

Low-input Modified Extensive Shrimp Culture Systems for *Penaeus monodon* Restrain Vibriosis

Sheryl Oliveira Fernandes¹, R.A. Sreepada², Shantanu S. Kulkarni², Sheetal V. Karekar², Resha R. Shirodkar², Christian Vogelsang³, P. A. LokaBharathi¹✉

1. Marine microbiology laboratory, CSIR-National Institute of Oceanography, Dona Paula, Goa - 403 004, India

2. Aquaculture laboratory, CSIR-National Institute of Oceanography, Dona Paula, Goa - 403004, India

3. Norwegian Institute for Water Research (NIVA), Brekkeveien 19, Kjelsaas-N-0411 Oslo, Norway

✉ Corresponding author email: loka@nio.org

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Abstract It was hypothesized that an outbreak of vibriosis can be restrained in ponds with a low stocking density (~5 post-larvae m⁻²) till harvest. Therefore, during a 135 day production cycle of *Penaeus monodon* at an aquaculture facility, we monitored (i) physico-chemical parameters (ii) abundance of total heterotrophic bacteria (THB), total *Vibrio* like organisms (TVLO) and luminescent bacteria (LB) (iii) alterations in environmental factors controlling *Vibrio* abundance and (iv) changes in composition of the *Vibrio* community. Physico-chemical parameters were within the optimum range recommended for culture of *P. monodon*. In source and pond water, THB abundance remained steady at 10³ CFU mL⁻¹ throughout the culture period whereas in sediments, they varied between 10²⁻⁴ CFU g⁻¹. The abundance of TVLO and LB in pond components were below the threshold levels of 10⁴ CFU mL⁻¹ and 10² CFU mL⁻¹ respectively. THB, TVLO and LB were more abundant in the shrimp hepatopancreas (10⁵ CFU g⁻¹) yet apparently below the threshold for the outbreak of vibriosis in the current set up. Changes in bacterial abundance were seemingly independent of changes in environmental parameters suggesting that the systems maintained TVLO below disease-causing threshold. The *Vibrio* community was represented mainly by *V. metschnikovii*, *V. fluvialis*, *V. mimicus* and the closely related *Aeromonas spp.* The vibrios in shrimp hepatopancreas were similar to other pond components with no dominance of particular species. A disease-free environment prevailed and shrimp yield was sustainable within the set framework perhaps due to the low abundance of vibrios represented by innocuous strains.

Keywords Abundance; Bacteria; Environmental conditions; Species diversity; Shrimp culture; Vibriosis

Introduction

In the recent years aquaculture has emerged as the world's fastest food-producing sector growing at an annual rate of almost 10% per year since 1970 (Subasinghe, 2005). Shrimp farming constitutes an important source of revenue and employment in many developing countries in Asia and Latin America. However, rapid expansion of this industry has spawned various technical, ecological, economical and social ordeals. A multitude of factors has contributed to the health problems currently faced by the industry. Between 1987 and 1994, the combined economic losses from shrimp diseases in 11 countries were estimated at US \$ 3019 million. In India, the loss during 1994 was estimated at US \$ 125 million (Israngkura and Sae-Hae, 2002). Therefore, the sustainability of shrimp culture industry largely

depends on disease control and health status of the shrimp. Outbreaks of vibriosis and low survival rates in hatchery and grow-out ponds (Lightner, 1988; Lightner, 1993; Brock and Lightner, 1990) have become a severe barrier to the development of shrimp aquaculture. Vibrios are among the most abundant cultivable microbes in aquatic environments (Heidelberg et al., 2002) and usually constitute a part of normal micro flora in farmed and wild penaeid shrimps. Opportunistic vibrios may turn pathogenic when shrimps are stressed due to deteriorated environmental conditions like sub-optimal or unstable environment, high stocking densities and inadequate management. When such stresses prevail, a large increase in the number and composition of *Vibrio* spp. in the culture environment is expected (Sung et al., 1999). Despite the opportunistic nature of most

pathogenic vibrios, findings by Costa et al. (1998) suggest that some diseases occurring in penaeid shrimps have been caused by *Vibrio* spp., which behave more like true pathogens rather than opportunists. Pathogenic vibrios usually invade the host through the hepatopancreas, the common target organ of most bacterial pathogens of shrimps (Chen et al., 1992; Frelie et al., 1992). They then proliferate and colonize the host digestive tract becoming pathogenic.

Studies on vibrios in shrimp ponds have focused on their isolation, characterization and identification (Gomez-Gil et al., 1998; Lavillo-Pitogo et al., 1998; Leño et al., 1998; Sung et al., 1999; Abraham and Palaniappan, 2004). A comparison of vibrios in water, sediment and shrimp samples from multiple shrimp ponds from east and west coast of India has been made by Gopal et al (2005). However, the changes in the abundance and composition of *Vibrio* spp. in shrimp pond components during the entire crop cycle from post larvae (PL) till the harvestable size has not been studied, particularly from modified extensive ponds. Elevated organic loading from shrimp farming activities can result in an increase in the abundance of heterotrophic bacteria (Zaccone et al., 2005) as they are active degraders of organic matter. Generic composition of heterotrophic isolates in an aquaculture pond has shown the predominance of *Vibrio* spp., (Ganesh et al., 2010). A comprehensive knowledge of the changes in the abundance and species composition of vibrios would therefore be inevitable for a chosen set of environmental conditions in aquaculture ponds. This approach would not only help understanding the dynamics but also plan strategies for disease containment and increasing shrimp yield. Therefore, during a 135 day production cycle of *Penaeus monodon* we aimed to (i) monitor changes in physico-chemical parameters along with the abundance of total heterotrophic bacteria (THB), total *Vibrio* like organisms (TVLO; potential pathogens) and luminescent bacteria (LB; confirmed pathogens) in different pond components (rearing water and sediment, pond water and sediment and shrimp hepatopancreas) (ii) examine the relationship between environmental variables and abundance of vibrios and (iii) assess changes in the composition of the *Vibrio* community in different components of shrimp pond. Results obtained during the study revealed prevalence of a healthy

environment within the culture ponds with no outbreak of pathogenic vibrios during the culture period. The *Vibrio* diversity within the shrimp hepatopancreas was similar to the aquatic system with no detection of pathogenic LB towards the end of the culture. Thus, this study enabled us to establish a framework within which the modified extensive shrimp ponds operated optimally leading to a sustainable yield.

