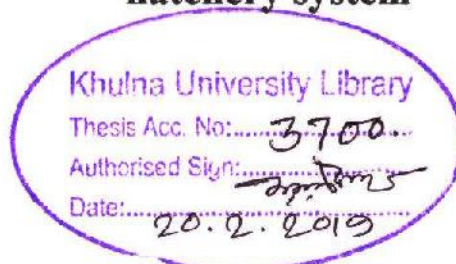


Antibiotic resistance profile of *Vibrio* spp. from freshwater prawn hatchery system



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Session: 2016-2017



Fisheries & Marine Resources Technology Discipline

Khulna University

Khulna, Bangladesh

November, 2018

Antibiotic resistance profile of *Vibrio* spp. from freshwater prawn hatchery system

A Thesis

By

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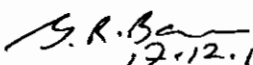
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Antibiotic resistance profile of *Vibrio* spp. from freshwater prawn hatchery system

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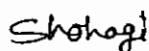
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Declaration

Antibiotic resistance profile of *Vibrio* spp. from freshwater prawn hatchery system

The results presented in this thesis with the above mentioned title are entirely of the candidate's own investigations and no part of the results has been accepted for any degree nor is it being concurrently submitted for any degree.

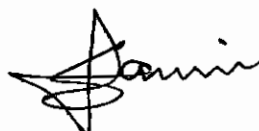


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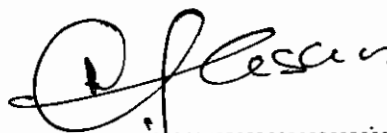


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November, 2018

Dedicated To

.....

My Beloved Parents

Acknowledgement

At first, all my admiration goes to The Almighty, the supreme creator and ruler of the universe, whose mercy keeps me alive and enables me to pursue my education in Fisheries and Marine Resource Technology Discipline and to complete thesis for the degree of M.Sc. in Fisheries.

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Abstract

This study was undertaken to enumerate total bacterial and total *Vibrio* loads at different steps of a freshwater prawn hatchery system with particular focus on identification of the members of *Vibrio* up to species level and investigation of their antibiotic resistance profile so that a link to persistent mass mortality in prawn hatcheries can be established. Among all the samples tested *Artemia* were identified as the key source of total bacteria (1.15×10^7 CFU/g) and total *Vibrio* (6.25×10^6 CFU/g) contamination in the hatchery followed by brood eggs (2.75×10^6 CFU/g and 9.59×10^4 CFU/g for total bacteria and total *Vibrio*, respectively). Nevertheless, river water, mixed water (pond water mixed with brine) were also found to contain bacteria in similar order of magnitudes.

Through different biochemical tests 11 *Vibrio* species were identified from 246 randomly picked *Vibrio* colonies. *V. ordalii* comprised the highest percentage (20%) of member belonging to *Vibrio* genus followed by *V. salmonicida* (11%), *V. cholerae* (9%), *V. orientalis* (8%), *V. mediterranei* (4%), *P. shigelloides* (4%), *V. campbellii* (3%) and *V. splendidus* II (2%). Besides, *V. cincinnatiensis*, *V. marinus*, *V. pelagius* I were also isolated in few numbers from some samples. Antimicrobial susceptibility of 9 out of the 11 isolated *Vibrio* species involving 25 different antibiotics revealed that almost all *Vibrio* species were susceptible to chloramphenicol, nitrofurantoin and norfloxacin. Tetracycline was able to inhibit the growth of all but two *Vibrio* spp despite its wide use as this drug is approved for use in aquaculture. *V. splendidus* and *V. salmonicida* appeared to be resistant to tetracycline. On the other hand two commonly used drugs Erythromycin and Novobiocin were almost completely ineffective against all *Vibrio* spp. Similarly, a high percentage of intermediate susceptibility to neomycine, kanamycine and gentamycine was detected suggesting a potential resistance trend.

All *Vibrio* spp. tested exhibited Multi Drug Resistance phenotypes, notably including the four *Vibrio* species associated with disease in farmed finfish and shellfish. The pathogenic *V. salmonicida*, *V. ordalii* and *V. splendidus* showed the highest MDR followed by *V. campbellii* and others. These results suggest that improper use of antibiotics might cause reduction of beneficial bacteria, which paves the way for pathogenic *Vibrio* to propagate at later stages of hatchery operation. Thus, this study suggests that adhering to better hygiene and improved management practices should be ensured instead of improper use of antibiotics for prophylactic purposes.

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Chapter 1

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Introduction

1. Introduction

1.1 Background of the study

Bangladesh is considered one of the most suitable regions for fisheries in the world, with the world's largest flooded wetland and the third largest aquatic biodiversity in Asia after China and India. The favorable geographic position of Bangladesh comes with a large number of aquatic species and provides plenty of resources to support fisheries potential. Fisheries and aquaculture play a vital role in the socio-economic development of Bangladesh by alleviating protein shortage, creating employments and earning foreign currencies. Fish alone is supplementing about 60% of animal protein in the daily dietary requirement of the people and generates about 15 million employments throughout the value chain (DoF, 2017). The sector has maintained an average contribution of 4-6% to the national GDP and within agriculture, the contribution of fisheries has increased significantly from 7% in 1975-76 to over 23% in 2015-16 (DoF, 2017). With an estimated current total annual production of about four million metric tonnes, Bangladesh remains among the top five fish producing countries of the world (FAO, 2016). It is also a major export earning sector of the country that realized over US\$ 500 million in the last fiscal year (EPB, 2017). The rise and development of shrimp and prawn industry as an exclusively export-oriented entrepreneurial activity provided much of the impetus for the fisheries sector to emerge as a major export earning industry, only second to the garments industry.

Shrimp and prawn industry, nowadays, plays a central role in the fisheries sector of Bangladesh. It alone contributes more than 70% to the total export earnings from all the agro-based products. Of the US\$ 530 million export earnings from the fish and fish products in 2016-17 fiscal year, shrimp alone contributed about 85% of the total value (EPB, 2017). Not only does this crop earn valuable foreign exchange, but the sector also employs about 1.2 million throughout the value chain and livelihoods of a further 4.8 million household members are linked to the sector (Gammage *et al.*, 2006). Therefore, any disruption in the production, processing, marketing and export of these valuable shellfish is bound to have severe and important implications for their livelihoods as well as negative multiplier impact for the country's economic growth.

Shrimp and prawn are decapod crustaceans covering about 33 genera with over 2500 species identified worldwide, of which less than 300 species are of economic interest. However, the two main species cultured in Bangladesh are Black Tiger Shrimp (*Penaeus monodon*), locally known as bagda and Giant Freshwater Prawn (*Macrobrachium rosenbergii*), locally known as galda. Starting from a few sub-districts of Satkhira during 70s, the commercial shrimp culture practice gradually spread to entire Satkhira, and later in other coastal districts of greater Khulna (see Ahsan, 2009 and references cited therein for an overview on the evolution of shrimp and prawn industry in Bangladesh). Approximately 80% of the total shrimp culture of the country continues to occur in Khulna-Satkhira-Bagerhat region and the rest in Cox's Bazaar. Interestingly, at approximately the same period, an entrepreneurial farmer in Bagerhat district began to experiment with the giant freshwater prawn in a public freshwater body. Encouraged by positive results, he then began to construct ghers in low-lying areas. Eventually other farmers adopted the gher technology and the 'revolution' ensued. Culture of freshwater prawn mainly takes place in the southwest but is expanding to other interior districts as well (Ahmed, 2014; Ahmed, 2008).

There is evidence that the expansion of black tiger shrimp farming has resulted in the eviction of smallholder farmers and landless fishermen and the illegal use of public lands and water bodies often involving violent confrontation and corruption (Ahsan, 2009). In contrast, the freshwater prawn is the economic mainstay of small-scale farmers, which is apparently better off in terms of negative environmental and social consequences (Ahmed, 2001). Culture of freshwater prawn mainly takes place in the southwest but is expanding to other interior districts as well. Given the already large increase since 1995 there is probably little scope for further increases in cultivation area for black tiger shrimp. In contrast, there is scope to increase freshwater prawn farming, which to date has expanded at a rate of about 10-20% annually (Biswas and Finan, 2004).

As was the case for black tiger shrimp, the freshwater prawn fry were initially either caught from the wild, or came into the ponds naturally with incoming water during tidal exchanges. However, the first hatchery, located in Cox's Bazar, was not built until 1986, after which the prawn hatchery business increased and spread to other parts of Southern Bangladesh (mostly around the Khulna and Barisal divisions). This was partially a result of a Government-imposed ban on catching wild post-larvae (PL) (in order to avoid possible deleterious environmental problems) in 2000, a ban on importation of foreign "guest" PL from India, and increased demand for PL from

the growers. Thus, by 2007, there were estimated to be 81 prawn hatcheries in Bangladesh, although only few (about 21) (FRSS 2014) of them were actually operational at that time (Brigs, 2013).

Even with the rapid establishment of prawn hatcheries across Khulna and Barisal divisions in response to increased farming areas, the hatcheries could supply only a small fraction of an estimated total demand of over 1.5 billion PL (Kennedy *et al.*, 2006). Thus despite a steady growth of prawn hatcheries farmers were still relying heavily on wild PLs. Since 2011, however, mass mortalities PLs started to occur in most hatcheries forcing them to slow down or abandon the operations. Initially the problem started in prawn hatcheries of Khulna regions but within the next few years, those operating in Barisal and Patukhali districts were also affected and by 2015 to 2016 almost all hatcheries of the country experienced the same production loss and eventually they stopped operating.

The sustainability of prawn sub-sector is currently at stake due to persistent production problems in prawn hatcheries. It is now severely undermining the government initiative to ban wild PL collection from the rivers. Besides, PLs of neighboring countries with unknown quality are entering illegally into the country that poses another dimension to the already dire situation the prawn industry has been going through. The illegally introduced PLs are believed to carry a number of pathogens in our pristine environment. It has already been over six years since mass mortalities of PL in prawn hatchery was first observed, but to date none has managed to identify the precise cause(s) of these production losses.

A complex mix of environmental factors, microbiological profiles and management practices can be responsible for such mass mortalities. In case of the problems occurring in the prawn hatcheries of Bangladesh, the disease symptoms are closest to known bacterial problems associated with heavy infections by pathogenic bacterial species, particularly those belonging to the genus *Vibrio*. There are not many reports regarding the normal bacterial flora associated with *M. rosenbergii* hatcheries. Miyamoto *et al.* (1983) identified several genera of bacteria from *M. rosenbergii* larvae including *Aeromonas*, *Pseudomonas* and *Vibrio* as the dominant flora while Anderson *et al.* (1989) reported *Alcaligenes* spp. and *Vibrio* spp. to be the most frequently encountered genera. In both studies, a large number of *Vibrio* spp was encountered. Bacterial disease due to *Vibrio* spp. is well known to cause heavy mortalities in systems employing saline

waters such as *M. rosenbergii* (Tonguthai, 1995) and *P. monodon* hatcheries (Karunasagar *et al.*, 1994; Kennedy *et al.*, 2006).

The larval rearing practices in hatchery system favour the rapid multiplication of bacteria in the system. However, most bacteria are part of the commensal flora and only some of them may be opportunistic pathogens. Each step of *Macrobrachium* larval development is characterised by different type of associated microbiota, which is introduced in to the system by different ways such as source water (in eggs and yolk-sac larvae) and live feed (*Artemia* and rotifers), brood stock (berried female) and hatchery water (pond, mix and brine) at different stages of hatchery operation.

Understanding the bacteria responsible for disease and/or the route of contamination can be an excellent tool to circumvent the problem through use of specific antibiotics at specific dose (Rodgers and Furones, 2009). However, in most cases, hatchery operators try a range of antibiotics both prophylactically and as a blind treatment once the problems start. The antibiotics used are usually oxytetracycline (OTC), often in combination with ciprofloxacin, each at 2-3ppm or more for 24 hours, but a host of other antibiotics, including erythromycin, chloramphenicol, furazolidone and a host of other undisclosed antibiotics (Brigs, 2013; Hua and Apun, 2013). Most of these remedial measures implemented by the hatchery operators have been unsuccessful which ultimately forced many operators to shut down their hatcheries.

The use of antibiotics to overcome disease is a common practice in hatcheries. Different types of antibiotics like- Ampicillin, Amoxycillin, Erythromycin, Penicillin, kanamycin, Novobiocin, Ciprofloxacin, streptomycin etc. may have used to treat bacterial diseases and to control bacterial population in the hatcheries and prawn farms (Hua and Apun, 2013). Antibiotics, which have capability of obstructing the disease problems are no longer the ultimate way for the treatment of infections because bacteria have continued to develop multiple resistance towards many different types or classes of the drugs (Son *et al.*, 1997).

Frequent use of many antibiotics by the hatchery operators appeared to be incapable of preventing or treating the problem. It is therefore plausible to hypothesize the presence of antibiotic resistant *Vibrio* spp. Continuous and improper use of antibiotics favours the selection of resistant isolates and the dissemination of resistance genes within bacterial populations in the

environment (Tendencia and Pena, 2002). Many cases of multiple antimicrobial resistances have been reported from shrimp farms in countries where widespread use of antibiotics is prevalent such as China (Dang *et al.*, 2006), Korea (Kang *et al.*, 2005) and Chile (Miranda and Rojas, 2007). The situation is aggravated by the emergence of antibiotic-resistant strains (Karunasagar *et al.*, 1994) and the ability of *Vibrio* spp. to form biofilms (Karunasagar *et al.*, 1996). Hence, it is necessary to understand the bacterial flora associated with freshwater prawn hatchery system with particular focus on *Vibrio* spp. and their antibiotic profiles because the host-microbe interactions have far reaching implications on larval health, development and outbreaks of disease.

1.2 Objectives

The objectives of this study were:

1. To assess the bacterial flora in brood eggs, water, larvae, *Artemia* and other sources associated with different stages of prawn hatchery.
2. To determine the quantitative and qualitative aspects of *Vibrio* spp. at different stages of hatchery operation.
3. To understand the antibiotic resistance profile of *Vibrio* spp.

