A STUDY OF THE ECOLOGY OF BIOLUMINESCENT BACTERIA IN A MARINE ENVIRONMENT

A Thesis

Presented to

The Faculty of the Department of Biology

University of Houston

In Partial Fulfillment

of the Requirement for the Degree

Master of Science

By

Catherine Harvey O'Brien

December 1978

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ABSTRACT

Bioluminescent bacteria were readily isolated from several niches in an estuarine environment of the East Lagoon in Galveston, Texas. These sites included the water column, sediment, shrimp and gastrointestinal tract of marine fishes. In this semi-tropical estuary, the numbers of luminous bacteria in the water column were observed to fluctuate with the water temperatures, with highest counts detected during the warmest months. During the cold weather, a larger percentage of luminous bacteria were found in the sediment. This niche may serve as a resevoir for "free-living" luminous bacteria in cold months.

Unlike other studies, *Beneckea harveyi* was found to be the dominant, if not the only, species of bioluminescent bacteria isolated. The highest numbers and highest percentage of luminous organisms in the total bacterial population were isolated from the gastrointestinal tract of marine fishes. Bioluminescent bacteria were found to survive well in the fish intestines for up to five days of starvation. It was proposed that the gut may well be the preferred natural habitat of luminescent bacteria.

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INTRODUCTION

Luminescent bacteria are found saprophytically and parasitically on marine animals and live symbiotically in specialized organs in certain marine fish and cephalopods (Baumann, *et. al.*, 1971). Although the biochemistry, physiology and, most recently, the taxonomy of luminous bacteria have been studied extensively (Harvey, 1952; Strehler, 1955; McElroy, 1961, Hastings, 1968, Reichelt and Baumann, 1973; 1974), the ecology of these organisms has to some extent been neglected. The role of luminescence in symbiotically associated bacteria can be appreciated. However, in luminous bacteria that live as parasites or saprophytes, the functional importance of bioluminescence is less obvious. As parasitic symbionts on crustaceans, luminous bacteria could be ingested by potential predators. Within the gut of such a predator, the bacteria could replicate and survive as enteric symbionts.

Three groups of bioluminescent bacteria exist, of which two(*Photobacterium*) has been found in specific association with light organs in fish. The third group (*Beneckea*) has not (Reichelt and Baumann, 1973). The goal of the research reported in this thesis was to examine alternate modes of existence for "free-living" bacteria in an estuarine environment. The specific objectives of this study were: (1) to enumerate the bacterial flora in the water

column, sediment, penaeid shrimp and gastrointestinal contents of several marine fish; (2) to compare enumerations with luminous bacterial incidence and estimate percent luminescence; (3) and to estimate through feeding experiments the retention time and rate of multiplication of luminous bacteria within the intestinal tract of fish.

REVIEW OF LITERATURE

Bioluminescent Bacteria

General Characteristics. The ability to produce light is widely distributed among bacteria, fungi, algae and animals. The bacteria were the first of the bioluminescent organisms to be made the subject of scientific research (Boyle, 1667). Boyle was unaware that the light emitted by what he referred to as "stinking Fish" was bacterial in origin (Boyle, 1672). It was not until 1853 that a description of a species of luminous bacteria, *Sarcina notiluca*, was published (Heller, 1853).

Beijerinck was the first to note that there was a close relationship between luminescent bacteria and cholera vibrios (Beijerinck, 1916). Bioluminescent bacteria possess morphological, physiological and biochemical properties similar to genera within the Enterobacteriaceae and Vibrionaceae families (Cohen, *et. al.*, 1969). Bacteria with the ability to emit light are, in general, gram-negative, non-spore forming, facultative anaerobic, straight or curved rods, which are usually motile by polar flagella (Hendrie, *et. al.*, 1970; Reichelt and Baumann, 1973; Bergey's, 1974). Peptone is the main source for nitrogen intake in laboratory media. They can obtain carbon from glucose, fructose, maltose, galactose, calcium lactate, man-

nose, glycerol and utilize N-acetylglucosamine. Luminous bacteria also produce an extracellular chitinase (Reichelt and Baumann, 1973). They prefer a neutral to slightly alkaline medium for growth and reproduction (Beijerinck, 1889; Hendrie, *et. al.*, 1970). Beijerinck noted that a trace of acid, such as is formed by bacterial activity in media containing 2% or more glucose, is enough to prevent light emission (Beijerinck, 1889).

With one exception, *Vibrio cholerae biotype albensis*, all luminescent bacteria are of marine origin and demonstrate a specific requirement for sodium (Reichelt and Baumann, 1973; 1974).

In experiments conducted by Boyle, it was demonstrated that "air" was necessary for the emission of light (Boyle, 1667). The required component in "air" is oxygen (Johnson and Shimomura, 1975). Beijerinck (1889) stated that growth of luminous bacteria without free oxygen was impossible regardless of the conditions. However, dark mutants have been shown to "survive" under anaerobic conditions (Dietrich and Nealson, 1972). Strains of the genus *Photobacterium*, which were isolated as symbionts from the luminous organs of fish, show an increase in the synthesis of the bioluminescent system at low oxygen tension; whereas, the genus *Beneckea*, a group of luminous organisms not known to be symbiotically associated, exhibits a decrease in the systhesis of components in the luminescent system at low oxygen tension. Growth at low oxygen is decreased in both organisms (Nealson and Hastings, 1977).

Bacterial bioluminescence is visible only during the exponential phase of the growth curve (Nealson, *et. al.*, 1970). In freshly inoculated cultures, the luciferase gene (or operon) is repressed or inactive. During the exponential phase, its activation occurs and luciferase is then rapidly synthesized. This phenomenon is referred to as "autoinduction" since the bacteria themselves induce the enzyme (Nealson, *et. al.*, 1970: Nealson, 1977). The autoinducer appears to accumulate in the medium at a constant rate and stumulates the production of luciferase once a critical concentration of autoinducer has been reached. Luciferase synthesis may be repressed by the addition of glucose and derepressed by c-AMP (Nealson, *et. al.*, 1972). Such an inducible system permits the bacteria to maintain either a luminous or non-luminous existence. In a dilute condition, such as one provided by the open ocean, "free-living" luminous bacteria are unable to synthesize luciferase or emit light (Hastings and Nealson, 1977).

Fully induced luminous bacteria emit light somewhere between 10^3 and 10^5 photons per second per cell, depending upon the strain (Harvey, 1952). The blue green light emitted by bioluminescent bacteria has an emission maximum near 490 nm. Recently a strain of *Photobacterium fischeri* has been isolated that emits a yellow light at 545 nm. The exact reason for this yellow light emission is not known (Ruby and Nealson, 1977).

In many organisms, enzymatic oxidoreductions take place in which the free energy change is utilized to excite a molecule to a high energy state. This is followed by the return of the excited molecule to the ground state, a process accompanied by the emission of visible light. This phenomenon is bioluminescence (Lehninger, 1975). The bioluminescent reaction in bacteria differs from other types of luminescence both in the chemistry of the reaction and in the cellular organization and control of the luminous system. The reaction, which is intracellular and continuous, is the result of oxidation of a reduced flavin cofactor, flavin mononucleotide. This system uses a pathway whereby electrons are shunted directly to oxygen, with the production of light rather than ATP, which would be the normal product of oxidative phosphorylation (Brown, 1973).

Biochemistry. Biochemical studies have revealed most of the mechanism involved in the bioluminescent reaction. The enzyme luciferase, which is a mixed function oxidase, is required as well as three substrates: reduced flavin mononucleotide ($FMNH_2$), molecular oxygen and a long chain saturated aldehyde containing more than eight carbons (RCHO). An electron donor, either NADH or NADPH, is also necessary. The proposed reaction pathway is shown in Figure 1. The luciferase-bound $FMNH_2$ (intermediate I) undergoes a reaction with O_2, leading to the formation of a peroxide (intermediate II). The flavin peroxide reacts with aldehyde to form a flavin peroxy-hemiacetal (intermediate IIA).

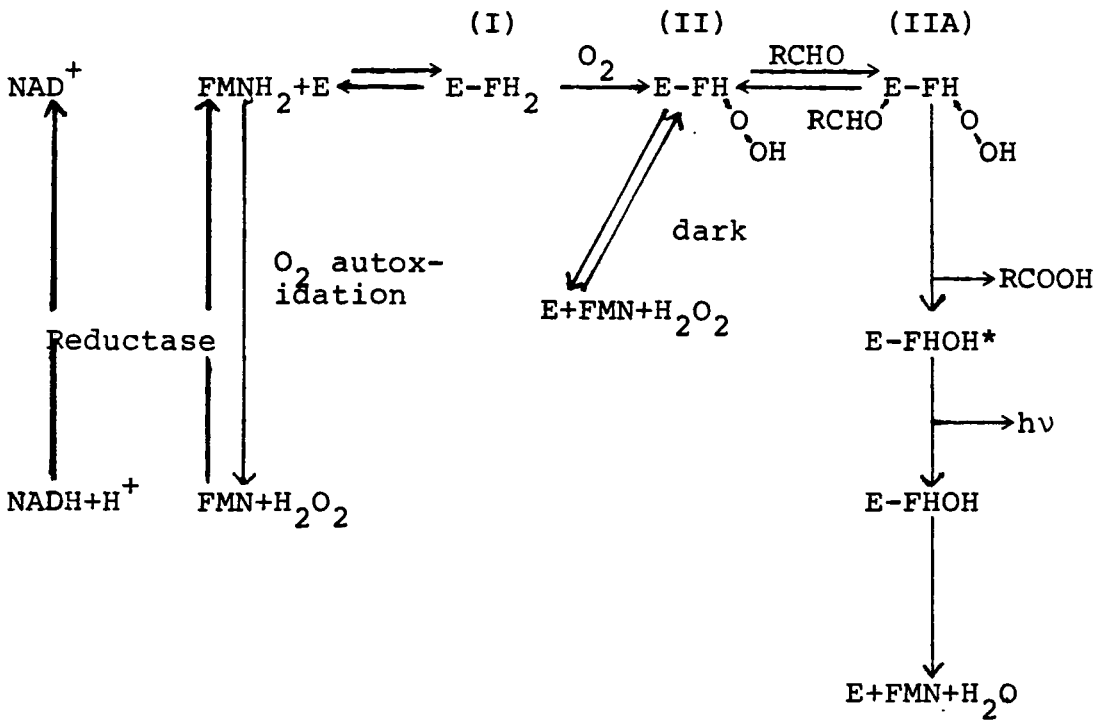

Figure 1. Postulated reaction mechanism and intermediates in bacterial bioluminescence (Hastings and Nealson, 1977).

The intermediate IIA subsequently decays to produce a flavin species, postulated to be a 4a-hydroxydihydroflavin, at the excited state. As the excited flavin returns to the ground state, light is emitted and FMN is reformed (Brock, 1977; Hastings and Nealson, 1977). Bacterial luciferase is a simple protein with molecular weight of 79,000. It is comprised of two different subunits: α (42,000) and β (37,000). The catalytic site is located on α and the function of β is unknown. Bioluminescence competes with normal electron transport for the electron of NADH. One consequence of this competition is that if the activity of the cytochrmoe system is blocked by cyanide or some other inhibitor (cytochrome a $\longrightarrow\!\!\!\!\!/\longrightarrow O_2$), the intensity of luminescence is increased (Brock, 1970).