1 Results

1.1 Physico-chemical characteristics

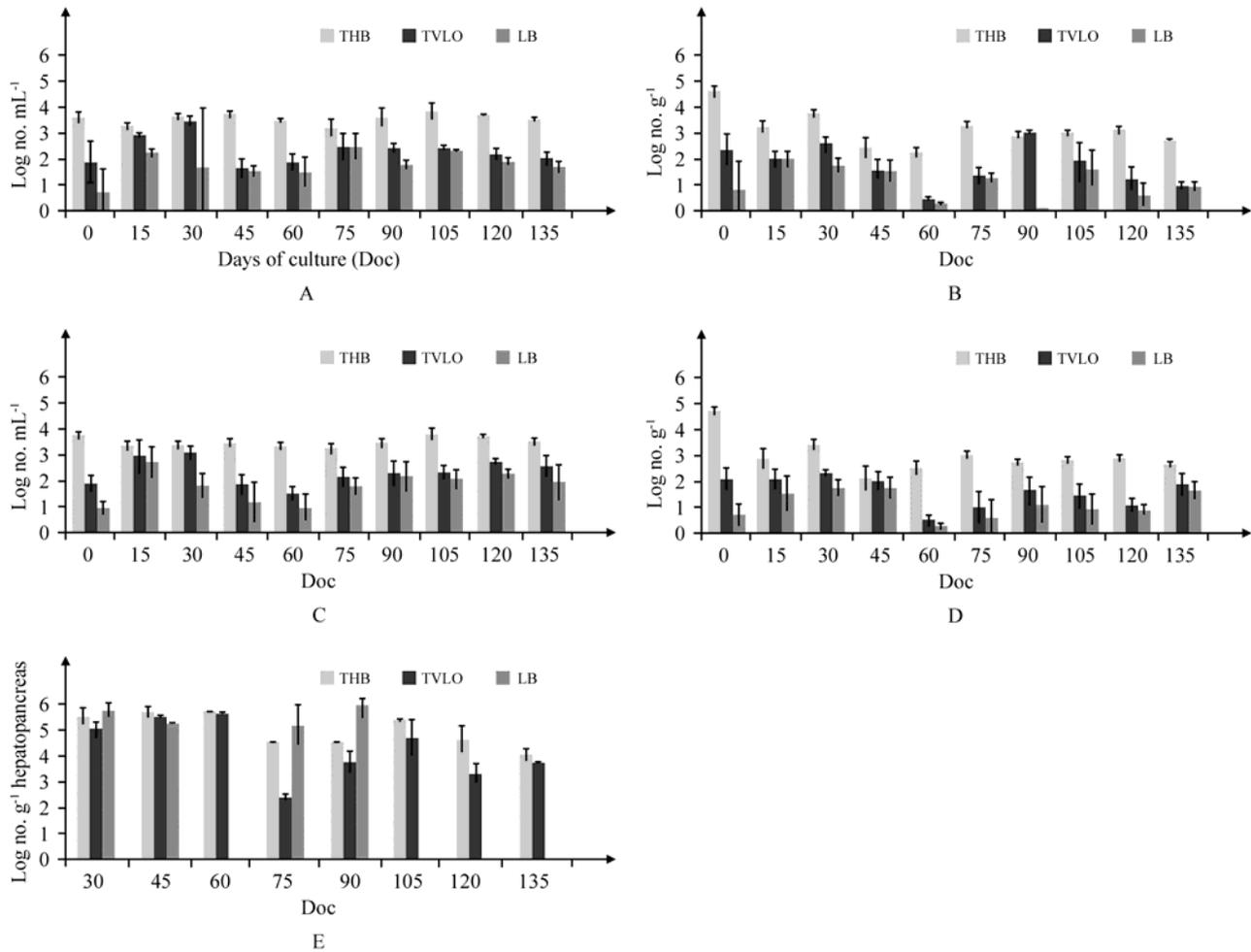
Results of physico-chemical characteristics analyzed in the pond water and sediment during the culture of *P. monodon* have been tabulated in Table 1a. Between ponds, no significant differences in physico-chemical parameters were observed. Hence, the values from two ponds were pooled ($n = 6$) to obtain an average (\pm SD). A gradual increase in the temperature and salinity of the pond water through the culture period was noted. Temperature of pond water increased from 22.2 ± 0.4 °C at the start of the cycle reaching to a maximum of 32.5 ± 0.4 °C at the end of 135 days. Similarly, salinity increased from 8.7 ± 0.2 at 0-doc to 34.0 ± 0.2 at the end of the cycle. Near neutral pH (7.3 ± 0.2 to 8.0 ± 0.1) was recorded in the pond water whereas the pH of sediment in the ponds was more acidic with little variation through the culture period (6.2 ± 0.2 to 6.7 ± 0.2). Eh values in the pond water ranged between 27.90 ± 0.19 and 142.77 ± 3.25 whereas in the pond sediments the range was relatively lower varying from 26.78 ± 2.57 to 127.73 ± 1.60 . Dissolved oxygen generally showed a decreasing trend as the culture period progressed. The lowest dissolved oxygen content of 4.4 ± 0.3 mg L⁻¹ was recorded at 120-doc. POC in the pond (Table 1a) and creek water (Table 1b) followed a similar trend with a steady increase in concentration until 60-doc to reach about 25.2 ± 8.4 g C m⁻³. Thereafter, POC in the pond and creek varied with doc. The organic matter content in the pond sediments increased with the progress of the culture period. A maximum of 58.1 ± 10.6 mg g⁻¹ was recorded on 120th day of observation.

In the water/sediment sourced from the nearby creek, the trend in the variation of temperature, salinity, pH, Eh, dissolved oxygen and sediment organic matter were similar to those observed in the culture ponds (Table 1b).

1.2 Bacterial counts

No significant difference in THB, TVLO and LB abundance was found between ponds. Hence, the bacterial counts from the two ponds were pooled to obtain average

values (n = 6). Fortnightly variations in counts of THB, TVLO and LB in different shrimp pond components (source water and sediment, rearing water and sediment and shrimp hepatopancreas) are depicted in Figures 1 a-e.



