APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1989, p. 559–567
0099-2240/89/030559-09$02.00/0

Vol. 55, No. 3

# Occurrence and Distribution of *Vibrio* spp., *Listonella* spp., and *Clostridium botulinum* in the Seto Inland Sea of Japan

KASTHURI VENKATESWARAN,[1]* HIROYUKI NAKANO,[1] TADASHI OKABE,[1] KIYOSHI TAKAYAMA,[1] OSAMU MATSUDA,[2] AND HIDEO HASHIMOTO[1]

*Department of Food Microbiology and Hygiene[1] and Department of Aquatic Environmental Biology,[2] Faculty of Applied Biological Science, Hiroshima University, Higashi-Hiroshima 724, Japan*

Received 22 August 1988/Accepted 5 December 1988

**The distribution of *Vibrio* species in samples of surface water, bottom water (water 2 m above the sediment), and sediment from the Seto Inland Sea was studied. A simple technique using a membrane filter and short preenrichment in alkaline peptone water was developed to resuscitate the injured cells, followed by plating them onto TCBS agar. In addition, a survey was conducted to determine the incidence of *Clostridium botulinum* in sediment samples. Large populations of heterotrophs were found in surface water, whereas large numbers of total vibrios were found in bottom water. In samples from various water sampling regions, high counts of all bacterial populations were found in the inner regions having little exchange of seawater when compared with those of the open region of the inland sea. In the identification of 463 isolates, 23 *Vibrio* spp. and 2 *Listonella* spp. were observed. *V. harveyi* was prevalent among the members of the *Vibrio* genus. *Vibrio* species were categorized into six groups; an estimated 20% of these species were in the so-called "pathogenic to humans" group. In addition, a significant proportion of this group was hemolytic and found in the Bisan Seto region. *V. vulnificus*, *V. fluvialis*, and *V. cholerae* non-O1 predominated in the constricted area of the inland sea, which is eutrophic as a result of riverine influence. It was concluded that salinity indirectly governs the distribution of total vibrios and analysis of variance revealed that all bacterial populations were distributed homogeneously and the variance values were found to be significant in some water sampling regions. Of 26 sediment samples (12%), 3 harbored *C. botulinum*; one was typed as C, while the toxin type of the other two could not be determined. All *C. botulinum*-positive samples were collected from inshore regions and riverine effluents would have influenced the higher incidence of clostridial spores. The present investigation concludes that there is considerable contamination with pathogenic organisms, which will challenge the inhabitants of the Seto Inland Sea, as well as their dependence on natural resources.**

Mariculture in Japan includes commercially important fish, shellfish, as well as seaweed. About 30% of all mariculture products are from the Seto Inland Sea. Oysters are the major mariculture product and account for 80% of the total mariculture industry in Japan; the coastal area of Hiroshima prefecture alone accounts for 85% of the Seto Inland Sea oyster culture production. Next in importance in the mariculture of this area after oysters are shrimp (34%), as well as seaweeds, such as nori (*Porphyra* sp. [39%]) and wakame (*Undaria pinnatifida* [20%]) (2), which are frequently eaten raw. Therefore, the recovery of pathogenic vibrios, listonellas, and *Clostridium botulinum* from regions where shellfish are harvested has become a great concern from the public health viewpoint, but to date, no research on this has been done.

The genus *Vibrio*, as presently defined in *Bergey's Manual of Systematic Bacteriology* (4), consists of 26 species, all of which have been isolated from or are associated with the aquatic environment. This genus has received much attention in recent years; additional descriptions have been made, resulting in 32 currently recognized species (9, 13, 39). On the basis of rRNA phylogenic data, the species formerly known as *V. anguillarum*, *V. damsela*, and *V. ordalii* have been transferred into the newly proposed genus *Listonella* (28). A total of 10 species have been associated with gastroenteritis, wound infections, or septicemia of humans (5), and seven species have been described as pathogens of fishes (3).

The status of botulism as a world health problem is well known (29), and the organism responsible for botulism, *C. botulinum*, is found in soil, even from virgin and forest environments. Extensive surveys of *C. botulinum* were made in sediments of limnetic (49) and marine (52) environments. Although *C. botulinum* was reported in the sediments of Hokkaido, Japan (20), no work has attempted to determine whether this organism is present in the Seto Inland Sea of Japan.

Knowledge of the ecology of *Vibrio* species is mainly focused on the coastal and pelagic areas of Japan (45), but microbiological examination of the Seto Inland Sea area has not been attempted. However, localized investigations, i.e., of only Hiuchi Nada (15, 44), as well as species-specific research, i.e. *L. anguillarum* distribution (32), are available. In addition, a number of bacteriological studies on various rivers which shed their effluents into the Seto Inland Sea were carried out (44, 50). These studies indicated that considerable contamination with pathogenic organisms exists, posing potential hazards to the inhabitants of the Seto Inland Sea as well as their dependence on natural resources. The present investigation was carried out to understand the ecology and distribution of the predominant genus of marine heterotrophic bacteria, i.e., *Vibrio*, and the influence of abiotic factors on their abundance. Furthermore, an alarmingly increasing incidence of infant botulism in Japan (33) makes it important to know the ecology of *C. botulinum* in various environments.

* Corresponding author.

