Microbial Ecology 4:135–155 (1978)

MICROBIAL ECOLOGY

The Annual Cycle of *Vibrio Parahaemolyticus* in Chesapeake Bay

*Tatsuo Kaneko and Rita R. Colwell

Department of Microbiology, University of Maryland, College Park, Maryland 20742

Abstract. An ecological study of *Vibrio parahaemolyticus* was carried out from December 1970 to December 1971 in the Rhode River of Chesapeake Bay. The annual cycle of the organism was elucidated and factors restricting its distribution in the estuary were described, including the association of the organism with zooplankton. Numerical taxonomy was employed for identification and classification of *V. parahaemolyticus* and related organisms on the basis of substrate utilization tests. From characteristics recorded for *V. parahaemolyticus*, it is concluded to be an estuarine organism.

Introduction

In the time since the suggestion was put forward that the pathogenic, halophilic bacterial species *Vibrio parahaemolyticus* was of marine origin, extensive studies on its isolation and distribution in rivers, coastal waters, and the open sea have been carried out by a number of investigators. Historically, the isolation and distribution of *V. parahaemolyticus* in the marine environment was first reported by Takikawa (35). The first major survey of the seasonal occurrence of *V. parahaemolyticus* in seawater and association with plankton in Tokyo and Sagami Bay was undertaken by Miyamoto (22). From the early 1960s on, a number of surveys of the incidence of this pathogenic halophilic bacterium in seawater and marine sediment and of its association with marine animals were carried out. Aiso (1) demonstrated an incidence of 20% of the aerobic, heterotrophic bacterial population to be *V. parahaemolyticus* in coastal seawater throughout the year. Zen-Yoji et al. (42), Sakai et al. (28), and Terayama (37) isolated *V. parahaemolyticus* during the summer months from plankton, as well as from seawater samples collected off Oshima Island near Tokyo Bay. Horie et al. (12) collected relatively large numbers of bacteria possessing characteristics very nearly identical to those of *V. parahaemolyticus* samples during the summer in Tokyo and Sagami Bay. Other workers (24,27) also reported a high incidence of the organism in coastal regions and estuaries in the summer.

Results of studies of the incidence of *V. parahaemolyticus* in the open sea

* Present address: Department of Biology, University of Louisville, Louisville, Kentucky 40208.

0095–3628/78/0004–0135 $04.20

are, however, contradictory. Yasunaga and Kuroda (41) reported isolating *V. parahaemolyticus* in the open sea in southeast Asia, but Horie et al. (11,13) were not able to show the occurrence of *V. parahaemolyticus* in seawater or associated with plankton collected from open ocean locations.

Yasunaga (40) reported the occurrence of *V. parahaemolyticus* in the supratidal zone of a city river and suggested that the river may have been the source of contamination of coastal water with *V. parahaemolyticus*, whereas Ose and Ikeda (26) suggested that the source of *V. parahaemolyticus* was raw sewage. Yet Horie et al. (14) could not detect *V. parahaemolyticus* in freshwater of the supratidal zone of a river, but did report comparatively large numbers (as many as 7×10^5/liter) at the mouth of a river and in coastal water during the months of July, August, and September.

V. parahaemolyticus has rarely been isolated from the marine environment during the winter months, especially during the colder months of the year. Asakawa et al. (5) and Noguchi and Asakawa (25) reported isolating the organism from scavenger fish and shellfish, but at very low levels, in Lake Hamana estuary during the winter. Wagatsuma et al. (38) described the isolation of Kanagawa phenomenon-positive strains of *V. parahaemolyticus* from sediment of an oyster bed in the winter. These observations suggest that *V. parahaemolyticus* can survive in sediment or associated with animals during the colder periods of the year, although the numbers of surviving strains are extremely low.

Of the problems associated with the isolation and distribution of *V. parahaemolyticus*, the most important are the efficiency of isolation of *V. parahaemolyticus* from the natural environment and the difficulties faced in identification and classification. To state the situation kindly, until recently, the identification and classification of *V. parahaemolyticus* were confused, with the taxonomic confusion persisting over a rather long period, i.e., at least 20 years. Hence, misidentification in the earlier published studies added to the generally conflicting nature of results reported for the many isolation and distribution studies.

There were several objectives established for the project reported here. It was intended that the annual cycle of *V. parahaemolyticus* in Chesapeake Bay be established and the natural habitat of the organism and its ecological role in nature be clarified. Finally, we wished to apply numerical taxonomy, already shown to be useful for identification and classification of microorganisms, in microbial ecology. Preliminary results were reported (16), and this article presents results of the most complete study of the ecology of *V. parahaemolyticus* to date (15).

Materials and Methods

Sampling

Sampling on a regular basis was carried out in the Rhode River of Chesapeake Bay from December 1970 through December 1971. The site of the study is shown in Fig. 1. The depth of the sampling area, a relatively shallow subestuary, is 2 to 3 m, on average, and the bottom sediment is composed of thick mud.

Water samples were collected at a depth of 50 cm, using an aseptic procedure and employing presterilized 250- to 500-ml glass bottles; sediment samples were collected with a core sampler fitted

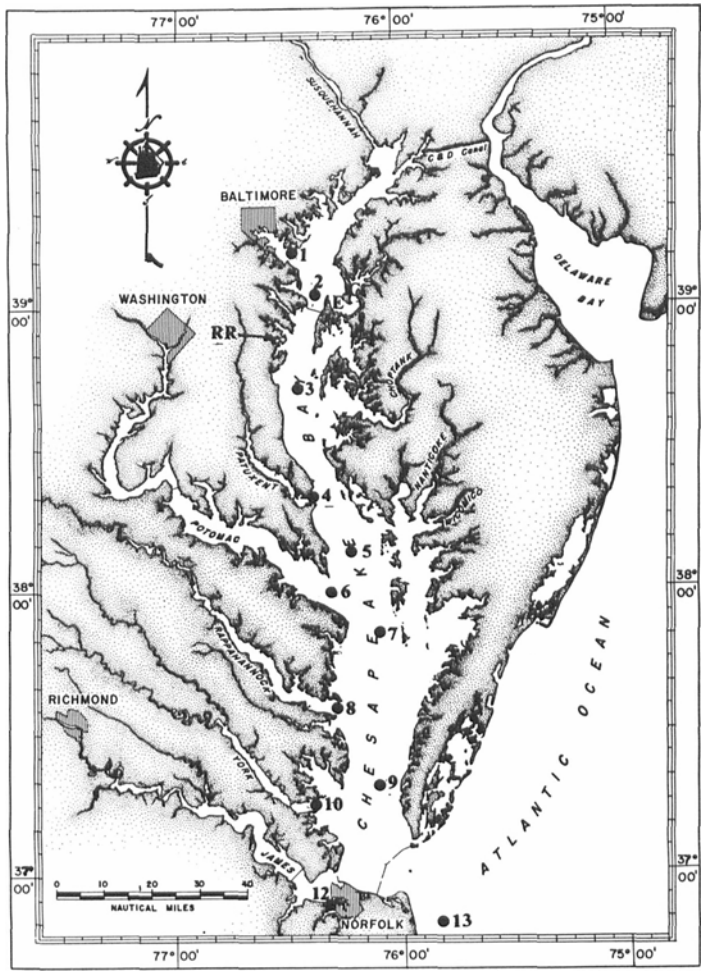

Fig. 1. Map of Chesapeake Bay showing the Rhode River area.

with presterilized core liners (Wildlife Supply Company, Saginaw, Michigan). A No. 20 plankton net (77-μm opening) was used to collect plankton samples. The plankton samples were transferred to presterilized wide-mouth tubes after collection.

Environmental parameters, i.e., water temperatures, salinity, dissolved oxygen, pH, and turbidity, were measured using, respectively, a reversing thermometer, a titration method for salinity (34), a YSI oxygen meter (Yellow Springs Instrument Co., Yellow Springs, Ohio), a portable Corning pH meter, Model 6 (Corning, New York), and a Secchi disk.

Bacteriological Methods

Water and plankton samples were separately homogenized before bacteriological analysis by blending for 10 min in a Sorvall Omnimixer. Plankton samples were homogenized in a four-salts solution consisting of 2.4% NaCl, 0.7% $MgSO_4 \cdot 7H_2O$, 0.07% KCl, and 0.53% $MgCl_2 \cdot 6H_2O$, pH 7.6 $\pm$ 0.2.