Figures 1a-e Fortnightly changes in the abundance of total heterotrophic bacteria (THB), total *Vibrio* like organisms (TVLO) and luminescent bacteria (LB) in creek water (a), creek sediment (b), pond water (c), pond sediment (d) and shrimp hepatopancreas (e) during the cultivation of *P. monodon*. Vertical bars denote standard deviation

Counts of total heterotrophic bacteria (THB) in the pond water did not vary much and remained at 10^3 CFU mL⁻¹ throughout the culture period. In the pond sediments, their abundance varied from 10^4 CFU g⁻¹ at the beginning of the culture period decreasing to 10^2 CFU g⁻¹ towards the end of the culture period. The abundance of THB in the source water also followed a similar pattern as in the ponds. However, in shrimp hepatopancreas, their numbers were 1-2 orders higher at 10^{4-5} CFU g⁻¹.

The abundance of TVLO observed in rearing water was not significantly different from that of source

water. In the culture ponds and source water, TVLO were low on 0-doc (0.02×10^3 CFU mL⁻¹) but increased by 2 orders at 30-doc. Thereafter, their numbers reduced by 60-doc coinciding with water exchange. The abundance of TVLO in creek and pond sediments followed a similar pattern of abundance through the culture period varying from 10^{1-3} CFU g⁻¹. In shrimp hepatopancreas, the TVLO abundance was significantly higher and varied from 10^{4-5} CFU g⁻¹.

The LB in the source water and ponds followed a similar pattern of abundance through the culture period varying between $0.01-1.46 \times 10^3$ CFU mL⁻¹. In

the creek and pond sediments, their abundance was far lesser varying between non-detectable levels to 0.10×10^3 CFU g^{-1} . Counts of LB were found to be below detectable levels in hepatopancreas between 105 to 135-doc.

1.3 Correlation analysis

None of the measured environmental variables (temperature, salinity, pH, DO) showed any significant correlation with bacterial abundance either in rearing water and/or in the pond sediments.

1.4 Bacterial characterization

A total of 114 and 42 bacterial isolates from shrimp pond components and source water respectively were retrieved from TCBS plates. The representative isolates were identified according to cultural, morphological and biochemical characteristics. Species composition of *Vibrio* communities recovered from shrimp pond components and source water have been presented in Table 2. Among the pond components, maximum isolates were retrieved from rearing water (42%), followed by pond sediment (18%) and shrimp hepatopancreas (13%).

The *Vibrio* community remained fairly diverse throughout the culture period. Within the shrimp culture system, closely related *Aeromonas* spp., *V. fluvialis*, and *V. metschnikovii* were most dominant (Table 3). Species such as *V. mimicus*, *V. mediterranei*, *V. gazogenes*, *V. harveyi* and *V. hollisae* contributed less than 10% of the total isolates. Relatively low species diversity was noticed in source water with a dominance of *Aeromonas* spp., *V. fluvialis*, and *V. metschnikovii*. In rearing water, *Aeromonas* spp. showed complete dominance at 34% although *V. fluvialis* (15%) and *V. metschnikovii* (12%) were consistently present during the culture period. In pond sediment, *Aeromonas* spp. dominated followed by *V. fluvialis* (13%) and showed a greater species diversity in the middle and later stages of the culture. The *Vibrio* community in the hepatopancreas had limited diversity through the culture period with *V. metschnikovii* and *V. salmonicida* being some of the dominant strains. The presence of *V. logei* and *V. marinus* was also observed in the hepatopancreas.

Table 2 Composition of vibrios and closely related pathogenic species isolated from shrimp pond components during cultivation of *Penaeus monodon*

Species	Number of isolates			
	Source water	Pond water	Pond sediment	Shrimp hepatopancreas
<i>Aeromonas</i> spp.	9 (23)	23 (35)	12 (41)	5 (25)
<i>Phenon 10/85</i>	2 (5)	–	–	1 (5)
<i>Phenon 21</i>	2 (5)	–	–	–
<i>Plesiomonas shigelloides</i>	–	–	3 (10)	–
<i>V. fluvialis</i>	6 (15)	8 (12)	4 (14)	1 (5)
<i>V. furnissii</i>	–	–	2 (7)	–
<i>V. gazogenes</i>	1 (2)	2 (3)	1 (3)	1 (5)
<i>V. harveyi</i>	–	2 (3)	–	–
<i>V. hollisae</i>	–	2 (3)	1 (3)	–
<i>V. logei</i>	–	–	–	1 (5)
<i>V. marinus</i>	–	–	–	2 (10)
<i>V. mediterranei</i>	2 (15)	4 (6)	1 (3)	1 (5)
<i>V. metschnikovii</i>	11 (28)	10 (15)	3 (10)	5 (25)
<i>V. mimicus</i>	6 (15)	6 (9)	2 (7)	–
<i>V. natriegenes</i>	–	1 (2)	–	–
<i>V. ordalii</i>	1 (2)	2 (3)	–	1 (5)
<i>V. salmonicida</i>	–	4 (6)	–	2 (10)
<i>V. splendidus II</i>	–	1 (2)	–	–
Total no. of isolates	42	65	29	20
Percentage of isolates	27	42	18	13

Note: Isolates retrieved from shrimp pond components = 114 and source water = 42; total isolates = 156. Percentage of isolates is given in parenthesis

Table 3 Changes in the composition of *Vibrio* spp. community (% of total *Vibrio* isolates) and closely related pathogenic species in different shrimp pond components during cultivation of *Penaeus monodon*

