Will Microbial Manipulation Sustain the Ecological Balance in Shrimp (*Penaeus monodon*) Hatcheries?

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ABSTRACT: A shift in preferred methods employed to contain bacterial diseases in the hatchery phase of shrimp culture has resulted largely from the unsuccessful control by and deleterious effects of chemotherapy. Manipulation of hatchery microbial ecology has gained popularity, but for successful implementation, this niche-filling approach requires a thorough understanding of the epidemiology of bacterial diseases in the hatchery. This study examined the responses of *Vibrio harveyi* populations, (associated with luminescent vibriosis in shrimp larvae) to various physico-chemical factors and various hatchery components. Results showed that *V. harveyi* had a wider range of tolerance to environmental parameters than larvae of *Penaeus monodon,* such that control measures based on manipulation of these parameters might not be feasible. However, it was evident from the results that there were components in the shrimp hatchery environment that could be manipulated to control high populations of *V. harveyi*. The natural microflora of seawater, as well as the microbial flora associated with the diatoms *Skeletonema costatum* and *Chaetoceros calcitrans* negatively affected the survival of *V. harveyi* in experimental mixed cultures. The successful manipulation of such benign microbial components to compete with and exclude potential pathogens is necessary to sustain ecological balance in the shrimp hatchery environment.

KEY WORDS: *Vibrio harveyi*, luminescent vibriosis, shrimp hatchery, *Penaeus monodon*

INTRODUCTION

Luminescent vibriosis due to the bacteria *Vibrio harveyi* is one of the major causes of failure in shrimp, *Penaeus monodon*, larval rearing in the Philippines (Lavilla-Pitogo et al. 1990). Chemical control of the disease based on efficacy of available drugs appears to be limited because of the restricted tolerance of shrimp larvae to drugs and because of the possible development of resistant strains of bacteria (Baticados et al. 1990). Preventive measures are, therefore, necessary. The effect of chlorine as water treatment against *V. harveyi* was tested to establish effective levels for use in hatcheries (Baticados & Pitogo 1990). Furthermore, the sources of *V. harveyi* in shrimp hatcheries were investigated to unravel part of its epidemiology (Lavilla-Pitogo et al. 1992). A detailed analysis of the possible origin of luminescent bacterial disease outbreaks in the shrimp hatchery was reviewed recently (Lavilla-Pitogo & dela Peña 1998) and factors like shifting husbandry and feed practices leading to ecological imbalance were pointed out. From successful efforts in land-based animal production industries, it is generally recognized that environmental management to reduce disease impacts is more reliable for increasing profitability than "pathogen" hunts for most diseases (Stoskopf 1996).

Armed with this view and with growing evidence for feasible microbial manipulation in the larval rearing environment of various aquatic species (Dopazo et al. 1988, Nogami & Maeda 1992, Austin et al. 1995, Garriques & Arevalo 1995, Riquelme et al. 1997, Skjermo et al. 1997), some aspects in the larval rearing of *Penaeus monodon* were examined to give sound basis for effective control of bacterial pathogens in the shrimp hatchery.

The study measured the tolerance of *Vibrio harveyi* to various physico-chemical factors, determined the hatchery conditions and components that could control its growth, and tested its growth in mix cultures with diatoms and other bacteria. All these were aimed at identifying an ecologically sound preventive practice against luminescent vibriosis. This work was based on the premise that sound management of the microbial balance in the hatchery will prevent the creation of a niche for opportunistically pathogenic bacteria.

MATERIALS AND METHODS

Growth response of *Vibrio harveyi* to different salinity, pH and temperature

The growth response of *Vibrio harveyi* to various temperatures, salinity, and pH levels were studied using nutrient broth (NB) medium. Standardized bacterial inoculum contained an initial bacterial density of 10^1 colony forming units (cfu)/ml. For salinity and pH tolerance tests, cultures were incubated at room temperature (27-29°C). Inoculated NB

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for temperature tolerance tests was incubated in a temperature gradient incubator (Toyo Kagaku Sangyu Co. Ltd.) set at 10-39°C with minimum shaking. Viable cell counts were monitored by the spread plate method on nutrient agar (NA) with 1.5% NaCl. Monitoring of bacterial populations was done daily for 7 consecutive days.

