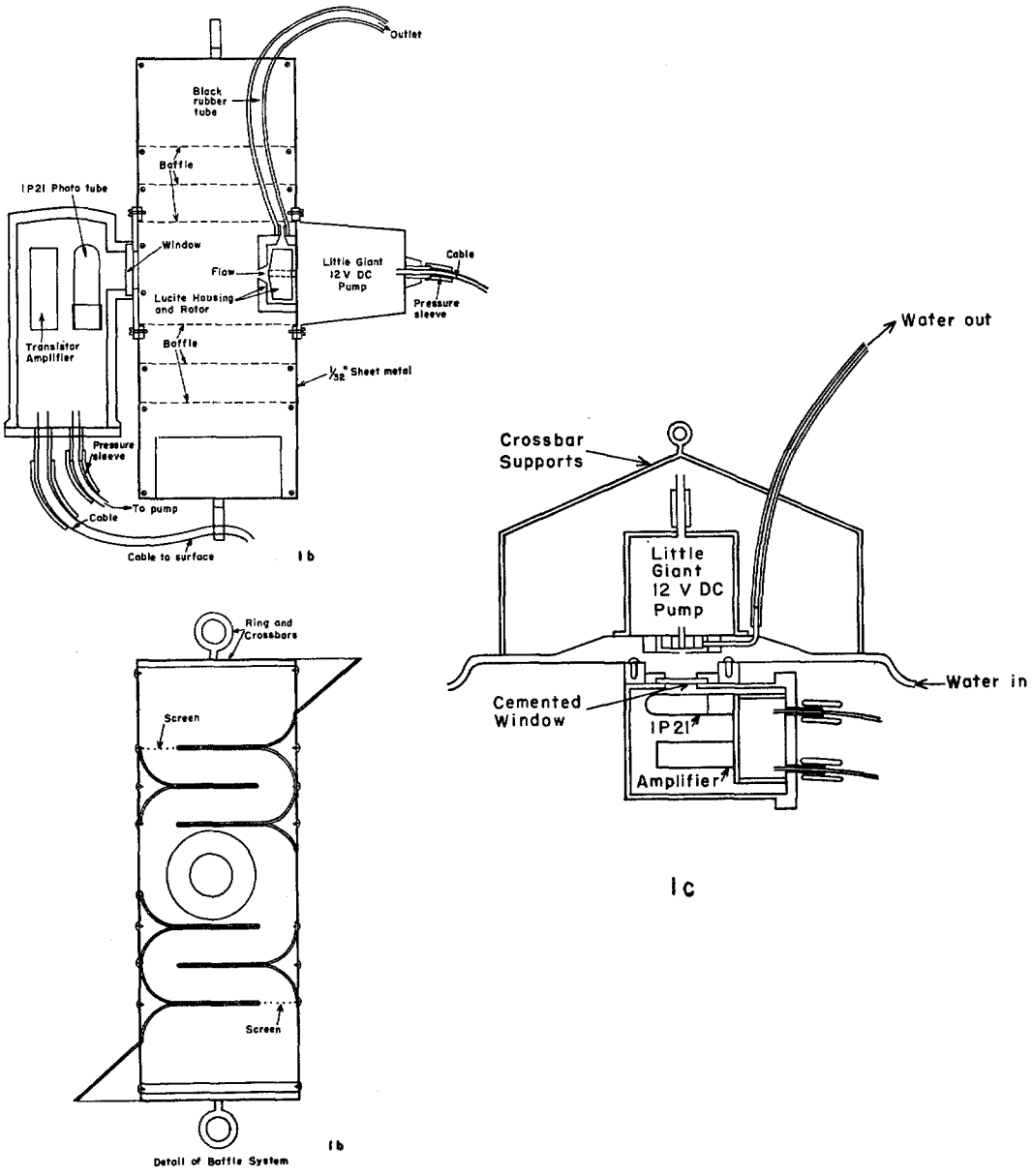
In Fig. 1 is shown the evolution of the three types of underwater units which have been developed. The unit in Fig. 1 *a* was used in Chesapeake Bay; that in Fig. 1 *b* was used in Oyster Bay and Great Harbor and in Fig. 1 *c* is a unit that has recently been compared with Fig. 1 *b* in Chesapeake Bay. In all cases, an electron multiplier phototube is mounted in a water-tight housing with a gasketed glass window facing the inside of a light-tight sample volume. All measurements were of phototube current using a transistorized DC amplifier contained inside the phototube housing. The response time of the DC amplifier was 10^{-3} sec. This is one order of magnitude lower than the rise time of a light flash from a single cell, thus permitting the simultaneous observation of individual flashes on an oscilloscope and the integrated light intensity on a miniature chart recorder. The complete electronic circuit is shown in Fig. 2.