TABLE 1. General description of the sampling stations

| Collection date and station no.[a] | Station location | | Depth (m) | Water sampling region | River(s) | Relevant characteristic |
|---|---|---|---|---|---|---|
| | Latitude N | Longtitude E | | | | |
| 9/02/87 | | | | | | |
| 1 | 34°28.0′ | 133°40.7′ | 9 | Bisan Seto | Takahashi and Asahi | Shallow and strait |
| 2 | 34°25.1′ | 133°38.2′ | 14 | Bisan Seto | Takahashi and Asahi | |
| 3 | 34°22.6′ | 133°36.8′ | 21 | Bisan Seto | Takahashi and Asahi | |
| 4 | 34°20.3′ | 133°35.4′ | 24 | Bisan Seto | Takahashi and Asahi | |
| 10/13/87 | | | | | | |
| 5 | 34°24.4′ | 133°24.8′ | 8 | Bingo Nada | Ashida and Nuta | Like a basin |
| 6 | 34°23.4′ | 133°22.8′ | 12 | Bingo Nada | Ashida and Nuta | |
| 7 | 34°22.4′ | 133°21.0′ | 8 | Bingo Nada | Ashida and Nuta | |
| 6/14/88 | | | | | | |
| 8 | 34°19.0′ | 133°26.3′ | 23 | Hiuchi Nada | Ashida and Nuta | |
| 9 | 34°14.8′ | 133°18.8′ | 22 | Hiuchi Nada | Ashida and Nuta | |
| 10 | 34°08.5′ | 133°13.0′ | 26 | Hiuchi Nada | Ashida and Nuta | |
| 11 | 34°05.0′ | 133°05.0′ | 42 | Hiuchi Nada | Ashida and Nuta | |
| 12 | 34°06.7′ | 132°50.3′ | 38 | Aki Nada | | |
| 13 | 34°03.6′ | 132°38.6′ | 45 | Aki Nada | | |
| 5/20/88 | | | | | | |
| 14 | 34°08.5′ | 132°21.0′ | 27 | Hiroshima Bay | Ohta | Cesspool of pollutants |
| 15 | 34°00.5′ | 132°28.0′ | 37 | Hiroshima Bay | Ohta | |
| 12/09/87 | | | | | | |
| 16 | 34°07.2′ | 132°44.0′ | 41 | Aki Nada | | |
| 17 | 34°07.2′ | 132°37.0′ | 39 | Aki Nada | | |
| 18 | 33°48.0′ | 132°31.8′ | 59 | Iyo Nada | None (no freshwater influence) | Influence of open sea |
| 19 | 33°38.2′ | 132°19.2′ | 60 | Iyo Nada | | |
| 20 | 33°26.0′ | 132°02.7′ | 110 | Iyo Nada | | |
| 21 | 32°58.6′ | 131°54.6′ | 44 | Bungo Strait | None (no freshwater influence) | |
| 5/20/88 | | | | | | |
| 22 | 33°57.2′ | 131°55.3′ | 17 | Suo Nada | Shimada | Shallow and stagnant |
| 23 | 33°55.3′ | 131°54.0′ | 32 | Suo Nada | Shimada | |
| 24 | 33°53.0′ | 131°52.7′ | 40 | Suo Nada | Shimada | |
| 25 | 33°50.0′ | 131°51.5′ | 47 | Suo Nada | Shimada | |

[a] Stations 1 to 4, 5 to 11, 12 to 17, 18 to 21, and 22 to 25 represent Bisan Seto, Hiuchi Nada, Hiroshima Bay, Iyo Nada, and Suo Nada water sampling regions, respectively, as discussed in the text.

## MATERIALS AND METHODS

**Study area and sample collection.** General characteristics and pollution level of the Seto Inland Sea have been described previously (51). Four cruises were made on *Toyoshio Maru*, a research vessel of Hiroshima University, to collect samples; Table 1 shows the dates of collection, water sampling regions, and geographical locations of the sampling stations. Care was taken to cover as many water sampling regions of the Seto Inland Sea as possible (Fig. 1).

Samples of surface and bottom water (water found 2 m above bottom sediments), as well as two sets of sediment samples of 25 stations (one set was kept at 4°C for *C. botulinum* isolation), were collected and transferred aseptically into the sterile containers. Bacteriological procedures were carried out on board not later than 2 h after sample collection. Appropriate sediment samples were aseptically weighed into 50% seawater and mechanically stirred for 30 min to dissociate the adhered bacterial population. Further, the large particles were allowed to settle and suitable samples of the supernatant were subjected to various bacteriological procedures as described below.

Total viable counts (TVC) were determined by using duplicate samples and the spread plate method on Anderson agar containing Bacto-Peptone (0.25%), yeast extract (Difco Laboratories, Detroit, Mich.) (0.25%), ferric phosphate (0.01%), and agar (1.5%) in artificial seawater (pH 7.4 to 7.6). The plates were incubated at 25°C for 5 to 7 days, and CFU were counted.

**Vibrios.** A simple, short-preenrichment technique was developed to enumerate total vibrios in order to resuscitate the stressed cells, if any, due to unfavorable conditions. In this technique, large quantities of the samples (1 liter and 100 ml for water; 10 ml and 1 ml for sediment) were membrane filtered (0.45-μm pore size; Millipore Corp., Bedford, Mass.) and the filter disks were placed over a pad that had been soaked in alkaline peptone water (pH 8.6) for 30 min. After being incubated at 30°C for 6 h, the membranes were transferred onto thiosulfate-citrate-bile salts (TCBS) agar (Eiken, Tokyo, Japan) plates for a combined incubation of 18 to 20 h at 30°C. The CFU were counted for both sucrose-negative (green) and total vibrios. Three strains each of green and yellow colonies were picked, and stocks were grown on $T_1N_1$ agar for identification.

**Biochemical tests.** Vibrios were identified as described by West and Colwell (54). Initially, all the media were prepared with 50% seawater, but superior results prompted us to use