Sediment samples were weighed and diluted with sterile four-salts solution and mixed for 10 min with a magnetic stirrer.

For TVC (total, heterotrophic, aerobic, viable bacterial count), YE medium (1.0% Difco Proteose Peptone, 0.3% Difco Yeast Extract, 0.5% NaCl, pH 7.2 to 7.4) and SWYE medium (1.0% Difco Proteose Peptone, 0.3% Difco Yeast Extract, four-salts solution, pH 7.2 to 7.4) were employed. TVC was determined by the most probable number (MPN) method, after incubation of the inoculated media at 25°C for 48 hours.

Isolation of V. parahaemolyticus

TCBS medium (BBL, Division of Bioquest, Cockeysville, Maryland), which contains 0.5% Yeast Extract, 1.0% polypeptone peptone, 1.0% sodium citrate, 1.0% sodium thiosulfate, 0.5% oxgall, 0.3% sodium cholate, 2.0% sucrose, 1.0% sodium chloride, 0.1% iron citrate, 0.004% thymol blue, 0.004% bromothymol blue, and 1.4% agar, pH 8.6 ± 0.2, was used as a selective medium for *V. parahaemolyticus*.

SWYE broth MPN tubes showing growth were streaked onto TCBS agar plates, which were incubated at 37°C for 24 to 48 hours after inoculation. In this study, colonies appearing on TCBS agar plates were regarded as presumptive vibrios (PV), corresponding to the *Vibrio*-like organisms (VLO) described in an earlier paper (16), and typical green colonies on TCBS agar plates were regarded as presumptive *V. parahaemolyticus* (PVP), corresponding to *Vibrio parahaemolyticus*-like organisms (VPLO). The term "presumptive" is employed in this study with the same meaning as in the coliform test series, i.e., "presumptive" *Escherichia coli;* therefore, there is no direct taxonomic translation to genus or species.

Identification of *V. parahaemolyticus* was achieved using the following characteristics: gram negative; motile; good growth in media containing 3.0% and 7.0% sodium chloride; no growth or slight growth at 0 and 10% added sodium chloride; growth at 43°C; cytochrome oxidase positive; catalase positive; acid production in glucose, under aerobic and anaerobic conditions; acid from mannitol; no acid from sucrose and lactose; no H_2S produced in Triple Sugar Ion medium (Difco); Voges-Proskauer test negative; indole production positive; nitrate reduction positive; starch hydrolyzed under both aerobic and anaerobic conditions; gelatin liquefied; methyl red positive; lysine decarboxylase positive; beta-haemolysis on human blood agar; and nonbioluminescence. The completed identification was confirmed by numerical taxonomy of the isolates including representative reference strains of *V. parahaemolyticus*.

For antibiotic sensitivity tests, a total of 21 antibiotics (see Table 4) were employed and the sensitivity disc procedure was used (BBL, Cockeysville, Maryland). The tests were carried out using SWYE agar and 15 hour SWYE broth cultures for inocula.

Coliform tests were also done on the samples collected during the study. These involved inoculation of lactose broth (Difco), and incubation of the inoculated broth tubes at 37°C for 48 hours, and EC broth (Difco) for the EC test, incubated at 45.5 ± 0.5°C in a water bath (8).

Numerical Taxonomy

Numerical taxonomy was done on organisms classified as PVP including *V. parahaemolyticus*, and the set of strains analyzed also included reference strains (*vide infra*). Substrate utilization tests, essentially according to the procedures described by Stanier et al. (33), were done for each strain. The results were coded and the data analyzed by the methods of numerical taxonomy.

A total of 154 substrates (15) were employed in the study. The number and kind of substrates employed were not exactly as listed by Stanier et al. (33), with differences noted as follows. The basal medium employed was composed of 0.05% $NH_4H_2PO_4$, 0.05% K_2HPO_4, 1.5% refined Ion Agar (Difco), and four-salts solution. The pH was adjusted, using Tris buffer [Tris (hydroxymethyl) aminomethane], to 7.2. Buffer solutions were autoclaved separately, as was the agar–four-salts mixture, in order to avoid formation of precipitates. After autoclaving, the buffer solution and agar–four-salts solution were mixed and 154 organic compounds were added as individual substrate tests, as required.

Inoculation was done following the replica plating method (20), with the number of inocula sites

Table 1. Seasonal variations in the environmental parameters measured in the Rhode River

Date	Surface Water Temp. (°C)	Bottom Water Temp. (°C)	Surface Water Salinity (‰)	Surface Water Dissolved Oxygen (ppm)	Surface Water pH	Transparency (m)
12/11/70	5.5	5.5	10.8	12.7	8.3	0.75
2/17/71	−0.2	1.0	—[a]	—	—	—
3/ 3/71	6.0	5.0	5.8	12.4	7.2 (6.4)[b]	0.50
4/13/71	14.0	15.1	5.8	—	—	—
6/ 1/71	19.0	20.0	5.5	7.9	—	0.80
7/ 8/71	31.2	28.2	8.7	11.4	—	0.60
7/22/71	27.2	27.0	10.1	12.2	—	0.75
8/11/71	30.1	—	10.0	8.1	—	0.35
10/ 7/71	21.0	21.5	9.6	8.7	—	0.40
12/ 9/71	8.0	6.5	4.2	—	—	1.15

[a] No data.
[b] pH of the bottom water.

on each plate limited to a maximum of 16. A "patch" of each culture serving as inoculum was from a 24-hour MSWYE agar plate (0.1% Difco Proteose peptone, 0.1% Difco Yeast Extract, four-salts solution, 1.5% Bacto Agar, pH 7.4 ± 0.2). Incubation of the inoculated test media was at 25°C. All inoculated plates were observed and results recorded daily for 2 weeks. For chitin utilization, the following medium was used: 0.1% K_2HPO_4, 3.0% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$), 0.01% $CaCl_2 \cdot 2H_2O$, 0.1% NH_4Cl, and pulverized chitin, pH 7.0. Incubation was for 3 weeks at 25°C.

Results obtained in the substrate utilization tests were recorded as negative if the reaction was questionable or if it was too difficult to determine whether the small amount of growth observed represented utilization of the substrate.

The set of strains employed in the numerical taxonomy analyses included a total of 80 strains isolated from the Rhode River, i.e., organisms classified as PVP including *V. parahaemolyticus*. Reference strains included: *V. parahaemolyticus* SAK 3, 23, FC 1101, K 4 (Iida), Bainbridge 4203 and 10734; *V. ichthyodermis* NCMB 407; *V. marinopraesens* ATCC 19648; *V. aliginolyticus* ATCC 17749; *V. cholerae* ATCC 14035; *V. anguillarum* ATCC 14181; *V. marinofulvus* ATCC 14395; *V. ponticus* ATCC 14391; *V. haloplanktis* ATCC 14393; *V. marinagilis* ATCC 14398; *V. algosus* ATCC 14390; *Lucibacterium harveyi* ATCC 14126; *Photobacterium pierantonii* ATCC 14546; *Beneckea campbellii* ATCC 25920; *Beneckea neptuna* ATCC 25919; *Beneckea nereida* ATCC 25917; and *Beneckea pelagia* ATCC 25916.

The numerical analyses were done using the Jaccard (S_J) similarity coefficient (negative matches excluded) on an IBM 360/40 system with disc and tape drives located in the Computation Center, Georgetown University. Groups were formed by a single-linkage clustering method (31). The programs used were GTP-1, -2, -3, -4, and -5 (Georgetown Taxonomy Programs 1–5), written by R. D' Amico and R. R. Colwell.

Results

Environmental Parameters

Environmental parameters measured over the full annual cycle are given in Table 1. The minimum surface water temperature recorded was −2°C in February

1971, and the maximum, 31.2°C in July 1971. The surface of the water in the Rhode River was covered with ice about 1 inch thick in January and February 1971. After disappearance of the ice, the water temperature rose rapidly, reaching the maximum temperature of 31.2°C in July and remaining between 27°C and 32°C during the summer, and dropping to 8°C in December 1971. Since the Rhode River is relatively shallow, a significant difference in temperature between surface and bottom water was not observed.

The annual variation in salinity is also shown in Table 1. Relatively low salinities were recorded, i.e., < 6 ‰ during March through June. The salinity gradually increased during July to August, with a maximum of 10.1 ‰ recorded in July, thereafter decreasing to 4.2 ‰ in December 1971.