The light-baffled cells were designed so that bioluminescence intensity measurements could be made in bright sunlight at the sea surface and to depths of 150 feet. Thus, data could be obtained in shallow waters and continuous horizontal mapping measurements could be made throughout the day and night. Bioluminescence was observed as a result of mechanical stimulation of the organisms, providing three to four orders of magnitude more luminescence than the spontaneous luminescence observed by Clark's group. Two types of mechanical stimulation were used. In Fig. 1 *a* an impeller type of water pump was mounted opposite the phototube face with its exit nozzle adjusted so that a jet of water was directed at the phototube window. The turbulence in this jet was sufficient to stimulate light emission in those organisms within the stream. The 9 × 12 × 12 inch sample volume was sufficiently large that non-stimulated organisms were being continually replenished by convection to the jet stream. In this manner reasonably constant light intensities over a period of 1 to 2 minutes could be obtained from the same sample volume. After this time the intensity decreased with time due to exhaustion of the organisms. The reproducibility was within ± 10 per cent so it is reasonably certain that the observed stimulated bioluminescence could be correlated with the density of bioluminescent organisms. In Figs. 1 *b* and 1 *c* the mechanical stimulation technique represents an improvement over the jet stream in several ways. First the Nylon impeller blade and impeller blade housing have been replaced with components made of clear lucite. In this case the water sample is drawn in through the one-half inch diameter center hole of the impeller blade housing and is forced around and out of the exit nozzle, this time into a black rubber tube which snakes out of the baffled cell and discharges the spent organisms into the surrounding water several feet from the baffled cell. The effluent is dark and