Taxonomy. In 1889, Beijerinck proposed that all luminescent bacteria be placed in the genera *Photobacterium* (Beijerinck, 1889). His criterium for being placed in this genera was that the organism have the ability to emit light. Breed and Lessel proposed that all luminous bacteria should be placed in two genera: *Photobacterium* in the family Pseudomonadaceae, and *Vibrio* in the family Vibrionaceae Breed and Lessel, 1954). Hendrie and her coworkers recommended that all luminous bacteria be classified in three genera: *Photobacterium, Vibrio*, and a new genus *Lucibacterium* (Hendrie, *et. al.*, 1970). Bergey's Manual of Determinative Becteriology (1974) currently recognizes five species of bioluminescent bacteria: *Photobacterium phospho-*

reum, P. mandapamensis, Vibrio fischeri, V. cholerae biotype albensis, and *Lucibacterium harveyi*. Although the specific epithets used vary, most if not all recent studies in classification have concluded that there are three major groups of luminous bacteria (Hendrie, *et. al.*, 1970; Reichelt and Baumann, 1973; Bergey's, 1974). The separation into three groups is based on (1) the mode of flagellation (2) the moles percent guanine + cytosine (G+C) composition of the DNA (Reichelt and Baumann, 1973), and the DNA/DNA hybridization (Reichelt and Baumann, 1976). The three groups of bacteria may also be separated on the basis of nutritional and enzymatic characteristics (Reichelt and Baumann, 1973). In the procedure established by Reichelt and Baumann, the production of extracellular enzymes and the ability of the bacteria to utilize one of several carbohydrates and amino acids serving as the sole source of carbon and energy in a minimal media are examined (Table 1).

The group *Photobacterium fischeri* (designated *Vibrio fischeri* in Bergey's) is characterized by rod-shaped cells, a yellow cell-associated pigmentation and polar tuft comprised of two to eight sheathed flagella (Hendrie, *et. al.*, 1970; Allen and Baumann, 1971; Reichelt and Baumann, 1973; Hastings and Nealson, 1977). The marine bacteria *P. fischeri* demonstrates a specific requirement for 200 mM sodium ion for growth (Reichelt and Baumann, 1974). The species is found either free-living in seawater (Ruby and Nealson, 1976) or as the specific luminous symbiont of monocentrid fishes, such as *Monocentris japonica* (Ruby and Nealson,

1976) and *Cleidopus gloriamarus* (Graham, *et. al.*, 1972; Fitzgerald, 1977). *Photobacterium fischeri* usually grows at 4°C but not at 35°C. Growth occurs from pH 6 to pH9. DNA base ratio has been reported as 43 to 45 moles % G+C (Hendrie, *et. al.*, 1970) for *Vibrio fischeri* and 39.8 $\pm$ 1.1 moles % for *P. fischeri* (Reichelt and Baumann, 1973; 1976).

The kinetics of *in vitro* light emission by luciferase when using dodecanol is different between bacteria taxa. They are designated either as "slow" or "fast" luciferase kinetics. *P. fischeri* possess the trait referred to as fast enzyme kinetics (Hastings and Mitchell, 1971; Reichelt and Baumann, 1973).

Photobacterium leiognathi and *Photobacterium phosphoreum* compose the second group of luminous bacteria (Reichelt and Baumann, 1973; Hastings and Nealson, 1977). These species are coccobacilli possessing one to three unsheathed polar flagella. When grown on glucose, they produce bright refracfile granules of poly-β-hydroxybutyric acid (Boisvert, *et. al.*, 1967; Hendrie, *et. al.*, 1970; Eberhard and Rouser, 1971; Reichelt and Baumann, 1973; 1975). An additional characteristic which differentiates between group one and group two is the production of acetoin and/or diacetyl which occurs in *P. phosphoreum* and *P. leiognathi* but not in *P. fischeri* (Reichelt and Baumann, 1973). Like *P. fischeri*, *P. phosphoreum* and *P. leiognathi* demonstrate fast enzyme kinetics. The type species of the genus, *P. phosphoreum*, has a specific sodium ion requirement and

can be isolated from seawater by enrichment at low temperatures (4°C) (Reichelt and Baumann, 1973). There is not growth of this organism at 37°C (Hendrie, *et. al.*, 1970). *P. phosphoreum* is also found to occur symbiotically in a number of specific hosts: the argentinoid fish *Opistroproctus* (Herring, 1975), *Nezumia* (Nealson and Hastings, 1977), *Winteria* and *Sphagemacrurus* (Hastings and Nealson, 1977). The species *P. leiognathi*, formally designated *P. mandapamensis* (Hendrie, *et. al.*, 1970; Bergey's, 1974), has been isolated directly from seawater (Hendrie, *et. al.*, 1970; Hastings and Mitchell, 1971; Reichelt and Baumann, 1973) and as the specific luminous symbiont in leiognathid fishes: *Leiognathus*, *Secutor*, and *Gazza* (Boisvert, *et. al.*, 1967; Hastings and Mitchell, 1971; Reichelt, *et. al.*, 1977). *P. leiognathi* exhibits a specific requirement of 250 mM sodium for growth (Reichelt and Baumann, 1974) and grows at 35°C, but not at 4°C (Hendrie, *et. al.*, 1970; Reichelt and Baumann, 1973). *P. phosphoreum* and *P. leiognathi* can also be separated on the basis that *P. phosphoreum* does not produce extracellular lipase as observed in *P. leiognathi*; but excretes pyruvate, which is not typical of *P. leiognathi* (Reichelt and Baumann, 1973). *P. phosphoreum* has a DNA base ratio of 41.2 - 41.8 moles % G+C and 42.8 - 43.8 moles % G+C is the ratio for *P. leiognathi* (Chumakova, *et. al.*, 1972; Reichelt and Baumann, 1973; 1976; Hastings and Nealson, 1977). Both species exhibit growth from pH 6 to pH 9 (Hendrie,*et. al.*, 1970).

The third group of bioluminescent bacteria contains *Beneckea (Lucibacterium) harveyi*, *Beneckea (Photobacterium) splendida*, and *Vibrio cholerae biotype albensis* (Reichelt and Baumann, 1973). All species within this group are rod-shaped cells, with *B. harveyi* being straight rods while *B. splendida* and *V. cholerae* are curved. All possess one to three sheathed polar flagella (Das, 1966; Reichelt and Baumann, 1973). When grown on solid media, *B. harveyi* produces unsheathed peritrichous flagella in addition to the sheathed polar flagella (Hendrie, *et. al.*, 1970; Reichelt and Baumann, 1973). Baumann and coworkers, (Baumann, *et. al.*, 1971; Baumann and Baumann, 1977; Reichelt and Baumann, 1976; 1977) have separated terrestrial *Vibrio* and marine *Vibrio* (*Beneckea*). Their separation is based on the production of both peritrichous and polar flagella on solid media and the sodium requirement. The inclusion of *Vibrio* isolates into the genus *Beneckea* has not universally been accepted (Kaneko and Colwell, 1973), and throughout literature *Beneckea* and *Vibrio* are often used interchangeably. *Beneckea harveyi* may be isolated from coastal seawater in high concentrations and from the surfaces of non-luminous marine fish (Reichelt and Baumann, 1973). All strains are capable of growth at pH 6 to 9 and some are able to grow at 40°C. They usually grow at 35°C, but not at 4°C (Hendrie, *et. al.*, 1970). *Beneckea harveyi* demonstrates a specific requirement for 300 mM sodium ion for growth (Reichelt and Baumann, 1973). All organisms within this group have slower luciferase kinetics and a wider nutritional versatility than do *Photobacterium*

species (Reichelt and Baumann, 1973). The DNA base ratio range is 46.0 - 47.2 moles % G+C for *B. harveyi*, 45.0 - 45.9 moles % G+C for *B. splendida*, and 47.8 moles % G+C for *V. cholerae* (Chumakova, *et. al.*, 1972; Reichelt and Baumann, 1973; 1974; Hastings and Nealson, 1977).

In vitro DNA/DNA homology studies have shown *B. harveyi* and *B. splendida* to be two distinct species (Reichelt and Baumann, 1976). *B. splendida* has been further divided into biotype I, consisting of luminous strains, and biotype II, nonluminous strains (Reichelt and Baumann, 1976; Reichelt, *et. al.*, 1977). Non-luminous strains isolated from fish surfaces and directly from seawater are genetically comparable to *B. harveyi* (Baumann, *et. al.*, 1971; Reichelt and Baumann, 1976). *Beneckea harveyi* is closely related to the marine bacteria *Vibrio parahaemolyticus*, *Vibrio alginolyticus* and *Vibrio natriegens*, which are designated as the genus *Beneckea* by Baumann and his coworkers (Baumann and Baumann, 1977). These non-luminous marine species also have specific sodium ion requirements for growth (Reichelt and Baumann, 1974).

Vibrio cholerae biotype albensis is the only luminous bacteria known to occur outside the marine environment. It is indistinguishable both genotypically and phenotypically from non-luminous strains of *Vibrio cholerae* (Hendrie, *et. al.*, 1970; Reichelt and Baumann, 1976). The non-luminous strains of *V. cholerae* can be grown on media without added sodium. However, both the growth rate and cell yield may be increased through the addition of low amounts of NaCl (Reichelt and Baumann, 1974).

Table 1. Characters employed to classify luminous bacteria[a,b]

	SPECIES			
	P. fischeri	*P. phosphoreum*	*P. leiognathi*	*B. harveyi*
Utilization of:				
D-Xylose	-	-	-	-
Maltose	+	+	-	+
Cellobiose	+	-	-	+
D-Gluconate	-	+	+	+
D-Glucuronate	-	V	-	+
Mannitol	+	-	-	+
DL-Lactate	-	V	+	+
[d]Pyruvate	V	V	+	+
Acetate		-		+
Propionate	-	-	-	+
L-Proline	+	-	+	+
D-α-Alanine	-	-	-	+
Production of extracellular enzymes:				
Lipase	+	-	+	+
Amylase	-	-	-	+
Gelatinase	-	-	-	+
Growth at 35°C	V	-	+	+
Luciferase kinetics	fast	fast	fast	slow

[a]Data derived from Reichelt and Baumann (1973) except where indicated.

[b]+ = 80% or more of the strains tested were positive; - = less than 5% of the strains were positive; V = between 5% and 80% of the strains tested were positive.

[d]Data from Reichlet, Nealson and Hastings (1977).

Ecology. The two *Photobacterium* groups contain members (*P. leiognathi*, *P. phosphoreum* and *P. fischeri*) known to be symbiotically associated with higher organisms (Boisvert, *et. al.*, 1967; Reichelt and Baumann, 1975; Herring, 1975; Ruby and Nealson, 1976). They are found as endosymbionts in the light organs of marine fish (Hastings and Mitchell, 1971), but may also be isolated from a range of habitats, including decaying marine organisms and directly from seawater (Harvey,1952). There appears to be absolute specificity between the bacterial species cultures from the luminous organ and a given species of fish (Reichelt, *et. al.*, 1977).

