CHAPTER – V

CONTROL OF *VIBRIO* IN MATURATION AND LARVAL TANKS USING GREEN ALGAE *TETRASELMIS SUECICA*

The hatchery production of postlarvae is severely affected by the infection from bacteria, virus, parasites etc., resulting in severe mortalities of larvae and postlarvae. But, among these, the most frequent problem is caused 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. campbellii* 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).

The 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 *Vibrio* and thus may act as vector (Olafsen, 2001). Interestingly, *Vibrio* 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. 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 favour 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 by introducing microalgae (Igarashi *et al.*, 1990), vaccination (Alabi *et al.*, 2000), use of probiotics (Garriques and Arevalo, 1995; Verschuere *et al.*, 2000; Sung *et al.*, 2001) and immunostimulants (Sung *et al.*, 1996).

The green alga, *Tetraselmis suecica*, has been reported to inhibit *in vitro* growth of pathogenic *Vibrio*. Austin and Day (1990) used spray-dried *T. suecica* and reported the same to inhibit prawn pathogenic strains of *Vibrio* spp. Austin *et al.* (1992) reported that extracts from *T. suecica* inhibited the growth of number of bacterial fish pathogens of Atlantic Salmon (*Salmo salar*). The same authors also reported that the use of algae as food supplement resulted in the reduction of bacterial numbers in the culture water. Olsen *et al.* (2000) reported reduction in pathogenic bacteria (*Vibrio* spp.) in bacterial flora associated with juvenile *Artemia franciscana* by short term feeding of *Tetraselmis* sp.

In this study, *Tetraselmis suecica* was used as feed, both in the maturation and larval rearing tanks of the Indian white prawn (*Fenneropenaeus indicus*). 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 analysed 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

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 were maintained axenically in batch culture method and mass cultures were maintained semi-continuously in 200 litre 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 acclimatized to hatchery conditions for ten days. After acclimatization, 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 and the stocking density was at $8/m^2$; 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), squid fractions after processing and before feeding were marinated in algal paste at 2% level (2 g algal dry weight/100 g squid) for twelve hours at 8 °C. The control tanks received similar percentage of squid processed and stored similarly, but without algae. Everyday, in the early morning and evening, the remaining feed and faecal matter in the maturation tanks were siphoned out. The daily water exchange was 100%, by slow, continuous flow-through. Usually, aeration was kept mild and water exchange 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 density. The different larval stages were fed following the normal commercial hatchery feeding regime (Table 21). 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 larvae were fed 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.

Table 21. Larval feeding regime with microalgae, artificial feed and *Artemia*.

Substage	*Chaetoceross muelleri* (cells/ml)	*Tetraselmis suecica* (cells/ml)	Artificial feeds (mg/l/day)	*Artemia* (nauplii/ml/day)
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

Sampling and Analysis

Dry weight of the concentrate was determined by resuspension of concentrate in pre-weighed tubes with 5 ml of ammonium formate (0.5 M) to remove non-volatile 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 28-30 °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* counts, but, were incubated for 48 hours at 28-30 °C.

During the 25 days of maturation experiment, a total of 36 samples each (water, gut content, egg and nauplii) were collected from control and tetraselmis-fed 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 28-30 °C for 48 hours.

The larvae of different stages and postlarvae were collected from the rearing tanks (each treatment in two tanks) after the metamorphosis of the required larval stage. The water and larval samples (collected before water exchange) 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/ml or cfu/g) and all colonies appeared were assumed to be *Vibrio*. Plates were observed at 6 hours interval in total darkness for two days to determine the number of luminescent colonies. With all 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 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.

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/l. The mean *Vibrio* counts obtained with various samples collected from the individual control and tetra-fed tanks throughout the experiment period are given in table 22. No

Table 22. 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 tank.