Growth response of *Vibrio harveyi* to various hatchery components

The effect of various hatchery components on the survival of *Vibrio harveyi* was determined. This experiment was done in 3.5 l glass jars containing 2 l autoclaved seawater. A treatment using untreated seawater which was obtained directly from SEAFDEC's seawater line, was included to measure the effects of natural components of seawater. *Vibrio harveyi* was added to the following: (a) autoclaved seawater, (b) untreated seawater, (c) mixed diatoms (*Skeletonema costatum* and *Chaetoceros calcitrans*) in autoclaved seawater, (d) mixed diatoms and larvae in autoclaved seawater, and (e) larvae in autoclaved seawater. Diatoms were obtained from peak cultures and added into the vessels to obtain initial cell densities of 30,000 - 50,000 cells/ml. Larvae were washed gently to remove surface-attached bacteria and debris. Inoculated bacterial suspensions resulted to an initial density of 10^4 cells/ml. The jars were aerated and incubated under static conditions at room temperature. *Vibrio harveyi* populations were monitored daily for 7 days by the spread plate method using NA with 1.5% NaCl.

Determination of the bacterial load of *Skeletonema costatum*

One-liter samples from *Skeletonema costatum* cultures at different phases of growth were obtained, serially diluted and spread plated on NA with 1.5% NaCl to determine the total bacterial count and luminescent bacterial count. To determine the number of presumptive *Vibrio*, samples were plated on thiosulfate citrate bile sucrose (TCBS) agar, a selective medium for *Vibrio*. Plates were incubated at room temperature (28-30°C) and colonies were counted after 24 h.

Mixed culture tests

The growth pattern of *Vibrio harveyi* in various mixed cultures and in cell-free diatom filtrates was tested further to determine the factors that influence its population. *Vibrio harveyi* cultures used in all tests were grown overnight on nutrient agar with 2.0% NaCl, harvested, and suspended in sterile seawater. Initial densities in test containers were not more than 10^2 cfu/ml in all cases. The following mixed culture tests were done.

Skeletonema costatum and *Chaetoceros calcitrans* populations

Two levels of *Skeletonema* and *Chaetoceros* were used: peak cell cultures and feeding levels. The test was done using 500 ml sterile flasks with 250 ml of test mixture. Preparation of feeding densities was accomplished by adding portions of peak cell cultures to sterilized seawater to obtain a density of 30,000 - 50,000 cells/ml. Control flasks contained sterilized seawater only. Inoculation was done as described above.

Cell-free diatom filtrate

To know which component of the diatom culture had an influence on populations of *V. harveyi*, peak cultures of *C. calcitrans* and *S. costatum* were centrifuged to remove the cells. The supernatants were sterilized by filtration using 0.22µ Millipore filters. The filtrate was then distributed at 250 ml/flask. Control flasks contained sterile seawater only. *Vibrio harveyi* was inoculated as previously described.

Commercial bacterial products

The growth response of *V. harveyi* to the presence of two commercially available bacterial products was tested. Aqua Bacta Aid (ABA, Argent Laboratories) is a suspension reportedly containing 7 species of live complementary bacteria for use in hatcheries to improve water quality. NS Series HF is advertised as a scientifically blended concentration of selected, adapted and cultured bacteria formulated with enzymes and special buffers to help minimize problems in prawn hatcheries. Product ABA was available in liquid form while HF was available in granular form. Sterile seawater was used as the test medium and control. The manner of preparation of solutions and product concentration followed the manufacturers' directions. *Vibrio harveyi* was prepared and inoculated as previously described.

Mixed cultures were kept with minimum shaking throughout the tests. Bacterial counts was carried out upon inoculation of test containers and daily for 3 to 5 days thereafter, applying standard spread plate methods using nutrient agar with 1.5 % NaCl. All cultures were maintained at room temperature (28-30°C). Luminescent colonies of *V. harveyi* were counted in a dark room.

RESULTS

The growth patterns of *Vibrio harveyi* at various temperature, pH, and salinity levels are shown in Figs. 1, 2, and 3, respectively. Fig. 1 shows that *V. harveyi* can grow well at salinity levels of 5 to 70 parts per thousand (ppt), although it took two days for the bacteria to reach peak populations at 5 and 70 ppt. High numbers of colony forming units were obtained at various salinity levels up to and beyond 7 days. However, bacteria inoculated into NB without NaCl and those at 80 ppt died within one day of inoculation.

