ABSTRACT

Title of Dissertation / Thesis:	INTERACTIONS OF *VIBRIO CHOLERAE* SEROGROUPS O1 AND O139 AND COPEPODS
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Vibrio cholerae O1 El Tor and O139 Bengal have caused cholera epidemics throughout Bangladesh and elsewhere in the world with a seasonal regularity. This has raised questions about whether clinical presentations of cholera caused by these two epidemic serogroups of *V. cholerae* reflect similar responses to their aquatic habitat. The association of *V. cholerae* with plankton has been suggested to be an important factor in transmission of the disease. In this study, differences in resource utilization of copepods and chitin by *V. cholerae* O1 and O139 were analyzed using laboratory microcosm experiments.

When occurring separately, *V. cholerae* O1 and O139 were able to colonize copepods, including *Acartia tonsa* and *Eurytemora affinis*. However, *V. cholerae* O1 had a higher affinity for colonizing adults of both copepod species, as well as the multiple life stages of *E. affinis*, than *V. cholerae* O139.

In sympatry, colonization of copepods by *V. cholerae* O1 and O139 did not result in specific exclusion of one serogroup by the other. Results of this study indicate that cells that are already established may facilitate attachment through new biofilm formation, notably by *V. cholerae* O139.

Soluble chitin, employed as a nutrient source, supported growth of *V. cholerae* O1 and O139. Growth of both serogroups with the addition of chitin was significantly greater than in river water alone. In competition assays, *V. cholerae* O139 had a deleterious effect on *V. cholerae* O1 growth, but not vice versa.

Together, these data indicate that *V. cholerae* O1 and O139 respond differently to copepods as habitat, as well as nutrient resources. Such differences may play a role in cholera epidemics. The spatiotemporal dynamics of *V. cholerae* in the environment is complex, and understanding what drives cholera outbreaks requires explicit consideration of population responses and interactions of multiple serogroups.

INTERACTIONS OF *VIBRIO CHOLERAE* SEROGROUPS O1 AND O139 AND COPEPODS

By

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Dedication

I dedicate this work to my parents, Gilbert and Marti Rawlings, who have supported me in every endeavor since my inception.

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Chapter 1: General Introduction

1.1 Cholera as a Paradigm

Research on cholera epidemics requires a multi-disciplinary approach in order to understand the dynamics of the disease (Colwell, 1996). Cholera provides a useful model for how ecology, combined with medical microbiology and epidemiology, can enhance the understanding of the disease and the disease agent, *Vibrio cholerae*. Once believed to have only an human reservoir, *V. cholerae* is now known to be naturally occurring in aquatic environments globally. The classical research of Robert Koch (1884), taken together with the microbial ecology studies of Colwell et al. (1977), provide overwhelming evidence for the aquatic existence of *V. cholerae*. Recent studies of *V. cholerae* ecology (Lobitz, 2000; Jiang and Fu, 2001; Pascual et al., 2002; Louis et al., 2003) demonstrate the influence of abiotic factors, including sea surface temperature, sea surface height, salinity, and pH, as important to the onset of cholera "seasons." These abiotic factors, in part, explain the cycle of bacterial abundance in the system. However, biotic interactions also influence the dynamics of *V. cholerae* (Huq 1983, 1984b, 1995; Islam, 1994; Tamplin, 1994). In particular, association with zooplankton has been noted as an important factor, both in the survival and persistence of *V. cholerae* in the aquatic environment (Amako et al., 1987; Carman, 1994; Dawson et al., 1981; Huq et al., 1983, 1984; Nalin, 1979). Chitin was also suggested to provide facilitated passage through the highly acidic gastric barrier of the human gut by Nalin (1979).

Molecular studies of *V. cholerae* suggest that proteins used in attachment to zooplankton may be a part of the disease cascade, once it reaches the human gut (Tarsi and Pruzzo, 1999; Meibom et al., 2004). Specifically, cholera toxin and other virulence factors may influence the association of *V. cholerae* with zooplankton in the aquatic environment, thereby contributing to its transmission to susceptible human hosts. Thus, components influencing the persistence of *V. cholerae* in the aquatic environment may play a role in human infection.

Research across many disciplines provides a composite model of transmission (Colwell and Huq, 1994), demonstrating how the cycling of *V. cholerae* between aquatic and human environments may be significant in epidemic cholera. Such a model can provide a mechanism for viewing physicochemical parameters and epibiotic interactions with plankton that involve *V. cholerae* in the aquatic environment as an agent of disease.

Despite these advances, several gaps remain in understanding V. cholerae dynamics in the environment. Below the current knowledge about the association of *V. cholerae* and plankton is reviewed briefly, providing the background and basis for my thesis research.

1.2 Ecological and Epidemiological Background of Vibrio cholerae with an emphasis on the association of serogroups O1 and O139 with zooplankton

Past research explored the association between *V. cholerae* and plankton and provided a foundation for understanding the relationship between ecological dynamics of *V. cholerae* in the aquatic environment and human epidemics. The body

of literature that provides a background for the work presented in the following chapters is briefly reviewed here. This section is divided into three parts: 1) the zooplankton-vibrio association, 2) the emergence and re-emergence of *V. cholerae* O1 El Tor and O139 Bengal., and 3) the association of *V. cholerae* with zooplankton in the aquatic environment and its possible role in shaping the characteristics of a cholera epidemic.

1.2.1 Importance of zooplankton

The association of plankton and *Vibrio cholerae* has been reported in aquatic systems globally. *V. cholerae* blooms have been correlated with plankton abundance and warm temperatures, whereby *V. cholerae* achieves greatest abundance during the summer months, often attached to zooplankton (Venkateswaran et al., 1989; Tamplin et al., 1990; Heidelberg, 2000). Laboratory studies (Huq et al., 1983, 1984) further demonstrated that zooplankton contribute to the growth of *V. cholerae*. These studies suggest that zooplankton provide a resource for *V. cholerae* during inter-epidemic and epidemic initiating periods that may play a role in the ubiquitous occurrence of *V. cholerae* in the aquatic environment.

The chitinous exoskeleton of zooplankton has been reported to provide a protective environment for *V. cholerae*, contributing to transmission of cholera when ingested by humans. Research on *V. cholerae* exposed to low temperatures in the presence of chitin indicates that attachment to zooplankton increased survivorship (Amako et al., 1987). Attachment to chitin particles has provided protection for *V. cholerae* O1 from acidic environments with chitin acting as a buffer (Nalin, 1979). Research involving human subjects showed that adequate dosages of *V. cholerae* O1 did not stimulate disease symptoms unless administered with a buffer or with food (Levine et al., 1981). Thus, attachment to zooplankton is hypothesized to enhance the

probability of disease initiation by increasing the chance of survival past the acidic human gastric barrier (Nalin, 1979).

An advantage of epibiotic organisms, such as *V. cholerae*, on biotic substrates, is their proximity to available nutrients. *V. cholerae* that live on highly mobile zooplankton are less likely to be nutrient limited than "free-living" or planktonic forms of organisms in the environment. Zooplankton hosts have their own nutrient requirements, and provide a regular nutrient source for *V. cholerae* via exudates, creating an environment suitable for growth and colonization (Wahl, 1989). Furthermore, nutrients accumulate at solid-liquid interfaces (Marshall, 1976), making them more accessible to *V. cholerae* that live on host organisms than those that occur unattached as planktonic cells. During periods when readily available nutrients are limited for both host and bacterium, chitin can act as a sole source of C and N for vibrios since vibrios posses a chitinase (Kaneko and Colwell, 1975; West and Colwell, 1984), breaking down chitin into its constituents, fructose-6-P, acetate, and NH_3.

Attachment to plankton may also affect osmolarity with important consequences for survival of *V. cholerae*. Despite an unequivocal requirement for sodium (Na+) and optimal growth stimulated by conditions typical of an estuarine habitat (Cavari and Colwell, 1981; Singleton et al., 1982a,b), *V. cholerae* is found in aquatic environments ranging from freshwater to river water. Colwell (1984) suggested that *V. cholerae* can sequester Na+ in the human intestinal environment and similarly acquire Na+ when associated with aquatic metazoans.

It has been suggested that a function of cholera toxin (CT) may be osmoregulation. Once in the human intestines, environmental signals (i.e., a change in osmolarity) can promote a cascade that stimulates the up- regulation of virulence factors (DiRita et al., 1992; Häse and Mekalanos, 1999). The sodium-sensitive

flagellar motor of *V. cholerae* is driven by sodium motive forces, and any decrease in flagellar rotation, i.e. contact with the high media viscosity of the epithelial lining or a change in osmolarity, increases expression of the cholera toxin gene *(ctxab)* regulated by a series of transcriptional activators (Häse and Mekalanos, 1999). Cholera toxin, once acting on and internalized by the epithelial cells of the intestines, stimulates intestinal secretions of electrolytes by blocking the uptake of Na+ (Field et al., 1972), causing a build up of Na+ in the intestinal space. The subsequent increase of electrolytes in the intestines induces the release of fluids from cells and consequently the diarrhea that characterizes cholera.

The broad aquatic distribution of *V. cholerae* suggests that these mechanisms, when associated with epithelial cells of aquatic metazoans, may act to assist in its survival during periods of low sodium concentrations in the aquatic environment. The *vibrio*-plankton relationship may help *V. cholerae* survive across a wide range of salinities and periods of rapid fluctuations, and may possibly benefit plankton by playing a role in osmoregulation.

Although the association of *V. cholerae* with various plankton species has been reported, the relationship between *V. cholerae* and copepods may be especially important. Not only are copepods the predominant macroplankton taxon in most estuaries (Herman et al., 1968; Conover, 1956), but there is also a significant presence of *V. cholerae* cells in size fractions of filtered water where copepods are most numerous (Huq et al., 1984; Lipp et al., 2003; Louis et al., 2003). Attachment of *V. cholerae* in and on copepods may be an essential component in the initiation of epidemics, as large numbers of copepods are frequently present during the bimodal cholera peaks, as observed in the Chesapeake Bay (Heidelberg et al., 2002; Louis et al., 2003) and the Bay of Bengal (Cockburn and Cassanos, 1960; Oppenheimer et al., 1978; Glass et al., 1982; Lobitz et al., 2000). Furthermore, *V. cholerae* has been reported attached to single calanoid copepods in concentrations that can serve as

infectious doses (Huq et al., 1983). Thus, copepods may prove important in facilitating transmission and causing disease, since cholera is dose-dependent, with *ca*. 10^4-10^6 cells being required for infection, depending on the health of the individual (Cash et al., 1974).

1.2.2 Epidemiology and Molecular Biology of *V. cholerae* O1 El Tor and O139 Bengal.

V. cholerae comprises a diverse group of genotypes. There are over 206 serogroups, two of which, serogroups O1 and O139, are known to cause epidemic cholera (Faruque et al., 1998). With regard to clinical presentation and general modes of transmission, serogroup O139 does not differ from O1 (Morris et al., 1995). But the shifting predominance of *V. cholerae* O1 El Tor and O139 Bengal as the etiologic agent of cholera suggests that aquatic populations are dynamic, a factor that this research explores.

Historically, the *V. cholerae* O1 serogroup has been shown to be the causative agent of cholera (Pacini, 1854; Koch, 1884; Pollitzer, 1959). The "Classical" biotype is responsible for at least the fifth and sixth pandemics, whereas *V. cholerae* O1 El Tor biotype, which originated in Indonesia, was isolated and described as the etiologic agent of the seventh pandemic. Recently, it has been suggested that we are entering the eighth pandemic, with the emergence of the Bengal biotype, serogroup O139.

V. cholerae O139 was initially isolated in Madras, India in 1992 (Ramamurthy et al., 1993). *V. cholerae* O139 likely derived from *V. cholerae* O1 El Tor as a clonal lineage, with the exception of the deletion of O1 specific DNA and the insertion of *V. cholerae* non-O1 specific DNA (Bik et al., 1995). This genetic recombination appears to give *V. cholerae* O139 the ability to cause cholera outbreaks (Islam et al.,

1993). *V. cholerae* O139 expresses a thin polysaccharide capsule specific to *V. cholerae* non-O1 types that may thwart human immunity, giving O139 a possible advantage, compared to O1, as the etiologic agent of cholera (Weintraub et al., 1994). Although polysaccharide layers protect many bacteria against leukocyte phagocytosis (Weintraub et al., 1994), the layer of polysaccharides surrounding *V. cholerae* O139 cells differs enough that it rarely contributes to establishment of septicaemic infection (Albert et al., 1999).

In Bangladesh, the shift from cholera epidemics caused by *V. cholerae* O1 to those caused by *V. cholerae* O139 is thought to be a result of the inefficient protection of immunity acquired from *V. cholerae* O1 infections and the pathogenic properties of *V. cholerae* O139 (Morris et al., 1994; Nair et al., 1994). Most adults in endemic areas have *V. cholerae* O1 antibodies and are thus partially immune, while children are traditionally the group most susceptible to *V. cholerae* O1 infection (Glass et al., 1982; Cholera Working Group, ICDDR,B, 1993). The Cholera Working Group, International Centre for Diarrhoeal Disease Research, Bangladesh (1993) showed, however, that neither adults nor children had immunity to *V. cholerae* O139, increasing the number of susceptibles to include adults. In fact, 74% of patients during the initial wave of cholera caused by *V. cholerae* O139 in Bangladesh were greater than 15 years of age (Cholera Working Group, ICDDR,B, 1993). Increased proportions of susceptibles in Bangladeshi populations facilitated the emergence and rapid spread, replacing both *V. cholerae* O1 Classical and El Tor, largely in southern coastal regions and northeastern districts. In northwestern districts, however, both *V. cholerae* O1 and O139 were detected (Siddique et al., 1996). Spread of *V. cholerae* O139 varied seasonally by location, coinciding with the dry season in coastal areas and the wet season in the northern areas. Siddique et al. (1996) detected substantial differences in *V. cholerae* redistribution, appearing nearly two years after the initial spread of serogroup O139 in Bangladesh. In coastal regions *V. cholerae* O1 El Tor

re-emerged and coexisted with O139 but a new clone of *V. cholerae* O1 El Tor replaced O139 in the northern districts (Siddique et al., 1996). Therefore, conditions, i.e., temperature, salinity, biotic interactions, experienced by *V. cholerae* O1 and O139 in the aquatic environment, combined with immunity and possible coinfections occurring in humans, may influence emergence and resurgence by altering regional persistence of clones, genetic reassortment, abundance, and pathogenicity.

Scientists have indicated molecular changes associated with the annual fluctuations in the populations of *V. cholerae* O1 and O139 in Bangladesh (Faruque 1997a, 1997b, 2000; Basu et al., 2000). Since the initial emergence of *V. cholerae* O139, a significant amount of genetic reassortment has occurred in O139 populations, and to a lesser degree in the O1 populations (Faruque et al., 2003). Early studies indicated that O139 strains were closely related to O1 El Tor strains and the initial O139 strain emerged from serotype-specific genetic changes in an ancestral El Tor strain (Nair et al., 1994; Bik et al., 1995). However, since the initial spread of *V. cholerae* O139, rapid genetic changes have resulted in the origination of new clones (Albert et al., 1997; Faruque et al., 1999, 2000). Initial appearance of *V. cholerae* O139 was caused by strains from two different ribotypes, BI and BII. Since 1992, at least seven ribotypes have been documented (Faruque et al., 1999, 2000).

Past and recent *V. cholerae* O139 strains also contain different copies of CTX prophage that carries the cholera toxin gene. Strains isolated during 1992-1993 had the El Tor type CTX prophage (CTX^{ET}) and variants associated with epidemics since contain CTX^{ET}, as well as a new type of CTX prophage, referred to as the Calcutta type (CTX^{cal}) (Faruque et al., 2003). Clonal differences, recent susceptibility to antibiotics, and additional cholera toxin (CTX) prophages indicate that *V. cholerae* O139 continues to evolve.

In addition, Faruque et al. (1997b) showed differences in genes for conserved rRNA, cholera toxin, and zonula occludens toxin between *V. cholerae* O1 El Tor strains preceding and following the 1992-1993 epidemic of *V. cholerae* O139. The newly emerged *V. cholerae* O1 El Tor strain proceeded to replace O139 in northern Bangladesh. It is unclear what is driving these genetic reassortments. However, Faruque and his colleagues suggest that interactions in the aquatic environment conferring mechanisms of survival may be factors in the changing genotypes of epidemic cholera (Faruque et al.,1997a,b, 2003).

Some scientists have interpreted the genetic variation within the O139 genotype as attributed to multiple origins (Faruque et al., 2000), however, others suggest that the substantial genetic diversity reflects "founder flush" (Garg et al., 2003). Founder flush occurs when similar genotypes descendent from the founder population but not identical to the founder produce a sufficient amount of offspring that become established in the population during times of population expansion (Garg et al., 2003). Thus, genetic diversity is not necessarily produced by separate origins but can be created by the variation in genotypes originating from the founder. The subsequent annual changes in genotypes within the two serogroups may demonstrate that population dynamics are correlated with the severity of an epidemic being affected by selection pressures from unidentified ecological factors, as well as similar pressures due to the immunity of the human host population.

1.2.3 Interactions in the aquatic environment influencing *V. cholerae* distribution

Genotypic replacement of one epidemic variant by another appears to be a critical component in the defining character of cholera pandemics, i.e., *V. cholerae* O1 Classical replaced by O1 El Tor and possibly *V. cholerae* O1 El Tor replaced by O139 Bengal. Furthermore, the shifting predominance and coexistence of *V.*

cholerae O1 and O139 in both the human and aquatic reservoir suggests that dynamics in the aquatic habitat may affect the relative importance of a specific biotype in cholera infection. Nevertheless, the question of how ecological dynamics of these variants in the aquatic environment have an impact on cholera epidemiology remains unanswered. Like any organism, *V. cholerae* genotypes experience variation in habitat suitability that could influence population survival and distribution. Dynamic biotic interactions, some of which are already recognized to influence survival and persistence of *V.cholerae* in the aquatic environment (Nalin, 1979; Amako et al., 1987; Dawson et al., 1981; Huq et al., 1983, 1984; Islam, 1994; Carman, 1994; Tamplin, 1990), perhaps contribute to shifts in epidemic agents. Small shifts in genotype may go unrecognized until the character of an epidemic changes significantly, as was the case in the emergence of *V. cholerae* O139. Adults considered immune to cholera infections were susceptible to the newly emerged *V. cholerae* O139. In particular, biotic interactions (vibrio-vibrio or vibrio-plankton) that influence aquatic persistence and water to human transmission could contribute strongly to shifts in the composition of genotypes.

Persistence of microbial species in dynamic aquatic systems suggests that they must respond to shifts in environmental parameters through a wide variety of genotypic and phenotypic mechanisms. Previous research on the ecology of *V. cholerae* include examinations of the response of *V. cholerae* to varying salinities, temperatures, and inter- and intra-species interactions (Amako et al., 1987; Singleton et al., 1982; MacDonell et al., 1984). *V. cholerae* has shown tolerance to a wide range of salinities and temperatures by its presence in fluctuating tidal, estuarine, and riverine systems around the world, as well as in laboratory and field studies (Singleton et al., 1982; Jiang and Fu, 2001; Louis et al., 2003). *V. cholerae* has also shown a suite of physiological and biotic adaptations to adverse environmental

conditions, such as size reduction, metabolic rate increase, and attachment to substrates (MacDonell et al., 1984; Dawson et al., 1981; Ostling et al., 1993).

Significant genotypic and phenotypic variation may exist in the ability of *V. cholerae* to respond to the presence of shifting biotic interactions, such as inter- and intra-species competition, fluctuating substrate availability, and predation. As already mentioned, *V. cholerae* occurs in large numbers attached to zooplankton relative to those in the water column (Huq et al., 1984; Heidelberg et al., 2000), however, not all strains may be able to compete equally for space on all zooplankton species. The epibiotic nature of *V. cholerae* also exposes these cells to fluctuations within the zooplankton host environment. Of the *Vibrio* species that attach to and comprise the zooplankton microhabitat, most are pathogenic for humans (Heidelberg et al., 2000), suggesting that pathogenicity, i.e., selected properties of *V. cholerae* associated with the epibiotic existence may contribute to pathogenicity in humans. Faruque et al. (1998) suggested that the acquisition of the CTX genetic element provides a selective advantage to epidemic forms of *V. cholerae* by facilitating selective enrichment in the intestinal environment, conferring increased evolutionary fitness. If pathogenicity is linked to association with zooplankton, whether passive or active, then colonization and competition within this habitat could affect the character of an epidemic by affecting the abundance of a particular genotype of *V. cholerae* and possibly the evolution of clones.

Within the discourse of the ecology of *V. cholerae* and the association of *V. cholerae* with zooplankton, it is often assumed implicitly that where *V. cholerae* is present in the aquatic environment it has an even and constant distribution. But

zooplankton, an important vehicle of transmission of *V. cholerae*, like many other aquatic organisms, are distributed in patches that change over space and time (Haury, 1977), and epibiotic bacteria that utilize zooplankton as a substrate are likely to be affected by this variation. Theory suggests that organisms exposed to the stochastic loss of local habitat patches will depend on their ability to colonize new patches in order to persist (Slatkin, 1974; Roughgarden, 1977; Hanski, 1983; Neuhauser, 1998). Some populations may experience local extinctions and the ability to disperse to new patches will facilitate regional existence. Populations that have a greater affinity to disperse and colonize new patches may have an advantage during periods of habitat loss or competitive exclusion. *V. cholerae* may experience similar loss of habitat when attached to molting copepods or thinning copepod patches. Differences between genotypes of *V. cholerae* in their ability to disperse, colonize, and compete for nutrients and space on zooplankton may lead to one genotype outcompeting another for use of zooplankton resources, especially in coastal environments that are also subject to fluctuations in temperature, salinity, and nutrient availability.

The use of the zooplankton substrate by pathogenic *V. cholerae* species raises questions, explored in the research reported here: whether serogroups O1 and O139 differ in response to zooplankton species, and how such responses may contribute to cycles of endemicity. Zooplankton as a resource and endemic cycles of *V. cholerae* appear to be inextricably linked. Zooplankton are utilized by *V. cholerae* as habitat as well as nutritional or protective resources, and they are simultaneously inadvertent vehicles of human infection. Thus, interactions that influence the colonization ability of various genotypes could affect the characteristics of an epidemic. If one genotype

of *V. cholerae* demonstrates a colonization rate more rapid and/or extensive than others during epidemic-initiating periods when "empty patches" of newly emerged zooplankton increase in numbers, then that genotype could have a numeric advantage during transmission. Differences between epidemic variants in the degree of colonization and resource utilization of zooplankton microhabitats should give insight into the pattern of emergence and re-emergence of *V. cholerae* O1 and O139.

Variation in the strength of association with specific zooplankton species could also influence transmission, spread, and seasonal patterns of cholera occurrence. Plankton species vary in life histories and have seasonal cycles themselves. They can be found in a number of habitats and are often designated by their existence in one, i.e. benthic, demersal., or planktonic. They can brood their eggs in sacs on the outside of their bodies or they can scatter them one by one. Furthermore, most plankton species are highly influenced by a combination of salinity and temperature tolerances and tidal movement, often limiting their occurrence to certain regions, seasons, and years. Depending on species, the distribution of metapopulations can be geographically extensive or very restricted, and abundance can fluctuate greatly. Due to the seasonality of *V. cholerae* and its affinity for plankton, it appears likely that *V. cholerae* dynamics are associated and causally linked to dynamics of particular zooplankton species or a number of co-occurring species.