<i>Vibrio</i> species	Days of culture (doc)										
	0	15	30	45	60	75	90	105	120	135	Av. abundance (%)
(a) Source water											
<i>Aeromonas</i> spp.	50	20	20	29	0	25	33	0	20	33	23
<i>Phenon 10/85</i>	0	20	0	0	0	0	0	0	0	33	5
<i>Phenon 21</i>	0	0	0	14	0	0	17	0	0	0	3
<i>V. fluvialis</i>	0	20	20	14	0	25	17	33	0	0	13
<i>V. gazogenes</i>	0	0	0	0	0	0	0	0	20	0	2
<i>V. mediterranei</i>	0	0	0	0	0	50	0	0	0	0	5
<i>V. metschnikovii</i>	50	0	20	14	100	0	33	33	60	33	34
<i>V. mimicus</i>	0	40	40	29	0	0	0	0	0	0	11
<i>V. ordalii</i>	0	0	0	0	0	0	0	33	0	0	3
(b) Pond water											
<i>Aeromonas</i> spp.	46	0	60	57	25	29	17	14	40	50	34
<i>V. fluvialis</i>	0	33	20	0	25	14	0	14	40	0	15
<i>V. gazogenes</i>	0	0	0	0	13	0	0	0	20	0	3
<i>V. harveyi</i>	0	0	0	0	0	29	0	0	0	0	3
<i>V. hollisae</i>	0	0	0	0	0	0	17	14	0	0	3
<i>V. mediterranei</i>	0	33	0	0	13	0	17	14	0	0	9
<i>V. metschnikovii</i>	31	0	20	14	13	14	17	14	0	0	12
<i>V. mimicus</i>	15	33	0	29	13	0	0	0	0	0	9
<i>V. natriegens</i>	0	0	0	0	0	14	0	0	0	0	1
<i>V. ordalii</i>	0	0	0	0	0	0	17	14	0	0	3
<i>V. splendidus II</i>	0	0	0	0	0	0	0	0	0	25	3
<i>Vibrio</i> unknown spp.	8	0	0	0	0	0	17	14	0	25	6
(c) Pond sediment											
<i>Aeromonas</i> spp.	100	33	80	0	0	33	33	0	33	33	35
<i>Plesiomonas shigelloides</i>	0	17	0	0	33	33	0	0	0	0	8
<i>V. fluvialis</i>	0	0	0	0	0	33	67	0	33	0	13
<i>V. furnissii</i>	0	17	20	0	0	0	0	0	0	0	4
<i>V. gazogenes</i>	0	0	0	0	0	0	0	0	0	33	3
<i>V. hollisae</i>	0	0	0	0	0	0	0	0	0	33	3
<i>V. mediterranei</i>	0	0	0	0	33	0	0	0	0	0	3
<i>V. metschnikovii</i>	0	17	0	0	33	0	0	0	33	0	8
<i>V. mimicus</i>	0	17	0	100	0	0	0	0	0	0	12
(d) Shrimp hepatopancreas											
<i>Aeromonas</i> spp.	n.s	n.s	n.s	0	0	40	20	0	20	50	19
<i>Phenon 10/85</i>	n.s	n.s	n.s	0	0	0	20	0	0	0	3
<i>V. fluvialis</i>	n.s	n.s	n.s	0	0	0	0	50	0	0	7
<i>V. gazogenes</i>	n.s	n.s	n.s	0	0	0	20	0	0	0	3
<i>V. logei</i>	n.s	n.s	n.s	0	0	0	0	50	0	0	7
<i>V. marinus</i>	n.s	n.s	n.s	0	0	20	20	0	0	0	6
<i>V. mediterranei</i>	n.s	n.s	n.s	0	0	0	0	0	20	0	3
<i>V. metschnikovii</i>	n.s	n.s	n.s	0	0	20	20	0	40	50	19
<i>V. ordalii</i>	n.s	n.s	n.s	0	0	20	0	0	0	0	3
<i>V. salmonicida</i>	n.s	n.s	n.s	0	100	0	0	0	20	0	17

Note: ns = not sampled; total isolates retrieved from source water = 42, pond water = 65, pond sediment = 29 and shrimp hepatopancreas = 20

1.5 Average weight, feed conversion ratio and survival

Cultured shrimps attained an average weight ranging from 0.05 ± 0.01 g on 0-doc to 32.0 ± 4.0 g on 135-doc with a total production of 650 ± 50 kg per pond. Average survival was about 78% and the feed conversion ratio (FCR) was 1.5 ± 0.1 .

2 Discussion

Monitoring of bacterial abundance and assessment of the taxonomic diversity of potential pathogens is essential to avoid viral outbreaks in aquaculture ponds. Very few studies have been carried out to systematically monitor the bacterial composition with special reference to vibrios in shrimp ponds. In the present study, we examined the abundance of THB along with potential pathogens like TVLO and LB. The abundance of THB in pond water recorded in this study were lower by 2-orders of magnitude than those previously reported from shrimp culture ponds (Putro et al., 1990; Ruangpan et al., 1995; Prem Anand et al., 1996; Bright Singh et al., 1998; Otta et al., 1999) and by an order of magnitude higher than those reported by Fonseka (1990), Nayyarahamed et al (1995) and Sharmila et al (1996). Such differences may be attributed to the species cultured, intensity of the culture system, feed used and media used to enumerate bacteria. The bacterial load of source water is also known to influence that of rearing water as reported from semi-intensive shrimp aquaculture farms by Nayyarahamed et al (1995) and Sharmila et al (1996). In the present study, no significant difference between THB in source/rearing water was observed. This could be due to the modified extensive system followed wherein the feed inputs and stocking rates are relatively lower than semi-intensive/intensive culture systems.

Vibrios constitute a major portion of the micro biota in brackish water pond ecosystems. In shrimp farms from India, vibrios account for 1 - 10% of the total bacterial CFU mL⁻¹ (Otta et al., 1999). Compared to the total heterotrophs enumerated in the present study, the retrievability of TVLO in pond water and pond sediment were high varying between 1 - 100% and 1 - 10% respectively. Considering a total bacterial load of 10^7 cells mL⁻¹ (unpublished data) in the ponds during the culture period, the TVLO form only 0.01% of the total bacterial population in the pond water whereas in

pond sediments, they constituted ~ 0.1%. The abundance of TVLO and LB during the culture were below the threshold levels of 10^4 CFU mL⁻¹ and 10^2 CFU mL⁻¹ respectively (Leano et al., 1998; Vaseeharan and Ramasamy, 2003). Hence, it can be implied that in low-input modified extensive shrimp culture systems and well managed shrimp ponds, the usage of antibiotics could be avoided.

Bacterial densities of 1-3 orders of magnitude higher in pond sediment as compared to rearing water have been reported (Moriarty, 1986; Allan et al., 1995; Ruangpan et al., 1997; Janakiram et al., 2000). However, in this study, the average bacterial counts in rearing water were significantly higher than in pond sediments (*t*-test, $P < 0.05$). This observation is unexpected as one could anticipate higher bacterial counts in pond sediments which contain nutrients in higher concentration resulting from the accumulation of leached and/or unconsumed feed, phytoplankton/zooplankton detritus and chitin deposition as a result of moulting. Gopal et al (2005) have reported a higher relative abundance of vibrios by up to 2-orders of magnitude in rearing water than in aquaculture pond sediments located along the west coast of India. Such differences have not been observed in shrimp ponds on the east coast where farmers stock relatively higher PL per m². Shrimps are known to be deposit feeders. It is possible that they ingest bacteria adhered to settled organic particles. Hence, a lower bacterial density in the sediments could be expected.