Results of the pH tolerance test are shown in Fig. 2. The growth patterns of *Vibrio harveyi* at pH 6 to 9 were very similar. However, at pH levels of 5 and 10, no colonies were recovered after one day. The result of the temperature tolerance test is shown in Fig. 3. The bacteria survived at temperatures of 14-37°C, but rapid growth occurred from 17-35°C (Fig. 3). High populations of bacteria remained viable in the tests even after 7 days of inoculation.

The growth patterns of *Vibrio harveyi* exposed to various components in the hatchery are shown in Fig. 4. Bacterial density in all treatments after inoculation was 10^4 cfu/ml. It is interesting to note that in the absence of competitors in sterilized seawater, *V. harveyi* sustained high populations of up to 10^6 cfu/ml, even after 7 days. By contrast, the population of *V. harveyi* inoculated into untreated seawater obtained directly from SEAFDEC's seawater line started to

Figure 1. Growth patterns of *Vibrio harveyi* at different NaCl concentrations.

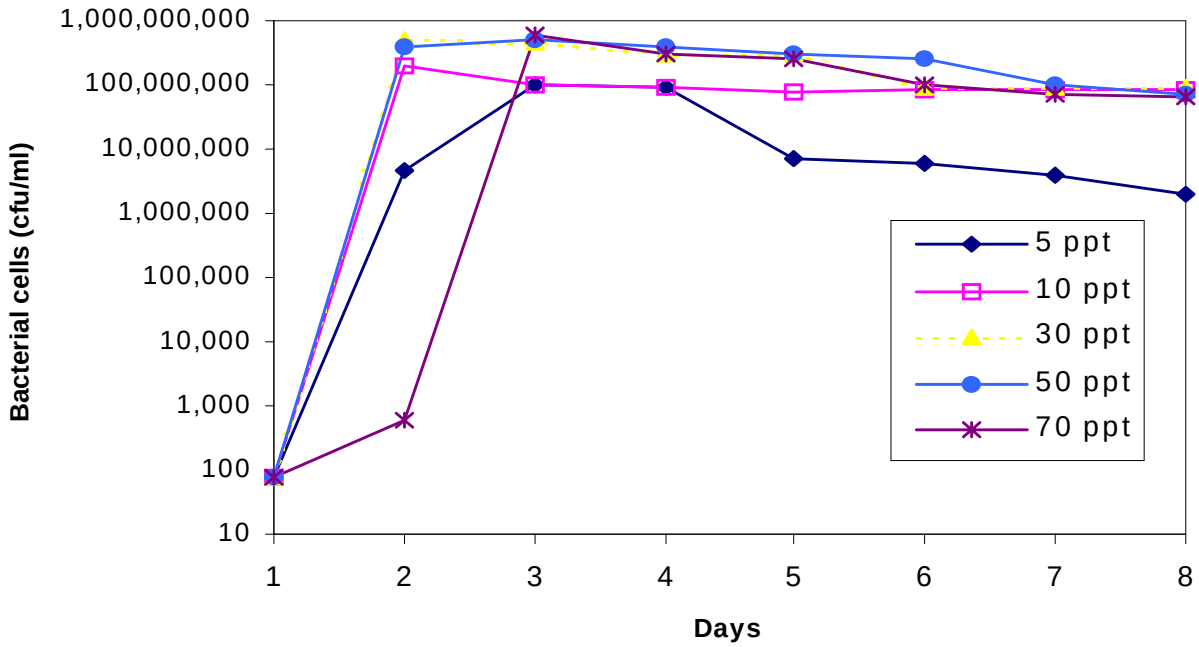
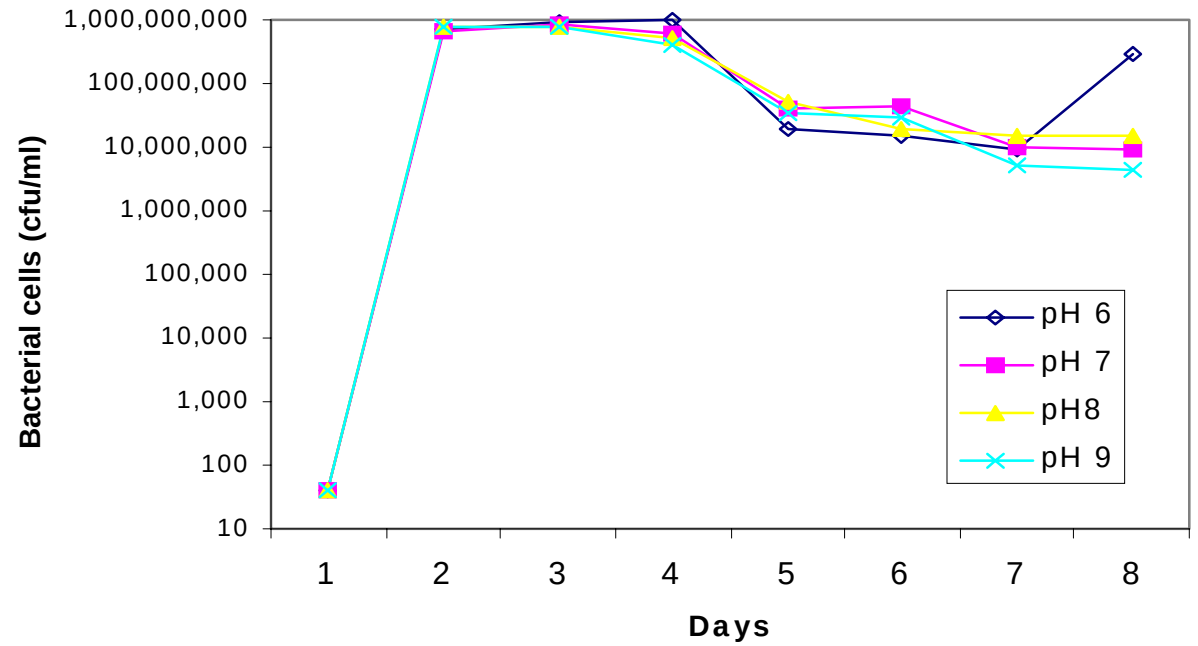