1.3 Association of V. cholerae with Copepods

Though copepods have been reported as important to *V. cholerae*, only a few studies have shown an association of *V. cholerae* with specific genera or species (Huq

et al., 1990; Tamplin et al., 1990). Several additional reports demonstrate plankton-*V. cholerae* associations. These reports identify the plankton that are present in samples by species, large taxonomic groupings, or size fractions (Table 1.1). However, the association of plankton and *V. cholerae* was made on the composite and not on specific species or groupings. Although, most studies present results of multiple plankton species and not specific associations, investigations from different global regions show that *V. cholerae* is consistently found in size fractions where copepods are predominant (Heidelberg et al., 2002a; Lipp et al., 2003; Louis et al., 2004). Furthermore, the zooplankton genera reported as associated with *V. cholerae* in Bangladesh can be found worldwide with similar seasonal occurrences (Table 1.1), particularly, *Acartia*

Chesapeake Bay provides an example of zooplankton composition and species-level differences that may influence vibrio dynamics. Copepods comprised 98% of zooplankton in an analysis for Chesapeake Bay (Herman et al., 1968), as well as 95% in Long Island Sound (Conover, 1956), two estuarine ecosystems in areas of historic cholera epidemics in the United States. *Acartia tonsa* and *Eurytemora affinis* are two of the predominant copepod species in both estuaries, and these species occur commonly in estuaries throughout the Northern Hemisphere.

TABLE 1.1 Zooplankton associated with environmental *V. cholerae* in plankton surveys.

Region	Plankton taxon or size class identified in plankton samples where *V. cholerae* was present	SOURCE	Reference
Bangladesh	ZOOPLANKTON		
	(*V. cholerae* found)		
	Copepods		
	Acartia sp.		Tamplin et al., 1990
	Acartia chilaensis	River	
	Acartia sewelli	River	
	Cyclops sp.	River	
	Diaptomus sp.	Pond	
		Pond	
	Cladocerans		
	Bosmina sp.		
	Daphnia sp.	River	
	Ceriodaphnia sp.	Pond	
	Diaphanosoma sp.	River	
	Bosminopsis sp.	River	
	Rotifers (*Brachionus* sp.)	Pond	
		River,	
	2 Unidentified copepods	Pond	
		River	Huq et al., 1983
	Copepods		
	Cladocera	River	
	Rotifera	River	Huq et al., 1990
		River	
Gulf of Mexico	**Copepods**		
	(*Vibrio* sp. found)		
	Acartia tonsa		Sochard et al., 1979
	Labidocera aestiva		
	Pontellopsis regalis		
	Centropages furcatus		
Maryland			
Chesapeake Bay	COPEPODS		
	(*V. cholerae* found)		
	Adults/copepodites		
	Nauplii	River	Heidelberg et al., 2002a;
		River	Louis et al., 2004
	Acartia tonsa		
	Eurytemora affinis	River	Huq et al., 1983
	Scottolana canadensis	River	
		River	
Japan			
Fukuyama Port	ZOOPLANKTON		
	(*V. cholerae* non-O1 found)		
		Seto	Venkateswaran et al., 1989
	comprised of 81% copepods	Inland Sea	
Italy			
Gulf of Naples	COPEPODS		
	(*V. cholerae* non-O1 found)		
		Coastal	Dumontet et al., 1996
	Temora stylifera	lagoon	
	Acartia clausi		
	Acartia margalefi		
	Centropages typicus		
	Paracalanus parvus		
Coastal Peru			
	V. cholerae especially prevalent in plankton >202 µm		Lipp et al., 2003

Differences in physical characters and life history distinguish them. Although *A. tonsa* and *E. affinis* are both considered pelagic, calanoid copepods, *E. affinis* is also known to be demersal (attaches to substrates such as rocks and pilings), whereas *A. tonsa* is largely planktonic. Egg production is another feature that differentiates these copepods. *A. tonsa* is a broadcast spawner, releasing eggs one at a time, essentially "scattering" them, and *E. affinis* copepods brood their eggs in a exterior assemblage (egg sac) near the anal pore.

Finally, these two species also differ in salinity distribution and seasonal abundance, as reported for the Patuxent River of Chesapeake Bay (Heinle, 1969). *A. tonsa* is continuously present in the Patuxent River, where it is the most abundant copepod, comprising 55-60% of the total zooplankton and occurring at all salinities. Population densities are commonly $1x10^5$-$2x10^5$ individuals per m^3 and sometimes 10^6 individuals per m^3 during its peak (Heinle, 1969). *E. affinis*, however, is the second most abundant copepod (37%); it occurs in a range of salinities but usually dominates the lower salinity waters. Its population density is similar to that of *A. tonsa,* but at times exceeds $3x10^6$ individuals per m^3. *Acartia* makes up the largest fraction in the warmer months, whereas *Eurytemora* increases in abundance during the winter and occurs, commonly, until late spring. There is a seasonal shift in dominance between *Acartia* and *Eurytemora* which results in a period of sympatry, coinciding with a shift from the cooler, less saline months to warmer, more saline months (Brownlee and Jacobs, 1987).

For *V. cholerae* dynamics, the significance of biological., ecological., and seasonal differences among these and other zooplankton species remains virtually unexplored.

1.4 Scope of the Present Research

In a "Special Feature" of the journal Ecology entitled *Disease Ecology (1996)*, several investigators illustrated the importance of applying ecological principles in investigations of community dynamics influencing ecological and evolutionary consequences of pathogens, emphasizing a multi-disciplinary approach to understand disease transmission, vector production, emergence, and maintenance of disease foci. Real (1996) proposed a set of questions upon which disease ecology has and should focus to elucidate disease processes and disease management practices, asking: how processes of populations account for patterns of disease emergence; what genetic processes account for evolutionary patterns in resistance and virulence; does the ecology of hosts, vectors, and pathogens enter into the design of immunization programs; will diseases respond to global climate change.

This dissertation examines one of the questions put forth by disease ecologists as fundamental in the field, namely: whether ecological and population dynamics can account for a pattern of disease emergence. In applying this construct, the dynamics within the zooplankton habitat were studied to obtain an understanding of why the epidemic variants of *V. cholerae*, serogroups O1 and O139, fluctuate in a pattern of infection in cholera endemic Bangladesh. The pattern of shifting prevalence between *V. cholerae* serogroups O1 and O139 over the past decade has raised many questions about whether their coexistence is a result of competition for resources or whether

they occupy completely separate niches. Microcosm experiments, modeled to replicate environmental conditions of cholera endemic areas, were used to test whether serogroups O1 and O139 utilize spatial and nutritional resources similarly as a means of finding out whether they can occupy the same niche.

In Chapter Two, differences are reported between *V. cholerae* O1 and O139 in their ability to colonize adult copepods, *A. tonsa* and *E. affinis*, as well as eggs and nauplii of *E. affinis*. Similarities or differences in how the serogroups O1 and O139 utilize different copepod species should give perspective on possible niche differences.

Chapter Three explores the question of whether the extent of resource utilization, both colonization and nutrient acquisition, by *V. cholerae* O1 and O139 in a copepod habitat may facilitate or inhibit coexistence. Sympatric colonization of copepods by *V. cholerae* O1 and O139 and utilization of chitin by these serogroups was examined to test the dynamics of *V. cholerae* O1 and O139 when co-occurring. Coexistence and competitive interactions of *V. cholerae* O1 and O139 in ecological environments are intriguing, as interactions between serogroups within their natural aquatic habitat may help explain their role in epidemics of cholera.

Chapter Four discusses the implications of the copepod-*V. cholerae* relationship based on results presented in experimental chapters, results of previous *V. cholerae* investigations, spatiotemporal patterns of copepods, and ecological theories of interactions in stochastic environments.

Chapter Five discusses the shift towards integrative studies addressing disease science and possible directions for future research.

Chapter 2: Association of *Vibrio cholerae* serogroups O1 El Tor and O139 Bengal with the copepods *Acartia tonsa* and *Eurytemora affinis*

2.1 INTRODUCTION

Vibrio cholerae O1 El Tor and O139 Bengal are recognized as causative agents of cholera and are responsible for cholera epidemics in India and Bangladesh. More than ten years ago, *V. cholerae* O139 Bengal was recognized as a newly emerged epidemic variant when it replaced *V. cholerae* O1 El Tor for two successive cholera seasons. It is not clear what factors contributed to the emergence of serogroup O139 as an epidemic variant or its present coexistence with O1. However, results from research provide strong evidence that horizontal transfer of genes among environmental strains of *V. cholerae* was a mechanism for its origin (Bik et al., 1995; Comstock et al., 1995). Since the emergence of *V. cholerae* O139 in the Gangetic Delta region, both *V. cholerae* O1 and O139 have been the cause of cholera infections with a regular seasonality but with temporal variation in the prevalence of the two serogroups (Mukhopadhyay et al., 1996; Basu et al., 2000). The observed fluctuations in the reported numbers of people infected by either *V. cholerae* O1 or O139 may suggest differences in responses of the two serogroups to stochastic aquatic habitats, resource utilization, immunity of susceptible human populations and possibly in mechanisms of transmission.

Vibrios are found associated with various aquatic organisms, which serve as microhabitats, ranging from algae to crustaceans. Simidu et al. (1985) showed that different vibrios aggregate in microhabitats based on phenotype, suggesting that

habitat segregation among vibrios corresponds to temporal and spatial changes in the abiotic and biotic aquatic environments. *V. cholerae* has been reported attached to or associated with phytoplankton, zooplankton, and other crustaceans. Survey data of Bangladeshi aquatic habitats reveal that *V. cholerae* association with phytoplankton does not demonstrate the same corresponding bacterial blooms as it shows when associated with zooplankton just prior to cholera epidemics (R.R. Colwell, personal communication). Furthermore, zooplankton have been demonstrated as particularly important habitats for epidemic forms of *V. cholerae*. Zooplankton have a protective effect for attached cholera vibrios, especially during inter-epidemic and epidemic periods (Nalin, 1979; Dawson et al., 1981; Huq et al., 1983, 1984; Amako et al., 1987; Carman, 1994). Furthermore, Colwell and Huq (1994) proposed transmission of the cholera vibrios via zooplankton to be significant in cholera epidemics in developing countries. Copepods, in particular, have been reported to be important in this regard because they are the most numerous and widely dispersed zooplankton with which the incidence and distribution of *V. cholerae* have been found associated (Huq et al., 1983, 1984; Tamplin et al., 1990). Thus, the seasonal and regional fluctuations of copepod populations will have an impact on attached *V. cholerae* populations.

Attachment to an organic substrate like the copepod also provides a bacterial cell the advantage of nutrients from the copepod itself (Marshall, 1976; Dawson et al., 1981). In fact, *V. cholerae*, like most vibrios, is both chitinolytic and proteolytic, allowing it to break down and utilize constituents of chitin and zooplankton egg protein as nutrient resources (Kaneko and Colwell, 1975; Nalin, 1976; Bassler et al.,

1991; Guthrie and Cofie, 1991; Halpern et al., 2003; Meibom et al., 2004). During plankton blooms, the use of such enzymes may be unnecessary as copepods produce nutrient plumes by "sloppy-feeding," exuding high and low molecular weight molecules, and evacuating fecal pellets (Moller and Nielsen, 2001). The extent of nutrients released by copepod blooms may be largely responsible for the increased numbers of *V. cholerae* during and after blooms. Some models suggest that increased aquatic populations of *V. cholerae* are integral in transmission of cholera and, ultimately, in cholera outbreaks in areas of cholera endemicity (Colwell and Huq, 1994).

Results of previous investigations studying the colonization, interactions, and organization of zooplankton epibiont communities suggest that there is a premium on efficient location of suitable substrates, especially when such substrates are periodically renewed and sometimes occur in widely dispersed patches (Threlkeld and Willey, 1993). Areas on the copepod or within copepod patches that provide optimal growth conditions by proximity to nutrients may be limited, possibly creating competitive interactions between *V. cholerae* variants. Thus, differences in dispersal and colonizing abilities may affect relative inter-strain abundances, persistence during inter-epidemic periods, and transmission of cholera.

Publications that compare *V. cholerae* O1 and O139 largely emphasize molecular distinctions (Faruque et al., 1994; Johnson et al., 1994; Khetawat et al., 1998; Dziejman et al., 2002), and surveys of their distributions (Huq et al., 1995, Siddique et al., 1996; Ali et al., 2001), rather than explore the ways in which these genotypes may differ in their resource use. Different copepod species or life stages

may not provide equally suitable resources for *V. cholerae*, since space, molt frequency, nutrients, and patch distribution can differ greatly between species and life stages (Carman and Dobbs, 1997). It is unknown whether *V. cholerae* colonizes copepod species or copepod life stages in similar concentrations, or if one serogroup is better than the other at colonizing these substrates.

The objective of this study was to analyze the interaction of *V. cholerae* epidemic serogroups, O1 and O139, with two copepod species that represent different life histories as well as to explore any differences in affinity to copepod life stages in order to elucidate resource use of *V. cholerae* O1 and O139. Microcosms were used to compare the colonization of two species copepods, *Acartia tonsa* and *Eurytemora affinis* by *V. cholerae* O1 and O139. The copepods included in the study are predominant in the Chesapeake Bay system, have been associated with *V. cholerae* cell attachment, and may have been a significant factor in historic cholera epidemics in the Mid-Atlantic region of the United States.

2.2 MATERIALS AND METHODS

2.2.1 Effect of Copepod Species and Serotype on Colonization

Microcosms contained between 15-40 adult copepods of either *A. tonsa* collected from Baltimore Harbor or *E. affinis* collected from the Patuxent River, a tributary of the Chesapeake Bay. The copepods were cultured as single species in containers to acclimate them to the experimental conditions. After washing once with phosphate buffered saline, the harvested copepods were placed into 200 ml tall form berzelius beakers (Kimble-Kontes, Inc., New Jersey) containing 100 ml of filter

sterilized river water collected during the spring from the Rhode River, Maryland, which served as the microecosystem. The river water was filtered in a step series filtration, employing 10 µm, 5 µm, 1 µm filters (Filterite Inc., Timonium, MD), and 0.22 µm poretic polycarbonate filters (OSMONIC Inc., Livermore, CA) and served as the suspension medium for the microcosms, adjusted to a salinity of 15‰ and pH 8.5 and (Huq et al., 1994).

Prior to inoculation, the two strains of *V. cholerae* were incubated individually at 37 ºC, with shaking, for 6 hours in 20 ml of APW (1% w/v Peptone, Difco, 1% w/v NaCl, pH 8.5). Both serogroups grew to ca. 10^7 cells per ml, determined by direct fluorescent counts (Hasan et al., 1994). The cells were harvested by centrifugation, washed twice, resuspended in filter sterilized river water, and inoculated into microcosms.

For each copepod species, microcosms (n=13) were inoculated with *V. cholerae* O1 El Tor strain C6709 and the same number with *V. cholerae* O139 Bengal strain MO10 to a final concentration of ca. 5 x 10^4 cells per ml. For each copepod species, additional microcosms (n=13) were used as controls, containing copepods without addition of *V. cholerae* to detect *V. cholerae* present on cultured copepods.

Microcosms were incubated at 25 ºC for 24 h. At 24 h, copepods from each microcosm were collected by filtration, using a 153µm sieve. The copepods were rinsed to disassociate loosely attached bacteria. All samples were prepared for direct viable counts (DVC) described elsewhere (Hasan et al., 1994; Chowdhury et al., 1995) and fixed with 5% formalin.

The number of copepods in each sample was counted and the copepod surface area estimated for each microcosm. Surface area was determined by measuring the length and width of a subsample of each copepod species (n=50, per species) using the Scion Imaging Program (NIH, Bethesda, MD). To estimate the surface area, the cephalothorax was modeled as a right angle cylinder ($\pi\sqrt{(r^2 + l^2)}$). After the copepods in each microcosm had been counted, they were sonicated for 120 seconds and homogenized in a tissue grinder. Direct fluorescent antibody reagent (New Horizons, Columbia, MD) specific for O1 and O139 was added to each sample, permitting enumeration of *V. cholerae* O1 and O139 cells (both non-culturable and culturable cells), using an epifluorescent microscope (Leitz Dialux 20, Germany).

Microcosm experiments were employed in a Randomized Complete Block Design (RCBD) with a 2x2 factorial treatment structure. A two-way ANOVA (Mixed Model, SAS Institute, 8.0) was used to determine differences, if any, in the number of *V. cholerae* cells per mm^2 that were associated with *A. tonsa* and *E. affinis*. Multiple replicates for each copepod-serogroup treatment (including the control) were run on different days to obtain an overall total of thirteen replicate microcosms per treatment. Replicates were pooled because there was no significant covariate effect of day in the Mixed Model ANOVA. Day and day x treatments were considered random effects in the mixed model. Further analysis (two–way ANOVA) was performed to determine whether there were differences in total surface area contributing to any differences between copepods. Neither *V. cholerae* O1 nor O139 was present in any of the controls and thus the controls were not included in any of the analyses. Cell counts were log-transformed to satisfy homoscedacity.

2.2.2 Colonization of *E. affinis* Life Stage

Time Series: *V. cholerae* O1 and O139 colonization of adults and eggs

V. cholerae O1 El Tor strain C6709 and O139 Bengal strain MO10 were introduced into microcosms containing *E. affinis* adults. *E. affinis* copepods were collected from the Patuxent River, Maryland, a tributary of the Chesapeake Bay. They were cultured in flasks from which they were collected, washed once, and placed into the microcosms (as described above), preceding bacterial inoculation. Water, collected in the spring from the Rhode River, a tributary of the Chesapeake Bay, Maryland, was filtered though 10 µm, 5 µm, 1 µm filters (Filterite Inc, Timonium, MD), and finally a 0.22 µm polycarbonate filter (OSMONICS, Livermore, CA) and served as the suspension medium for the microcosms. Microcosms consisted initially of *ca.*50-100 adult copepods of*E. affinis* collected. Copepods distributed to microcosms were a mixture of males and both ovigerous and non-ovigerous females. Initial counts of eggs ranged from between 30-150 per microcosm.

V. cholerae O1 and O139 were incubated at 37ºC, with shaking at 150 rpm, for 6 h in 20 ml of APW (1% w/v Peptone, 1% w/v NaCl, pH 8.5). Both serogroups grew to ca. 10^7 cells per ml, determined by direct fluorescent counts (Hasan et al., 1994). The cells were harvested, washed twice, resuspended in filter sterilized water, and inoculated into microcosms containing copepods at a final concentration of *ca.* 5 x 10^4 cells per ml. Microcosms were run without the addition of bacteria to detect any inherent *V. cholerae* O1 or O139 present in or on cultured copepods that may

contribute to differences in the results. All microcosms were adjusted to a salinity of 15‰ and pH 8.5 and incubated at 25ºC, conditions shown to be representative of endemic and epidemic conditions (Huq et al., 1994). Microcosms were sampled at 0, 24, and 48 h, such that each microcosm was sampled only once, representing a single independent replicate. There were five independent microcosms for each serogroup + five microcosms controlling for inherent *V. cholerae* at each time point (0, 24, and 48). Neither *V. cholerae* O1 nor O139 was present in any of the controls and thus the controls were not included in the analyses.

Adults and eggs were collected from each microcosm by filtering the copepod fractions with a 63 μm sieve. Copepods were rinsed with filter sterilized water to disassociate loosely attached bacteria. All samples were prepared for direct viable counts (DVC) obtained following the method described in detail elsewhere (Hasan et al., 1994; Chowdhury et al., 1995) and then fixed with 5% formalin.

Each life stage was separated out manually. Adults and eggs were analyzed as independent components. The number of adult copepods and eggs were counted. Once counted, each replicate was sonicated for 120 seconds and homogenized in a tissue grinder. Direct fluorescent antibody reagent (New Horizons, Columbia, MD), specific for O1 and O139, was added to the samples for counting. *V. cholerae* O1 and O139 cells (both non-culturable and culturable cells, combined) were enumerated by epifluorescent microscopy using a Leitz Dialux microscope (Wetzlar, Germany). Any nauplii present were not included in this analysis.

Two-way ANOVA (General Linear Model, SAS 8.0) was used to analyze the effect of time on the attachment of *V. cholerae* O1 and O139 on adults and eggs.

Adults and eggs, however, were analyzed separately to avoid violating the assumptions of independence of the ANOVA model. Cell counts were log-transformed to satisfy homocesdascity

Effect of nauplii on colonization of adults and eggs by *V. cholerae* O1 and O139

Analysis of the effect of nauplii on the association of *V. cholerae* O1 and O139 with adults and eggs over 24 h was performed. Microcosms were prepared and analyzed as above, where each microcosm contained between 15-40 adults, including males as well as ovigerous and non-ovigerous females. The presence of nauplii was a function of their hatching out during the experiment and they were not produced in all microcosms. Replicates were employed for each serogroup treatment.

Adults, nauplii, and eggs were collected from each microcosm by filtering the copepod fractions with a 63μm sieve. Each life stage was separated out manually and each was analyzed as an independent component. The number of adult copepods and eggs were counted. Nauplii were treated as a qualitative measure (present/absent).

Microcosms were set-up in a RCBD with a 2x2 factorial treatment structure to examine the effect of nauplii on the colonization of adults and eggs by *V. cholerae* O1 and O139. Experimental runs were performed on different days and combined using Mixed Model ANOVA statistics (SAS 8.0). Replicates employed were different between days, three for the run on the first day and four on the second. Day and day x serogroup treatment were considered random effects. There was no significant covariate effect of day so results were combined from two experimental runs (run on separate days), forming an overall total of seven replicate microcosms for adults and

eggs. Adults and eggs were analyzed separately to avoid violating the assumptions of the ANOVA model. Cell counts were log-transformed to satisfy homoscedascity. Neither *V. cholerae* O1 nor O139 was present in any of the controls and thus were not included in the analyses.

Colonization of adults and nauplii by *V. cholerae* O1

Following the methods described above, *V. cholerae* O1 El Tor was added to microcosms containing either 10-20 *E. affinis* adults or 20-45 nauplii (all stages, N1-N6) to obtain a quantitative measure of the attachment of *V. cholerae* to adults and nauplii as separate entities. As above, all microcosms were inoculated with *V. cholerae* O1 to a final concentration of *ca.* 5 x 10^4 cells per ml. All microcosms were adjusted to a salinity of 15‰ and pH 8.5, incubated at 25ºC, and sampled after 24 h, each representing a single independent replicate. Again, controls consisted of microcosms without addition of *V. cholerae* bacteria. Replicates were run, two for the first two runs of the experiment and four for the third, for each treatment. Experimental runs were performed on different days.