Chapter 2



Literature Review

2. Literature Review

There is a considerable body of works related to *P. monodon* hatchery but those focusing on *M. rosenbergii* hatchery are few and far between. The following review of literatures are based on the reports available for *M. rosenbergii* hatchery with some from shrimps *P. monodon*, *P. japonicas* and *Litopenaeus vannamei* which are thought to be of relevance to the present study.

Miyamoto *et al.* (1983) identified 13 genera of bacteria from *M. rosenbergii* larvae, including *Aeromonas*, *Pseudomonas* and *Vibrio* as the dominant flora while Anderson *et al.* (1989) studied the aerobic heterotrophic microflora in *M. rosenbergii* hatcheries in Malaysia and reported *Alcaligenes* spp. and *Vibrio* spp. to be the most frequently encountered genera.

Kennedy *et al.* (2006) investigated the microbial flora associated with the hatchery system of freshwater prawn, during an entire rearing cycle. They found that the bacterial genera were predominantly Gram-negative and comprised of *Aeromonas* spp., *Pseudomonas* spp., *Vibrio* spp. and *Bacillus* spp. whereas non-spore formers (NSF) were the dominant Gram-positive bacteria. The study enumerated total bacterial loads in well water, seawater and inlet water varied from 10^1 to 10^5 CFU/ml with higher counts seen in larval rearing tank (LRT) water. The *Vibrio* count ranged between 10^1 and 10^3 CFU/ml. Larvae samples harbored a bacterial load of 10^6 to 10^7 CFU/10 larvae. The bacterial load in *Artemia* hatching water ranged from 4.90×10^4 to 5.63×10^6 CFU/ml while *Artemia* had a load ranging from 1.08×10^7 to 2.09×10^9 CFU/g. *Vibrio* count in the LRT water ranged from 10^1 – 10^3 CFU/ml while the count in larvae ranged from 10^2 to 10^4 CFU/10 larvae.

Shakibazadeh *et al.* (2009) estimated bacteria flora associated with different body parts of hatchery reared juvenile *P. monodon*, tank water and sediment. In their study eight different genera were isolated in which 7 genera were identified. *Vibrio* was the most dominant genera followed by *Shewanella* and *Burkholderia*. On the other hand, *Clavibacter* followed by *Staphylococcus* were the most dominant gram positive bacteria. No coliform bacteria was detected in the shrimp body parts and rearing environment. Incidence of *Shewanella* in the digestive system was significantly higher than sediment, rearing water and muscles. This may be implied its ability to colonize in the digestive tract of juvenile *P. monodon*.

Prakash and Karmagam (2013) found a statistically significance correlation between Total Coliform Count (TCC) and Total Bacterial Count (TBC) in freshwater prawn ($r=0.40$; $p<0.01$ and $r=0.55$; $p<0.01$). The positive regression line suggested an increase in TCC with the increase of TBC. The principal bacterial genera encountered in freshwater prawn comprised of *E. coli*, *Pseudomonas* spp., *Enterobacter* spp., *Vibrio* spp., *Aeromonas* spp., and *Staphylococcus aureus*. The antibiotic sensitivity showed Oxytetracycline antibiotic was highly sensitive to most of the bacterial isolates from the prawn samples.

Zheng *et al.* (2017) conducted an experiment on bacterial community associated with healthy and diseased pacific white shrimp (*L. vannamei*) larvae and rearing Water across different growth stages. In this study, the author investigated bacterial communities associated with *L. vannamei* larvae at different healthy statuses (diseased and healthy) and growth stages (i.e., zoea, mysis, and early postlarvae periods) were examined using 454-pyrosequencing of the 16S rRNA gene. Bacterial communities with significant difference were observed between healthy and diseased rearing water, and several bacterial groups, such as genera *Nautella* and *Kordiimonas* could also distinguish healthy and diseased shrimp. *Rhodobacteraceae* was widely distributed in rearing water at all growth stages but there were several stage-specific groups, indicating that bacterial members in rearing water assembled into distinct communities throughout the larval development. However, Gammaproteobacteria, mainly family *Enterobacteriaceae*, was the most abundant group (accounting for more than 85%) in shrimp larvae at all growth stages. The bacterial 16S rRNA gene abundance ranged from 1.5×10^6 to 4.7×10^7 copies/ml in water and 2.4×10^7 to 3.1×10^9 copies/g in shrimp larvae. There was significant difference ($P< 0.05$) of 16S rRNA gene abundance in the rearing water between zoea (1.5×10^6 – 6.9×10^6 copies/ml) and mysis (7.3×10^6 – 3.0×10^7 copies/ml) stages, but no significant difference ($P> 0.05$) was observed between mysis and postlarva (6.0×10^6 – 4.7×10^7 copies/ml) stages. The 16S rRNA gene copy numbers in shrimp increased with growth stages, but with no significant difference

Oxley *et al.* (2002) examined the gut of the wild and cultured banana shrimp, *Penaeus merguensis*. Quantitative and qualitative comparisons of the bacterial flora from the digestive system of the wild and cultured prawn were done in their study. They found in both wild and cultured shrimp remarkably similar bacterial floral compositions, which included members from the genera *Aeromonas*, *Plesiomonas*, *Photobacterium*, *Pseudoalteromonas*, *Pseudomonas* and

Vibrio. Members of the genus *Vibrio* were quantitatively dominant. A number of *Vibrio* species were recovered solely from cultured prawns. Of these, *Vibrio gazogenes* was the most notable (numerically dominating in all but the midgut). The opportunistic pathogen *V. parahaemolyticus* was also recovered.

Lalitha and Surendran (2004) analyzed microflora associated with farmed freshwater prawn *M. rosenbergii* and the aquaculture environment. This study conducted a total of 367 randomly selected isolates which were characterized and identified. Prawn samples yielded mean microbiological counts of 4.92 Log CFU/g of shell with muscle and 7.78 Log CFU/g of intestine. Bacterial numbers in the intestine of prawn were much higher than those in the pond water. Motile and non-motile aerobic Gram-negative bacteria together with Enterobacteriaceae accounted for 60-70% of them esophiles isolated from water and prawn. Among Gram-positive bacteria, *Micrococcus* *Bacillus*, coryneforms, and *Arthrobacter* were found. Faecal coliforms and enterococci were found in significant numbers in *M. rosenbergii*.

Vandenbergh *et al.* (1999) assessed different *Vibrio* spp. associated with *L. vannamei* larvae, post-larvae, broodstock, and hatchery probionts. They identified 11 major *Vibrio* species and one *Photobacterium* spp. by Biolog fingerprinting. Identified species were *V. alginolyticus* (84 strains), *V. harveyi* (57 strains), *V. parahaemolyticus* (18 strains), *V. hollisae* (9 strains), *V. mimicus* (6 strains), *V. diazotrophicus/V. mytili* (6 strains), *V. fluvialis* (2), *V. piscium* (1 strain), *V. orientalis* (1 strain), *V. proteolyticus* (1 strain), *V. mediterranei* (1 strain), *Vibrio* sp. (5 strains), and *Photobacterium damsela* (11 strains).

Sharshar and Azab (2008) studied on diseased freshwater Prawn *M. rosenbergii* infected with *Vibrio vulnificus*. They aimed at isolation and characterization of the pathogenic bacterium from diseased freshwater prawn and also investigated the effect of the bacterial pathogen on hepatopancreas, gills and exoskeleton. The diseased prawn showed dark brown focal lesions and necrosis of appendage tips. The causative bacterial pathogen was isolated from haemolymph and hepatopancreas of the diseased prawn. Based on the morphological, biochemical and physiological characteristics, in addition EPI 20E test, the isolated pathogen was characterized as *V. vulnificus*. In this study the pathogen, *V. vulnificus* showed different degrees of sensitivity to different antimicrobial agents. It was highly sensitive to each of the antibiotics rifadin,

virbamycin, oflaxcin, garamycin, flummox and trimethoprim/sulfamethoxazole) and resistant to nalidixic acid, unasin, velosef, claforan, negram and amoxicillin.

Yasuda and Kitao (1980) analyzed bacterial flora in the digestive tract of prawn, *P. japonicus*. They observed that, *Pseudomonas* spp. were the most abundant in the flora of the digestive tract of adult prawn. The bacterial floras in the water and mud environment were also investigated. In the water of the prawn culture tank, the highest bacterial number was counted in the zoea stages (1.8×10^5 CFU/ml) and the numbers decreased from the end of the mysis stage to the postlarval stage. They showed in shrimp that grew poorly had large numbers of the *Aeromonas* and *Vibrio* groups in the digestive tract.

Hoa *et al.* (2014) studied on diseases in giant freshwater prawns (*M. rosenbergii*). Based on the results of this study, a variety of pathogens had been found in larval, juvenile and adult *M. rosenbergii*, including fouling protozoans such as Epistylis, Zoothamnium, and Vorticella; and pathogenic bacteria such as *Vibrio*, *Aeromonas*, *Pseudomonas*, *Edwardsiella*. These pathogens were isolated from healthy and diseased prawns at different stages as well as from habitats such as the shell of *Artemia*, and rearing water.

Rao and Surendran (2013) observed pathogenic Vibrios in *P. monodon* shrimp hatcheries and aquaculture farms. Presumptive *Vibrio* species isolated from hatchery water (105), post-larvae (105), farm water (75), farm sediment (75) and shrimp (100) from aquaculture pond were screened for the presence of pathogenic *Vibrio* species. *V. alginolyticus* was the most dominant pathogenic *Vibrio* species in both hatchery and aquaculture farm environments. *V. parahaemolyticus*, *V. vulnificus* and *V. harveyi* were detected only in hatcheries and *V. cholerae* was detected only in aquaculture farms. *Vibrio* loads were higher in *P. monodon* hatchery samples than in aquaculture pond samples. Post-larvae had maximum mean loads of Vibrios (2.1×10^5 CFU/g). Shrimp cephalothorax (head) portion had relatively higher mean counts of Vibrios (3.5×10^4 CFU/g) than shrimp muscle portion (1.4×10^4 CFU/g). Hatchery water had higher *Vibrio* loads (2.4×10^2 CFU/ml) than farm water (150 CFU/ml).

Schryver *et al.* (2014) studied on early mortality syndrome outbreak which is a microbial management issue in shrimp farming. A recent disease of farmed *Penaeid* shrimp, commonly referred to as “early mortality syndrome” (EMS) or more technically known as “acute

hepatopancreatic necrosis disease" (AHPND), was first reported in southern China in 2010 and subsequently in Vietnam, Thailand, and Malaysia. The causative agent of EMS/AHPND has been reported to be a bacterium—more specifically a pathogenic *Vibrio* belonging to the Harveyi clade, presumably *Vibrio parahaemolyticus*.

Karunasagar *et al.* (1998) performed a study on disease problems affecting cultured *Penaeid* shrimp in India. Some of the major disease problems affecting cultured shrimp (*P. monodon*, and *P. indicus*) in India were observed. In hatcheries, mortalities due to *Vibrio* infection were common. A high incidence of occlusion bodies indicative of monodon baculovirus (MBV) had been found in larvae obtained from various hatcheries. However mortalities due to MBV infections were observed only occasionally. In grow out system white spot syndrome was the major problem causing serious mortalities.

Regunathan and Wesley (2004) conducted a study on control of *Vibrio spp.* in shrimp hatcheries using the green algae *Tetraselmis suecica*. 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.

Zulkifli *et al.* (2009) tested antibiotic resistance and plasmid profiling of *V. parahaemolyticus* isolated from cockles in Padang, Indonesia. In their study 32 isolates of *V. parahaemolyticus* isolated from cockles were tested for sensitivity to 16 antibiotics and the presence of plasmids. All the isolates were multi-resistance, defined as resistant to at least three different antibiotics with multiple antibiotic resistance indexes ranging from 0.31 to 0.69, indicating the isolates originate from high risk sources of contamination where antibiotics are often used. In the plasmid profiling test, only 15 isolates (47%) harbored plasmid DNA, which ranged in size from 2.7 to 56.2 kb, separating the isolates into 14 plasmid profiles.

Krishnika and Ramasamy (2013) carried out their study on antimicrobial resistance profile of *Vibrio* species isolated from the hatchery system of *M. rosenbergii*. They observed *Vibrio* spp. are responsible for high mortality and severe economic loss in prawn industry in all the prawn producing countries. The incidence of high antibiotic resistance is reported in hatcheries. They isolated 23 *Vibrio* species from the larvae, larval rearing water, *Artemia* and *Artemia* rearing water from the hatchery system of *M. rosenbergii*. The isolates were characterised and nine different species were identified viz., *V. harveyi*, *V. parahaemolyticus*, *V. damsela*, *V. splendidus*, *V. anguillarum*, *V. alginolyticus*, *V. fluvialis*, *V. vulnificus* and *V. campbelli*. Fifteen commonly used antibiotics in the hatchery system were tested against the isolated *Vibrio* species. The results showed that all the isolates (100%) were resistant to ampicillin, erythromycin, doxycycline hydrochloride and sensitive to amikacin, chloramphenicol, cefaclor, gentamycin and streptomycin.

Reboucas *et al.* (2011) experimented antimicrobial resistance profile of *Vibrio* species isolated from marine shrimp farming environments (*L. vannamei*) at Ceara, Brazil. Their study aimed to evaluate the phenotypic resistance profile among *Vibrio* isolates from hatcheries water samples and from cultivated marine shrimp hepatopancreas (*L. vannamei*). Antimicrobial susceptibility testing was carried out by a standard disc diffusion method and the minimum inhibitory concentration (MIC) of oxytetracycline (OTC) for resistant *Vibrio* isolates was determined by broth dilution method. The results showed a high incidence of resistance to ampicillin (45.2%) and to the tetracycline class (38.7%). Florfenicol and nitrofurantoin were 100% effective against *Vibrio* isolates. In their study, the OTC-resistant *Vibrio* spp. showed MIC values of more than 400 mg/L and the presence of seawater did not influence the oxytetracycline bioactivity. The occurrence of antimicrobial multi resistance patterns was observed in 29% of *Vibrio* isolates. Fifty-five percent of multi resistant isolates of *Vibrio* lost one or more antibiotic resistance phenotype after procedure to curing of resistance plasmids. The oxytetracycline resistance was the phenotype most often lost among plasmid-cured isolates.