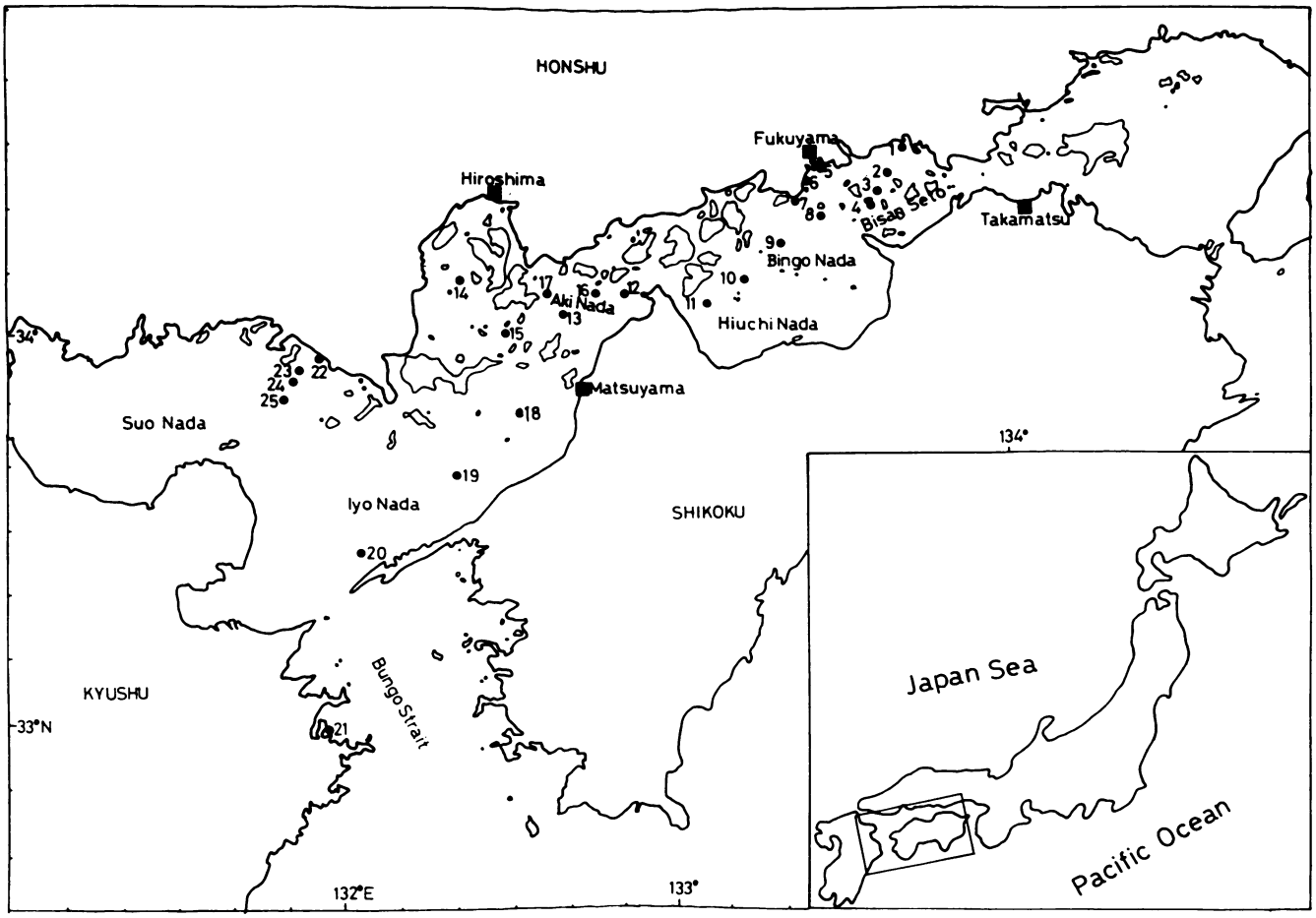


FIG. 1. Map of the Seto Inland Sea, showing various sampling stations and water sampling regions.

ORI medium (46) for the biochemical tests later in the study. The ability to grow at a NaCl concentration of 3, 6, 8, or 10% was determined on agar medium, and the ability to grow without NaCl was determined on CLED agar (Nissui, Japan) plates. Sugars were tested on broth at a concentration of 1%. Arginine dihydrolase, lysine, and ornithine decarboxylase tests were carried out in a modified decarboxylase-dihydrolase medium containing 1 g of protease peptone 3 (Difco), 1 g of yeast extract (Difco), 0.5 g of Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), 0.2 g of sodium thiosulfate, 0.05 g of sodium sulfite, 0.04 g of ferric citrate, 0.5 g of glucose, 0.005 g of pyridoxal-5-phosphate, 0.05 g of phenol red, and 2 g of agar dissolved in a solution of 900 ml of aged seawater and 100 ml of distilled water, and the pH was adjusted to 6.5. Many isolates which showed negative reactions in the decarboxylase and dihydrolase tests in the earlier part of the study showed a positive reaction for one or another in this medium. Hemolytic activity and growth at 37°C were recorded on Trypticase soy agar supplemented with 5% defibrinated sheep blood.

***C. botulinum.*** Sediment samples collected during the cruises were qualitatively examined for *C. botulinum* in the laboratory not more than 5 days after sample collection. A sample (50 g) was mixed with 250 ml of 0.1% peptone water in a measuring cylinder, and the contents were vigorously shaken upside down and allowed to settle for 20 to 30 min. The supernatants were centrifuged at 9,000 × *g* for 30 min at 4°C, and the precipitated pellet was suspended in 2 ml of sterile distilled water and treated as a sediment sample. Two sets (0.1 and 0.9 ml) of these samples were inoculated into 10 ml of cooked meat medium (CMM; Difco) fortified with 0.1% glucose and 0.2% soluble starch. One set of tubes was heated to 80°C for 15 min, and another set was heated to 60°C for 30 min (in an oil bath) and then rapidly cooled. All the tubes were incubated at 30°C for 7 days in an anaerobic jar (GasPack Systems [BBL]). The culture supernatant fluid was centrifuged at 5,000 rpm (KM-15200; Kubota, Tokyo, Japan) for 15 min, and 0.2 ml of the resulting supernatant was diluted with 0.8 ml of gelatin phosphate buffer solution (pH 6.2). A 0.5-ml sample of this dilution was injected intraperitonially in a 4- to 5-week-old mouse (ddY, male) and observed for 4 days. If it died with typical botulinum symptoms, a neutralization test was carried out by using

TABLE 2. Environmental characteristics of the Seto Inland Sea

| Water sampling region | Salinity[a] (‰) | | Temp[a] (°C) | | pH[a] | |
|---|---|---|---|---|---|---|
| | Surface water | Bottom water | Surface water | Bottom water | Surface water | Bottom water |
| Bisan Seto | 31.79 | 32.19 | 26.93 | 26.00 | 8.10 | 8.09 |
| Hiuchi Nada | 31.75 | 32.02 | 22.03 | 18.98 | 8.33 | 8.03 |
| Hiroshima Bay | 32.70 | 32.89 | 16.36 | 15.61 | 8.12 | 8.16 |
| Iyo Nada | 33.71 | 33.78 | 17.71 | 17.63 | 8.34 | 8.32 |
| Suo Nada | 32.65 | 33.60 | 16.66 | 13.82 | 8.14 | 8.10 |

[a] Arithmetic mean.

TABLE 3. Microbiological characteristics of the Seto Inland Sea

| Microbial population | No. (CFU[a]) of organisms in: | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Bisan Seto | | | Hiuchi Nada | | | Hiroshima Bay | | | Iyo Nada | | | Suo Nada | | |
| | S | B | M | S | B | M | S | B | M | S | B | M | S | B | M |
| Total viable counts ($10^4$) | 19.05 | 4.17 | 0.91 | 66.07 | 5.25 | 2.40 | 10.47 | 3.80 | 6.76 | 20.89 | 9.55 | 7.76 | 5.01 | 1.07 | 1.70 |
| Total vibrios | 10 | 8 | <2 | 90 | 130 | 80 | 35 | 75 | 60 | 15 | 10 | 65 | 50 | 30 | 20 |
| Sucrose-negative vibrios | 4 | 5 | <2 | 18 | 40 | 15 | 20 | 50 | 35 | 9 | 10 | 15 | 14 | 18 | 10 |

[a] CFU/100 milliliters for surface (S) and bottom (B) water and CFU/gram for sediment (M) samples.

specific antitoxins (types A, B, C, E, and F) obtained from the Chiba Serum Institute, Ichikawa, Japan.

To isolate the organism, a loopful of the original CMM culture (which was lethal to the mice after an intraperitoneal injection) was transferred onto fresh CMM. If the neutralization test revealed the presence of type C toxin, then $CaCO_3$-fortified CMM (24) was used as the culture medium and incubated at 30°C for 2 days under anaerobic conditions. A loopful from the second culture was streaked over egg yolk GAM (Nissui, Japan) agar supplemented with 0.1% cysteine hydrochloride and incubated anaerobically at 30°C for 2 to 3 days and the colonies with pearly layers that had developed were isolated. These strains were tested for their lethality on mice and the type of *C. botulinum* was determined by neutralization testing as described above. If colonies could not be screened in the above procedure, a colony blotting hydrophobic grid membrane filter-enzyme-linked immunosorbent assay method was carried out to obtain pure colonies (manuscript in preparation).

