

## Bacteria of the $\gamma$ -Subclass *Proteobacteria* Associated with Zooplankton in Chesapeake Bay

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**The seasonal abundance of  $\gamma$ -subclass *Proteobacteria*, *Vibrio-Photobacterium*, *Vibrio cholerae-Vibrio mimicus*, *Vibrio cincinnatiensis*, and *Vibrio vulnificus* in the Choptank River of Chesapeake Bay associated with zooplankton was monitored from April to December 1996. Large (>202- $\mu$ m) and small (64- to 202- $\mu$ m) size classes of zooplankton were collected, and the bacteria associated with each of the zooplankton size classes were enumerated by fluorescent oligonucleotide direct count. Large populations of bacteria were found to be associated with both the large and small size classes of zooplankton. Also, the species of bacteria associated with the zooplankton showed seasonal abundance, with the largest numbers occurring in the early spring and again in the summer, when zooplankton total numbers were correspondingly large. Approximately 0.01 to 40.0% of the total water column bacteria were associated with zooplankton, with the percentage of the total water column bacteria population associated with zooplankton varying by season. A taxonomically diverse group of bacteria was associated with zooplankton, and a larger proportion was found in and on zooplankton during the cooler months of the year, with selected taxa comprising a larger percent of the *Bacteria* in the summer. *V. cholerae-V. mimicus* and *V. vulnificus* comprised the bulk of the large and small zooplankton-associated *Vibrio-Photobacterium* species. In contrast, *V. cincinnatiensis* accounted for less than 0.1 to 3%. It is concluded that water column and zooplankton bacterial populations vary independently with respect to species composition since no correlation was observed between taxa occurring with highest frequency in the water column and those in association with zooplankton.**

*Vibrio* spp. comprise a significant portion of the natural bacterial flora of zooplankton, especially zooplankton with a chitinous exoskeleton, e.g., copepods (8, 10, 24, 38, 40, 42, 43, 45). In general, larger numbers of vibrios are associated with zooplankton than are found in the surrounding water column (24, 26, 43), and this finding has led to the conclusion that *Vibrio* spp. have a competitive advantage in the chitinous exoskeleton microenvironment of zooplankton.

A great deal of attention in marine microbial ecology has been focused on microorganisms associated with particles, especially marine snow (9, 14, 29, 30). However, pelagic particle-associated bacterial populations are different, in both metabolic activity and taxonomic diversity, from free-living oceanic bacteria (9, 14, 30). The differences arise, at least in part, because marine particles provide stable, localized microhabitats for colonization by bacteria (2, 14, 30). For example, particle-associated bacteria are generally larger and more active metabolically and occur in higher population densities than free-living oceanic bacteria. In the open ocean, with respect to total bacterial abundance (or total bacterial carbon), free-living bacteria often comprise the dominant communities, primarily because fewer particles are present in the water column (14, 41, 44). In freshwater, coastal, and estuarine systems,

where particles are more abundant, particle-associated microorganisms are present in higher (per volume) concentrations than in the open ocean (7, 16, 29).

The taxonomic compositions of free-living and particle-associated bacteria differ (7, 14). In the open ocean, *Cytophaga*, *Planktomyces*, and the  $\gamma$ -*Proteobacteria* are dominant in the particle-associated *Bacteria* populations, while the  $\alpha$ -*Proteobacteria* comprise the majority of the free-living *Bacteria* (14). However, this is a more complex relationship (1, 13, 36). It is understood that marine snow provides a very different microecosystem from the exoskeleton surfaces of zooplankton, but the data to date suggest that the taxonomic compositions of *Bacteria* associated with zooplankton and those living free in the water column may be significantly different.

Zooplankton-associated *Vibrio* populations play an important role in the mineralization of chitin by binding to the chitin (6, 24, 27, 43, 45, 46) and utilizing it as a sole source of both carbon and nitrogen (5, 48). Several *Vibrio* spp. are also pathogenic for aquatic animals, including *Vibrio vulnificus* biotype 2 and *Vibrio alginolyticus*, both of which cause significant losses of commercially important finfish and shellfish (4, 15, 33).

The *Vibrio*-zooplankton association may be important in the occurrence of *Vibrio* spp. during colder months of the year, since *Vibrio cholerae* can persist at 0°C in the environment, especially when associated with chitin. The cryoprotective property of chitin has been postulated to maintain *V. cholerae* in the environment during the winter (3, 39).

Culture methods have routinely been employed for detection and enumeration of *Vibrio* spp. in the gut or on the surfaces of zooplankton, but these methods do not always

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succeed in enumerating attached bacterial populations (46). Part of the difficulty is that many bacteria, including *Vibrio* spp., enter a viable but nonculturable (VBNC) state under environmental conditions adverse to active cell metabolism and division (37). When a large portion of *Vibrio* spp. are VBNC, culture methods significantly underestimate bacterial populations present in a sample (47). Therefore, in this study, both in situ and direct bacterial detection methods were used to study the bacteria associated with zooplankton (25, 26).

The objectives of this study were to determine whether *Bacteria*,  $\gamma$ -*Proteobacteria*, including *Vibrio-Photobacterium*, *V. cholerae-Vibrio mimicus*, *Vibrio cincinnatiensis*, and *V. vulnificus* are associated with zooplankton in the environment, whether the composition of zooplankton-associated bacterial communities is the same as or different from that of the water column, whether water column and zooplankton-associated bacterial populations are related, whether bacterial communities found on small (64- to 202- $\mu$ m) and large (>202- $\mu$ m) zooplankton are the same or differ, and, finally, what percentage of these taxa in the total samples is zooplankton associated.

#### MATERIALS AND METHODS

**Sample collection.** Water and zooplankton samples were collected 15 cm below the surface off the dock in the Choptank River at the University of Maryland Horn Point Laboratory, Cambridge, Md. Samples were collected within 1 h of high tide on the rising tide, so that they were collected when tidal mixing at the site was greatest, reducing the chance of bacteria entering the collection site with flow from the tidal marsh. Zooplankton samples were collected using a Hormelite model AP-125 water pump with approximately 1,000 liters of water pumped through a 64- $\mu$ m plankton net at each sampling. The water intake hose was placed inside a wire enclosure (15 by 15 by 15 cm, 1-cm<sup>2</sup> mesh) to exclude gelatinous zooplankton during sample collection. To reduce physical damage to the zooplankton, the water was pumped through a net suspended in a bucket of water (D. Nemazie, personal communication). The zooplankton samples were transported immediately after collection (<15 min) to the laboratory and processed.

In the laboratory, zooplankton samples were split, using a Folsom plankton splitter (Aquatic Research Instruments, Lemhi, Idaho). Each zooplankton sample was further divided into 64- to 202- $\mu$ m and >202- $\mu$ m size fractions by filtration through Nytex screens. One sample of each size class was used to enumerate zooplankton, and the other was used to enumerate zooplankton-associated bacteria. Samples for zooplankton enumeration were stored in 5% formalin. Zooplankton-associated bacterial samples were processed immediately.