Hossain *et al.* (2012) revealed antimicrobial susceptibility of *Vibrio* species isolated from brackish water shrimp culture environment. In their study total *Vibrio* counts were found to range up to 2.5×10^3 CFU/gm and 60 CFU/gm in shrimp and Gher water samples, respectively. They showed that the total *Vibrio* count were found to be higher in samples taking from the market shops in

Dhaka city than Gher shrimp samples and the live shrimps were not contaminated with *Vibrio* species but dead shrimps were found contaminated, which were collected from shrimp Gher. In the antimicrobial susceptibility test, highest 28.57% resistance among total *Vibrio* isolates was observed to penicillin and cephalexin. Among the seven isolates of *Vibrio* species from Gher water sample one isolate displayed multidrug resistance (MDR).

Otta *et al.* (2001) conducted a study on bacteriological status of shrimp, *P. monodon* hatcheries in India. The total plate counts of raw sea water on tryptic soya agar ranged from 10^2 to 10^4 CFU/ml, whereas it ranged from 10^4 to 10^6 CFU/ml in larval tanks. In the larval tanks, the proportion of *Vibrio* species ranged from 50% to 73%, as compared to 31% in raw sea water. Over 90% of the strains were resistant to amoxycillin, ampicillin, cephalexin, cephalosporin, cloxacillin and sulphafurazole. Most strains showed sensitivity to tetracycline, chloramphenicol, and quinolones such as norfloxacin and ciprofloxacin.

Thakur *et al.* (2003) analyzed pathogenicity and antibiotic susceptibility of *Vibrio* species isolated from moribund shrimps. In their study bacterial identification based on morphological and biochemical characteristics showed that four groups of *Vibrios* were present in shrimp hepatopancreas namely *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum* and *V. vulnificus*. The diseased shrimps displayed poor growth, lethargic movements, red discoloration and mortality. Antibiotic resistance profiles of the isolated *Vibrios* were investigated. All the four species were sensitive to Erythromycin, Chloramphenicol and Streptomycin. However, *V. parahaemolyticus* and *V. anguillarum* showed resistance to Oxytetracycline and Polymyxin-B respectively and *V. parahaemolyticus* and *V. vulnificus* were resistant to ampicillin.

Albuquerque *et al.* (2015) determined antibiotic-resistant *Vibrios* in farmed shrimp. Antimicrobial susceptibility pattern was determined in 100 strains of *Vibrio* isolated from the *L. vannamei* shrimp and identified phenotypically. A high antibiotic-resistance index (75%) was observed, with the following phenotypic profiles: mono resistance (n=42), cross-resistance to β -lactams (n=20) and multiple resistance (n=13). Plasmid resistance was characterized for penicillin (n=11), penicillin + ampicillin (n=1), penicillin + aztreonam (n=1), and ampicillin (n=1). Resistance to antimicrobial drugs by the other strains (n=86) was possibly mediated by chromosomal genes.

Amiri *et al.* (2014) surveyed of the sensitivity of *Vibrio* spp. isolated from *L. vannamei* to different antibiotics. The *Vibrio* spp., were *V. alginolyticus*, *V. parahaemolyticus*, *V. fluvialis*, *V. splendidus*, *V. proteolyticus*, *V. anguillarum* II, *V. natriegenes*, *V. harveyi*, *V. nereis*, *V. gazogenes*, *V. pelagius* and *Vibrio* spp. The *Vibrio* spp., isolated were survived for measure of sensitivity to seven broad-spectrum antibiotics. The results showed that the bacterial resistance to tetracycline, chloramphenicol, furazolidone, erythromycin, neomycin, ciprofloxacin and gentamicin were 20.69, 44.83, 58.62, 37.93, 45.45, 0 and 16.64% in hatcheries and 1.755, 0, 1.79, 58.93, 52.27, 0 and 27.90% in shrimp farms, respectively.

Tan *et al.* (2017) experimented prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from short mackerels (*Rastrelliger brachysoma*) in Malaysia. In this study, short mackerels (*Rastrelliger brachysoma*) obtained from different retail marketplaces were monitored with the presence of total and pathogenic strains of *V. parahaemolyticus*. Out of 130 short mackerel samples, 116 (89.2%) were detected with the presence of total *V. parahaemolyticus* and microbial loads of total *V. parahaemolyticus* ranging from <3 to $>10^5$ MPN/g. Prevalence of total *V. parahaemolyticus* was found highest in wet markets (95.2%) followed by minimarkets (89.1%) and hypermarkets (83.3%). Pathogenic *V. parahaemolyticus* strains (tdh+ and/or trh+) were detected in 16.2% (21 of 130) of short mackerel samples. The density of tdh+ *V. parahaemolyticus* strains were examined ranging from 3.6 to $>10^5$ MPN/g and microbial loads of *V. parahaemolyticus* strains positive for both tdh and trh were found ranging from 300 to 740 MPN/g. Assessment of antimicrobial susceptibility profile of *V. parahaemolyticus* revealed majority of the isolates were highly susceptible to ampicillin sulbactam, meropenem, ceftazidime, and imipenem, but resistant to penicillin G and ampicillin. Two isolates (2.99%) exhibited the highest multiple antibiotic resistance (MAR) index value of 0.41 which shown resistance to 7 antibiotics.

Oramadike and Ogunbanwo (2015) studied about prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from seafoods in Lagos Lagoon Nigeria. In this study, a total of 90 seafood samples; croaker fish (*Pseudotolithus senegalensis*), shrimps (*Penaeus notialis*) and blue crab (*Callinectes sapidus*) were collected. Biochemical identification of the isolates was confirmed by Polymerase Chain Reaction (PCR). The presence of the virulence-associated *tdh* (thermo stable direct haemolysin), *trh*₁ (thermo stable-related haemolysin) and *trh*₂ genes in the

V. parahaemolyticus isolates was also detected by the PCR method. PCR products from the V.16S primers were sequenced. Antibiotics susceptibility of the isolates was also determined. About, eight isolates were presumptively identified as *V. parahaemolyticus*, PCR identified five and none of the isolates were positive for the genes *tdh* or *trh*. The five isolates were identified as different strains of *V. parahaemolyticus*. *V. parahaemolyticus*_RIMD_2210633 = 2MKSHa remained resistant to all antimicrobials tested. However, only *V. parahaemolyticus*_MP-2_AY911391 = TBSHy showed strong sensitivity to all the antimicrobials with ampicillin (minimum inhibitory concentration-4 µg/ml). In addition, the other three isolates showed sensitivity for Tetracycline, Ciprofloxacin, Gentamicin and Ceftazidime.

Akinbowale O.L, Peng H. and Barton M. D. (2006) revealed antimicrobial resistance in bacteria isolated from aquaculture sources in Australia. In this study all the isolates were tested for sensitivity to 19 antibiotics and Minimal Inhibitory Concentrations. Resistance to ampicillin, amoxycillin, cephalixin and erythromycin was widespread; resistance to oxytetracycline, tetracycline, nalidixic acid and sulfonamides was common but resistance to chloramphenicol, florfenicol, ceftiofur, cephalothin, cefoperazone, oxolinic acid, gentamicin, kanamycin and trimethoprim was less common. All strains were susceptible to ciprofloxacin. Multiple resistance was also observed and 74.4% of resistant isolates had between one and ten kbp. plasmids with sizes ranging 2–51.

Interaminense *et al.* (2014) experimented *Vibrio* spp. control at brine shrimp, *Artemia* hatching. They evaluated different prophylactic methods to control the bacterial load in brine shrimp, *Artemia* hatching. The first experiment evaluated three treatments to control *Vibrio* spp. during the *Artemia* hatching: microalgae (*Chaetoceros calcitrans*), probiotic (*Bacillus* spp.), and antibiotic (Florfenicol). In the second experiment, *Artemia* metanauplius were enriched in distinct treatments with *C. calcitrans*, probiotic, and emulsion rich in docosahexaenoic and eicosapentaenoic fatty acids. Enriched *Artemia* metanauplius and nauplii (control) were offered to white shrimp, *L. vannamei*, postlarvae (PL7–PL19). Presumptive *Vibrio* were quantified in *Artemia*, PL, and rearing water. Microalgae and probiotic were effective to control *Vibrio* spp. in *Artemia* nauplii. The enrichment process increased the *Artemia* bacterial load but did not affect *Vibrio* load in *L. vannamei*.

Chapter 3



Materials & Methods

3. Materials and Methods

3.1 Study area and period

The study was conducted to examine the total bacterial load and total *Vibrio* load of a government prawn hatchery named Fish Seed Multiplication Farm situated just beside the Khulna University during January to July 2018.

Biological study was carried out at Fish Molecular Biology and Biotechnology Laboratory of Fisheries and Marine Resource Technology Discipline of Khulna University, Khulna.

The target of the study was to identify the different *Vibrio* spp. associated with different operational points viz. different water source, brood eggs, feed (*Artemia*), larvae of that hatchery during the PL production and also to evaluate the sensitivity or susceptibility of *Vibrio* spp. to different antibiotics.

3.2 Collection of samples

Samples were taken out based on two categories and these were live samples and water samples as shown in Table 1.

Table 1. Type and source of samples investigated in the present study

Source	Sample specification
River	River water from where broods were collected
Brood	Eggs of brood
Brine	Concentrated salt solution used in mixing tank
Larvae rearing tank water(LRT)	Water sample where larvae were reared
Larvae	Dead larvae and live larvae
<i>Artemia</i>	Live feed for larvae
Mixed water	Pond water mixed with brine
Pond water	Used in mixing tank

The hatchery operation was started in mid March and again in early June 2018. Samples were collected from both cycles of operation.

3.3 Investigation of the levels of bacteria in different steps of hatchery operation

3.3.1 Sterilization of different equipments

Test tube, eppendorf tube, micropipette, tips, petri dishes, isolating loops and other glassware were sterilized by autoclaving at temperature 121 °C (1.06 kg/cm²) for 15 minutes (Barrow and Feltham, 1993).

3.3.2 Enumeration of Total Viable Bacterial Count (TBC)

3.3.2.1 Nutrient agar preparation

The procedure followed to prepare the media is as follows (Barrow *et al.*, 1993):

- The amount of Nutrient agar (HiMedia Laboratories Pvt.LTD) media was accurately weighed and taken in volumetric flask containing distilled water.
- The final volume was adjusted by pouring additional distilled water.
- Then the mixer was heated to boiling to dissolve the medium completely.
- Then the media was sterilized by autoclaving at 121 °C for 15 minutes.
- After autoclaving the agar media was kept under bio-safety cabinet and poured to required number of Petri dishes by 25 ml volume.
- Then Petri dishes were cooled for 20-30 min to solidify the media properly.

3.3.2.2 Sample preparation & dilution (water Samples)

- 1ml of sample water was taken especially from the each sample and was transferred to cotton plugged bottle containing 9 ml alkaline peptone water.
- The sample was mixed properly by shaking the bottle carefully by using Vortex Machine.
- Then the total amount became 10 ml. This made 1st decimal dilution (10⁻¹).
- From 1st dilution, 1ml was transferred into other tube containing 9 ml alkaline peptone water. Then 2nd dilution (10⁻²) was prepared.

- Following the same process each dilution of next was prepared from previous dilution and prepared as needed like 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} and so on.

3.3.2.3 Sample preparation & dilution (larvae, brood eggs and *Artemia* Samples)

- Samples were collected by pipette and taken into a eppendrof tube.
- Centrifuged at 3000 rpm (rotation per minute) for five minutes
- Upper layer water was collected with micropipette
- Then solid portion of the samples were grinded through grinder into eppendrof tube
- Then required portion of distilled water was added for creating 1ml of sample
- 1ml of sample water was taken especially from the each sample and was transferred to cotton plugged bottle containing 9 ml alkaline peptone water.
- The sample was mixed properly by shaking the bottle carefully by using Vortex Machine.
- Then the total amount became 10 ml. This made 1st decimal dilution (10^{-1}).
- From 1st dilution, 1ml was transferred into other tube containing 9 ml alkaline peptone water. Then 2nd dilution (10^{-2}) was prepared.
- Following the same process each dilution of next was prepared from previous dilution and prepared as needed dilution like 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} and so on.

3.3.2.4 Total Bacterial Count by spread plate technique

Total Bacterial Count (TBC) was done according to the Bacteriological Analytical Manual of United States Food and Drug Administration. Prepared samples were serially diluted for required dilution (like 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5}) and plated at 1 ml on each Petri dish containing nutrient agar to determine the TPC. Then the plates were incubated at 35-37 °C for 18-24 hours. Plates with 30-300 colonies were taken into consideration for counting (FDA, 2001).

3.3.3 Enumeration of Total *Vibrio* Count (TVC)

3.3.3.1 Preparation of Thiosulfate Citrate Bile Salt-sucrose (TCBS) Agar Media

- Media was available commercially in powdered form. The procedure to prepare media was labeled on media container. The procedure followed was as described below-
- The amount of TCBS agar (HiMedia Laboratories Pvt.LTD) media was accurately weighed and taken in volumetric flask containing distilled water.
- The final volume was adjusted by pouring additional distilled water.
- Then the volumetric flask containing agar solution was kept on hot plate for boiling and dissolving fully at 100 °C.
- After dissolving the agar media was kept under bio-safety cabinet and poured to required number of Petri dishes by 25 ml volume.
- Then Petri dishes were cooled for 20-30 min to solidify the media properly.

3.3.3.2 Sample preparation & dilution (water Samples)

- 1ml of sample water was taken especially from the each sample and was transferred to cotton plugged bottle containing 9 ml alkaline peptone water.
- The sample was mixed properly by shaking the bottle carefully by using Vortex Machine.
- Then the total amount became 10 ml. This made 1st decimal dilution (10^{-1}).
- From 1st dilution, 1ml was transferred into other tube containing 9 ml alkaline peptone water. Then 2nd dilution (10^{-2}) was prepared.
- Following the same process each dilution of next was prepared from previous dilution and prepared as needed dilution like 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} and so on.

