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Control of *Vibrio* spp. in Shrimp Hatcheries Using the Green Algae *Tetraselmis suecica*

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Abstract

The *Vibrio* spp. are the most common and harmful shrimp pathogenic bacteria causing serious diseases of larval and postlarval stages in the hatchery. The *Vibrio* inhibitory activity of green algae *Tetraselmis suecica* has been reported *in vitro* (Austin and Day 1990).In these experiments, the inhibitory activity was assessed *in vivo* in a commercial shrimp hatchery of the white prawn *Fenneropenaeus indicus*. The concentrate of the algae was used as a food supplement for the broodstock and also as partial live larval feed in the larval rearing tanks. Results showed that use of algae resulted in significant reduction of *Vibrio* numbers in maturation and spawning tank water, broodstock gut contents, egg and nauplii samples. The spawning tank water *Vibrio* count showed a negative correlation with egg hatching rate. The algae when used in larval tanks were found to reduce *Vibrio* count in both rearing water and larval samples, resulting in improved nauplii 5 to postlarvae 1 survival. Rearing tank experiments with axenic algae proved that the inhibitory effect was by *Tetraselmis* and not by associated bacteria. Usage of this algae in the hatcheries will be advantageous as it suppresses the dominance of *Vibrio* spp. may be including the potentially pathogenic ones.

Introduction

Though the shrimp hatchery technology has advanced over the decades, the hatchery production is more often hampered by severe mortalities caused mostly by bacteria. Different bacterial genera have been associated with infections of penaeid shrimp larvae, but gram negative *Vibrionacea* undoubtedly represent the most harmful pathogenic bacteria. *Vibrio* spp.

have been identified as the dominant genera of the normal bacterial flora of larval and adult shrimp (Yasuda and Kitao 1980, Jayakumar and Ramasamy 1999).

The term vibriosis refers to diseases associated with bacteria of the genus *Vibrio*. *Vibrio* sp. such as *V. harveyi, V. splendidus, V. parahaemolyticus, V. alginolyticus, V. vulnificus, V. penaeicida* and *V. campbell*i are some important species that have been reported to be pathogenic to shrimp (Lavilla-Pitogo et al. 1990, Chen et al. 1992, Lightner et al. 1992, Limsuwan 1993; Ruangpan et al. 1995, Hameed et al. 1996). Increasing *Vibrio* population in larvae and rearing tank water has been reported to reduce the survival rate of larvae and postlarvae (Singh 1986; Hameed 1993). Luminous species *V. harveyi* have been associated with causing mass mortalities in shrimp hatcheries (Sunaryanto and Mariam 1986; Baticados et al. 1990; Karunasagar et al. 1994).

Ways of *Vibrio* entry into hatchery include seawater, faecal matter and exoskeleton of spawners, and *Artemia* water (Lavilla-Pitogo et al. 1992). Moreover, in maturation tanks, marine bivalves, used as diet for the broodstock, may also harbour opportunistic or potentially pathogenic vibrios and thus may act as vector (Olafsen 2001). Interestingly, *vibrios* persisted in bivalve haemolymph and soft tissues even after depuration in UV-treated seawater (Tamplin and Capers 1992).

Treating broodstock with antibiotics, swift separation of eggs from spawners and spawners' faeces after spawning, effective egg rinsing with sterile seawater, sterilization of the eggs and spawning tank water are advised to avoid fouling of eggs with pathogens. But, even with these measures pathogenic *Vibrio* manages to enter the larval rearing system. Similarly, techniques to sterilize the eggs to get rid of adherent microflora disturb the balance of microbial communities and favor exponential growth of opportunistic bacteria (Baticados and Pitogo 1990). In the rearing tanks, in addition to the above, antimicrobial drug induced deformities in larvae (Baticados et al. 1990) and had the potential for development of antibiotic resistant strains (Baticados et al. 1990, Karunasagar et al. 1994, Pillai and Jayabalan 1996).

It is recommended therefore, to adopt different husbandry practices to eschew outbreak of vibriosis. This could include controlling the bacterial flora of the culture water through introducing microalgae (Igarashi et al. 1990), vaccination (Alabi et al. 2000), use of probiotics (Sung et al. 2001) and immunostimulants (Sung et al. 1996).

