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Luminous bacteria of a monocentrid fish (*Monocentris japonicus*) and two anomalopid fishes (*Photoblepharon palpebratus* and *Kryptophanaron alfredi*): population sizes and growth within the light organs, and rates of release into the seawater

M. G. Haygood, B. M. Tebo and K. H. Nealson

Marine Biology Research Division, A-002, Scripps Institution of Oceanography; La Jolla, California 92093, USA

Abstract

The light organs of monocentrid and anomalopid fishes consist of bacteria-filled tubular invaginations of the epidermis which are connected to the surrounding seawater by ducts. We used the release of bacteria from the light organs to estimate bacterial rates of growth in the light organs. For one monocentrid fish (4 specimens of *Monocentris japonicus* collected at Jogashima, Japan in 1980) and for two anomalopid fishes (2 specimens each of *Photoblepharon palpebratus* collected at Sebu, Phillipines in 1981 and Grand Comore Island in 1975 and *Kryptophanaron alfredi* collected at Parguera, Puerto Rico in 1982) we measured rates of release of bacteria into the surrounding seawater and the bacterial population sizes in the light organs; from this information we calculated doubling times of bacteria in the light organs. In addition, we determined the luminescence of bacteria after their release into the seawater. For *M. japonicus,* two specimens released 1.1 to 6×10^6 and 2×10^7 bacteria h^{-1}, respectively; the light organs contained about 1.5×10^8 bacteria. For *P. palpebratus,* one specimen released 2.2×10^8 bacteria h^{-1}; a second specimen had light organs containing 5.2×10^9 bacteria. For *K. alfredi,* one specimen released 7×10^7 bacteria h^{-1} and had light organs containing 5.6×10^8 bacteria; a second specimen released 3.6×10^7 bacteria h^{-1} and had light organs containing 7.3×10^8 bacteria. Bacterial doubling times in the light organs of these three fishes were variable and ranged from 7.5 to 135 h in *M. japonicus* and 8 to 23 h in the anomalopids. Bacteria released from *M. japonicus* into the seawater remained viable, but bacteria from all of the fishes soon ceased to emit light.

Introduction

Fishes of the families Monocentridae and Anomalopidae luminesce because they have light organs containing symbiotic, luminous bacteria (Yasaki, 1928; Haneda and Tsuji, 1971). A monocentrid has a pair of light organs at the anterior angle of the lower jaw, and an anomalopid has a suborbital light organ ventral to each eye. Monocentrids harbor *Vibrio fischeri* (formerly *Photobacterium fischeri*) (Ruby and Nealson, 1976), but the bacteria from anomalopid fishes have not yet been cultured or identified. Each light organ consists of many tubular invaginations of the epidermis, and the bacteria are located in the tubular lumina, which connect via ducts with the surrounding seawater (Kessel, 1977; Tebo *et al.*, 1979).

While a previous study has shown that luminous bacteria are released from the light organs of the monocentrids *Cleidopus gloriamaris* and *Monocentris japonicus* and the anomalopid *Photoblepharon palpebratus* (Nealson *et al.*, in press), the rates of release could not be determined due to division of the bacteria after release. Thus, no conclusion could be drawn regarding growth rates of bacteria in the light organs. Therefore, our main purposes were to measure release rates and to calculate bacterial growth rates within the light organs. (This calculation is based on the assumption that the fish maintains a fixed population of bacteria in the light organs and, as the bacteria divide, they are released into the seawater; if bacteria do not divide after leaving the light organ, then the time in which the number of released bacteria equals the light organ population is the "estimated doubling time".) In addition, we observed light production in the released bacteria to determine if bacteria released from these fishes contribute to bioluminescence of seawater.