## RESULTS

Environmental characteristics of various sampling stations are shown in Table 2. A normal pattern of high saline and low thermal conditions was observed in the bottom water layers when compared with that of surface water samples. Table 3 shows characteristics of the bacterial populations of various water sampling regions of the Seto Inland Sea. TVC were highest in surface water layers, followed by bottom water and sediment samples, with the highest densities in Hiuchi Nada. The population seems to decrease with distance from the coast, which was more evident in the Bisan Seto, Hiuchi Nada, and Suo Nada regions.

**Total vibrios.** Ranges of 10 to 90 CFU/100 ml, 80 to 130 CFU/100 ml, and <2 to 80 CFU/g in surface water, bottom water, and sediment samples, respectively, were recorded. A high concentration of total vibrios was found in Hiuchi Nada in all stratified samples compared with those of other

TABLE 4. Distribution of various species of vibrios and listonellas in the Seto Inland Sea

| Species | No. of isolates from sample[a] from: | | | | | | | | | | | | | | | Total no. of isolates | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Bisan Seto | | | Hiuchi Nada | | | Hiroshima Bay | | | Iyo Nada | | | Suo Nada | | | | | |
| | S | B | M | S | B | M | S | B | M | S | B | M | S | B | M | S | B | M |
| *V. alginolyticus* | | 1 | | 5 | 5 | 1 | 11 | 4 | 5 | | 1 | | 9 | 4 | 1 | 25 | 15 | 7 |
| *L. anguillarum* | | 1 | | | | | 8 | 3 | | 4 | | 1 | 4 | 5 | | 16 | 9 | 1 |
| *V. campbellii* | | | | 1 | 1 | | 1 | 3 | | | 4 | | | | | 2 | 8 | |
| *V. cholerae* non-O1 | 5 | 2 | | | | | | | | | | | 1 | | | 6 | 2 | |
| *V. costicola* | | | | | | | | | | | 1 | | | | | | 1 | |
| *V. diazotrophicus* | | | | | | | | 1 | | | | | | | | | 1 | |
| *V. fischeri* | | | | | | | 1 | | | | | 1 | | | | 1 | | 1 |
| *V. fluvialis* | | | | 2 | 3 | 2 | 3 | 6 | 3 | 2 | | | 1 | 3 | 4 | 8 | 12 | 9 |
| *V. furnissii* | | | | | | | | | | | | | | 1 | | | 1 | |
| *V. gazogenes* | 3 | 1 | | | | | | | | | | | | | | 3 | 1 | |
| *V. harveyi* | | | | 5 | 6 | 5 | 9 | 4 | 7 | 9 | 18 | 16 | | 1 | | 23 | 29 | 28 |
| *V. logei* | | | | | | | 1 | | | | | | | | | 1 | | |
| *V. marinus* | | | | 3 | 3 | | | | | | | 2 | | | 1 | 3 | 3 | 3 |
| *V. metschnikovii* | | 4 | 2 | | | | | | | | | 1 | | 1 | | | 5 | 3 |
| *V. nereis* | 1 | 3 | | | | | 4 | 2 | 3 | | | | | | | 5 | 5 | 3 |
| *V. nigripulchritudo* | | | | 1 | | | 5 | 1 | | | | 1 | 1 | | | 7 | 1 | 1 |
| *L. ordalii* | | | | | | | | | | | | | | | 1 | | | 1 |
| *V. parahaemolyticus* | | 1 | | 1 | 3 | | 2 | | | | 2 | | | 3 | | 3 | 9 | |
| *V. pelagius* biovar I | 3 | | | 2 | 1 | | 4 | 3 | 3 | 9 | 3 | 3 | 8 | 12 | 2 | 26 | 19 | 8 |
| *V. pelagius* biovar II | | | | | 1 | | | | | | | | | | | | 1 | |
| *V. proteolyticus* | | | | | | | | | | | 2 | 2 | | 2 | 2 | | 4 | 4 |
| *V. splendidus* biovar I | | | | 2 | 1 | 3 | 8 | 12 | 6 | 1 | | 2 | 1 | 5 | 7 | 12 | 18 | 18 |
| *V. splendidus* biovar II | 4 | 1 | 1 | 4 | 3 | | 3 | | | 2 | 1 | | 5 | 6 | | 18 | 11 | 1 |
| *V. vulnificus* | 1 | | | 5 | 3 | 2 | 2 | 2 | 3 | 1 | 1 | | 4 | | | 13 | 6 | 5 |
| *Vibrio* spp. | 10 | 4 | | 8 | 3 | 3 | 4 | 2 | | 1 | 2 | | | | | 23 | 11 | 3 |

[a] S, Surface water; B, bottom water; M, sediment.

TABLE 5. Distribution of *C. botulinum* in various regions of the Seto Inland Sea[a]

| Water sampling region | No. of samples tested | No. (%) of samples positive | Heat treatment[b] | | Toxin type |
|---|---|---|---|---|---|
| | | | 80°C | 60°C | |
| Bisan Seto | 4 | 0 | − | − | |
| Hiuchi Nada | 4 | 1 (25) | − | + | ND[c] |
| Hiroshima Bay | 9 | 2 (22) | − | + | C, ND |
| Iyo Nada | 4 | 0 | − | − | |
| Suo Nada | 5 | 0 | − | − | |

[a] A total of 26 samples were tested, of which 3 (12%) were positive for *C. botulinum*.
[b] See text for details of heat treatment. +, Positive; −, negative.
[c] The mouse died with the typical botulism symptoms but the toxin type could not be determined.

water sampling regions of the Seto Inland Sea. Among the different sampling locales, the bottom water samples showed the highest population of total vibrios except for Iyo Nada, where sediments had a higher population. Sucrose-negative vibrios showed a pattern of distribution similar to that of total vibrios both for sampling stations and strata.

The distribution of various species of vibrios in different water sampling regions is shown in Table 4. A total of 463 strains were isolated and identified to the species level. *V. harveyi* (17.3%) was the predominant species of the genus *Vibrio* throughout the sampling period. *V. alginolyticus* (10.2%) was isolated frequently, along with *V. splendidus* biovar I (10.4%). All the vibrios and listonellas were categorized into the following six groups; (i) those that are capable of growth in 10% NaCl (*V. alginolyticus*, *V. harveyi*, and *V. nereis*); (ii) those that are pathogenic to humans (*V. campbellii*, *V. cholerae*, *V. fluvialis*, *V. metschinikovii*, *V. parahaemolyticus*, and *V. vulnificus*); (iii) those that are pathogenic to fish (*L. anguillarum* and *L. ordalii*); (iv) those that are luminous (*V. fischeri*, *V. logei*, *V. marinus*, and *V. splendidus* biovar I); (v) those that are decarboxylase and dihydrolase negative (*V. gazogenes*, *V. nigripulchritudo*, *V. pelagius* biovars I and II and *V. splendidus* biovar II; and (vi) others (species not listed in the above groups).