As described above, total cells attached per microcosm were analyzed using a one-way ANOVA (Mixed Model ANOVA, SAS Institute 8.0) to measure the attachment of *V. cholerae* O1 to adults and nauplii. Neither *V. cholerae* O1 was present in any of the controls and thus the controls were not included in the analyses. Since experimental runs were performed on different days, treatments were combined where day and day x copepod life stage were considered random effects in the ANOVA. There was no significant covariate effect of day. Thus, replicates were

combined from the three experiments, forming an overall total of eight replicate microcosms for adults and nauplii. Cell counts were log-transformed to satisfy homoscedascity.

2.3 RESULTS

2.3.1 Effect of Copepod Species and Serogroup on Colonization

After incubation for 24 h, results revealed a significant difference in attachment of *V. cholerae* to copepod species and a significant difference in colonization by strain (Table 2.1). More *V. cholerae* colonized *A. tonsa* than *E. affinis* per mm^2 of surface area (Fig. 2.1). Furthermore, *V. cholerae* O1 attached to both copepod species in significantly larger numbers than O139 per mm^2 of surface area (Table 2.1), greater than an order of magnitude difference between the two strains (Fig. 2.1). There was no significant difference in the amount of copepod surface area present between *V. cholerae* O1 and O139 microcosms that would contribute to differences in cells attached per mm^2 (Table 2.2.).

TABLE 2.1: Two-way ANOVA (Mixed Model) for the colonization of *A. tonsa* and *E. affinis* copepods by *V. cholerae* O1 and O139. Colonization of copepods by *V. cholerae* resulted in a significant difference of cells per surface area in bacterial attachment to *A. tonsa* as compared with *E. affinis*. Density of strains, *V. cholerae* O1 and O139, attached to copepods were also significantly different.

Source	NDF[a]	DDF[b]	F	P
Strain	1	41.2	6.11	0.0478
Copepod	1	6.09	37.72	<.0001
Strain*Copepod	1	41.2	0.06	0.8154

[a]NDF denotes the numerator degrees of freedom

[b]DDF denotes the denominator degrees of freedom

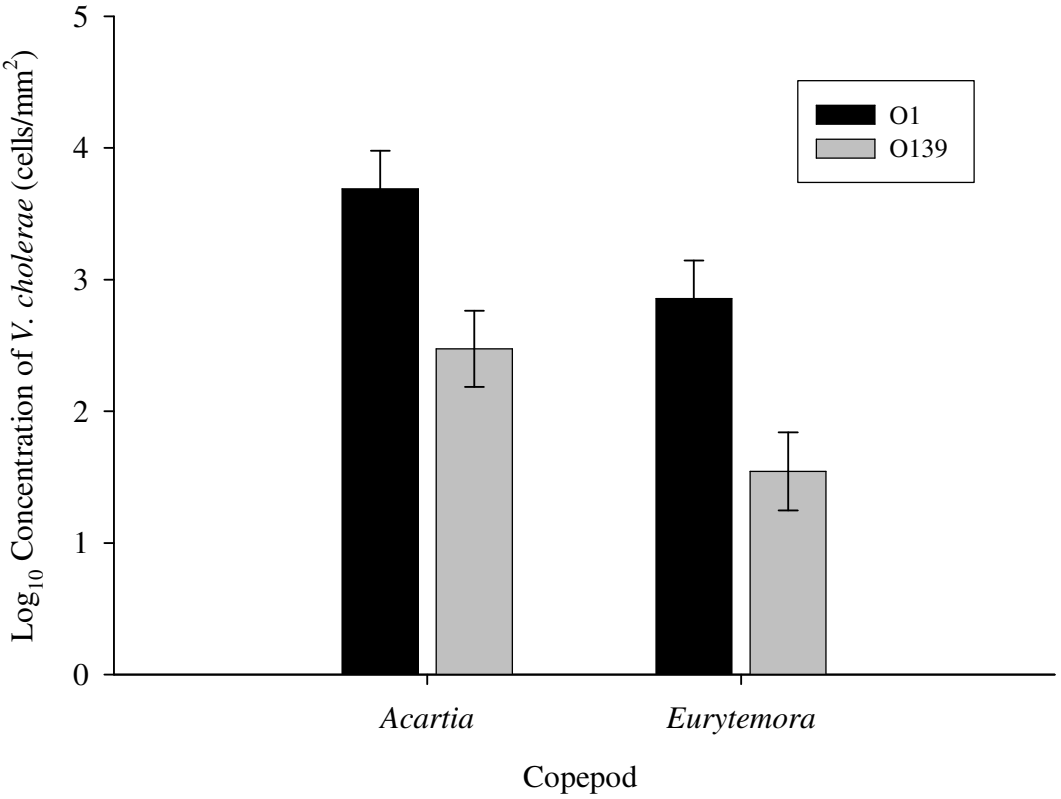

FIG 2.1. *V. cholerae* O1 and O139 attached to *A. tonsa* and *E. affinis*. Bar values represent mean counts of cells per mm^2 of adult carapace surface area. Error bars represent 1 ± standard error of the mean.

A significant difference in total surface area between *A. tonsa* and *E. affinis*, however, may account for the difference reported in attachment per mm^2 (Table 2.2), as there was no significant difference when re-analyzing attachment to *A. tonsa* and *E. affinis* using total cells attached as the dependent variable (one-way ANOVA; Table 2.3 and Table 2.4). Significantly greater surface area of *E. affinis* (Fig. 2.2) and a non significant difference in total cells attached to both *E. affinis* and *A. tonsa* present in microcosms revealed a lower concentration of cells attached per mm^2 to *E. affinis*. *V. cholerae* O1 and O139 were analyzed separately due to differences in population variances between the two serogroups.

There were no significant copepod by species interactions in any of the statistical models, suggesting that copepod type did not significantly affect colonization differences between *V. cholerae* O1 and O139.

TABLE 2.2: Two-way ANOVA (Mixed Model) for the difference in total surface area between *A. tonsa* and *E. affinis* copepods by *V. cholerae* O1 and O139. A significant difference in total surface area occurred between *A. tonsa* and *E. affinis* copepods. Strains, *V. cholerae* O1 and O139, did not differ by surface area.

Source	NDF[a]	DDF[b]	F	P
Strain	1	2.63	0.26	0.6502
Copepod	1	3.18	32.75	0.0090
Strain*Copepod	1	38.5	1.86	0.1800

[a]NDF denotes the numerator degrees of freedom

[b]DDF denotes the denominator degrees of freedom

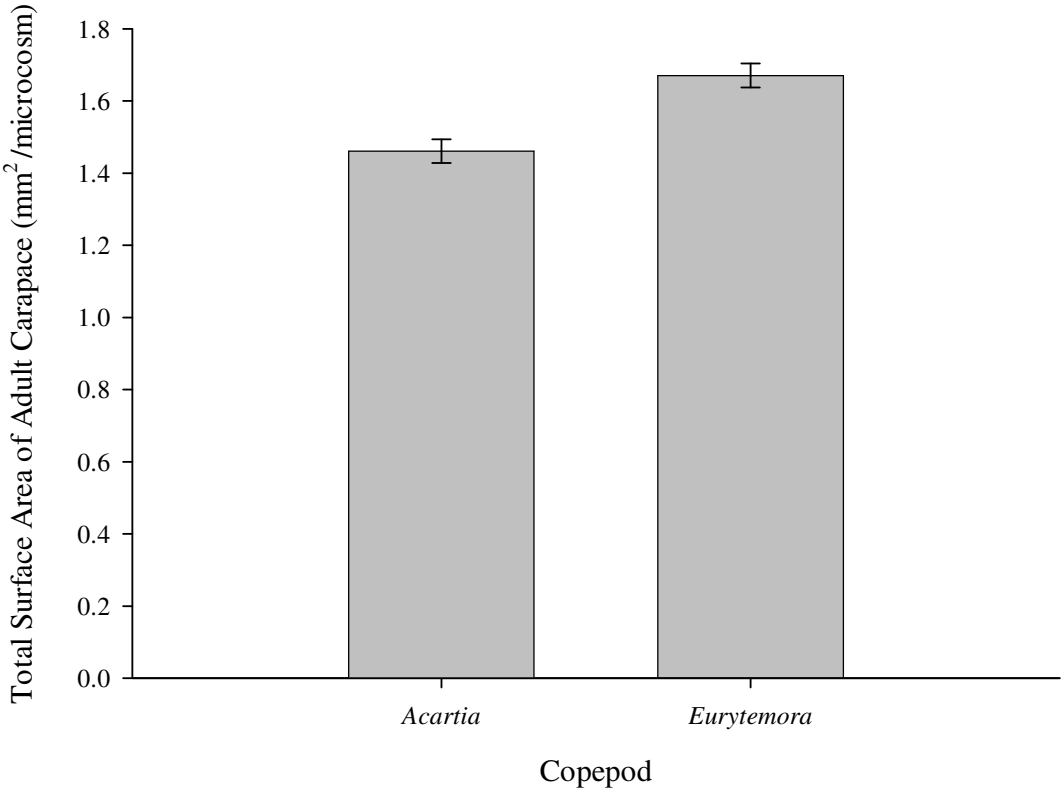

FIG 2.2. Difference in total surface area per microcosm between *A. tonsa* and *E. affinis*. *Acartia* = *A. tonsa*. *Eurytemora* = *E. affinis*. *E. affinis* copepods showed significantly greater total surface area compared to *A. tonsa*. Error bars represent ± 1 standard error of the mean. Mean$_{Acartia}$, n=26 and Mean$_{Eurytemora}$, n=22. Difference in sample number between *Acartia* and *Eurytemora* represent missing values.

TABLE 2.3: One-way ANOVA (Mixed Model) for the colonization of *A. tonsa* and *E. affinis* copepods by *V. cholerae* O1. Colonization of copepods by *V. cholerae* O1, measured by total cells attached, was not significantly different between *A. tonsa* and *E. affinis*.

Source	NDF	DDF	F	P
Copepod	1	5.81	4.20	0.0877

TABLE 2.4: One-way ANOVA (Mixed Model) for the colonization of *A. tonsa* and *E. affinis* copepods by *V. cholerae* O139. Colonization of copepods by *V. cholerae* O139, measured by total cells attached, was not significantly different between *A. tonsa* and *E. affinis*.

Source	NDF	DDF	F	P
Copepod	1	6.28	2.99	0.1326

2.3.2 Colonization of *E. affinis* Adults, Eggs, Nauplii

Time Series: *V. cholerae* O1 and O139 colonization of adults and eggs

Time series data showed significant differences between cell counts of attached *V. cholerae* O1 and O139 over 48 h to *E. affinis* adults (Table 2.5), revealing more *V. cholerae* O1 cells attached than O139 (Fig 2.3). Colonization of *V. cholerae* O1 and O139 to adult *E. affinis* copepods, although different in abundance, increased by over an order of magnitude within the first 24 h of incubation and remained at the same cell counts after another 24 h (Fig 2.3). Attachment to eggs by *V. cholerae* showed a significant difference between strains but not between time periods (Table 2.7), whereby *V. cholerae* O1 attaches in greater numbers than O139 over 48 h (Fig 2.5).

TABLE 2.5: Two-way ANOVA (GLM) for colonization of *E. affinis* adults by *V. cholerae* O1 and O139 over 48 h.

Source	df[c]	MS[d]	F[e]	P[f]
Strain[a]	1	6.98294767	35.01	<.0001
Time[b]	2	4.93140613	24.73	<.0001
Time*Strain	2	0.38142554	1.91	0.1715

[a] *V. cholerae* O1 or O139, attached to copepods.

[b] Time = 0, 24, 48 h of incubation. Actual time points were equal to t + 1 hour.

[c] df = degrees of freedom

[d] MS = mean square error or the mean squared deviation from the mean

[e] F statistic= test of the equality of the means, a one-tailed variance ratio of the groups MS/error MS

[f] P = probability of rejecting the null hypothesis at 0.05

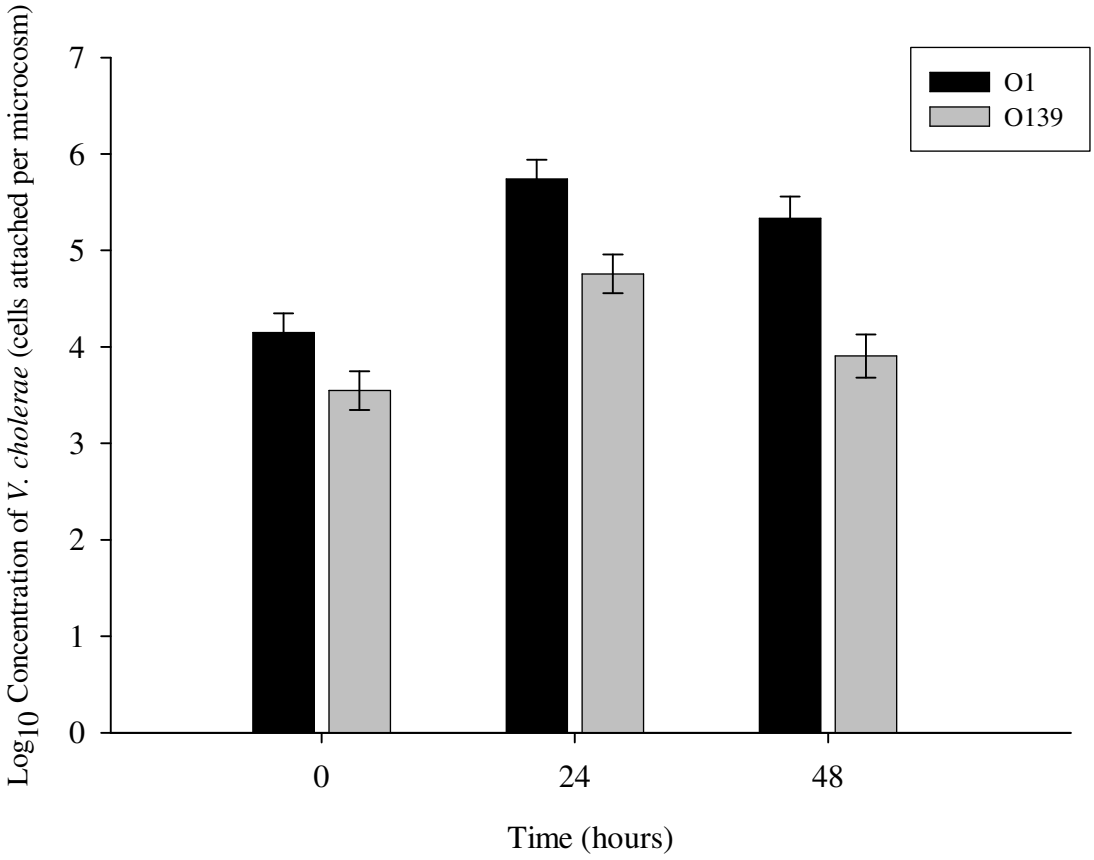

FIG 2.3. Colonization of *E. affinis* adults by *V. cholerae* O1 and O139, measured over 48 h. Bar values are means of total cells attached per microcosms ± 1 SE (n = 5 for means at time 0 and 24; n = 4 for means at time 48).

TABLE 2.6: Two-way ANOVA (GLM) for colonization of *E. affinis* eggs by *V. cholerae* O1 and O139 over 48 h.

Source	df[c]	MS[d]	F[e]	P[f]
Strain[a]	1	13.24906899	16.70	0.0005
Time[b]	2	0.43120080	0.54	0.5886
Time*Strain	2	2.61098147	3.29	0.0571

[a] *V. cholerae* O1 or O139, attached to copepods.

[b] Time = 0, 24, 48 h of incubation. Actual time points were equal to t + 1 hour.

[c] df = degrees of freedom

[d] MS = mean square error or the mean squared deviation from the mean

[e] F statistic= test of the equality of the means, a one-tailed variance ratio of the groups MS/error MS

[f] P = probability of rejecting the null hypothesis at 0.05

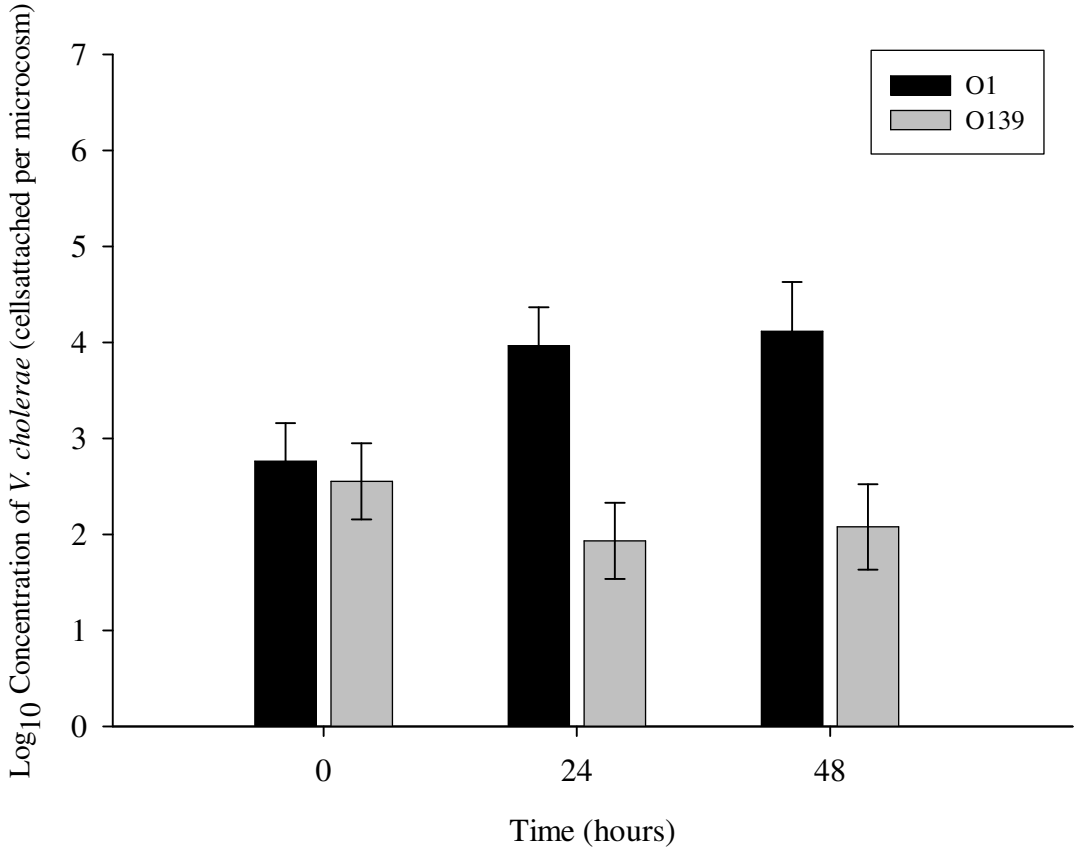

FIG 2.4. Colonization of *E. affinis* eggs by *V. cholerae* O1 and O139, measured over 48 h. Bar values are means of total cells attached per microcosms ± 1 SE (n = 5 for means at time 0 and 24; n = 4 for means at time 48).

Effect of nauplii on colonization of adults and eggs by *V. cholerae* O1 and O139

As shown in Table 2.7, there was an effect of presence or absence of nauplii on the numbers of *V. cholerae* but the effect of nauplii was not the same for serogroups O1 and O139, as indicated by the significant interaction of main effects. Tukey's HSD test, used for *post hoc* multiple comparisons of strain and nauplii, showed that the presence or absence of nauplii did not affect numbers of *V. cholerae* O1 (Fig. 2.4). However, *V. cholerae* O139 attached in greater numbers to adults in the absence of nauplii (Fig. 2.4).

There was also a significant difference between numbers of *V. cholerae* O1 and O139 attached to *E. affinis* eggs (Table 2.8). Again, *V. cholerae* O1 attached in significantly greater numbers than *V. cholerae* O139 (Fig. 2.6). Furthermore, *V. cholerae* attached to eggs in greater numbers when nauplii were present, as compared to attachment when nauplii were absent (Fig. 2.6). Unlike attachment of *V. cholerae* to adults, there was no significant interaction between serogroups and the presence of nauplii when attaching to eggs.

TABLE 2.7: Two-way ANOVA (Mixed-Model) for the effect of the presence of nauplii on the colonization of *E. affinis* adults by *V. cholerae* O1 and O139.

Effect	NDF[a]	DDF[b]	F	P
Strain	1	9.09	116.42	<.0001
Nauplii	1	9.55	11.51	0.0073
Strain*Nauplii[c]	1	9.09	15.28	0.0035

[a]NDF denotes the numerator degrees of freedom

[b]DDF denotes the denominator degrees of freedom

[c]Nauplii had a significant effect on the attachment of *V. cholerae* strain, demonstrated by the Strain*Nauplii interaction. Multiple Pairwise Comparisons were performed on Strain*Nauplii interaction and adjusted using Tukey-Kramer.

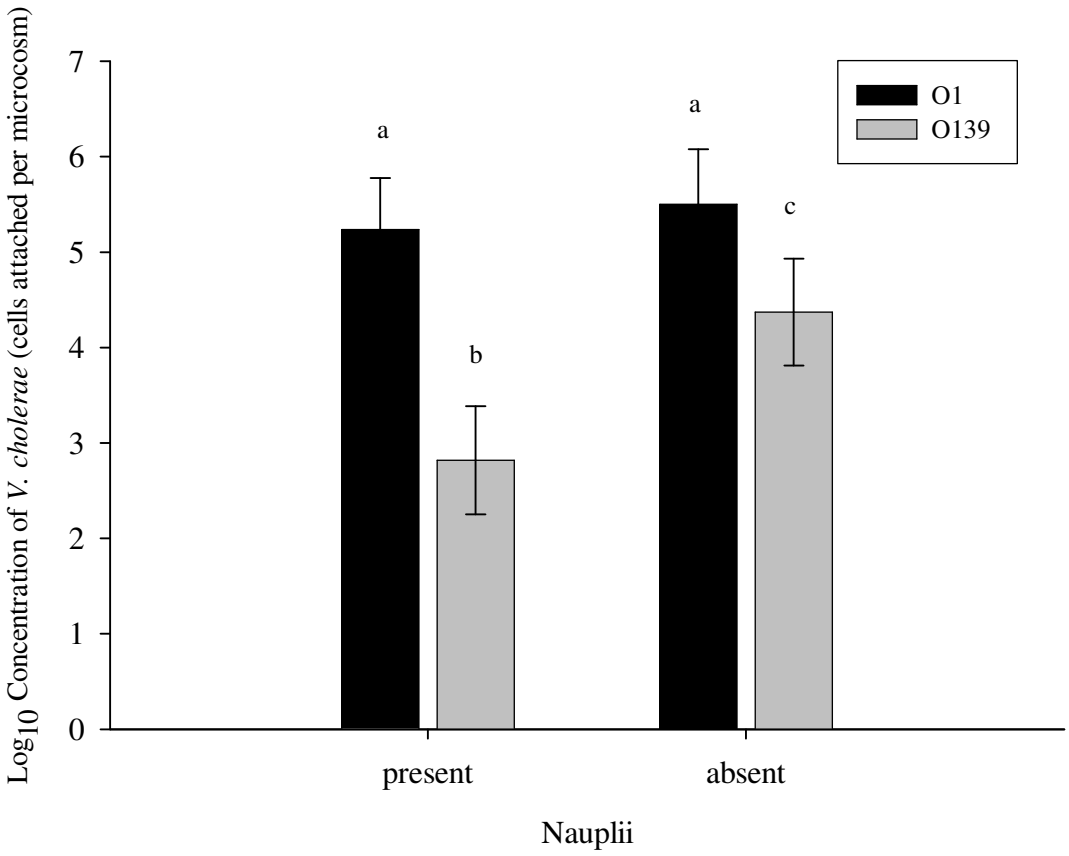

FIG 2.5. Association of *V. cholerae* O1 and O139 with *E. affinis* adults in the presence or absence of hatched nauplii. Bar values are means of total cells attached per microcoms ± 1 SE (n = 4). Letters denote differences between means, adjusted by Tukey-Kramer (significance level $\alpha = 0.05$). Means with different letters are significantly different.