Other environmental parameters, viz., dissolved oxygen, pH, and transparency, are given in Table 1.

Bacteriological Results

Changes in the bacterial population in the water column occurring throughout the year are shown in Fig. 2. TVC of the water fluctuated between 10^4 and 10^6/100 ml throughout the year. Counts of PV, however, were influenced by seasonal factors. Minimum counts of 10^2 were obtained during the winter when the water temperature was below 6°C. The counts of PV gradually increased in the period from the middle of March onward, which was matched by an increase in the water temperature. Counts of 10^4 were obtained in July (when the water temperature was 31.2°C). The PV counts stayed between 10^4 and 10^5 until October, with counts of 3.5×10^3 recorded in December, when the water temperature was 8°C.

The PVP counts were below detectable levels in the water column from January to early April, until the water temperature rose to 14°C. However, a

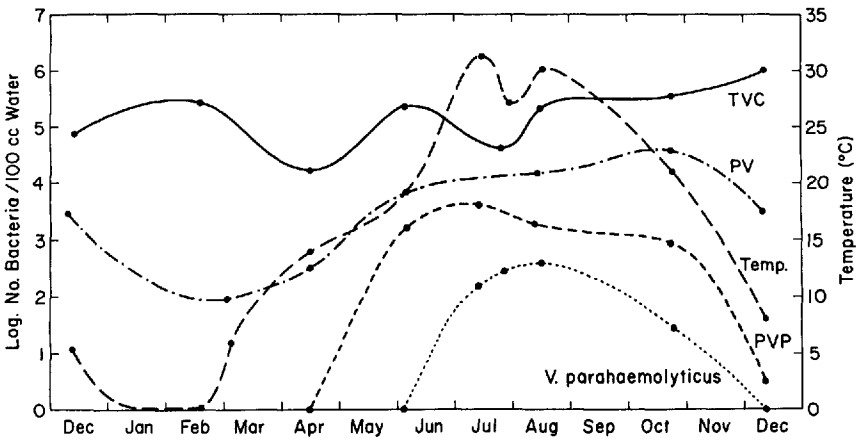

Fig. 2. Distribution, by season, of total viable, aerobic, heterotrophic bacteria (TVC), presumptive vibrios (PV), presumptive *Vibrio parahaemolyticus* (PVP), and *V. parahaemolyticus* in water samples (per 100 ml) collected in the Rhode River area (December 1970 to December 1971).

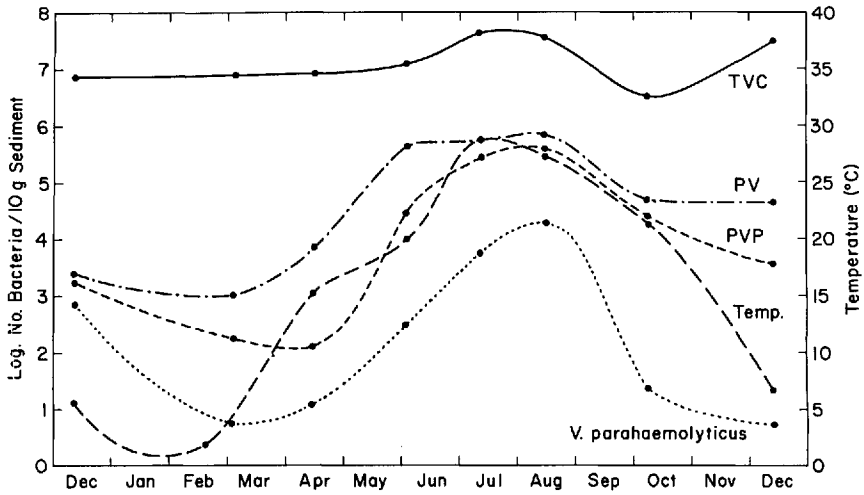

Fig. 3. Seasonal change in the population of total viable, aerobic, heterotrophic bacteria (TVC), presumptive vibrios (PV), presumptive *Vibrio parahaemolyticus (PVP)*, and *V. parahaemolyticus* in sediment samples (per 10 g) in the Rhode River area (December 1970 to December 1971).

rapid increase in the counts, from zero to 10^3, was observed in the period from April to early June, when the water temperature rose from 14°C to 19°C. Maximum counts were 6.2×10^3 in the middle of July, when the water temperature was also at a maximum of 31.2°C. The counts of PVP stayed at approximately 10^3 until October, then decreased rapidly to below 10 in December, when the water temperature was 8°C.

Although the PVP counts increased in April, the *V. parahaemolyticus* counts were below detectable levels until early June, when the water temperature was 19°C. However, the number of *V. parahaemolyticus* rapidly increased with increase in temperature to approximately 30°C. Counts of 10^2 were recorded in early July and a maximum count of 4×10^2 in August, after which the *V. parahaemolyticus* showed a decrease, reaching numbers of less than 10^2 in October and below a detectable level in December. Thus, *V. parahaemolyticus* was detectable in the water column from early summer to fall.

During the period when *V. parahaemolyticus* and PVP were isolated, luminescent PVP were also isolated from the water column samples. A fluctuation in the counts of the luminescent PVP was also observed (Fig. 5). Luminescent PVP were detected in the water column earlier than *V. parahaemolyticus* and reached maximum counts of 10^2, approximately the same population level as *V. parahaemolyticus,* in July. However, the counts of luminescent PVP decreased earlier than those of *V. parahaemolyticus,* the former finally disappearing from the water column in October.

Bacterial counts of sediment are shown in Fig. 3. The TVC of sediment samples held relatively constant, i.e., approximately 10^7/g throughout the year. Counts of PV in sediment samples, however, were found to be influenced by season. The lowest counts of PV, 10^3/10 g, were recorded during the winter, when the bottom temperature was below 5°C. Thereafter, the counts rose gradually, reaching 5×10^5/10 g in early June, when the bottom temperature was

20°C. Counts held at 10^5 to 10^6 during June through August, when the bottom temperature was 27°C. Thereafter, the counts gradually decreased to 10^4 to 10^5 in December when the bottom temperature was 6.5°C.

PVP counts during the winter were 10^2 to 10^3, after which the counts increased in April and very rapidly increased thereafter until August. During this time the sediment counts increased from 10^2 to 4×10^5/10 g maximum, after which the counts decreased, coupled with a decrease in the bottom temperature to 4×10^3/10 g in December.

Although *V. parahaemolyticus* counts were detectable in the sediment during the winter, the counts were extremely low (less than 10/10 g of sediment). Counts of *V. parahaemolyticus* increased in April and continuously thereafter until August, i.e., the *V. parahaemolyticus* counts rose from approximately 10 to 5.7×10^4/10 g during this period. After maximum counts were reached, the counts decreased rapidly with decrease in bottom temperature. The counts dropped from 10^4 to $< 10^2$ in October, and to less than 10 in December. Thus, *V. parahaemolyticus* was found to survive throughout the year, but winter conditions were observed to be detrimental, in terms of total viable counts of the organism.

Luminescent PVP were also detected in sediment at the same time that *V. parahaemolyticus* was isolated, as shown in Fig. 5. The changes in the counts of these organisms resembled those of *V. parahaemolyticus*, although the time period when luminescent PVP could be detected was limited to April through

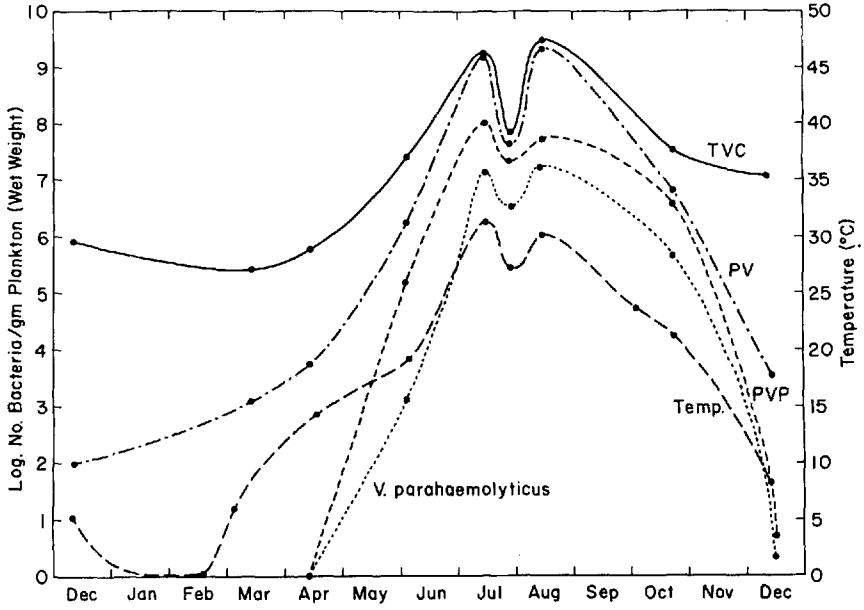

Fig. 4. Seasonal change in the population of total viable, aerobic, heterotrophic bacteria (TVC), presumptive vibrios (PV), presumptive *Vibrio parahaemolyticus* (PVP), and *V. parahaemolyticus* associated with plankton (per gram wet weight) in the Rhode River area (December 1970 to December 1971).