Maturation tank	Maturation tank water (cfu/ml)	Gut content (cfu/g)	Spawning tank water (cfu/ml)	Eggs (cfu/g)	Nauplii (cfu/g)
CONTROL 1	$4.36 \pm 0.44 \times 10^3$	$5.52 \pm 0.72 \times 10^5$	$5.07 \pm 0.33 \times 10^3$	$4.02 \pm 0.27 \times 10^4$	$4.27 \pm 0.64 \times 10^4$
CONTROL 2	$5.09 \pm 0.40 \times 10^3$	$8.55 \pm 1.31 \times 10^5$	$6.45 \pm 0.59 \times 10^3$	$2.17 \pm 0.58 \times 10^4$	$7.29 \pm 0.36 \times 10^4$
CONTROL 3	$3.68 \pm 0.42 \times 10^3$	$1.09 \pm 0.15 \times 10^6$	$3.91 \pm 0.51 \times 10^3$	$3.21 \pm 0.29 \times 10^4$	$5.71 \pm 0.28 \times 10^4$
ALGAE 1	$4.59 \pm 0.34 \times 10^2$	$1.19 \pm 0.07 \times 10^3$	$2.87 \pm 0.25 \times 10^2$	$6.46 \pm 0.59 \times 10^2$	$1.03 \pm 0.13 \times 10^3$
ALGAE 2	$6.30 \pm 0.49 \times 10^2$	$2.33 \pm 0.14 \times 10^3$	$3.26 \pm 0.26 \times 10^2$	$5.75 \pm 0.54 \times 10^2$	$8.27 \pm 0.75 \times 10^2$
ALGAE 3	$7.52 \pm 0.36 \times 10^2$	$5.46 \pm 0.27 \times 10^3$	$4.56 \pm 0.34 \times 10^2$	$3.92 \pm 0.41 \times 10^2$	$7.04 \pm 0.55 \times 10^2$

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

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 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 in the maturation and spawning water. A reduction of two to three log units was obtained in gut content *Vibrio* and a little less than two log reduction in egg and nauplii. Analysis with reproductive performance data indicated that the values for mean egg hatching percentage (61.4% and 72.1%) and broodstock survival (79.4% and 88.2%) from control and *Tetraselmis*-fed treatments respectively, differed significantly ($P<0.01$).

A significant positive correlation was noticed between spawning tank water *Vibrio* count and egg *Vibrio* count ($r=0.57$; $P<0.01$; Fig. 9). The egg *Vibrio* count showed a negative correlation with relation to hatching rate ($r=-0.51$; $P<0.01$; Fig.10).

Larval rearing experiment

The tank water temperature ranged from 29.4 °C to 30.8 °C, pH varied from 8 to 8.2 and oxygen always above 6.5 mg/l. In the case of xenic algae, the overall mean total heterotrophic bacterial count in *Chaetoceros* was 3.1×10^2 and with *Tetraselmis* concentrate 9.4×10^1. The mean *Vibrio* counts of *Chaetoceros* and *Tetraselmis* concentrates were 7×10^1 and 4.2×10^1 respectively. No luminescent colonies were observed in the inoculated plates of NA and TCBS.

Fig. 9. Correlation between *Vibrio* count of spawning tank water and egg *Vibrio* count

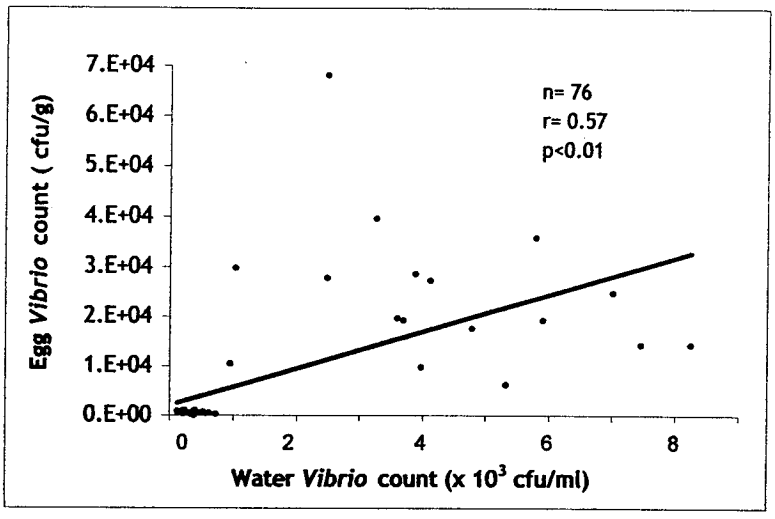
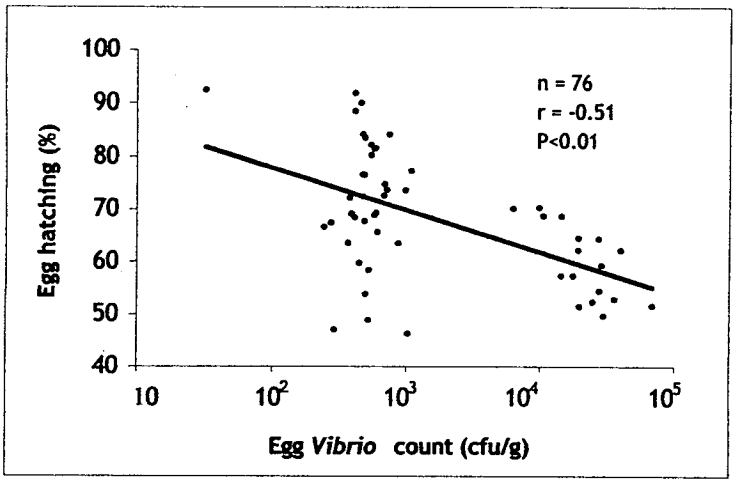