Materials and methods

Table 1 summarizes collection information for *Monocentris japonicus, Photoblepharon palpebratus* and *Kryptophanaron alfredi. M. japonicus* were collected in 1980 and maintained in a 750-liter aquarium containing filtered, recirculated seawater at 20 °C at the Misaki Marine Bio-

Table 1. *Monocentris japonicus, Photoblepharon palpebratus and Kryptophanaron alfredi.* Collection information, release rates and doubling times of bacterial symbionts. SCUBA collections of anomalopids took place at night; use of underwater lights was avoided except to stun fish for capture (Colin *et al.*, 1979; McCosker, 1982). nk: not known; na: not applicable; nd: not determined

Family and species	Specimen No.	Collection location	Depth (m)	Method	Time since collection (mo)	Days starved	Cells per fish	Mean release rate (cells h^{-1})	Doubling time (h)
Monocentridae									
Monocentris japonicus	M1	Jogashima Island, Japan	20	set net[a]	2 – 6	na	1.5×10^8	nd	nd
	M2				7 – 12	8	nd	2×10^7	7.5
	M3				7 – 12	11	nd	1.1×10^6	135
	M3				7 – 12	9	nd	6×10^6	25
	M4				7 – 12	10	nd	nd	nd
Anomalopidae									
Photoblepharon palpebratus	P1	Grand Comore Island	nk	SCUBA	0	na	5.2×10^9	nd	nd
	P2	Sebu, Phillipines	nk	SCUBA	6	5	nd	2.2×10^8	23
Kryptophanaron alfredi	K1	Parguera, Puerto Rico	30	SCUBA	1	4	7.3×10^8	3.6×10^7	20
	K2		30	SCUBA	2	4	5.6×10^8	7.0×10^7	8

[a] Obtained from local fishermen by collectors of University of Tokyo, Misaki Marine Biological Station, Misaki, Japan

logical Station for 1 to 6 mo; they were then transported to Scripps Institution of Oceanography (SIO), where they were maintained in a 750-liter running seawater aquarium at 17° to 18 °C. A living specimen of *P. palpebratus* (Specimen P2), collected in 1981, was obtained from the Steinhart Aquarium, San Francisco; after study at SIO for 1 wk (at 25.5 °C in a 284-liter recirculating aquarium in the dark) this fish was returned. *K. alfredi* were collected in 1982 and held for approximately 1 wk in running seawater aquariums in the dark at the Department of Marine Sciences of the University of Puerto Rico on Isla Mayagues, Puerto Rico, and then transported to SIO, where they were maintained at 17 °C in a 50-liter aquarium with running seawater in a darkroom.

All three species were fed live brine shrimp (*Artemia* sp.) and were starved before experiments to reduce contamination by gut flora. After release experiments with *Kryptophanaron alfredi* had been completed, the bacterial populations in the light organs were measured. Specimens of *Monocentris japonicus* and *Photoblepharon palpebratus* that had been used for release experiments were not sacrificed; in these instances, bacterial populations were determined in a specimen of the same size. In the present study, all the living fishes had light organs that were luminous (sometimes such fishes lose their luminescence in captivity).

To measure release of bacteria from light organs, we passed seawater through a bed of activated charcoal, and then filter-sterilized it (0.2 μm membrane filters Gelman Sciences, Inc.). 5.1 liters of the sterilized seawater were placed in an autoclaved covered cylinder of Pyrex glass (7 liters). The seawater was gently stirred with a magnetic stirrer and aerated with a cotton-plugged, sterile, gas-dispersion tube. Before the start of each experiment, 100 ml were removed for control measurements. Starved fish were vigorously washed in several changes of sterile seawater prior to placement in the cylinder. Bacterial release was followed for at least 10 h in *Monocentris japonicus* and at least 3 h in the anomalopids.

To study release of bacteria from *Monocentris japonicus,* we measured the appearance of particles in the seawater in duplicate samples (10 ml) with a Coulter Counter (Coulter Electronics Model ZBI) equipped with a 30 μm aperture tube. For plate counts, bacteria were grown on a seawater complete medium (SWC): 75% seawater, 5 g bactopeptone (Difco), 3 g yeast extract (Difco), 3 ml glycerol and 15 g agar l^{-1} (Nealson, 1978). Plate counts of water samples from the cylinder (5 replicas) were performed by direct-plating or dilution-plating of 0.1 ml of the sample. Plates were incubated at 18 °C for 24 to 48 h before counting luminous and total colony-forming units (CFUs).

The bacterial symbionts have not been cultured from any anomalopid species; thus, for *Photoblepharon palpebratus* and *Kryptophanaron alfredi,* the release of bacteria from the light organs was measured by Coulter counts and epifluorescence. For epifluorescence, samples were fixed in 5% formalin, stained with acridine orange, filtered onto 0.2 μm Nuclepore filters, and counted directly by fluorescence microscopy (epifluorescence; Hobbie *et al.*, 1977). For *K. alfredi,* bacterial release was examined in two specimens (designated K1 and K2) both before and after removal of the light organs under quinaldine anesthesia. K1 was retested 5 d after removal of the light organs. K2 was retested 30 and 37 d after removal of the light organs.