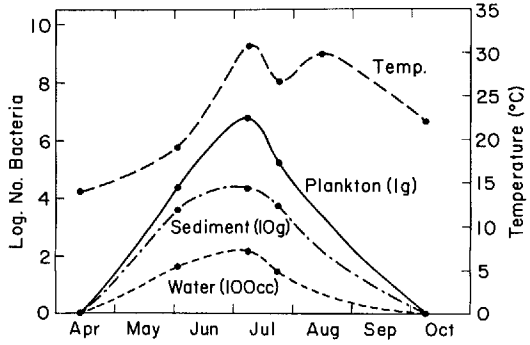

Fig. 5. Seasonal change in the luminescent PVP population in water (per 100 cc), sediment (per gram) and plankton (per gram wet weight) in the Rhode River area (April to October 1971).

October. *V. parahaemolyticus*, on the other hand, was present at detectable levels throughout the year.

Results of plankton analyses are shown in Fig. 4. Plankton collected in the Rhode River were predominantly adults or juveniles of the copepod *Acartia tonsa* throughout the year. Bacterial counts of plankton samples, expressed per gram wet weight, revealed a striking seasonal change, quite different from that observed for the water and sediment samples. The TVC of the plankton samples during the winter was between 10^5 and 10^6, increasing in April, when the water temperature was 14°C, then continuously increasing to a maximum of 3×10^9 in August, when the water temperature was 30.1°C, the increased numbers following closely the increase in water temperature. The TVC gradually dropped, along with the decrease in water temperature, indicating close ties between the bacteria and copepod biology.

PV associated with plankton were detectable during the winter, although counts were low, if counts of individual plankton are considered. The PV counts increased rapidly from April to June, when the water temperature rose from 14°C to 19°C, reaching 6×10^3 to 2×10^6. The numbers continuously increased to 2×10^9 in July, in parallel with the increase in water temperature, which rose to 31.2°C. PV were the dominant component of the plankton TVC during the summer months, after which PV rapidly decreased to 10^4 in December.

PVP counts for plankton were below detectable levels from winter to early spring, when the water temperature rose to 14°C. However, PVP increased rapidly, from zero to $> 10^3$, when the water temperature reached 19°C to 20°C, and were approximately 10^7 during the summer. Thereafter, the PVP gradually decreased after the water temperature reached 22°C to 23°C, to counts of less than 10 in December.

The change in the *V. parahaemolyticus* counts followed the same pattern as that of the PVP; that is, *V. parahaemolyticus* were below detectable levels during the winter months, rapidly increasing in number as the temperature increased. A maximum for *V. parahaemolyticus* was recorded during the summer months, and thereafter there was a gradual decrease until October, at which point the decrease was rapid, to barely detectable levels in December.

Luminescent PVP were also detected in plankton samples, as shown in Fig. 5. Although the pattern of change in the luminescent PVP population resembled

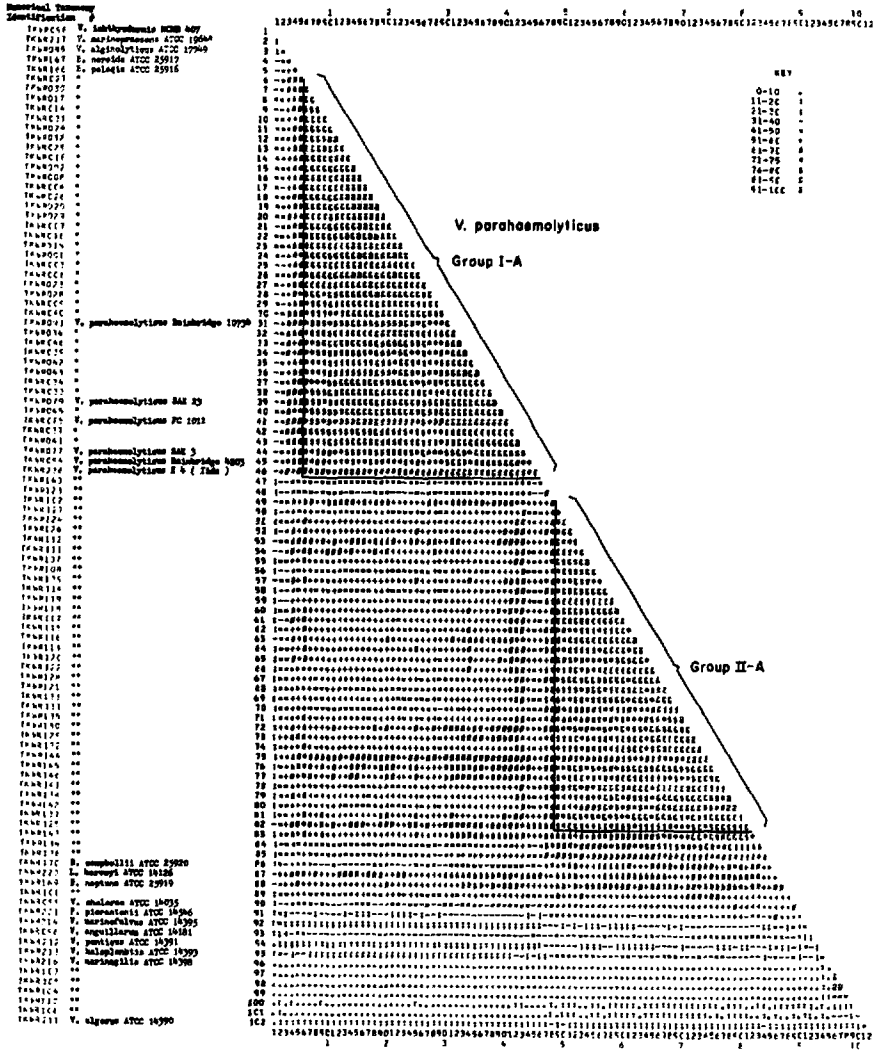

Fig. 6. Numerical taxonomy analysis of Chesapeake Bay isolates. * = *V. parahaemolyticus;* ** = luminescent strains.

that of *V. parahaemolyticus*, these organisms disappeared from the plankton earlier than *V. parahaemolyticus*.