In this study, the green algae *Tetraselmis suecica,* which has been reported to inhibit *in vitro* growth of pathogenic *Vibrio* spp. (Austin and Day 1990) was used as feed both in the maturation and larval rearing tanks of the prawn *Fenneropenaeus indicus* (Indian white shrimp, *Penaeidae*). The objectives were to investigate the effect of feeding broodstock with algal supplement on reducing the *Vibrio* levels in the broodstock gut, maturation tank water, spawning tank water and egg epiflora. The larval rearing experiment analyzed the effect of maintaining a particular concentration of *Tetraselmis* as feed in the tanks on *Vibrio* count of various larval stages and in larval rearing water.

Materials and Methods

These experiments were carried out in the commercial shrimp hatchery facility located in the Arabian Sea port city, Al Mukalla in the Republic of Yemen. The algae *Tetraselmis suecica* (Kylin) Butch (CS-187) and *Chaetoceros muelleri* Lemmermann(CS-176) were sourced from CSIRO Collection of Living Microalgae, CSIRO Marine Research, Hobart, Tasmania, Australia. Algae were maintained in f_2 medium (Guillard and Ryther 1962). The starter cultures maintained axenically in batch culture method and mass cultures maintained semi-continuously in 200 l capacity translucent vertical tubes (Solar Components Corp., USA) were used for the experiments. The algae were grown at 23 ± 2°C at a salinity of 29 ppt. The required quantity of algae was harvested at the log growth phase and concentrated by centrifugation at 8000 g (Tredici et al. 1996) resulting in a paste with a moisture content of 60 to 70%.

Maturation tank experiment

The *F. indicus* broodstock sourced from wild (average weight of females 52.5 g and males 41.0 g) were first given formalin bath (200 ppm) for one hour and acclimatized to hatchery conditions for ten days period. The healthy females selected were transferred to 3.5 meter diameter circular concrete maturation tanks, after getting subjected to unilateral eyestalk ablation using red hot scissors. The sex ratio was 1:1 with a stocking density of $8 \cdot m^2$; light regime was 14:10 light: dark maintained using blue fluorescent tubes.

The broodstock were fed five times a day with fresh or fresh-frozen squid (three feedings), cuttlefish (one feed) and oysters (one feed), the total accounting for 12% of total wet weight biomass per day. For the experimental tanks (algae fed, T), squid fractions after processing and before feeding were marinated in algal paste at 2% level (2 g algal dry weight per 100 g squid) for twelve hours at 8°C. The control tanks (C) received similar percentage quantity of squid processed and stored similarly but without algae. Every day, the remaining feed and faecal matter in the maturation tanks were siphoned out in the early morning and evening. The daily water exchange was 100%, by slow, continuous flow-through. Usually, water exchange was stopped for one hour following feeding. From the fourth day of post-ablation, every alternate day in the evening, sourcing for ready to spawn females was carried out using an underwater flashlight. The ripe females were transferred to individual 250 l spawning tanks.

Larval rearing experiment

In the larval rearing tanks (1000 L), the larvae at nauplii 5 stage were stocked at 100 larvae l^{-1}. The different larval stages were fed following normal commercial hatchery feeding regime (Table 1).The treatments included larval tanks fed with 1. xenic *Chaetoceros* and artificial feeds (C), 2. xenic

Chaetoceros, xenic *Tetraselmis* and artificial feeds (CT_X) and 3.xenic *Chaetoceros*, axenic *Tetraselmis* and artificial feeds (CT_{AX}). The feeding was six times per day, each time after observing the residual algal cell concentration and artificial feed (AF). AF included micro-encapsulated diet (MED), *Spirulina* and brine shrimp flakes. The water exchange rate was 30% for Zoea 3 (Z_3), 50% for Mysis (M) and 70% for Postlarval (PL) stage.