TABLE 2.8: Two-way ANOVA (Mixed-Model) for the effect of *V. cholerae* strain and presence of nauplii on the colonization of *E. affinis* eggs by *V. cholerae* O1 and O139.

Effect	NDF[a]	DDF[b]	F	P
Strain	1	10	12.80	0.0050
Nauplii	1	10	5.81	0.0366
Strain*Nauplii	1	10	1.97	0.1904

[a]NDF denotes the numerator degrees of freedom

[b]DDF denotes the denominator degrees of freedom

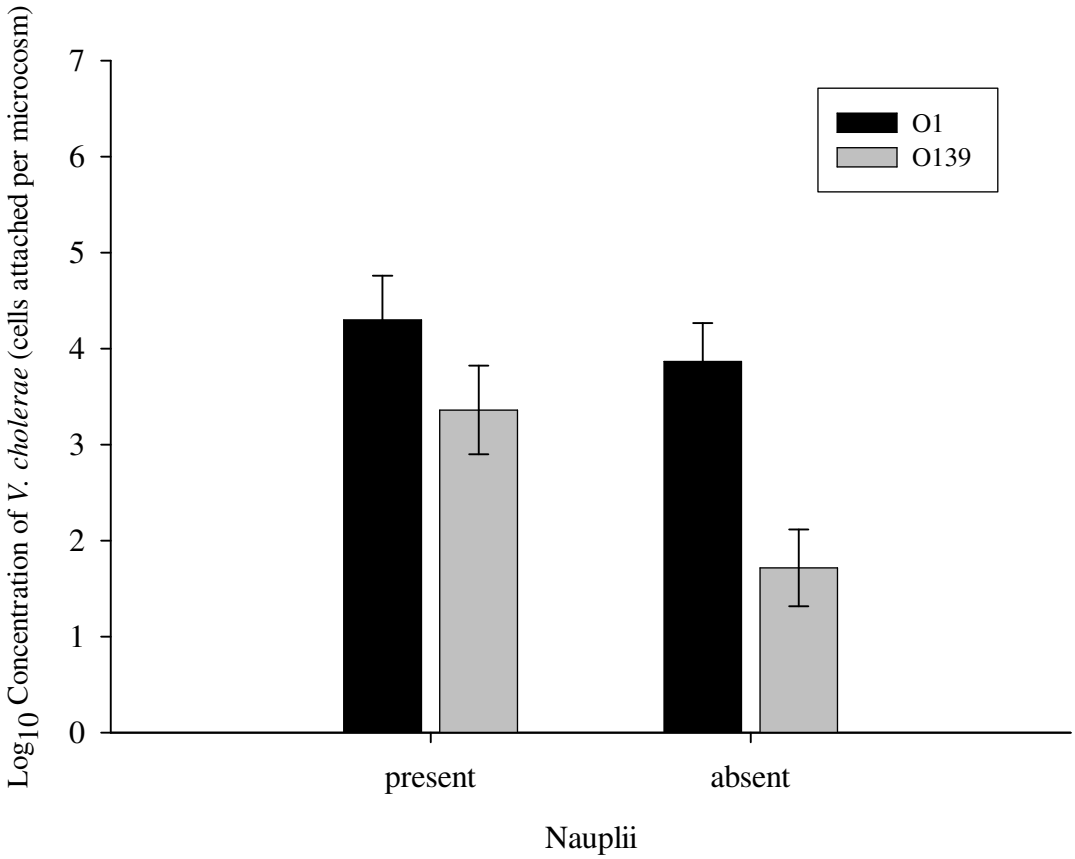

FIG 2.6. Association of *V. cholerae* O1 and O139 with *E. affinis* eggs in the presence or absence of hatched nauplii. Bar values are means of total cells attached per microcosms ± 1 SE (n = 3 for means where nauplii were present; n = 4 for means where nauplii were absent). The difference between strain and the difference between presence and absence of nauplii were significant.

Colonization of adults and nauplii by *V. cholerae* O1

When exploring the attachment of *V. cholerae* O1 to adults and nauplii, occurring in separate microcosms, there was a significant difference in colonization numbers (Mixed Model one-way ANOVA, $f_{df(19)} = 4.37$, $p=0.05$), showing greater attachment to nauplii (Fig 2.7). Total cell numbers were used as the dependent variable, as total surface area between the two life stages was not significantly different (Mixed Model ANOVA, $f_{df(2)} = 4.52$, $p=0.1673$).

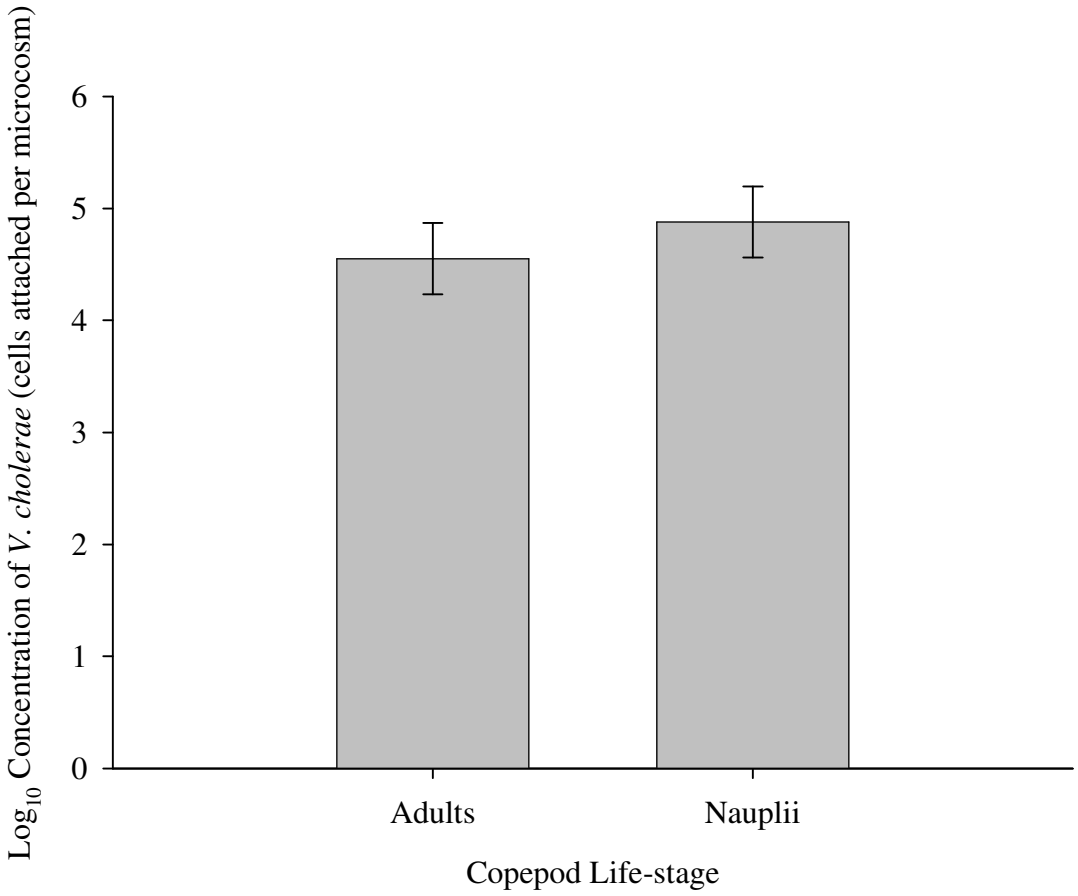

FIG 2.7. Association of *V. cholerae* O1 with *E. affinis* adults and nauplii in separate microcosms. Bar values are means of total cells attached per microcosms ± 1 SE (n = 8).

2.4 DISCUSSION

2.4.1 Effect of Copepod Species and Serogroup on Colonization

Understanding persistence of epidemic forms of *V. cholerae* can be complicated, especially as distributions can be far reaching and can fluctuate temporally or seasonally. Annual shifts in predominance of *V. cholerae* O1 El Tor and O139 Bengal in Bangladesh are an example of how aquatic populations of *V. cholerae* are dynamic. The coexistence of these serogroups has raised many questions about whether they occupy similar or completely separate niches. Despite some phenotypic and genotypic differences, both *V. cholerae* O1 and O139 are quite similar in their molecular characteristics (Johnson et al., 1994), suggesting similarities in response to resources and overlapping regions of their fundamental niches. Both serogroups have caused epidemic cholera in Southeast Asia (Faruque et al., 1998) and the clinical presentation and general modes of transmission, i.e. ingestion of contaminated water, do not differ (Morris et al., 1995). The two serogroups exist in the same environmental landscape, based on GIS mapping and zooplankton sampling data (Huq et al., 1995; Ali et al., 2001).

Results, after 24 h incubation in river water microcosms, demonstrated that *V. cholerae* O1 El Tor and O139 both colonize *A. tonsa* and *E. affinis*. However, *V. cholerae* O1 does so with a higher affinity. The difference in colonization suggests that *V. cholerae* O139 was less efficient at colonizing these copepods. Chiavelli et al. (2001) presented similar qualitative results when comparing attachment of mannose-sensitive hemagglutinin mutants of *V. cholerae* O1 El Tor and O139 Bengal to

Daphnia exoskeletons, but it was unclear whether differences between serogroups were statistically significant. Moreover, Chiavelli et al. (2001) proposed that deficient colonization by O139 may be associated with exopolysaccharide, which contributes to biofilm formation. Although exopolysaccharides provide O139 with the ability to form biofilms, it may hinder initial attachment to plankton. That is, the exopolysaccharide produced by *V. cholerae* O139, which is thicker than that of *V. cholerae* O1, shields outer membrane components involved in zooplankton colonization, i.e. membrane-associated chitin binding proteins (Chiavelli et al., 2001).

Studies of the emergence and preeminence of *V. cholerae* O1 El Tor, as compared to *V. cholerae* O1 Classical and *V. cholerae* O139 Bengal strains, suggest that genotypes that caused the recent cholera epidemics are those that are better adapted to survival in a stochastic aquatic environment (Dziejman et al., 2002). Differential efficiencies of resource use of *V. cholerae* serogroups would thus influence the character of an epidemic because serogroups that are unable to concentrate themselves in a manner in which they can be transmitted, i.e., colonizing copepods efficiently, may be selected against as epidemically viable. It would logically follow that an enhanced affinity *of V. cholerae* O1 El Tor to colonize copepods, compared to *V. cholerae* O139 Bengal., may in part contribute to the dominance of *V. cholerae* O1 El Tor in cholera epidemics.

The difference in resource use between *V. cholerae* O1 and O139 presented here combined with other research may indicate some resource partitioning between *V. cholerae* O139 and O1. During *V. cholerae* blooms when nutrients are abundant, the ability of *V. cholerae* O139 to form biofilms may enable it to aggregate in the

aquatic environment without attaching to a substrate. This is evident in a study of *V. cholerae* O139 during an outbreak in Calcutta, where O139 was found in surface waters in significantly greater numbers than in sediment and on plankton (Ghosh et al., 1994). The potential to cause an epidemic would seem less in this case, but may still be substantial under certain conditions.

Furthermore, a recent comparison of attachment of *V. cholerae* O1 and *V. cholerae* O139 to harpacticoid copepods from the Adriatic Sea showed *V. cholerae* O1 to be less successful in attachment (Pruzzo, personal communication). That is, *V. cholerae* O1 attached in fewer numbers to the copepod populations than *V. cholerae* O139. Harpacticoid copepods are primarily benthic, spending a large portion of their life history dwelling in bottom habitats of aquatic bodies. Calanoid copepods, like the two species studied here, are largely planktonic, existing in the water-column, although *E. affinis* can also be found attached to rocks and pilings.

Despite colonization differences between *V. cholerae* O1 and O139, there was no preferential association of *V. cholerae* with either *A. tonsa* or *E. affinis* when measured as total cells attached in the results presented here. However, Heidelberg et al. (2002b) showed that *Vibrio cholerae-Vibrio mimicus* populations in the Chesapeake Bay were most abundant in both the small and large size classes of plankton sampled during the summer months when *A. tonsa* is the predominant copepod. The average temperature and salinity in the Chesapeake Bay during the summer months when this survey was conducted was 25ºC and 12‰, respectively, optimum growth conditions for *V. cholerae* (Singleton et al., 1982a). Furthermore, Heidelberg et al. (2002a) showed fluctuating abundance of *V. cholerae* in relation to

seasonal shifts in salinity and temperature in the Chesapeake Bay, with greater abundance during summer months. Huq et al. (1994) reported similar seasonal patterns for *V. cholerae* during the cholera season in an area of endemicity, Bangladesh. Thus, similar attachment to *A. tonsa* and *E. affinis* obtained in the results of the present study may be attributed, in part, to the temperature-salinity regime, factors chosen to correlate with *V. cholerae* abundance during summer months. If any species-specific attachment occurs in the aquatic environment during cholera season, specificity may correlate with seasonal cycles of specific zooplankton and seasonal and climatic events.

2.4.2 Colonization of *E. affinis* Adults, Eggs, Nauplii

One benefit of epibiosis is the proximity of the epibiont to nutrients and newly emerging surfaces created by the host (Wahl, 1989). The copepod host provides new, unoccupied habitat patches by molting, as well as by production of eggs and nauplii.

Within the microcosm environment presented in this study, *V. cholerae* attachment to adults and eggs of *E. affinis* became saturated after 24 h of incubation. Thus, any factors limiting attachment may be important in the transmission of genotypes. It is unclear in the results presented in this study what limits attachment. However, saturation of attached *V. cholerae* O1 and O139 cells may not be associated explicitly with spatial limitations, as differences in surface area between the two copepods yet similar total attached cell numbers suggest that total area is not necessarily a limiting factor in attachment. Other factors such as the abundance of nutrients available and the optimal proximity to those nutrients may influence

attached cell abundance. For example, Huq et al. (1983) reported that *V. cholerae* largely accumulated in areas of copepods where exudates were concentrated, e.g., around the mouthparts and on the egg sacs (which are located adjacent to the anal pore). Similarly, results of earlier research revealed that vibrios, including *V. cholerae,* attached to and used the chitinaceous exoskeleton and eggs of aquatic invertebrates as nutrient substrates (Kaneko and Colwell, 1975; Nalin, 1979; Guthrie and Cofie, 1991; Yu et al., 1991; Broza and Halpern, 2001; Halpern et al., 2003; Meibom et al., 2004). The cooperation of bacterial cells through quorum-sensing in biofilms is limited by the size of the three-dimensional structure, based on nutrient diffusion, consumption rates by cells present, and local saturation of growth (Ward et al., 2003). Thus, the ability to attach to copepods in "hot spots" such that *V. cholerae* cells can efficiently use nutritional resources, in microcolonies or biofilms, may be crucial for incidence and distribution during periods of nutrient limitations and may be an important factor in the persistence and/or ascendance of one genotype over another. Furthermore, contact rates of copepods and *V. cholerae* may limit attachment, particularly in the microcosm environment in this study.

Results revealed here showed that the hatching of nauplii affects the distribution of *V. cholerae* attachment to both adults and eggs, especially *V. cholerae* O139. Presence of hatched nauplii influenced *V. cholerae* cell abundance on eggs, such that eggs had greater numbers of cells attached when nauplii were present as compared to when nauplii were absent. One possible explanation for this difference may be production of chemoattractants produced by prehatched nauplii, drawing *V. cholerae* to the egg sac. In this case, the ability of *V. cholerae* to metabolize chitin

and mucin may contribute to hatching of copepod eggs by weakening the integrity of the egg sac and egg casing. The close proximity of *V. cholerae* to hatching nauplii by colonization of the egg sac could further facilitate colonization and distribution of *V. cholerae* to new emerging substrate of the nauplius. The effect of nauplii on the decrease in numbers of *V. cholerae* O139 colonizing adults may be associated with the redistribution from the carapace to eggs, as noted above.

In addition, results revealed greater colonization of nauplii by *V. cholerae* O1, compared to colonization of adults when adults and nauplii occurred in separate microcosms. Although the difference in attachment between adults and nauplii was slight but significant, production of a large number of nauplii that occurs during copepod blooms suggests that nauplii may serve as an amplifying mechanism for *V. cholerae* during cholera season. Similar results were observed for plankton samples collected from the Bangladeshi aquatic environment, demonstrating that at times when nauplii are the majority of the copepod population, *V. cholerae* will continue to be associated with copepods, perhaps at even greater abundance (Huq et al., in preparation). Increases in copepods and consequent offspring production coincide with phytoplankton blooms, which sequentially precede the onset of cholera seasons in Bangladesh (Colwell and Huq, 1994). It would, thus, fit that a period of bacterial transmission, whereby cells are dispersed to nauplii, could facilitate a regional *V. cholerae* bloom.

Similarly, studies of other vibrios have shown initial juvenile stages attracting and providing an important substrate on which vibrio bacteria can settle. The relationship between *Vibrio fischeri* and its squid host*, Euprymna scolopes,* is a good

example, whereby *V. fischeri* from adults colonize the crypt epithelia of squid juveniles. The crypt epithelia of the squid provide an array of amino acids, peptides, and proteins, supporting the proliferation of *V. fischeri* (Graf and Ruby, 1998). Constant proliferation of *V. fischeri* renders the adult host a source of expelled cells that subsequently colonize newly hatched juveniles. Adult squid provide a source habitat of *V. fischeri* for juveniles, which, in time, also become source habitats and maintain the persistence of *V. fischeri* (Lee and Ruby, 1994). Similarly, copepod eggs, particularly those that hatch into nauplii and go though the multiple-molting juvenile life stages may be critical to *V. cholerae* in its regional dispersal and persistence during epidemic-initiating and epidemic periods.

Estuarine zooplankton blooms, in particular, create a situation in which large numbers of eggs and juveniles are produced and often outnumber adults (Miller, 1984). Thus, the abundance of juvenile copepod stages is among the many factors that may significantly influence the patterns of occurrence and distribution of *V. cholerae* in the aquatic environment. Temperature and salinity regulate many of the physiological changes that occur during the life cycle of copepods, i.e., hatching and molting, hence it becomes difficult to uncouple the many abiotic and biotic factors influencing cholera epidemics. It is clear, nevertheless, that the role of juvenile stages of the copepod in cholera transmission merits further investigation.

Chapter 3: Sympatric existence of *V. cholerae* O1 and O139 in copepod habitats

3.1 INTRODUCTION

Since the emergence of *V. cholerae* O139 Bengal in 1992 as an epidemic strain, both *V. cholerae* O1 and O139 have caused epidemic cholera in South East Asia (Faruque and Mekalanos, 1998). In some areas, *V. cholerae* O139 replaced both the Classical and El Tor forms of *V. cholerae* O1 and, in others, *V. cholerae* O139 and O1 El Tor continue to coexist (Siddique et al., 1996). In the coastal areas of Bangladesh, where they coexist, annual fluctuations suggest that populations of each serogroup are highly dynamic. In fact, data accumulated to date show a shift in predominance of infection prevalence between *V. cholerae* O1 El Tor and O139 Bengal (Mukhopadhyay et al., 1996), which has raised questions about whether similar clinical presentations translate into related responses to the natural aquatic habitat (Zo et al., 2002). If so, the dynamics of coexisting serogroups in the aquatic reservoir may involve direct interactions, i.e. competition or facilitation.

The molecular characteristics of *V. cholerae* and its association with zooplankton provide evidence that *V. cholerae* O1 and O139 have the potential to occupy the same niche and possibly compete for the same resources (Johnson et al., 1994; Huq et al., 1995; Ali et al., 2001). To date, it has been difficult to find both serogroups in a given environment at the same time, especially during inter-epidemic periods, which makes it even more difficult to demonstrate whether the two

serogroups are sympatric. Investigators have, however, detected both serogroups attached to some of the same aquatic metazoans (Bhanumathi et al., 2003; Huq et al., 1983, 1995; Islam and Aziz, 1981) but only one of the studies has revealed simultaneous occurrence on the same zooplankton assemblage (Huq et al., 1995).

Although the available evidence reveals that occupation of the same habitat can occur for *V. cholerae* O1 and O139, patterns of resource utilization that may explain the coexistence and yearly fluctuation in prevalence of epidemic serogroups have not been established. If a numerical advantage of one serogroup could prevent the attachment of another to a copepod, attachment ability could favor transmission of the first serogroup. Furthermore, because cholera is dose-dependent (Cash et al., 1974), survival and proliferation of *V. cholerae* in the human gut and subsequent release into the water in large numbers via fecal discharge would affect transmission. Thus, the serogroup that is efficient in colonizing copepods would increase transmission prevalence.

Attachment to copepods may provide *V. cholerae* with the nutrients created by a copepod in its own feeding, making attachment advantageous. In fact, Yu et al. (1991) demonstrated that adhesion to immobilized chitin oligosaccharides by *V. furnissii* required trace amounts of amino acids. Channels circulating nutrients that are formed by the three-dimensional structure of cells in biofilms would enable *V. cholerae* to take advantage of nutrient capture when attached to copepods as compared to cells that are planktonic.

During inter-epidemic periods, when plankton blooms are infrequent or not as pronounced, *V. cholerae* cells may rely on their ability to sequester nutrients straight

from the structural elements of copepods. Vibrios, including *V. cholerae* posses chitinolytic and proteolytic enzymes that degrade the chitin exoskeleton and egg protein of the copepod to use as a nutrient resource (Kaneko and Colwell, 1974; Nalin, 1976; Guthrie and Cofie, 1991; Yu et al., 1991; Halpern et al., 2003). Bassler et al. (1991a) showed that starvation of *Vibrio furnissii* resulted in a 2- to 3-fold increase in chemotaxis to chitin oligomers. It is probable that *V. cholerae* will be similarly attracted to chitin when small molecular weight nutrients are scarce.

Attaching to and breaking down the structural matrix of insoluble chitin substrates into its constituents for use is a highly regulated process (Yu et al., 1991; Bassler et al., 1991a, 1991b). In fact, Meibom et al. (2004) demonstrated a complex regulation of genes, elucidated by Heidelberg et al. (2002), in processing chitin. Chitin utilization involves regulation of genes that enable sensing of, attachment to, and degradation of natural chitin surfaces (Yu et al., 1991; Bassler et al., 1991a, 1991b; Meibom et al., 2004). Furthermore, Guthrie and Cofie (1991) demonstrated that chitin exoskeletons of marine organisms also stimulate cholera toxin production. Any differences between *V. cholerae* O1 and O139 in chitin utilization, i.e., attachment to and acquisition of chitin, could be a significant factor in determining success of one serogroup over the other in copepod habitats, transmission to humans, and the character of cholera epidemics.