Light emission from seawater samples was measured photometrically in 1 ml subsamples (4 to 7 replicas) (SAI Technology Co., Photometer Model 2000). This photometer can detect luminescence of a single fully induced bacterial cell (Nealson *et al.*, in press).

Bacterial populations in light organs were estimated in several ways. For *Monocentris japonicus,* the lower jaw was removed from one specimen (designated M1), which had been anesthetized in MS 222 (tricane methane sulfonate).

The jaw was rinsed 4 times in sterile seawater, and the light organs were sterilely removed and homogenized in a hand-held glass homogenizer. Samples were serially diluted in sterile seawater and plated on SWC. After incubation for 24 to 48 h at 20 °C, the colonies were counted; all colonies were uniform in size and luminescence. For *Photoblepharon palpebratus,* a formalin-fixed light organ of one specimen (designated P1) was thoroughly homogenized in filter-sterilized (0.2 μm) artificial seawater (ASW) in a ground-glass hand-held homogenizer, and formalin was added to 5%. One liter of ASW stock contains: 31.1 g of NaCl, 1.5 g of KCl, 24.7 g of $MgSO_4 \cdot 7\,H_2O$, and 2.9 g of $CaCl_2 \cdot H_2O$. 50% ASW stock is used for media (Nealson, 1978). Dilutions were made into filter-sterilized ASW. Diluted samples were counted by epifluorescence as above. For *Kryptophanaron alfredi,* an homogenized, fresh, light organ from one specimen (K1) was formalin-fixed and counted by epifluorescence as described above, and the light organ of a second specimen (K2) was frozen at −70 °C for 2 mo, then thawed on ice and homogenized and counted as above.

Results

Monocentris japonicus

The number of luminous bacteria in the water, as determined by particle (Coulter) counts and plate counts increased after introduction of a specimen of *Monocentris japonicus* (Fig. 1). Release rates ranged from 1.1×10^6 to

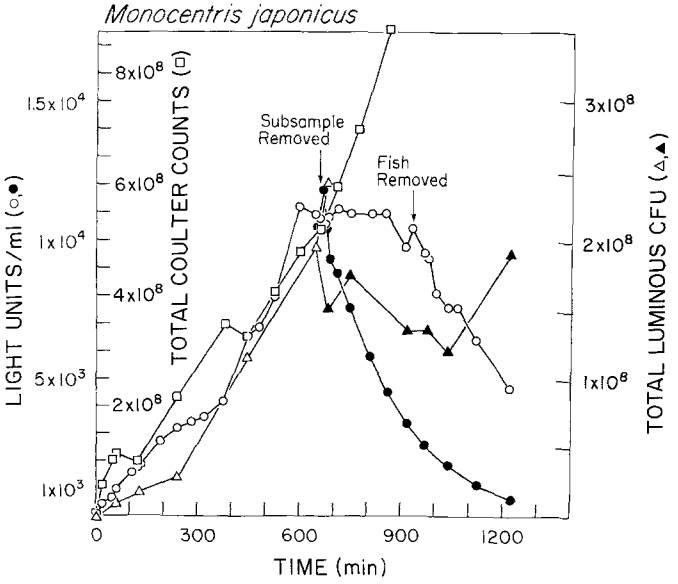

Fig. 1. *Monocentris japonicus.* Release of luminous bacteria by Specimen M2 in a vessel containing 5 liters of sterile charcoal-filtered seawater. □: total Coulter counts in experimental vessel; △: total luminous colony-forming units (CFUs) in experimental vessel; ▲: total luminous colony-forming units in a 30 ml subsample removed from experimental vessel; ○: light units per ml in experimental vessel; ●: light units per ml in a 30 ml subsample removed from experimental vessel. Contaminants (non-luminous colonies) began to appear at time fish was removed