3.3.3.3 Sample preparation & dilution (larvae, brood eggs and *Artemia* Samples)

- Samples were collected by pipette and taken into a eppendorf tube.
- Centrifuged at 3000 rpm (rotation per minute) for five minutes
- Upper layer water was collected with micropipette
- Then solid portion of the samples were grinded through grinder into eppendorf tube
- Then required portion of distilled water was added for creating 1ml of sample
- 1ml of sample water was taken especially from the each sample and was transferred to cotton plugged bottle containing 9 ml alkaline peptone water.
- The sample was mixed properly by shaking the bottle carefully by using Vortex Machine.

- Then the total amount became 10 ml. This made 1st decimal dilution (10^{-1}).
- From 1st dilution, 1ml was transferred into other tube containing 9 ml alkaline peptone water. Then 2nd dilution (10^{-2}) was prepared.
- Following the same process each dilution of next was prepared from previous dilution and prepared as needed dilution like 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} and so on.

3.3.3.4 Isolation and counting of *Vibrio* Species

Prepared samples were serially diluted for required dilution (like 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5}) and plated at 1 ml on each Petri dish containing TCBS agar to determine the TVC. Then the plates were incubated at 35-37 °C for 18-24 hours. After incubation the plates were examined for the presence of typical colonies of presumptive *Vibrio* spp. Then the colonies of each plate were counted to assess the total *Vibrio* in samples.

3.4. Identification of *Vibrio* spp.

3.4.1 Colonies selection

To identify the spp. colonies were selected from the TCBS plate through some criteria like color, size etc.

3.4.2 Pure culture

Media and reagents

- Nutrient agar
- Sodium chloride (NaCl)
- Distilled water

Procedure

- The amount of Nutrient agar (HiMedia Laboratories Pvt.LTD) media was accurately weighed and taken in volumetric flask containing distilled water.
- The final volume was adjusted by pouring additional distilled water.
- Then the mixer was heated to boiling to dissolved the medium completely.
- Then the media was sterilized by autoclaving at 121 °C for 15 minutes.

- After autoclaving the agar media was kept under bio-safety cabinet and poured to required number of Petri dishes by 25 ml volume.
- Then Petri dishes were cooled for 20-30 min to solidify the media properly.
- A small amount of a colony was picked up with a loop from the TCBS plate.
- The loop was streaked on the solid nutrient media.
- Then the inoculated nutrient agar plates were incubated at 37 °C for 24 hours.

3.4.3 Biochemical test

Different biochemical test were performed to indentify the *vibrio* spp. by using the identification matrix provided by Alsina and Blanch (Alsina and Blanch, 1994) like-

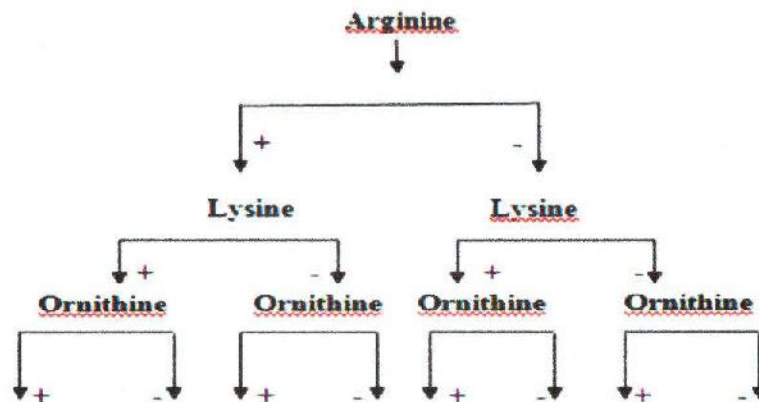


Fig 1: The main key defined by arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase (A/L/O).

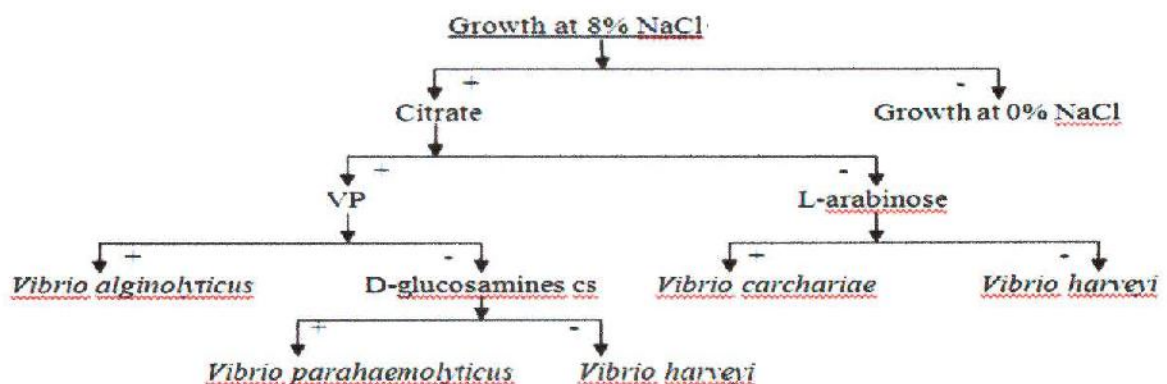


Fig 2: Identification key for cluster A-/L+/O+

Procedure of some biochemical tests that were used were following-

3.4.3.1 Oxidase test

Reagents

- N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride
- water

Procedure

- A piece of filter paper was soaked in the reagent solution
- Some fresh growth from the culture plate were scraped with a disposable loop
- A positive reaction was determined by violet or deep purple color within 5-10 sec
- Negative test: light pink (no color change)

3.4.3.2 Indole test

Media & reagents

- Tryptophan Broth
- Kovac's reagent

Procedure

- Tryptophan broth was inoculated with samples with the help of loop
- Incubated at 20-24 °C for 24-48 hours
- After 24 hours incubation 2 ml of media was placed in another tube for further 24 hours incubation if the test shows the negative result after 24 hours.
- Following incubation 5 drops of Kovac's reagents were added.
- Positive result shows red ring in the top layer

3.4.3.3 Voges Proskauer test (VP test)

Media & reagents

- MR/VP broth
- Alpha-naphthol 5% color intensifier
- 40% potassium hydroxide

Procedure

- Pure cultured bacteria were inoculated with the loop into the test tube containing media
- Incubation for 24–48 hours at 37 °C
- 6 drops of 5% alpha-naphthol and 2 drops of 40% potassium hydroxide were added respectively
- A pink red color at the surface within 30 min gives positive result.

3.4.3.4 Growth at 0%, 8%, 10%

Media and Reagents

- Nutrient agar
- Sodium chloride (NaCl)
- Water

Procedure

- The test was done on nutrient agar media supplemented with varying amounts of NaCl (0%, 8%, 10%)
- This was performed to test the salt tolerance range of the isolated species and the optimum concentration.
- A positive reaction was indicated by appearance of colonies.

3.4.3.5 Gelatin Hydrolysis Test

Media and Reagents

- Gelatin
- Peptone
- Beef extract

- Distilled water

Procedure

- Several well-isolated colony was picked up with a sterile needle.
- Inoculated the nutrient gelatin medium with a test inoculums.
- Incubated the test and an un-inoculated tube for 48 hour at 37°C.
- Gently removed the inoculated and uninoculated tubes from the incubator and placed in ice bath or refrigerator for at least 30 min.
- A positive reaction was indicated by partial or complete liquefaction of the inoculated tube at 4°C.
- And the negative reaction was indicated by completely solidified media.

Besides these tests some other tests were also performed. They were following-

- ✓ Arginine dihydrolase test
- ✓ Lysine Decarboxylase Test
- ✓ Ornithine Decarboxylase Test
- ✓ Citrate test
- ✓ Sucrose Fermentation Test
- ✓ Arabinose Fermentation Test

3.5 Evaluation of the susceptibility of different antibiotics to the identified *Vibrio* spp.

Identified *Vibrio* spp. under this study was tested for susceptibility of different 25 antibiotics using the Kirby Bauer disc diffusion method.

Table 2. Antibiotics used in this study-

Name of antibiotics	Abbreviation	Disk potency (µg)
Ampicillin	AMP	10
Penicillin G	P	10
Penicillin V	PV	10

Streptomycin	S	10
Kanamycin	K	30
Chloramphenicol	C	30
Nitrofurantoin	F	300
Neomycin	N	30
Colistin sulphate	CT	10
Novobiocin	NV	30
Mecillinam	MEL	25
Erythromycin	E	15
Polymyxin B	PB	300
Bacitracin	B	10
Norfloxacin	NOR	10
Amoxicillin	AML	10
Cephalexin	CL	30
Ciprofloxacin	CIP	5
Gentamicin	CN	10
Vancomycin	VA	30
Sulphamethoxazole	SXT	25
Trimethoprim	W	5
Tetracycline	TE	30
Nalidixic	NA	30
Amikacin	AK	30

3.5.1 Inoculum Preparation

Purified cultured colony of the test species (*Vibrio*) was picked with a wire loop and introduced into a test tube containing 4-5 ml nutrient broth. Then the tube was incubated for 20-24 hours to produce a bacterial suspension of moderate cloudiness.

3.5.2 Preparation of McFarland Turbidity Standards

McFarland turbidity standards were prepared by mixing various volumes of 1% sulfuric acid and 1% barium chloride to obtain solutions with specific optical densities. 0.5 McFarland turbidity standard provides an optical density comparable to the density of a bacterial suspension 1.5×10^8 colony forming units (CFU/ml).

For performing antimicrobial susceptibility testing using Kirby Bauer disc diffusion method, a cell suspension of organism equivalent to a 0.5 McFarland standard was used in this study.

Procedure

- Prepared a 1% solution of anhydrous barium chloride (BaCl_2).
- Prepared a 1% solution of sulfuric acid (H_2SO_4)
- Combined and completely mixed the barium chloride (0.05 ml) and sulfuric acid (9.95 ml) solutions to form a turbid suspension to the density of 1.5×10^8 colony forming units (CFU/ml).
- Place the resulting mixture in a foil-covered screw cap tube.

3.5.3 Inoculum standardization

- Density of the suspension of bacterial cells were standardized to the 0.5 McFarland turbidity standard by holding the suspension and McFarland turbidity standard in front of a light against a white background with contrasting black lines (If the density is too heavy, the suspension should be diluted with broth. If the density is not sufficient, additional bacteria should be added to the suspension).
- The adjusted suspension was used as inocula within 15 minutes.

3.5.4 Disk Diffusion Method

The disk diffusion method was performed using Mueller-Hinton Agar (MHA), which is the best medium for routine susceptibility tests because it has good reproducibility and gives satisfactory growth of most bacterial pathogens.

3.5.4.1 Preparation of agar medium

- ✓ Powder form of MHA was weighed according to the manufacturer's instructions.
- ✓ Media was prepared using distilled water or deionized water.
- ✓ Heated with frequent agitation and boiled to dissolve the medium completely.
- ✓ Sterilized by autoclaving at 121°C for 15 min.
- ✓ Cooled the agar medium to 40-50°C.
- ✓ Poured the agar into petri dish on a flat surface to a uniform depth of 4 mm.
- ✓ Allowed to solidify.

3.5.4.2 Procedure of disk diffusion method

- ✓ The standardized broth suspension was spread onto the surface of the MHA medium with a spreader.
- ✓ Drying after the inoculation, the antibiotics disks were placed on the agar with flamed forceps and gently pressed down to ensure contact.
- ✓ Then the plates were inverted and incubated at 37 °C.
- ✓ After 16-18 hours of incubation, the plates were examined.
- ✓ The diameter of the zones of inhibition read and recorded using a ruler graduated to 0.5 mm.
- ✓ The zone measurement rounded up to the nearest millimeter.
- ✓ The zone diameter for individual antimicrobial agents was then translated into susceptible, intermediate and resistant categories according to the interpretation table of the Oxoid Ltd., Basingstoke, UK.

3.6 Data analysis

During the experiment the data was recorded, accumulated, grouped and interpreted according to the objectives. Data were analyzed by using Microsoft Office Excel 2007 computer program.

Chapter 4



Results

4. Results

The study was conducted to enumerate and isolate the total bacterial load and total *Vibrio* load from different sources of water related to prawn hatchery (like river water, brine, mixed water (12 ppt saline water used in LRT)) larvae rearing tank (LRT) water, brood tank water), brood's egg, larvae, treated and non treated *Artemia* (which was used as a live feed for larvae). Different *Vibrio* species (11 types) were identified from isolated bacteria and also their antibiotic profile were examined by using the 25 different antibiotics.

4.1. Total viable bacterial count (TBC) and total presumptive *Vibrio* count (TVC)

In case of different source waters from 1st cycle of production total bacterial count (TBC) from river water showed the highest load of bacteria (9.25×10^6 CFU/ml) followed by mixed water (1.70×10^6 CFU/ml) and pond water (1.61×10^5 CFU/ml). Brine water (1.46×10^4 CFU/ml) had the lowest number of TBC (Fig 3).

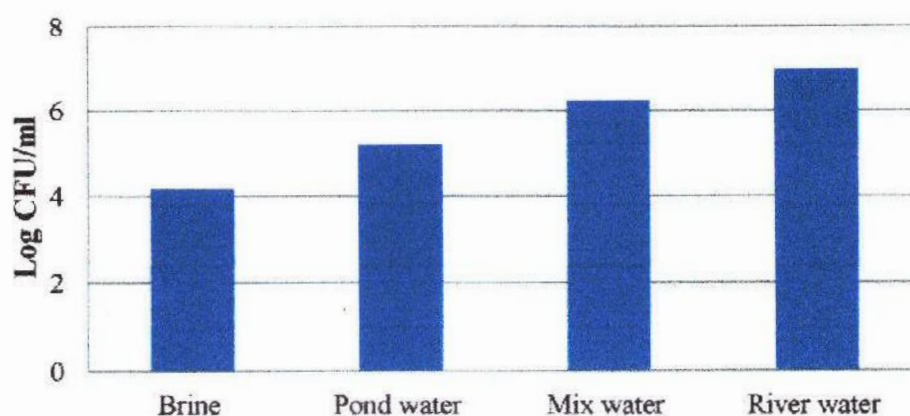


Fig. 3: Total bacterial load in different source water in 1st cycle

Non treated brood eggs and non treated *Artemia* showed higher amount of bacterial load than the treated ones. Non-treated brood and non treated *Artemia* showed 1.51×10^9 CFU/g and 6.29×10^8 CFU/g bacteria, respectively while the treated samples had one order lower magnitude of counts (Fig. 4).