Sampling and analysis

Dry weight of the concentrate was determined by resuspension of concentrate in preweighed tubes with 5 ml of ammonium formate (0.5 M) to remove nonvolatile salts, centrifuging (5000 g) and oven-drying of tubes at 105°C to constant weight after the supernatant was discarded. The cell density of the concentrate was determined using a haemocytometer after diluting the concentrate with 0.2 µ filtered seawater. The sterility of starter culture algal concentrate was checked by plating 0.1 ml of diluted samples (to 10^{-4} in sterile seawater) in Tryptic soy agar (TSA) and Thiosulphate citrate bile salt sucrose agar (TCBS), both with 1.5% NaCl incubated at 28 to 30°C for 5 days. The algae from mass cultures were also inoculated in the same manner to get the total viable counts and viable *Vibrio* sp. counts, but, were incubated for 48 h at 28 to 30°C.

During the 25 days of maturation experiment, a total of 36 samples each (water, gut content, egg and nauplii) were collected from C and T tanks. Water samples from the maturation and spawning tanks were collected in sterile bottles and serially diluted to 10^{-5} and were processed immediately by plating 1 ml on TCBS. The eggs and nauplii were collected from the tanks as soon as possible after spawning and hatching. All the samples were rinsed gently with sterile seawater, weighed and macerated in 0.25 ml of sterile seawater. The macerated samples were diluted to 10^{-6} with sterile seawater and seeded on TCBS and Nutrient Agar (NA, with 1.5% NaCl).

Broodstock animals were sacrificed; surface disinfected with 70% ethanol, dissected and the gut was removed in its entirety. The gut contents were removed by squeezing and collected in sterile weighing bottles, homogenized and diluted to 10^{-5} in sterile saline and 0.1 ml of volumes of each dilution spread over the surface of triplicate plates of TCBS and NA. The inoculated plates of all the samples from maturation tanks were incubated at 28 to 30°C for 48 hours.

Table 1. Larval feeding regime for microalgae, Artificial feed and *Artemia*

Substage	*Chaetocerossmuelleri* (cells·ml^{-1})	*Tetraselmissuecica* (cells·ml^{-1})	Artificial feeds (mg·l^{-1}·day^{-1})	*Artemia* (nauplii·ml^{-1}·day^{-1})
Z_1	100000	30000	8	-
Z_2	100000	30000	8	-
Z_3	100000	30000	10	-
M_1	80000	30000	12	3
M_2	75000	30000	12	5
M_3	60000	30000	14	8
PL_1	40000	30000	16	10

The larvae of different stages and PL were collected from the rearing tanks (each treatment with two tanks) after the metamorphosis of the required larval stage. The water and larval sample were processed and inoculated in the same way as in the maturation experiment. In both maturation and larval rearing experiments, with samples inoculated in TCBS, yellow and green colonies were counted to obtain colony forming units per ml or g ($cfu \cdot ml^{-1}$ or $cfu \cdot g^{-1}$) and all colonies that appeared were assumed as *Vibrio*. Plates were observed at 6 h interval in total darkness for two days to determine the number of luminescent colonies. With all the samples, three replicates of each dilution were used.

The statistical significance of data was tested by Analysis of Variance (ANOVA) or Student's t-test whichever is appropriate. The percentage survival values were arcsine transformed. Correlation analysis was used to detect any relationship between water *Vibrio* count and egg hatching as well as survival of larvae and postlarvae. Significance of correlation was tested using t-test. The Standard Error of the Mean (SEM) was also calculated.

Results

Maturation tank experiment

During the experimental period the temperature in the maturation tanks ranged from 28.7 to 30°C, pH from 7.9 to 8.1 and dissolved oxygen levels were always above 7 $mg \cdot l^{-1}$. The mean *Vibrio* counts obtained with various samples collected from the individual C and T tanks throughout the experiment period are given in table 2. No luminescent bacteria were noticed in the samples seeded both in NA and TCBS. The axenic concentrated algae did not show any contamination.

Feeding broodstock with squid freshfeed marinated in *Tetraselmis* resulted in reduction of *Vibrio* count in all the samples. The algae-fed tanks when compared to controls showed a reduction of one log unit in *Vibrio* count with the maturation and spawning water. A reduction of two to three log units was obtained with gut content *Vibrio* and a little less than two log reduction with egg and nauplii. Analysis showed that with all the parameters the T tanks values differed significantly from the C tanks ($P<0.01$). The values for mean egg hatching percentage (61.4 and 72.1) and broodstock survival (79.4 and 88.2) from C and T treatments respectively, differed significantly ($P < 0.01$).