Strains of *Beneckea* have been isolated directly from seawater in high concentrations, as saprophytes on the surface of decaying fish and squid (Harvey, 1952) and from the surfaces of living marine animals (Reichelt and Baumann, 1973) and crustaceans (Haneda, 1955; Baumann, *et. al.*, 1971). To date, none of the three luminous species in the *Beneckea* group have been found to occur as endosymbionts in luminous organs. Luminous members of the genus *Beneckea* are commonly termed "free-living" (i.e. those isolated from a habitat not considered symbiotic). The term "free-living" would also include those species of *Photobacterium* that have been shed into seawater from luminous organs (Hastings and Mitchell, 1971). A recent study has shown that there are seasonal changes of the luminescent bacterial flora in surface waters off California. *Photobacterium fischeri* may be isolated from the water column throughout the year. How-

ever, *Photobacterium phosphoreum* was detected only in winter samples while *B. harveyi* dominated the summer collections. A correlation between the ambient surface water temperature and the abundance of *Beneckea harveyi* was observed (Ruby and Nealson, 1978).

The occurrence of light emission in bacteria that would otherwise be physiologically distinct, would seem to imply that bioluminescence would have a functional importance in more than one ecological situation or that bioluminescence may have more than one function. Fish containing luminescent bacteria in specialized organs use the light for several purposes: to attract prey, to evade predators, and for intraspecies communication (O'Day, 1974). The advantages of light emission to free-living and saprophytic luminous bacteria are less obvious. As a "free-living" organism, luminous bacteria can occupy the guts of fish (Liston, 1954; Reichelt and Baumann, 1973) as well as the surface of marine animals, including crustaceans (i.e. penaeid shrimp). The ingestion of an organism infected with luminous bacteria, as the result of nocturnal predation, would place the bacteria in an environment well-suited for propagation - the gut of a marine fish (Hastings and Nealson, 1977). The gut may be the optimal niche for survival and propagation of luminous bacteria (Hastings and Nealson, 1977).

Estuaries

An estuary is defined as a semienclosed part of the coastal ocean where freshwater from the land mixes with seawater (Gross, 1972). The bacterial numbers in shallow estuarine waters greatly exceed those found in the open ocean, a phenomenon which may be attributed to heavy organic matter and suspended solids in the estuarine water column (Zobell, 1954). Estuaries, being rich in such nutrients, usually support a large phytoplankton population as well. These in turn provide food for zooplankton, fishes, and benthic organisms. Estuaries play a major role in the productivity of the coastal ocean, serving as home, nursery and breeding ground for many marine organisms. Patterns of spawning and development vary among estuarine-dependent fishes and shellfish. Many coastal fishes, including Sciaenids, and crustaceans spawn offshore with the larvae entering the bays to develop. Within the bays, an intricate food web begins, with bottom-dwelling shrimp feeding on detrital material (Gross, 1972) and bacteria (Zobell and Feltham, 1938). Penaeid shrimp and other invertebtates are a major food source for marine fish within the family Sciaenidae (Pew, 1971).

Marine Fish

Sand trout (*Cynoscion nothus*), speckled trout (*C. nebulosus*), golden croaker (*Micropogon undulatus*), black drum (*Pogonias cromis*), star drum (*Stellifer lanceolatus*)

and spot (*Leiostomus xanthurus*) are representatives of the family Sciaenidae occurring commonly along the upper Texas coast (Pew, 1971; Walls, 1975; Hoese, 1977). None of these fish possess light organs. The only shallow water luminescent marine in the Galveston Bay system is the Atlantic midshipman (*Porichthys porosissimus*) (Walls, 1975). The midshipman is self-luminating by means of photophores (Hoese, 1977), which do not contain bacteria (Haneda, 1955). There are several representatives in the family Macrouridae found in Texas coastal waters which possess luminous organs. These fish, however, occur offshore in the Gulf of Mexico at depths ranging from 200 - 2000 m (Marshall and Iwamoto, 1973).

Luminous bacteria occur as parasites attached to shirmp and other curstacea (Reichelt and Baumann, 1973; Hastings and Nealson, 1977) and found attached to small suspended particles (Zobell, 1943). Due to the epiphytic nature of marine bacteria, an indeterminable quantity of bacteria are ingested by virtually all types of marine animals (Zobell and Feltham, 1938). The gut of a fasting fish if reported to be essentially sterile (Obst, 1919; Margolis, 1953). The sterilization of the gut can be explained by the digestion of the bacteria by the gastric juices (Wood, 1967). In carniverous fishes, gastric acidities of pH 2.4 to 3.6 have been measured, while the enzymes secreted by the small intestine produce a pH ranging from neutral to alkaline (Barrington, 1957; Lagler, *et. al.*, 1962). Thus, the bacterial flora of the gut would have to depend primarily on the fish's recent intake of food. The microorganisms in

the gastrointestinal tract reflect the degree of bacterial colonization of the food and water (Geldrich and Clarke, 1966; Seki, 1969).

When isolated, bacterial flora of fish, like bacterial flora of the ocean (Zobell, 1946), is composed principally of gram negative rods (Liston, 1957). Members of the genera *Pseudomonas*, *Achromobacter*, *Flavobacterium* and *Micrococcus* predominate and representatives of the genera *Proteus* *Sarcina*, *Bacillus*, *Corynebacterium*, *Serratia* and *Vibrio* are frequently encountered in the bacterial flora of marine fish in the natural environment (Stewart, 1932; Shewan, 1938; Thjøtte and Sømme, 1943; Aschehoug and Vesterhus, 1943; Liston, 1957; Georgala, 1958). The occurrence of luminous bacteria in the intestine of marine fish has been reported by several investigators (Stewart, 1932; Liston, 1954; Spencer, 1961; Reichelt and Baumann, 1973). Liston further suggested that the conditions of the fish gut exert a selective effect on the bacteria ingested and only the "Gut Group Vibrios" survive in numbers large enough to constitute a portion of the gut commensal flora (Liston, 1957).

METHODS AND MATERIALS

Sampling

Habitat description. The seasonal incidence of luminous bacteria was followed in East Lagoon. Christmas Bay was used as a secondary source of fish used in the feeding experiments. East Lagoon is a narrow body of water, 1.1 miles long and 230 feet wide at the narrowest point with a volume of approximately 75 million gallons (Zein-Eldin, 1961). The lagoon is on the eastern end of Galveston Island (Figure 2), with its mouth approximately ¼ miles north of the Gulf of Mexico. The head of the lagoon has a depth of approximately 4.4 meters and receives the greatest amount of drainage from the surrounding marsh. The mouth of the lagoon is 2.5 meters deep and subject to greater tidal action (Zein-Eldin, 1961). The lagoon supports a wide variety of small fish and crustaceans.

Sampling apparatus and procedure. Water and sediment samples, shrimp and fish were collected from East Lagoon over twelve months (June, 1977, to June, 1978). Water samples were collected in presterilized milk dilution bottles (100 ml) (Colwell, *et. al.*, 1975). The samples were capped after collection and immediately returned to the lab for processing.

Sediment samples were collected in a polypropylene cen-

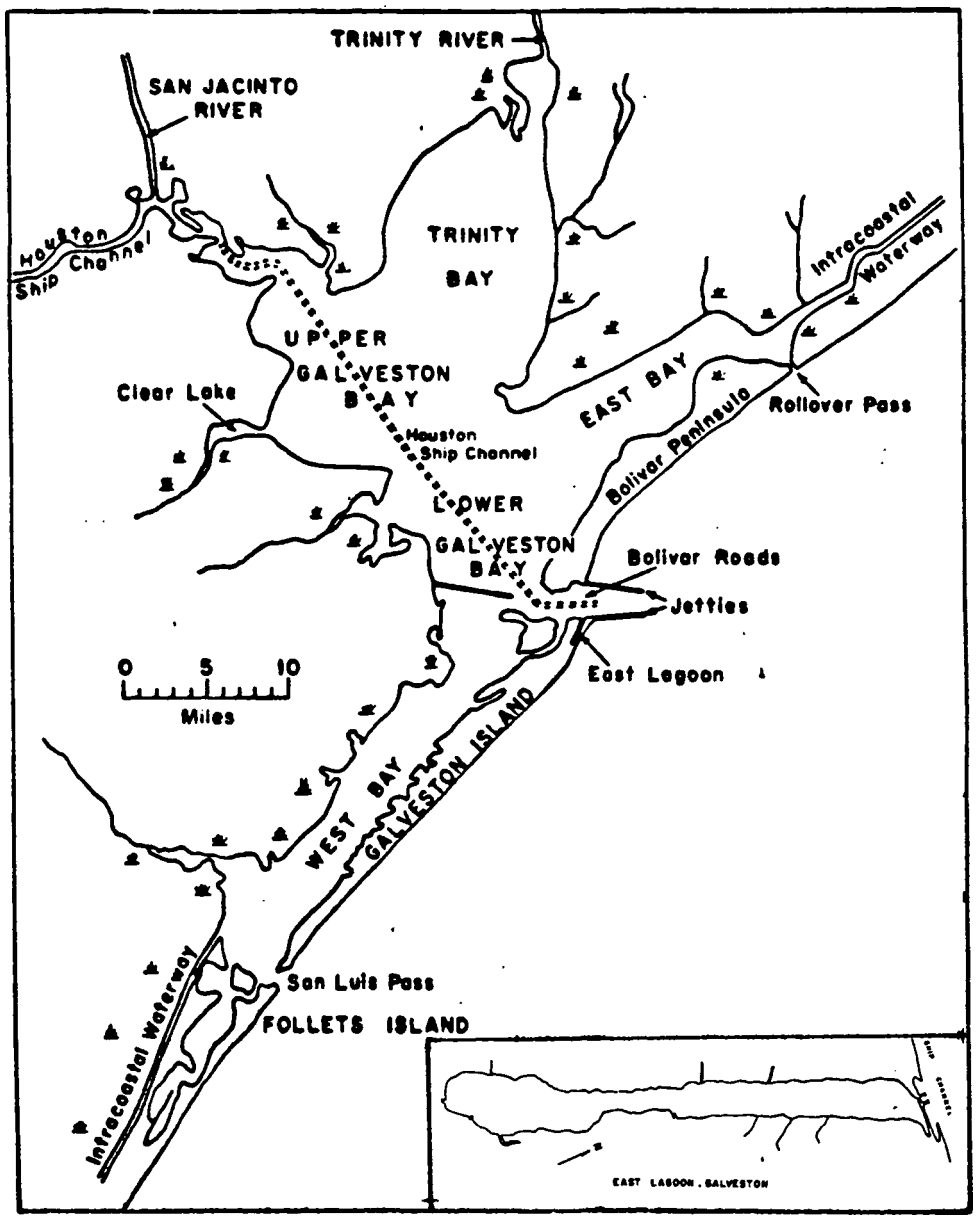

Figure 2. Map of Galveston Bay.

trifuge tube with a hole drilled in the bottom. The presterilized tube is forced into the sediment and a rubber stopper inserted in the hole. The sample was immediately transferred to a sterile glass jar (250 ml) and returned to the lab for plating.