**Enumeration of zooplankton.** Zooplankton samples (both 64- to 202- $\mu$ m and >202- $\mu$ m size classes) were randomly subsampled using a 5-ml Stemple pipette and enumerated by counting at least 100 individuals of the most abundant zooplankton. In the summer months, when >202- $\mu$ m zooplankton were less abundant, the entire sample was counted. Plankton were identified to order for copepods, barnacle nauplii, copepod nauplii, polychaetes, tintinids, rotifers, ciliates, or mollusks and measured, i.e., copepod cephalothorax length and midbody width, other plankton total body length and midbody width, using a version 4.5 Scan Array 2 image analysis system (Galai Corp., Gosford, Australia). External surface area was estimated by modeling zooplankters as right-angle cylinders (surface area =  $\pi \sqrt{r^2 + l^2}$ ). Zooplankton abundance was converted to number of individuals per cubic meter of Choptank River water (CRW) and per square millimeter of external surface area.

**Analysis of zooplankton-associated bacteria.** Zooplankton were concentrated from each sample by filtration through Nytex screens (202 or 64  $\mu$ m) and suspended in 10 ml of filter-sterilized (FS) phosphate-buffered saline (PBS). Zooplankton-associated bacteria were removed and fixed for fluorescent oligonucleotide direct count (FODC) by adding 30 ml of fresh FS 4% (wt/vol in phosphate-buffered saline) paraformaldehyde and 10  $\mu$ g of Tween 80 ml<sup>-1</sup>. The sample was incubated on ice with gentle shaking for 4 h and then sonicated (10 W) for 30 s. Large particles, which could obstruct the flow cytometer, were removed by filtration through a 25- $\mu$ m Nytex filter. The Nytex filter was washed twice with approximately 25 ml of FS CRW. The paraformaldehyde and Tween 80 were removed by centrifugation, and the sample was resuspended in 1 ml of

FODC hybridization buffer containing 50% ethanol. All samples were stored at -20°C until enumerated.

**Enumeration of probe-positive bacterial cells.** Total *Bacteria* (probe EUB 338),  $\gamma$ -*Proteobacteria* (probe GAM), *Vibrio-Photobacterium* (probe Vib/Pho), *V. cholerae-V. mimicus* (probe Vcho/mim), *V. cincinnatiensis* (probe Vcinc), and *V. vulnificus* (probe Vvul3) in each sample were enumerated by the FODC method (17, 18). The probes were generated for the study and are described in detail elsewhere (18, 19). Bacterial abundance was estimated using a Epics II flow cytometer (Coulter Corp., Miami, Fla.), and bacterial cells per cubic meter of CRW and per square millimeter of zooplankton external surface area were calculated.

**Data analysis.** SigmaStat (version 3.0; Jandel Scientific, Corte Madera, Calif.) was used for statistical analysis of the data. Pearson product moment correlation was used to determine relationships between zooplankton-associated and water column bacterial abundance. To equalize the variance, bacterial and zooplankton abundance were natural-logarithm transformed. Normality of transformed data was tested by the Komogorov-Smirnov test. Also, analysis of variance and regression analysis (linear and stepwise forward regression, model II) were used to determine the relationships among bacterial abundance and physical parameters.

#### RESULTS

**Large zooplankton (>202  $\mu$ m).** Total number of large zooplankton ranged from  $5 \times 10^2$  to  $1.3 \times 10^5$  zooplankters per m<sup>3</sup> of CRW. Zooplankton populations were numerically largest during the spring and, later, during the early winter (Fig. 1). Calanoid copepods, the dominant copepod taxonomic group, were most abundant in the spring (*Acartia* spp.) and later in the year during early winter (*Eurytemora* spp.). Cyclopoid copepods (*Oithona* spp.) and unidentified harpacticoid copepods were present intermittently in lesser numbers throughout the year. Copepod nauplii were most abundant during the spring and late summer, barnacle nauplii were most abundant in the early summer and fall, and polychaetes were most abundant during the fall and early winter (Fig. 1).

The large-zooplankton external surface area data showed a pattern similar to that of total zooplankton abundance (Fig. 1). Zooplankton surface area ranged from  $2.5 \times 10^1$  to  $2.8 \times 10^4$  mm<sup>2</sup> external surface area per m<sup>3</sup> of CRW, with a larger zooplankton total surface area observed during spring and later in the year in the early winter. Copepods accounted for the majority of the total external surface area during the spring and contributed significantly (along with polychaetes) to the total external surface area in the fall and early winter (Fig. 1).

**Small zooplankton.** The total number of small zooplankton (64 to 202  $\mu$ m) ranged from  $2.6 \times 10^4$  to  $1.4 \times 10^6$  zooplankters per m<sup>3</sup> of CRW, with the greatest abundance observed in samples collected during the spring (Fig. 2). Copepod nauplii were dominant among the small-zooplankton populations and were most abundant during the spring. Barnacle nauplii were most frequently observed in the early summer and fall, ciliates were most frequently observed during the fall, mollusks were most frequently observed during the summer, and tintinids were most frequently observed during late spring and early fall. Adult copepods and copepod nauplii comprised a portion of the population throughout the year but were not a dominant part of the 64- to 202- $\mu$ m size class (Fig. 2).

The pattern of the small-zooplankton surface area distribution was similar to that of the total small-zooplankton abundance (Fig. 2). The external surface area of the small zooplankton ranged from  $6.4 \times 10^2$  to  $6.2 \times 10^4$  mm<sup>2</sup> per m<sup>3</sup> of CRW, with greater total surface area during the spring. Copepod