A significant correlation was noticed between spawning tank water *Vibrio* count and egg *Vibrio* count ($r = 0.57$; $P < 0.01$) (Fig. 1). The egg *Vibrio* count showed a negative correlation with hatching rate ($r = 0.51$; $P < 0.01$) (Fig. 2).

Larval rearing experiment

Results from the larval rearing experiments are given in table 3. The tank water temperature ranged from 29.4°C to 30.8°C, pH 8 to 8.2 and

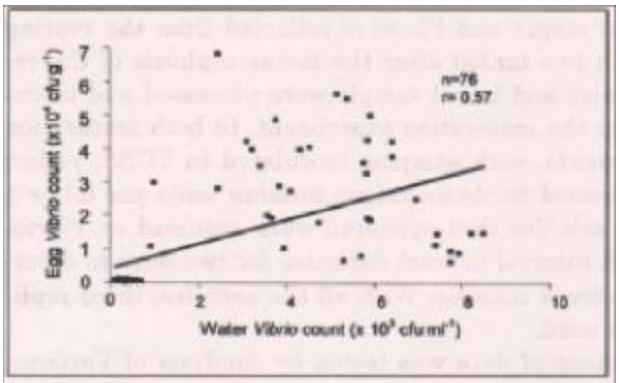

Fig. 1. Correlation between spawning tank water *Vibrio* count (cfu·ml^{-1}) and egg *Vibrio* count (cfu·g^{-1}) of *P. indicus*, for all the samples collected during the experminetal period ($P < 0.01$)

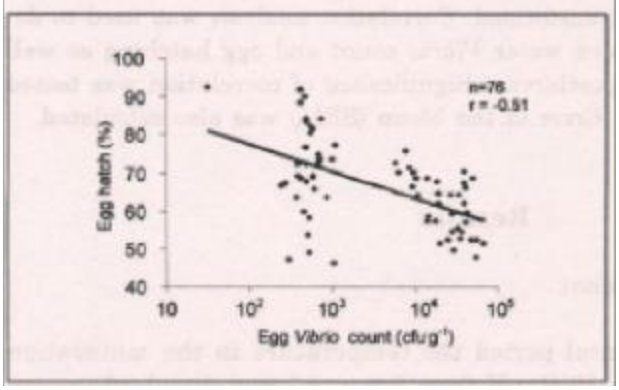

Fig. 2. Correlation between *P. indicus* egg *Vibrio* count (cfu·g^{-1}) and egg hatching (%) for all the samples collected during the experminetal period ($P < 0.01$)

Table 2. Mean Vibrio counts (± s.e)* in the gut content, maturation tank water, spawning tank water, eggs and nauplii collected from control (no *Tetraselmis* feeding) and *Tetraselmis* fed prawn *F. indicus*, their maturation and respective spawning tanks

Maturation tank	Maturation tank water (cfu·ml^{-1})	Gut content (cfu·g^{-1})	Spawning tank water (cfu·ml^{-1})	Eggs (cfu·g^{-1})	Nauplii (cfu·g^{-1})
CONTROL1	$4.36\pm0.44\times10^{3}$	$5.52\pm0.72\times10^{5}$	$5.07\pm0.33\times10^{3}$	$4.02\pm0.27\times10^{4}$	$4.27\pm0.64\times10^{4}$
CONTROL2	$5.09\pm0.40\times10^{3}$	$8.55\pm1.31\times10^{5}$	$6.45\pm0.59\times10^{3}$	$2.17\pm0.58\times10^{4}$	$7.29\pm0.36\times10^{4}$
CONTROL3	$3.68\pm0.42\times10^{3}$	$1.09\pm0.15\times10^{6}$	$3.91\pm0.51\times10^{3}$	$3.21\pm0.29\times10^{4}$	$5.71\pm0.28\times10^{4}$
ALGAE 1	$4.59\pm0.34\times10^{2}$	$1.19\pm0.07\times10^{3}$	$2.87\pm0.25\times10^{2}$	$6.46\pm0.59\times10^{2}$	$1.03\pm0.13\times10^{3}$
ALGAE 2	$6.30\pm0.49\times10^{2}$	$2.33\pm0.14\times10^{3}$	$3.26\pm0.26\times10^{2}$	$5.75\pm0.54\times10^{2}$	$8.27\pm0.75\times10^{2}$
ALGAE 3	$7.52\pm0.36\times10^{2}$	$5.46\pm0.27\times10^{3}$	$4.56\pm0.34\times10^{2}$	$3.92\pm0.41\times10^{2}$	$7.04\pm0.55\times10^{2}$