Shrimp and fish were collected in a 10-foot otter trawl. The specimens were transferred to sterile wide mouth jars (250 ml) and placed on ice. They were aseptically dissected upon return to the laboratory, usually within one to three hours. Fish collected from East Lagoon and Christmas Bay for feeding experiments were transported live to the laboratory in ice chests aerated with a 12-volt compact air compressor (W. W. Grainger, Inc., Houston, Tx).

Water temperature, salinity and dissolved oxygen were measured with each water-sediment sampling. Dissolved oxygen and surface water temperatures were taken using the YSI model dissolved oxygen meter (Yellow Springs Instrument, Co. Yellow Springs, Ohio). Salinity measurements were made with a hand refractometer (American Optics).

Media: Isolation and Maintenance. A variety of media was used for the isolation, maintenance and taxonomic identification of bioluminescent bacteria. The media were prepared with either three salts solution or artificial seawater (ASW). The three salts solution was prepared in bulk quantities and retained as stock solutions in the laboratory. The three salts solution was prepared so that each liter of distilled water contained NaCl (0.4 M), $MgSO_4 \cdot 7H_2O$ (0.028 M)

and KCl (0.01 M). The principal marine media for isolation and maintenance used in this study were Sea Water Yeast Extract Agar (SWYE) and Modified Sea Water Yeast Extract Agar (MSWYE). SWYE contained 1% proteose peptone (Difco Laboratories, Detroit, Mich.), 0.3% yeast extract, three salts solution and pH 7.2 - 7.4. MSWYE was similar to SWYE except 0.1% proteose peptone and 0.1% yeast estract were used. To the SWYE or MSWYE broth, 20 g of Bacto Difco agar per liter may be added to produce SWYE or MSWYE agar (Colwell and Wiebe, 1970).

Media: Taxonomic Identification. Taxonomic idnetification of the luminous bacterial isolates was accomplished using selected criteria established by Reichelt and Baumann (1973). Specifically, the nutritional versatility of the isolates on minimal media (BMA) containing one of ten carbohydrates (D-xylose, maltose, cellobiose, D-gluconate, D-glucuronate, mannitol, DL-lactate, pyruvate, acetate and propionate) and two amino acids (L-proline and D-α-alanine) as the sole sources of carbon and energy was tested. One-half strength artificial seawater (ASW) was prepared by adding 0.2 M NaCl, 0.05 M $MgSO_4 \cdot 7H_2O$, 0.01 M KCl and 0.02 M $CaCl_2 \cdot 2H_2O$ to distilled water. The basal medium contained 50 mM Tris(Hydroxyethyl)aminomethane (Sigma Chemical Co.), 19 mM NH_4Cl, 0.33 mM $K_2HPO_4 \cdot 3H_2O$, 0.1 mM $FeSO_4 \cdot 7H_2O$ and half-strength ASW (pH 7.5). Basal medium agar (BMA) was prepared by sterilizing separately and then mixing equal volumes of double-strength BM and 20 g/l Noble Agar (Difco Laboratories). The filter

sterilized solution of the carbon source of amino acid was added to cooled basal medium agar to give a final concentration of 0.2% for the carbohydrate and 20 mg/l for the amino acid.

The production of three extracellular enzymes (lipase, gelatinase and amylase) was monitored. Lipolytic activity was measured by adding 1 ml of sterilized Tween 80 to each 100 ml of SWYE. A positive reaction was opacity or cloudiness around the colony. Gelatin liquification was measured with nutrient agar prepared with three salts solution and 0.4% gelatin. After growth, the plated is flooded with a gelatin precipitant prepared from 15 g $HgCl_2$, 20 ml concentrated HCl and 100 ml distilled water. After flooding the plate, if a clear zone appears around the growth with a border of white precipitant around the clear zone, the reaction is considered to be positive. The production of amylase was tested by adding starch solution to MSWYE agar for a final concentration of 0.2%. After growth, the plate was flooded with Lugol's iodine solution. A clear zone around the colony is considered to be the positive reaction, and the absence of a zone is a negative reaction.

Plating procedure. The spread plate method was the primary procedure for determining the number of bacteria either per ml of water or per gram of sediment, shrimp, stomach or intestine. All samples were diluted in sterile three salts solution and one-tenth ml aliquots were placed on the surface of SWYE agar plates. To determine dry weight for each

sediment sample, 1 ml of sediment slurry was placed in a preweighed weigh boat and dryed for three days. The weigh boat was then reweighed, and the difference between the final weight and the initial weight was calculated to be the dry weight of 1 ml of sediment slurry.

The penaeid shrimp taken in trawls and returned to the lab were weighed in presterilized weigh boats and homogenized in a Waring blender with 100 ml of sterile three salts solution for one minute. Aliquots of the slurry were then spread on the surface of SWYE agar plates.

Marine fish were returned to the lab for dissection. The fish were placed on sterile cloths and the stomach and intestines of the fish were separately removed. Precautions were taken during the dissection to prevent contamination of the guts with flora from the fish surface by flaming each piece of dissection equipment immediately before it was used. The removed gut sections were each weighed, homogenized in 100 ml sterile three salts solution and spread onto SWYE media.

Incubation and colony counting. All inoculated plates were incubated at 25°C for 24 hours. The plates were observed in a dark room at 24 and 48 hours for luminescent colonies. Plates were counted twice - once to determine the number of luminous bacteria and once to determine the total viable count of the sample.

Isolation and storage of strains. Luminescent colonies which appeared within 24 hours after plating were selected

and purified by further streaking onto SWYE and incubated at 25°C for another 24 hours.

Isolated luminescent strains were frozen away for later taxonimic studies. Cultures for freezing were prepared by suspending the bacterial lawn of a 24 hour SWYE plate in a 1% sterile milk solution. The suspension was transferred to sterile vials and stored in a Kelvinator freezer (Kelvinator Commercial Products, Inc., Manitowac, Wis.) at -90°C until needed. The majority of the strains used for this study were isolated directly from seawater, sediment, shrimp and gastrointestinal tracts of marine fish. *Photobacterium fischeri* (strain MJ-1), *P. phosphoreum* (strain NZ-1), *P. leiognathi* (strain L720), and *Beneckea harveyi* (strain B392) were obtained from Dr. Kenneth Nealson (Scripps Institute of Oceanography). For some experiments naladixic acid resistant luminous bacteria were needed. *Beneckea harveyi* was made resistant to naladixic acid by sequentially transferring the strain on antibiotic media with increasing amounts of naladixic acid (5γ/ml, 30γ/ml, 100γ/ml and 250γ/ml).

Starvation experiment. To examine the survival rate of luminous bacteria in fish guts, two experiments were designed using 35 and 50 fish respectively. The fish were collected and returned live to the laboratory, where they were maintained in an aquarium without food up to twelve days. On a daily basis, up to ten fish were sacrificed. The stomach and intestines were aseptically dissected, weighed, homogenized and plated by techniques previously described. Incubations and counting procedures were performed as with the

field data. Luminescent bacteria were enumerated either as counts per gm of intestines or as plates which were scored as positive or negative for the presence of luminous bacteria.

Feeding experiments. Several types of feeding experiments were conducted to estimate the retention time and rate of multiplication of luminous bacteria within the intestinal tracts of fish. To determine whether chitin might significantly affect the retention time of luminous bacteria in the gut, two experiments were designed in which fish were fed two varieties of shrimp food. The shrimp pieces used in the experiments were either void of an exoskeleton or had the exoskeleton in tact. Both food types were inoculated with a naladixic acid resistant strain of *Beneckea harveyi*. The fish, which had been starved for two weeks prior to the experiment, were fed one dose of inoculated shrimp. After thirty minutes, all uneaten portions were removed from the tank. The fish were sacrificed on a daily basis for six days in one experiment or between 48 and 72 hours in the second. Total viable counts and luminescent counts per gm were made.

A third experiment was conducted to determine the rate at which luminous bacteria enter the intestines after feeding. Dissection and plating procedures were the same, and the plates were scored positive or negative for the presence of luminous bacteria.

pH. To determine the optimum pH over which luminous bac-

teria will grow, 16 test tubes filled with 15 ml of MSWYE broth were inoculated with *Beneckea harveyi*. The pH of the tubes ranged from 2 to 9.5 at 0.5 intervals. No buffer was added to the media. The tubes were incubated in a shaker bath at 25°C. Growth was measured by a Spectrophotometer (Bausch and Lomb, Co.) at eight hour intervals for 24 hours.

RESULTS

Field Study

Water - Sediment samples. Over a twelve month period, 27 water samples were collected from East Lagoon, 15 of which (55.6%) contained bioluminescent bacteria. Temperature, salinity and dissolved oxygen measurements were collected for each sample. Total viable counts of the water column were found to fluctuate from 3.4×10^2 to 3.6×10^4 bacteria per ml throughout the year, with an average of 5.2×10^3 (Table 2). The incidence of bioluminescent bacteria tended to vary with water temperature, with less than one (or none) per ml observed for water temperatures of 7°C (i.e. midwinter). The number of bioluminescent organisms gradually increased around a water temperature of 14°C (Figure 3). Maximum bioluminescent counts of 38.3 per ml were obtained during the summer months.

The percentage of bacteria which were luminescent was calculated by dividing the number of luminescent cells per ml by the total viable counts per ml (Table 3). The results of this conversion also indicate the relationship between luminescent counts and water temperature (Figure 4).

If the luminescent bacterial counts are separated on the basis of temperature, it can be observed that 12 of the

Table 2. Yearly average bacterial counts for each site examined.