The research reported here was undertaken to compare chitin utilization by *V. cholerae* O1 and O139 when they occur sympatrically in and on copepod habitats. Chapter two reported colonization of live copepods, by *V. cholerae* O1 El Tor and O139 Bengal when occurring allopatrically, indicating that *V. cholerae* O1 colonized

the copepod substratum in greater numbers than O139. This study tests whether sympatric colonization of copepods by *V. cholerae* O1 and O139 affected serotype composition in and on the copepod and it examines the ability of *V. cholerae* O1 and O139 to utilize and compete for chitin

Several different laboratory experiments were performed, whereby *V. cholerae* O1 and O139 were introduced into laboratory microcosms in either the presence of copepods or soluble chitin. First, for each serogroup, *V. cholerae* was introduced as an "invader" to copepods, with a pre-established population of *V. cholerae* of the alternate serogroup, to determine if the previously established strain would inhibit colonization of the newly introduced strain and whether colonization by the "invading" serogroup would influence total abundance of the established serogroup. Second, growth of *V. cholerae* O1 and O139 were compared, with soluble chitin as the nutrient source, under the same environmental conditions as the colonization assays. Third, *V. cholerae* O1 and O139 were inoculated in different ratios into aquatic microcosms containing soluble chitin to determine whether relative fitness, a measure of the relative ability to convert nutrient resources into more cells, changed over time when they occurred sympatrically. Fourth, mixed cultures of *V. cholerae* O1 and O139 were compared to their respective unmixed cultures to determine the presence of any competitive interactions.

3.2 MATERIAL AND METHODS

3.2.1 Sympatric colonization of copepods

Microcosms were prepared, consisting of either 10-50 adult copepods of *Acartia tonsa* collected from the Baltimore Harbor or *Eurytemora affinis* collected

from the Patuxent River, both contributing bodies of water of the Chesapeake Bay, Maryland. Stock cultures of copepods were kept in flasks at 20ºC from which they were collected using a 202 µm sieve, washed once with filter sterilized water, and placed into microcosms. Microcosms, 200 ml tall form berzelius beakers (Kimbel-Kontes, Inc., New Jersey), contained 100 ml of filter sterilized river water collected from the Rhode River, a tributary of the Chesapeake Bay, Maryland. Water was filtered through 10 µm, 5 µm, 1 µm filters (Filterite Inc, Timonium, MD), and finally a 0.22 µm polycarbonate filter (OSMONICS, Livermore, CA) and served as the suspension medium for the microcosms, adjusted to a salinity of 15‰ and pH 8.5 (Huq et al., 1994).

Prior to inoculation, *V. cholerae* strains were grown separately in 20 ml tubes of akaline peptone water (1% w/v Peptone, 1% w/v NaCl, pH 8.5) at 37ºC, with shaking at 150 rpm, for six hours. Both serogroups grew to ca. 10^7 cells per ml, determined by direct fluorescent counts (Hasan et al., 1994). The cells were harvested by centrifugation, washed twice, resuspended in filter sterilized river water, inoculated into microcosms to a final concentration of ca. 5 x 10^4 cells per ml.

Microcosms were set-up in a replicated Randomized Incomplete Block Design with a 2x2x2 factorial treatment structure (2 copepod species, 2 initial strains of *V. cholerae*, 2 subsequent additions (+/-) of the other strain). Each copepod-strain treatment was replicated twice and run on two different days, representing a total of four microcosms for each copepod-strain treatment (Fig 3.1).

Microcosms of each copepod species were inoculated with either an initial aliquot of *V. cholerae* O1 El Tor strain C6709 or inoculated with *V. cholerae* O139

Bengal strain MO10. For each species of copepod, additional microcosms contained copepods without addition of either *V. cholerae* serogroup to control for the presence of any naturally associated *V. cholerae* O1 and O139. After incubation for 24 h at 25ºC, half of the treatment microcosms consisting of the initial serogroup, represented here as the "established strain," were inoculated with the other *V. cholerae* serogroup, the "invading strain", such that the final concentration of the “invading strain” was ca. 5 x 10^4 cells per ml. For example, microcosms with *V. cholerae* O1 as an "established strain" received O139 as the "invading strain" and vice versa. However, the other half of the treatment microcosms with pre-established strains was not exposed to “invaders” of the same strain type (Fig 3.1). The microcosms were then incubated for an additional 24 h at the same temperature before final sampling.

Each microcosm was sampled at 48 h with each microcosm representing an independent observational unit. Copepods from each microcosm were collected by filtration using a 153µm sieve and analyzed as independent components. The copepods were rinsed to disassociate loosely attached bacteria. All samples were prepared for direct viable counts (DVC) (Hasan et al., 1994; Chowdhury et al., 1995) and fixed with 5% formalin.

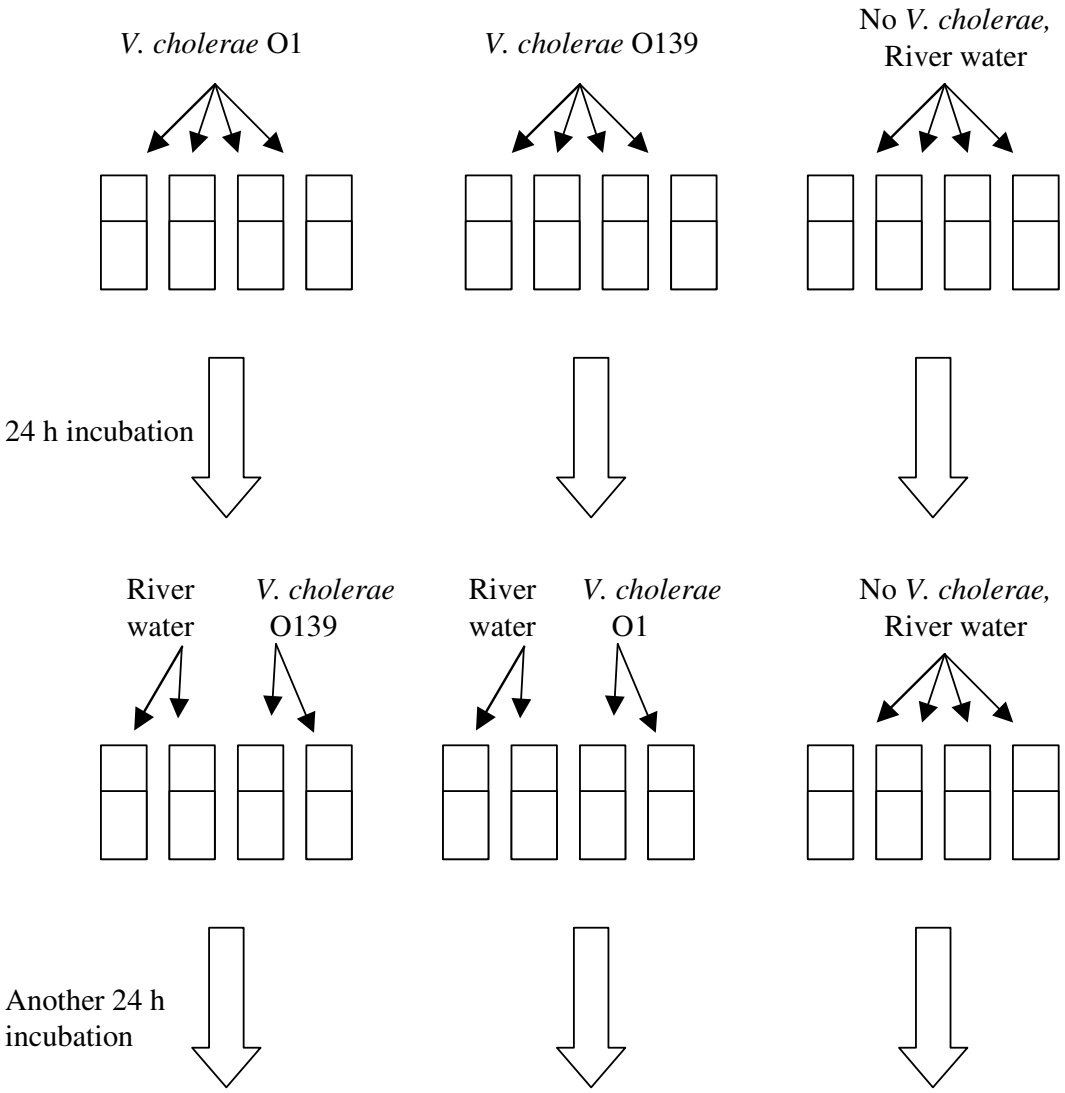

FIG 3.1. Experimental design of sympatric colonization of *A. tonsa* and *E. affinis* by *V. cholerae* O1 and O139. The above design was the same for each copepod species, replicated twice for each treatment and blocked by day. Samples were analyzed after 48 h. Aliquots of river water equaling the aliquot volume of *V. cholerae* cells resuspended in river water were added to microcosms that did not receive *V. cholerae*.

The copepods from each microcosm were counted. Eggs were removed from ovigerous females and these eggs were not included in the analyses. After the copepods in each microcosm had been counted, they were sonicated for 120 sec and homogenized in a tissue grinder. Direct fluorescent antibody reagent (New Horizons, Columbia, MD), specific for O1 and O139, was added to each sample. *V. cholerae* O1 and O139 cells (both non-culturable and culturable cells, combined) were enumerated using an epifluorescent microscope (Leitz Dialux 20, Germany).

Three-way analysis of variance (Mixed Model ANOVA, SAS Institute, Version 8.0) was used to determine differences, if any, in *V. cholerae* strains attached as established bacteria on *A. tonsa* and *E. affinis* copepods in the presence or absence of an "invader" strain. Two-way analysis of variance (Mixed Model, SAS 8.0) was used to further analyze whether attachment to copepods by "established" and "invader" strains were significantly different. To determine whether established and invader strains attach in different numbers, *V. cholerae* O1 and O139 were analyzed separately to avoid violating assumptions of the ANOVA model. Day and day x treatment was included in these Mixed Models as a random effect. There was a small covariate effect of day. It, however, was insignificant. Thus, treatments were combined to obtain a total of four replicate microcosms per treatment. Cell counts were log-transformed to satisfy homoscedacity

3.2.2 Chitin Utilization

V. cholerae O1 El Tor strain C6709 and O139 Bengal strain MO10 were inoculated into separate, 20 ml tubes of Akaline Peptone Water (1% w/v Peptone, 1% w/v NaCl, pH 8.5), incubated at 37ºC, with shaking at 150 rpm, for 6 hours. Both

serogroups grew to ca. 10^7 cells per ml, determined by direct fluorescent counts (Hasan et al., 1994). The cells were harvested, washed thrice, and resuspended in filter sterilized water collected from the Rhode River, a tributary of the Chesapeake Bay, MD [water filtered through 10 µm, 5 µm, 1 µm filters (Filterite Inc, Timonium, MD), and a 0.22 µm polycarbonate filter (OSMONICS, Livermore, CA)]. *V. cholerae* O1 and O139 cells were inoculated into 200 ml beakers as microcosms, containing 100 ml of river water, to a final concentration of *ca.* 5 x 10^4 cells per ml. Aliquots of a soluble chitin oligosaccharide, chitobiohexoase - $GlcNAc_6$, (Seikagaku America, East Falmouth, MA) described as able to induce two extracellular chitinases and a porin that senses oligosaccharides (Meibom et al., 2004), were then added to microcosms containing either *V. cholerae* O1 or O139 so that the final chitin concentration was 56 µg/ml. Controls consisted of microcosms without the chitin oligosaccharide. Treatments and controls were replicated (n=4). Salinity of the microcosms was adjusted to 15‰, pH 8.5. The microcosms were incubated at 25ºC for 72 h, conditions representative of those in endemic and epidemic cholera countries, e.g. Bangladesh and India (Huq et al., 1994).

At 24, 48, and 72 h, 1 ml samples were taken from each microcosm. All samples were prepared for direct viable counts (DVC) (Hasan, 1994; Chowdhury, 1995) and fixed with 2% formalin. After fixation, samples were sonicated for 60 seconds to disrupt any aggregation due to biofilm formation. Aliquots were fixed to slide wells and direct fluorescent antibody reagent (New Horizons, Columbia, MD) for O1 and O139 was applied for DVC. All antigen-antibody associated *V. cholerae* O1 and O139 cells (both non-culturable and culturable cells combined) were

enumerated by epifluorescent microscopy using a Leitz Dialux microscope (Wetzlar, Germany).

Repeated measures analysis (Mixed Model ANOVA, SAS Institute) was used to determine differences in the influence of soluble chitin and river water nutrients on growth of *V. cholerae* O1 and O139 in single culture microcosms over 72 h. Heterogeneous Toeplitz structure was chosen as the best-fit variance-covariance matrix, in part due to the experimental design and fit statistics. Tukey's HSD test was used for *post hoc* multiple comparisons of a significant interaction between time, serogroup, and nutrient (significant at $\alpha = 0.05$). Cell counts were log-transformed to satisfy homocedacity.

3.2.3 Competition for Soluble Chitin

For fitness assays, *V. cholerae* O1 and O139 and media components were prepared as above. However, the two serogroups were inoculated into microcosms, 200 ml beakers containing 100 ml of river water, at different O1:O139 ratios. *V. cholerae* O1 and O139 were present together in three different initial O1:O139 ratios (0.25, 0.50, 0.75) totaling a final concentration of *ca.* 5×10^4 cells per ml. Aliquots of a soluble chitin oligosaccharide were added to all treatments so that the final concentration was 56 µg/ml. Microcosms were adjusted to a salinity of 15‰, pH 8.5, and were incubated at 25ºC for 72 h (Huq et al., 1994). Fitness assays were replicated four times. Samples (1 ml) were taken from each microcosm at 24, 48, and 72 h and were prepared and analyzed as above.

An estimate of relative fitness was used to determine whether different initial ratios of *V. cholerae* O1 and O139 facilitate competitive coexistence between the two serogroups when present in mixed cultures. Differences in fitness between *V. cholerae* O1 and O139 may be caused by differences in the duration of lag phase, growth rates, affinity for resources as they become limiting, survival at stationary phase, or a combination of these factors. Relative fitness between *V. cholerae* O1 and O139 was measured by calculating the ratio of the realized Malthusian parameter for each serogroup (Lenski et al., 1991), a measure of the average rate of change of a genotype, $m_i = \ln[N_i(1)/N_i(0)]/(t)$, where $N_i(0)$ and $N_i(1)$ are the initial density of a genotype and density after some time, respectively. Relative fitness is, thus, the ratio of Malthusian parameters during direct competition of genotypes or $W_{ij} = m_i/m_j$.

Competition was analyzed using a subset of the above experiment, whereby mixed cultures, those inoculated in a 1:1 ratio of *V. cholerae* O1 and O139, were compared to single cultures. Mixed and unmixed cultures were analyzed to determine the effect of a competing strain on growth of *V. cholerae* O1 or O139. Competition assays were designed so that in unmixed microcosms an equal amount of the same serogroup was added as compared to the amount of competitor serogroup added to mixed microcosms. Aliquots of a soluble chitin oligosaccharide were added to all treatments so that the final concentration was 56 µg/ml. Microcosms were adjusted to a salinity of 15‰, pH 8.5, and were incubated at 25ºC for 72 h (Huq et al., 1994). Competition assays were replicated four times. Samples (1 ml) were taken from each microcosm at 24, 48, and 72 h and were prepared and analyzed as above.

Repeated measures analysis (Mixed Model ANOVA, SAS Institute 8.0) was used to determine differences in the relative fitness and differences in growth curves of mixed and single serogroup cultures of *V. cholerae* O1 and O139 to determine the extent of competition between 24 and 72 h. Compound Symmetry structure was chosen as the best-fit variance-covariance matrix, in part due to the experimental design and fit statistics for the analyses determining a change in relative fitness between *V. cholerae* O1 and O139 over time. Compound Symmetry structure was also chosen as the best-fit variance-covariance matrix for analysis of growth curve differences of *V. cholerae* O1 when present in single and mixed cultures to determine the extent of competition. However, the Heterogenous Compound Symmetry structure was used to analyze growth curves of *V. cholerae* O139 in single and mixed cultures. *V. cholerae* O1 and O139 were analyzed separately when comparing differences between mixed and single strain growth curves to avoid non-independence of the mixed cultures. Cell counts were log-transformed to satisfy homoscedacity.

3.3 RESULTS

3.3.1 Sympatric colonization of copepods

Patterns of colonization showed that both *V. cholerae* O1 and O139 colonized copepods in significantly different numbers in the presence or absence of an invader strain (Table 3.1; Fig. 3.2). Overall, *V. cholerae* O1 attached to copepods an order of magnitude greater than O139. Despite the significance in numbers attached, the addition of either *V. cholerae* O1 or O139 positively influenced the numbers of the

established strain, whether *V. cholerae* O1 or O139 had previously colonized the copepods. As for attachment to *A. tonsa* and *E. affinis*, the extent to which *V. cholerae* colonized the two was not significantly different (Table 3.1).

When each serogroup was examined separately, to elucidate colonization patterns between established and invading *V. cholerae*, there were differences present. The extent of colonization by established and invading *V. cholerae* O1 did not differ significantly regardless of copepod species (Table 3.2). However, established and invading forms of *V. cholerae* O139 attached to copepods in significantly different numbers (Table 3.3). In fact, numbers of attached *V. cholerae* O139 were greater as invaders than as established strains (Fig. 3.3) and attached in greater numbers to *E. affinis* than to *A. tonsa* (Fig. 3.4).

TABLE 3.1: Three-way ANOVA (Mixed-Model) for sympatric colonization of *A. tonsa* and *E. affinis* by *V. cholerae* O1 and O139. Pre-established *V. cholerae*, either O1 or O139, was exposed to its opposite as an 'invader' strain in the copepod microhabitat.

Effect	NDF[a]	DDF[b]	F	P
Strain	1	8	60.24	<.0001
Treatment[c]	1	8	16.07	0.0039
Copepod	1	8	2.16	0.1796
Treatment*Strain	1	8	2.62	0.1440
Copepod*Strain	1	8	0.16	0.7038
Copepod* Treatment	1	8	3.07	0.1176
Copepod* Trt*Strain	1	8	1.20	0.3053

[a] NDF denotes the numerator degrees of freedom

[b] DDF denotes the denominator degrees of freedom

[c] Treatment represents whether a strain was invaded or not.

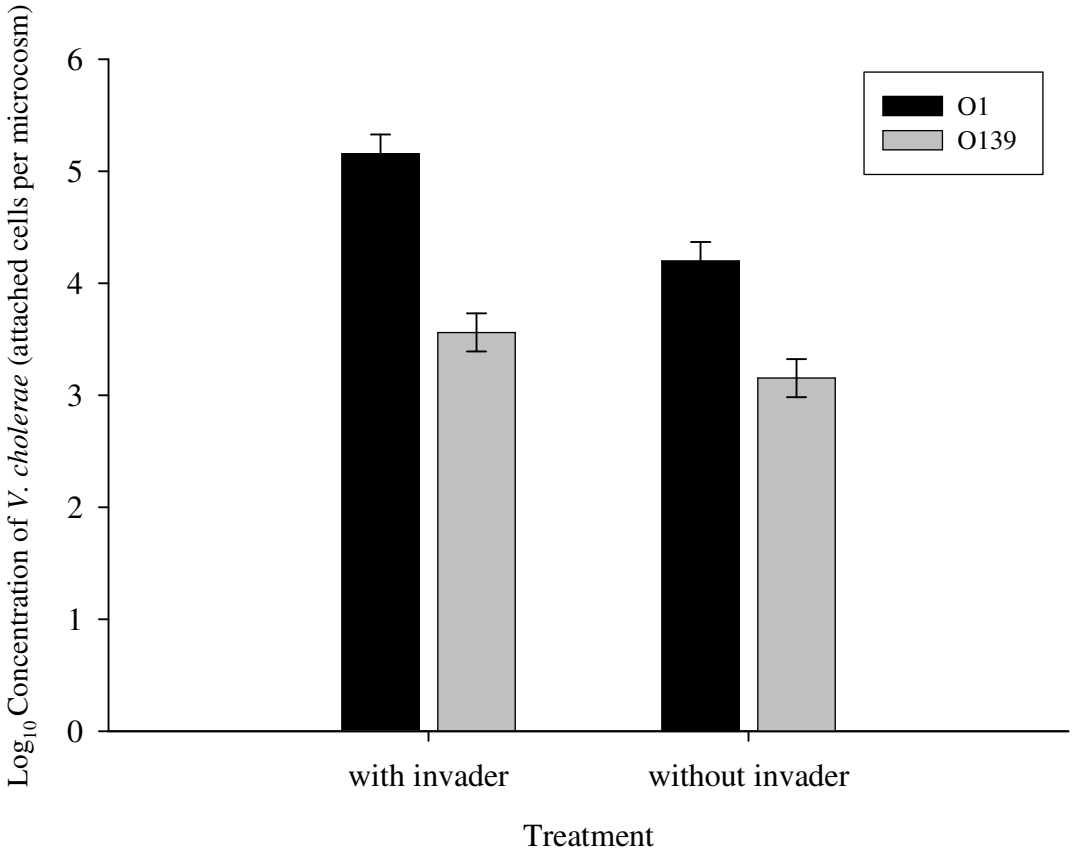

FIG 3.2. Influence of "invader" strains by *V. cholerae* previously "established" on copepods. Error bars represent ± 1 SE of the mean (n = 8).

TABLE 3.2: Two-way ANOVA (Mixed-Model) for sympatric colonization of *A. tonsa* and *E. affinis* by *V. cholerae* O1. The analysis demonstrates attachment of *V. cholerae* O1 as an established strain compared to attachment as an invading strain to *A. tonsa* and *E. affinis*.

Effect	NDF[a]	DDF[b]	F	P
Copepod	1	2	3.35	0.2085
Form[c]	1	10	0.02	0.9006
Copepod*Form	1	10	0.04	0.8387

[a] NDF denotes the numerator degrees of freedom

[b] DDF denotes the denominator degrees of freedom

[c] Form represents whether a strain was an "established" or "invader" strain.