No clear-cut trend in TVLO abundance was observed in rearing waters. Absence of any particular trend may be attributed to the 'artificial' nature of culture system where the manipulation of the pond environment by the way of water quality management and pond bottom management (water exchange, liming) leads to frequent changes in biotic and abiotic factors in the pond. The only major change in TVLO abundance observed in the rearing water, was an increase of these bacteria by about two orders of magnitude until 30-doc. At the end of 60-doc, their numbers were restored to those recorded at the commencement of the culture. This change during the first 30-doc could be attributed to the lack of water exchange accompanied by input of feed and accumulation of fecal matter in the ponds. Once the water exchange

was complete, the TVLO abundance decreased to match that of the source water.

The TVLO in shrimp hepatopancreas were significantly higher than in rearing water and pond sediment ($P < 0.05$, t -test) during the corresponding period. This may be because of their known close association with the digestive tract. Lalitha and Surendran (2004) have also recovered intestinal bacterial density much higher ($> 3.5 \log \text{CFU g}^{-1}$) than those in rearing waters growing *Macrobrachium rosenbergii*. The abundance and composition of bacteria associated with intestinal tract is related to the environment as well as to the food the shrimps consume. It has been reported that the food materials concentrated in the intestine of marine animals can provide a favorable ecological niche for symbiotic heterotrophic populations, and these can be of nutritional importance to the host organisms by producing enzymes that help in digestion or by supplying part of its nutrients (Cahill, 1990). Some authors have claimed that extensive bacterial flora is not commonly found in the hepatopancreas because they are prevented from entering by the gastric sieve (Hopkin and Nott, 1980) in combination with digestive enzymes (Raissy et al., 2011). Damage to gastric sieve by artificial pellet-formed foods has been suggested to occur in cultivated animals (Gomez-Gil et al., 1998) leading to the colonization of bacteria in the internal organs (Raissy et al., 2011). Modified extensive culture method followed in the present study relies on natural productivity to some extent at least in the initial days of culture in contrast to semi-intensive and intensive culture methods. Additional work is required to determine whether the differences in colonization are in fact, due to the occurrence of particular lining and/or digestive mechanisms in different culture systems. A wide variation in the weight of hepatopancreas of shrimps was also observed during analysis (data not shown) which could be attributed to the differential amounts of ingesta in shrimps sampled in spite of our best efforts to collect specimen with full stomach. Further, insignificant correlation between the numbers of bacteria retrieved and weight of the hepatopancreas indicates that the majority of the bacteria were associated with the digestive tract itself rather than with the diet. This is in accordance with studies by Gomez-Gil et al. (1998). It has also been documented that shedding of the chitinous lining of the hindgut

due to frequent moulting in shrimps during a rapid growth phase can greatly influence the bacterial number and type of bacteria in the digestive system of shrimps (Dempsey et al., 1989).

Representative *Vibrio* isolates collected from different shrimp pond components in this study were identified using standard biochemical tests. It may be noted that molecular methods for taxonomic identification of *Vibrio* spp. have not been used in this study as they would be useful at a secondary level when there are indications of disease outbreaks or the number of vibrios are above threshold limits. The rearing waters and pond sediments had a fairly diverse *Vibrio* community with a larger prevalence of species such as *V. metschnikovii*, *V. fluvialis* and those belonging to the genus *Aeromonas*. These species were also found in the shrimp hepatopancreas suggesting that the bacterial flora colonizing the shrimp digestive system is normally drawn from the aquatic system either in the rearing water or the pond sediment. Some *Vibrio* species recorded solely from the shrimp hepatopancreas included *V. logei*, *V. marinus* and *V. salmonicida*. The halophilic and psychrophilic bacterium *V. salmonicida* (*Aliivibrio salmonicida*) is closely related to the luminous bacteria *V. fischeri* and *V. logei* (Fidopiastis et al., 1999). It is known to be a causative agent of cold water vibriosis in marine aquaculture causing tissue degradation, hemolysis and sepsis *in vivo* (Hjerde et al., 2008). In the present study, the average abundance of *V. salmonicida* in shrimp hepatopancreas was about 17% through the culture period. It is unlikely that they would have reached a threshold level to affect the shrimps. The presence of *V. gazogenes* was also recorded in shrimp hepatopancreas. This strain is generally found in the mid and hind gut of the shrimp and its presence could be due to cross contamination with other parts of shrimp digestive system while removing the hepatopancreas. Reports of infection by particular members of the genus *Vibrio* within cultured penaeid shrimp populations leading to disease are numerous (Lightner, 1988; Lavilla-Pitogo et al., 1990; Ruangpan and Kitao, 1991; Sung et al., 1999; Liu et al., 1996). Sung et al (2001) have even reported growth retardation. *V. gazogenes* is known to have antagonistic activity towards a panel of shrimp pathogenic vibrios causing a significant decline in the number of *Vibrio*-like bacteria in the fore and hind gut

(Thompson et al., 2010). Compared to many other opportunistic pathogenic *Vibrio* species, the occurrence of *V. gazogenes* in the shrimp culture system should not be a cause of concern to farmers and diagnosticians as this species has not been found to cause or occur in association with a disease outbreak when compared to other *Vibrio* species (Egidius, 1987).