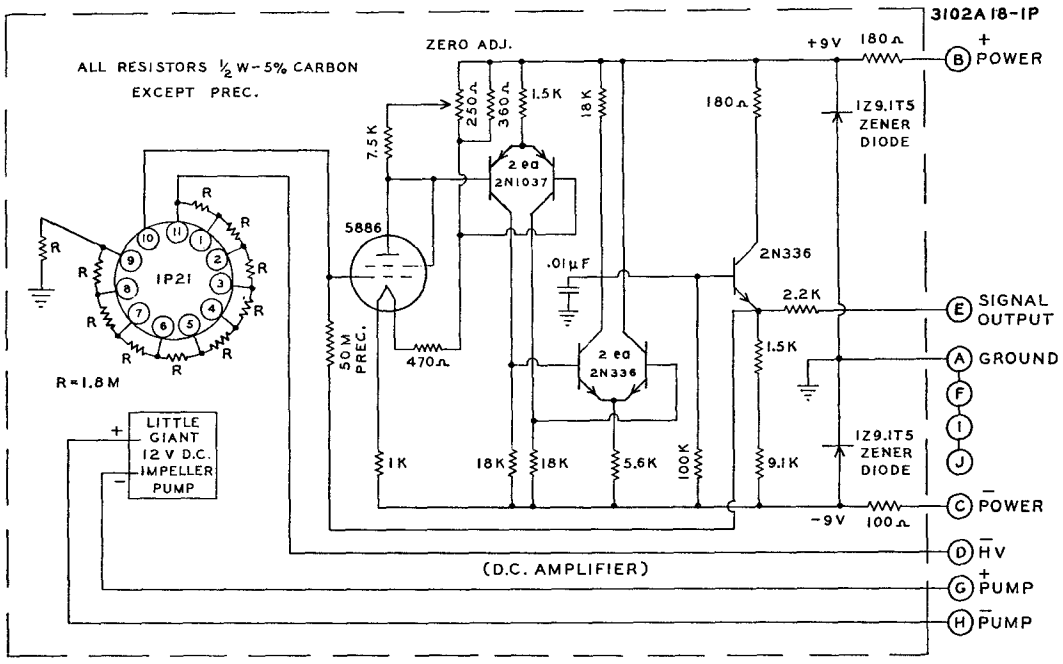


1 a

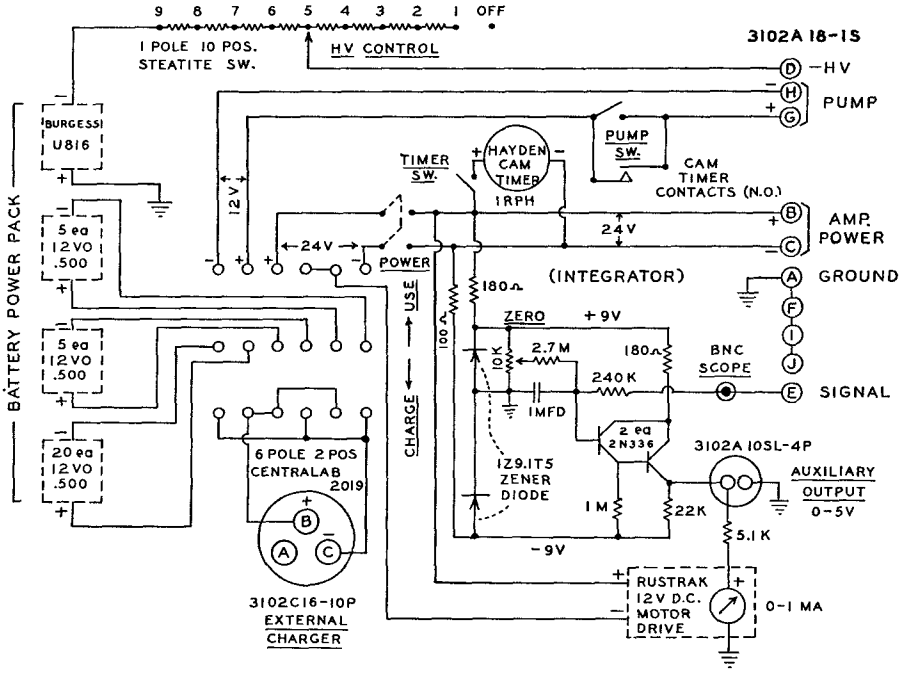
FIGURE 1. Drawings of the underwater sample cells used in the measurements. In Figs. 1 *b* and 1 *c* the impeller housing and the impeller blade are clear lucite. The positive sealing boots used to lead in the electrical cables were cut from one-quarter inch wall rubber vacuum tubing.



non-stimulable, providing direct evidence that the organisms have been maximally stimulated inside the lucite impeller housing and that the observed bioluminescence intensity is directly proportional to the total available light. Second, the sample available to the impeller blade housing is being continually replenished by a gradual flow through the much larger, cross-sectional area of the baffled cell. Thus, continuous light intensity measurements can be made at a fixed depth over day-night periods



UNDERWATER PHOTOTUBE HOUSING



SURFACE CONTROL BOX

with no decrease in intensity due to exhaustion of the organisms in a fixed sample volume such as that in the cell in Fig. 1 *a*. Third, since only the lucite housing emits light the problem of absolute calibration of the phototube geometry becomes simple and can be related directly to the light emission by exactly the same volume of luminous bacteria. The cell in Fig. 1 *c* uses the identical continuous flow stimulation of Fig. 1 *b*. However, the narrow horizontal circumferential slot through which the sample enters into the apparatus provides much better depth resolution, from 6 inches in the case of the cell in Fig. 1 *b* held in a horizontal position to one-half inch. In addition the phototube is much closer to the lucite housing.