Numerical Taxonomy

The results of the numerical taxonomy analyses, based on substrate utilization data, are shown in Fig. 6. Two main clusters (groups I-A and II-A) were observed. Group I-A consisted of *V. parahaemolyticus* strains, including the six

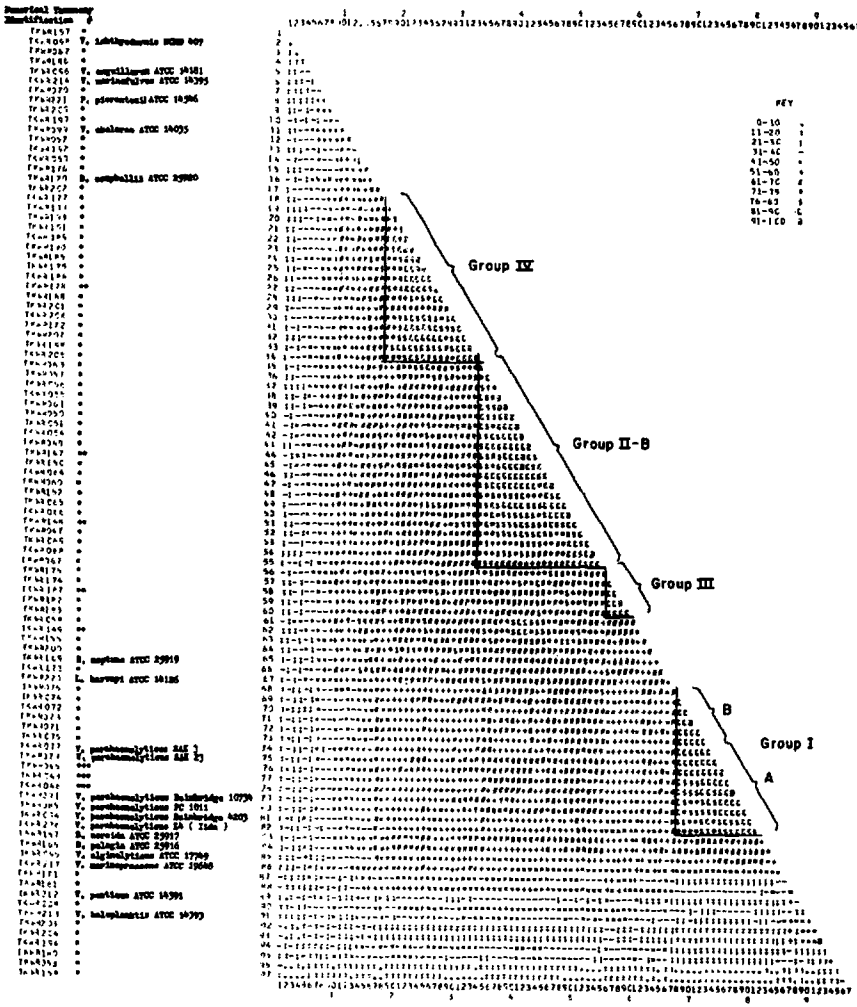

Fig. 6 (Continued)

reference strains of *V. parahaemolyticus* and 35 Chesapeake Bay strains isolated during the present study. Group I-A was easily differentiated from Group II-A, which was composed of 34 strains. Not all of the PVP strains were included in group I-A or group II-A. Eight bioluminescent strains did not fall into any group, and two bioluminescent strains formed a small, separate group, including *V. marinagilis* strain ATCC 14398 (see Fig. 6).

In Table 2 are given feature frequency data for those tests employed by most investigators for identification of *V. parahaemolyticus*.

Table 3 lists feature frequencies for substrate utilization tests for those organisms comprising groups I-A and II-A, with uniformly negative results excluded.

In Table 4, feature frequencies for antibiotic sensitivity tests for 21 antibiotics are listed.

Table 2. Computed frequencies for those characteristics most commonly used in the identification of *V. parahaemolyticus* for the two clusters obtained in this analysis and for the *V. parahaemolyticus* reference strains

Feature	Reference Strains[a]	Group I-A	Group II-A
Gram negative	1.00	1.00	1.00
Motility positive	1.00	1.00	1.00
Cytochrome oxidase	1.00	1.00	1.00
Catalase	1.00	1.00	1.00
Growth at 5°C	—[b]	0.81	0.10
Growth at 10°C	1.00	1.00	1.00
Growth at 25°C	1.00	1.00	1.00
Growth at 37°C	1.00	1.00	1.00
Growth at 43°C	1.00	1.00	1.00
Growth in peptone water of 0% NaCl	0.00	0.00	0.00
Growth in peptone water of 3% NaCl	1.00	1.00	1.00
Growth in peptone water of 7% NaCl	1.00	1.00	0.00
Growth in peptone water of 10% NaCl	0.13	0.59	0.00
Urease	0.25	0.31	0.14
Indole	1.00	1.00	1.00
Methyl red	1.00	1.00	1.00
Voges-Proskauer	0.00	0.00	0.00
Citrate utilization	1.00	0.94	1.00
Malonate utilization	0.00	0.00	0.00
Phenylalanine deaminase	0.00	0.28	0.62
Nitrate reduction	1.00	1.00	1.00
Gelatin liquefaction	1.00	1.00	1.00
Starch hydrolysis	1.00	1.00	1.00
β-Haemolysis	1.00	1.00	0.90
Lysine decarboxylase	1.00	1.00	1.00
Arginine dehydrolase	0.00	0.00	0.00
Ornithine decarboxylase	0.88	0.88	0.38
Gas from glucose	0.00	0.00	0.00
Acid from glucose	1.00	1.00	1.00
Acid from adonitol	0.00	0.00	0.00
Acid from cellobiose	0.00	0.12	1.00
Acid from galactose	0.50	0.67	0.95
Acid from inositol	0.00	0.00	0.00
Acid from lactose	0.00	0.00	0.00
Acid from maltose	1.00	1.00	1.00
Acid from mannitol	1.00	1.00	0.10
Acid from rhamnose	0.00	0.00	0.00
Acid from ribose	1.00	1.00	1.00
Acid from salicin	0.00	0.00	0.00
Acid from sorbitol	0.00	0.00	0.00
Acid from sucrose	0.00	0.00	0.00
Acid from trehalose	1.00	0.95	1.00
Acid from xylose	0.00	0.00	0.00
NH_3 from peptone	1.00	1.00	1.00
Bioluminescence	0.00	0.00	1.00

[a] Eight reference strains.
[b] Not tested.

Table 3. Frequencies of occurrence of selected substrate utilization features of groups I-A and II-A strains

	Group I-A	Group II-A
L-Arabinose	1.00	0.00
D-Ribose	0.91	1.00
D-Fructose	0.97	0.48
D-Galactose	1.00	1.00
D-Glucose	1.00	1.00
D-Mannose	1.00	0.95
D-Melibiose	0.09	0.33
Lactose	0.19	0.81
Trehalose	1.00	0.52
Maltose	1.00	0.90
Cellobiose	0.91	1.00
Salicin	0.09	0.00
Acetate	1.00	1.00
Oleate	0.59	0.00
Laurate	0.97	0.00
DL-Malate	0.78	0.00
Citrate	0.94	1.00
α-Ketoglutarate	0.97	1.00
Gluconate	1.00	0.95
Mannitol	1.00	0.19
Dulcitol	0.06	0.00
Adonitol	0.03	0.00
Phenol	0.06	0.00
Phenylacetate	0.22	0.00
N-Acetylglucosamine	1.00	0.48
Protamine sulfate	1.00	1.00
D-Mannosamine	1.00	0.00
Glycine	0.78	0.00
L-Alanine	1.00	0.67
L-Serine	0.78	0.00
L-Threonine	0.97	1.00
L-Leucine	1.00	0.30
Isoleucine	0.03	0.00
L-Glutamate	0.13	0.00
L-Lysine	1.00	1.00
L-Arginine	0.63	0.14
L-Ornithine	0.97	0.95
Cystine	0.84	0.05
Hydroxyproline	1.00	0.00
L-Tyrosine	0.13	0.14
L-Histidine	0.13	0.00
L-Tryptophan	0.03	0.00
L-Phenylalanine	0.22	0.10
Adenine	0.88	0.00
Naphthalene	0.44	0.00
Hexane	0.38	0.00
Starch	1.00	1.00
Glycogen	1.00	0.95
Chitin	1.00	0.00
Pectin	0.75	0.00

Table 3 *(Continued)*

	Group I-A	Group II-A
Carrageenan	0.19	0.00
Casein	0.96	1.00
Collagen	1.00	1.00
Tween 20	1.00	1.00
Tween 40	1.00	1.00
Tween 60	1.00	1.00
Tween 80	1.00	0.95

Discussion

Identification and Classification of V. parahaemolyticus

Although many papers have been published concerning the ecology of *V. parahaemolyticus*, most of the data deal with incidence or distribution of this organism in a given area over a very limited time period. Data in the literature are generally not parallel in time or geographical location so that it is almost impossible to sort out the ecology of *V. parahaemolyticus* from the literature alone. Identification and classification of *V. parahaemolyticus* have changed from a broad, very nearly all-inclusive definition to a much narrower one as a result of the excellent contributions of Sakazaki (29) and others. Therefore, papers published before 1967 show confusion, with some misidentifications. Even at the present time, if insufficient descriptive tests are applied, errors will arise, particularly in cases where the focus of the work is on the ecology of *V. parahaemolyticus*. Furthermore, misidentification of marine bacteria phenetically similar or closely related to *V. parahaemolyticus* also creates problems.