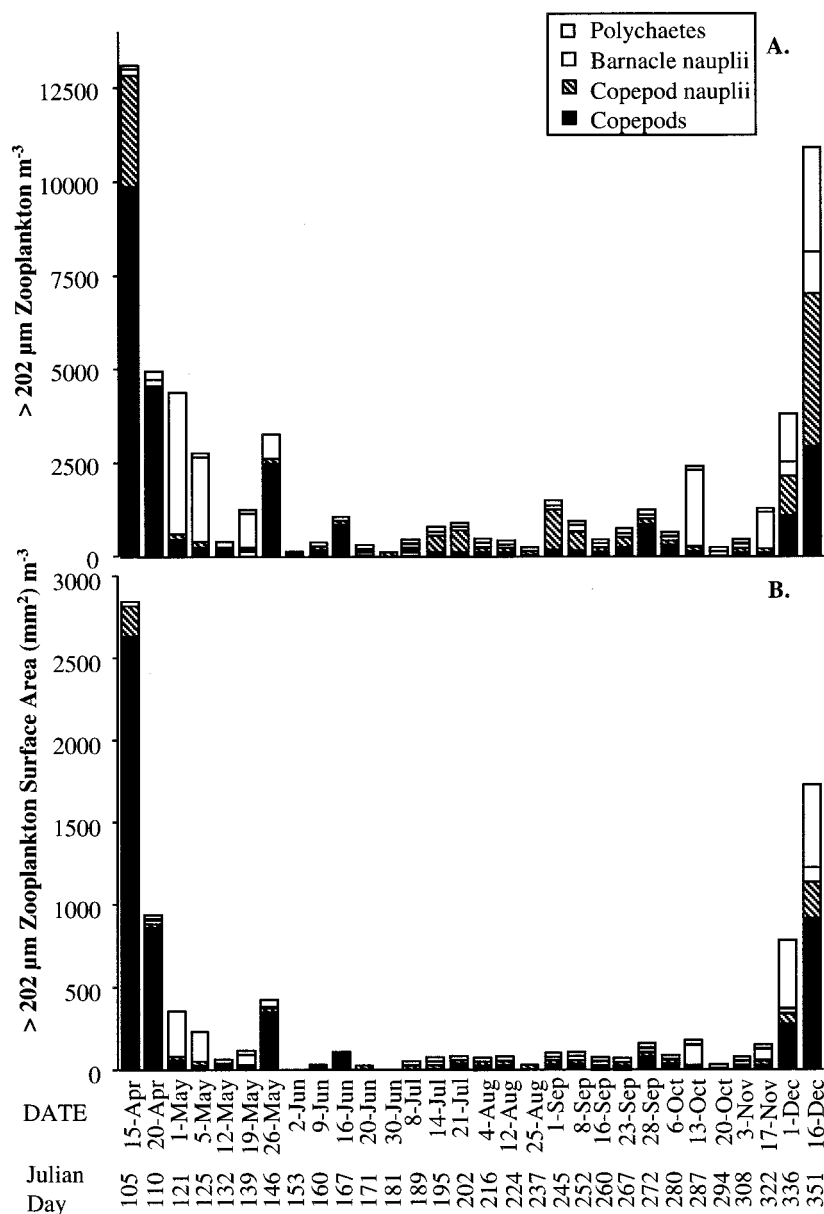


FIG. 1. Composition of  $>202\text{-}\mu\text{m}$  zooplankton. (A) Number of zooplankton per cubic meter of CRW; (B) surface area of zooplankton per cubic meter of CRW.

nauplii dominated the total surface area, i.e.,  $1.4 \times 10^1$  to  $5.9 \times 10^4 \text{ mm}^2$  (external surface area) per  $\text{m}^3$  of CRW (Fig. 2).

**Acridine orange direct counts of CRW samples.** The total number of bacteria, estimated by AODC, in centrifuge-concentrated CRW samples collected at the dock site between 15 April and 16 December 1996 ranged from  $2.0 \times 10^9$  to  $24 \times 10^9$  cells  $\text{liter}^{-1}$ . The highest bacterial concentrations in the CRW samples were obtained during the warm summer months, and the lowest concentrations were obtained during the winter and early spring.

Bacterioplankton abundance, enumerated using centrifuge-concentrated water samples, ranged from 64 to 93% of the total cell abundance measured by direct counts of unconcentrated water samples. However, no more than 36% of the total number of bacteria was lost during concentration, and it was

observed that larger percentages of cells were lost when early-spring and winter water samples were analyzed than when summer water samples were analyzed. The effect of water temperature on centrifuge concentration efficiency may be a result of cell size, since large cells are more efficiently concentrated by centrifugation, and warm water may be associated with larger cell sizes (19). Measurement of the biovolume of the cells was not an objective of this study.

**Bacterial populations associated with zooplankton.** The number of *Bacteria* associated with the large zooplankton ranged from  $1.0 \times 10^8$  to  $3.8 \times 10^9$  cells per  $\text{m}^3$  of CRW, the total number of  *$\gamma$ -Proteobacteria* was  $1.9 \times 10^7$  to  $7.0 \times 10^8$  cells  $\text{m}^{-3}$ , the total number of *Vibrio-Photobacterium*  $1.9 \times 10^6$  to  $2.4 \times 10^8$  cells  $\text{m}^{-3}$ , the total number of *V. vulnificus* was  $2.4 \times 10^5$  to  $1.3 \times 10^7$  cells  $\text{m}^{-3}$ , the total number of *V. cholerae-V.*

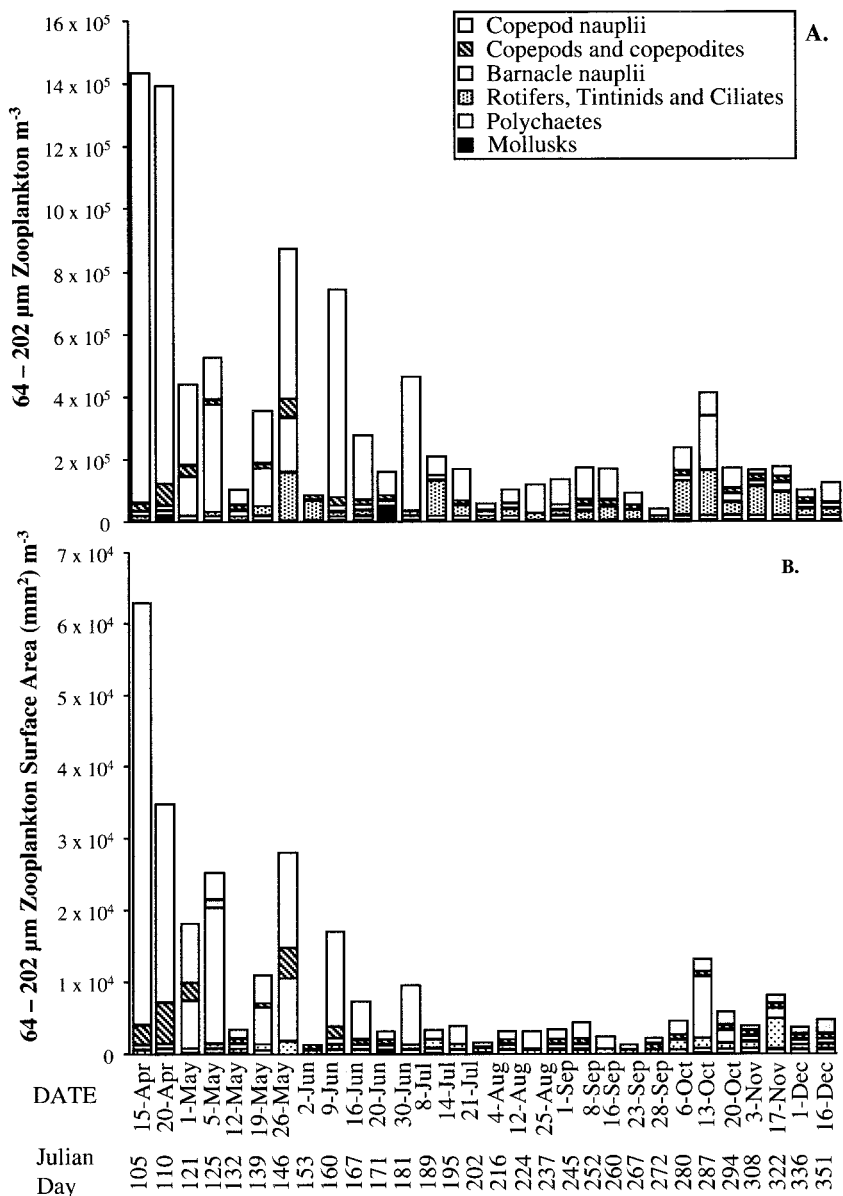


FIG. 2. Composition of 64- to 202-µm zooplankton. (A) Number of zooplankton per cubic meter of CRW; (B) surface area of zooplankton per cubic meter of CRW.