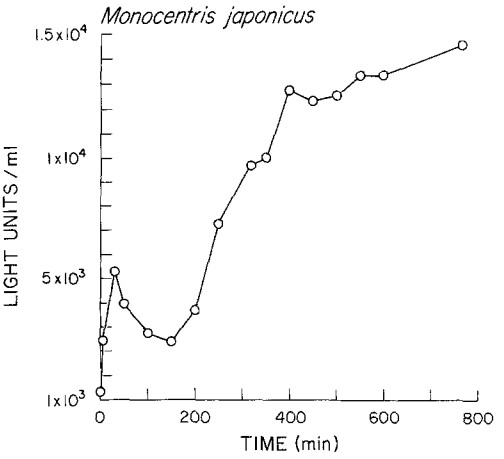

Fig. 2. *Monocentris japonicus.* Light emission by luminous bacteria released by Specimen M4 in a vessel containing 5 liters of sterile charcoal-filtered seawater. Note apparent "pulse" of bacteria released in first 30 min

2×10^7 cells (colony-forming units) h^{-1} in Specimens M2 and M3. The population of luminous bacteria in a 30 ml subsample from the experimental vessel did not continue to increase; there was thus no detectable growth or death after release (Fig. 1). The total population of both light organs of *M. japonicus* Specimen M1 was approximately 1.5×10^8 cells. This estimate could be low, since clumping or incomplete recovery would increase the estimated doubling times. Estimated doubling times calculated with the population size from M1 and release rates from M2 and M3 ranged from 7.5 to 135 h (Table 1).

The light in the water increased after addition of the specimen of *Monocentris japonicus* due to the release of bright bacteria (Fig. 1). In some experiments the light did not increase continuously, but showed plateaus and occasional decreases, suggesting that the bacteria may be released in pulses (Fig. 2). As shown in Fig. 1, the light reached a steady-state level where the rate of decline in light emission by previously released bacteria was evidently balanced by the rate of release of brightly luminous bacteria by the fish. The light in a subsample decayed exponentially ($t_{1/2} = 185$ min), although the number of luminous bacteria present, as determined by plate counts, remained approximately constant. When the fish was removed from the experimental vessel, the light diminished at approximately the same rate as in the subsample.

Photoblepharon palpebratus

Although plate counts were not possible in release experiments with *Photoblepharon palpebratus,* Coulter counts indicated a steady increase in particles of a size appropriate for bacteria (thresholds were set using laboratory-grown *Vibrio fischeri*), which ceased increasing when the fish was removed (see data for Specimen P2: Fig. 3). A sample counted by epifluorescence provided a conversion factor between Coulter counts and cell number of 5.7×10^3

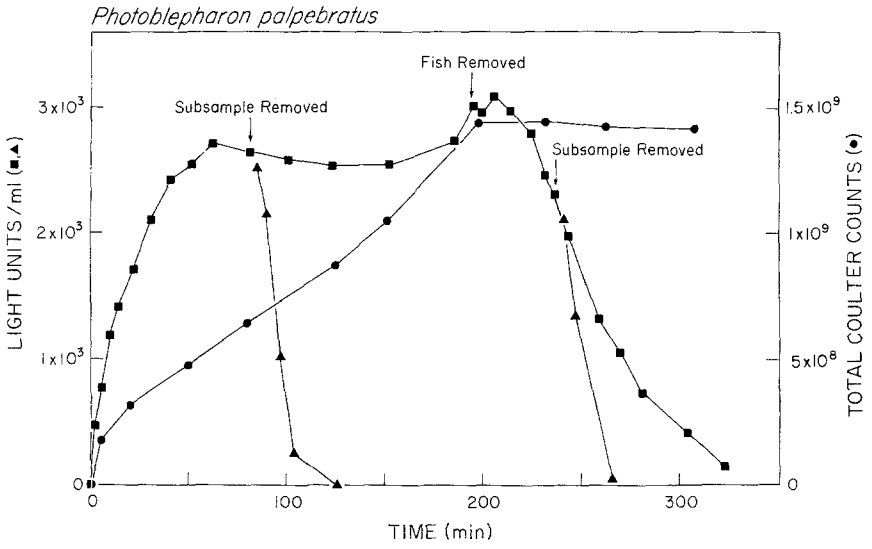

Fig. 3. *Photoblepharon palpebratus.* Release of luminous bacteria by Specimen P2 in a vessel containing 5 liters of sterile charcoal-filtered seawater. ●: total Coulter counts; ■: light units per ml in experimental vessel; ▲: light units per ml in 1 ml subsamples removed from experimental vessel