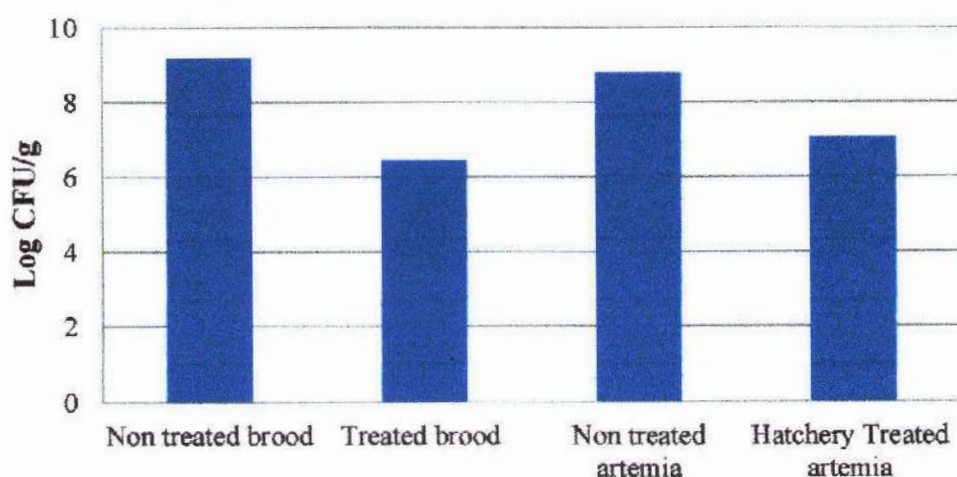


Fig. 4: Total bacterial load in Brood eggs and *Artemia* in 1st cycle

Antibiotic treatments in larval rearing tanks lowered the TBC to one to two order of magnitudes as shown in Fig. 5. Total bacterial load in LRTs before and after antibiotic treatment ranged from 6.2×10^5 to 4.15×10^6 CFU/ml.

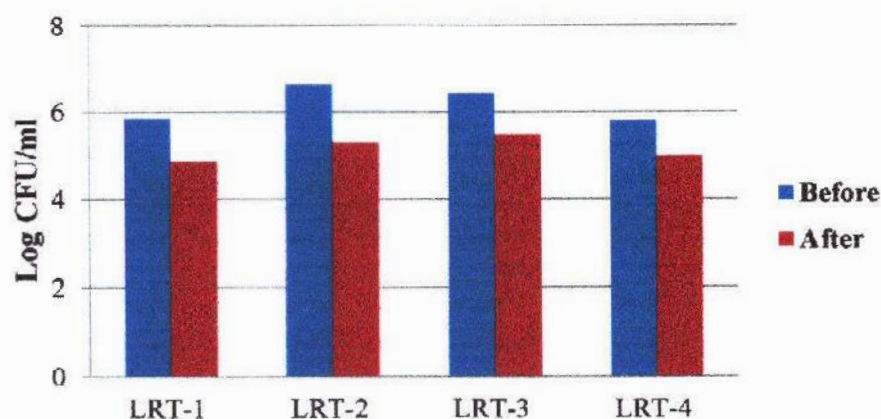


Fig. 5: Total bacterial load in LRT water in 1st cycle

As shown in Fig. 6, a slightly lower count but in the same order of magnitude was observed for larvae in each of the corresponding LRT and the count varied from 3.77×10^5 CFU/g to 4.43×10^6 CFU/g.

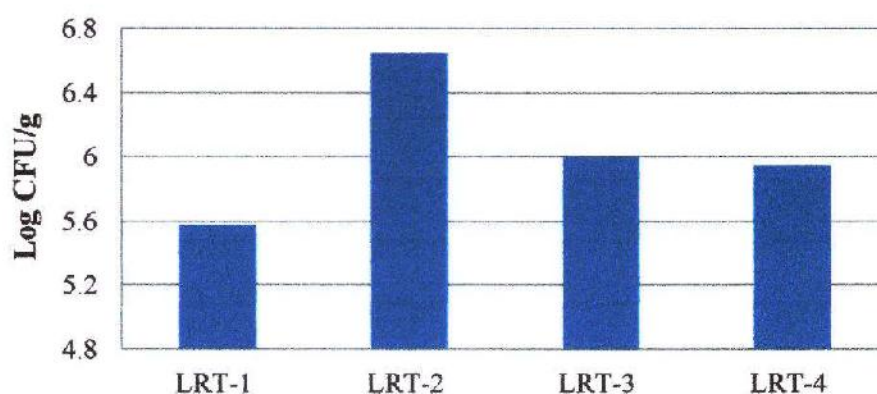


Fig. 6: Total bacterial load in LRT larvae in 1st cycle

In case of total *Vibrio* count (TVC) on specific TCBS plate both green and yellow colonies were enumerated which provided some rough estimation of the type of *Vibrio* spp present.

Samples obtained from the 1st cycle of production TVC from mix water showed a high load of bacteria than the other water sources (like pond water, river water and brine solution) and that was 1.3×10^4 CFU/g whereas the lowest TVC was obtained from brine solution. Considering all water sources the TVC ranged 0.9×10^2 CFU/g to 1.3×10^4 CFU/g (Fig. 7). Green *Vibrio* colonies were predominant in brine and river water samples whereas *Vibrio* colonies from mix water samples were mostly yellow in color.

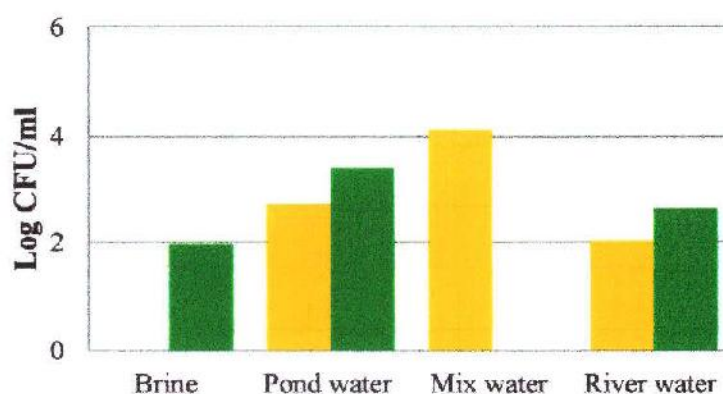


Fig. 7: Total *Vibrio* load in different source waters in 1st cycle

Vibrio count from both treated and non-treated brood eggs and *Artemia* were examined which showed a high load of bacteria in both samples (2.85×10^6 CFU/g and 2.61×10^7 CFU/g,

respectively) which were reduced substantially following prophylactic treatments by antibiotic (Fig. 8).

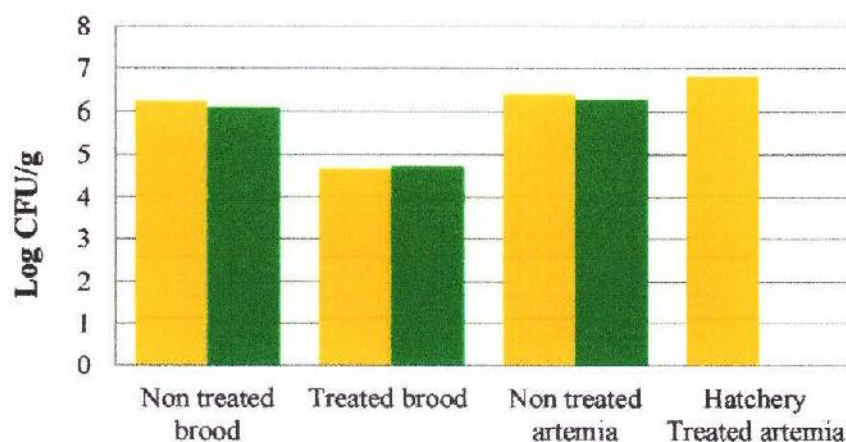


Fig. 8: Total *Vibrio* load in brood eggs and *Artemia* 1st cycle

In case water samples from LRT, the TVC was highest in LRT-2 both before and after antibiotic treatment following partial water exchange (Figs 9 and 10). Before water change and treatment the load varied from 1.5×10^4 CFU/ml to 2.98×10^5 CFU/ml (Fig. 9) whereas water exchange and antibiotic treatment reduced the TVC to one order lower magnitude in all LRTs (Fig. 10). In line with eggs and *Artemia* yellow colonies were predominant in all LRTs.

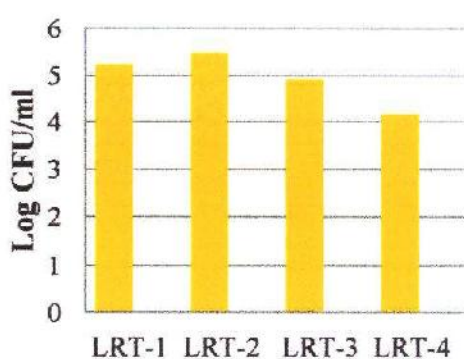


Fig. 9: Total *Vibrio* load in LRT water before water change and treatment in 1st cycle

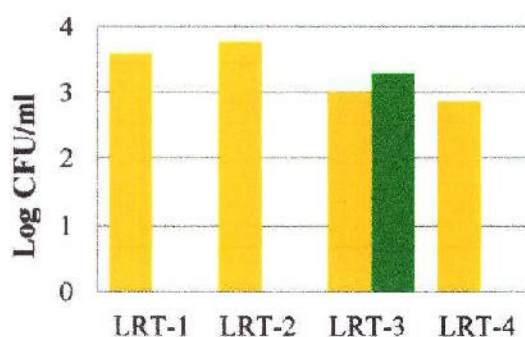


Fig.10: Total *Vibrio* load in LRT water after water change and treatment in 1st cycle

Fig. 11 illustrates that the TVC LRT larvae ranged from 8.0×10^2 CFU/g to 5.5×10^4 CFU/g where LRT-1 showed the highest load and there the count of green colonies were more. LRT larvae were less in count for *Vibrio* than the LRT water.

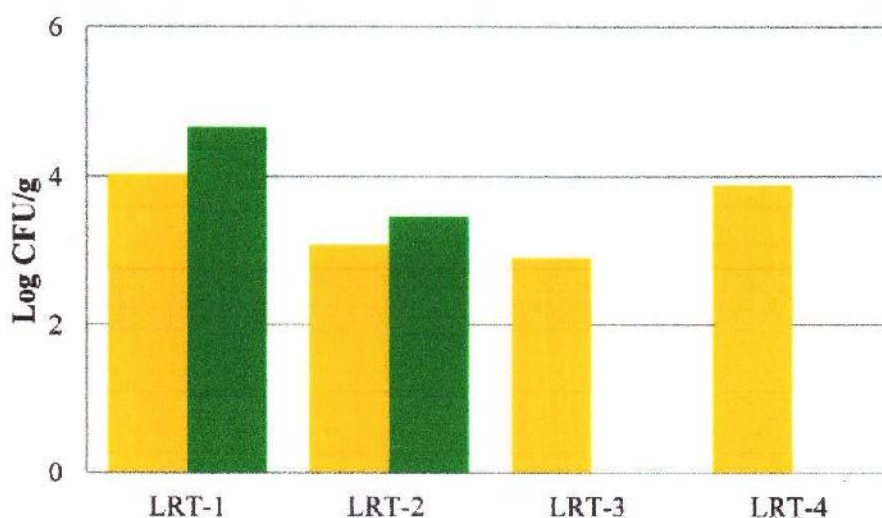


Fig.11: Total *Vibrio* load in LRT larvae in 1st cycle

In 2nd cycle of production, the range of TBC and TVC loads was quite wide being 2.16×10^3 to 3.23×10^9 and 0.6×10^2 to 1.17×10^9 CFU/ml or CFU/g depending on the samples (different water samples, *Artemia*, live and dead larvae), respectively. Nevertheless, the trend of counts was similar to that of samples obtained from the 1st production cycle and the results are summarized in Table 3. As shown in the table, shrimp PL showed large counts for both TBC and TVC resulting in, understandably, the highest counts among all samples investigated followed by *Artemia*. Also shown in the table is the composition of yellow (Y) and green (G) colonies of *Vibrio*, which is almost similar in all samples investigated except water samples from LRTs.

Table 3: Total bacterial (TBC) and total *Vibrio* counts (TVC) for different samples from 2nd cycle of production.

Samples	TBC	TVC		
Mix water	1.84×10^5	3.5×10^2	Y	0
			G	3.5×10^2
Pond water	2.16×10^3	1.07×10^2	Y	0.44×10^2
			G	0.63×10^2
River water	5.28×10^4	2.29×10^4	Y	0.64×10^4
			G	1.65×10^4
Brine	4.1×10^3	0.6×10^2	Y	0.5×10^2
			G	0.1×10^2
Brood's egg	1.43×10^5	4.16×10^4	Y	2.69×10^4
			G	1.47×10^4
<i>Artemia</i>	5.09×10^8	1.55×10^7	Y	0.84×10^7
			G	0.71×10^7
LRT-1 water	1.91×10^6	1.7×10^5	Y	1.7×10^5
			G	0
LRT-2 water	2.7×10^6	1.17×10^6	Y	1.17×10^6
			G	0
Live larvae	1.97×10^8	1.38×10^7	Y	1.08×10^7
			G	0.30×10^7
Dead larvae	3.23×10^9	1.17×10^9	Y	1.1×10^9
			G	0.07×10^9

4.2 Identification and composition of *Vibrio* spp.

The presumptive *Vibrio* colonies were randomly picked and subjected to a set of biochemical tests, as summarized in Table 4, to identify them up to species level.