Fig. 10. Correlation between the egg *Vibrio* count and egg hatching



Results from the larval rearing experiments are given in table 23. 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 at the minimum single log unit less in CT_X and CT_{AX} tanks, with two log reductions during the mysis 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.

In Z, M and PL samples, a significant ($P<0.05$) reduction in *Vibrio* count was noticed between the *Tetraselmis*-fed tanks and control treatments, but not with nauplius 5 stage (N_5) ($P>0.05$). While the Z and M samples from *Tetraselmis*-fed tanks recorded one log count less than control, it was two log with PL samples. The *Vibrio* count (cfu/g) between N, Z, M and PL samples differed, depicting a 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. However, in 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* count (Fig.11,12 and 13) showed a significant negative correlation ($r = -0.53$, -0.78 and -0.70; t - test; $P<0.05$).

Table 23 . Mean *Vibrio* counts (± S.E.)* of larvae and postlarvae of *F. 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}).

Treatment	Nauplii (cfu/g)	Larval rearing tank water (cfu/ml)	Zoea (cfu/g)	Larval rearing tank water (cfu ml)	Mysis (cfu/g)	Larval rearing tank water (cfu/ml)	Postlarvae (cfu/g)	Larval rearing tank water (cfu/ml)
C1	$2.8 \pm 0.24 \times 10^3$	$1.6 \pm 0.30 \times 10^3$	$5.4 \pm 0.07 \times 10^4$	$5.7 \pm 0.28 \times 10^3$	$1.3 \pm 0.28 \times 10^5$	$1.4 \pm 0.08 \times 10^4$	$3.3 \pm 0.32 \times 10^6$	$7.6 \pm 0.22 \times 10^4$
C2	$6.2 \pm 0.32 \times 10^3$	$5.9 \pm 0.15 \times 10^3$	$9.2 \pm 0.24 \times 10^4$	$9.8 \pm 0.30 \times 10^3$	$3.6 \pm 0.37 \times 10^5$	$5.0 \pm 0.16 \times 10^4$	$5.1 \pm 0.27 \times 10^6$	$5.6 \pm 0.18 \times 10^4$
CT_x	$4.7 \pm 0.28 \times 10^3$	$4.4 \pm 0.17 \times 10^2$	$6.4 \pm 0.32 \times 10^3$	$9.9 \pm 0.12 \times 10^2$	$1.3 \pm 0.22 \times 10^4$	$8.8 \pm 0.20 \times 10^2$	$7.8 \pm 0.16 \times 10^4$	$2.1 \pm 0.20 \times 10^3$
CT_X	$5.1 \pm 0.18 \times 10^3$	$7.8 \pm 0.18 \times 10^2$	$5.9 \pm 0.18 \times 10^3$	$6.4 \pm 0.31 \times 10^2$	$9.7 \pm 0.22 \times 10^3$	$1.3 \pm 0.31 \times 10^3$	$6.6 \pm 0.17 \times 10^4$	$3.1 \pm 0.31 \times 10^3$
CT_{AX}	$9.2 \pm 0.16 \times 10^2$	$2.7 \pm 0.22 \times 10^2$	$7.6 \pm 0.21 \times 10^3$	$1.0 \pm 0.24 \times 10^3$	$1.0 \pm 0.18 \times 10^4$	$9.4 \pm 0.16 \times 10^2$	$8.7 \pm 0.16 \times 10^4$	$4.3 \pm 0.28 \times 10^3$
CT_{AX}	$2.2 \pm 0.14 \times 10^3$	$6.2 \pm 0.28 \times 10^2$	$9.3 \pm 0.33 \times 10^3$	$8.3 \pm 0.24 \times 10^2$	$1.5 \pm 0.15 \times 10^4$	$5.7 \pm 0.22 \times 10^3$	$9.2 \pm 0.22 \times 10^4$	$7.6 \pm 0.18 \times 10^3$