Light intensity measurements were made as a function of depth over short time intervals as well as over day-night periods at a fixed depth. In Oyster Bay these light intensity measurements were correlated with measurements of temperature, salinity, and other ionic concentrations, photosynthetic activity, species identification, and organism concentration.

Calibration with Luminous Bacteria

The use of a lucite impeller housing as a defined volume in which stimulated bioluminescence occurs allows a direct standardization of the efficiency of the light-detecting system through the use of luminous bacteria. We have been making measurements of the quantum yield of bacterial luminescence similar to our measurements of the quantum yield of firefly bioluminescence (6). We can therefore determine quite accurately the total number of photons emitted per second by a given volume of luminous bacteria. If the internal volume (55 ml) of the impeller housing is filled with a known amount of a light-emitting bacterial solution, we have a direct calibration of the effective geometry of the photometer phototube. The major advantages of using luminous bacteria are (*a*) the light emission of a dilute solution is essentially continuous and constant, not requiring external stimulation and (*b*) the spectral emission of the particular strain of luminous bacteria used (A. *fischeri*) very closely matches that of the dinoflagellates, obviating the necessity for knowledge of the phototube spectral sensitivity.

The assumption made here is that the average spatial distribution of stimulated bioluminescence inside the impeller housing is the same as that of the continuously luminous bacteria. Using this technique we have found that a single dinoflagellate

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FIGURE 2. Circuit diagram of the photometer unit and surface control box. The system including batteries was portable and housed in a water-tight container. In order to conserve power, the pump, drawing approximately 2 amperes, was not operated continuously. A 1 RPH 24 volt DC synchronous timer was fitted with a four-step cam switch which could override the manual pump switch, allowing the pump unit to operate automatically overnight at a cycle of 2 minutes on, 13 minutes off. The optimum integration time was 0.24 second. There were no breakdown problems encountered in having voltages as high as 1200 volts sent through the connectors or the waterproof cable. At the present time we have a circuit design for an adjustable high voltage supply using a transistor amplifier inside the phototube housing and sending low voltage DC through the cable. The rechargeable battery packs permit continuous readings over a period of 30 hours. The high voltage battery has essentially shelf life.

emits of the order of 10^{10} light quanta per flash. With this number, it is possible to express observed integrated light intensities in terms of organism concentrations per liter, knowing the sample volume and the pumping speed. This method applied to data observed at Great Harbor in July, 1961, and in Spa Creek of the Severn River outside Chesapeake Bay gave peak organism concentrations of 100 and 200 per liter respectively, 100 times *smaller* than in Oyster Bay.

RESULTS AND DISCUSSION

The organisms in Oyster Bay responsible for the luminescence observed were identified as an essentially homogeneous dense culture of the marine dino-

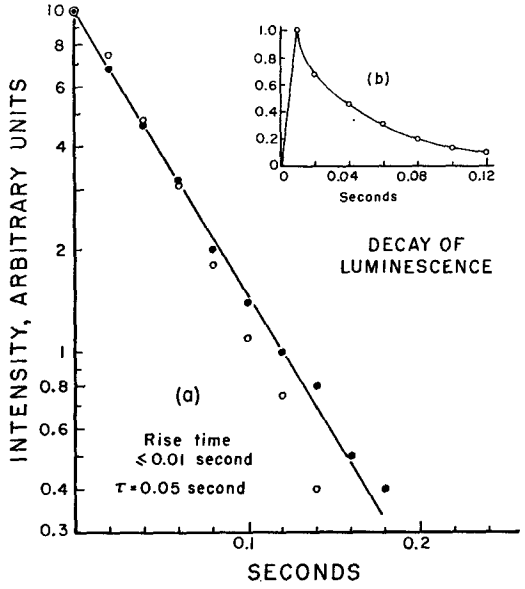

FIGURE 3. Rise and decay times of bioluminescent flashes.

flagellate *Pyrodinium bahamence.*[1] Four other species were observed but these occurred in very small numbers.