*Mean values of twelve samples collected through out the experimental period at regular intervals. Each sample seeded in triplicates in TCBS at 28-30° C for 48 hours

Table 3. Mean *Vibrio* counts (± s.e.)* of larvae and postlarvae of *Fenneropenaeus indicus* and in the larval rearing tank water. Treatments included tanks fed xenic *Chaetoceros* and artificial feeds (Control, C), xenic *Chaetoceros*, xenic *Tetraselmis* and artificial feeds (CT_x) and xenic *Chaetoceros*, axenic *Tetraselmis* and artificial feeds (CT_{ax})

	Nauplii (cfu·g^{-1})	Larval rearing tank water (cfu·ml^{-1})	Zoea (cfu·g^{-1})	Larval rearing tank water (cfu·ml^{-1})	Mysis (cfu·g^{-1})	Larval rearing tank water (cfu·ml^{-1})	Postlarvae (cfu·g^{-1})	Larval rearing tank water (cfu·ml^{-1})
C1	$2.8\pm0.24\times10^{3}$	$1.6\pm0.30\times10^{3}$	$5.4\pm0.07\times10^{4}$	$5.7\pm0.28\times10^{3}$	$1.3\pm0.28\times10^{5}$	$1.4\pm0.08\times10^{4}$	$3.3\pm0.32\times10^{6}$	$7.6\pm0.22\times10^{4}$
C2	$6.2\pm0.32\times10^{3}$	$5.9\pm0.15\times10^{3}$	$9.2\pm0.24\times10^{4}$	$9.8\pm0.30\times10^{3}$	$3.6\pm0.37\times10^{5}$	$5.0\pm0.16\times10^{4}$	$5.1\pm0.27\times10^{6}$	$5.6\pm0.18\times10^{4}$
CT_x	$4.7\pm0.28\times10^{3}$	$4.4\pm0.17\times10^{2}$	$6.4\pm0.32\times10^{3}$	$9.9\pm0.12\times10^{2}$	$1.3\pm0.22\times10^{4}$	$8.8\pm0.20\times10^{2}$	$7.8\pm0.16\times10^{4}$	$2.1\pm0.20\times10^{3}$
CT_X	$5.1\pm0.18\times10^{3}$	$7.8\pm0.18\times10^{2}$	$5.9\pm0.18\times10^{3}$	$6.4\pm0.31\times10^{2}$	$9.7\pm0.22\times10^{3}$	$1.3\pm0.31\times10^{3}$	$6.6\pm0.17\times10^{4}$	$3.1\pm0.31\times10^{3}$
CT_{AX}	$9.2\pm0.16\times10^{2}$	$2.7\pm0.22\times10^{2}$	$7.6\pm0.21\times10^{3}$	$1.0\pm0.24\times10^{3}$	$1.0\pm0.18\times10^{4}$	$9.4\pm0.16\times10^{2}$	$8.7\pm0.16\times10^{4}$	$4.3\pm0.28\times10^{3}$
CT_{AX}	$2.2\pm0.14\times10^{3}$	$6.2\pm0.28\times10^{2}$	$9.3\pm0.33\times10^{3}$	$8.3\pm0.24\times10^{2}$	$1.5\pm0.15\times10^{4}$	$5.7\pm0.22\times10^{3}$	$9.2\pm0.22\times10^{4}$	$7.6\pm0.18\times10^{3}$

*Mean of colonies from triplicate plates of TCBS agar after incubation at 28-30 °C for 48 hours

oxygen always above 6.5 $mg \cdot l^{-1}$. In the case of xenic algae, the overall mean total heterotrophic bacterial count with *Chaetoceros* was 3.1 x 10^2 and with *Tetraselmis* concentrate 9.4 x 10^1. The mean *Vibrio* counts were 7 x 10^1 and 4.2 x 10^1 respectively. No luminescent colonies were observed in the inoculated plates of NA and TCBS.