	Total viable counts	Luminous counts	% luminescence
Water (per ml)	5.2×10^3	4.9×10^0	0.09
Sediment (per gm dry wt)	5.7×10^5	2.6×10^2	0.05
Shrimp (per gm wet wt)	7.7×10^5	2.9×10^3	0.38
Stomach (per gm wet wt)	4.8×10^6	2.1×10^4	0.44
Intestines (per gm wet wt	1.8×10^7	3.7×10^6	20.55

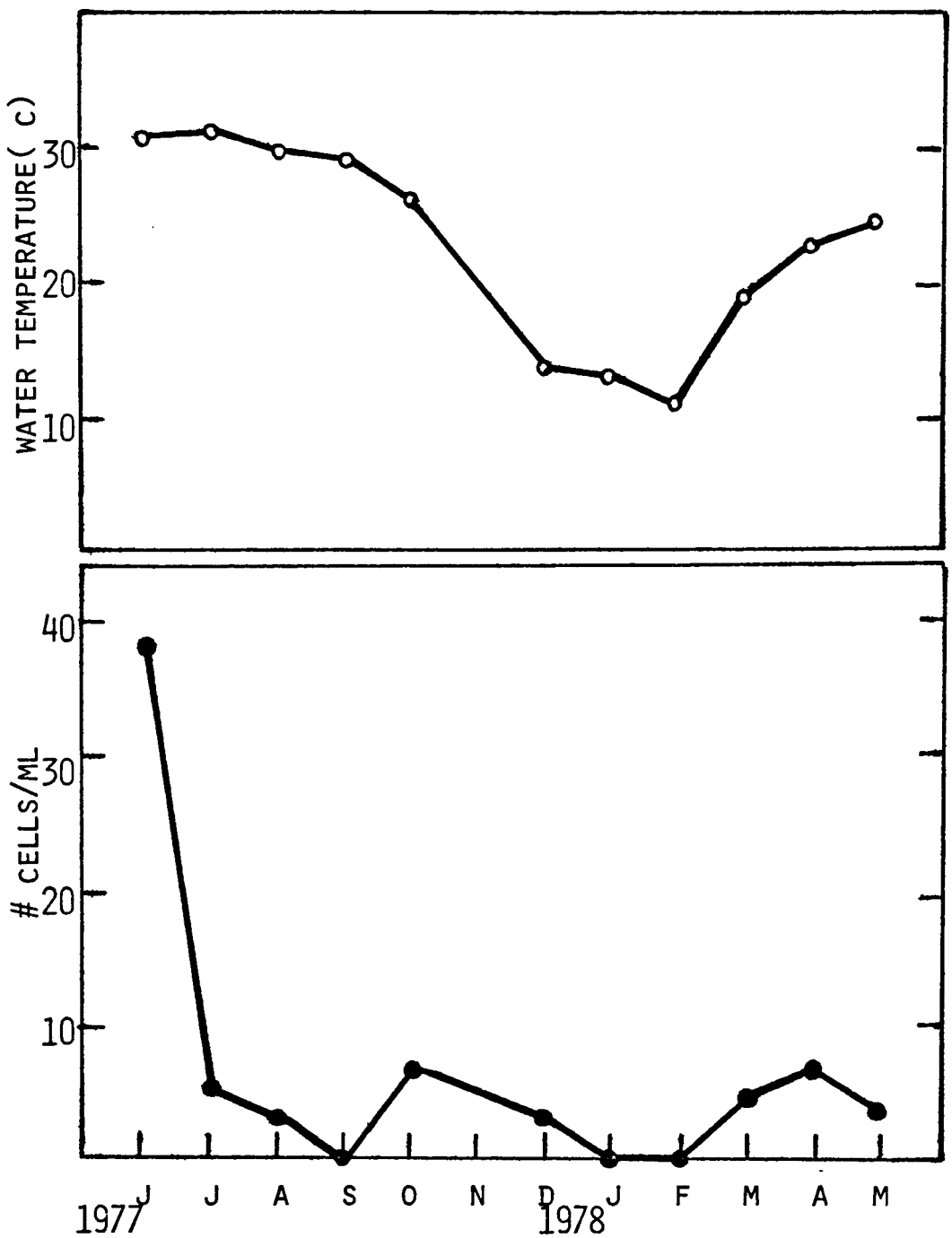

Figure 3. Luminescent bacterial counts and surface and water temperature over a one-year period.

Table 3. Monthly average total viable counts and luminescent counts per ml of water.

	Luminous counts	Total viable counts	Water temp. (°C)	% luminescence
1977				
Jun	38.3	3.4×10^2	31.00	1.13
Jul	5.5	5.9×10^2	31.50	0.95
Aug	3.3	9.9×10^2	30.00	0.34
Sep	0	3.9×10^3	29.50	0
Oct	6.7	4.9×10^3	26.50	0.14
Dec	3.3	1.9×10^3	14.00	0.18
1978				
Jan	0	1.5×10^3	13.50	0
Feb	0	1.0×10^4	11.20	0
Mar	4.9	1.1×10^4	19.38	0.05
Apr	6.7	6.5×10^3	23.00	0.10
May	3.3	3.4×10^3	24.75	0.10

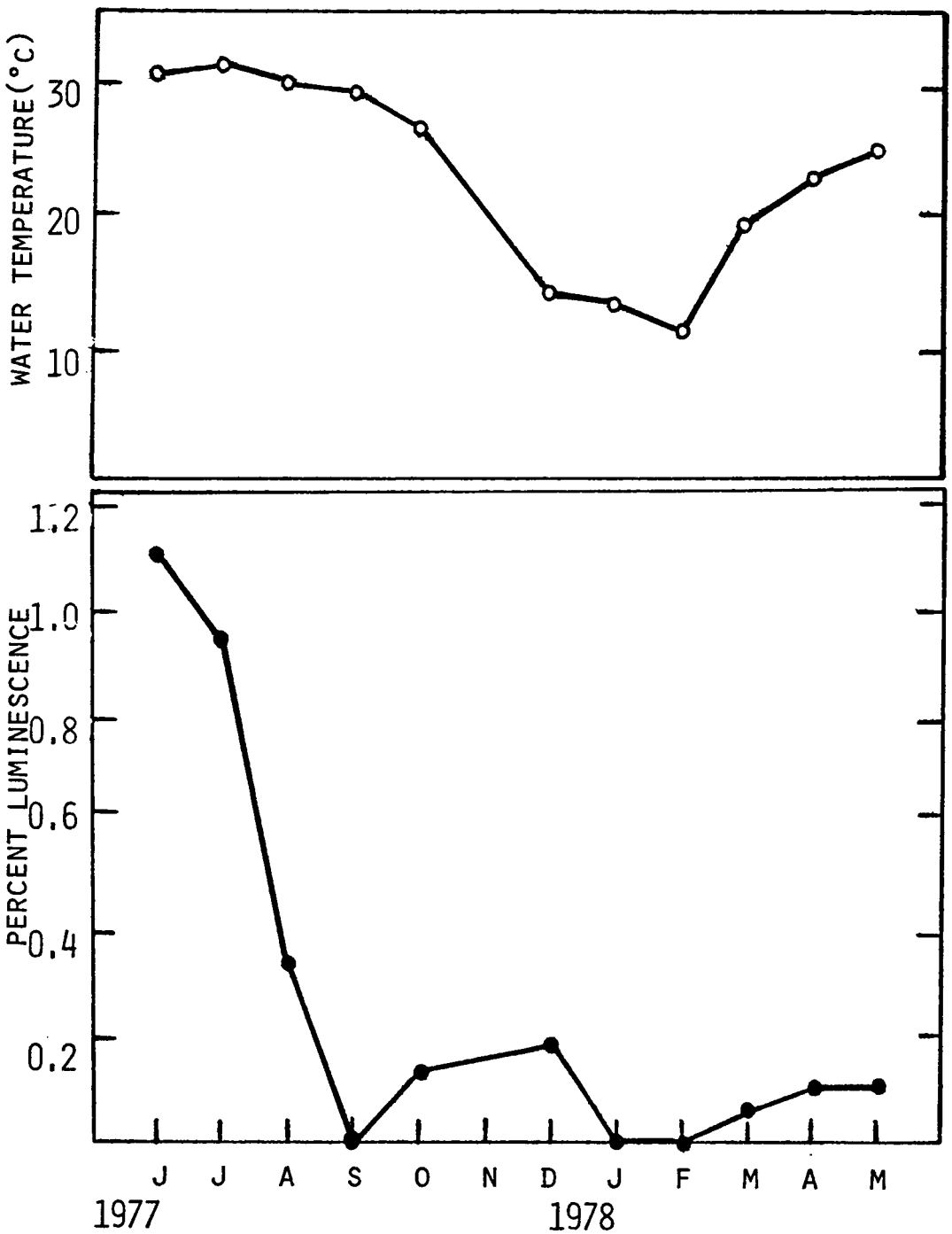

Figure 4. Monthly percent luminescence as compared to water temperature over a one-year period.

18 samples (66.7%) collected when temperatures were greater than 15°C contained luminescent bacteria. In contrast, only 2 of 9 samples collected when temperatures were $\leq$ 15°C (22.9 %) contained luminescent bacteria (Table 4).

Of the 28 sediment samples collected during this same period, 24 (85.7%) exhibited luminescent growth. Total viable counts for the sediment varied from 1.8×10^4 to 2.4×10^6 cells per gram dry weight throughout the year. The total concentration of luminous cells, when detectable, varied from 1.6×10^0 to 1.3×10^3 cells per gram of dry weight. The percentage luminescent values for the sediment were calculated by dividing luminescent counts per gram of sediment by the total viable counts (Table 5).

Fluctuations in the counts per gram of luminous bacteria and in percent luminescence for the sediment are greater than the water column and any correlation with water temperatures is more difficult to observe. However, during the winter months (i.e. January and February), the incidence of luminous bacteria occurring in the sediment is greater than for the water column (Tables 3 and 5).

Shrimp. Of the 25 shrimp examined 20 (80%) contained luminescent bacteria. Total viable counts for the shrimp were observed to fluctuate from 1.3×10^4 to 2.7×10^6 cells per gm of shrimp (Table 6). The highest number of bioluminescent bacteria in shrimp were obtained during the summer months. Fish feeding on shrimp during the summer would then presumably get the largest inoculum of luminous bacteria into

Table 4. The presence of luminous bacteria in samples at water temperatures above and below 15°C

Sample Date	Presence of[a] luminescence	Water temperature[b] above 15°C
6-22-77	+	+
7- 6	+	+
7- 6	-	+
7-25	+	+
8-23	+	+
9-21	-	+
10-19	+	+
10-19	-	+
10-27	+	-
10-30	+	+
1- 4-78	-	-
1- 5	-	-
2- 6	-	-
2- 9	-	-
2-23	-	-
2-23	-	-
2-27	-	-
3- 6	+	-
3-13	-	+
3-20	+	+
3-27	-	+
4- 6	-	+
4-10	+	+
4-17	+	+
4-24	+	+
5- 2	+	+
5- 8	+	+

[a]indicates presence of luminous bacteria in sample.

[b]indicates water temperature above 15C at the time the sample was collected.

Table 5. Monthly average total viable counts and luminescent counts per gram of sediment (dry wt).

	Luminous counts	Total viable counts	% luminescence	Water temp. (°C)
1977				
Jun	1.9×10^2	2.5×10^5	0.08	31.00
Jul	*	*	0.15	31.50
Aug	*	*	0.18	30.00
Sep	2.4×10^1	2.3×10^5	0.01	29.50
Oct	8.6×10^2	8.8×10^5	0.10	26.50
Dec	2.1×10^2	1.6×10^5	0.13	14.00
1978				
Jan	3.0×10^2	6.3×10^5	0.05	13.50
Feb	9.7×10^1	3.4×10^5	0.03	11.20
Mar	1.3×10^2	3.0×10^5	0.04	19.38
Apr	2.6×10^2	1.8×10^6	0.01	23.00
May	5.1×10^2	5.1×10^5	0.10	24.75

* = Counts/gm were unavailable for these months. The percent luminescence was calculated using counts/ml of sediment slurry.

Table 6. Monthly average total viable counts and luminescent counts per gram of shrimp (wet wt).