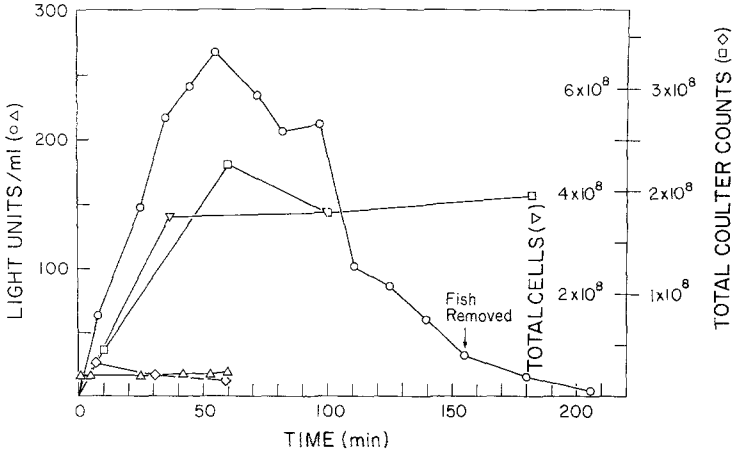

Fig. 4. *Kryptophanaron alfredi.* Release of luminous bacteria by Specimen K1 before and after light organ removal in a vessel containing 5 liters of sterile charcoal-filtered seawater. □: total Coulter counts before light organ removal; ◇: total Coulter counts after light organ removal; ▽: total cells (epifluorescence direct counts) before light organ removal; ○: light units per ml before light organ removal; △: light units per ml after light organ removal

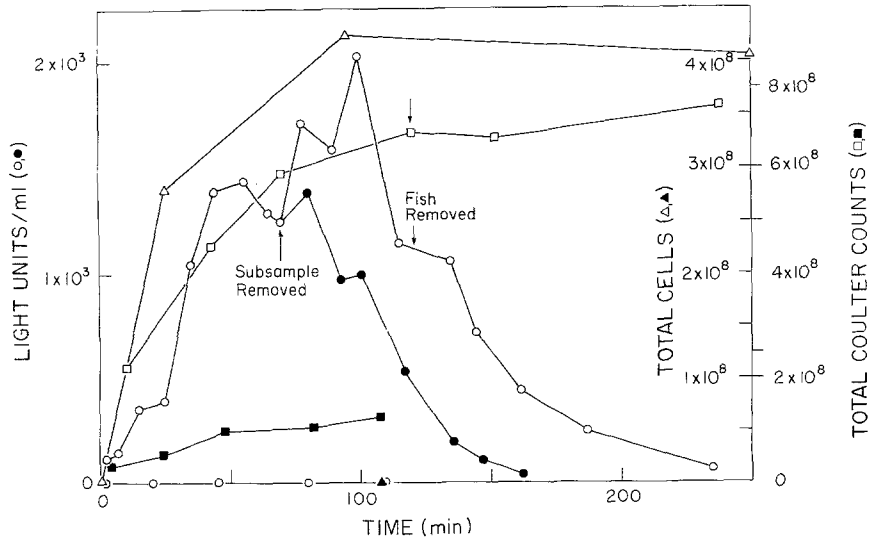

Fig. 5. *Kryptophanaron alfredi.* Release of luminous bacteria by Specimen K2 before and after light organ removal in a vessel containing 5 liters of sterile charcoal-filtered seawater. □: total Coulter counts before light organ removal; ■: total Coulter counts after light organ removal; △: total cells (epifluorescence direct counts) before light organ removal; ▲: total cells (epifluorescence direct counts) after light organ removal; ○: light units per ml in experimental vessel before and after light organ removal (after light organ removal all measurements were zero); ●: lights units per ml in a 30 ml subsample removed from experimental vessel

cells ml^{-1} 1 000 $counts^{-1}$. Cells were released at the rate of 2.2×10^8 cells h^{-1}. The total population of both light organs of Specimen P1 was 5.2×10^9 bacteria (Table 1). The estimated doubling time calculated with this value was 23 h. As in *Monocentris japonicus,* light in the water increased after introduction of P2, reached a steady state, and declined in subsamples or when the fish was removed (Fig. 3). The decline, which was not exponential, was very fast in the subsamples ($t_{1/2}=13$ min), and somewhat slower in the experimental vessel ($t_{1/2}=60$ min).