The presence of *Tetraselmis* (either xenic or axenic) was found to reduce the *Vibrio* count in the rearing water as well as in larval samples. Rearing water *Vibrio* counts, when compared to control were to the minimum single log unit less in CT_X and CT_{AX} tanks, with two log reductions during the M stages. All the water samples collected during the three larval stages and at PL stage showed that the *Vibrio* count in samples from CT_X and CT_{AX} tanks were significantly lower ($P<0.05$) than control.

With Z, M and PL samples, a significant ($P<0.05$) reduction in *Vibrio* count was noticed between the Tetra-fed tanks and control treatments, but not with N_5 ($P>0.05$).While the Z and M samples from Tetra-fed tanks recorded one log count less than control, it was two log with PL samples. The *Vibrio* count (cfu · gÉ¹) from N, Z, M and PL samples differed, depicting an increasing trend with advancement of stage.

Comparison between the CT_X and CT_{AX} treatments showed that all the water as well as larval samples from them recorded counts that were close and did not differ significantly ($P>0.05$).The survival from N_5 to PL_1 stage of C, CT_X and CT_{AX} tanks averaged 31.52, 48.31 and 38.59 % respectively. But, at all larval stages analysis of survival values did not show any significance between the three treatments. Correlation between the survival rates of Z, M and PL and their respective *Vibrio* numbers (Figs. 3, 4 and 5) showed a significant negative correlation ($r = 0.53, -0.78$ and -0.70 respectively; t-test; $P < 0.05$).

Discussion

Maturation tank experiment

Among the different sources of *Vibrio* contamination in a hatchery, spawners may very much serve as vector for pathogenic *Vibrio* especially through its gut contents. Dominance of *Vibrio* in the *F. Indicus* gut contents and its possible harmful role in the life of animal has been reported (Singh et al. 1998). Lavilla-Pitogo et al. (1992) reported 10^8 levels of *Vibrio harveyi* $cells \cdot ml^{-1}$ of spawner (*Penaeus monodon*) midgut contents. Fish faeces have been shown to be a source of infection and medium for bacterial proliferation (Ruby and Morin 1979).

The ability of *Vibrio* to undergo division in the gut of prawn and mollusc has been reported (Singh et al. 1998, Prieur 1981).Interestingly, results from this experiment show that inclusion of *Tetraselmis* as supplement in the regular maturation diet significantly reduced the *Vibrio* count in the gut contents. The reduction must have been achieved by the inhibition of their

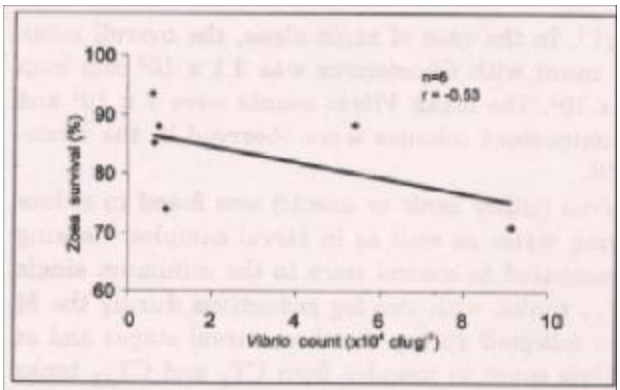

Fig. 3. Correlation between mean *Vibrio* count (cfu·g^{-1}) of *P. indicus*, zoea larvae and its survival percentage ($P < 0.05$)