In these categories, vibrios capable of growing in 10% NaCl make up 30.2% of the total vibrios and were found to be predominant in all the water sampling regions. In addition, the members of this group were found to exist more frequently in surface water layers compared with those of other samples. The next most abundant group was the decarboxylase and dihydrolase-negative group (22%), in which *V. pelagius* (54 strains), the most common species, *V. alginolyticus* (1 strain), which was found sporadically, as well as *V. campbellii* (3 strains), were found.

A higher incidence (19.7%) of the so-called vibrios pathogenic to humans found in the present study threatens the inhabitants of this area. Of these pathogenic vibrios, eight strains were identified as *V. cholerae* non-O1, with seven strains occurring in the Bisan Seto region. *V. vulnificus* (24 strains) was the most prevalent; 80% of these strains showed hemolytic activity with sheep erythrocytes. Substantial proportions (35%) of *V. fluvialis* exhibited hemolytic activity. Eight strains of oxidase-negative vibrios, i.e., *V. metschnikovii*, were encountered and were relatively abundant in bottom water and sediment samples. It is noteworthy that no isolate from the surface water samples was identified as *V. metschnikovii*. All *V. parahaemolyticus* strains (12) isolated, which were common in bottom water layers, showed negative reactions with Kanagawa phenomenon. In addition, the 15 strains isolated from Bisan Seto, which fall into the vibrios pathogenic to humans group, all showed hemolytic activity except for one strain of *V. parahaemolyticus*. This anomaly needs further study.

In group 4, i.e., vibrios pathogenic to fish, *L. anguillarum* frequently appeared in the water column and only one strain of *L. ordalii* could be isolated in the sediment sample of station 22. About 13% of the total strains appeared to be luminous and of the luminous species, *V. splendidus* biovar I recurred frequently, with a high incidence in bottom water samples. Other species not mentioned above were grouped together with unidentified *Vibrio* spp. (37 strains), making up about 10% of the total number of species. In this group, one strain each of *V. furnissii* and *V. costicola* were included and were found to be isolated from bottom water layers of Iyo Nada and Suo Nada, respectively.

***C. botulinum.*** The distribution of *C. botulinum* is shown in Table 5. Among various water sampling regions, one sample from Hiuchi Nada (25%) and two samples from Hiroshima Bay (22%) were found to have this toxic organism. While heat shocking the samples to kill vegetative bacteria at 80 and 60°C, it was observed that the latter produced toxin; 80°C heat treatment is probably lethal to the stressed spores of *C. botulinum*. The toxin was known to be type C in one case, i.e., station 14, and for the samples from another two stations (stations 9 and 16), the mouse died with typical botulism symptoms but the toxin type could not be determined by neutralizing with antitoxin. In all three cases, *C. botulinum* colonies were not recovered by using either egg yolk GAM agar plates or the hydrophobic grid membrane filter-enzyme-linked immunosorbent assay.

## DISCUSSION

Extensive surveys on the distribution of heterotrophic bacteria in the Indian Ocean and South China Sea (47), as well as pelagic areas of Tokyo Bay (45), were carried out. These two studies reported that no clear vertical distribution of total heterotrophic bacteria was observed. However, the observance in the present study of a clear distribution in the heterotrophic bacterial population might be attributed to the number of sampling layers, which was restricted to surface and bottom waters, as well as to the relative quiescence of the environment. In addition, the depth of the sampling area did not exceed 47 m, except at Iyo Nada. A river discharge with high nutrient effluents would have enhanced the growth of these bacterial populations in the surface waters and is evidenced by the decrease in the densities of heterotrophic bacteria from the distance of the coast. Analysis of variance (Table 6) indicated that the organisms (measured by TVC) were homogeneously distributed and converged at the surface water and were significant at the 1% level.

TABLE 6. Analysis of variance for various bacterial populations of the Seto Inland Sea

| Microbial population | Analysis of variance value[a] for bacterial population from: | | | | | | | | | | Total analysis of variance | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Bisan Seto | | Hiuchi Nada | | Hiroshima Bay | | Iyo Nada | | Suo Nada | | | |
| | A | B | A | B | A | B | A | B | A | B | A | B |
| Total viable counts | 4.90[b] | 0.14 | 8.11[c] | 6.91[c] | 1.78 | 3.33[b] | 9.63[b] | 7.71[b] | 2.33 | 1.7 | 3.00 | 9.44[c] |
| Total vibrios | 2.90 | 3.21 | 5.30[c] | 1.61 | 9.00[c] | 1.07 | 1.20 | 5.41[b] | 1.21 | 2.50 | 6.70[b] | 0.10 |
| Sucrose-negative vibrios | 0.30 | 1.60 | 3.03[b] | 1.18 | 7.80[c] | 1.67 | 6.37[b] | 3.11 | 12.11[c] | 8.81[b] | 20.50[c] | 3.50 |

[a] A, Variation between samples; B, variation between sampling stations.
[b] Significant at 5% level.
[c] Significant at 1% level.

The distribution of *Vibrio* spp. and their ecology in some bodies of water in Japan are well understood. However, such investigations on the most fertile shellfish-growing ground of Japan, i.e., the Seto Inland Sea, were limited to only certain sites, such as Hiuchi Nada (15), or to some pathogenic vibrios, such as *V. parahaemolyticus*, etc. (K. Venkateswaran, S. W. Kim, T. Onbe, and H. Hashimoto, Syst. Appl. Microbiol., in press). Unless the ecology of vibrios is fully understood, complete control of disease(s) caused by these microorganisms will not be possible. In the present study, clear-cut zonation in the sample stratification of total vibrios was observed. Previous studies also suggested that members of the family *Vibrionaceae* were found less frequently in surface water. Also, in the identification of total heterotrophs in this region (K. Venkateswaran, H. Nakano, and H. Hashimoto, Nippon Suisan Gakkaishi, in press), a small population of members of *Vibrionaceae* was found in surface water samples. Analysis of variance also showed a significant variation from homogeneous distribution and *Vibrionaceae* were found to be distributed mainly in bottom water layers. The relationship between total vibrios and other biotic and abiotic factors was analyzed (Table 7). The inverse relationship with salinity shows that this factor is a crucial factor governing the distribution as salinity was found to be statistically significant in most water sampling regions. This supports the hypothesis that river discharge with high nutrients perhaps influenced the proliferation of vibrios, which in turn reduced the salinity of the environment. Similar observations were made by Kaper et al. (21) on salinity and *V. cholerae* in Chesapeake Bay and by Oliver