*mimicus* was  $1.3 \times 10^5$  to  $6.4 \times 10^7$  cells  $m^{-3}$ , and the total number of *V. cincinnatiensis* was  $1.9 \times 10^3$  to  $7.9 \times 10^6$  cells  $m^{-3}$  (Fig. 3). The number of *Bacteria* associated with the large zooplankton was largest in the early spring and fall, whereas  $\gamma$ -*Proteobacteria*, *Vibrio-Photobacterium*, and *V. vulnificus* populations were largest in the early spring and late summer. *V. cholerae-V. mimicus* was most abundant in the early spring and summer, and *V. cincinnatiensis* was most abundant in the early spring.

The number of *Bacteria* per individual zooplankton of the >202-µm size class ranged from  $2.8 \times 10^3$  to  $9.6 \times 10^5$ , that of  $\gamma$ -*Proteobacteria* ranged from  $1.0 \times 10^3$  to  $2.3 \times 10^5$ , that of *Vibrio-Photobacterium* ranged from  $1.6 \times 10^2$  to  $1.2 \times 10^5$ , that of *V. vulnificus* ranged from  $3.6 \times 10^1$  to  $1.0 \times 10^4$ , that of *V. cholerae-V. mimicus* ranged from  $3.0 \times 10^0$  to  $3.3 \times 10^3$ , and

that of *V. cincinnatiensis* ranged from  $9.3 \times 10^{-2}$  to  $1.2 \times 10^2$ . Total populations of *Bacteria*,  $\gamma$ -*Proteobacteria*, and *Vibrio-Photobacterium* associated with the large zooplankton demonstrated a clear seasonality, with the largest numbers of these taxa per zooplankton occurring during the summer. The *Vibrio* spp. varied in total number but demonstrated seasonality in annual distribution, with a trend toward larger populations in the summer.

The number of *Bacteria* attached to the >202-µm zooplankton ranged from  $1.8 \times 10^4$  to  $1.7 \times 10^7$  cells per  $mm^2$  of zooplankton external surface area, the number of  $\gamma$ -*Proteobacteria* ranged from  $6.6 \times 10^3$  to  $4.4 \times 10^6$ , the number of *Vibrio-Photobacterium* ranged from  $1.3 \times 10^3$  to  $4.3 \times 10^6$ , the number of *V. vulnificus* ranged from  $1.7 \times 10^2$  to  $1.9 \times 10^5$ , the number of *V. cholerae-V. mimicus* ranged from  $1.9 \times 10^1$  to  $6.3$

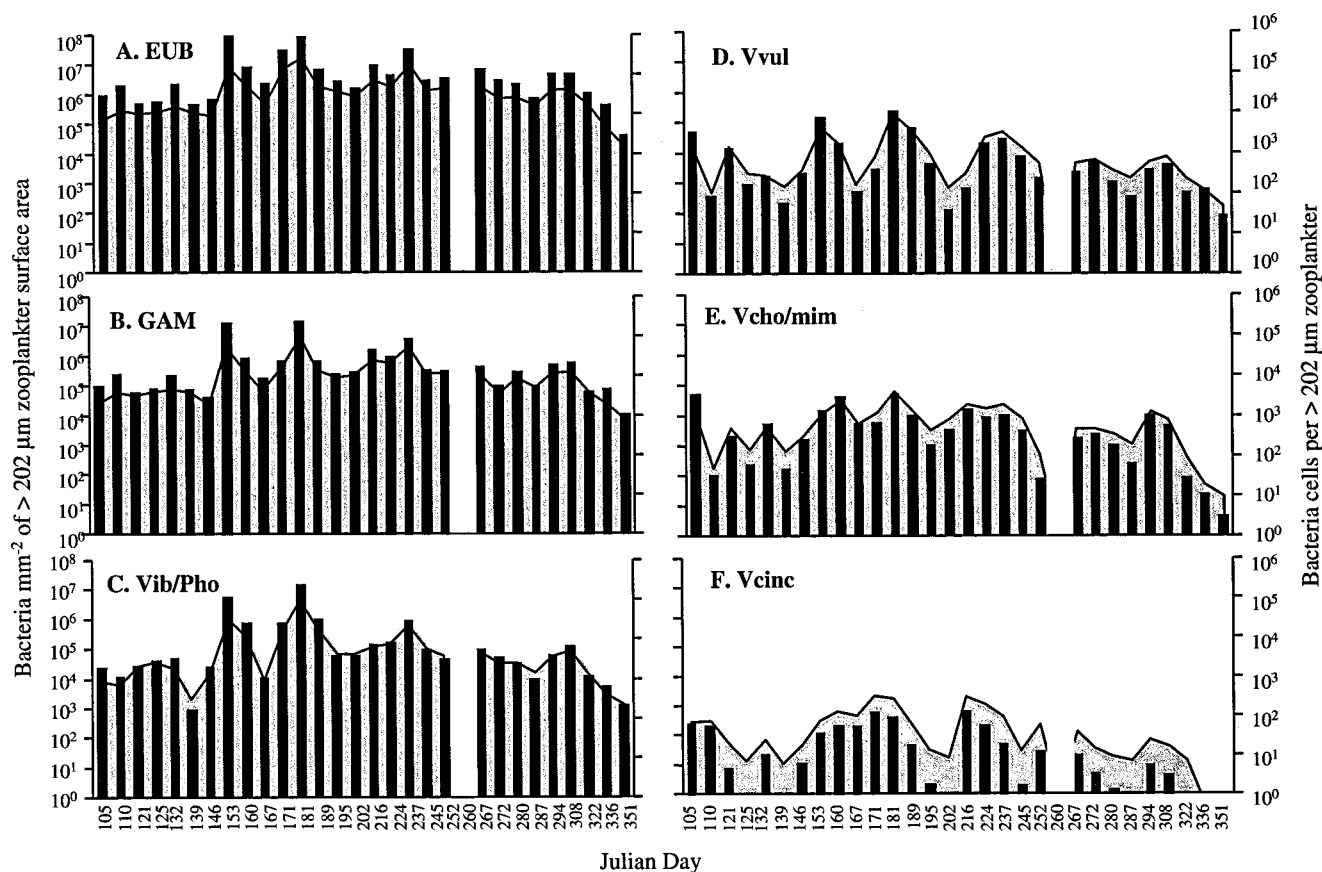


FIG. 3. Number of bacteria associated with  $>202\text{-}\mu\text{m}$  zooplankton per cubic meter of CRW. EUB, *Bacteria*; GAM,  $\gamma$ -*Proteobacteria*; Vib/Pho, *Vibrio-Photobacterium*; Vvul, *V. vulnificus*; Vcho/mim, *V. cholerae-V. mimicus*; Vcinc, *V. cincinnatiensis*. Shaded areas represent the number of cells per square millimeter of surface area; bars represent the number of cells per zooplankton.