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

Fig. 11. Correlation between mean *Vibrio* count of zoea larvae and its survival

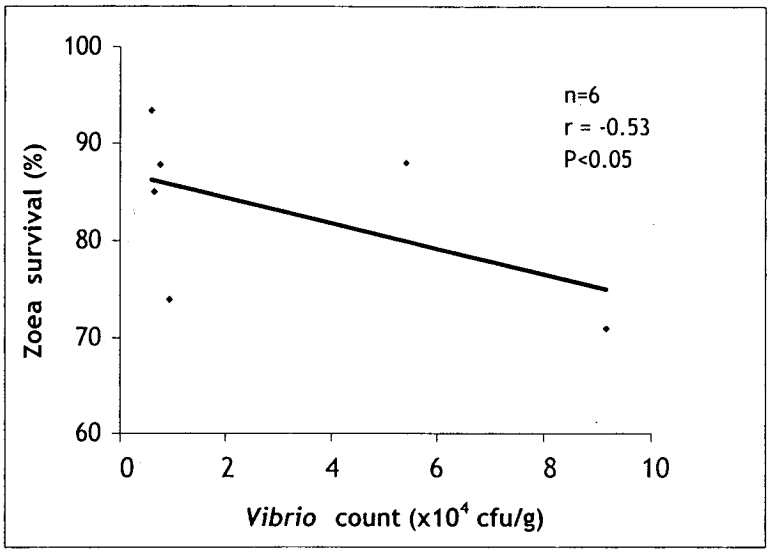


Fig. 12. Correlation between mean *Vibrio* count of Mysis larvae and its surviva

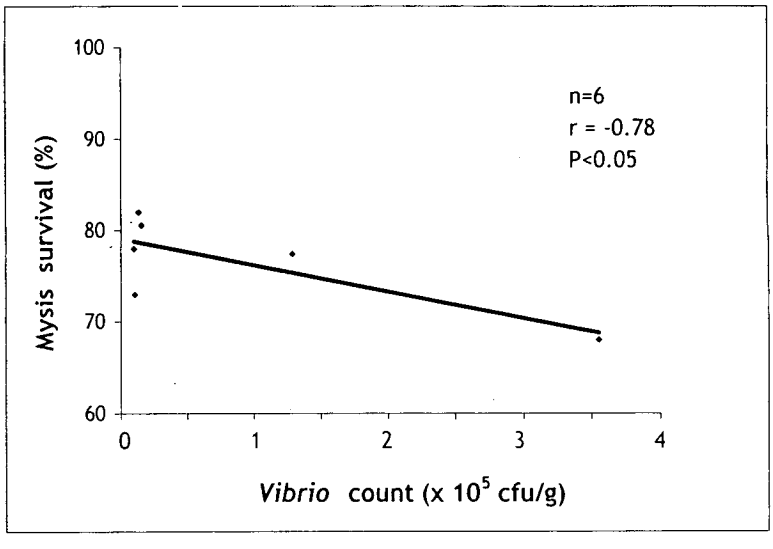
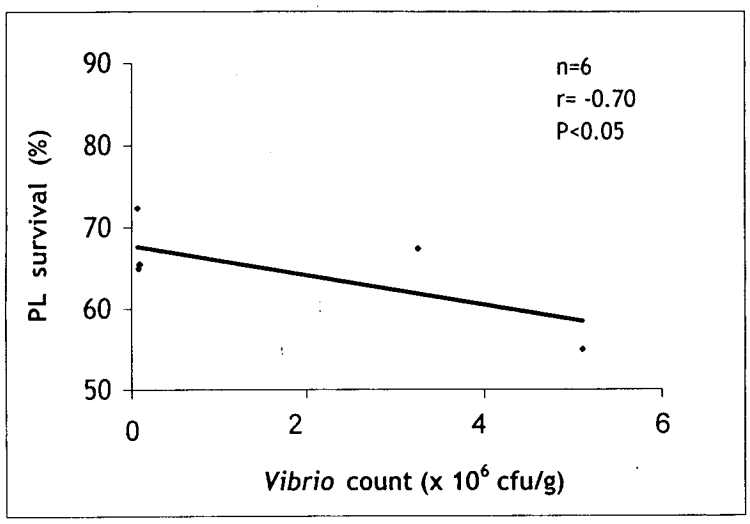