In this study numerical taxonomy was applied and the definition of *V. parahaemolyticus* was extended. Two main groups, shown in Fig. 6, were observed: groups I-A and II-A. Group I-A included *V. parahaemolyticus* strains, including six reference strains, and group II-A consisted of the luminescent PVP strains. Most of the characteristics identifying *V. parahaemolyticus* were shared by strains of group II-A, with some exceptions (Table 2). When substrate utilization data were included, some clear differences between the two groups were noted (Table 3). Group I-A (intrasimilarity value, 82.4%) and group II-A (intrasimilarity value, 80.8%) yielded an intersimilarity value of 55.1%, based on substrate utilization data (15). Organisms of group I-A were capable of utilizing a wider array of substrates (25.1%) than those of group II-A (15.8%). Other differences noted were antibiotic sensitivity (Table 4), with organisms of group II-A found to be sensitive to a wider selection of antibiotics.

The range of DNA base composition for selected organisms of groups I-A and II-A was found to be 46 to 47% (15). DNA homology of a strain from each of groups I-A and II-A with a reference strain of *V. parahaemolyticus* was found to be 90.6% and 26.4%, respectively (32). Obviously, much more work is needed, but these preliminary data suggest that groups I-A and II-A are different,

although sharing phenetic characteristics. Organisms of group II-A yielded relatively lower similarity values with *L. harveyi* strain ATCC 14126 and *P. pierantonii* strain ATCC 14546.

A study of the Atlantic coastal waters off Georgia and South Carolina (17) yielded many PVP organisms, but these were not related to group II-A (15). Strains possessing many of the generally accepted identifying characteristics of *V. parahaemolyticus* were isolated, but were found not be be *V. parahaemolyticus* on further analysis, a situation noted above (15). That is, when a wider array of substrates was employed in characterizing those organisms presumptively identified as PVP, clear differences in intergroup similarity values with *V. parahaemolyticus* were obtained by numerical taxonomy (17).

Thus, the numerical taxonomy results of substrate utilization data analyses for strains presumptively identified as *V. parahaemolyticus* proved very helpful in the classification and confirmation of identification of strains seemingly related to *V. parahaemolyticus*.

Annual Cycle of V. parahaemolyticus

Preliminary data showing seasonal changes in the populations of *V. parahaemolyticus* in water and sediment and its association with plankton were described in

Table 4. Frequency of occurrence of antibiotic sensitivity in the bacterial strains examined in this study

Antibiotic		Reference strains[a]	Group I-A	Group II-A
Chloramphenicol	30 μg	1.00	1.00	1.00
Furacin	100 μg	1.00	1.00	1.00
Furoxone	100 μg	1.00	1.00	1.00
Neomycin	30 μg	1.00	1.00	0.90
Erythromycin	15 μg	0.83	0.92	1.00
Novobiocin	30 μg	1.00	0.81	1.00
Kantrex	30 μg	1.00	0.85	0.95
Streptomycin	10 μg	1.00	0.88	0.57
Dehydroxystreptomycin	10 μg	1.00	0.88	0.86
Polymixcin B	30 units	1.00	0.58	0.00
Colimycin	10 μg	0.50	0.42	0.00
Terramycin	30 μg	0.50	0.31	0.90
Sulfacetamide	1.0 mg	0.50	0.23	0.95
Elkosin	1.0 mg	0.17	0.19	0.90
Aureomycin	30 μg	0.17	0.12	1.00
Penicillin	10 units	0.17	0.08	1.00
Madribon	1.0 mg	0.17	0.08	0.95
Gantrisin	2.0 mg	0.00	0.08	0.90
Thiosulfil	1.0 mg	0.33	0.08	0.86
Doxycycline	5 μg	0.00	0.00	0.00
Oxacillin	1 μg	0.00	0.00	0.00

[a] Six reference strains of *V. parahaemolyticus* were examined.

an earlier publication (16). It is generally accepted that the distribution of *V. parahaemolyticus* is restricted by low temperatures, and some workers speculated that *V. parahaemolyticus* disappears during the winter months, reappearing in late spring or summer. In this study *V. parahaemolyticus* was not detected in either the water column or associated with plankton during January through March 1971, when the water temperature varied between subzero and 6°C. However, the organism was detected in sediment during the same period, although the numbers of the organism were extremely low (Fig. 3). Most probably, *V. parahaemolyticus* surviving the winter were released into the water column to become attached to plankton, thereupon proliferating after initiation of the association with plankton. From April to early June, the sediment in the Rhode River area is well mixed with the water column by the wind, as well as by water movement resulting from runoff from the land, since the Rhode River is a relatively shallow site. Once the microorganisms are attached to plankton, which undergo vertical movement, they can proliferate on the new substrata provided by the plankton. In this study the number of *V. parahaemolyticus* on the plankton remained below detectable levels and did not show great proliferation from the time the microorganism was detected in the sediment until it could be found in the water column. Adsorption of *V. parahaemolyticus* onto plankton or chitin materials occurs with higher efficiency under conditions of low salinity (18), and in the Rhode River, salinities are low when absorption of *V. parahaemolyticus* onto the plankton occurs.

When the water temperature rose to 19°C, *V. parahaemolyticus* was easily detected in the water column. At this point, release of *V. parahaemolyticus* resulting from growth on plankton probably occurs, along with continued release of *V. parahaemolyticus* from sediment. During June and July, the first peak in bacterial counts, including counts of *V. parahaemolyticus* on plankton, was observed. The maximum counts for *V. parahaemolyticus* associated with plankton were between 10^7 and 10^8/g wet weight at this time, and the maximum numbers of *V. parahaemolyticus* occurred in the summer months for plankton, water column, and sediment samples. During this time, bacteria, including *V. parahaemolyticus,* associated with the proliferating plankton population, are involved in mineralization of the plankton, eventually breaking down and disintegrating the plankton subsequent to the plankton bloom. Mechanical action via water movement very likely assists in the release of bacteria into the water column, where the released bacteria thereby are capable of reattachment to other, intact plankton. Some of the bacteria would be expected to be brought back to the bottom via attachment to fragments of plankton returned to the bottom by sedimentation. The latter is significant since increases in bacterial counts of PVP and/or *V. parahaemolyticus* in the sediment need not necessarily reflect only growth of those bacteria in sediment. Although few *V. parahaemolyticus* were detected in plankton samples collected in December, and no *V. parahaemolyticus* in the water column, it was possible to isolate *V. parahaemolyticus* from sediment.

Seasonal changes occurring in numbers of bioluminescent PVP organisms in water, sediment, and plankton, similar to those of *V. parahaemolyticus,* were observed. The association of bioluminescent organisms with plankton was unequivocally demonstrated, as was the fact that the bioluminescent bacterial population peaked in the summer months.

Distribution of V. parahaemolyticus

It is generally accepted that the distribution of *V. parahaemolyticus* is restricted to the coastal regions, especially at the mouth of rivers. The occurrence of *V. parahaemolyticus* in areas between the ocean and where rivers empty into the ocean suggests that the origin or source of *V. parahaemolyticus* is terrestrial. However, field surveys at the supratidal zones of rivers contradict such a hypothesis (14). Some workers reported the incidence of *V. parahaemolyticus* in the middle of the Pacific Ocean and in the Indian Ocean (3,4,41). However, these results might be ascribed to misidentification of *V. parahaemolyticus* because of the superficial resemblances between marine vibrios and *V. parahaemolyticus* (*vide supra*).

In a study of the distribution of *V. parahaemolyticus* in the open sea, *V. parahaemolyticus* (group I-A) and organisms belonging to group II-A were not isolated from any of the water column, plankton, or sediment samples collected from the South Carolina and Georgia coasts during the summer months of 1971, when surface water temperatures were approximately 30°C, although other PVP type organisms were isolated (17).