$\times 10^4$ , and the number of *V. cincinnatiensis* ranged from  $1.1 \times 10^{-1}$  to  $2.1 \times 10^3$ . The largest populations of all groups were associated with  $>202\text{-}\mu\text{m}$  zooplankton surface area,  $\text{mm}^{-2}$ , during the summer months (Fig. 4).

Approximately 6 to 37% of the total large-zooplankton-associated *Bacteria* were  $\gamma$ -*Proteobacteria*, and the *Vibrio-Photobacterium* spp. comprised 1 to 26%. The three other species included in this study comprised 0 to 7% of the total *Bacteria*. Seasonally, the percent composition of *Bacteria* of the  $\gamma$ -*Proteobacteria* attached to large zooplankton was essentially constant throughout the year. However, *Vibrio-Photobacterium* spp. comprised a larger percentage of the *Bacteria* during the spring and summer, with a pronounced seasonal trend.

Overall, *V. vulnificus* comprised 1 to 57% of the total *Vibrio-Photobacterium* population, *V. cholerae-V. mimicus* comprised 1 to 60%, and *V. cincinnatiensis* comprised 0.01 to 5%, with significant variation in species composition observed from week to week (Fig. 5).

Taxa associated with the small size class (64 to 202  $\mu\text{m}$ ) of zooplankton did not show the same variation in abundance as did taxa associated with the large zooplankton. The total number of *Bacteria* was  $8.6 \times 10^7$  to  $1.1 \times 10^9$  cells  $\text{m}^{-3}$ , the total number of  $\gamma$ -*Proteobacteria* was  $2.2 \times 10^7$  to  $2.1 \times 10^8$ , the total number of *Vibrio-Photobacterium* was  $4.7 \times 10^6$  to  $1 \times 10^8$ , the total number of *V. vulnificus* was  $3 \times 10^5$  to  $2.9 \times 10^7$ , the total

number of *V. cholerae-V. mimicus* was  $4.2 \times 10^5$  to  $1.8 \times 10^7$ , and the total number of *V. cincinnatiensis* was  $3.2 \times 10^2$  to  $5 \times 10^5$ . Taxa associated with the smaller zooplankton showed a distinct seasonality, with larger numbers detected in the spring and summer months (Fig. 6).

The number of *Bacteria* associated with an individual small-size-class (64- to 202- $\mu\text{m}$ ) zooplankton was 260 to 28,606 cells, the number of  $\gamma$ -*Proteobacteria* was 88 to 4,690, the number of *Vibrio-Photobacterium* was 25 to 1,980, the number of *V. vulnificus* was 2 to 188, the number of *V. cholerae-V. mimicus* was 1 to 265, and the number of *V. cincinnatiensis* was 0 to 18. *Bacteria* associated with the smaller zooplankton, namely,  $\gamma$ -*Proteobacteria* and *Vibrio-Photobacterium*, showed a clear seasonal trend, with greater populations observed to occur in the summer months.

The number of *Bacteria* associated with the small-size-class (64- to 202- $\mu\text{m}$ ) zooplankton ranged from  $1 \times 10^4$  to  $1.2 \times 10^6$  cells per  $\text{mm}^2$  of zooplankton surface area, the number of  $\gamma$ -*Proteobacteria* ranged from  $2.7 \times 10^3$  to  $1.9 \times 10^5$ , the number of *Vibrio-Photobacterium* ranged from  $8.7 \times 10^2$  to  $8 \times 10^4$ , the number of *V. vulnificus* ranged from  $7.2 \times 10^1$  to  $7 \times 10^3$ , the number of *V. cholerae-V. mimicus* ranged from  $4.3 \times 10^1$  to  $9.9 \times 10^3$ , and the number of *V. cincinnatiensis* ranged from  $9.7 \times 10^{-2}$  to  $7.3 \times 10^2$ . All of the taxonomic groups analyzed in this study were present in larger numbers on the smaller zoo-

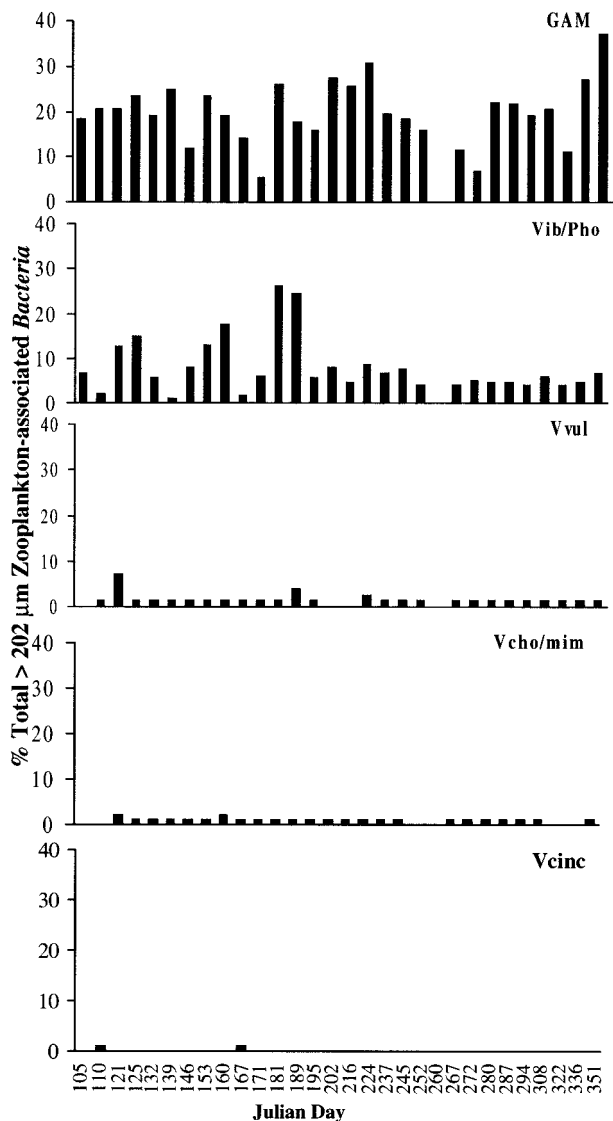


FIG. 4. Percentage of total bacteria associated with >202- $\mu$ m zooplankton. Results are given as number of cells per zooplankton. EUB, *Bacteria*; GAM,  $\gamma$ -*Proteobacteria*; Vib/Pho, *Vibrio-Photobacterium*; Vvul, *V. vulnificus*; Vcho/mim, *V. cholerae-V. mimicus*; Vcinc, *V. cincinnatiensis*.

plankton, especially during the summer months. However, total numbers per square millimeter of surface area were significantly smaller than those of the large zooplankton (Fig. 6).