Table 4. Biochemical characteristics of several members Vibrionaceae family

<i>Vibrio</i> species	Growth in TCBS	AD test	LD test	OD test	8% NaCl	0% NaCl	Sucrose test	Citrate test	Indole test	Gelatinage test	VP test	NO ₂ test	Oxidase test	Arabinose test
<i>V. cholerae</i>	Y	-	+	+	-	+	+	ND	ND	ND	ND	ND	ND	ND
<i>V. campbellii</i>	G	-	+	-	ND	ND	-	ND	+	+	-	ND	ND	ND
<i>V. splendidus ii</i>	G	-	-	-	ND	ND	-	ND	+	ND	ND	+	ND	-
<i>V. orientalis</i>	Y	-	+	-	ND	ND	+	ND	+	+	-	ND	ND	ND
<i>Plesionomas shigelloides</i>	G	+	+	+	ND	ND	ND	ND	ND	-	ND	ND	ND	ND
<i>V. ordalii</i>	Y/G	-	-	-	ND	ND	+	ND	-	ND	ND	-	+	ND
<i>V. mediterranei</i>	Y	-	-	-	ND	ND	+	ND	+	-	ND	ND	ND	ND
<i>V. cincinnatiensis</i>	Y/G	-	+	-	ND	ND	+	ND	-	+	ND	ND	ND	ND
<i>V. salmonicida</i>	Y	-	-	-	ND	ND	-	ND	-	-	ND	-	+	ND
<i>V. Pelagius i</i>	Y	-	-	-	ND	ND	+	ND	-	ND	ND	+	ND	-
<i>V. marinus</i>	Y	-	+	-	ND	ND	ND	ND	-	-	ND	ND	ND	ND

As a result, 11 *Vibrio* species were identified from the hatchery system from 246 presumptive *Vibrio* spp. that were collected randomly from TCBS plates from different samples. The identified *Vibrio* species were *V. cholerae*, *V. ordalii*, *V. orientalis*, *V. campbellii*, *V.*

mediterranei, *V. salmonicida*, *V. marinus*, *V. pelagius* I, *V. cincinnatiensis*, *V. splendidus* II, *P. shigelloides* and their absence and presence at different stages of hatchery operation are shown in Table 5 whereas the compositions of these *Vibrio* species in individual samples are shown in Figs 12 through 19.

Table 5. Occurrence of *Vibrio* species at different stages of prawn hatchery operation

source	No of colony	<i>V. cholerae</i>	<i>V. campbellii</i>	<i>V. splendidus ii</i>	<i>V. orientalis</i>	<i>Plesionomas shigelloides</i>	<i>V. ordalii</i>	<i>V. mediterranei</i>	<i>V. cincinnatiensis</i>	<i>V. salmonicida</i>	<i>V. Pelagius i</i>	<i>V. marinus</i>
River water	55	+	+	-	+	+	+	+	-	-	-	-
Pond water	16	+	+	-	+	-	-	-	-	+	-	-
Brood's egg	40	+	-	-	+	-	-	+	-	-	-	-
<i>Artemia</i>	15	-	-	+	-	-	+	-	+	+	+	-
LRT water	50	-	-	+	-	-	+	-	-	+	-	+
Live larvae	50	-	-	+	+	-	+	+	-	+	-	-
Dead larvae	20	-	-	+	-	+	+	+	-	+	-	-

Fig. 12 shows the composition of these species where *V. ordalii* comprised the highest percentage (20%) followed by *V. salmonicida* (11%), *V. cholera* (9%) and *V. orientalis* (8%). The remaining seven *Vibrio* spp. comprised 2-3% of the total composition.

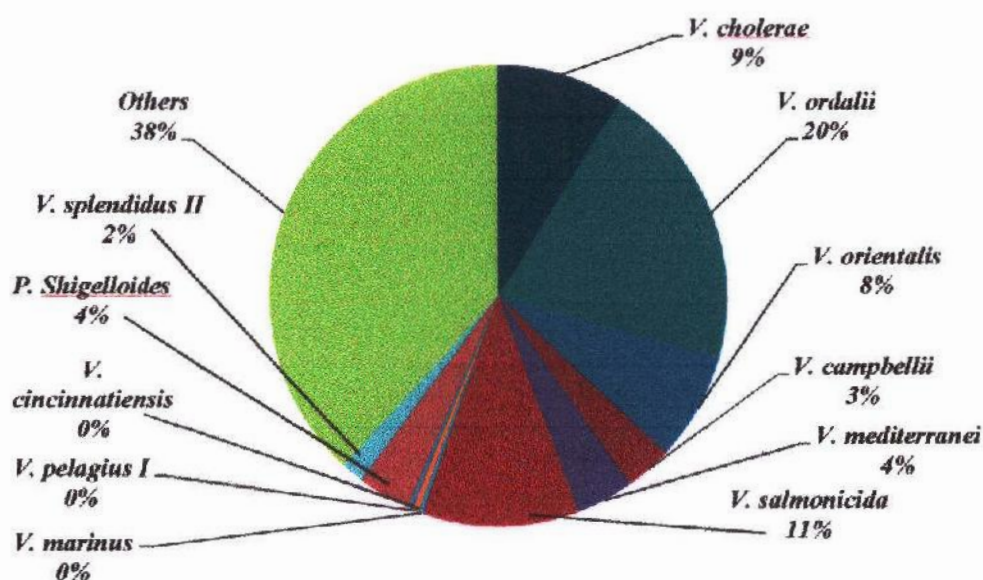


Fig 12: Composition of identified *Vibrio* species

In case of pond water *V. orientalis* and *V. salmonicida* comprised a little over 60% of the *Vibrio* isolates tested (Fig. 13). In case of *Artemia* samples, *V. orientalis* could not be detected; instead *V. ordalii* (20%) and *V. splendidus* occurred in considerable percentages (20% and 13%, respectively; Fig. 14).

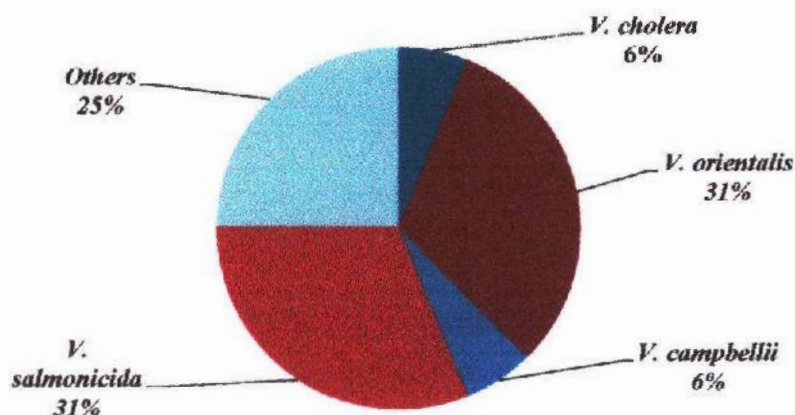


Fig 13: Identified *Vibrio* Species in pond water

The highest *Vibrio* spp. encountered from LRT comprised *V. ordalii* (30%) and *V. salmonicida* (20%) and *V. splendidus* (8%) (Fig.15).

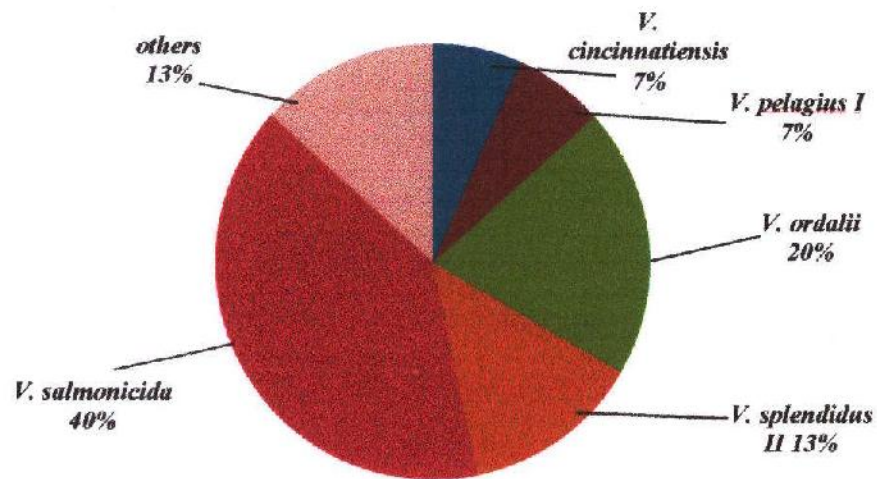


Fig 14: Identified *Vibrio* Species in *Artemia*

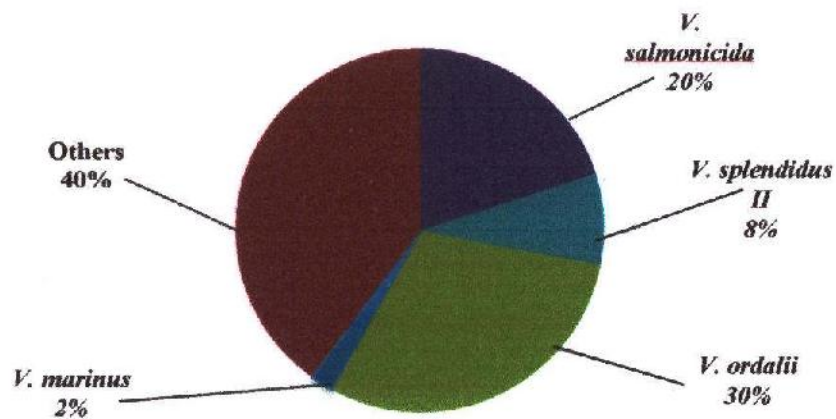


Fig 15: Identified *Vibrio* Species in LRT water

Fig. 16 shows the percentage of identified *Vibrio* species from brood eggs. *V. cholera* was the highest in percentage, which was 30% followed by *V. orientalis* (5%) and *V. mediterranei* (3%).

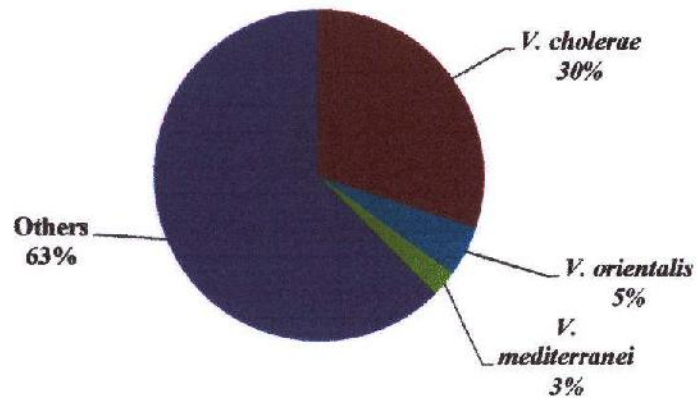


Fig 16: Identified *Vibrio* Species in brood eggs

According to Fig. 17 a variety of *Vibrio* spp. were isolated from river water samples including *V. ordalii*, *V. orientalis*, *V. campbellii*, *V. mediterranei*, *P. shigelloides* and among those *V. Cholera* comprised the highest percentage (17%) followed by *V. campbellii* (13%) and *V. orientalis* (10%).

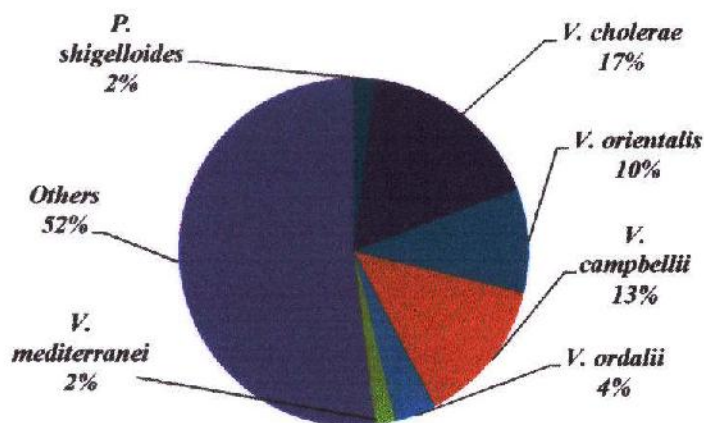


Fig 17: Identified *Vibrio* Species in river water

Figs 18 and 19 illustrate that the identified *Vibrio* spp. Identified from live and dead larva were almost similar. *V. ordalii* appeared to be highest in both samples with 32% in the live and 58% in the dead samples of larva. Interestingly, *V. orientalis* (14%) was not detected in dead samples.

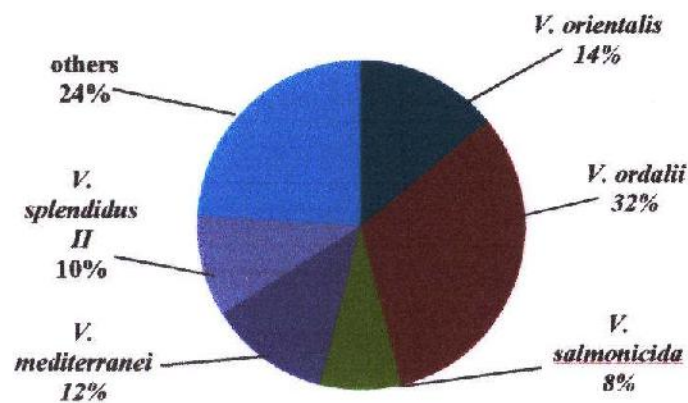


Fig 18: Identified *Vibrio* Species in live larvae

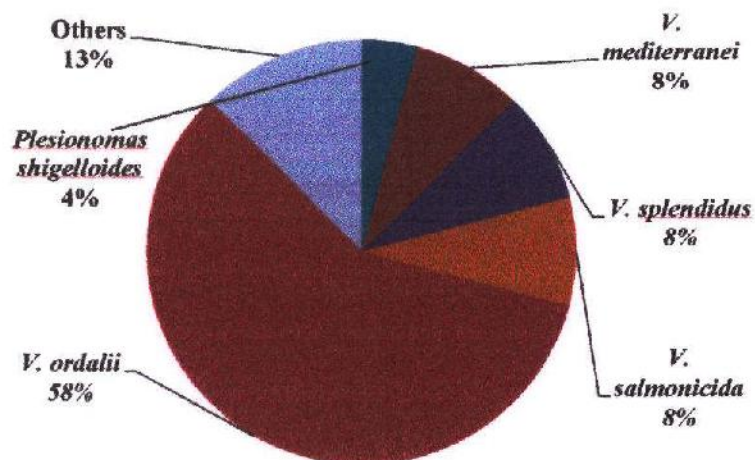


Fig 19: Identified *Vibrio* Species in dead larvae

4.3. Antibiotic susceptibility profile

In present study, discs containing 25 antibiotics were used to examine the susceptibility of 9 out of the 11 identified *Vibrio* species. Among these, 13 antibiotics are specific to G- bacteria (*Vibrio*

spp.) and they belong to Polymixins, Aminoglycosides, Chloramphenicol, fluoroquinolones, Quinolones and Urinary tract antiseptic. Eight antibiotics belonging to Glycopeptide, Cephalosporins, β -Lactam, mainly work against G+ bacteria. The remaining four antibiotics used work against both G+ and G- bacteria (Table 6).