The important consideration for aquaculture is to understand the influences of physiological stress and environmental factors on gut floral composition in cultured animals (Oxley et al., 2002). Ingestion of the free-living bacterial community in environments other than natural stable conditions may lead to establishment of abnormal gut floral species (Lynch and Hobbie, 1988), pre-disposing the host to pathogens (Munro et al., 1994). Moreover, the digestive system of unhealthy or diseased prawns may be found to support a gut flora where one or two species pre-dominate. Researchers have shown that stressed environment result in a decrease in the diversity of the microbial community and an increase in the numbers of functionally specific microbial groups (Atlas et al., 1991; Geiselbrecht et al., 1996). Outbreaks of disease in shrimp are poorly related to the increased number of vibrios in the culture environment *per se* but are strongly influenced by the significant increase in the proportion of potentially pathogenic *Vibrio* community in the rearing water as well as in the shrimp hepatopancreas (Sung et al., 1999; Sung et al., 2001). Furthermore, a close relationship between the incidence of disease and pathogen population of the surrounding water has also been documented (Amaro et al., 1995). Gomez-Gil et al (1998) reported a diverse population of vibrios associated with healthy *P. vannamei*. This differs from the situation where in one or two species pre-dominate in diseased animals. In the present study, different species of vibrios were found throughout the production cycle and there was no sudden increase in potentially pathogenic species, indicating that these were a part of the normal flora. Absence of a dramatic decrease in the diversity of *Vibrio* community either in rearing water, pond sediment or shrimp hepatopancreas during the culture suggests that the environmental conditions were apparently stress-free.

In the present study, proper pond management practices were followed with lime application and

water exchange during the culture period. Physico-chemical characteristics did not vary significantly during the culture period and were within the optimum range recommended for culture of *P. monodon* (Chien, 1992). Though some strong correlations were expected, statistical analysis did not provide any insights into the relationship between bacterial abundance and other environmental variables in rearing water and sediment. Overall, the relationships between abiotic and biotic variables were low. Similar results have been reported by Prem Anand et al (1996) and Janakiram et al (2000). Previous studies have attributed differences in density of vibrios to changes in environmental variables like temperature and salinity (Johnson et al., 2010). Bright Singh (1998) suggested that increase in the salinity of ambient water could enhance the proliferation of vibrios in *Penaeus indicus* culture system. Felix (2000) hypothesized that higher salinity and nutrient content could result in a 'blooms' of *Vibrio* spp. In the present study, *Vibrio* counts increased with an increase in salinity at certain period of the culture. However, this relationship was found to be inconsistent and statistically insignificant.

In conclusion, regular water exchange and liming maintained the water quality of the shrimp ponds during the culture period. Though the abundance of pathogenic bacteria in the pond waters was significantly higher compared to the sediments, their numbers were maintained below the threshold levels cited in literature. No dependency between abiotic and biotic components was observed during the culture period suggesting that the environmental parameters were within the threshold did not influence the abundance of bacteria. Culture-dependent approaches to examine the composition of the *Vibrio* community through the culture period revealed that many species recorded from shrimp hepatopancreas were also observed in other pond components. This observation indicates that the pathogenic micro-flora is derived from the ambient aquatic system. More importantly, the dominance of particular *Vibrio* spp. was not observed in the hepatopancreas which was indicative of a stress-free environment for the shrimps. Thus, timely monitoring of water quality parameters along with abundance/composition of pathogenic bacteria in different pond components can help detect disease outbreaks if any. Farmers could be trained to use such

routine analyses using minimal cost-effective resources at the field laboratory. As in this study, farmers would attain a high quality shrimp yield at the end of the culture period. Moreover, the release of water from the culture ponds at the end of the culture would not alter the balance of autochthonous micro-flora in the adjacent water bodies. Future studies would be directed towards increasing the stocking density by introducing aerators without compromising the environmental quality. However, this has to be carried out in a step-wise manner to understand the grid of interactions in detail as it would help us scale the yield gradually.

3 Materials and methods

3.1 Culture ponds, farm management and approach

Two identical shrimp culture ponds (P1 and P2; Figure 2) in Kumta taluka of Karnataka state, west coast of India (14.42 °N and 74.40 °E) were chosen as part of the Indo-Norwegian co-operation project entitled “Environmental management Strategy for Coastal Aquaculture Practices in South-western Coastal India”. Kumta experiences very hot summer with temperature varying between 28-36 °C. The impact of winter is less with temperature ranging from 20-26 °C. Rainfall in the region is seasonal (June to September), but heavy with annual precipitation exceeding 4000 mm. The shrimp farm had an easy access from national highway no. 17 and a good power supply. The site was also equipped with a field laboratory to carry out physical, chemical and biological analysis of pond water and sediment. The culture ponds had a water

spread area of ~ 0.60 ha and an average depth of 1.2 m. The source of water to the culture ponds was an adjacent tidal fed creek which was located about 3 km away from the sea.

Pond management practices as applicable to modified extensive culture method were followed. Before stocking, the ponds were sun dried for a period of one month and lime was applied to the bottom of the pond. The ponds were filled with water five days prior to stocking during high tide with the help of sluice gates and electric pump. White spot syndrome virus (WSSV) negative post-larvae (PL) of tiger shrimp *P. monodon* as confirmed by the polymerase chain reaction test were procured from a local hatchery. Larvae were actively moving around with no visible signs of disease or morbidity and were transferred into the two production ponds at a stocking density of 5 PL m⁻². No water exchange was done for the first 30- day of culture (doc). Due to the close physical proximity, the climatic conditions in both the ponds were almost similar. No artificial aeration was used during the culture period. An additional dose of lime was applied regularly after 40 days of culture (doc) to adjust the sediment pH around 7.0. Shrimps were fed with commercial shrimp pellets procured from C P Aquaculture (India) Pvt. Ltd. at 10% of their body weight twice a day during the juvenile stage. Precautions were taken to adjust the feeding according to the biomass and survival. Towards the end of the culture period, high organic load and nutrients in aqua farms can lead to eutrophic conditions. Further, oxygen-depletion and production of sulfide in sediments and water column can stress free-swimming fish (Bagarinao and Lantin-Olaguer, 1998). Therefore, the feeding rate was gradually reduced to 2% to prevent accumulation of organic matter within the ponds.

The ponds were monitored for one shrimp harvest cycle covering 135 days to assess the variation in physico-chemical parameters along with the abundance and diversity of culturable vibrios.