V. parahaemolyticus utilizes a wider variety of substrates than other marine bacterial strains examined in this study, viz., PVP organisms (15), and is found in Chesapeake Bay where the total organic content of the water is higher than that of the open sea (7). It is uncertain whether the total dissolved organic content of either estuarine or open seawater is high enough to support growth of heterotrophic bacteria, although salinity effects are well known. Particulate organic matter, such as plankton and planktonic detritus, can carry larger numbers of organisms, but bacterial populations associated with plankton were not significantly different for the estuarine and open sea samples, despite the large differences in the total numbers of bacteria in estuarine water compared with open seawater (15). Nutritional differences between estuarine and open ocean waters, alone, do not appear to restrict the distribution of *V. parahaemolyticus*. Sewage and industrial drainage have been reported by several workers to affect the incidence of *V. parahaemolyticus*, with correlations with incidence of *E. coli* suggested (26,27). However, no discernible correlation between *E. coli* and *V. parahaemolyticus* counts was noted in the Rhode River (16).

There is no doubt that temperatures below 10°C are detrimental to the growth of *V. parahaemolyticus*, as observed by Tenmei and Yanagisawa (36). However, some strains of *V. parahaemolyticus* may grow at 5°C under laboratory conditions, after very long periods of incubation (see Table 2). The number of *V. parahaemolyticus* in samples collected during the winter was at barely detectable levels, except in the case of sediment samples. Nevertheless, even in the sediment samples, the number of *V. parahaemolyticus* was extremely low during the winter (Fig. 3). Divalent ions, such as Ca^{2+} and Mg^{2+}, may act to protect *V. parahaemolyticus* against effects of low temperatures, i.e., < 5°C, as has been found for *E. coli* (30). Thus divalent ions and nutrients in sediment may protect *V. parahaemolyticus*, permitting survival at low temperatures.

V. parahaemolyticus has been isolated from marine animals during the winter (5,25). *V. parahaemolyticus* was found in oysters (*Crassostrea gigas*) and soft-shell clams (*Mya arenaria*) in Chesapeake Bay during the winter, but no general trend was noted and the numbers were extremely low (15). Benthic organisms

may harbor *V. parahaemolyticus* during periods of low temperature, picking up the bacteria from the sediment in which they live.

V. parahaemolyticus cannot survive over extended periods in freshwater, and, in fact, has been shown to die off rapidly in distilled water (21). If river water becomes heavily polluted, such as river runoff from large cities, *V. parahaemolytics* may be found as far up a river as the supratidal zone. At the mouths of rivers or in zones below the supratidal regions, conditions favor growth of *V. parahaemolyticus*. Horie et al. (14) observed significantly high counts of *V. parahaemolyticus*, i.e., 1.5×10^5/liter at sites where the salinity was as low as 5 $^0/_{00}$. Therefore, it is clear that low salinity, *per se*, will not necessarily be detrimental to *V. parahaemolyticus* but may favor growth and survival if the water is rich in organic matter.

V. parahaemolyticus will grow under laboratory conditions in media with 3% NaCl or in seawater media at 37°C; in fact, in its earliest isolations, *V. parahaemolyticus* was referred to by Japanese workers as the "so-called halophilic pathogenic bacteria." However, it is also known that the optimum concentration of NaCl for growth of *V. parahaemolyticus* is affected by temperature, as has been shown for *V. marinus* (23). Aiso et al. (2) found that *V. parahaemolyticus* grew at NaCl concentrations between 0.1 and 7%, with an optimum concentration of 0.5 to 1.0% at 20°C, 3% at 37°C, and no growth at 0.05% NaCl. If these observations are extrapolated to the situation in the natural environment where, for example, the surface water temperatures in Chesapeake Bay or off the Georgia and South Carolina coasts during the summer months have been recorded at approximately 30°C, the optimum concentration of NaCl for growth of *V. parahaemolyticus* would be 1 to 3% NaCl. Salinity also affects the adsorption of *V. parahaemolyticus* onto substrata (18).

About 80% of the TVC in the water column was found to be derived from plankton. Only 20% of the TVC were free cells in the water column, the rest being attached to plankton or particulates (16). A seasonal change in the populations of *V. parahaemolyticus* associated with zooplankton was evident, both quantitatively and qualitatively (see Fig. 5 and Table 4). In Chesapeake Bay, copepods comprise a majority of the zooplankton population (10), with *Acartia tonsa* the most abundant species (9). The copepod population is highest during the warmer months and lowest during the colder months, with the summer population consisting mainly of immature stages or juveniles and the winter population mainly adults (9). A striking drop in the population of adult copepods is observed from spring to summer (10), with the decrease in population of adult copepods ascribed to predation by animals of higher tropic levels. However, direct decomposition by microbial action should also be considered, as in direct decomposition of marsh grass by microbial action (7). The direct cause of death may, or may not, be microbially mediated. However, if copepods are weakened or moribund as a result of molting, microorganisms associated with plankton may prove harmful even though they may not be truly pathogenic. Most probably the exoskeleton of adult copepods is mineralized via microbial action. Clearly, the association of bacteria with the copepods is significant, both ecologically and in terms of the biological associations.

The nutritional value of the copepod includes about 70 to 80% of the nitrogen as amino acid nitrogen and a variety of lipid and phosphorous compounds, as

well as other nutrients (6). *V. parahaemolyticus* can utilize a relatively large variety of substrates (see Table 3), so plankton would serve as an adequate source of food and energy. *V parahaemolyticus* is capable of utilizing chitin (39), hence, the decomposition of copepods by microbial action should be important in the recycling of chitin, with chitinoclastic organisms, such as *V. parahaemolyticus,* playing a significant ecological role as pioneers in the initial colonization of copepods during the spring and summer months, eventually effecting the decomposition of the copepods. The organic matter of copepods is not utilized solely by the chitinase-producing organisms. Group II-A organisms, also associated with the copepods, do not possess a chitinase, but can utilize *N*-acetyl-glucosamine, a derivative of chitin, as well as other organic compounds (see Table 3). Nonchitinase-producing organisms, thus, may also be important in the complete decomposition or mineralization of copepods.

There is no clear and unequivocal evidence showing that organisms associated with copepods are, or are not, pathogenic for copepods, and the association of bacteria examined in this study was predominantly external to the copepod (16). Metabolites produced on the exoskeleton may be important in the bacteria-plankton relationship (43). From the evidence gathered to date, the association of *V. parahaemolyticus* and related vibrios with the copepod is considered to be ecologically significant, particularly in recycling of organic matter of which those planktonic elements are composed.

Acknowledgments This work was supported by a grant from the National Science Foundation (NSF Grant No. GB-35261X) and by Sea Grant Project No. 04-3-158-7 funded by the Sea Grant Program, National Oceanic and Atmospheric Administration. Facilities were made available for this work at the Smithsonian Institution Environmental and Estuarine Studies Center for the Rhode River work, for which we gratefully acknowledge the cooperation of Dr. Francis Williamson, Director, and the Rhode River Program Participants (funded through Chesapeake Research Consortium RANN Grant No. GI-38973).

References

1. Aiso, K.: Survey of food poisoning outbreaks caused by pathogenic halophilic bacteria along the Chiba Prefecture coast. Shokuhin Eisei Kenkyu **10**, 21–25 (1960) (in Japanese)
2. Aiso, K., U. Shimidu, H. Katoh, K. Tatsumi, F. Sawada, and S. Katoh: *Pseudomonas enteritis* and related bacteria isolated from sea water in the Pacific coast area. Ann. Rept. Inst. Food. Micro., Chiba Univ. **15**, 12–20 (1963) (in Japanese)
3. Aoki, K.: Survey of the distribution of *Vibrio parahaemolyticus* in the Southwest Asia Sea. In: T. Fujino and H. Fukumi (Eds.): *Vibrio parahaemolyticus,* Ed. 2, pp. 329–331. Nayashoten, Tokyo, Japan (1967) (in Japanese)
4. Aoki, K.: A survey of the distribution of *Vibrio parahaemolyticus* in the Middle Pacific Ocean. In: T. Fujino and H. Fukumi (Eds.): *Vibrio parahemolyticus,* Ed. 2, pp. 325–327. Nayashoten, Tokyo, Japan (1967) (in Japanese)
5. Asakawa, Y., S. Akabane, and M. Noguchi: Studies of *Vibrio parahaemolyticus*. 5. Distribution of the organism in Lake Hamana and overwintering experiments. Jap. J. Pub. Health **13**, 158 (1966)
6. Corner, E. D. S., and C. B. Cowey: Biochemical studies on the production of marine zooplankton. Biol. Rev. **43**, 393–426 (1968)
7. Darnell, R. M.: Organic detritus in relation to the estuarine ecosystem. In: G. H. Lauff (Ed.):