Individual Flashes The rise and decay times of the dinoflagellate-stimulated bioluminescence were identical in Chesapeake Bay, Oyster Bay, and Great Harbor. The decay of light intensity by over a factor of 25 appears to be first order and is shown in Fig. 3. The luminescence occurs inside the cell, unlike the *Cypridina* bioluminescence where the luciferase and luciferin are ejected separately into the surrounding medium and mix by diffusion. The rise time was determined with a tektronix oscilloscope to be 0.01 second

[1] A satisfactory growth-supporting medium for *Pyrodinium bahamence* has been reported recently by J. J. McLaughlin and P. A. Zahl, *Science*, 1961, **134**, 1878.

±10 per cent. The mean life for decay of 0.05 second most likely indicates a pseudo first order reaction due to an excess of luciferin inside the cell. Upon stimulation of samples of very low organism concentration the light flashes could be seen individually. These flashes varied in height by as much as a factor of 10. Since the phototube was approximately 6 inches removed from the impeller housing sample volume (Fig. 1 *b*) it can be assumed that within ±30 per cent the variation in light flash height was not an inverse square geometry effect but a real effect due to variations among the organisms.

Except for an occasional very weak flash we found that freshly collected dinoflagellates will emit no observable light in the laboratory in perfectly still water. These same dinoflagellates will emit brilliant flashes if the container is tapped by a pencil or by a finger or if the laboratory table is jarred. After this flashing they will again be dark until they are tapped again. In all cases the total light emitted as a result of tapping is very much less than the total available light so that the effect is not one of exhaustion and replenishment of reactants. Sweeney and Hastings (7) have recently succeeded in culturing pure colonies of *Gonyaulax polyedra*, and in these laboratory cultures there appears to be a continuous low background of light emission ascribed to "leakage" during cell reproduction. We have not observed this in our *in situ* measurements in Oyster Bay. This may be a species difference or a culture medium difference.[2]

Integrated Intensity Measurements

Fig. 4 shows a set of measurements of stimulated bioluminescence intensity data for roughly a 24 hour period in Oyster Bay. The cell in Fig. 1 *b* was fixed in a horizontal position at a 2 foot depth below a small rowboat anchored in the Bay. Each point is the sum of the integrated light intensity readings over a period of 10 minutes. The curve is drawn unsmoothed to illustrate the nature of variation in the measurements. At approximately 3:00 a.m. and for about 30 minutes afterward there were very wide oscillations in stimulated light intensity which were correlated with a small local shower. The increase to the minor peak of luminescence at 5:00 a.m. is believed to have been due to the recovery of the organisms from this local disturbance.

We can draw several conclusions from these data: (*a*) The amount of light that can be emitted by these organisms upon maximal stimulation can vary over a factor of approximately 100 from night to day. (*b*) The peak intensity observed occurs approximately 4 hours after sunset and decreases even in the dark of night. (*c*) The bioluminescence intensity shows several distinct peaks;

[2] Hastings has stated that his solution concentrations were 100 times as dense as our *in situ* concentrations. This may account for the fact that he was able to observe this effect. However, it seems more likely that these dense growths were sufficient for mutual interaction of the dinoflagellates themselves which would indicate that the light emission is not an all or none phenomenon.

the first maximum occurring within 30 minutes of sunset, followed by a second maximum 2 hours after sunset.