3.2 Collection of samples

Samples of source/creek water and pond water were collected one day prior to transfer of post-larvae and thereafter at fortnightly intervals after stocking from 0-doc until 135-doc between September 2003 and January 2004 at three pre-determined sampling

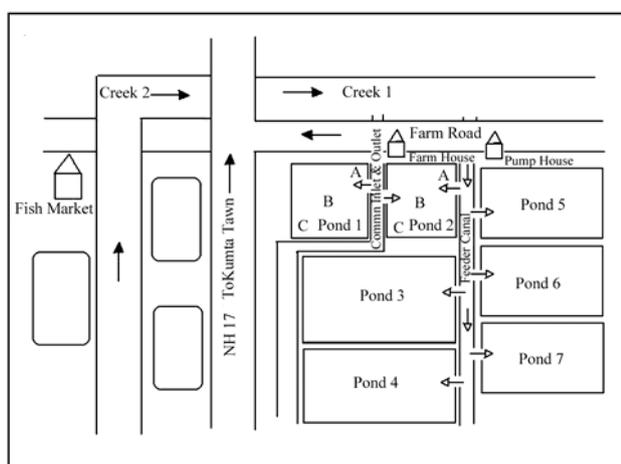


Figure 2 Layout of the shrimp farm (not to scale) near Alvekodi village in Karnataka state, west coast of India. Sampling points in the ponds have been denoted as A, B and C

locations in two ponds (pond 1 and pond 2; sampling points denoted as A, B and C in each pond) and two locations in the source water (creek 1 and creek 2) as shown in Figure 2.

A Niskin water sampler was used to collect water samples from ponds and the source water (creek) between 0800 and 0900 h. The samples were transferred aseptically into sterile BOD bottles. The sediment samples were collected with the help of a hand held acrylic core (400 mm long, 40 mm dia) and were transferred immediately into new sterile polythene bags. All samples were transferred immediately to an ice box and transported to the laboratory for analysis. From 30-day onwards, for enumeration of bacteria in the hepatopancreas, five shrimps from each pond were collected at every fortnight from feed-check trays. Collected shrimp were transferred immediately to polythene bags containing seawater which was filled with oxygen gas prior to packing. Samples were processed for microbiological analysis within 3-4 h after collection.

3.3 Physico-chemical analysis

Environmental parameters such as temperature, salinity and pH were measured with the help of centigrade thermometer, hand-held refractometer (Atago, Japan) and calibrated pH meter (Lab-India, PHAN) respectively. The oxidation-reduction potential (Eh) was measured with the help of microprocessor-based analyzer (Lab-India, model PHAN) fitted with an *Orion* combination platinum/Ag/AgCl electrode. Dissolved oxygen (DO) content was estimated by Winkler's method (Parsons et al., 1984). For the estimation of particulate organic carbon (POC), a known volume of water sample was filtered under vacuum (1/3 atmospheric pressure) through a Whatman GF/F filter (47 mm diameter; 1.2 µm pore size) which was previously ignited in a muffle furnace at 450°C for 3 h. Two mL of sodium sulphate solution (4.5% v/v) was added separately to remove interference of chloride ions. Filters were then dried and stored in an aluminum foil until further analysis. The material retained on the filter paper was oxidized by acid-chromate solution and the reduction in optical density (OD) was measured spectrophotometrically (spectrophotometer model UV-1201; Shimadzu, Japan). Standardization was carried out using glucose as the carbon source. The

POC has been expressed as g C m^{-3} . Organic matter in the sediment was estimated following the method described by El Wakeel and Riley (1956).

3.4 Bacterial quantification

Total heterotrophic bacteria (THB) were estimated using half-strength nutrient agar (NA; HiMedia, India) as preliminary tests showed highest retrievability on 50% NA. Total *Vibrio* like organisms (TVLO) were enumerated on *Vibrio*-selective medium- thiosulphate citrate bile-salt sucrose agar (TCBS; HiMedia, India). Luminescent bacteria (LB) were quantified using half-strength NA supplemented with 1% glycerol. An inoculum of 100 µL pond water was directly spread plated onto respective media. For isolation of vibrios in sediment, 10 g of wet sample was diluted in 90 mL of 50% sterile seawater (SSW). Appropriate dilutions were carried out and 100 µL were spread plated onto media.

For quantification of bacteria in the hepatopancreas, live shrimps were surface-disinfected by swabbing with 75% alcohol. Hepatopancreas from shrimps were aseptically removed, pooled and weighed. They were then washed twice with 50% sterile seawater (SSW) to remove loosely adhering particles and homogenized. A known quantity of hepatopancreas was suspended in 50% SSW (w/v) and serially diluted (up to 10^{-7}) and plated onto TCBS and NA media. The LB plates were stored in the dark and colonies were counted after 18-24 h of incubation at room temperature. After spread-plating, TCBS and NA plates were incubated at room temperature for 48-96 h and counts in source/rearing water, sediment and hepatopancreas of shrimps were expressed as CFU mL^{-1} , CFU g^{-1} dry weight and CFU g^{-1} wet weight respectively.

3.5 Bacterial isolation and identification

To analyze the composition of the TVLO in each sample, 1 to 3 numerically dominant colonies from each plate containing about 20-200 colonies were selected based on their morphological appearance and were further purified on NA plates. Identification of *Vibrio* spp. was based on their morphological, physiological (growth, temperature and NaCl tolerance or requirements) and biochemical characteristics (oxidase, catalase, glucose utilization, nitrate reduction, indole production, Voges-Proskauer test, methyl red, citrate, arginine dihydrolase,

gelatinase production, acid production from carbohydrates) as described by Ruangpan and Kitao (1991). Additional biochemical tests were performed as per standard methods and compared with standard reactions for confirmation (Alsina and Blanch, 1994; Holt et al., 1994). Species level identification was carried out wherever possible using the Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

3.6 Food conversion ratio and average survival of shrimps

Weight (g) of thirty shrimps collected from each pond was recorded at fortnightly intervals from 30-days onwards. The following calculations were carried out:

Food conversion ratio (FCR) = total feed used in the culture pond / net production of shrimps

Average survival (%) = (total no. of shrimps survived / total no. of post-larvae stocked) × 100

3.7 Statistical analysis

Temporal and spatial fluctuations in the variables (bacteriological and environmental variables) were assessed by Analysis of Variance (ANOVA) with time (days of culture) and space (ponds and/or sources water and/or with stations) as sources of variation. Because the variances were not homogenous and/or the residual departed from normality as indicated by Cochran's test, all the bacteriological data were transformed into $\log_{10}(x+1)$. Student's *t*-test was performed to detect significant differences in bacterial counts between shrimp pond components. The degree of correlation between abiotic and biotic variables was analyzed by means of Pearson's correlation for rearing water and sediment samples. All statistical analyses were performed using PC based Minitab statistical package (Minitab Inc., USA).