Figure 2. Growth patterns of *Vibrio harveyi* at various pH levels.



decline 3 days after inoculation and were undetectable after 7 days. *Vibrio harveyi* counts in treatments with diatoms, larvae or a combination of both, started to decrease after 3 days, finally reaching levels similar to those from near shore seawater populations after 5 days. In treatment combinations with larvae, *V. harveyi* populations were also reduced starting on the fourth day, but total larval mortality occurred within 3 days.

Table 1 shows the mean bacterial populations (cfu/ml) associated with *Skeletonema costatum* at various phases of culture. Total plate counts of bacteria on NA were constant at 10^4 cfu/ml throughout the diatom culture period, which normally lasts for three days. Presumptive *Vibrio* colonies decreased from 7.5% on the first day of diatom culture to 2% after three days. This period corresponds to the period when peak *Skeletonema* cell density is attained. A similar trend was observed for *Chaetoceros* (Table 1).

Figs. 5a and 5b show the reduction of *Vibrio harveyi* populations exposed to peak cell densities of *Skeletonema costatum* (922,500 cells/ml) and *Chaetoceros calcitrans* (2,700,000 cells/ml). Effective reduction of *V. harveyi* to levels below 10^2 cfu/ml were achieved in one day in both mixed

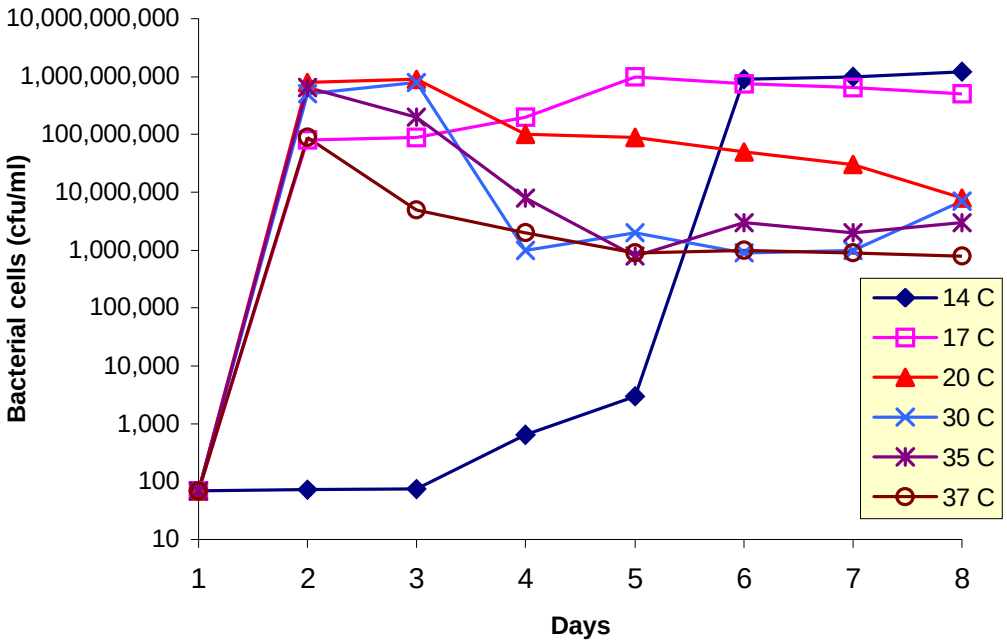


Figure3. Growth patterns of *Vibrio harveyi* at different temperatures.

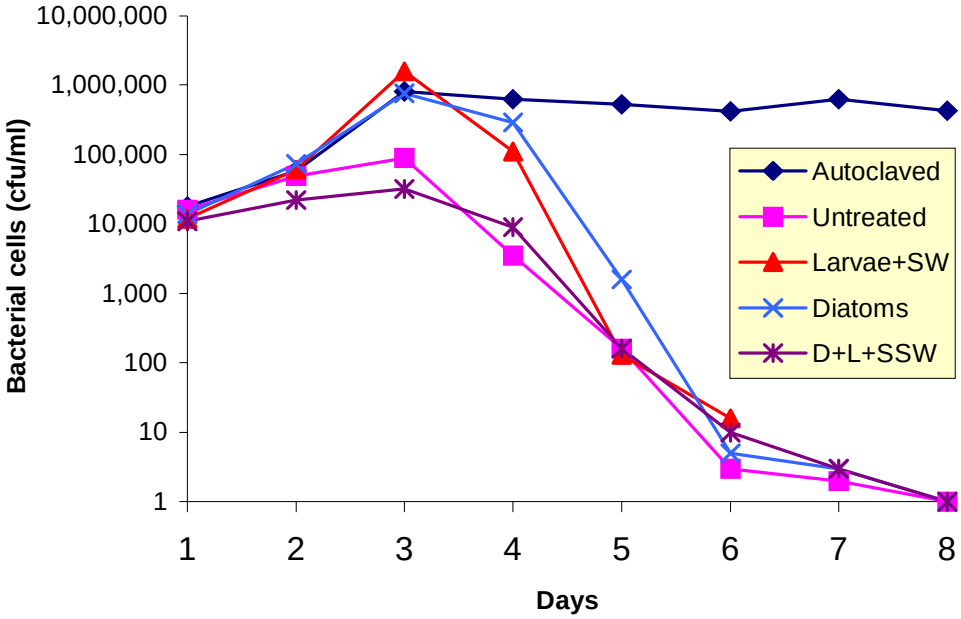


Figure 4. Reduction in *Vibrio harveyi* populations exposed to various hatchery components (Autoclaved = sterilized sea water or SSW; Untreated = untreated sea water; D = diatoms; L = larvae).

Table 1. Mean bacterial counts obtained from various phases of *Skeletonema costatum* and *Chaetoceros calcitrans* cultures. Data for *Chaetoceros calcitrans* was obtained from Lavilla-Pitogo et al. (1992) and presented here for comparison.

	Skeletonema			*Chaetoceros*		
Day	TPC*	% *Vibrio*	% *V. harveyi*	TPC*	% *Vibrio*	% *V. harveyi*
0	2.5 X 10^4	7.5	0	1.2 X 10^4	5.0	0
1	3.2 X 10^4	6.0	0	1.4 X 10^4	3.5	0
2	3.4 X 10^4	4.5	0	3.7 X 10^4	3.0	0
3	3.7 X 10^4	2.0	0	3.8 X 10^4	0.9	0