Fig. 13. Correlation between mean *Vibrio* count of post larvae (PL) and its survival percentage



DISCUSSION

The antibacterial activity exhibited by algae has been widely reported. The microalgae namely *Skeletonema costatum* (Kogure *et al.*, 1979; Cooper, 1983; Kitto and Regunathan, 1997; Naviner *et al.*, 1999), *Chattonella marina* (Riquelme and Ishida, 1988), *Chlorella* (Tendencia and de la Pena, 2003), *Phaeodactylum tricornutum* (Cooper *et al.*, 1983) and *Tetraselmis suecica* (Viso *et al.*, 1987; Kellam and Walker, 1989; Austin and Day, 1990; Olsen *et al.*, 2000) have been confirmed for their antibacterial activity. In all the above mentioned algae, except for *Chattonella marina*, all the rest have shown anti-*Vibrio* activity.

Maturation tank experiment

Among the different sources of *Vibrio* contamination in a hatchery, spawners may very much serve as vector, especially through its gut contents. Dominance of *Vibrio* in *F. indicus* gut contents and its possible harmful role in the life of animal have been reported by Singh *et al.* (1998). Lavilla-Pitogo *et al.* (1992) reported 10^8 levels of *Vibrio harveyi* cells/ml of tiger prawn 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 shows 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 multiplication by *Tetraselmis*.

Austin *et al.* (1992) using *T. suecica* as feed supplement for Atlantic salmon, reported that after a seven day feeding regime, an increase in the numbers of Enterobacteriaceae representatives in the fish 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.

Olsen *et al.* (2000) reported that feeding *Artemia franciscana* with *Tetraselmis* influenced the numbers and composition of the associated flora of *Artemia*. In their study, incubating the 2-day old *Artemia* in *Tetraselmis* culture resulted in lower numbers of pathogenic bacteria (*Vibrio alginolyticus*) as well as in a more diverse bacterial community.

Austin *et al.* (1992) noted a two log level drop in total heterotrophic bacterial numbers in tank water after a seven day period, when Atlantic Salmon were fed with food supplemented with *Tetraselmis* algal cells. In this experiment also, log reduction in *Vibrio* numbers were noticed in the maturation tank water and might 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 count of *Vibrio* in water samples and eggs. Microbial community of the ambient water has been shown to influence the composition of the

bacterial egg epiflora (Olafsen, 2001). It may be that reduction in *Vibrio* in the spawning tank resulted in non-pathogenic egg community composition that restricted 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 trout (*Salmo gairdneri*) and brown trout (*Salmo trutta*) eggs.

The increment in broodstock survival may be due to the presence of less *Vibrio* in the gut of animals from *Tetraselmis*-fed tanks. Yasuda and Kitao (1980) observed an abundant *Pseudomonas* population in the gut of healthy, cultured and wild adult prawn *Marsupenaeus 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

In larval rearing water samples, the log reduction of *Vibrio* count in the CT_X and CT_{AX} treatments proved the inhibitory effect of *Tetraselmis*. As, even 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.* (1999a) while experimenting with *Skeletonema* and *Tetraselmis* on rearing of *F. indicus* from Z_I to M_I 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 microencapsulated diet 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 favour 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 to be very rare in algal cultures (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.

In 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 (25-32 %). 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 this study between *Vibrio* population and larval survival corroborate with the reports of Hameed (1993) and Singh (1986).

Austin and Day (1990) with antibiograms 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 the same to be a 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 in a number of microalgae (Viso *et al.*, 1987; Naviner *et al.*, 1999). Many authors have reported that the antibacterial activity of microalgae was due to fatty acids (Findlay and Patil, 1984; Viso *et al.*, 1987; Kellam *et al.*, 1988).

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.*, 1989b).

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 even better than probiotics in that the growth media (rich in organic nutrients) added with probionts may boost growth of unwanted micro organisms too. Usage of algae does not pose such problems and reduces the expenditure on probiotics. So, it is very much recommended to use the algae as a maturation diet supplement and also as a ingredient in the regular feeding regime of larval rearing.