	Luminous counts	Total viable counts	% luminescence	Water temp.(°C)
1977 Jul	8.5×10^2	4.8×10^5	0.18	31.50
Sep	0	3.6×10^4	0	29.50
Oct	2.1×10^3	2.0×10^5	1.05	26.50
Dec	2.2×10^3	8.6×10^5	0.26	14.00
1978 Mar	4.8×10^2	2.8×10^5	0.17	19.38
May	5.3×10^3	1.9×10^6	0.28	24.75
Jun	7.9×10^3	1.4×10^6	0.56	31.00
Jul	4.4×10^3	1.0×10^6	0.44	-

their stomach. Neither the percent of luminescent bacteria or the monthly counts per gram appear to be closely related to the water temperatures. A higher percentage of luminous bacteria, however, occurs in shrimp, on the average, than in the water column or the sediment. Shrimp are bottom-dwelling marine animals. No correlation was noted between the number of luminous bacteria in the sediment and the numbers found on shrimp.

Marine fish. The total viable counts in the gastrointestinal tracts of marine fish range from 2.3×10^3 to 8.1×10^7 cells per gram for the stomach and 0 to 1.7×10^8 cells per gram for the intestine (Table 7). Luminous bacteria were observed in four fish stomachs (19%) and eight intestines (38%). In two fish luminous bacteria occurring the the stomach were not detected in the intestine. The reverse situation was observed in five fish. Bioluminescent concentrations fluctuated from 1.3×10^2 to 4.2×10^5 cells per gram in the stomach and 1.2×10^3 to 5.0×10^7 cells per gram in the intestines, when detectable.

By separating the fish into species, the abundance of luminous bacteria in the gut of a particular species can be examined. The differences observed, however, appear to be due more to the season in which the fish were collected rather than a basic physiology of the different guts (Table 7).

Results of the field data on fish counts were examined as average monthly counts per gram and average monthly percentages (Tables 8 and 9). The highest concentrations of

Table 7. Luminescent and total viable counts/gm of the stomach and intestines of the various fish examined.

	STOMACH		INTESTINES		
Date	Luminous counts	Total viable counts	Luminous counts	Total viable counts	Species*
7/ 6/77	4.2×10^5	8.1×10^7	3.0×10^6	1.7×10^8	Cno
7/ 6/77	1.2×10^4	7.8×10^5	3.3×10^5	5.1×10^6	Cno
7/ 6/77	0	8.3×10^3	0	0	Cno
7/25/77	0	2.5×10^6	0	0	Cno
8/23/77	0	5.9×10^4	1.8×10^7	1.3×10^8	Cno
8/23/77	0	2.6×10^5	5.0×10^5	3.3×10^7	Cno
8/23/77	0	5.0×10^5	7.3×10^6	5.3×10^6	Cno
9/21/77	1.3×10^2	1.1×10^7	0	4.3×10^7	Cno
10/30/77	5.3×10^3	6.3×10^5	0	3.0×10^4	Lx
2/10/78	0	1.1×10^5	0	3.0×10^4	Cno
2/10/78	0	1.1×10^5	0	1.6×10^4	Mu
2/10/78	0	2.4×10^4	0	1.1×10^4	Mu
2/10/78	0	3.6×10^6	0	4.6×10^3	Mu

Table 7 continued

	STOMACH		INTESTINES		
Date	Luminous counts	Total viable counts	Luminous counts	Total viable counts	Species*
2/10/78	0	5.7×10^5	0	4.1×10^4	Mu
3/21/78	0	2.1×10^4	1.7×10^4	1.0×10^5	Pc
3/21/78	0	2.3×10^3	1.8×10^3	9.9×10^4	Pc
3/21/78	0	5.1×10^3	1.2×10^3	1.5×10^5	Pc
3/21/78	0	7.8×10^3	0	1.2×10^4	Pc
3/21/78	0	2.9×10^3	0	1.2×10^4	Pc
3/21/78	0	1.6×10^4	0	1.9×10^5	Cno
3/31/78	0	2.6×10^4	0	1.3×10^5	Mu

*Cno - Cynoscion nothus, Mu - Micropogon undulatus, Pc - Pogonias cromis
Lx - Leiostomus xanthurus

Table 8. Monthly average total viable counts and luminescent counts per gram in fish stomach (wet wt).

	Luminous counts	Total viable counts	% luminescence	Water temp.(°C)
1977				
Jul	1.1×10^5	2.1×10^7	0.52	31.50
Aug	0	2.7×10^5	0	30.00
Sep	1.3×10^2	1.1×10^7	0.001	29.50
Oct	5.3×10^3	6.3×10^5	0.84	26.50
1978				
Feb	0	8.8×10^5	0	11.20
Mar	0	1.1×10^4	0	19.38

Table 9. Monthly average total viable counts and luminescent counts per gram of fish intestine(wet wt)

	Luminous counts	Total viable counts	% luminescence	Water Temp.(°C)
1977 Jul	8.3×10^5	4.4×10^7	1.89	31.50
Aug	2.5×10^7	5.6×10^7	44.64	30.00
Sep	0	4.3×10^7	0	29.50
Oct	0	3.0×10^4	0	26.50
1978 Feb	0	1.5×10^4	0	11.20
Mar	2.9×10^3	9.9×10^4	2.93	19.38

luminous bacteria in the stomach and intestines occur during the summer months and the lowest in midwinter. Whether this is due to water temperatures or food availability was not determined.

In examining the percentage of luminescent bacteria present in the gut, the bacteria, when present in the stomach, are found in consistently lower percentages than in the intestines.

A percentage of fish possessing bioluminescent bacteria in their guts was calculated by dividing the number of fish with luminous bacteria present by the number of fish collected for the month. The results again indicate higher percentages of fish with luminous bacteria in their intestines than in their stomach.

Laboratory Data

Starvation experiment. The results from thirty-five starved fish indicates a decrease in the number of bioluminescent bacteria per gram of intestines and the percentage of fish retaining luminous bacteria as the period of starvation increased (Figure 5). Short term (5 day) comparison of the percentage of fish with luminous bacteria in the intestines and stomachs of 50 starved fish showed a rapid decrease in both areas (Table 10), with bacteria being readily lost from the stomach (Figure 6). It is unclear as to why an increase in counts occurs on day five in both experiments.

Feeding experiment I. Sixty starved fish were fed shrimp

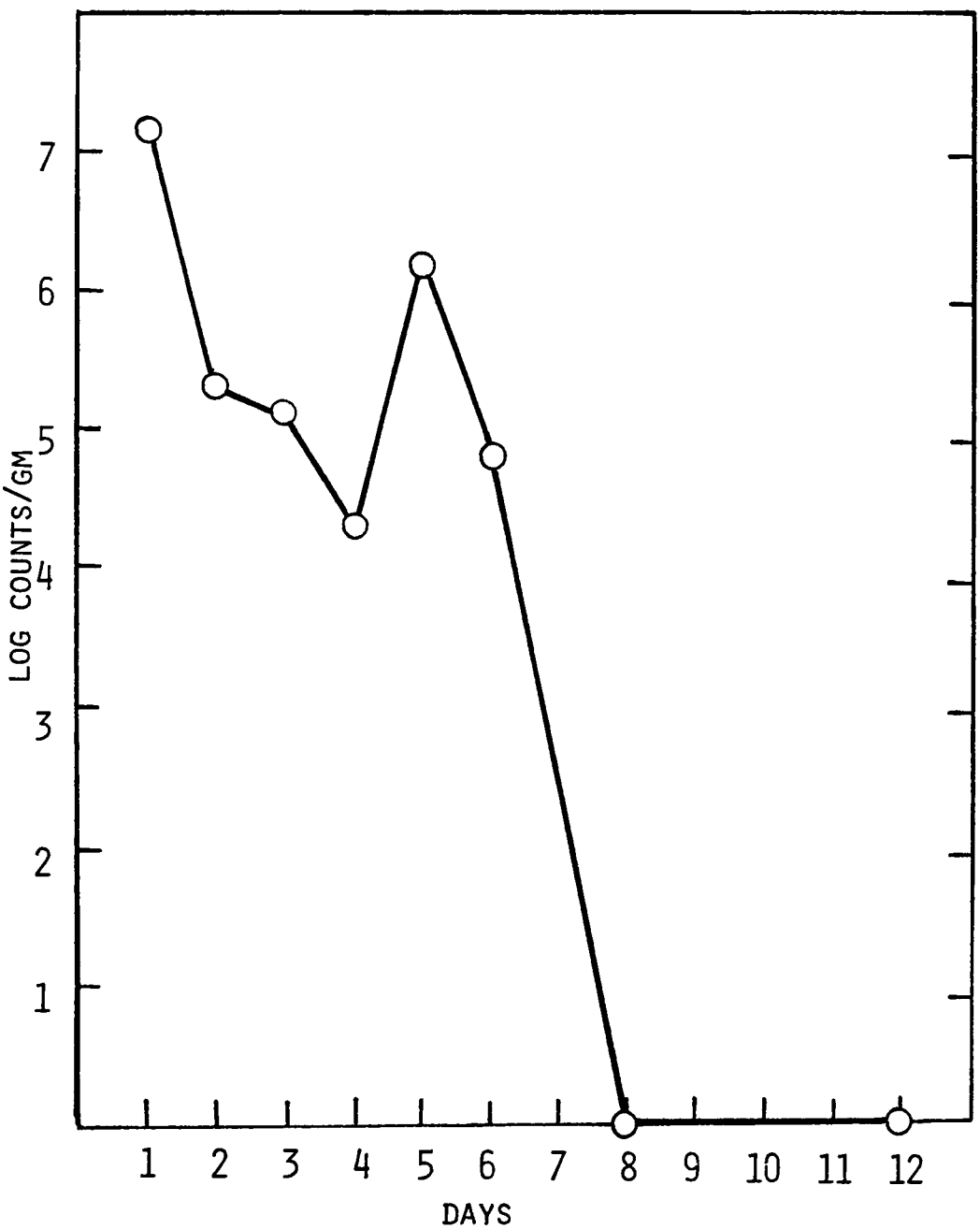

Figure 5. Average luminescent counts per gram of intestines in starved fish over time.