Authors' contributions

PAL and RAS conceived the study. RAS, SSK, SVK and RRS participated in the sampling and sample analysis. SOF analyzed the data and drafted the manuscript.

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Table 1 Average values of physico-chemical characteristics in (a) rearing water and sediment (\pm SD; n = 6), (b) Source water (creek) (\pm SD) during the cultivation of *Penaeus monodon*

Days of Culture (doc)	Water						Sediment		
	Temperature ($^{\circ}$ C)	Salinity	pH	Eh	Dissolved oxygen (mg L^{-1})	Particulate organic carbon (g C m^{-3})	pH	Eh	Organic matter (mg g^{-1} dry sed.)
(a). Shrimp ponds									
0	22.2 \pm 0.4	8.7 \pm 0.2	8.0 \pm 0.1	68.9 \pm 2.1	7.7 \pm 0.0	4.2 \pm 0.1	6.2 \pm 0.2	95.5 \pm 1.1	10.2 \pm 2.0
15	23.3 \pm 0.4	13.2 \pm 0.5	8.0 \pm 0.1	42.2 \pm 13.7	5.3 \pm 0.4	4.6 \pm 0.3	6.7 \pm 0.2	26.8 \pm 2.6	17.6 \pm 3.9
30	28.3 \pm 0.4	13.9 \pm 0.4	8.0 \pm 0.1	104.5 \pm 0.2	4.9 \pm 0.2	4.5 \pm 0.0	6.6 \pm 0.3	100.7 \pm 8.1	22.2 \pm 5.6
45	28.3 \pm 0.4	14.1 \pm 1.2	7.3 \pm 0.2	27.9 \pm 0.2	5.5 \pm 0.2	8.8 \pm 1.7	6.4 \pm 0.2	56.6 \pm 3.5	18.4 \pm 4.4
60	29.0 \pm 0.0	20.4 \pm 1.4	7.7 \pm 0.2	119.4 \pm 4.3	4.7 \pm 0.3	25.2 \pm 8.4	6.6 \pm 0.3	116.7 \pm 2.0	9.6 \pm 6.6
75	29.3 \pm 0.4	23.5 \pm 2.8	7.7 \pm 0.1	142.8 \pm 3.2	5.2 \pm 0.5	12.0 \pm 7.3	6.5 \pm 0.2	127.7 \pm 1.1	46.9 \pm 8.2
90	28.3 \pm 0.4	29.4 \pm 2.8	7.7 \pm 0.1	108.9 \pm 4.1	6.1 \pm 0.7	12.4 \pm 2.3	6.7 \pm 0.1	100.1 \pm 5.1	44.8 \pm 21.5
105	31.3 \pm 0.4	30.5 \pm 0.0	7.5 \pm 0.2	98.6 \pm 0.5	5.4 \pm 0.4	12.7 \pm 3.4	6.4 \pm 0.1	107.1 \pm 10.7	38.8 \pm 14.7
120	31.3 \pm 0.4	28.5 \pm 0.3	7.7 \pm 0.0	100.2 \pm 1.6	4.4 \pm 0.3	22.4 \pm 0.8	6.5 \pm 0.2	122.9 \pm 3.3	58.1 \pm 10.6
135	32.5 \pm 0.4	34.0 \pm 0.2	7.8 \pm 0.1	102.6 \pm 1.8	5.1 \pm 0.3	22.6 \pm 6.0	6.2 \pm 0.3	105.3 \pm 6.9	56.1 \pm 6.7
(b). Source water (creek)									
0	23.0 \pm 0.0	13.2 \pm 0.1	7.5 \pm 0.2	62.9 \pm 12.2	6.2 \pm 1.1	4.8 \pm 0.7	6.5 \pm 0.4	74.3 \pm 12.8	12.2 \pm 1.2
15	24.0 \pm 0.0	13.5 \pm 0.4	7.6 \pm 0.0	28.8 \pm 4.8	4.7 \pm 0.7	6.0 \pm 1.9	7.1 \pm 0.0	42.4 \pm 24.4	8.8 \pm 8.9
30	29.0 \pm 0.0	17.6 \pm 1.5	8.0 \pm 0.0	99.9 \pm 3.4	5.0 \pm 0.4	4.2 \pm 0.2	6.9 \pm 0.1	106.1 \pm 2.0	22.6 \pm 7.1
45	28.0 \pm 0.0	18.4 \pm 1.6	7.5 \pm 0.1	30.2 \pm 3.4	4.8 \pm 0.0	7.7 \pm 3.6	5.8 \pm 0.7	53.2 \pm 9.9	12.6 \pm 3.6
60	29.5 \pm 0.0	23.0 \pm 0.6	7.6 \pm 0.1	113.9 \pm 2.2	4.6 \pm 0.5	24.3 \pm 2.7	6.1 \pm 1.0	107.3 \pm 4.9	47.7 \pm 7.1
75	29.5 \pm 0.0	29.8 \pm 3.9	7.9 \pm 0.1	133.4 \pm 1.9	4.9 \pm 0.6	17.9 \pm 7.0	6.8 \pm 0.0	124.0 \pm 1.8	67.8 \pm 35.5
90	28.2 \pm 0.3	35.0 \pm 0.4	7.8 \pm 0.1	97.3 \pm 2.9	5.1 \pm 0.0	11.9 \pm 4.9	6.6 \pm 0.3	86.1 \pm 4.6	116.8 \pm 1.8
105	31.5 \pm 0.0	30.7 \pm 0.3	7.7 \pm 0.2	96.9 \pm 0.5	6.0 \pm 0.7	7.8 \pm 1.0	6.6 \pm 0.2	96.8 \pm 5.2	50.2 \pm 3.5
120	32.0 \pm 0.0	28.7 \pm 0.8	8.0 \pm 0.2	99.2 \pm 2.7	4.6 \pm 0.5	38.3 \pm 21.0	6.5 \pm 0.2	121.2 \pm 4.4	54.0 \pm 19.5
135	33.5 \pm 0.0	31.0 \pm 0.2	7.9 \pm 0.0	104.6 \pm 3.5	5.2 \pm 0.1	8.6 \pm 2.2	6.4 \pm 0.3	104.1 \pm 4.2	70.3 \pm 3.5