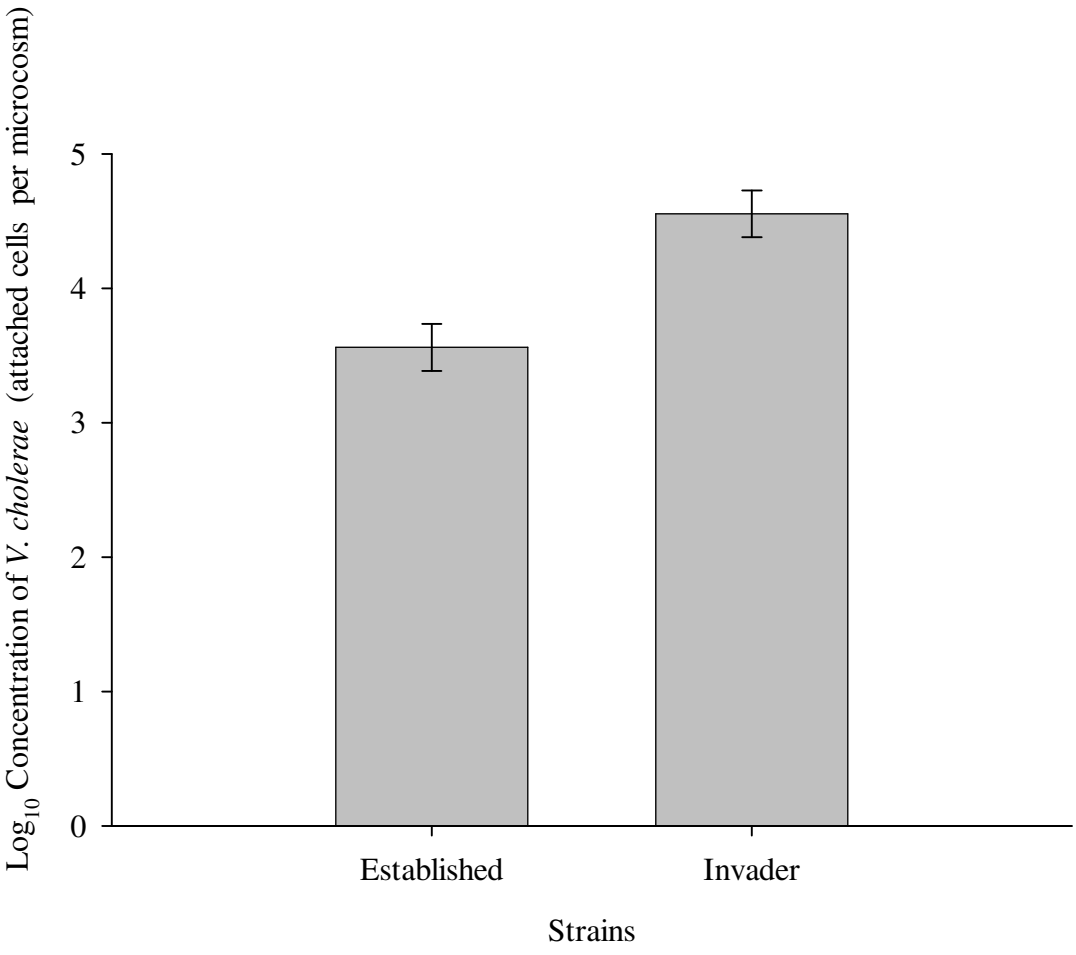

FIG 3.3 Colonization of copepods by "established" and "invader" forms of *V. cholerae* O139. Error bars represent ± 1 SE of the mean (n = 8).

TABLE 3.3: Two-way ANOVA (Mixed-Model) for sympatric colonization of *A. tonsa* and *E. affinis* by *V. cholerae* O139. The analysis compares the attachment of *V. cholerae* O139 as an established and an invading strain to *A. tonsa* and *E. affinis*..

Effect	NDF[a]	DDF[b]	F	P
Copepod	1	12	6.49	0.0255
Form[c]	1	12	16.28	0.0017
Copepod*Form	1	12	0.65	0.4361

[a] NDF denotes the numerator degrees of freedom

[b] DDF denotes the denominator degrees of freedom

[c] Form represents whether a strain was an "established" or "invader" strain.

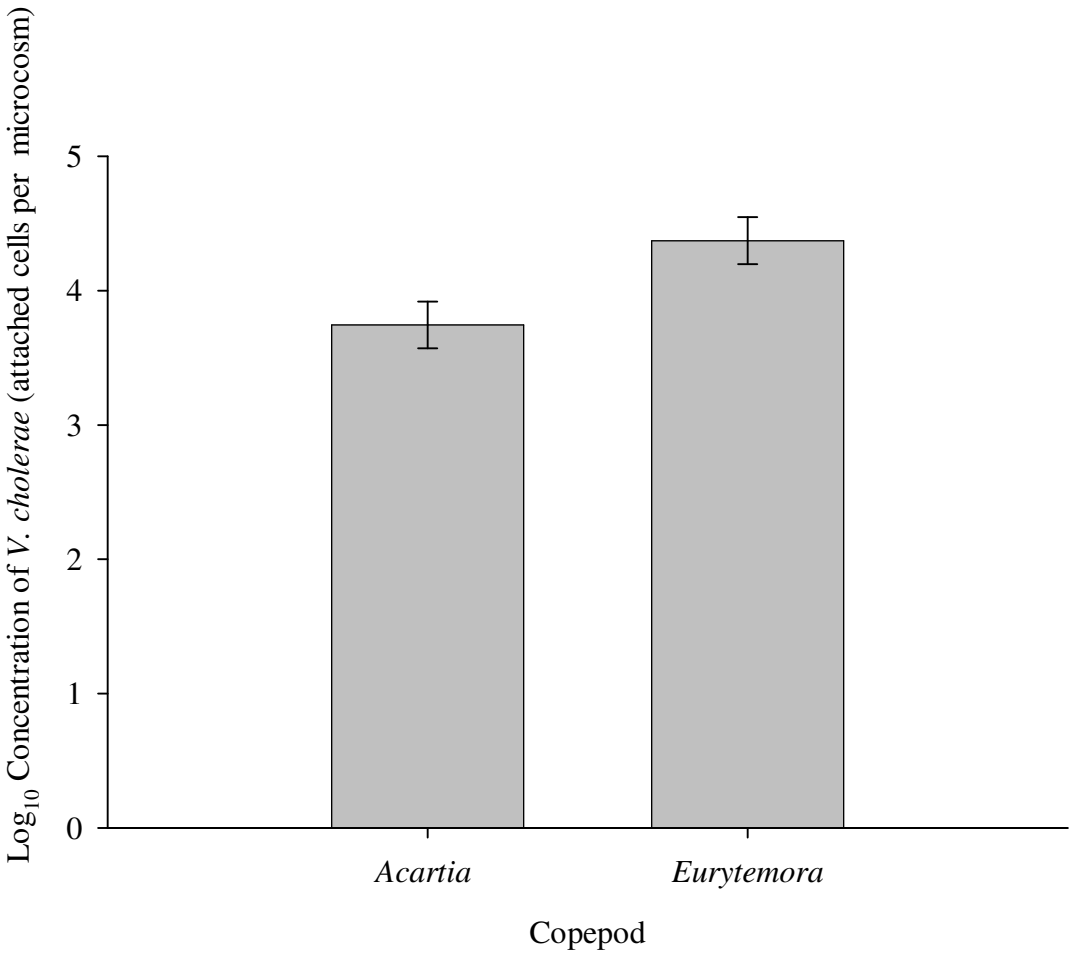

FIG 3.4. Colonization of *A. tonsa* and *E. affinis* by "established and "invader" forms of *V. cholerae* O139. Error bars represent ± 1 SE of the mean (n = 4).

3.3.2 Chitin Utilization

Results showed that soluble chitin contributed to the growth of both *V. cholerae* O1 El Tor and O139 Bengal (Fig. 3.5). The presence of a third level interaction demonstrates that time has an effect on the growth of *V. cholerae* O1 and O139 when in the presence or absence of soluble chitin. It is clear that growth for both *V. cholerae* O1 and O139 was significantly greater in the presence of soluble chitin than in filtered river water alone (Table 3.4). Multiple pairwise comparisons adjusted using Tukey-Kramer to control experimentwise error showed that O1 and O139 were not significantly different when grown using soluble chitin as growth substrate.

Exponential growth of both *V. cholerae* O1 and O139, utilizing soluble chitin as a resource, demonstrated a faster rate of increase as seen by the steeper slope of soluble chitin compared to the slope of exponential growth using river water nutrients (Fig. 3.5). *V. cholerae* O1 and O139 also entered stationary growth phase quicker in soluble chitin microcosms.

TABLE 3.4: Repeated Measures analysis for 72 h growth of *V. cholerae* O1 and O139 using either soluble chitin or micronutrients present in filtered river water as substrate. There was no significant difference between strains. Nutrient type, however, significantly affected growth.

Source of variation	NDF[a]	DDF[b]	F[c]	P[d]
Strain	1	12.2	0.95	0.3484
Nutrient[e]	1	12.2	1507.33	<.0001
Time	2	13.5	50.09	<.0001
Strain*Nutrient	1	12.2	7.07	0.0206
Strain*Time	2	13.5	0.66	0.5329
Nutrient*Time	2	13.5	50.92	<.0001
Strain*Nut*Time	2	13.5	4.61	0.0299

[a] df = degrees of freedom

[b] MS = mean square error or the mean squared deviation from the mean

[c] F statistic= test of the equality of the means, a one-tailed variance ratio of the groups MS/error MS

[d] P = probability of rejecting the null hypothesis at 0.05

[e] Nutrient represents soluble chitin and micronutrients present in filtered river water (collected from the Rhode River, Maryland), serving as suspension medium.

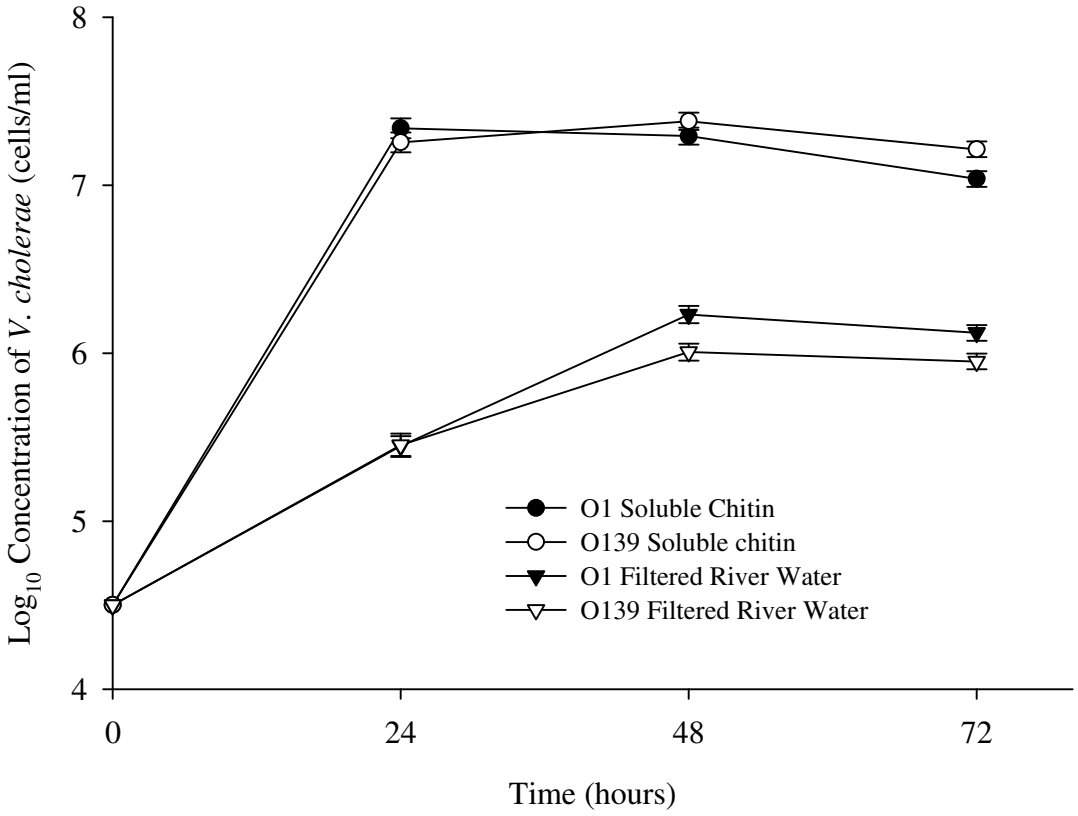

FIG 3.5. Growth of *V. cholerae* O1 and O139 with soluble chitin as substrate. Stimulated growth occurred in microcosms containing soluble chitin. Cells in microcosms without added chitin utilized micronutrients in the river water, collected from the Rhode River, Maryland, serving as substrate but to a lesser extent. Error bars represent ±1 standard error of the mean (n = 4).

3.3.3 Competition for Soluble Chitin

Relative fitness assays between the two serogroups, showed no significant difference between ratios of mixed treatments but a significant difference between relative fitness of *V. cholerae* O1 compared to O139 measured from 0-24 and 24-72 hours (Table 3.5). After 24 h, there was a one to one relationship between *V. cholerae* O1 and O139 (Fig 3.6). Relative fitness after 72 h, however, showed a significant change in fitness between *V. cholerae* O1 and O139 (Table 3.5), which demonstrated either a decrease in the growth rate of *V. cholerae* O1, an increase in the growth rate of *V. cholerae* O139, or both (Fig 3.6).

To evaluate the change in relative fitness, the absolute rate of change in population density was computed between 24 to 72 h for both *V. cholerae* O1 and O139 (adapted from Turner et al., 1996). Results showed that *V. cholerae* O139 had net growth in the presence of O1, whereas O1 had a negative growth rate when mixed with O139 (Fig. 3.7; ANOVA, $f = 33.01$, $p < 0.0001$). Similarly, single strain microcosms containing only *V. cholerae* O1 showed negative rates of change but microcosms with only *V. cholerae* O139 bacteria demonstrated no net change (Fig. 3.8).

TABLE 3.5: Repeated Measures analysis for the relative fitness of *V. cholerae* O1 and O139 using soluble chitin as substrate. There was no significant difference between the mixed treatments. Time, however, significantly affected fitness.

Source of variation	NDF[a]	DDF[b]	F[c]	P[d]
Treatment[e]	2	8.38	3.73	0.0695
Time	1	8.21	29.24	0.0006
Treatment*Time	2	8.14	2.27	0.1644

[a] NDF denotes the numerator degrees of freedom

[b] DDF denotes the denominator degrees of freedom

[c] F statistic= test of the equality of the means, a one-tailed variance ratio of the groups MS/error MS

[d] P = probability of rejecting the null hypothesis at 0.05

[e] Treatment represents the different mixed ratios of O1:O139 (0.25, 0.5, 0.75).

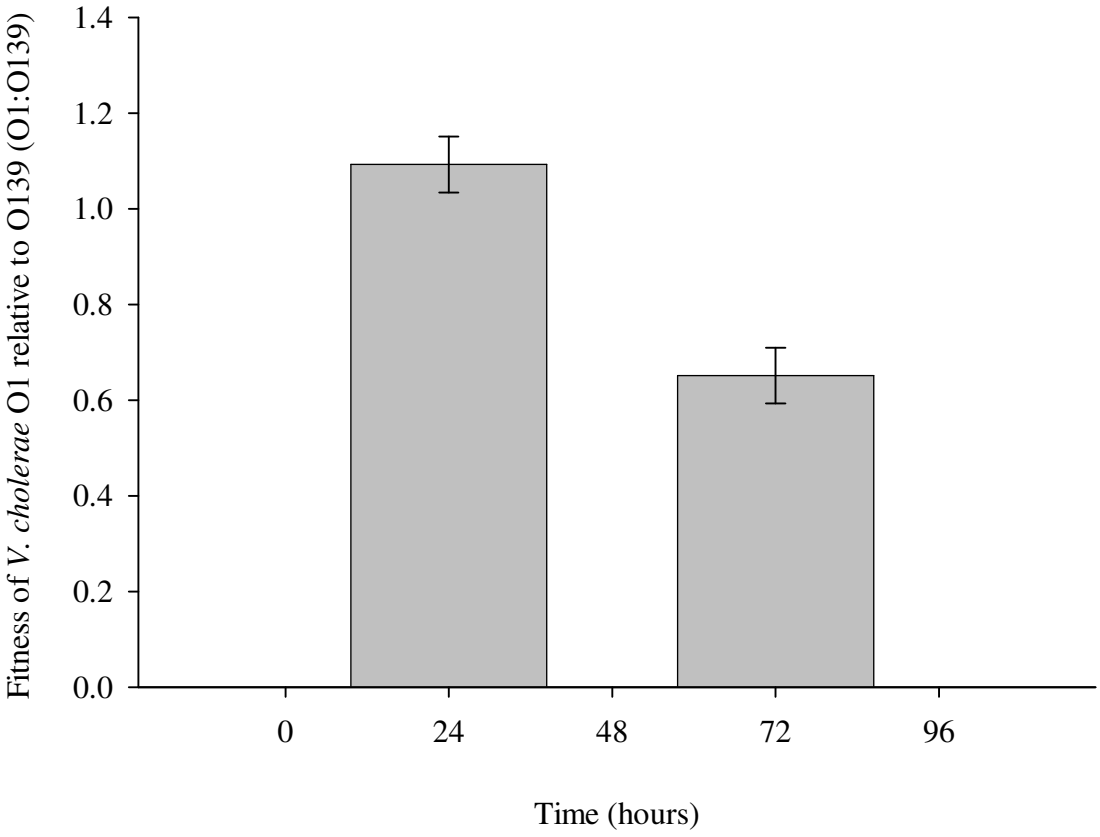

FIG 3.6. Change in fitness of *V. cholerae* O1 and O139 measured at 0-24h and 24-72h, designated as 24 and 72, here respectively. Fitness of *V. cholerae* O1, defined as the ability to utilize the resources available (soluble chitin) for growth purposes, changes significantly over time relative to O139.

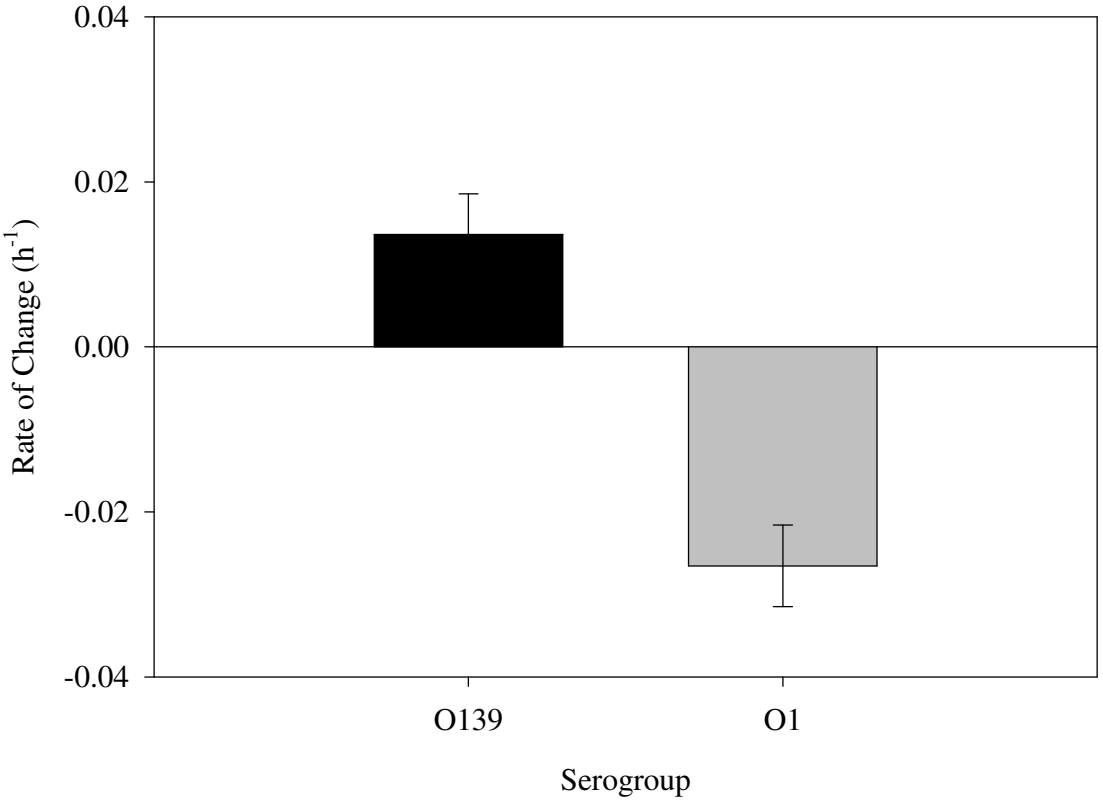

FIG 3.7. Net rate of change in cell concentration of *V. cholerae* O1 and O139 in mixed strain microcosms. Rate of change was measured between 24 and 72 h. *V. cholerae* O139 benefitted from the presence of *V. cholerae* O1, seen here as net growth. However, *V. cholerae* O1 experienced negative growth in the presence of O139. Error bars represent ±1 standard error of the mean (n = 4).

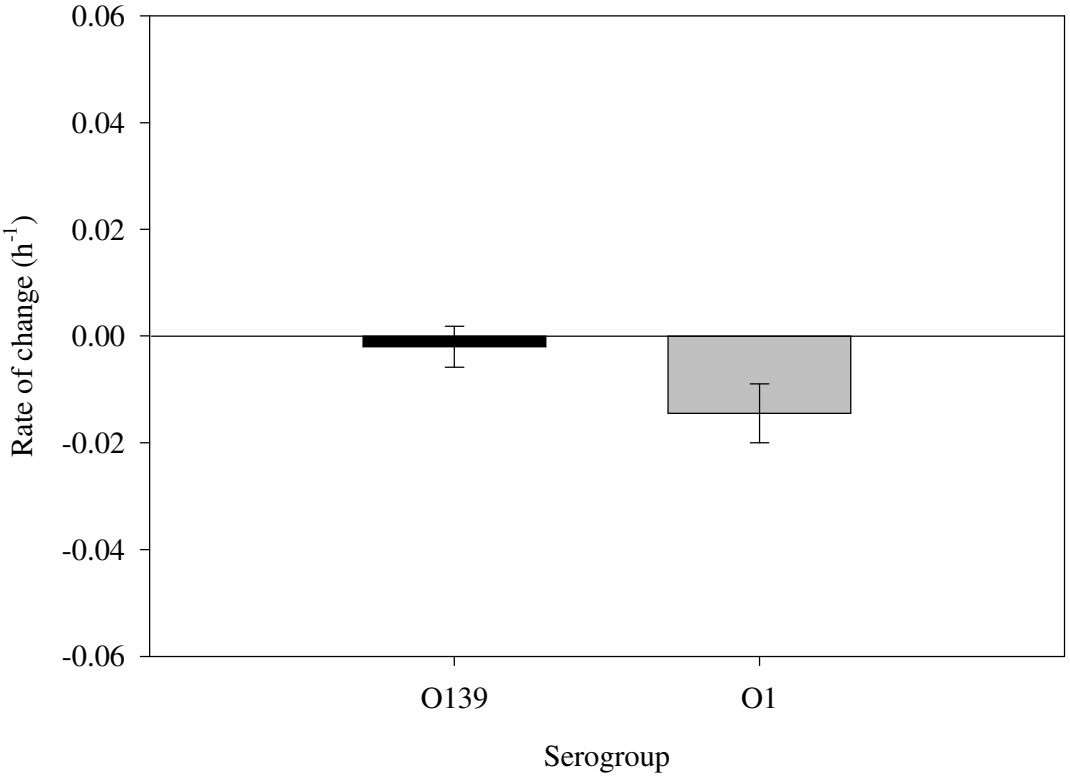

FIG 3.8. Net rate of change in concentration of *V. cholerae* O1 and O139 in single strain microcosms. Rate of change was measured between 24 and 72 h. Neither serogroups expressed net growth in the absence of the other. Error bars represent ±1 standard error of the mean (n = 4).