*Bacteria* associated with the smaller zooplankton included 7 to 80%  $\gamma$ -*Proteobacteria*, 1.3 to 27% *Vibrio-Photobacterium*, 0.04 to 3.3% *V. vulnificus*, 0.07 to 3.5% *V. cholerae-V. mimicus*, and 0 to 0.11% *V. cincinnatiensis*. Seasonally, the percent composition of all of these taxonomic groups was higher during the summer months (Fig. 7).

*V. vulnificus* comprised 0.5 to 51% of the total *Vibrio-Photobacterium* population, *V. cholerae-V. mimicus* comprised 1 to 36%, and *V. cincinnatiensis* comprised 0.002 to 1.1% (Fig. 8).

**DISCUSSION**

The association of *V. cholerae* with zooplankton is of interest for several reasons, the most important being public health,

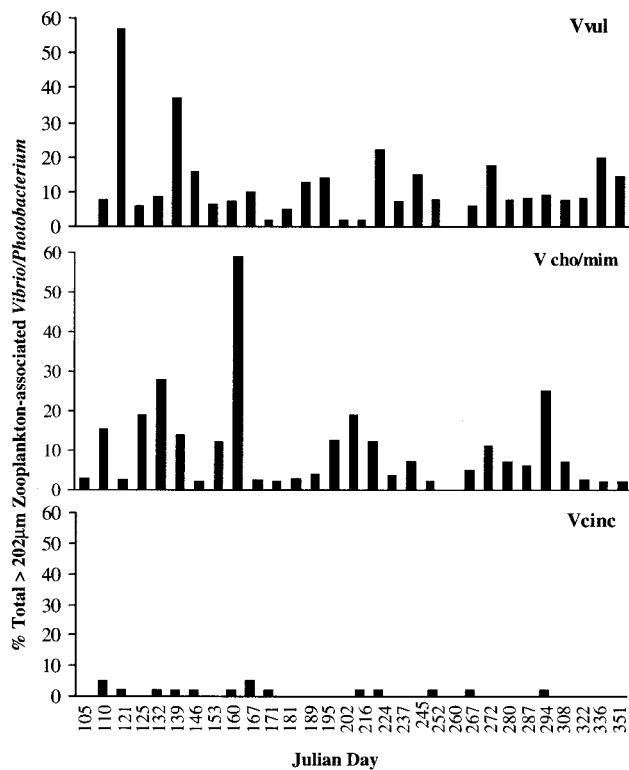


FIG. 5. Percentage of total *Vibrio-Photobacterium* associated with >202- $\mu$ m zooplankton; Vvul, *V. vulnificus*; Vcho/mim, *V. cholerae-V. mimicus*; Vcinc, *V. cincinnatiensis*.

since this ecological relationship can have significant implications for initiation and spread of cholera epidemics, notably in developing countries (11, 21, 22). The role of zooplankton in the initiation of cholera epidemics can be deduced from the correlations observed between copepod abundance and onset of epidemics. In Bangladesh, cholera epidemics occur biannually, during the spring and fall, and the seasonal cycle of cholera is closely correlated with copepod abundance. Furthermore, in years when copepod populations were small, fewer cholera cases were recorded (20, 21). Specifically, zooplankton appear to amplify *V. cholerae* populations at the time of zooplankton blooms, thereby either initiating or exacerbating cholera epidemics, or both (24, 26, 43). Chitin, part of the exoskeletal structure of zooplankton, can protect *V. cholerae* from stomach acid if the bacteria are ingested by humans along with plankton present in untreated water used for drinking (31, 35). It has been shown that by removing zooplankton from drinking water, nearly all of the pathogenic *Vibrio* spp. are removed (23).

*Bacteria*,  $\gamma$ -*Proteobacteria*, *Vibrio-Photobacterium*, *V. cholerae-V. mimicus*, *V. cincinnatiensis*, and *V. vulnificus* were associated with both the large and small zooplankton size classes in the Choptank River of the Chesapeake Bay, confirming the earlier work of other investigators (11, 28).

There are two important factors to consider in analyzing microbial populations associated with zooplankton, namely, the patchy distribution of zooplankton (32) and the limits of zooplankton surface area estimates. Thus, accurate estimates of populations of bacteria associated with zooplankton per