*Total plate count

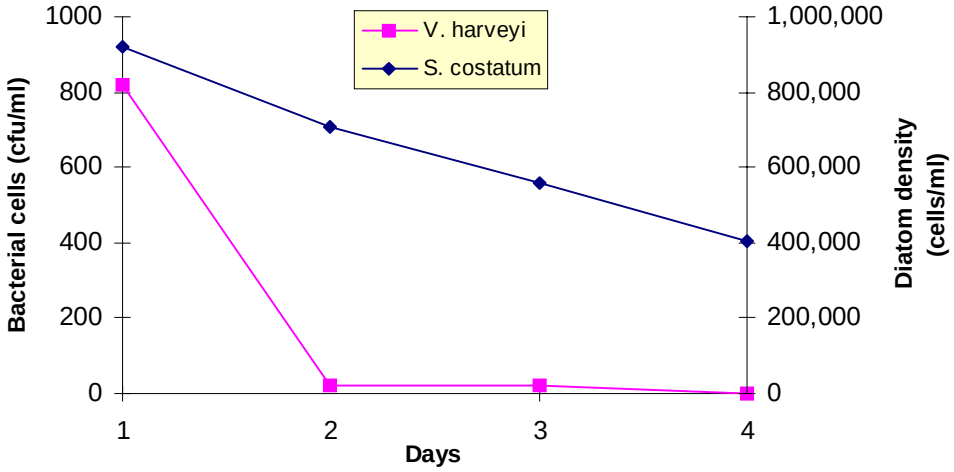


Figure 5a. Reduction of *Vibrio harveyi* population in mixed cultures with *Skeletonema costatim.*

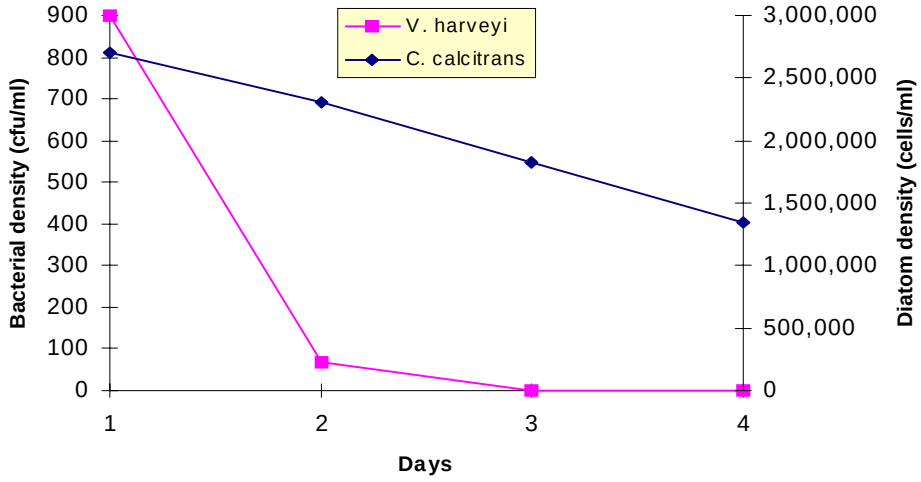


Figure 5b. Reduction of *Vibrio harveyi* population in mixed cultures with *Chaetoceros calcitrans.*

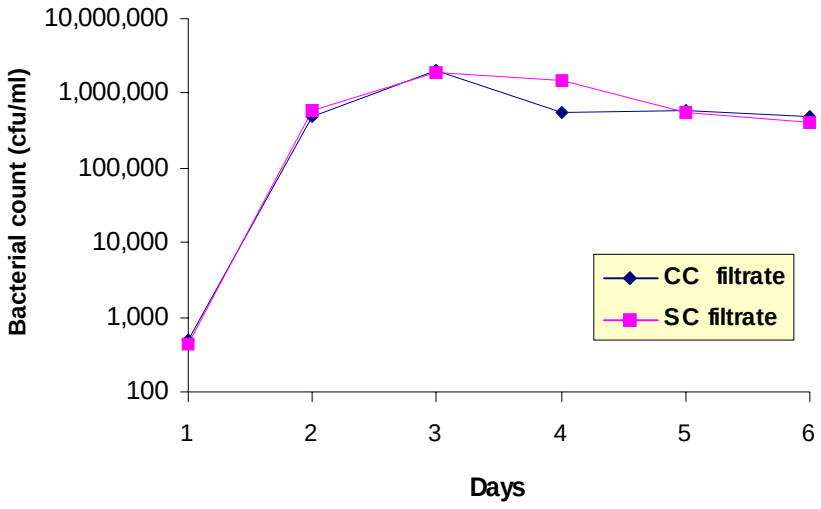


Figure 6. Growth patterns of *Vibrio harveyi* inoculated into cell-free diatom filtrates (CC = *Chaetoceros calcitrans;* SC = *Skeletonema costatum).*