Results of the competition assay showed that *V. cholerae* O1 grown in mixed and single cultures changed significantly in concentration from each other over time (Table 3.6), whereby the presence of *V. cholerae* O139 influenced the growth of *V. cholerae* O1, such that O1 begins to sharply decline after 24h as compared with single cultures of *V. cholerae* O1 (Fig. 3.9). However, the presence of *V. cholerae* O1 did not have the same effect on *V. cholerae* O139 (Table 3.7). In fact, by 48 h *V. cholerae* O139 in mixed cultures was not significantly different than *V. cholerae* O139 in single cultures (Fig 3.10).

TABLE 3.6: Repeated Measures analysis of growth curves of *V. cholerae* O1 in single and mixed competitive cultures, measured at 24 to 72 h, with soluble chitin as substrate.

Source of variation	NDF[a]	DDF[b]	F[c]	P[d]
Treatment[e]	1	6	23.92	0.0027
Time	2	12	18.35	0.0002
Treatment*Time	2	12	4.53	0.0342

[a] NDF denotes the numerator degrees of freedom

[b] DDF denotes the denominator degrees of freedom

[c] F statistic= test of the equality of the means, a one-tailed variance ratio of the groups MS/error MS

[d] P = probability of rejecting the null hypothesis at 0.05

[e] Treatment represents single and mixed cultures of *V. cholerae* O1. Mixed cultures were mixed in a 1:1 ratio with *V. cholerae* O139.

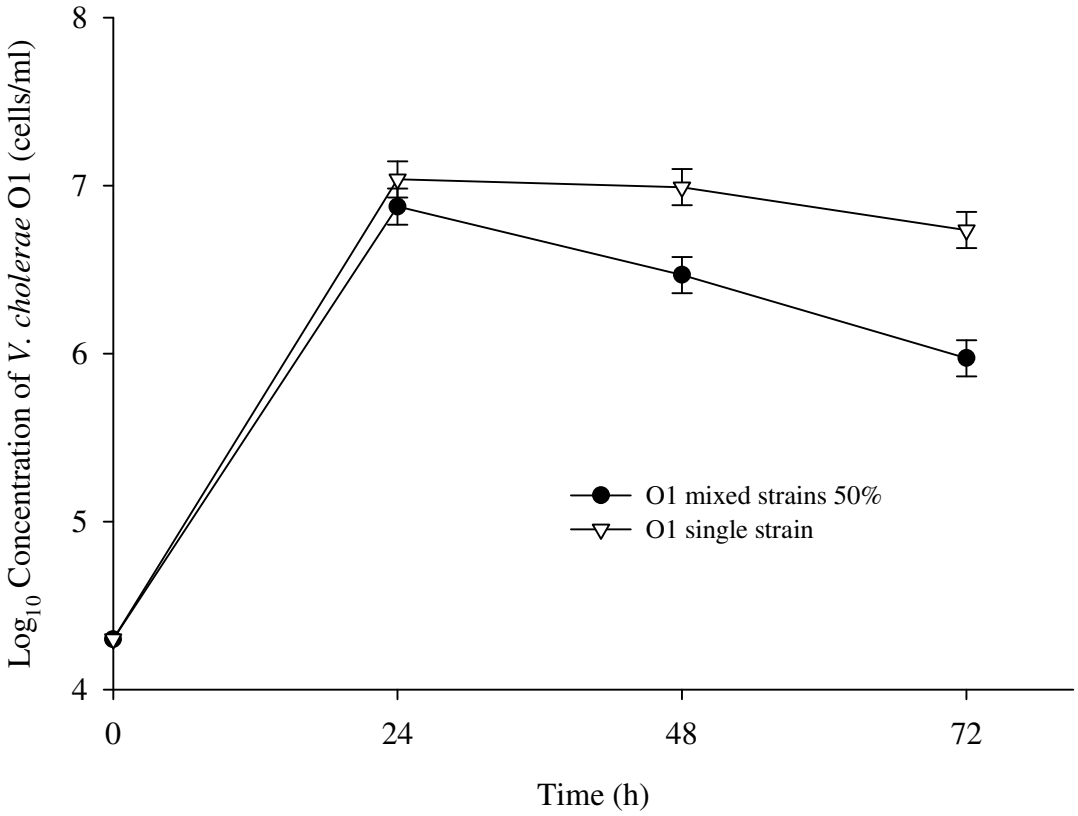

FIG 3.9. Comparison of single and mixed cultures of *V. cholerae* O1. Growth curves demonstrate the effect of *V. cholerae* O139 on the growth of *V. cholerae* O1 over 72 h. *V. cholerae* O1 and O139 were mixed in a 1:1 ratio, represented here in terms of O1 as "O1 mixed strains 50%." Error bars represent ± 1 SE of the mean (n=4).

TABLE 3.7: Repeated Measures analysis of growth curves of *V. cholerae* O139 in single and mixed competitive cultures, measured at 24 to 72 h, with soluble chitin as substrate.

Source of variation	NDF[a]	DDF[b]	F[c]	P[d]
Treatment[e]	1	6.06	28.43	0.0017
Time	2	8.09	2.43	0.1492
Treatment *Time	2	8.09	1.78	0.2295

[a] NDF denotes the numerator degrees of freedom

[b] DDF denotes the denominator degrees of freedom

[c] F statistic= test of the equality of the means, a one-tailed variance ratio of the groups MS/error MS

[d] P = probability of rejecting the null hypothesis at 0.05

[e] Treatment represents single and mixed cultures of *V. cholerae* O139. Mixed cultures were mixed in a 1:1 ratio with *V. cholerae* O1.

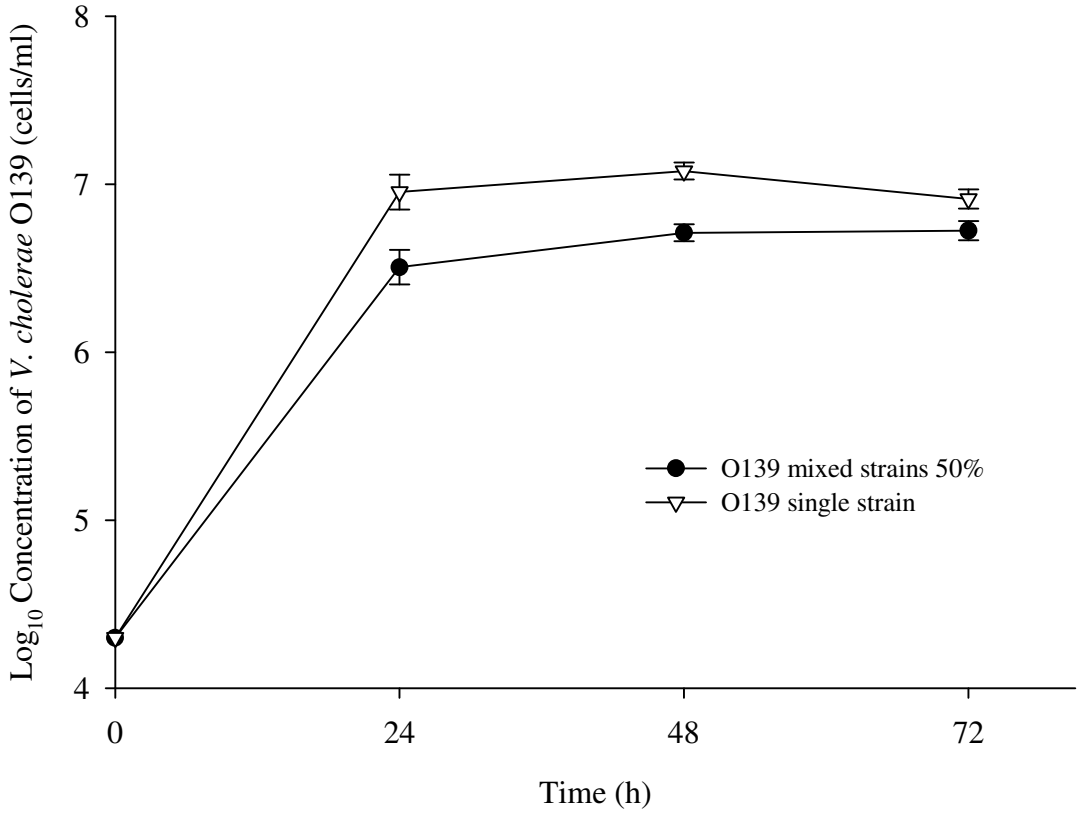

FIG 3.10. Comparison of single and mixed cultures of *V. cholerae* O139. Growth curves demonstrate the effect of *V. cholerae* O1 on the growth of *V. cholerae* O139 over 72 h. *V. cholerae* O139 and O1 were mixed in a 1:1 ratio, represented here in terms of O139 as "O139 mixed strains 50%." Error bars represent ± 1 SE of the mean (n=4).

3.4 DISCUSSION

3.4.1 Sympatric colonization of copepods

A difference in colonization of copepods demonstrated by *V. cholerae* O1 El Tor and O139 Bengal., as presented in results of this study, supports reported differences in zooplankton associated populations of the two serogroups from earlier studies, whereby O1 was found in more plankton samples than O139 (Huq et al., 1995). In the same study by Huq and colleagues, both serogroups also occurred together. Co-occurrence, as reported here, demonstrated that colonization of the copepod by one serogroup did not preclude attachment of another serogroup, nor did the addition of more cells hinder growth or attachment of the previously established cells. In fact, the data suggest that one serogroup already established on the copepod may facilitate the colonization of the other serogroup. Since there are more than 200 serogroups of *V. cholerae* (but only two epidemic serogroups), this phenomenon will need to be extended to other serogroups in the future. Possible mechanisms of facilitation of bacterial attachment include cross-feeding on secondary metabolites, transfer of genetic material., and biofilm formation and intra-biofilm cooperation. Results reported here appear to belong in the category of biofilm cooperation but do not rule out other forms of facilitation.

The ability to attach to copepods even when an established population of *V. cholerae* may be present would be important, especially, during a cholera epidemic when *V. cholerae* cells are returned to the aquatic environment through fecal shedding. Passage through the human gut increases infectivity of *V. cholerae* O1,

with enhanced pathogenicity retained upon reentry into the aquatic environment (Merrell et al., 2002). Increased patch density of copepods during blooms and increased numbers of *V. cholerae* cells through proliferation in the aquatic environment and proliferation in the human gut can facilitate increased colonization of cells that have greater infectivity. With this in mind, it is evident that not only is initial transmission important in defining an epidemic, but also the physiological state of cells returned to the aquatic system from the human gut and their ability to colonize plankton.

It is unclear what stimulated additional attachment or growth of *V. cholerae* cells already established in the microcosm. However, research has shown that biofilm formation on solid substrates are especially important in oligotrophic environments, enabling bacteria to take advantage of the trapping of nutrients by channels formed through three dimensional structure of the biofilm (Marshall, 1976; Geesey et al., 1978). Thus, *V. cholerae* added to nutrient poor microcosms that already contained *V. cholerae* may have stimulated an increase in cell attachment to copepods. Additional *V. cholerae* cells added to microcosms would also increase the probability of contact between copepods and *V. cholerae*, possibly facilitating an increase in cells attached. Furthermore, such additions revealed that the association of copepods and *V. cholerae* cells that were already established in the system was not deterred, suggesting that colonization may protect attached cells from biotic perturbations.

3.4.2 Chitin Utilization

Results from the chitin utilization assay demonstrated similar growth patterns in the presence of soluble chitin for the strains of *V. cholerae* O1 and O139 examined in this study. Low molecular weight nutrients present in river water could account for the growth in control microcosms. Results from previous investigations of *V. cholerae* in sterilized estuarine water showed significant growth, proposed as a result of available micronutrients and absence of competing organisms (Hood and Ness, 1982). Additionally, *V. cholerae* has been shown to survive under various salinities and temperatures simulating estuarine conditions, even when nutrient concentrations were limited or absent (Singleton et al., 1982a, 1982b; Miller et al., 1984).

Although soluble chitin stimulated increased *V. cholerae* cell growth, both serogroups reached carrying-capacity 24 h before *V. cholerae* grown on river water nutrients, suggesting different efficiencies and pathways used to metabolize the nutrients. Differences in the amount of waste build-up in the two systems may also contribute to differential onset of stationary growth, as a greater number of cells would produce more secondary metabolites.

3.4.3 Competition for Soluble Chitin

At these densities and chitin concentration, different ratios of the two serogroups did not demonstrate strong frequency-dependent competition that could suggest stabilizing coexistence. However, a change in relative fitness over time demonstrated differences in ability to utilize chitin between the two strains. The change of relative fitness was evident in competition assays showing that the presence

of *V. cholerae* O139 influenced a decline in *V. cholerae* O1 but not vice versa, suggesting that *V. cholerae* O139 is a better competitor for soluble chitin in the presence of O1. Results presented here also showed different rates of change of the two serogroup strains between 24 and 72 h, suggesting that the change in relative fitness results from both a decrease in *V. cholerae* O1 cells and an increase in *V. cholerae* O139 cells. Increase of the latter, although positive in direction, was insignificant in magnitude, as revealed by nonsignificant growth of *V. cholerae* O139 in mixed cultures from 24 to 72 h. Extending the time frame of the experiment may reveal significant growth of *V. cholerae* O139 during the latter time periods but it was not evident here.

Soluble chitin as substrate in this experiment may not represent the complete activity of serogroups on insoluble chitin exoskeletons in the aquatic environment. In chitin degradation, *V. cholerae* most probably attaches to the chitin polymer, degrades it to soluble oligosaccharides extracellularly, further hydrolyzes the oligosaccharides to N-acetylglucosamine which is converted to glucosamine-6-P, and finally to fructose-6-P, intracellularly, in the periplasmic space, as shown for *V. furnissii* (Yu et al., 1991; Bassler et al., 1991b). Furthermore, Kaneko and Colwell (1975) suggested that adsorption of *Vibrio parahaemolyticus* to copepod exoskeletons may be complicated by materials secreted by the copepod, i.e., the epicuticle secreted by the tegumental gland, which covers the exoskeleton. Therefore, differences in the use of copepods as resource occurring between *V. cholerae* O1 and O139 may begin at steps involved in attaching to and breaking-down the insoluble form, such as the differences revealed between the two serogroups in this and the previous chapter

when comparing their ability to colonize copepod substrates. Once attached, however, superior uptake and efficient integration of chitin and other nutrients into new cells by one serogroup may generate a situation where there is a competitive dominant and a competitive inferior.

3.4.4 Possible Mechanisms of Coexistence

Ecological theorists are beginning to recognize the importance of facilitation, i.e., positive interactions, among organisms as influential in structuring communities (Bruno et al., 2003). Studies of microbial biofilms and surface-attached bacterial physiology and structure are providing evidence to support facilitation as a factor meriting inclusion in niche theory. For instance, Palmer et al. (2001) reported enhanced growth of biofilm forming species involved in dental plaque formation as a result of interspecies and intergeneric facilitation, whereby dental plaque-forming species flourished in numbers together that they were unable to achieve individually. Furthermore, Nielsen et al. (2000) showed that metabolic commensalism of bacterial species, i.e., cross-feeding, where one species metabolizes the secondary metabolites of the other, can influence the spatial structure of biofilms. When cross-feeding occurred, so did mixed biofilm formation. However, when cross-feeding did not occur, species grew in unassociated microcolonies.

If sympatric existence of *V. cholerae* O1 and O139 involves facilitation, then interactions may be contact dependent, whereby cells that are already established in the copepod microhabitat provide a three-dimensional structure, potentially making it easier for further attachment and biofilm formation by other strains. An established

three-dimensional biofilm structure may be more important for attachment by *V. cholerae* O139. That is, its performance as an invader versus as an established strain, reported in this study, suggests that its ability to form biofilms to previously attached *V. cholerae* biofilms may be superior to its ability to attach directly to live calanoid copepod carapace. Furthermore, this study and the study in the previous chapter show that *V. cholerae* O139 attaches in smaller numbers compared to O1.

Research has shown that *V. cholerae* O139 develops biofilm more rapidly than *V. cholerae* O1 El Tor (Watnick et al., 2001). While *V. cholerae* O139 may produce biofilm more effectively, the results of this study and of other investigations (Chiavelli et al., 2001) showed that *V. cholerae* O1 El Tor colonized zooplankton more efficiently. It has been suggested that the exopolysaccharide produced by *V. cholerae* O139 may shield much of the chitin attachment proteins responsible for attachment to zooplankton, making it difficult to colonize zooplankton in greater numbers (Chiavelli et al., 2001). Secondary stages of biofilm formation, characterized by surface accumulation of cellular aggregates and mediated by polysaccharide production, as demonstrated by *Staphyloccus epidermidis* (Heilmann et al., 1996), may be more important in facilitating the association of *V. cholerae* O139 with copepods, than attachment proteins alone.

If facilitation of biofilm by pre-established *V. cholerae* O1, in fact, does occur, then *V. cholerae* O1 may provide *V. cholerae* O139 with the opportunity to attach to calanoid copepods in greater numbers. Such facilitative interactions would promote increased use of a portion of the fundamental niche space already occupied, resulting in periods of sympatry (Bruno et al., 2003). Bruno et al. (2003) further suggested that

the presence of facilitative interactions does not necessarily exclude competitive interactions. In fact, Griffin et al. (2004) demonstrated that the amount of cooperation in pathogenic bacteria can depend on the scale of competition and relatedness, such that the influence of relatedness on cooperation begins to decay as competition becomes more local. The degree of relatedness between *V. cholerae* O1 and O139 may have an influence on facilitative colonization presented here but it is unclear to what extent. Possible facilitative interactions, however, do not entirely explain coexistence or the yearly fluctuations in prevalence between *V. cholerae* O1 and O139, as there seems to be conditions in which one is favored over the other.

Frequency-dependent selection has been hypothesized as one of many mechanisms to maintain genetic polymorphisms and stable coexistence in natural populations, whereby each genotype has the ability to invade an established population when it is rare (Haldane and Jayakar, 1963; Ayala and Cambell, 1974; Levin, 1988). It is unclear whether frequency dependence can explain the coexistence between *V. cholerae* O1 and O139 in the aquatic environment or whether stable coexistence exists within copepod patches. A colonization-competition trade-off more appropriately fits the results of this study. Metapopulation theory suggests that coexistence between species will occur, if there is a trade-off between colonization and competition, such that the species that has superior colonization ability is an inferior competitor, comparatively (Hanski, 1983, 1994, Amarasekare and Nisbet, 2001). This trade-off enables successional use of patches in habitats without regional extinction of either genotype. Colonization results obtained in this study showed that *V. cholerae* O1 attached to copepods in greater numbers over 24 h

whereas fitness assays demonstrated that *V. cholerae* O139 continued stationary growth or persistence on nutrients for a greater period of time.

Without further experimentation, mechanisms of coexistence between the two serogroups in the copepod microhabitat remain hypothetical. Other factors that can contribute to the dynamics of *V. cholerae* and cholera outbreaks may also influence coexistence of epidemic serogroups. For instance, coexistence may, in part, be correlated with the changes in the relative competitive abilities of clones. Results of molecular investigations demonstrated that there is sufficient genetic variation within these populations that annual competitive abilities may fluctuate (Faruque et al., 1997a). For example, the *V. cholerae* O1 El Tor clone that emerged as the epidemic-causing strain in 1996, after the emergence of *V. cholerae* O139 Bengal., was shown to be genetically distinct from the El Tor strain that occurred pre-Bengal (Faruque et al., 1997a). Genetic changes have also been revealed for *V. cholerae* O139 since its initial emergence. Furthermore, genetic and phenotypic differences i.e., in sugar profiles and in polysaccharide production may suggest that *V. cholerae* O1 and O139 largely use different parts of the aquatic habitat, enabling them to coexist regionally in bays, estuaries, and river systems.

Interactions of genotypes of *V. cholerae* both within the aquatic environment and in the human gut, combined with selection, may drive genetic change, influencing the structure and function of the population and ultimately feeding back into genetic differences and emergence of new clones. Simply stated: subtle genetic differences among individual strains can have broader implications for ecological

interactions among populations, community fluxes, and evolutionary dynamics (Bohannan and Lenski, 2000).

Although the results presented here give some insight into the ecology of *V. cholerae* and copepods, to understand more fully the relationship between the two serogroups, copepods, and endemic patterns of cholera will require further research. Determining growth and competition in the presence of zooplankton exudates would provide useful information about the use of such nutrients by the epidemic serogroups of *V. cholerae*. Distinguishing between colonization and growth on copepods may reveal different results for competition of nutrients that could disprove the detrimental effect of *V. cholerae* O139 on the growth of O1, as differences in acquisition of and metabolizing nutrients by bacteria when attached to a substrate compared to bacteria that are planktonic are often different (Costerton et al., 1987). Furthermore, investigations into the effect of seasonal factors, i.e., temperature, salinity, and pH that have been shown to influence the patterns of cholera epidemics, on the *V. cholerae* O1/O139-plankton relationship, could help elucidate further the nature of epidemics caused by *V. cholerae* O139.

Chapter 4: Implications of copepod patchiness on *V. cholerae* distribution

Ecology of *Vibrio cholerae* is inherently complex due to the multi-environment existence of *V. cholerae*. Biotic and abiotic factors and interactions within fluctuating aquatic environments and in the human intestines expose *V. cholerae* to a range of conditions far more extensive than the experiments presented in this dissertation were able to explore. Results here are limited in their description of the *V. cholerae*-copepod relationship both in time and with respect to specific particular biotic and abiotic conditions. The results, however, showed that different copepod species and life stages can have different influences on colonization by *V. cholerae* O1 and O139. Furthermore, when *V. cholerae* O1 and O139 were sympatric, there were no obvious competitive influences on colonization of copepods but competitive effects were present when the serogroups used soluble chitin as a nutrient source under the conditions employed in this study. These results warrant further investigation and discussion, as the copepod-*V. cholerae* relationship is complex at many different spatiotemporal scales and may change if different strains of *V. cholerae* O1 and O139 are explored.

The cholera problem encompasses fundamentals of biocomplexity, defined as, "properties emerging from the interplay of behavioral., biological., chemical., physical., and social interactions that affect, sustain, or are modified by living organisms" (Michener et al., 2001). *V. cholerae* bacteria are influenced by factors at

multiple scales and in multiple environments, i.e., genetic, ecological., local., regional., diurnal., seasonal., and annual. As described in previous chapters, *V. cholerae* bacteria are affected by interactions in the aquatic environment and within the human gut. Additionally, anthropogenic and large-scale climatic effects can directly and indirectly influence *V. cholerae* distribution, transmission, and cholera severity (Lobitz et al., 2000; Pascual et al., 2002).