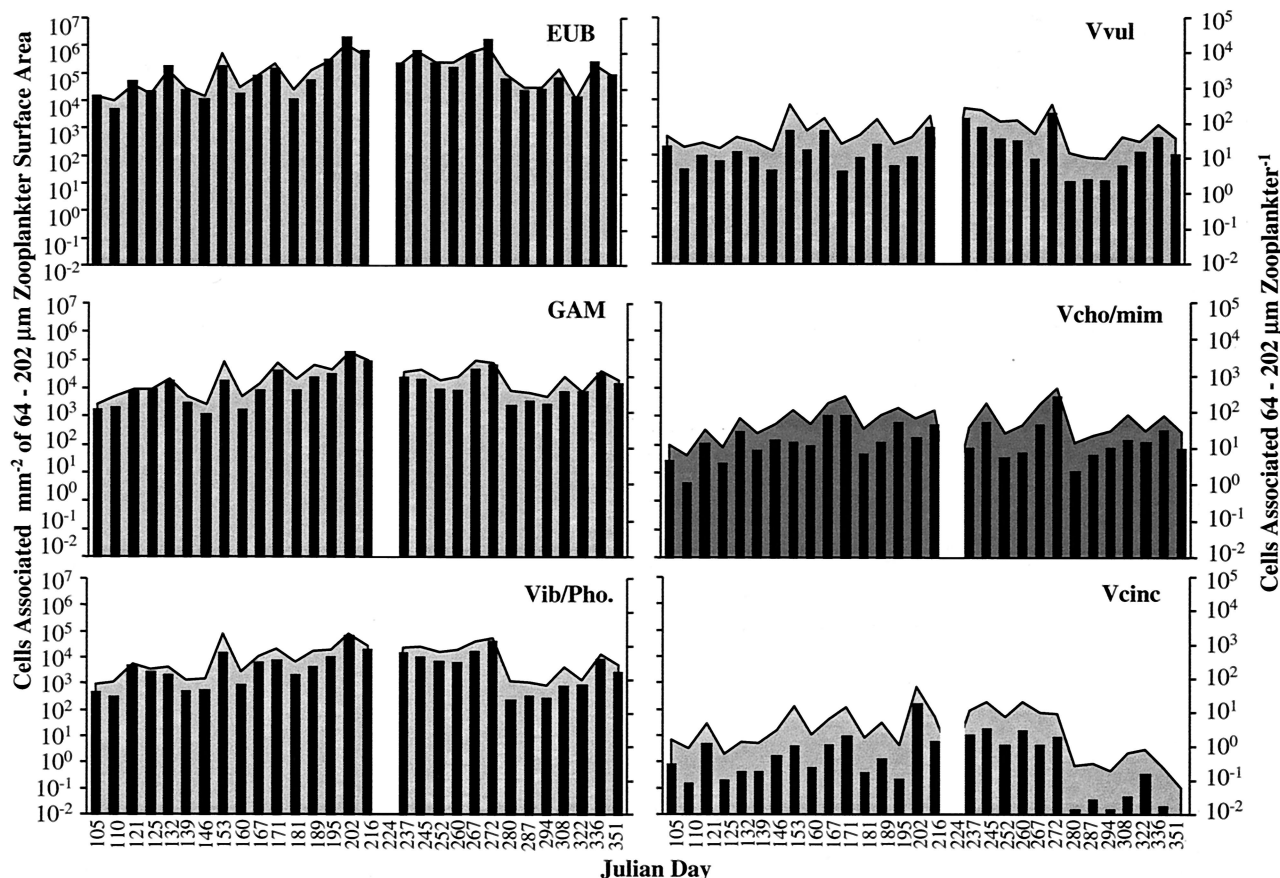


FIG. 6. Bacterial cells associated with 64- to 202- $\mu\text{m}$  zooplankton per cubic meter of CRW. EUB, *Bacteria*; GAM,  $\gamma$ -*Proteobacteria*; Vib/Pho., *Vibrio-Photobacterium*; Vvul, *V. vulnificus*; Vcho/mim, *V. cholerae-V. mimicus*; Vcinc, *V. cincinnatiensis*. Shaded areas represent the number of cells per square millimeter of surface area; bars represent the number of cells per zooplankter.

volume of water or with a single zooplankter are difficult to obtain. During sampling, if zooplankton abundance is high (or low) locally, the sampling protocol will overestimate (or underestimate) the number of zooplankton per cubic meter of water and, consequently, the zooplankton-associated bacterial populations per cubic meter water. Thus, comparisons between total-water-column bacterial abundance and zooplankton-associated bacterial abundance are, at best, estimates. In this study, potential error was reduced by collecting samples for both water column and zooplankton-associated bacterial populations simultaneously.

Based on this study, zooplankton-associated *Bacteria* comprised only a fraction of the total-water-column *Bacteria* in the Choptank river ecosystem. Only 1:1,000 to 1:10,000 of the total *Bacteria* per cubic millimeter of CRW were concluded to be attached to, or associated with, zooplankton. In the Choptank River, this relatively low percentage of total *Bacteria* associated with zooplankton may be a consequence of the total-water-column bacterial populations being relatively large. In ecosystems where nutrient availability limits bacterial abundance and total bacterial abundance is low, selection for specific bacterial taxa would be expected in the zooplankton exoskeleton microecosystem, since bacteria attached to copepods will have chitin available as a substrate. The largest percentage of bacterial populations associated with zooplankton were *Vibrio-Photo-*

*bacterium*, *V. cholerae-V. mimicus*, and *V. vulnificus*. All, incidentally, are chitin digesters.

In microcosms inoculated with *V. cholerae*, the number of *V. cholerae* cells attaching to a single calanoid copepod is large enough to serve as an infectious dose (12, 21, 22, 24). In the present study, the number of *V. cholerae-V. mimicus* cells per single copepod was not determined directly but was estimated by dividing the number of *V. cholerae-V. mimicus* per square millimeter of zooplankton surface area by the surface area of the calanoid copepod population in the sample. From this estimation, it was calculated that 2.5 to 4,000 (and a high of 7,100 on 30 June 1996) *V. cholerae-V. mimicus* organisms were associated with the exoskeleton of a calanoid copepod. However, because the total copepod surface area is an approximation and because of a possible preference of *V. cholerae-V. mimicus* for specific zooplankton species as well as the copepod gut (24), this number can be considered a very conservative estimate. Even within these limitations, it is clear that relatively large numbers of *V. cholerae-V. mimicus* are associated with the zooplankton of the Choptank River in Chesapeake Bay.

All bacterial taxa examined in this study were zooplankton associated but to different degrees. Very large populations of *Bacteria*,  $\gamma$ -*Proteobacteria*, *Vibrio-Photobacterium*, *V. cholerae-V. mimicus*, and *V. vulnificus* were associated with both the large

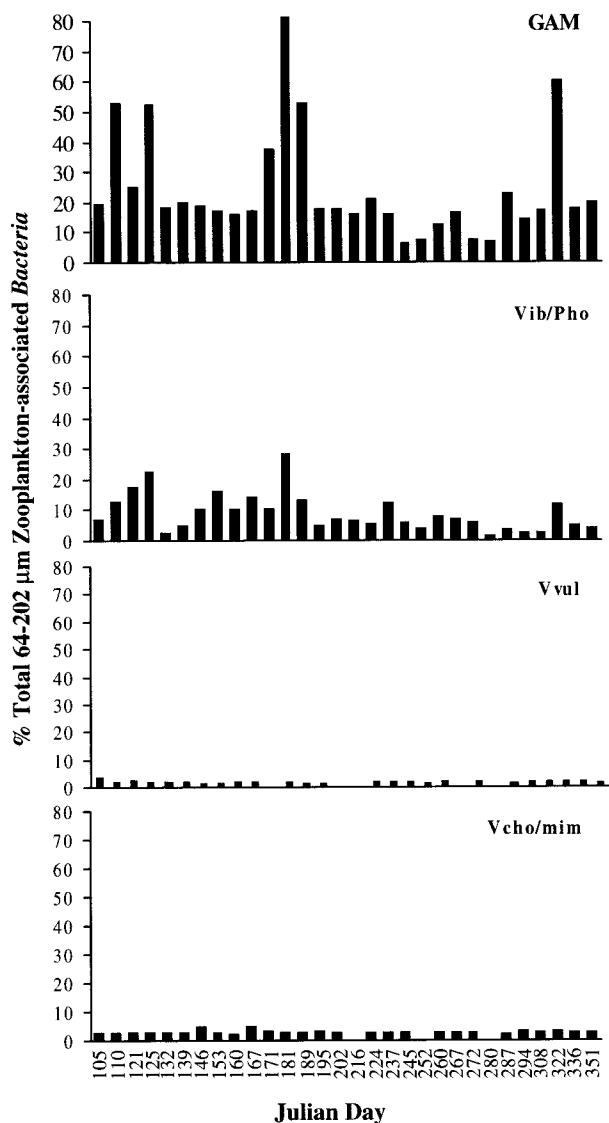


FIG. 7. Percentage of total bacterial cells associated with 64- to 202-µm zooplankton. GAM,  $\gamma$ -Proteobacteria; Vib/Pho, *Vibrio-Photobacterium*; Vvul, *V. vulnificus*; Vcho/mim, *V. cholerae-V. mimicus*.

and small size classes of zooplankton, especially during the summer. In contrast, *V. cincinnatiensis* comprised only a minor portion of the zooplankton-associated bacterial community. *V. cincinnatiensis* either is not able to compete with other bacterial species comprising this community, is present in small numbers consistently, or may be preferentially associated with other components of plankton, e.g., phytoplankton, in the water column.