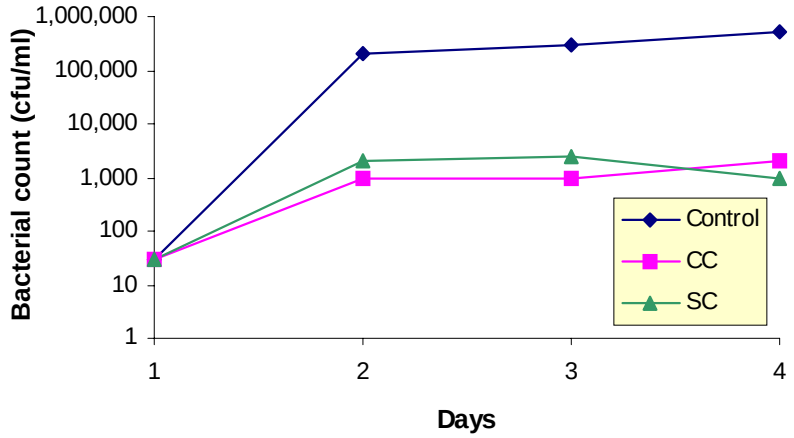


Figure 7. Growth patterns of *Vibrio harveyi* in mixed culture with diatoms at 30,000-50,000 cells/ml. (CC = *V. harveyi* with *Chaetoceros calcitrans;* SC = *V. harveyi* with *Skeletonema costatum).*

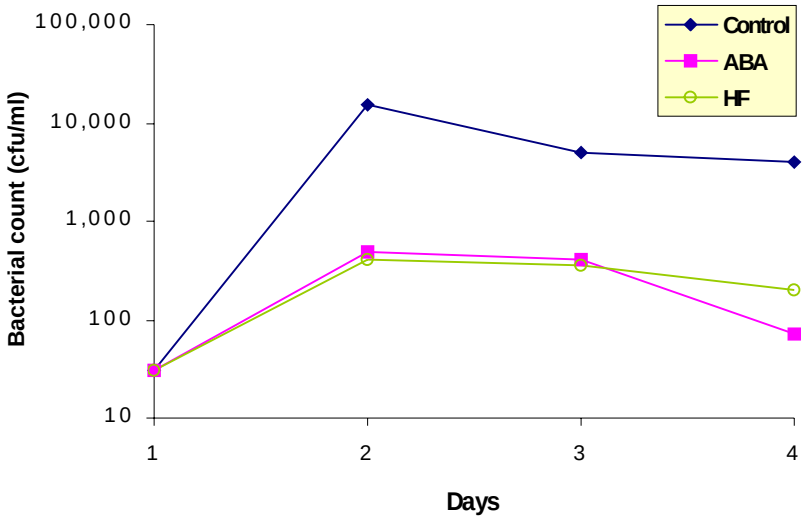


Figure 8. Growth patterns of *Vibrio harveyi* in mixed culture with commercially available bacterial products (ABA = *V. harveyi* with ABA; HF = *V. harveyi* with HF).

cultures. After 3 days with *C. calcitrans* and 4 days with *S. costatum*, *V. harveyi* reached undetectable levels. In cell-free filtrates of the same diatoms, however, the cells of *V. harveyi* maintained high populations even after five days (Fig. 6). In mixed cultures with feeding-density levels of diatoms, no reduction in *V. harveyi* population was obtained and the population maintained a plateau count at 10^3 cfu/ml for more than three days. *Vibrio harveyi* populations in the control reached more than 10^5 cfu/ml.

In mixed cultures with commercially available bacterial preparations, *Vibrio harveyi* populations were not reduced to levels lower than the initial inoculum of 10^1 cfu/ml after three days, but they were lower than the population of 10^4 cfu/ml reached in the controls (Fig. 8).

DISCUSSION

The responses of *Vibrio harveyi* to various salinity, pH and temperature levels conform to those reported for the type strain by Krieg and Holt (1984), and they show that there are no constraining environmental conditions that *Penaeus monodon* larvae can tolerate (Parado-Estepa et al. 1996). Control measures based on the manipulation of these parameters would therefore be unlikely or impossible in the hatchery. However, due to the halophilic nature of *V. harveyi*, luminescent vibriosis may not be expected in freshwater culture facilities.