The complexity of cholera epidemics derives at least in part from the occurrence of *V. cholerae* as an epibiont in the fluctuating ecosystem of estuaries, rivers, and other bodies of water. Field surveys and experimental investigations demonstrate the importance of the *V. cholerae*-copepod relationship on the incidence and distribution of *V. cholerae* in the aquatic environment and on cholera epidemics (Huq et al., 1983, 1984, 1994; Tamplin et al., 1990; Heidelberg et al., 2002a). However, in the conventional view of *V. cholerae* distribution in the aquatic environment, it is assumed implicitly that *V. cholerae* populations are evenly distributed wherever and whenever present in the environment. Very little attention has been given to the heterogeneity of its epibiont host (copepod) distribution and possible patchy existence of *V. cholerae* as well. Any bacterial species that inhabits a transient habitat, such as copepods will experience changes in number, quality, and spatial arrangement of habitat patches over time. The life cycle of copepods, in turn, can exhibit strong seasonal variation in distribution, size/age structure, and abundance.

Although attachment to copepods may facilitate dispersal greater distances as compared with distances that *V. cholerae* could disperse alone, specific spatiotemporal patterns of copepods that could influence cholera outbreaks have not

been fully explored, in part, due to the difficulty in finding endemic populations of *V. cholerae* and the logistics and costs of large-scale sampling. Speirs et al. (2004) developed a spatially explicit demographic model of copepod distributions that could be beneficial to the on-going study of the complexity of cholera by elucidating spatial and temporal maps of copepod recruitment and correlating them with epidemic *V. cholerae* distributions. A brief discussion of the implications of copepod dynamics on cholera epidemics, based on the results presented in this dissertation, results of previous *V. cholerae* investigations, spatiotemporal patterns of copepods, and ecological theories of interactions in stochastic environments, is provided as follows.

Plankton, by definition, are incapable of directly controlling their position in relation to water current, and copepods are notoriously spatially heterogeneous. Populations are distributed in patches, rather than distributed evenly, in the water column, in part, influenced by the distribution of resources in stochastic aquatic environments (Roughgarden, 1977). How patches are distributed can change throughout the day due to vertical migration and can change seasonally due to biotic and abiotic factors influenced by weather patterns. Copepod patches can be defined by a number of attributes, i.e., patch intensity, spacing, species composition, longevity, and spatial dimensions.

To address more effectively the ecological and evolutionary consequences of plankton patchiness, Haury (1977) developed a conceptual framework that divided the plankton aggregation continuum into categories at which spatial and temporal patterns can be observed. These categories describe different scales of plankton interaction and behavior, ranging from the large biogeographic patterns of oceans to

the micro-scale patterns of the individual. Each category encompasses different aspects of copepod ecology, as different processes happen at various spatiotemporal scales. Haury (1977) discussed how these categories could be used to explore copepod dynamics. However, in this chapter these categories are used to explore how different scales of copepod distribution could be used to determine how the copepod-*V. cholerae* relationship at various scales could influence cholera. The epibiotic nature of epidemic *V. cholerae* exposes the bacteria to patterns of copepod dynamics at various scales, which may directly or indirectly influence local interactions and regional persistence of endemic genotypes.

Of the categories presented by Haury (1977), meso, coarse, fine, and micro-scale patterns are most appropriate to the discussion of physical and biological influences of copepod aggregation on *V. cholerae* ecology. Meso to coarse scale effects are large physical processes, i.e., upwelling events, tidal fronts and river currents that can distribute copepods over long distances. Extensive spatial dispersal could distribute *V. cholerae* from water bodies that contain endemic *V. cholerae* populations to uncontaminated water bodies, spreading the possibility of infection from one region to the next. The fine scale category comprises copepod dynamics at the level of population and interactions of individuals. Reproduction rates, migration among local populations, and other biological processes of copepods at this scale affecting patch density and composition will have an impact on metapopulation dynamics of *V. cholerae*. Micro-spatial scales occur, however, at the level of the individual copepod. At this scale, local interactions of *V. cholerae* could affect

ecological and evolutionary trajectories of epidemic genotypes through cell-to-cell interactions, influencing selection.

4.1 Large Geographic Patterns: meso- to coarse-scale

Large-scale influences on the spatiotemporal heterogeneity of copepods are often associated with oceanic frontal zones and river currents. Processes that are particularly significant to copepod distribution at this scale are long-distance dispersal of patches and recruitment of juvenile stages. Meso-scale eddies, frontal zones, and river currents can carry copepod patches over hundreds of kilometers (Haury et al., 1977). This dispersal may be a significant factor explaining the transport of *V. cholerae* and the regional spread of cholera across hundreds of kilometers in Bangladesh. According to results presented here on the association of *V. cholerae* with copepod nauplii, as well as plankton surveys employed in Bangladesh (Huq et al., in preparation), large-scale dispersal of juvenile stages may be important, as the recruitment of juvenile copepods may determine the onset of cholera.

Additionally, stochastic weather events can influence the magnitude of processes at this scale. Siddique et al. (1996) suggested changes in climate, severe weather patterns, and anthropogenic impacts on the environment may influence patterns of cholera epidemics in Bangladesh:

> In the past few decades, soil erosion and construction of barrages and dams in the river system have affected the ecological balance of the area. Increasingly severe flooding and prolonged waterlogging affect mostly the northern and north-eastern regions. The north-western region of the country is becoming

> more and more arid due to reduced river flow. In the southern region, a reduction of freshwater flow has changed the hydrodynamics and resulted in an increase in salinity and incursion of brackish water deeper inland. The Bay of Bengal is subject to violent atmospheric turbulence in the form of cyclones. The last major cyclone occurred in April 1991, when a great tidal surge flooded the entire southern coast. This may have affected the estuarine water quality by mixing sediments dislodged by the force of the tidal surge. The changing ecological balance of different regions of the country and variations in their salinity and nutrient concentration were thought to be associated with geographic clustering of El Tor and classic biotypes of *V. cholerae* O1 prior to the emergence of O139 strains. This may also be the case for the difference in the distribution and dynamics of spread of O139 strains in Bangladesh.
>
> Siddique et al. (1996)

Increases in salinity along rivers, such as the incursion reported by Siddique et al. (1996), can extend the distribution of copepod species up-stream. Furthermore, severe weather events, such as cyclones, can potentially distribute coastal species farther inland than usual. If inland habitats are already perturbed by physical changes, then redistributed species could influence the composition of the endemic copepod population. Overall, changes in the physical attributes of an ecosystem and consequent influx of copepods carrying *V. cholerae* could affect the ecological balance and influence the emergence of novel strains of *V. cholerae*, especially if serogroups associate preferentially to a specific species, genus, or seasonal grouping

of copepods. For instance, if *V. cholerae* O139 attaches preferentially to benthic copepods, as recent research suggests (Pruzzo, personal communication), then a cyclonic event, such as the event Siddique et al. (1996) reported in 1991, may have helped distribute copepods and the attached *V. cholerae* farther then they might otherwise have migrated.

4.2 Population Interactions: fine-scale

Patch density and longevity caused by processes measured on the scale of individual interactions, such as competition, coexistence, niche partitioning, predation, and food acquisition may affect cholera spread, timing of peak infection prevalence, and the biogeography of cholera outbreaks. For instance, *V. cholerae* abundance positively correlates to the density of copepods (personal observation; Colwell, 1996), possibly due to the fact that density facilitates easier dispersal and colonization as dispersal distances from individual to individual are shorter. Therefore, during seasons when copepod patch densities are thin or robust, cholera prevalence may be similarly thin or robust.

Fine scale interactions can also influence species composition and distribution of copepods and their epibionts. Threlkeld et al. (1993) demonstrated that the prevalence of plankton infection by epibiotic ciliates correlated with the presence of a specific zooplankton taxon, such that the abundance of that taxon strongly influenced the abundance and seasonality of the cilitates. Thus, interactions like competition and selective predation that might limit the distribution of specific copepods would be important for epibionts as well.

Seasonal peaks of cholera outbreaks in Bangladesh are correlated with geographical location, demonstrating that *V. cholerae* populations are distributed as a metapopulation, whereby populations are regionally distributed in patches that are separated in space rather than distributed evenly. Additionally, results from investigations showing regional variation in genetic composition of phage types, characterizing individual strains, similarly support the suggested distribution (Glass et al., 1983). Persistence of regional distributions of patchy populations depends on a number of factors, such as the availability of resources, habitat patch size, the degree of isolation of patches, local interactions within patches, and the ability to disperse from patch to patch (Levins, 1969; Hanski, 1983, 1994). As plankton may facilitate nutrient acquisition by *V. cholerae* and its dispersal., colonizing mobile plankton populations would enhance the regional distribution of *V. cholerae.* Furthermore, close proximity to new colonizable substrate, i.e., egg sacs or embryos, may also be important to both local and regional distribution of epidemic *V. cholerae*. Dwyer (1991) demonstrated a similar dynamic while exploring the importance of host density, patchiness, and larval presence in transmission of nuclear polyhedrosis virus (NPV), an insect virus, from infected to healthy hosts. The results suggested that larval stages were particularly important in the transmission and spread of NPV, concluding that transmission models would benefit from including age-specific dispersal rates as it pertains to biological controls (Dwyer, 1991). Similarly, eggs and juvenile copepods provide a means for spread in the aquatic environment by increasing available copepod surface area per cubic meter of water.

4.3 Individual Copepod: micro-scale

Molting rates, chemical cues, and bacterial interactions that could affect *V. cholerae* distribution occur within the copepod spatiotemporal micro-scale. *V. cholerae* growth and distribution may be affected by age-structured molting rate of juvenile stages. Molting rates, which are linked to developmental and growth phenomena of the copepod, change at different developmental stages due to physiological processes, food availability, and temperature (Carlotti and Nival., 1992). Chemicals produced by physiological changes of copepod juveniles may have positive or negative effects on growth of *V. cholerae*. Certain attached genotypes may be more or less sensitive to these chemicals than others, potentially generating genotypes selection. Furthermore, molting could alleviate local interactions, such as competition, by producing more epibiotic surface area. Therefore, different rates of molting by juvenile copepod stages could influence the coexistence of genotypes, depending on the duration of the stage (molting usually occurs within 2-6 days, according to stage), as compared to the generation and competition period of *V. cholerae*.

Close proximity of cells in and on copepods due to sympatric biofilm formation enable horizontal gene transfer, facilitating ecological and possible evolutionary changes mediated through sharing of genetic material (Souza et al., 1991). Examples of gene transfer among *V. cholerae* genotypes are provided in studies of molecular influences of cholera. For instance, results from research demonstrates that *V. cholerae* O139 originated as a *V. cholerae* O1 El Tor genotype and differentiated through a suite of deletions and genetic acquisitions (Bik et al.,

1995; Comstock et al., 1995). One particularly important genetic transfer event was the acquisition of *rfb* genes from O22, O141, and other related non-O1 *V. cholerae* that enable *V. cholerae* O139 to produce a polysaccharide capsule, differentiating it from O1 (Bik et al., 1995, 1996; Comstock, 1995; Dumontier and Berche, 1998). Additionally, cholera toxin is acquired by transduction of DNA from a filamentous phage carrying the CTX genetic element, which can then be transferred laterally between strains, influencing genetic variation and consequent selection of epidemic genotypes (Waldor and Mekalanos, 1996). Cholera toxin can also be transferred to non-epidemic strains, enabling them to cause disease.

Local interactions of *V. cholerae,* such as competition and facilitation on copepods will influence the regional persistence of particular genotypes in the aquatic environment and the quality and distribution of copepod patches will influence the magnitude of *V. cholerae* spread, thus impacting cholera character and prevalence. Furthermore, dynamics in the aquatic environment combined with amplification and dispersal mechanisms that humans provide specifically impact the persistence of the epidemic branch of *V. cholerae*.

Local interactions demonstrated in the results presented in this dissertation, such as colonization, use of newly emerged substrate, and resource utilization, may influence the composition of regional persistence as density of plankton patches and concentrations of limiting nutrients fluctuate (Amarasekare and Nisbet, 2001). Metapopulation models such as that proposed by Ruxton and Rohani (1998) describe dispersal as fitness dependent, such that emigration from a site depends on relative fitness at that site. If dispersal were dependent on fitness, it would help maintain

coexistence between *V. cholerae* O1 and O139 and sustain regional persistence of *V. cholerae* O1, especially during inter-epidemic periods when nutrients are scarce. Ruxton and Rohani further suggest that this type of fitness-dependent dispersal could reduce chaotic fluctuations to simple cycles, synchronizing local population dynamics across patches (Ruxton and Rohani, 1998). As copepod population densities and diversity dwindle, coexistence between *V. cholerae* O1 and O139 may change.

Although scaling up from local interactions occurring in laboratory experiments to large-scale ecosystem dynamics is limited in accuracy, the implications are important to explore. Epidemiological models of cholera suggest that results could benefit from the inclusion of ecological interactions between *V. cholerae* and its environment. For example, Codeço (2001) demonstrated how the reproduction rate of *V. cholerae* in both the aquatic reservoir and the human host is important to epidemic and endemic occurrence. Models of cholera infection presently provide a starting point for further investigations. However, generalities in model coefficients and unknowns in the ecology and epidemiology of cholera cause models to fall short as a predictor (Bouma and Pascual., 2001; Codeço, 2001). Further assessments of these factors could help contribute to more accurate estimations of model coefficients, lending to clearer interpolation of emergence and resurgence. Once variables influencing reservoir persistence and transmission are uncovered for both *V. cholerae* O1 and O139, better assessments can also be made about variant coexistence and thus the future of pandemics.

Chapter 5: Future directions and Conclusion

5.1 Future Directions

To understand disease emergence, it is important to investigate the disease agent as well as its interactions with its environmental reservoir, vector, and other animal hosts (Schrag and Wiener, 1995; Galvani, 2003). Knowledge of the complete life history of disease-causing organisms, what Skelly and Weinstein (2003) call "pathogen survival trajectories", will improve our understanding and approach to disease prevention, control, and surveillance. This "eco-environmental" approach models the pathogen population as it moves through those environments that define its life history and includes human disease surveillance, epidemiological case-control studies, and microbiological and ecological investigations (Skelly and Weinstein, 2003).

Collins (2003) also asserts that an integrative approach is a way to discard the myopia of past paradigms in disease science to address the emergence and re-emergence of diseases such as cholera. He states that, "progress can be made through improved understanding of how health hazards arise through the changing ecology of disease agents [*V. cholerae*] in the environment and the human's relationship to that environment" (Collins, 2003). But, complexity in the interrelationships and linkages between climate, pathogens, and humans make it difficult to predict and manage disease outbreaks and their spread. Vector-borne diseases often interact with multiple hosts and vectors at various spatiotemporal scales. Conventional approaches to infectious disease have been limited in their ability to study parasite transmission

ecology and consequently the way transmission has been modeled (McCallum et al., 2001). Mathematical models of epidemic spread demonstrate that transmission coefficients are important factors in prediction of disease (Anderson and May, 1981; Keeling and Gilligan, 2000). For instance, researchers of bubonic plague demonstrate the importance of studying factors that maintain the pathogen cycle outside the human host (Hinnebusch et al., 1998; Perry, 2003). Understanding that genes affecting transmission of plague to humans also play key roles in maintaining the vector-pathogen relationship and that estimations of bacterial abundance can provide a reliable means to evaluate risk to human populations will be crucial for modeling and monitoring epizootic infections (Hinnebusch et al., 1998; Perry, 2003). Research uncovering specifics of pathogen interactions and mechanisms of disease ecology and evolution would enable better coefficients for predictive models. However, transmission values may be estimated improperly due to unknowns in how pathogen-vector/vehicle interactions influence transmission. When thinking about disease susceptibility and prevention, we should more consistently study aspects of the pathogen life cycle beyond the human host. Therefore, this study concludes with a description of several avenues for future studies of *V. cholerae* that would lead towards a more robust understanding of the ecology of this bacterial species.

Although there are concerns about a "crumbling foundation of infectious disease surveillance" (Berkelman et al., 1994), new tools and a shifting paradigm integrating multiple disciplines are rejuvenating the field of disease science. For instance, landscape ecologists are being brought into the fold of disease surveillance as scientists are recognizing the spatial complexities of vector- and water-borne

zoonoses (Kitron, 1998). Geographical Information Systems (GIS), Global Positioning Systems (GPS), remote sensing, and spatial statistics are tools that are used to gather, integrate, and analyze biotic and abiotic components of cholera on micro and macro-scales (Colwell, 1996; Lobitz et al., 2000; Ali et al., 2001; Pascual et al., 2002). Additionally, molecular biologists are studying how phylogeny can identify differences in ecological interactions of *V. cholerae* genotypes in various regions of Bangladesh, ultimately linking molecular insight to ecologically relevant traits (Zo, 2004 dissertation).

Integrating population biology into disease science is being recognized as an important aspect in studying the ecology of infectious disease agents that have an environmental reservoir or path (Schrag and Weiner, 1995; Schoolnik and Yildiz, 2000). Population biologists explore and describe the interaction of populations within their biotic and abiotic environments to elucidate the relative abilities of various genotypes to survive and reproduce, the relative "fitness" of the genotypes (Lenski et al., 1991). Specifically, they ask questions about whether environments that are stable or fluctuating in space and time and imposing fluctuating selection pressures can maintain polymorphisms. Typical disciplines exploring disease focus on gene function, transient nature of polymorphisms, and physiological mechanisms of disease symptoms. Multiscale and multispatial factors affecting disease symptomology, characteristics, and transmission make it difficult to elucidate clear cause and effect relationships; however studying molecular and physiological processes in their ecological context can help elucidate organismal development and performance (Jackson et al., 2002). Until now, the tools to address genetic and

proteomic responses in situ were not available. Advances in molecular technology, however, have made it possible to identify the expression of thousands of genes simultaneously, using microarray expression profiling. Microarrays exploit the preferential binding of complementary single-stranded nucleic acid sequences, whereby an unknown sample is hybridized to an ordered array of immobilized DNA molecules whose sequences are known. Unlike conventional nucleic-acid hybridization methods, microarrays allow researchers to determine genome-wide gene expression in a given cell type at a particular time and under particular conditions. Thus, when used in experimental settings, scientists can explore the genomic consequences of environmental conditions and population interactions. The combination of these two approaches, functional genomics and experimental population biology, will prove fruitful for exploring ecological and evolutionary principles involving such systems as host-parasite systems, competition, speciation, and transient polymorphisms (Gibson, 2002; Schoolnik, 2002).

The combination of exploring the ecology of infectious disease agents and then applying molecular techniques, such as microarray-based comparative studies, can further illuminate how specific gene function and plasticity of virulence may enhance the persistence of pathogenic lineages. Research including spatial dynamics, pathogen-vector interactions, and functional genomics of disease occurrence and its relation to abiotic factors and vector distribution should benefit significantly from genomic and proteomic microarray techniques. Comparative gene expressions can be assessed to determine whether virulence-associated genes are regulated differently in multiple environments such as the reservoir, vector, and host. Microarray analyses

can be used to assess the extent and nature of genes that are controlled by biochemical signatures in the host-vector microenvironments, which in turn could reveal new concepts about managing disease.

These approaches will yield a more robust understanding of the capabilities of infectious disease agents that interact with environments other than humans. Knowledge of factors underlying disease emergence can help focus resources on key situations and areas worldwide, and develop more effective prevention strategies. Conducting successful interdisciplinary collaborations will depend on *a priori* strategies to overcome potential discretions of knowledge structures among cooperating disciplines that could impede solving the cholera problem (Benda et al., 2002). Collaborating disciplines often approach problem solving at different space and time scales, with dissimilar interdisciplinary language, at different scales of accuracy and precision, and availability of data and predictive models. Therefore, practical considerations will be helpful in multi-scale approaches to cholera.

5.2 Summary and Conclusion

The objective of this study was to examine how *V. cholerae* O1 and O139 utilize the copepod as habitat, considering the role of copepods in the transmission of *V. cholerae* to humans (Colwell and Huq, 1994). Findings are summarized as follows.

1. Both *V. cholerae* O1 and O139 can attach to the copepods, *Acartia tonsa* and *Eurytemora affinis*. The extent of copepod colonization and chitin utilization,

however, suggest that the serogroups do not utilize copepods as a resource precisely the same.

2. *V. cholerae* O1 colonizes *A. tonsa* and *E. affinis* copepods to a larger total population than O139, irrespective of species or life stage.
3. The effect of nauplii on the attachment of *V. cholerae* O1 and O139 to adults and eggs suggest that eggs and nauplii may be important substrate for *V. cholerae* distribution.
4. *V. cholerae*, either O1 or O139, already established on copepods appears impervious to perturbation by the other serogroup. Furthermore, pre-established populations of *V. cholerae* O1 or O139 facilitates attachment of an "invader" strain.
5. The ability to utilize soluble chitin by *V. cholerae* O1 and O139 serogroups appears to be similar, when present in unmixed cultures.
6. However, results of competition assays demonstrate that, in mixed cultures, utilization of chitin by *V. cholerae* O1 and O139 is not the same. *V. cholerae* O139 appears to utilize chitin more efficiently then O1. *V. cholerae* O139 numbers remains in stationary phase from 24-72 h, whereas *V. cholerae* O1 declined sharply after 24 h.
7. Mulitscale and multispatial factors may contribute to the complexity of cholera, especially as they pertain to the ecology and evolution of epidemic forms of *V. cholerae*.

Results of this dissertation, unfortunately, do not elucidate why and how *V. cholerae* O139 has recently predominated as the etiologic agent of cholera. However, it does demonstrate differences in resource use between *V. cholerae* O1 and O139 that may prove to be significant in the ecology of epidemic *V. cholerae*. More precise analyses of the molecular genetic ecology of *V. cholerae* O1 and O139 may provide deeper insight into this phenomenon. The ability of *V. cholerae* O139 to form biofilms may enable it to aggregate more effectively in the aquatic environment, as suggested in a study of *V. cholerae* O139 during an outbreak in Calcutta, where *V. cholerae* O139 was found to be more abundant in surface waters than in sediment or on plankton (Ghosh et al., 1994).

Genetic variation within *V. cholerae* O1 and O139 populations and the varied responses to fluctuating conditions may also contribute to annual changes in prevalence. Bacteria, particularly those that attach to surfaces exposed to fluctuating environments, accommodate changes through complex regulation of genes that enable phenotypic plasticity (Costerton et al., 1987). Furthermore, genetic variation within populations provides plasticity to species, allowing for local extinctions while maintaining regional persistence. *V. cholerae*, like any epibiont, has to adapt to the changes in the copepod microhabitat, such as molting, growth, and temporal and spatial changes in density. *V. cholerae* also has to adjust to spatial and temporal changes in abiotic factors associated with tidal action in estuaries, for example, and the influence that these factors have on its epibiont host. Annual fluctuations in gene acquisition resulting in pathogenic genotypes suggest that population dynamics may be related to the stochasticity of the aquatic environment (Faruque et al., 1997a,b).

Thus, genetic diversity within pathogenic and nonpathogenic *V. cholerae* populations, as seen among the 206 serogroups, and the ability to adjust to changes in the environment can affect the character of an epidemic, by influencing the intensity of inter and intra-specific interactions.

Small shifts in genotypes, however, may go unrecognized until they change the character of an epidemic. This was the case in the emergence of *V. cholerae* O139, as human adults that were considered immune to cholera infections turned out to be susceptible to the newly emerged *V. cholerae* O139 infection. Understanding the synergism of abiotic and biotic interactions that influence aquatic persistence of *V. cholerae* and transmission to humans will help provide better understanding of the shifts in composition of genotypes and, potentially, mechanisms of endemic existence.

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