Kryptophanaron alfredi

Bacteria were released for less than 1 h by Specimen K1 (Fig. 4). The cell numbers then remained constant until the end of the experiment (fish removed after 155 min). The maximum rate of release was 1.4×10^8 cells h^{-1}, and the rate averaged over the entire length of the experiment was 3.6×10^7 cells h^{-1}. The population of both light organs was 7.3×10^8 cells. Estimated doubling times based on the above rates were 5 and 20 h, respectively (Table 1). Specimen K2 released bacteria at an average rate of 7.0×10^7 cells h^{-1} (Fig. 5). The total population of both light organs was 5.6×10^8 cells (Table 1). The estimated doubling time was 8 h. The decay of light was exponential (Figs. 4 and 5). After removal of Specimen K1, the half-time in the vessel was approximately 35 min. Following removal of Specimen K2 from the vessel, the half-time was 28 min; in the 30 ml subsample it was 15 min.

When the experiments were repeated after light organ removal from *Kryptophanaron alfredi,* no light could be detected and direct counts by epifluorescence showed no symbionts, which are long (5 to 25 μm) rods. In the seawater containing Specimen K2 a few particles did appear, as measured by Coulter counts. Direct counts showed some debris and small rods (approximately 1×10^3 cells ml^{-1} after 108 min). The rate of appearance of these contaminants was less than 4% of the rate of release of symbionts in the same fish.

Discussion

The shortest doubling times estimated for the bacteria in *Monocentris japonicus* light organs represent an increase of almost 10-fold relative to laboratory cultures of *Vibrio fischeri* (doubling time = 45 min in SWC). Growth repression is clearly occurring in the light organs, reducing the energetic cost of the symbiosis to the fish. There is no indication of how this repression is achieved, although Nealson (1979) suggested that oxygen limitation by the fish represses growth while maximizing luminescence of the bacteria. The wide range of doubling times measured in experiments with *M. japonicus,* even in repeated experiments on a single fish, as well as between individuals, demonstrates that the release rate, and possibly the growth rate, is not fixed. This variability may reflect the physiological or nutritional state of the fish.

Results from both *Monocentris japonicus* (Fig. 2) and *Kryptophanaron alfredi* (Fig. 4) indicate that bacteria may sometimes be released discontinuously, in "pulses". Thus, release rates must be measured over as long a period as possible for estimation of doubling times. In the present study we were limited by the eventual appearance of contaminants and the stress imposed on the fish by holding it in a small volume of water for long periods of time.

Although we cannot judge whether growth is repressed in anomalopid symbionts until they have been grown in the laboratory, the 23 h doubling time measured in the symbiont of *Photoblepharon palpebratus* is quite slow for heterotrophic bacteria and may represent growth repression. The mechanism of such repression is unknown; however, the bacteria contain inclusions resembling poly-β-hydroxybutyrate inclusions (Kessel, 1977) which typically appear under nitrogen or oxygen limitation (Stanier *et al.,* 1976).

In *Kryptophanaron alfredi,* the doubling times of 8 and 20 h were similar to those seen in *Monocentris japonicus* and *Photoblepharon palpebratus.* In one specimen of *K. alfredi* (K1), the calculated doubling time might be too short, since the fish released a large number of bacteria in the first hour of the experiment and none thereafter, and a longer experiment might have yielded a lower average release rate.

In spite of the relatively slow growth rates of the bacteria, anomalopids release numbers of bacteria (3.6×10^7 to 2.2×10^8 cells h^{-1}) that may represent a substantial energy drain for the fish. In *Monocentris japonicus,* the rate is lower (1.1×10^6 to 2×10^7 cells h^{-1}) and the bacteria are smaller.

The mechanism responsible for the extremely fast decline in light emission by bacteria released by anomalopids is unknown, although the failure of repeated efforts to culture these bacteria indicates that they are very sensitive to some environmental factors.

Our results indicate that *Vibrio fischeri* and anomalopid symbionts are only briefly luminous after release into seawater and probably make only negligible contribution to bioluminescence in seawater, except perhaps in areas with dense populations of fish with luminous symbionts. The release rates measured also suggest that symbiotic fish populations can make a significant contribution to planktonic populations of luminous bacteria in seawater.

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