TABLE 7. Correlation coefficients between total vibrios and various biotic and abiotic characteristics

| Sampling water regions and sample[a] | Correlation between no. of total vibrios and: | | | | |
|---|---|---|---|---|---|
| | Salinity | Temp | pH | TVC | Sucrose-negative vibrios |
| Bisan Seto | | | | | |
| S | −0.929[b] | 0.794 | −0.887 | 0.171 | 0.891[b] |
| B | −0.906[b] | 0.385 | 0.563 | 0.192 | 0.819 |
| M | 0.001 | 0.001 | 0.002 | 0.002 | 1.000[c] |
| Hiuchi Nada | | | | | |
| S | −0.158 | 0.650 | −0.624 | 0.587 | 0.904[c] |
| B | −0.358 | 0.588 | −0.341 | −0.016 | 0.843[c] |
| M | −0.301 | 0.302 | −0.510 | 0.764[b] | 0.539 |
| Hiroshima Bay | | | | | |
| S | 0.690 | 0.142 | −0.555 | 0.439 | 0.995[c] |
| B | −0.604 | 0.333 | 0.560 | 0.376 | 0.519 |
| M | −0.508 | 0.588 | −0.152 | 0.659 | 0.977[c] |
| Iyo Nada | | | | | |
| S | −0.283 | −0.732 | 0.740 | −0.273 | 0.937[b] |
| B | −0.745 | −0.701 | 0.723 | −0.917[b] | 0.947[b] |
| M | −0.876[b] | −0.560 | 0.888[b] | 0.606 | 0.993[c] |
| Suo Nada | | | | | |
| S | −0.930[b] | 0.424 | −0.318 | 0.585 | 0.981[c] |
| B | 0.712 | 0.831 | −0.524 | 0.890[b] | 0.999[c] |
| M | 0.629 | 0.977[c] | −0.244 | 0.391 | 0.983[c] |
| All regions | | | | | |
| S | −0.242 | −0.401 | 0.285 | 0.161 | 0.871[b] |
| B | −0.345 | −0.470 | −0.484 | −0.128 | 0.944[c] |
| M | 0.287 | −0.708 | 0.298 | 0.812[b] | 0.931[c] |

[a] Abbreviations: S, surface water; B, bottom water; M, sediment.
[b] Significant at 5% level.
[c] Significant at 1% level.

et al. (35) on lactose-fermenting vibrios in plankton samples from the coastal waters of the Southeastern United States, as well as from the East Coast of the United States (36). Sucrose-positive vibrios predominated over sucrose-negative vibrios in all the samples, as was found in the coastal waters of Hong Kong (7).

A higher incidence of *V. harveyi*, as seen in the present study, was also found in the subtropical waters of Hong Kong (7). O'Brien and Sizemore (34) found that all the luminescent bacteria isolated in a semitropical estuarine environment were identified as *Beneckea harveyi*. Furthermore, *B. harveyi* was reported to be abundant in the coastal strip, which is higher in nutrients and in productivity than the open waters (43). However, the luminescent properties of these strains were greatly reduced, possibly as a result of isolating the strains from TCBS. A consistently higher frequency of occurrence for *V. alginolyticus*, as seen in the present study, was reported for seawater and seafood samples collected in various countries (12, 30), including Japan (41). This indicates a public health problem, especially for wound infections resulting from exposure to seawater (37).

A higher percentage of vibrio strains (21.8%) not able to decarboxylate amino acids cast doubt upon identifying the strains belonging to the genus *Vibrio* on the basis of amino acid decarboxylases. West et al. (53) also questioned the use of Moller medium, and the limitations of the medium in detecting decarboxylase activity by coliform bacteria have also been noted previously (38). Early in the present study, the medium described by Smith and Fernandes (48) was used, but a high percentage of decarboxylase-negative reactions forced us to formulate a new medium which was described in Materials and Methods. With this new medium, some of the strains which showed negative reactions in previous media produced decarboxylases with either arginine or lysine and ornithine. This suggested that some deficiencies existed in the above-mentioned medium when it was adapted for use with marine isolates (53).

*V. cholerae* non-O1 and *V. metschnikovii* were observed to be more abundant in Bisan Seto, which might be due to the fact that only this region was flooded with a heavy inflow of fresh water ($5.7 \times 10^9$ $m^3/s$) from three major rivers (31). Also, the sampling season was the hottest season in the present investigation, which may explain the abundance of these pathogenic vibrios. The temperature-dependent distribution of *V. cholerae* non-O1 (K. Venkateswaran, T. Takai, I. M. Navarro, H. Nakano, H. Hashimoto, and R. J. Siebeling, personal communication) and *V. parahaemolyticus* of this region (Venkateswaran et al., in press), as well as in other parts of the world (19, 25) was well established. However, a high percentage of hemolytic activity and the presence of *V. cholerae* were great causes of concern about the safety of seafoods cultured in these areas, from the public health viewpoint.

A high percentage of *V. vulnificus*, which is reported to be responsible in causing septicemia in humans, was found. Except for a few classical studies in the Atlantic and Gulf Coasts of the United States (22, 23, 36), little attention has been paid to these lactose-fermenting vibrios; more research on their seasonality, environmental niche, and relationship to other similar vibrios in various parts of the world needs to be done. Also, the ecology of this organism is not fully documented in Japan and it is necessary to know its ecological behavior and aquatic environmental reservoir in a country which consumes raw seafoods. Although only Kanagawa phenomenon-negative *V. parahaemolyticus* was observed in the present study, recent descriptions of pathogenesis caused by these Kanagawa phenomenon-negative strains, such as gastroenteritis (16), synthesis of a new hemolysin (17), as well as wound infections (18), leads us to not underestimate their role in human safety.

*L. anguillarum* detection and distribution in waters of the Seto Inland Sea was thoroughly investigated (32). Annual losses exceeded 11 million pounds in Japan as a result of vibriosis in marine fish (3). Although only 26 strains of *L. anguillarum*, which is considered to be a facultative fish pathogen (10), were isolated during the present study, extensive research will detail its virulence and pathogenicity. In addition, *V. metschnikovii*, *V. fluvialis*, and *V. furnissii* which have been isolated but have been paid little attention (6, 26, 27) regarding their distribution, need detailed surveys to ascertain their ecological importance in various waters.

The incidence of botulism poisoning in humans in Japan is low when compared that of the United States, except for type E outbreaks (1). Use of vaccum-packed foods with improper preservation, and the high detection rate of botulism in honey in recent years, along with infant botulism cases (40), demonstrated that this toxic organism could be isolated from many environments. It is generally considered that *C. botulinum* type C is not hazardous for humans. However, human cases of type C in various countries (14) and its fastidiousness in experimental monkeys (8) showed that its potential risk should not be ignored. Hence, 12% recovery of this pathogen in the Seto Inland Sea is a matter of great concern for public health. In general, type E is associated primarily with aquatic habitats but research in recent years has suggested that type C can be recovered in birds as well as in river environments (11). The frequency of occurrence of *C. botulinum* appeared to be restricted to Hiuchi Nada and Hiroshima Bay and the basinlike topography of the former, as well as the unending discharge of pollutants in the latter region might be a cause in the growth of this pathogens. Spores of type C are reported to be susceptible to heat treatment when compared with spores of type A and B (42), and the absence of toxin in the samples heated to 80°C might be attributed to this fact. The failure to isolate *C. botulinum* colonies from the toxin-positive samples proved that some deficiencies still exist in the method employed for the selective isolation of this pathogen.