Table 10. Results of 5-day starvation experiment

# Days Starved	# Fish Sacrificed	# Fish with Luminous bacteria in intestines	# Fish with Luminous bacteria in stomach
1	10	5	2
2	10	8	1
3	10	2	0
4	10	0	0
5	10	2	1

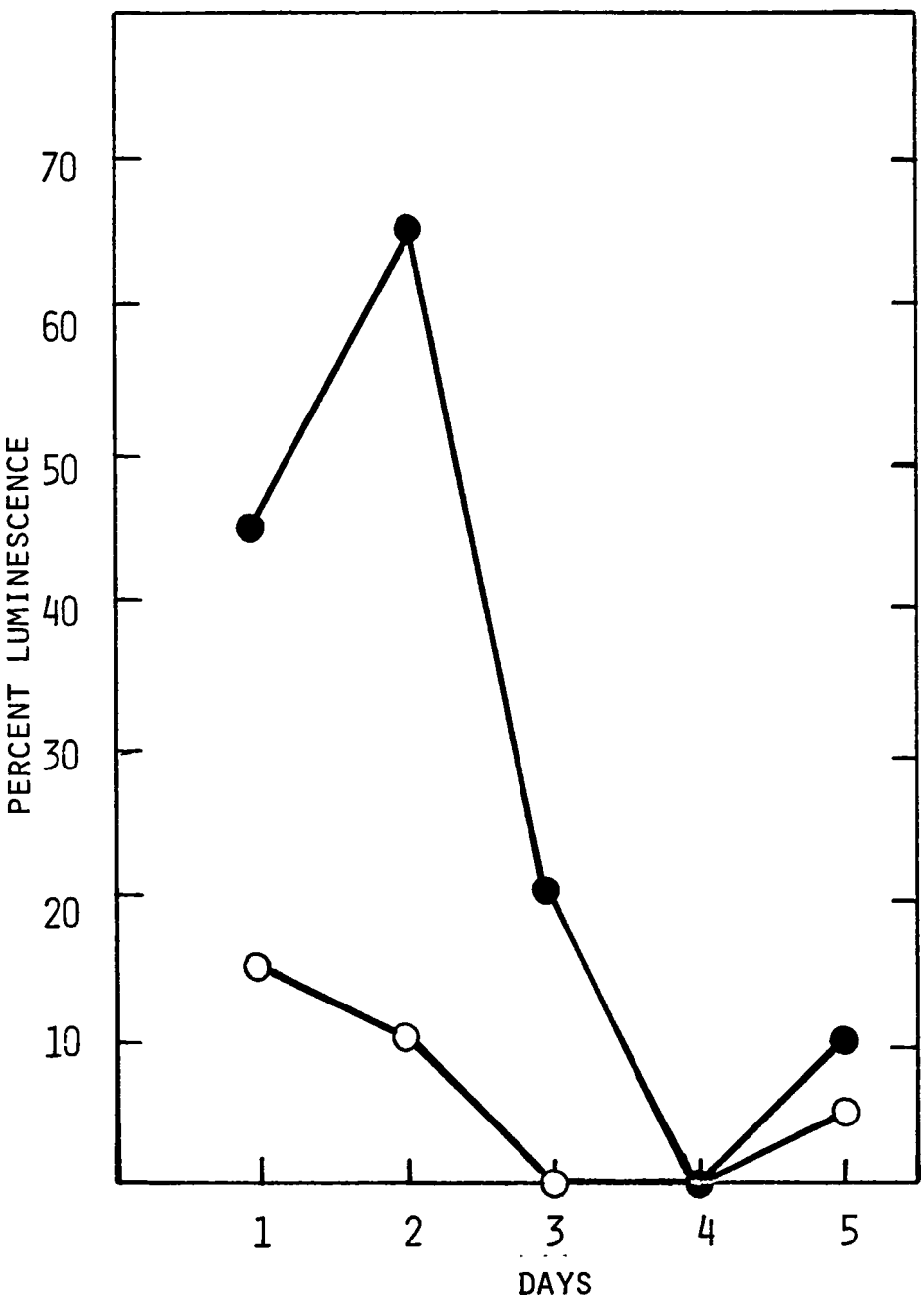

Figure 6. Percentage of fish with luminous bacteria present in the intestine (●) and stomach (O) during a 5-day period. Each dot = 10 fish.

inoculated with *Beneckea harveyi*. The luminous bacterial counts on the inoculated shrimp pieces ranged from 1.7×10^6 to 4.1×10^{11} cells per gram, with no difference between those possessing an exoskeleton and those lacking one. The result of the daily average luminescent counts per gram for the stomach and intestines of the fish fed the shrimp can be seen in Figure 7. An examination of the percentage of fish possessing luminous bacteria in the intestine yields the same result. As in the starvation experiment, there is a decrease in the numbers of bioluminescent bacteria present in the intestine over time with seemingly little difference between fish with and without chitin in their food. The numbers of luminous bacteria in the stomach decline at a faster rate than in the intestine. Again, as in the starvation experiment, there is an unexplanable increase in numbers of luminous bacteria after several days. Since all uneaten remnants of food had been removed from the tank, there is no obvious source of gross contamination.

Feeding experiment II. An experiment was designed to examine numbers of bioluminescent bacteria in the fish gut during a short period of time (less than three days) when a source of chitin is either present of absent.

The luminescent bacterial counts were 2.0×10^3 and 8.2×10^3 cells per gram of stomach for fish fed food with or without chitin, respectively (Table 11). In the intestines of those fish receiving shrimp with a chitin source, the counts were 1.7×10^5 per gram. The counts in the intestines

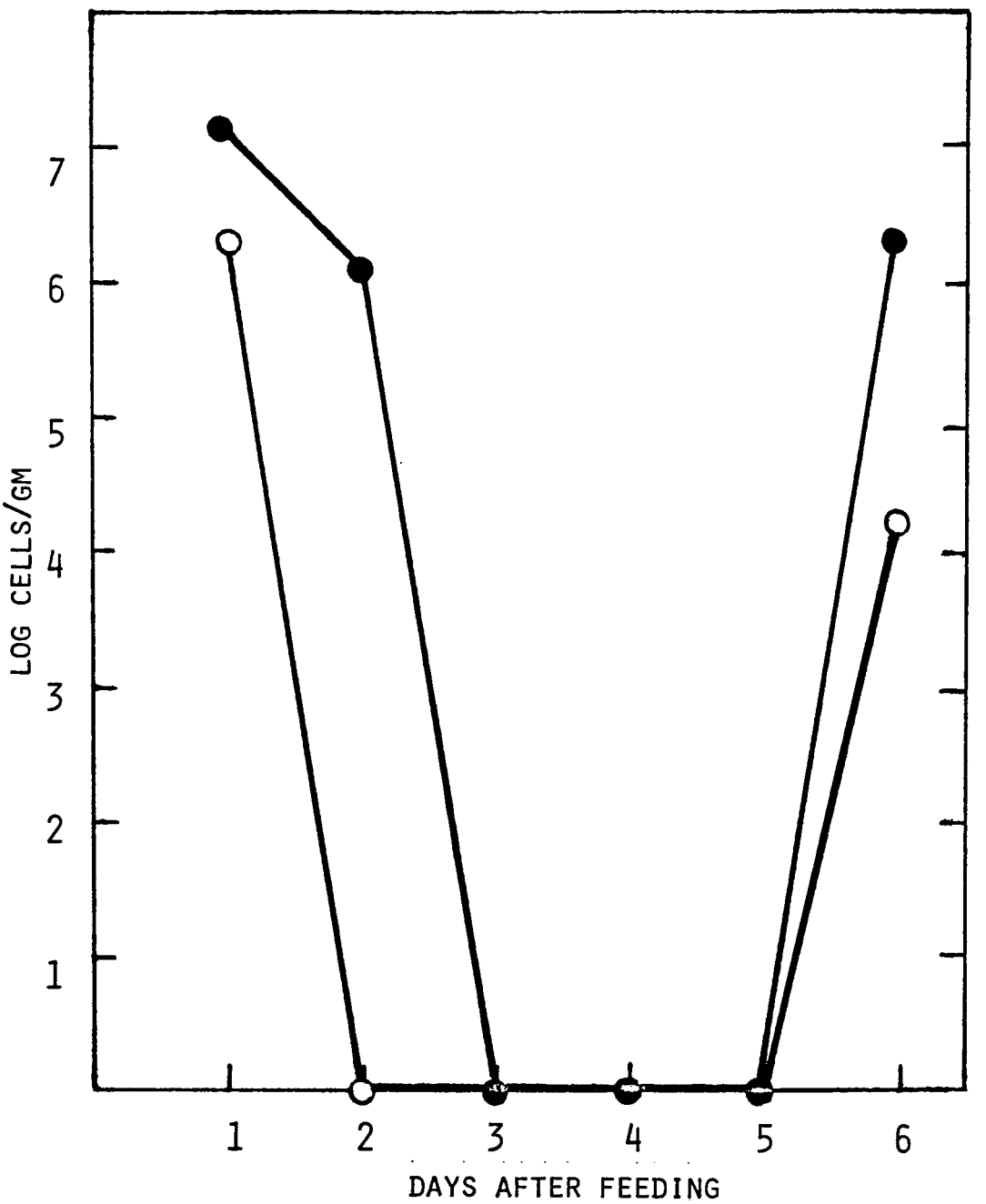

Figure 7. Luminous bacterial counts per gram wet wt of intestine compared over time in fish fed shrimp with (●) or without (O) exoskeleton. Each dot = 5 fish.

Table 11. Average luminescent bacterial counts in fish 48 hours after feeding with shrimp with or without exoskeleton.

	Stomach /gm wet wt	Intestine /gm wet wt
With Exoskeleton	2.0×10^3	1.7×10^5
Without Exoskeleton	8.2×10^3	6.4×10^4

of those fish lacking a chitin source were 6.4×10^4 per gram (37.6% higher with than without). Statistical analysis indicated a significant difference between fish receiving the different types of food.

If the percentage of fish possessing luminescent bacteria after 48 hours is examined, it can be seen that 45% of the fish fed shrimp with the exoskeleton and 40% of those fish fed shrimp without exoskeleton possessed luminescent bacteria.

Feeding experiment III. In the 12 hour feeding experiment, plates were scored as to whether or not they showed luminescent bacteria. Bioluminescent bacteria were immediately detectable in both the stomach and intestines after the 30-minute feeding time. Luminescent bacteria continued to be present in the gastrointestinal for the entire time period.

Taxonomic identification. Following the criteria set forth by Reichelt and Baumann (1973), taxonomic identification was made on the 79 luminous strains collected in the field study. Strains were characterized on the basis of nutritional versatility. Results from such tests indicate that all bioluminescent bacterial strains collected in East Lagoon from June, 1977, to June, 1978, belong to the genus *Beneckea harveyi* (Table 12). None of the *Photobacterium* spp. were isolated during this study.

The results from the experiment conducted on the growth of *B. harveyi* (strain B392) at various pH's show this organism is capable of growth from pH 4.5 to 9.5. The optimum

Table 12. Characters designated to classify luminous bacteria.

	Test Strains[b]	*B. harveyi*[d]
Utilization of[a]:		
D-Xylose	-[c]	-
Maltose	+	+
Cellobiose	+	+
D-Gluconate	+	+
D-Glucuronate	+	+
Mannitol	+	+
DL-Lactate	+	+
Pyruvate	+	+
Acetate	+	+
Propionate	+	+
L-Proline	V	+
D-α-Alanine	+	+
Production of extracellular enzymes:		
Lipase	+	+
Amylase	V	+
Gelatinase	+	+

[a]As sole source of carbon and energy.

[b]Results of 79 luminous test strains.

[c]+ = 80% or more of the strains tested were positive;
- = 5% or less of the strains tested were negative;
V = Between 5 and 80% of the strains tested were positive.

[d]Reichelt, Nealson and Hastings (1977).

at which *Beneckea harveyi* grows is pH 6 to 9, as reported by Hendrie (1970).

DISCUSSION

Bioluminescent bacteria exist as symbionts within specialized organs of certain marine fish and cephalopods. They also occur as "free-living" organisms in the water column and sediments. Often luminescent bacteria are found associated with penaeid shrimp and may be isolated from the gastrointestinal tracts of marine fish (Liston, 1954; Reichelt and Baumann, 1973). In this study luminous bacteria are found to occur throughout the year in East Lagoon, near Galveston, Texas. These bacteria were found to associate with penaeid shrimp, the gastrointestinal tracts of marine fish (i.e. Sciaenidae), and to occur "free-living" in the water column and sediment.