Table 6. General activity profile of antibiotics used in this study

Antibiotics	Group	Activity
Penicillin G	Penicillins (β -lactam)	Active against G+ bacteria
Penicillin		
Amoxicillin		
Mecillinum		
Ampicillin		
sulphomethoxazole	Trimethoprim	Mostly against G+ and some G- bacteria
Trimethoprim		
Polymixins B	Polymixins	Active against G- bacteria
Colistin sulphate		
Neomycin		
Streptomycin		

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Amikacin	Aminoglycosides	Active against G- bacteria
Gentamycin		
kanamycin		
Chloramphenicol	Chloramphenicol	Active against G- bacteria
Tetracycline	Tetracycline	Mostly against G+ and some G- bacteria
Erythromycin	Macrolides	Mostly against G+ and some G- bacteria
Norfloxacin	fluoroquinolones	Active against G- bacteria
Novobiocin		
Ciprofloxacin		
Nalidixic acid	Quinolones	Active against G- bacteria
Nitrofurantoin	Urinary tract antiseptic	Active against G- bacteria
Vancomycin	Glycopeptide	Active against G+ bacteria
Bacitracin		
Cephalexin	Cephalosporins	Good coverage against G+ bacteria

Table 7 shows the diameter of zone of inhibition (mm) of 9 *Vibrio* species against 25 antibiotics with their corresponding disc potencies. Typically, the raw data were interpreted based on the available CLSI (Clinical and Laboratory Standards Institute) data and zone diameter interpretive standards. All the antibiotics showed different inhibitory zones ranging from 6 to 33 mm.

Irrespective of *Vibrio* sp. Tested, Chloramphenicol (30 µg) showed the maximum inhibitory zone (33 mm) whereas the minimum inhibitory zone of 6 mm was produced by Novobiocin (30 µg).

Table 7: Inhibitory zone diameter (mm) of selected antibiotics against different *Vibrio* spp.

Antibiotics	Inhibitory zone (mm)								
	<i>V. cholerae</i>	<i>V. campbellii</i>	<i>V. splendidus II</i>	<i>V. orientalis</i>	<i>P. shigelloides</i>	<i>V. ordalii</i>	<i>V. mediterranei</i>	<i>V. cincinnatiensis</i>	<i>V. salmonicida</i>
Amplecillin (10 µg)	0	19	15	17	7	0	15	0	13
Penicillin G (10 µg)	0	0	15	0	0	0	0	0	12
Penicillin V (10 µg)	0	0	0	0	0	0	0	0	0
Streptomycin (10 µg)	17	9	19	7	9	0	10	12	18
Kanamycin (30 µg)	8	15	20	15	10	0	15	15	19
Chloramphenicol (30 µg)	27	33	31	27	26	0	31	23	30
Nitrofurantoin (300 µg)	20	22	20	22	15	16	22	18	20
Neomycin (30 µg)	15	16	19	15	13	11	15	13	18
Colistin sulphate (10 µg)	10	11	11	12	10	12	13	10	10
Novobiocin (30 µg)	7	12	0	12	0	6	10	8	0
Mecillinam (25 µg)	10	16	0	17	0	11	15	10	0
Erythromycin (15 µg)	14	12	12	0	0	7	10	12	12
Polymyxin B (300 µg)	11	12	12	12	12	13	10	11	12
Bacitracin (10 µg)	0	0	0	0	0	0	0	0	0

Norfloxacin (10 µg)	27	24	0	25	20	17	17	25	0
Amoxicillin (10 µg)	0	16	0	18	10	7	16	0	0
Cephalexin (30 µg)	0	12	10	11	9	12	0	7	10
Ciprofloxacin (5 µg)	27	26	0	25	20	18	22	30	0
Gentamicin (10 µg)	16	13	14	14	14	17	12	14	20
Vancomycin (30 µg)	10	7	7	0	0	0	0	0	0
Sulphamethoxazole (25 µg)	20	20	0	25	22	0	15	22	0
Trimethoprim (5 µg)	16	16	0	21	20	0	16	17	0
Tetracycline (30 µg)	22	22	8	25	20	20	23	20	8
Nalidixic acid (30 µg)	24	25	0	25	19	0	25	26	0
Amikacin (30 µg)	16	12	9	14	14	18	13	15	19

Based on disc diameter data shown in Table 7, the antibiotic susceptibility profile of these 9 *Vibrio* species was created following CLSI guideline for interpretation of inhibitory zone diameter and the results are shown in Table 8. From the view point of growth of bacteria, the antibiotics were categorized into three groups: antibiotics clearly inhibiting bacterial growth were denoted as susceptible (S), antibiotics having no measurable effects on bacterial growth as resistance (R) and those falling in between as intermediate (I).

In the present study all *Vibrio* species were resistant to Penicillins (Penicillin G, Penicillin V), Fluoroquinolones (Novobiocin), Glycopeptide (Bacitracin) and Cephalosporin (Cephalexin) whereas majority of them were highly susceptible to Chloramphenicol (89%) followed by Nitrofurantoin (78%), Norfloxacin (78%) and Tetracycline (78%). Other antibiotics were also found to be effective against several members of *Vibrio*.

Neomycin (67%) and Gentamicin (56%) appeared to be intermediate in terms of bacterial growth inhibition against all *Vibrio* species followed by Kanamycin (44%), Nitrofurantoin (22%), Amoxicillin (22%) and Ciprofloxacin (22%) (Table 6 and 7).

Table 8: Antibiotic resistance profile of *Vibrio* species

Antibiotics	Activity	Susceptibility of identified <i>Vibrio</i> species								
		<i>V. cholerae</i>	<i>V. campbellii</i>	<i>V. splendidus II</i>	<i>V. orientalis</i>	<i>P. shigelloides</i>	<i>V. ordalii</i>	<i>V. mediterranei</i>	<i>V. cincinnatiensis</i>	<i>V. salmonicida</i>
Colistin sulphate (10 µg)	Active against G-bacteria (<i>Vibrio</i> species)	R	S	S	S	R	S	S	R	R
Polymyxin B (300 µg)		R	S	S	S	S	S	R	R	S
Neomycin (30 µg)		I	I	S	I	I	R	I	I	S
Streptomycin (10 µg)		S	R	S	R	R	R	R	I	S
Kanamycin (30 µg)		R	I	S	I	R	R	I	I	S
Gentamicin (10 µg)		S	I	I	I	I	S	R	I	S
Amikacin (30 µg)		I	R	R	R	R	S	R	I	S
Chloramphenicol (30 µg)		S	S	S	S	S	R	S	S	S
Norfloxacin (10 µg)		S	S	R	S	S	S	S	S	R
Novobiocin (30 µg)		R	R	R	R	R	R	R	R	R
Ciprofloxacin (5 µg)		S	S	R	S	I	I	S	S	R
Nalidixic acid (30 µg)		S	S	R	S	S	R	S	S	R

Nitrofurantoin (300 µg)		S	S	S	S	I	I	S	S	S
Amplcillin (10 µg)	Active against G+ bacteria	R	S	I	S	R	R	I	R	R
Penicillin G (10 µg)		R	R	R	R	R	R	R	R	R
Penicillin V(10 µg)		R	R	R	R	R	R	R	R	R
Cephalexin (30 µg)		R	R	R	R	R	R	R	R	R
Bacitracin (10 µg)		R	R	R	R	R	R	R	R	R
Vancomycin (30 µg)		I	R	R	R	R	R	R	R	R
Mecillinam(25 µg)		R	S	R	S	R	R	S	R	R
Amoxicillin (10 µg)		R	I	R	S	R	R	I	R	R
Sulphamethoxazole (25 µg)	Active against both G+ and G- bacteria	S	S	R	S	S	R	I	S	R
Trimethoprim (5 µg)		S	S	R	S	S	R	S	S	R
Erythromycin (15 µg)		I	R	R	R	R	R	R	R	R
Tetracycline (30 µg)		S	S	R	S	S	S	S	S	R

[S= Susceptible; I= Intermediate; R= Resistance]

Table 9: Percentage of resistance/intermediate/susceptibility of *Vibrio* Species identified from hatchery against various antibiotics tested.

Antibiotics	Resistance (%)	Intermediate (%)	Susceptible (%)
Amplcillin (10 µg)	55	22	23
Penicillin G (10 µg)	100	0	0
Penicillin V(10 µg)	100	0	0
Streptomycin (10 µg)	55	12	33
Kanamycin (30 µg)	33	44	23

Chloramphenicol (30 µg)	11	0	89
Nitrofurantoin (300 µg)	0	22	78
Neomycin (30 µg)	11	67	22
Colistin sulphate (10 µg)	44	0	56
Novobiocin (30 µg)	100	0	0
Mecillinam(25 µg)	67	0	33
Erythromycin (15 µg)	89	11	0
Polymyxin B (300 µg)	33	0	67
Bacitracin (10 µg)	100	0	0
Norfloxacin (10 µg)	22	0	78
Amoxicillin (10 µg)	67	22	11
Cephalexin (30 µg)	100	0	0
Ciprofloxacin (5 µg)	22	22	56
Gentamicin (10 µg)	11	56	33
Vancomycin (30 µg)	89	11	0
Sulphamethoxazole (25 µg)	33	11	56
Trimethoprim (5 µg)	33	0	67
Tetracycline (30 µg)	22	0	78
Nalidixic acid (30 µg)	33	0	67
Amikacin (30 µg)	56	22	22

Chapter 5



Discussions

5. Discussion

The factors contributing to the establishment of a bacterial population in the hatchery environment include, among others, the quality of water, use of live feed supplements, rearing technique and the overall hygiene and sanitation practice. While a considerable body of works have been devoted to understand the bacteriological profile *P. monodon* hatchery system, little information is available on the levels of bacteria in *M. rosenbergii* larval rearing systems. In the present study total viable bacterial count (TBC) was determined for two consecutive cycles of production. In the 1st cycle TBC for different source of water ranged from 1.46×10^4 CFU/ml to 9.25×10^6 CFU/ml where the river water showed the highest count among all the water sources of 9.25×10^6 CFU/ml and the lowest bacterial count observed for brine (1.46×10^4 CFU/ml). In case of 2nd production cycle, however, pond water showed the lowest bacterial count (2.16×10^3 CFU/ml). According to the study of Sung *et al.* (2001) on total bacterial count of new and 3 years old grow out pond total bacterial count of recently constructed pond was 1.11×10^6 CFU/ml, while it was 6.25×10^6 CFU/ml for 3 years old pond. In the present study, the bacterial count in pond water was found to be less than that was reported by these authors. The comparatively lower bacterial count in pond water in the present study could be due to the fact that the pond was used only for the purpose of reserving water for the hatchery without rearing any fish whatsoever. Though the number bacteria were less it does necessarily rule out the presence of bacteria pathogenic to prawn due to improper sanitation of the surrounding environment. In fact, pond water contained substantial numbers of *V. salmonicida*, a known pathogen to fin fish and a few *V. Cholerae*, a pathogen known to cause disease in human.

There have been a few studies on bacterial flora associated with the freshwater prawn hatchery system. Kennedy *et al.* (2006) observed the total bacterial load in well water, seawater and inlet water varied from 10^1 to 10^5 CFU/ml, which is in accordance with the findings of the present study. Otta *et al.* (2001) in their study of hatchery system of *P. monodon* observed that both raw seawater and chlorinated seawater had consistently higher counts of 3.5×10^4 and 5.3×10^4 CFU/ml, respectively whereas Kanaujia *et al.* (1998) recorded a count of 1.48×10^3 CFU/ml and 2.54×10^3 CFU/ml in the tank inlet and outlet waters, respectively in *M. rosenbergii* hatchery system. The differences may be due to different water treatment systems used in different hatcheries.

The TBC in LRT was slightly higher than that of pond reservoir water and the count was reduced to one order magnitude following antibiotic treatment. However, in case of 2nd production cycle the TBC counts were one order higher suggesting an improper disinfection of the tanks after 1st production cycle. In case of 1st cycle, TBC varied from 7.2×10^5 CFU/ml to 4.15×10^6 CFU/ml before wash whereas the range was between 7.0×10^4 CFU/ml and 2.95×10^5 CFU/ml after wash. The range of LRT water varied from 1.91×10^6 CFU/ml to 2.7×10^6 CFU/ml for 2nd cycle where LRT-2 showed the highest count for both cycles. The total bacterial count in the LRT water was found to be similar to those observed in different source water (1.46×10^4 to 9.25×10^6 CFU/ml). The range of bacterial counts in larvae varied from 3.77×10^5 CFU/g to 4.43×10^6 CFU/g, a range similar to that of LRT suggesting that the number of bacteria increased as the rearing progressed. A similar observation was made by Miyamoto *et al.* (1983) and (Hameed, 1993). In contrast, Anderson *et al.* (1989) observed a decrease in bacterial population with age of the larvae in some larval tanks (as low as 3.7×10^3) but the authors did not mention the reason underlying such discrepancy in bacterial numbers from the same hatchery.

Eggs sampled from the non-treated brood and treated brood showed high count for TBC (1.51×10^9 CFU/g and 2.75×10^6 CFU/g respectively) which is not surprising as Hameed (1993) observed that when eggs come in contact with tank water upon their release they serve as a surface for bacterial attachment and proliferation. Similar to this finding, Colomi (1985) observed that egg homogenates were sterile, while newly hatched larvae less than 24 h old had a rich intestinal flora. In the present study, broods eggs might be potential source of bacteria although antibacterial treatment of broods reduced the TBC to three order of magnitudes.