In the absence of competitors in seawater, *Vibrio harveyi* sustained high populations for several days. This means that thorough cleaning and disinfection of rearing water may have negative effects, if added components like feed and larvae are contaminated. Since sources of the bacterium have been identified (Lavilla-Pitogo et al. 1992), care must be taken to prevent the introduction of grossly contaminated materials either during stocking, feeding, or water management, especially when water treatment has been carried out thoroughly to eradicate its microbial components. Re-population of dechlorinated seawater has been found to occur very rapidly (Baticados & Pitogo 1990). Hence, recontamination must be avoided, especially with known *V. harveyi*-carrying components. Reduction in *V. harveyi* populations exposed to untreated seawater after 96 h explains the benefits of aging seawater before use. Reported antibacterial activity of seawater has been ascribed to physico-chemical effects and biological factors, including predation, competition for nutrients, bacteriophage activity, and antibiotics produced by some marine organisms like the diatoms *Skeletonema*, *Chaetoceros*, *Nitzchia*, and *Licmophora*, among others

(Baslow 1969). The inhibition of bacterial pathogens by extracts and supernatants derived from spray-dried preparations of the microalga *Tetraselmis suecica* have been reported (Austin & Day 1990, Austin et al. 1992).

In the present study, effective reduction of *Vibrio harveyi* populations in mixed culture with diatoms was influenced mainly by the associated microflora rather than the diatoms or their metabolic products, as shown by the high bacterial population sustained in cell-free diatom filtrates. Sieburth (1968) found that high populations of the diatom *Skeletonema costatum* could inhibit *Vibrio*. Interestingly, however, the same study showed that dominant *Flavobacterium* associated with the diatom did not show inhibitory action against *Vibrio* in cross-streak plates. In another study (Rico-Mora & Voltolina 1995a), *Flavobacterium* was also among the five strains of bacteria (including *Plesiomonas*, *Aeromonas* and two strains of *Vibrio*) found in non-axenic cultures of *S. costatum*. In that study, however, bacteriostatic and antibiotic reactions among the strains were found. In another study by the same authors (Rico-Mora & Voltolina 1995b), it was shown that none of the *S. costatum*-associated bacteria were pathogenic to *Artemia franciscana* nauplii. Thorough analysis of bacterial associations is needed in order to exploit their potential. Aside from inhibitory activity, bacteria of choice should be benign to larval stages being cultured. In the present study, although bacterial populations associated with shrimp larvae also effectively reduced *V. harveyi*, they also caused mortality, thus limiting their potential as disease control agents.

Bacteria are among the most sought after marine organisms as potential sources of inhibitory compounds against fish diseases (Dopazo et al. 1988, Lemos et al. 1991, Nogami & Maeda 1992, Austin et al. 1995, Garriques & Arevalo 1995, Sugita et al. 1996, Gatesoupe 1997, Riquelme et al. 1997). Depending on how the bacteria or their inhibitory products are processed and applied, the action against target pathogens may be that of antibiosis, growth inhibition, competition for available nutrients, colonization leading to competitive exclusion, or habitat and rearing water conditioning. Probiotic application of defined or undefined bacterial populations has a long history of successful experimentation and implementation in the poultry industry and it is generally based on competitive exclusion of *Salmonella* (Stavric & D'Aoust 1993). Whether candidate probiotic marine bacteria can be be effective as feed additives or simply added to the water and whether they act as competitors or inhibitors should be studied further. There is no doubt that possible agents of control are already in the hatchery, but careful study and manipulation of their inherent control potential is needed to achieve the desired ecological balance in that system. The following items need consideration before bacteria or their products are applied as biological agents for disease control in the hatchery:

- The candidate bacteria must be benign
- They should provide antagonistic activity towards pathogens and maintain them at manageable levels in the rearing environment
- They should be able to re-establish rapidly in the rearing environment following regular water exchange
- To work as exclusion agents, they should be able to attach and divide on shrimp surfaces since *V. harveyi* is known to colonize and replicate on larval (Lavilla-Pitogo et al. 1990) and egg surfaces (Lavilla-Pitogo 1995)
- They should be stable under conditions for optimum larval rearing

The disease and pest problems that the aquaculture industry is facing are mirror images of the travails faced by other animal industries when they were starting. Thus, much can be learned from disease management protocols adapted by the poultry, cattle and hog industries. When formulating biological products and protocols for disease control, it is worthwhile to look beyond the present culture system and consider areas that will make its implementation successful and acceptable (Waage 1996).

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