There were large week-to-week variations in the number of bacteria associated with zooplankton, both per square millimeter of zooplankton surface area and per cubic meter of CRW (Fig. 3). These short-term temporal abundance variations create problems with respect to accuracy of monitoring the environment for public health purposes (20, 34). Any monitoring scheme involving infrequent environmental sampling may fail to detect an increase in bacterial abundance associated with a

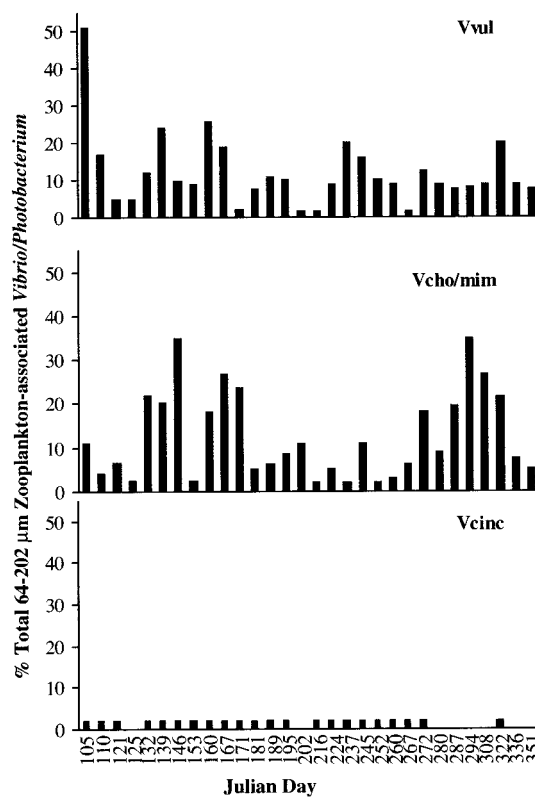


FIG. 8. Percentage of total *Vibrio-Photobacterium* associated with 64- to 202-µm zooplankton. Vvul, *vulnificus*; Vcho/mim, *V. cholerae-V. mimicus*; Vcinc, *V. cincinnatiensis*.

bloom in zooplankton. Based on the large temporal variation in abundance, there can be short periods during the year when zooplankton-associated bacterial pathogen populations are large enough to cause disease, as is the case in Bangladesh, where cholera outbreaks typically occur in April and May and again in August and September (23).

The contribution of each of the bacterial taxa included in this study to zooplankton-associated and water column populations of *Bacteria* was compared to determine whether each of the zooplankton exoskeleton and water column microhabitats harbored different bacterial populations. There was a 2- to 10-fold increase in the percentage of zooplankton-associated *Bacteria* represented by the taxonomic groups included in this study, compared to the proportion they comprised in water column *Bacteria* composition, indicating that the bacterial taxa associated with the zooplankton exoskeleton microhabitat differ from those in the water column, with the possibility of a coevolutionary history in the case of the bacterium-zooplankton association.

Zooplankton-associated *Vibrio-Photobacterium* showed a seasonal trend in percent composition opposite to that in the water column; i.e., *Vibrio-Photobacterium* made up a larger percentage of zooplankton-associated *Bacteria* during the summer but accounted for a larger percentage of total-water-column *Bacteria* during the spring and early winter, most probably a result of shedding from declining blooms of zooplankton. *V. vulnificus*, *V. cholerae-V. mimicus*, and *V. cincinnatiensis* showed seasonal trends similar to that of the *Vibrio-Photobac-*



terium genera. These species comprised a greater percentage of zooplankton-associated *Bacteria* during the summer months, whereas they were more abundant in the water column in the spring and early winter.

*V. vulnificus*, *V. cholerae-V. mimicus*, and *V. cincinnatiensis*, in total, comprised a greater relative portion of zooplankton-associated *Vibrio-Photobacterium* than of total-water-column *Vibrio-Photobacterium* populations. These three species, combined, comprised 10 to 25% of the zooplankton-associated *Vibrio-Photobacterium* populations but comprised up to 70% of the bacterial populations attached to the large zooplankton. *V. cholerae-V. mimicus* and *V. vulnificus* were present in significantly greater numbers as members of the zooplankton-associated *Vibrio-Photobacterium*, whereas *V. cincinnatiensis* was only a minor portion (0.0 to 5%) of this population.

A significant correlation between zooplankton and water column bacterial populations was observed only for *V. cincinnatiensis*. The lack of such correlation for other taxa included in this study leads to the conclusion that zooplankton-associated and water column bacterial populations are independent. If bacterial species moved frequently between these environments, strong correlations would be expected. The data do, furthermore, support the hypothesis that zooplankton provide an overwintering site for the bacteria, in accordance with the diapause exhibited by many species of zooplankton.

In conclusion, large populations of *Bacteria*,  $\gamma$ -*Proteobacteria*, *Vibrio-Photobacterium*, *V. vulnificus*, and *V. cholerae-V. mimicus* were found associated with both large and small size classes of zooplankton in the Choptank River of the Chesapeake Bay. In comparison, these zooplankton-associated bacterial populations comprised only a small fraction of the total-water-column bacterial populations. Furthermore, when the genera and species of bacteria associated with zooplankton were identified, the association was found to be significant. Zooplankton-associated bacterial populations showed seasonal trends, with larger populations associated with zooplankton being present in the environment during the spring and fall months of the year. Few differences were noted among taxa of bacterial populations associated with either large or small zooplankton size classes.

*V. cholerae-V. mimicus* and *V. vulnificus* comprised a significant portion of the total *Vibrio-Photobacterium* species found on both large and small class sizes of zooplankton. Also, these species comprised a greater portion of the numbers of *Vibrio-Photobacterium* in this microhabitat than in the water column, suggesting that these two species either selectively attach to, or are commensal and/or symbiotic with, zooplankton. Since a statistically significant correlation was not observed between either abundance or taxon distribution of the water column and zooplankton-associated bacterial populations, these populations are concluded to be independent and specific to their microhabitat.

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