On the basis of the present investigation, it could be concluded that the presence of pathogenic vibrios and *C. botulinum* in the inner region of the Seto Inland Sea poses potential hazards to the mariculture of this area as well as to the consumers of its seafood products.

## ACKNOWLEDGMENTS

We acknowledge, with a deep sense of gratitude, Takashi Onbe and Takuo Endo of Hiroshima University for infallible support during the cruises and the captain and crew of R/V *Toyoshio Maru* for helping with sampling.

## LITERATURE CITED

1. **Aibara, K.** 1985. Botulism. Bisebutsu **1**:12–19. (In Japanese.)
2. **Association of Agriculture and Forestry Statistics.** 1988. The 63rd statistical yearbook of Ministry of agriculture, forestry and fisheries, Japan, p. 314–401. Association of Agriculture and Forestry Statistics, Tokyo.
3. **Austin, B., and A. Austin (ed.).** 1987. Bacterial fish pathogens: disease in farmed and wild fish, p. 263–296. Ellis Horwood Ltd., Chichester, United Kingdom.
4. **Baumann, P., and R. H. W. Schubert.** 1984. Family II. *Vibrionaceae* Veron 1965, 5245, p. 516–550. *In* N. R. Krieg and J. G.

Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
5. **Blake, P. A.** 1984. Prevention of food borne disease caused by *Vibrio* species, p. 579–591. *In* R. R. Colwell (ed.), Vibrios in the environment. John Wiley & Sons, Inc., New York.
6. **Brenner, D. J., F. W. Hickman-Brenner, J. V. Lee, A. G. Steigerwalt, G. R. Fanning, D. G. Hollis, J. J. Farmer III, R. E. Weaver, S. W. Joseph, and R. J. Siedler.** 1983. *Vibrio furnissii* (formerly aerogenic biogroup of *Vibrio fluvialis*), a new species isolated from human feces and the environment. J. Clin. Microbiol. **18**:816–824.
7. **Chan, K. Y., M. L. Woo, K. W. Lo, and G. L. French.** 1986. Occurrence and distribution of halophilic vibrios in subtropical coastal waters of Hong Kong. Appl. Environ. Microbiol. **52**: 1407–1411.
8. **Dolman, C. E., G. E. Darby, and R. F. Lane.** 1961. *Clostridium botulinum* type F with recent observation on other types. J. Infect. Dis. **109**:107–128.
9. **Egidius, E., R. Wiik, K. Andersen, K. A. Hoff, and B. Hjeltnes.** 1986. *Vibrio salmonicida* sp. nov., a new fish pathogen. Int. J. Syst. Bacteriol. **36**:518–520.
10. **Evelyn, T. P. T.** 1971. First records of vibriosis in Pacific salmon cultured in Canada, and taxonomic studies of the responsible bacterium, *Vibrio anguillarum*. J. Fish. Res. Board Can. **28**: 517–525.
11. **Fujii, K., I. Ohishi, and G. Sakaguchi.** 1977. Distribution of *Clostridium botulinum* in the soils of Yodo river. Jpn. J. Bacteriol. **32**:949. (In Japanese.)
12. **Gjerde, J., and B. Boe.** 1981. Isolation and characterization of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* from the Norwegian coastal environment. Acta Vet. Scand. **22**:331–343.
13. **Grimes, D. J., J. Stemmler, H. Hada, E. B. May, D. Maneval, F. M. Hetrick, R. T. Jones, and M. Stoskopf.** 1984. *Vibrio* species associated with mortality of sharks held in captivity. Microb. Ecol. **10**:271–282.
14. **Haq, I., and G. Sakaguchi.** 1980. Prevalence of *Clostridium botulinum* in fishes from markets in Osaka. Jpn. J. Med. Sci. Biol. **33**:1–6.
15. **Hata, Y., and H. Miyoshi.** 1973. Bacterial biomass in Hiuchi Nada. Int. Rep. SETO, Japanese Committee for the International Biological Progam—Marine Production, 16–21. (In Japanese.)
16. **Honda, S., I. Goto, I. Minematsu, N. Ikeda, N. Asano, M. Ishibashi, Y. Kinoshita, M. Nishibuchi, T. Honda, and T. Miwatani.** 1987. Gastroenteritis due to Kanagawa negative *Vibrio parahaemolyticus*. Lancet **i**:331–332.
17. **Honda, T., Y. Ni, and T. Miwatani.** 1988. Purification and characterization of a hemolysin produced by a clinical isolate of Kanagawa phenomenon-negative *Vibrio parahaemolyticus* and related to the thermostable direct hemolysin. Infect. Immun. **56**:961–965.
18. **Johnson, D. E., L. Weinberg, J. Ciarkowski, P. West, and R. R. Colwell.** 1984. Wound infection caused by Kanagawa-negative *Vibrio parahaemolyticus*. J. Clin. Microbiol. **20**:811–812.
19. **Kaneko, T., and R. R. Colwell.** 1978. The annual cycle of *Vibrio parahaemolyticus* in Chesapeake Bay. Microb. Ecol. **4**:135–155.
20. **Kanzawa, K., T. Ono, T. Karashimada, and H. Iida.** 1970. Distribution of *Clostridium botulinum* type E in Hokkaido, Japan, p. 299–303. *In* M. Herzberg (ed.), Proceedings of the 1st U.S.-Japan Conference on Toxic Microorganisms. United States-Japan Cooperative Program in Natural Resources Joint Panels and U.S. Department of the Interior, Washington, D.C.
21. **Kaper, J., H. Lockman, R. R. Colwell, and S. W. Joseph.** 1979. Ecology, serology, and enterotoxin production of *Vibrio cholerae* in Chesapeake Bay. Appl. Environ. Microbiol. **37**:91–103.
22. **Kaysner, C. A., C. Abeyta, M. M. Wekell, A. DePaola, Jr., R. F. Stott, and J. M. Leitch.** 1987. Virulent strains of *Vibrio vulnificus* isolated from estuaries of United States West Coast. Appl. Environ. Microbiol. **53**:1349–1351.
23. **Kelly, M. T.** 1982. Effect of temperature and salinity on *Vibrio (Beneckea) vulnificus* occurrence in a Gulf coast environment. Appl. Environ. Microbiol. **44**:820–824.
24. **Kurazono, H., K. Shimozawa, and G. Sakaguchi.** 1985. Botulism among penned pheasants and protection by vaccination with $C_1$ toxoid. Res. Vet. Sci. **38**:104–108.
25. **Lee, J. V., D. J. Bashford, T. J. Donovan, A. L. Furniss, and P. A. West.** 1982. The incidence of *Vibrio cholerae* in water, animals and birds in Kent, England. J. Appl. Bacteriol. **52**: 281–291.
26. **Lee, J. V., T. J. Donovan, and A. L. Furniss.** 1978. Characterization, taxonomy and emended description of *Vibrio metschnikovii*. Int. J. Syst. Bacteriol. **28**:99–111.
27. **Lee, J. V., P. Shread, A. L. Furniss, and T. N. Bryant.** 1981. Taxonomy and description of *Vibrio fluvialis* sp. nov. (synonym group F vibrios, group EF 6). J. Appl. Bacteriol. **50**:73–94.
28. **MacDonell, M. T., and R. R. Colwell.** 1985. Phylogeny of the *Vibrionaceae* and recommendation for the new genera, *Listonella* and *Shewanella*. Syst. Appl. Microbiol. **6**:171–182.
29. **Meyer, K. F.** 1956. The status of botulism as a world health problem. Bull. W.H.O. **15**:281–298.
30. **Molitoris, E., S. W. Joseph, M. I. Krichersky, W. Sindhuhardja, and R. R. Colwell.** 1985. Characterization and distribution of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* isolated in Indonesia. Appl. Environ. Microbiol. **50**:1388–1394.
31. **Murakami, A. (ed.).** 1976. Marine ecology and fishing ground of Seto Inland Sea, p. 3–13. Fuji Techno-System, Tokyo. (In Japanese.)
32. **Muroga, K., M. Iida, H. Matsumoto, and T. Nakai.** 1986. Detection of *Vibrio anguillarum* from waters. Bull. Jpn. Soc. Sci. Fish. **52**:641–647.
33. **Noda, H.** 1988. Infant botulism. Media-Circle **33**:125–132. (In Japanese.)
34. **O'Brien, C. H., and R. K. Sizemore.** 1979. Distribution of the luminous bacterium *Beneckea harveyi* in a semitropical estuarine environment. Appl. Environ. Microbiol. **38**:928–933.
35. **Oliver, J. D., R. A. Warner, and D. R. Cleland.** 1982. Distribution and ecology of *Vibrio vulnificus* and other lactose-fermenting marine vibrios in coastal waters of the Southeastern United States. Appl. Environ. Microbiol. **44**:1404–1414.
36. **Oliver, J. D., R. A. Warner, and D. R. Cleland.** 1983. Distribution of *Vibrio vulnificus* and other lactose-fermenting vibrios in the marine environment. Appl. Environ. Microbiol. **45**:985–998.
37. **Pien, F., K. Lee, and H. Higa.** 1977. *Vibrio alginolyticus* infection in Hawaii. J. Clin. Microbiol. **5**:670–675.
38. **Pilsucki, R. W., N. W. Clayton, V. J. Cabelli, and P. S. Cohen.** 1979. Limitations of the Moeller lysine and ornithine decarboxylase tests. Appl. Environ. Microbiol. **37**:254–260.
39. **Pujalte, M. J., and E. Garay.** 1986. Proposal of *Vibrio mediterranei* sp. nov.: a new marine member of genus *Vibrio*. Int. J. Syst. Bacteriol. **36**:278–281.
40. **Sakaguchi, G.** 1988. Infant botulism and honey. Modern Media **34**:123–132. (In Japanese.)
41. **Sakazaki, R., T. Karashimada, K. Yuda, S. Sakai, Y. Asakawa, M. Yamazaki, H. Nakanishi, K. Kobayashi, T. Nishio, H. Okazaki, T. Doke, T. Shimada, and K. Tamura.** 1979. Enumeration of, and hygienic standard of food safety for, *Vibrio parahaemolyticus*. Arch. Lebensmittelhyg. **30**:103–106.
42. **Segner, W. P., and C. F. Schmidt.** 1971. Heat resistance of spores of marine and terrestrial strains of *Clostridium botulinum* type C. Appl. Microbiol. **22**:1030–1033.
43. **Shilo, M., and T. Yetinson.** 1979. Physiological characrteristics underlying the distribution patterns of luminous bacteria in the Mediterranean Sea and the Gulf of Elat. Appl. Environ. Microbiol. **38**:577–584.
44. **Shinoda, S., K. Itoh, Y. Hayashi, S. Miyoshi, Y. Yamasaki, M. Ikeda, T. Itoh, and T. Tsuchie.** 1985. Ecology of vibrios in estuarine region of Seto Inland Sea and distribution of some diarrhenogenic species. J. Hyg. Chem. **31**:220–226. (In Japanese.)
45. **Simidu, U., K. Kogure, and K. Tsukamoto.** 1987. Distribution of *Vibrionaceae* in the sea. Nihon Biseibutsu Seitai Gakkaiho **1**:65–74.