The shape of this diurnal variation curve can be readily explained if we assume that superimposed upon the single central peak due to diurnal variation there is an artifact in the experimental setup due to vertical migration of the organisms; first upward past the cell fixed at the 2 foot depth and then near dawn downward past the cell. This migration would give a three-peaked curve as shown in Fig. 4. Of more direct concern therefore are the data shown in Fig. 5 where the depth distribution of stimulated biolumines-

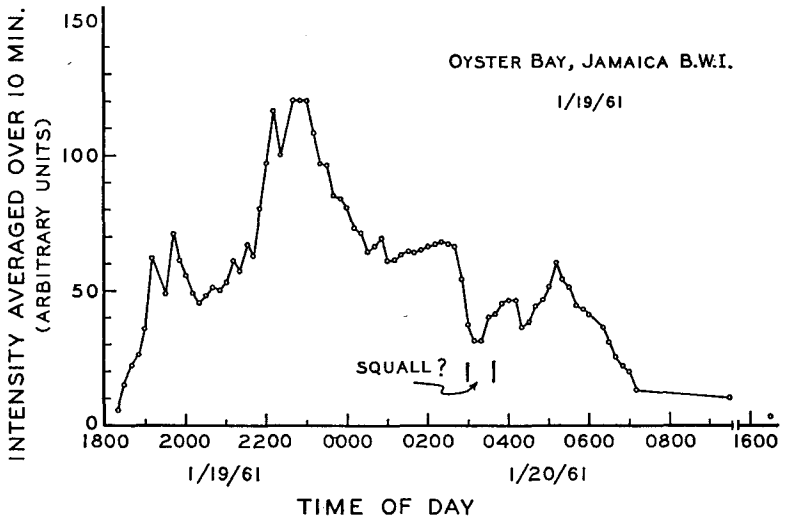

FIGURE 4. Measurements of stimulated bioluminescence intensities over roughly a 24 hour period. Curve is drawn unsmoothed. Oyster Bay, Jamaica, January, 1961.

cence intensity was measured over the period from just before sunset through to the time when the luminescence reached a maximum. The total depth of this small bay varied from 4 to 6 feet. At this particular spot the depth was 4½ feet. Curve 5 *a* taken at 6:20 p.m., about 20 minutes before sunset, actually shows a slight increase in luminescence with depth, although the intensity is very low, while curves 5 *b*, 5 *c*, and 5 *d*, taken at sunset and roughly 2½ and 4 hours after sunset respectively, show both the large diurnal increase as well as the migration to the surface. Data taken during daylight hours showed the same shape as curve 5 *a* and were even lower in intensity. At approximately the same times as the light intensity data were taken, samples were removed from the surface and from a 3 foot depth and the concentration of organisms was determined by microscopic count. Those data and the normalized ratios of bioluminescence intensities per organism are given in Table I. From this table we see that not only has the individual light emission

per organism increased by a factor of 40 or 50 but there is evidence of a migration of organisms to the surface and also from depths below 3 feet.

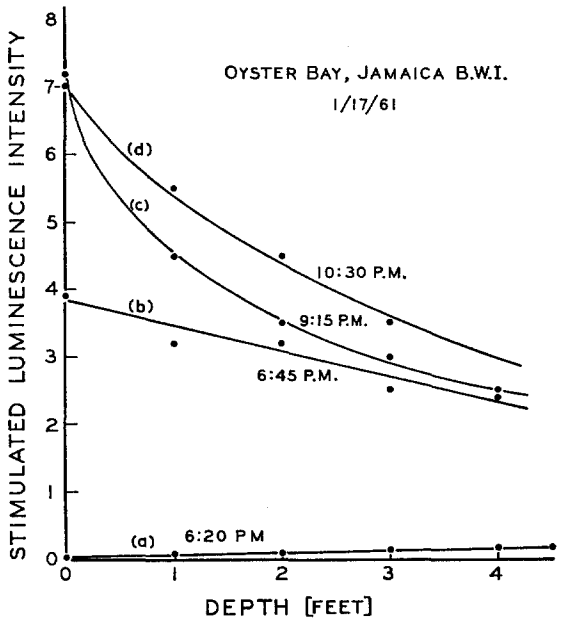

FIGURE 5. Depth distribution of bioluminescence intensity at various times from before sunset (*a*) to the time of maximum luminescence intensity (*d*). Oyster Bay, Jamaica, January, 1961.