The role of live feed like *Artemia* as a vector of bacterial contamination was suggested by D'Agostino and Dobblier (1980). A high load of total bacteria was observed in this study: counts 6.29×10^8 CFU/g and 1.15×10^7 CFU/g was observed in the hatched *Artemia* before and after antibiotic treatments, respectively. A high count of 1.15×10^7 CFU/g bacteria even in treated *Artemia* dose infact suggests that *Artemia* can play a role as a vector of bacterial contamination. Austin and Allen (1982) reported a count of 2.3×10^5 CFU/g on dry *Artemia* cyst surface while hatched cysts had a maximum count of 1.7×10^9 CFU/g after 72 h of hatching, a finding similar to the present study.

In the present study the count of total *Vibrio* from different water samples examined during the two production cycles ranged from 0.6×10^2 CFU/ml to 2.29×10^4 CFU/ml, which cannot be considered high or unusual considering the findings of other authors (Kennedy *et al.*, 2006; Prasad *et al.*, 2008; Sureshvarr *et al.*, 2011).

The total *Vibrio* count 8.0×10^2 to 5.5×10^4 CFU/g in larvae in the present study was in line with the observation of Prasad *et al.* (2008) who reported a similar count of 2.87×10^4 CFU/g from prawn larvae. Similar but a slightly lower count was reported by Sureshvarr *et al.* (2011). The authors reported the total *Vibrio* count to vary from 4.3×10^3 CFU/ml to 5.8×10^3 CFU/ml in their first set of prawn samples.

Vibriosis is caused by a number of *Vibrio* species of bacteria, including: *V. harveyi*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. penaeicida* (Brock, 1990; Ishimaru *et al.*, 1995). There have been occasional reports of vibriosis caused by *V. damsela*, *V. fluvialis* and other undefined *Vibrio* species (Lightner, 1996). None of these *Vibrio* species were isolated in the present study. Scheduled use of antibiotics might have eliminated these bacteria although they were not found in untreated water and prawn samples as well. This suggests that either these bacteria were not present in the samples or the number of presumptive colonies tested (246) were not sufficient enough to isolate all representative *Vibrio* species. However, Kanaujia *et al.* (1998) and Phatarpekar *et al.* (2002) observed the absence of *Vibrioin* *Macrobrachium* hatcheries. Among the 11 *Vibrio* species isolated in the present study *V. campbelli* has been implicated in disease of wild and farmed shellfish (Austin and Austin, 1993; Halder *et al.*, 2011) whereas *V. salmonicida* and *V. splendidus* have been reported to cause disease in fin fish (Toranzo *et al.*, 2005).

It is worth mentioning that *V. harveyi* and *V. parahemolyticus* have been frequently associated with mortalities both in hatcheries and grow out ponds of *penaeid* shrimps (Baticados *et al.*, 1990; Brock, 1990; Lavilla-Pitogo *et al.*, 1990; Kumazawa *et al.*, 1991; Ruangpan and Kitao, 1991; Krishnika and Ramasamy, 2013). Absence of these two species in the present study suggests that they are not responsible for mass mortalities in prawn hatcheries and that other member(s) of vibriaceae family might be involved in pathogenicity. In this regard, it is interesting to note that together with *V. Cambelli*, *V. harveyi* and *V. parahemolyticus* belong to Harveyi clade (Sawabe *et al.*, 2007). In the present study, the former was isolated from the source waters (river water and pond water). Previously, Sawabe *et al.* (2007) argued that a group

of transposase-coding sequence related with horizontal gene transference (HGT) might occur between sister species, such as *V. cholerae* and *V. mimicus* (Cholerae clade) or *V. harveyi* and *V. campbellii* (Harveyi clade). Subsequently, confirmation of the presence of HGT in *V. campbellii* and very recently in *Vibrio punensis*, a new member of Orinetalis clade by Restrepo *et al.* (2008). Although these studies were related to *penaeid* shrimps, a similar novel mechanism of disease occurrence in *M. rosenbergii* cannot be ruled out.

Vibrio species are part of the natural microflora of wild and cultured shrimps (Sinderman, 1990) and become opportunistic pathogens when natural defense mechanisms are suppressed (Brock, 1990). In addition to the pathogenic *Vibrio* spp. discussed above, *V. splendidus* has also been reported in association with disease outbreaks in shrimps (Baticados *et al.*, 1990; Lavilla-Pitoga, 1990; Krishnika and Ramasamy, 2013). Gomez-Leon *et al.* (2005) isolated *V. splendidus* from cultured carpet shell clam (*Ruditapes decussatus*) larvae associated with mass mortalities. Interestingly, in the present study this *Vibrio* species was not recovered from source waters but from the prawn and *Artemia* samples.

Krishnika and Ramasamy (2013) isolated *V. parahaemolyticus*, *V. damsela* and *V. splendidus* from the nauplii of *Artemia* and *V. splendidus*, *V. anguillarum*, *V. fluvialis* and *V. campbellii* from the *Artemia* rearing water. Abraham and Palaniappan (2004) reported the occurrence of *V. harveyi* in *Artemia* rearing water and *Artemia* nauplii. A number of other pathogenic non-vibrio bacteria were found in *Artemia* by many authors (Tanasomwang and Muroga 1990; Verdoncket *et al.*, 1991; Hameed *et al.*, 2000). Thus, it would be interesting to investigate whether *V. splendidus* in prawn samples in the present study was originated from *Artemia* and whether this could be responsible for mass mortality of PL in prawn hatcheries.

Another curious aspect worth mentioning is the presence of *V. ordalii* and *V. salmonicida* in high numbers in all shrimp and *Artemia* samples investigated in the present study. Since these two bacteria are frequently associated with disease in finfish (Sinderman, 1990; Brock, 1990) it would be interesting to investigate whether these two *Vibrio* spp. are related to mass mortalities in prawn hatchery. It would also be interesting to investigate whether any of these *Vibrio* species has acquired drug resistance due to indiscriminate use of antibiotics in hatchery operations for prophylactic purposes.

When faced with disease problems, the common response by the hatchery operators has been the application of antimicrobial drugs, almost always without any knowledge on the type of bacteria involved (Rodgers and Furones, 2009). The antibiotics used are usually oxytetracycline (OTC), often in combination with ciprofloxacin, erythromycin, chloramphenicol, furazolidone and a host of other undisclosed antibiotics (Brigs, 2013; Hua and Apun, 2013). Most of these remedial measures implemented by the hatchery operators have been unsuccessful which ultimately forced many operators to shut down their hatcheries. Frequent use of many antibiotics by the hatchery operators appeared to be incapable of preventing or treating the problem because bacteria have continued to develop multiple resistance towards many different types or classes of the drugs (Son *et al.*, 1997). It is therefore not unlikely that any of these *Vibrio* species has become resistant to multiple drugs. The results of the present study do, in fact, support this hypothesis.

In the present study, a comparative study on antimicrobial susceptibility of 9 out of the 11 isolated *Vibrio* species was carried out using 25 different antibiotics, of which 17 are supposed to work against *Vibrio* (Gram -ve bacteria). Almost all *Vibrio* species tested appeared to be susceptible to chloramphenicol, nitrofurantoin and norfloxacin. In Bangladesh, use of these drugs in hatchery and other aquaculture operations are prohibited, which may serve as an explanation of the result in this study. This finding correlates with others who also reported the susceptibility of *V. parahaemolyticus* to these drugs (He *et al.*, 2016; Xie *et al.* 2015). It should be mentioned, however, that *V. ordalii*, a species known to cause disease in finfish appeared to be resistant to chloramphenicol whereas *V. splendidus* and *V. salmonicida* were resistant to norfloxacin. On the other hand, tetracycline was able to inhibit the growth of all but two *Vibrio* spp despite its wide use as this drug is approved for use in aquaculture. Again, *V. splendidus* and *V. salmonicida* appeared to be resistant to tetracycline.

In the study of Hossain *et al.* (2012) the highest (28.57%) resistance among total *Vibrio* spp. was observed to penicillin, which is not surprising as this drug is effective against Gram +ve bacteria but the authors did not find any resistance to erythromycin in contrast to the findings of the present study. In the present study, erythromycin and novobiocin were almost completely ineffective against all *Vibrio* spp. tested despite the fact that these two drugs are supposed to work against Gram -ve bacteria. High incidence of resistance to streptomycin (55%) was also

observed in the present study, which is in accordance with the findings of several authors (He *et al.*, 2016; Kang *et al.*, 2015; Xie *et al.* 2015). Nevertheless, a high percentage of intermediate susceptibility to neomycine, kanamycine and gentamycine was detected in case of most *Vibrio* spp., suggesting a potential resistance trend.

Multidrug resistance (MDR) was defined as non susceptibility to at least one agent in three or more antimicrobial categories (Thapa Shrestha *et al.* 2015). Thus by definition all *Vibrio* spp. tested in the present study exhibited MDR phenotypes, which varied depending on the species and the antibiotics. Notably, the four *Vibrio* species associated with disease in farmed finfish and shellfish showed staggeringly high MDR phenotypes. The pathogenic *V. salmonicida*, *V. ordalii* and *V. splendidus* showed the highest MDR (9 out of 17 vibrio static drugs) followed by *V. campbelli* (4) and others. To the best of the knowledge, the comparative antibiotic resistance patterns of multiple *Vibrio* species involving 25 antibiotics have never been reported. Most of the past studies were conducted with *P. monodon* or *Litopenaeus vannamei* involving *V. parahaemolyticus* and few antibiotics and none with *M. rosenbergii* hatchery samples (de Melo *et al.* 2011; Albuquerque Costa *et al.* 2015; He *et al.*, 2016). Thus, a direct comparison is not possible. However, working with *V. parahaemolyticus* isolated from adult *M. rosenbergii* He *et al.* (2016) found MDR for 6% isolates involving three antibiotics.

The findings of this study support the conclusion that the emergence of resistant bacteria to antibacterial drugs may be indicative of the indiscriminate use of these drugs in prawn hatchery. Furthermore, the detection of multiple drug resistance in several *Vibrio* species commonly known to cause disease in farmed aquatic animals including shrimp serves as alert to the possibility of horizontal transfer of antibiotic resistance genes among bacteria. If this is true then an otherwise commensal nonpathogenic *Vibrio* might has become virulent and are proliferating to cause disease when antibiotics are applied to eliminate most of the beneficial bacteria from the system. Future studies could be designed to test this hypothesis using a systematic in depth analysis and molecular methods (rather than culture methods) for accurate assessment of the changes in microbial community along the hatchery production line.

Chapter 6



Conclusion

6. Conclusion

The objective of the present study was to assess the bacterial flora associated with different steps of freshwater prawn hatchery system with particular focus on isolation of *Vibrio* species and investigation of their antibiotic resistance profile so that a link to mass mortality of prawn PL can be understood. Among all the samples tested *Artemia* were identified as the key source of total bacteria (1.15×10^7 CFU/g) and total *Vibrio* (6.25×10^6 CFU/g) contamination in the hatchery followed by brood eggs (2.75×10^6 CFU/g and 9.59×10^4 CFU/g for total bacteria and total *Vibrio*, respectively). Nevertheless, river water, mixed water (pond water mixed with brine) were also found to contain bacteria in similar order of magnitudes. Nevertheless, the total bacterial counts obtained in the present study are within the range commonly encountered in hatchery operations. While this could be a good indication of better hygiene management in hatchery it does not reflect what happened to the individual members of Vibrionaceae family, particularly to those often associated with disease in farmed shrimp and prawn.

To investigate this proposition, a series of biochemical tests was followed that resulted in isolation and identification of 11 *Vibrio* species from 246 randomly picked presumptive *Vibrio* colonies representing both yellow and green colonies from different stages of hatchery operation. Among the identified *Vibrio* species, *V. ordalii* comprised the highest percentage followed by *V. salmonicida*, *V. cholerae*, *V. orientalis*, *V. mediterranei*, *P. shigelloides*, *V. campbellii* and *V. splendidus* II. Besides, *V. cincinnatiensis*, *V. marinus*, *V. pelagius* I were also isolated in few numbers from some samples. A critical analysis of relevant literatures suggest that *V. ordalii* and *V. salmonicida* are associated with disease in farmed aquatic animals on many occasions whereas *V. splendidus* and *V. cambelli* were found to cause disease in shrimp.

Usually the prawn hatcheries use different antibiotics at different doses without justifying its effectiveness and optimization of the chemicals. This might lead to develop antibiotic resistant bacteria strain(s) that could be a potential threat for successful hatchery operation. In fact the results of the present study support this statement. A comparative study on antimicrobial susceptibility of 9 out of the 11 isolated *Vibrio* species was carried out using 25 different antibiotics belonging to 11 generic groups, of which 17 are supposed to work against *Vibrio* (Gram-ve bacteria). Almost all *Vibrio* species tested appeared to be susceptible to chloramphenicol, nitrofurantoin and norfloxacin suggesting that these prohibited drugs are not

used. Tetracycline was able to inhibit the growth of all but two *Vibrio* spp. despite its wide use as this drug is approved for use in aquaculture. *V. splendidus* and *V. salmonicida* appeared to be resistant to tetracycline. Another commonly used drugs erythromycin and novobiocin were almost completely ineffective against all *Vibrio* spp. Similarly, a high percentage of intermediate susceptibility to neomycine, kanamycine and gentamycine was detected in case of most *Vibrio* spp., suggesting a potential resistance trend.

All *Vibrio* spp. tested in the present study exhibited MDR phenotypes, notably including the four *Vibrio* species associated with disease in farmed finfish and shellfish. The pathogenic *V. salmonicida*, *V. ordalii* and *V. splendidus* showed the highest MDR followed by *V. campbelli* and others.

Taken together, the results of this study indicate that indiscriminate use of multiple antibiotics and other unknown drugs by hatchery operators might pave the way for propagation of antibiotic resistant *Vibrio* spp. at later stages of hatchery operation leading to mass mortality of PL. Thus, this study suggests that adhering to better hygiene and improved management practices should be ensured instead of improper use of antibiotics for prophylactic purposes.

Chapter 7



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7. References

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