46. **Simidu, U., and K. Tsukamoto.** 1985. Habitat segregation and biochemical activities of marine members of the family *Vibrionaceae*. Appl. Environ. Microbiol. **50:**781–790.
47. **Simidu, U., K. Tsukamoto, and Y. Akagi.** 1982. Heterotrophic bacterial population in Bengal Bay and the South China Sea. Bull. Jpn. Soc. Sci. Fish. **48:**425–431.
48. **Smith, H. L., Jr., and P. B. Fernandes.** 1973. Modified decarboxylase-dihydrolase medium. Appl. Microbiol. **26:**620–621.
49. **Smith, L. D. S.** 1978. The occurrence of *Clostridium botulinum* and *Clostridium tetani* in the soil of the United States. Health Lab. Sci. **15:**74–80.
50. **Venkateswaran, K., and H. Hashimoto.** 1988. Influence of indicator bacteria on the incidence of *Salmonella* in aquatic environment. Nippon Suisan Gakkaishi **54:**253–258.
51. **Yanagi, T.** 1988. Preserving the inland sea. Mar. Pollut. Bull. **19:**51–53.
52. **Ward, B. Q., B. J. Carroll, E. S. Garrett, and G. B. Reese.** 1967. Survey of the U.S. gulf coast for the presence of *Clostridium botulinum*. Appl. Microbiol. **15:**629–636.
53. **West, P. A., P. R. Brayton, R. R. Twilley, T. N. Bryant, and R. R. Colwell.** 1985. Numerical taxonomy of nitrogen-fixing 'decarboxylase-negative' *Vibrio* species isolated from aquatic environment. Int. J. Syst. Bacteriol. **35:**198–205.
54. **West, P.A., and R. R. Colwell.** 1984. Identification and classification overview, p. 285–363. *In* R. R. Colwell (ed.), Vibrios in the environment. John Wiley & Sons, Inc., New York.