TABLE I

Time	Surface		3 ft.	
	No. of organisms per liter	Intensity per organism Arbitrary units	No. of organisms per liter	Intensity per organism Arbitrary units
6:00 p.m. pre-sunset	100,000	1	160,000	1
9:15 p.m. post-sunset	220,000	46	130,000	33
10:30 p.m. post-sunset	190,000	52	130,000	41

After sunset approximately 16 per cent of the organisms are below 3 feet while before sunset 35 per cent of the organisms are below 3 feet.

The flagella of these dinoflagellates cannot efficiently produce straight line motion. It is therefore rather unlikely that any vertical migration of these organisms is due to swimming. Rather a density change would be a much more efficient mechanism. Calculations using the Stokes formula for the viscous motion of a 25 micron diameter sphere indicate that a 6 per cent

change in effective mass due to ion diffusion through the rigid-walled organism could account for either the upward or downward migration observed.

These data, showing evidence for both a diurnal variation in stimulated light intensities and a vertical migration of organisms, agree in part with our previous Chesapeake Bay data, shown in Fig. 6. Here there appears to be a vertical migration but no diurnal variation as evidenced by the fact that the peak intensity at 2:00 p.m. on a bright sunny afternoon was even slightly higher than that at 10:00 p.m. Unfortunately, we were not able, in the Chesapeake Bay, to make concurrent organism density measurements or species

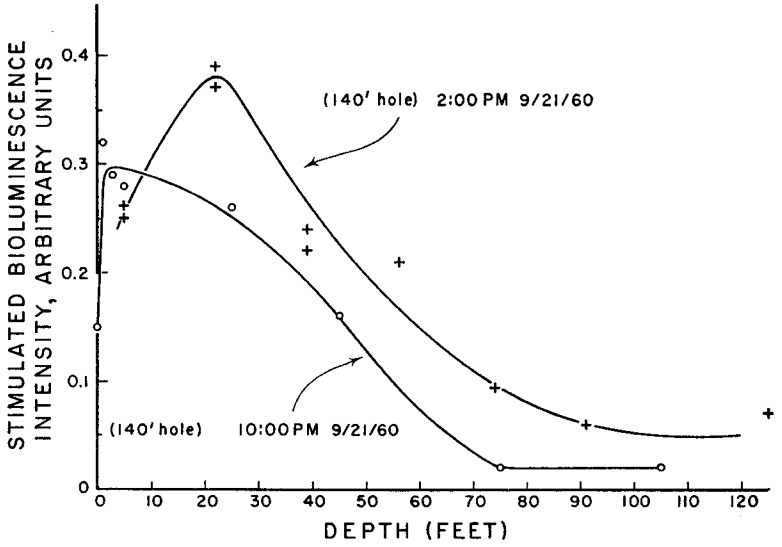

FIGURE 6. Depth distribution of stimulated bioluminescence intensity before sunset and after sunset. Chesapeake Bay, September, 1960.

identification. The temperature was constant to within a few tenths of a degree C to below 50 feet. We can only speculate that the diurnal difference observed is due to a species difference. This appears very likely since measurements made again this summer and fall (1961) in Chesapeake Bay and this summer in Great Harbor gave evidence for *both* a diurnal and vertical migration variation. There is the possibility that physiological variation occurs during the year and that diurnal variation in luminescence can be altered.