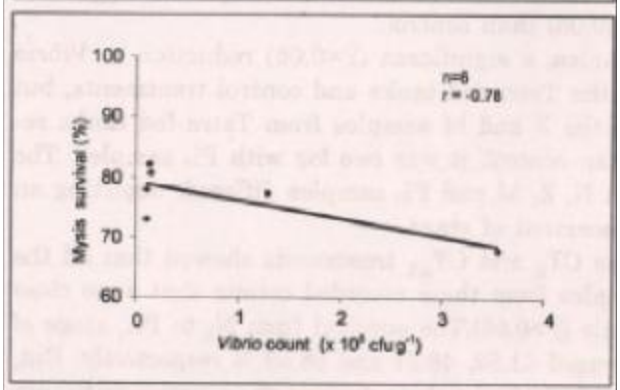

Fig. 4. Correlation between mean *Vibrio* count (cfu·g^{-1}) of *P. indicus*, mysis larvae and its survival percentage ($P < 0.05$)

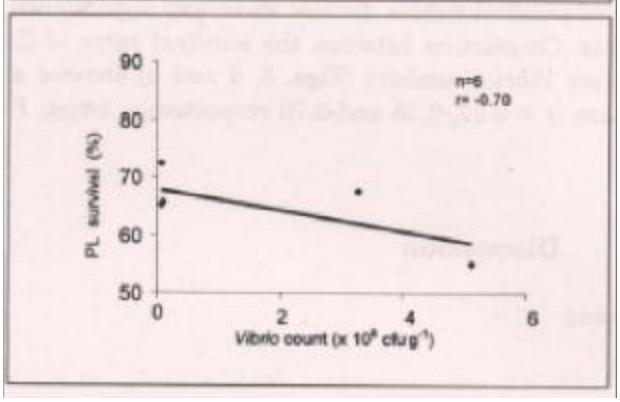

Fig. 5. Correlation between mean *Vibrio* count of *P. indicus*, postlarvae (PL) and its survival percentage ($P < 0.05$)

multiplication by *Tetraselmis*. Using *T. suecica* as feed supplement for *Salmo salar* (Atlantic salmon, *Salmonidae*), Austin et al. (1992) reported after a seven day feeding regime, an increase in the numbers of *Enterobacteriaceae* representatives in the digestive tract. The same genera have been reported in the alimentary canal of pond reared *F. indicus* (Singh et al. 1998). So, it may be postulated that similar phenomenon occurs in the case of shrimp gut also. Reduction of gut *Vibrio* count will be beneficial, as they are expected to behave as opportunistic pathogens invading tissues and haemolymph through the intestinal wall as reported by Herborg and Villadsen (1975) in fishes. Furthermore, the reductions in gut *Vibrio* naturally results in their decrease in faecal matter or if the initial concentration was lower even in the absence of pathogen.

Austin et al. (1992) noted a two log level drop in total heterotrophic bacterial numbers in tank water after a seven day period when Salmon were fed with food supplemented with *Tetraselmis* algal cells. In this experiment also a log reduction in *Vibrio* numbers were noticed in the maturation tank water and must have been due to their reduced presence in faecal matter.

The *Vibrio* counts from the spawning tanks, where the animal from algae fed tanks were spawned registered lower levels of *Vibrio* in water samples and eggs. Microbial community of the ambient water have been shown to influence the composition of the bacterial egg epiflora (Olafsen 2001). It may be that reduction in *Vibrio* in the spawning tank results in non-pathogenic egg community composition that restricts the adhesion of same and other harmful bacteria preventing the transfer of pathogens to later stages.

The negative correlation between the *Vibrio* count and the hatching rate in the control tanks well indicate the hampering effect of *Vibrio* on the latter parameter. Release of exoproteolytic enzymes from the adherent bacterial epiflora may damage the chorion (Hansen and Olafsen 1989). Some strains of *Vibrio* have been reported to produce a variety of extracellular proteases and toxins (Umbreit and Tripp 1975). A negative correlation between the egg hatching rate and total bacterial number was also reported by Hameed (1993). Interestingly, the *Vibrio* were the dominant genera isolated from the eggs. Barker et al. (1989) observed a marked correlation between egg surface bacteria and egg mortality in rainbow and brown trout eggs.