Estuaries, pp. 376–382. American Association for the Advancement of Science, Publication No. 82, Washington, D.C. (1967)
8. Fishbein, M.: The aerogenic response of *Escherichia coli* and strains of *Aerobacter* in EC broth and selected sugar broths at elevated temperatures. Appl. Microbiol. **10**, 79–85 (1962)
9. Heinle, D. R.: Production of a calanoid copepod, *Acartia tonsa*, in the Patuxent River estuary. Chesapeake Sci. **7**, 59–75 (1966)
10. Herman, S. S., J. A. Mihursky, and A. J. McErlean: Zooplankton and environmental characteristics of the Patuxent River estuary 1963–1965. Chesapeake Sci. **9**, 67–82 (1968)
11. Horie, S., K. Saheki, T. Kozima, and Y. Sekine: Oceanographic survey on the distribution of Takikawa's so-called pathogenic halophilic bacteria. Bull. Jpn. Soc. Sci. Fish. **29**, 37–43 (1963) (in Japanese)
12. Horie, S., K. Saheki, M. Nara, T. Kozima, Y. Sekine, and T. Takayanagi: Distribution of Takikawa's so-called pathogenic halophilic bacteria in the coastal sea area. Bull. Jpn. Soc. Sci. Fish. **29**, 785–793 (1963) (in Japanese)
13. Horie, S., K. Sakaki, T. Kozima, M. Nara, and Y. Sekine: Distribution of *Vibrio parahaemolyticus* in plankton and fish in the open sea. Bull. Jpn. Soc. Sci. Fish. **30**, 786–791 (1964) (in Japanese)
14. Horie, S., K. Sakeki, and T. Okuzami: Quantitative enumeration of *Vibrio parahaemolyticus* in sea and estuarine waters. Bull. Jpn. Soc. Sci. Fish. **33**, 126–130 (1967) (in Japanese)
15. Kaneko, T.: Ecology of *Vibrio parahaemolyticus* and related organisms in Chesapeake Bay. Ph.D. thesis, Georgetown University, Washington, D.C. (1973)
16. Kaneko, T., and R. R. Colwell: Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. J. Bacteriol. **113**, 24–32 (1973)
17. Kaneko, T., and R. R. Colwell: Distribution of *Vibrio parahaemolyticus* and related organisms in the Atlantic Ocean off South Carolina and Georgia. Appl. Microbiol. **28**, 1009–1017 (1974)
18. Kaneko, T., and R. R. Colwell: Adsorption of *Vibrio parahaemolyticus* onto chitin and copepods. Appl. Microbiol. **29**, 269–274 (1975)
19. Kaneko, T., and R. R. Colwell: Incidence of *Vibrio parahaemolyticus* in Chesapeake Bay. Appl. Microbiol. **30**, 251–257 (1975)
20. Lederberg, J., and E. M. Lederberg: Replica plating and indirect selection of bacterial mutants. J. Bacteriol. **63**, 399–406 (1952)
21. Lee, J. S.: Inactivation of *Vibrio parahaemolyticus* in distilled water. Appl. Microbiol. **33**, 166–167 (1972)
22. Miyamoto, Y.: Oceanographic survey and halophilic bacteria. Shokuhin Eisei Kenhyu. **119**, 23–30 (1960) (in Japanese)
23. Morita, R. Y., and R. D. Haight: Temperature effects on the growth of an obligate psychophilic marine bacterium. Limnol. Oceanogr. **9**, 103–106 (1964)
24. Nishio, T.: An ecological study of *Vibrio parahaemolyticus*. 3. A comparison of the K-antigens and haemolysis of strains isolated from the marine environment and from patients. Jpn. J. Bacteriol. **22**, 447 (1967) (in Japanese)
25. Noguchi, M., and Y. Asakawa: Distribution of *Vibrio parahaemolyticus*. In: T. Fujino and H. Fukumi (Eds.): *Vibrio parahaemolyticus*, Ed. 2, pp. 313–323. Nayashoten, Tokyo, Japan (1967) (in Japanese)
26. Ose, Y., and T. Ikeda: Studies on *Vibrio parahaemolyticus* from night soil. Isolation of *V. parahaemolyticus* from night soil. Shukuhin Eiseigaku Zasshi. **5**, 206–210 (1964) (in Japanese)
27. Oshiro, T.: The distribution of Vibrio parahaemolyticus in nature. In: T. Fujino and H. Fukumi (Eds.): *Vibrio parahaemolyticus*, Ed. 1, pp. 263–277. Isseido, Tokyo, Japan (1964) (in Japanese)
28. Sakai, S., T. Terayama, Y. Kudoh, and H. Zen-Yoji: A study of the pathogenic halophilic bacteria. VI. A survey of the marine and terrestrial environment. Jpn. J. Bacteriol. **18**, 253 (1963) (in Japanese)
29. Sakazaki, R.: *Vibrio parahaemolyticus* and related organisms—a taxonomic study. In: T. Fujino and H. Fukumi (Eds.): *Vibrio parahaemolyticus*, Ed. 2, pp. 83–115. Nayashoten, Tokyo, Japan (1967) (in Japanese)
30. Sato, M., and H. Takahashi: Cold shock of bacteria. II. Magnesium-mediated recovery from cold shock and existence of two critical temperature zones in various bacteria. J. Gen. Appl. Microbiol. **15**, 217–229 (1969)

31. Sokal, R. R., and P. H. A. Sneath: Principles of Numerical Taxonomy. W. H. Freeman and Co., San Francisco (1963)
32. Staley, T. E., and R. R. Colwell: Polynucleotide sequence relationship among Japanese and American strains of *Vibrio parahaemolyticus*. J. Bacteriol. **114**, 916–927 (1973)
33. Stanier, R. Y., N. J. Palleroni, and M. Doudoroff: The aerobic pseudomonads: A taxonomic study. J. Gen. Microbiol. **43**, 159–271 (1966)
34. Strickland, J. D. H., and T. R. Parsons: A Manual of Sea Water Analysis, Ed. 2. Fisheries Research Board of Canada, Ottawa, Canada (1965)
35. Takikawa, I.: Certain halophilic bacteria isolated from food poisoning patients. Jpn. J. Infect. Dis. **30**, 439–500 (1956) (in Japanese)
36. Tenmei, R., and F. Yanagisawa: Studies of the resistance of the pathogenic halophilic bacteria to cold temperatures. Nippon Kohshu Eisei Zasshi **9**, 447 (1962) (in Japanese)
37. Terayama, T.: Studies of the pathogenicity of *Vibrio parahaemolyticus*. II. Ecological survey and pathogenicity of isolates from various sources. Jpn. J. Bacteriol. **20**, 162–167 (1965) (in Japanese)
38. Wagatsuma, S., K. Tanji, K. Yuda, T. Shiratori, J. Konno, T. Watanabe, H. Monma, K. Hirama, K. Hayasaka, and M. Shimizu: Food poisoning caused by ingestion of raw oysters contaminated by *Vibrio parahaemolyticus* and survey of the source. Media Circle **16**, 168–171 (1971) (in Japanese)
39. Yanagisawa, F., Y. Tanaka, S. Hirose, and M. Yamaguchi: Studies of halophilic bacteria. XII. Halophilic chitinoclastic bacteria and the causative agent of food poisoning. Nippon Eiseigaku Zasshi **15**, 27 (1960) (in Japanese)
40. Yasunaga, N.: Studies of pathogenic halophilic bacteria. I. Pathogenic halophilic bacteria isolated from the river in Nagasaki city. Shokuhin Eisei Kenkyu **5**, 112–115 (1964) (in Japanese)
41. Yasunaga, N., and M. Kuroda: Studies of *Vibrio parahaemolyticus*. II. Distribution of *Vibrio parahaemolyticus* in marine sediment and on fish in Southeast Asia. Endemic Dis. Bull. Nagasaki Univ. **6**, 201–208 (1964)
42. Zen-Yoji, H. S., S. Sakai, Y. Kudo, T. Terayama, and T. Itoh: Study of pathogenic halophilic bacteria. VII. An ecological survey and examination of the relationship between biotypes and causative strains. Jpn. J. Bacteriol. **18**, 477–478 (1963) (in Japanese)
43. ZoBell, C. E.: The effect of solid surfaces upon bacterial activity. J. Bacteriol. **46**, 39–56 (1973)