Other Parameters

A description of the physical and chemical oceanography underlying the growth and light emission of these organisms with respect to temperature, salinity, photosynthesis, phosphate concentrations, and tides and basin structure will be published elsewhere (8). Temperature measurements were

made with a portable direct reading thermistor unit and chemical parameters were determined by standard techniques. *Pyrodinium* bioluminescence was found to be extremely temperature-sensitive. The normal temperature in Oyster Bay was 28°C and the measurements of Figs. 4 and 5 were made during periods when this temperature was constant as a function of depth. On other occasions there appear very strong temperature gradients at approximately a 2 foot depth; the surface temperature down to 2 feet could be 24°C and then in the space of a few inches the temperature would change to 28°C down to the bottom. In these cases, although the organisms were present, no bioluminescence was observed above the 2 foot depth. The bioluminescence measured at this 2 foot depth was within ±20 per cent of the previous maximum value at the surface when the temperature was uniform at 28° from top to bottom. The lack of bioluminescence at 24°C and the bioluminescence in Chesapeake Bay observed at 24.2°C point up a species difference between the two locations. *Pyrodinium* is a photosynthetic dinoflagellate as determined by C^{14}-uptake experiments. There apparently is some light requirement for replenishment of luminescence potential since of two samples, both of which had been stimulated to exhaustion, the one exposed to light for several hours recovered and the one kept in the dark remained dark.

The curve in Fig. 4 shows another interesting aspect. If we assume that the three positive peaks are due to the upward migration, diurnal variation, and downward migration respectively, then two alternative conclusions follow. Either the first maximum in the rate of light emission in less than 50 minutes following sunset is due to a rush of organisms toward the surface from depths below 2 feet, or there is a very rapid rate of increase of luminescent potential immediately following sunset, modified of course by the upward migration. In both of these cases the decrease of light intensity after 10:00 p.m. and the downward migration of the organisms are both much slower processes. The rapid increase of luminescent potential appears to be the more reasonable in that this could be explained easily on the basis of a light-inhibited step either in the final synthesis of the luciferase enzyme or in the release of an inhibitor from the enzyme surface or both. The decrease beginning at 10:00 p.m. in complete darkness would then be due to the gradual depletion of one of the reactants, which would exhibit the slower decay observed.

In our previous observations in the Chesapeake Bay, it was evident that vertical migration accounted for essentially all the variation in light intensity at the surface. Consequently, sampling at only one depth could give an apparent rhythm of luminescence even in the absence of diurnal rhythm.

Recently Backus, Yentsch, and Wing (9) have made observations of marine bioluminescence in Great Harbor at Woods Hole. The evidence presented suggests large diurnal variations of luminescence, which is in agreement with

our own observations. However, since they sampled at only one fixed depth, it is difficult to determine from their data whether diurnal migration also occurred. From the actual counts of the dinoflagellates, as given by Backus *et al.*, it is evident that large variations in numbers occurred from night to day for any one depth. For example, in one experiment between noon and midafternoon their light readings gave a value of 52 with 1425 dinoflagellates. In the late afternoon and evening the light readings were 6120 and the dinoflagellate count went up to 6500. Thus, it is evident that at this one depth the number of organisms varied in the same way as the light intensity did during the period of observation. It seems quite clear, however, that these changes in density of organisms are not sufficient to account for all the luminescent rhythm observed and that there is truly a diurnal variation.

We do feel that a second variable should be noted in the case of the observations of Backus *et al.* This refers to their method of sampling water for observation. As they indicate in their report, they pump the water a distance of several meters in a pipe or small tube prior to passing it in front of the photocell, the passage taking 1 minute for the shore installation and 5 minutes for the shipboard installation. Because of this rapid flow in a restricted area they observe that flashes occur prior to photocell observation. Thus, they are observing only a small proportion of the total light and one would need to know whether the tail end of the sampling is always proportional to the total population. A more serious problem relates to the threshold of stimulation which could vary with time of day due to temperature, salinity, ambient light intensity, and other reasons. The organisms in the luminous bay in Jamaica could not be pumped through a tube even at a very slow rate without reaching essentially complete luminescence exhaustion. It is for this reason that we have designed our equipment to eliminate any disturbance of the organisms until they are in front of the photocell.

It is evident that although most dinoflagellates exhibit a diurnal luminescent variation there is at least one species as yet unidentified that does not. This species was quite generally distributed in Chesapeake Bay in September, 1960. In 1961, however, measurements made again in Chesapeake Bay showed *both* vertical migration and a diurnal variation.

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