The increment with broodstock survival may be due to the presence of less *Vibrio* in the gut of animals from T-tanks. Yasuda and Kitao (1980) observed an abundant *Pseudomonas* population in the gut of healthy cultured and wild adult prawn *P. japonicus*. Another factor for the survival improvement could be the disease resistance boosting effect of *T. suecica*, as noticed by Austin et al. (1992).The authors noticed that when Atlantic salmon of 15 g size were fed prophylactically, the algal supplement was successful in preventing diseases caused by pathogens including *V. anguillarum* and *V. salmonicida*.

Larval rearing experiment

With larval rearing water samples, the log reduction of *Vibrio* count in the CT_X and CT_{AX} treatments prove the inhibitory effect of *Tetraselmis*. Although the addition of axenic *Tetraselmis* resulted in the bacterial reduction, it can be postulated that the bacteria associated with xenic algae (*Vibrio* as well as other heterotrophic bacteria) did not influence the inhibitory effect. Alabi et al. (1999) while experimenting with *Skeletonema* and *Tetraselmis* on rearing of *F. indicus* from Z_1 to M_1 stage noticed that treatment groups fed algae with or without its associated bacteria exhibited lower presumed viable *Vibrio* count values in culture water compared to only MED fed treatments. The *Vibrio* reduction in the larval samples could be due to their less concentration in the rearing water and also may be that *Tetraselmis* influenced gut microflora of larvae as noticed with adults in the maturation experiment.

The decline in *Vibrio* seems to favor the proliferation of other bacterial genera which support better larval survival. In this aspect, it could be explained that the non-*Vibrio* genera associated with xenic *Tetraselmis* concentrate in CT_X treatments had better chances of proliferation resulting in less *Vibrio* count than CT_{AX} treatment and higher overall survival. Studies have shown that microalgal cultures may serve as a source of antagonistic bacteria. Nevertheless, *Vibrio,* producing inhibitory substances have been found very rare (Riquelme et al. 1997) So, it could be well assumed that with the *Vibrio* inhibitory activity of *Tetraselmis* in effect, it is easy for other bacterial genera to dominate.

With all the treatments, gradual increase in the total numbers of *Vibrio* from egg to PL was observed both in control and experimental tanks. Hameed (1993) reported an increasing heterotrophic bacterial count from egg to PL where *Vibrio* was dominant (25 to 32 %).The *Vibrio* count in the larval rearing water was lower than the larvae and might be due to the fact that larval surface provide a suitable micro-environment for bacterial growth (Stevenson 1978). Negative correlation noticed in the experiments between *Vibrio* population and larval survival corroborate with reports of Hameed (1993) and Singh (1986).

Inhibitory activity

Austin and Day (1990) with antibiog confirmed that *Tetraselmis* supernatant or extracts developed inhibition zones against a number of pathogenic *Vibrio* species including *V. alginolyticus, V. parahaemolyticus and V. vulnificus.* Characterization of the inhibitor showed that the same to be polysaccharide (Austin et al. 1992). The method by which the algae or algal extracts exert bacteriostatic effect is unknown. Inhibition of *in vitro* development of *Vibrio* has also been reported with the number of microalgae (Cooper et al. 1983, Viso et al. 1987, Naviner et al. 1999).

The inclusion of *Tetraselmis* in addition to its bacteriostatic effect would also serve as an additional nutritional source to the larvae and the adult. *T. suecica* contains 20:4 (n-6) and 20:5 (n-3) highly unsaturated fatty acids (Volkman et al. 1989) essential for both maturation success (Middleditch et al. 1980) and acceptable larval survival (Kurmaly et al. 1989).

Conclusion

Tetraselmis with its anti-*Vibrio* activity reduced the *Vibrio* count in broodstock gut, eggs and larvae resulting in improved egg hatching and larval survival. Above all, the reduction would to a considerable extent bring down the chances of diseases caused by pathogenic *Vibrio* by inhibiting their growth. The algae thus work like probiotics or better than probiotics in that the growth media (rich in organic nutrients) added with probionts may boost growth of unwanted microorganisms, too. Usage of algae does not pose such problems and reduces the expenditure on costly probiotics. So, it is very

much recommended to use the algae as a maturation diet supplement and also as an ingredient in the regular feeding regime of larval rearing.

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