Members of the genus *Vibrio*, in particular *V. parahaemolyticus*, have been reported to occur in a yearly cycle in Chesapeake Bay with bacterial counts being directly related to water temperature. Furthermore, *V. parahaemolyticus* (designated *Beneckea parahaemolytica* by Baumann, *et. al.*, 1971; 1976) was found to overwinter in the sediments and to move from the sediments into the water column as the water temperature increased from 14° to 19°C (Kaneko and Colwell, 1973). A seasonal relationship between water temperature and bioluminescent bacteria in the water column has also been demonstrated off California (Ruby and Nealson, 1978).

In a Texas estuary, the correlation between water temperature and total luminous bacterial counts is less obvious. However, the precent luminescence seemed to be closely related to temperature. A pattern of seasonality was also reported for closely related *Vibrio* strains (Kaneko and Colwell, 1973). The highest concentration of bioluminescent bacteria in the water column of the study area occurs during the summer months and the lowest in the winter. Although there appears to be no seasonal variation in the numbers of luminous bacteria isolated from the sediment, the percentage of luminous bacteria in the sediment during the winter months is higher than the water column. Thus it appears that *B. harveyi* and *V. parahaemolyticus* follow a similar pattern of decreasing in the water column in cold weather. The percentage of luminous bacteria in the sediment also decreases, but not to the extent of the water column. The sediment may serve as a resevoir for these bacteria during the winter.

Seasonal variation of species composition of the bioluminescent bacteria population off the California coast has been reported with *Photobacterium fischeri* dominant in fall and winter samples and *Beneckea harveyi* dominant in the summer samples. In the same report, *P. phosphoreum* were observed in a few fall and winter samples and *P. leiognathi* was not present in detectable numbers (Ruby and Nealson, 1978). All bioluminescent bacteria isolated in this study were identified as *B. harveyi*. None of the *Photobacterium*

groups were detected in East Lagoon. Members of the genus *Beneckea* grow at higher temperatures than *Photobacterium* (Hendrie, *et. al.*,970), and this is probably involved in the dominance of *Beneckea* among the luminous bacteria in the semi-tropical Texas estuary. The yearly average water temperature for the Texas Gulf Coast during this study as 23°C, with only three months in which the average water temperatures are less than 15°C, hence the dominance of *Beneckea*. The average water temperature off California of 15°C (Gross, 1972) together with the seasonal low temperatures could select for the phychrotrophic *Photobacterium*.

Another explanation for the lack of *Photobacterium* in the study area is that *Photobacterium* found as "free-living" cells are assumed to be transients which survive a short period in seawater until they become associated with a new habitat (Ruby and Nealson, 1978). The source of *Photobacterium* in the Gulf of Mexico is probably marine fish generally found in water at depths of 200 - 2000 m (Marshall, 1973). However, with the gently sloping continental shelf of the Gulf, these depths are not found for several hundred miles off Galveston. It is unlikely that any luminous bacteria from these fish would be detected in the East Lagoon estuary. With the narrow continental shelf along the California coast (Gross, 1972), colder, deeper waters are closer inshore than in the Gulf of Mexico, and luminous bacteria shed by deep water fish would be found closer to shore.

The numbers of bioluminescent bacteria isolated during

this study were lowest in the water column, where the counts average 4.9 cells per ml, and highest counts were in the fish intestines with an average of 3.7×10^6 cells per gram (Table 2). The situation is not the same when the percentage of luminous bacteria in the bacterial population is examined. Bioluminescent bacteria on the average represent a larger percentage of the total bacterial population present in the water column (0.09%) than in the sediments (0.05%). However, luminous bacteria represent a larger percentage of the bacterial population associated with shrimp (0.38%) than in the water column or the sediment. The high numbers and a higher percentage of luminous bacteria associated with shrimp than "free-living" in the water column and sediment suggests that luminous bacteria survive at least transiently in or are attached to the surface of penaeid shrimp.

The average number of luminescent bacteria (2.1×10^4 per gm) and the percentage of luminous organisms in the bacterial population of a fish stomach (0.44%) are higher than those for the water column, sediment, or shrimp. Although the continuous presence of acidic digestive juices should destroy luminescent bacteria, the fish stomach contained larger numbers of luminous bacteria than obtained from a food source.

When luminous bacteria are present in the intestines of marine fish, they consistently represent a larger percentage of the total bacterial population in this habitat (20.55%)

than in the water, sediment, shrimp or stomach. The average number of bioluminescent bacteria is also higher ($3.7x10^6$ per gm). Luminous bacteria grow over a pH range comparable to that of the fish intestine (Barrington, 1957; Hendrie, *et. al.*, 1970). The pH range that exists in the intestines and the availability of nutrients suggest that this habitat is suitable for the survival and replication of luminous bacteria as enteric symbionts.

In microbiological work, the dilution factors and variations within samples tend to produce large statistical variances around the mean. If an F-test is used to compare the variances for water, sediment, shrimp, atomach and intestines, the indication is that there is no significant difference between these sampling sites. Results of statistical analysis on the percentages of luminous bacteria are the same. However, if a non-parametric test (i.e. Kruskal-Wallis test) is used in lieu of analysis of variance, the results are entirely differnt. The conclusion reached by the Kruskal-Wallis is the same as would have been reached could the regular method of analysis of variance have been used (Sokal and Rohlf, 1969). The field data has shown, with few exceptions, that the numbers and percentages of luminous bacteria increase in the water column, sediment, shrimp, stomach and intestines, respectively. Conclusions reached by the Kruskal-Wallis test, indicate a significant difference between the numbers of luminous bacteria when comparing water and sediment, sediment to shrimp, and shrimp

to stomach at the 0.01 level of significance. There is also a significant difference between the percentage of luminous bacteria occurring in the sediment to shrimp and shrimp to stomach. There is no significant difference between the numbers of luminous bacterial counts in the stomach and the luminous bacterial counts in the intestines. However, there is a significent difference between the percentage of luminous bacteria in the stomach and intestines (0.05 level of significance). Liston (1957) suggested a selective effect on ingested bacteria allowing numbers of the "Gut Group Vibrios" including luminous organisms, to survive in large enough numbers to constitute a portion of the commensal gut flora. The data collected in this study indicates that this situation does exist. Although the difference between the numbers of luminous bacteria found in the stomach and intestines was not significant, there was a very significant difference in the percentages, with bioluminescent bacteria representing a substantial precentage of the bacterial flora of the marine fish intestine.

The gut of fish under starvation conditions has been reported to be essentially sterile (Obst, 1919; Margolis, 1953). An examination of the incidence of bioluminescent bacteria in the intestines of starving fish yields identical results. As the length of time since the last feeding increased, the numbers and precentages of luminous bacteria in the gut decreases, and after one week, the intestine is virtually free of luminous bacteria. The numbers of lumi-

nous bacteria in the stomach decline at a faster rate than the intestine, a situation attributed to the acidic environment of the stomach.

The stomach of most vertebrates is provided with an acid barrier which is not condusive for the growth of bacteria (Davis and Dulbecco, 1973). The stomach of a fish has a pH range of 2 to 4 (Barrington, 1957). This does not prevent bacteria from entering the intestine, as was seen in the feeding experiment. Luminous bacteria were detected in both the stomach and intestine immediately after feeding and continued to be present in the intestine for as long as one week. Although the digestive secretions eliminate bacteria from the gastrointestinal tracts of marine fish under long-term starved conditions, they appear to have little effect on bacterial survival in the guts of a recently fed fish.

As with the starvation experiments, the feeding experiment demonstrates the decrease in the number of bioluminescent bacteria with time. There appears to be little long term difference in the survival of *Beneckea harveyi* in the gut between fish fed shrimp with or without a chitin source. However, if fish are examined between 48 and 72 hours after feeding, a short term difference in survival of *B. harveyi* in the gut is seen. When fish fed shrimp with the exoskeleton and fish fed shrimp without the exoskeleton are observed, higher numbers and percentages are found in those fed shrimp with chitin. Statistical analysis of the results

indicates that a significant difference does exist. Bioluminescent bacteria utilize N-acetylglucosamine and produce an extracellular chitinase, thus providing them with additional nutrient sources (Reichelt and Baumann, 1973). The ability to utilize available chitin may be advantageous temporarily as food passes through the gut, but does little to promote the maintenance of luminous bacteria in the intestines over time.

From the field data gathered on shrimp, it was found that the average luminous counts were 10^3 and the range was 10^4 to 10^6 cells per gram. The inoculated shrimp fed to fish in laboratory experiments ranged from 10^6 to 10^{11} luminous cells per gram. The range of luminous bacteria in the intestines of fish collected from the field ranged from 10^3 to 10^7 cells per gram over a one year period, with the average being 10^6 cells per gram. The ranges and averages during the first day of the starvation experiment and feeding experiments fall withing the range seen in the field data. This would appear to indicate that despite the amount of inoculum, the intestine of marine fish is capable of supporting a maximum of approximately 10^7 luminous cells per gram.

This study has shown several modes of existence for the "free-living" *Beneckea harveyi* in an estuarine environment. *B. harveyi* was found to exist in the water column and sediment in low numbers. These organisms were found to fluctuate with the yearly temperature cycle and to overwin-

ter in the sediments during the colder months. Higher numbers of luminous bacteria were observed associated with shrimp than existed in either the water column or the sediment. Shrimp serve as a major food source for Sciaenids, the marine fishes examined in this study. This provides one possible mode of entry into the fish stomach, where the numbers of luminous bacteria increased significantly. In a fish undergoing starvation, the numbers of luminous bacteria decrease as the length of the starvation period increases. Once luminous bacteria reach the intestine, the percentage increases significantly. Chitin, the major component in the exoskeleton of shrimp, affects the short term survival of luminous bacteria in the intestine, but does little to permit long term survival.

The inducible enzyme system of luminous bacteria permits the bacteria to maintain either a luminous or non-luminous existence. In a dilute condition, such as the ocean, "free-living" luminous bacteria are unable to systhesize luciferase or emit light (Hastings and Nealson, 1977). Most marine bacteria, however, demonstrate a preference for attachment to solid surfaces, a situation more beneficial to the bacteria than dilute nutrient solutions (Zobell, 1943). Luminous bacteria have been found to associate with such marine animals as penaeid shrimp (Reichelt and Baumann, 1973; Hastings and Nealson, 1977), where concentrated numbers could allow for light emission (i.e. as in a lesion). Such association, followed by ingestion by potential predators, would enable luminous bacteria to enter the gastroin-

testinal tract of marine fish (i.e. golden croaker). The acidic environment of the stomach is presumably not suitable for growth and reproduction. However, the pH of the intestines and nutrients available in the gut of well-fed fish would allow for the propagation of luminous bacteria. Such a proposed situation would establish a life history for bioluminescent bacteria, in which the preferred mode of existence would be as enteric symbionts, but the microorganisms could survive in alternate niches, such as on crustacean exoskeleton before reentry into the gut.

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