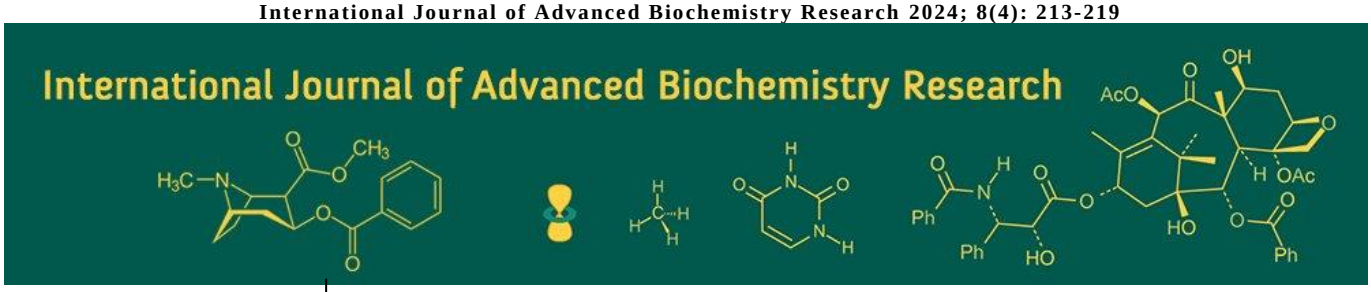

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Advanced techniques for quality control & quality monitoring of shrimp (*Litopenaeus vannamei*) seeds

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Abstract
Shrimp aquaculture plays a crucial role in India's economy and food production. In recent years, the diseases of shrimps hindered the development of shrimp culture. When shrimp farms are shut down due to disease outbreaks there are socioeconomic consequences, including loss of employment. To bring back the shrimp culture getting fast growth and maximum survival becomes crucial. Stocking of best quality seeds is the foremost requirement. As the demand for high-quality shrimp seedlings grows, adherence to rigorous standards becomes essential. Quality control is paramount in the successful operation of shrimp hatcheries. In this study, we delve into the critical aspects of quality control within shrimp hatcheries includes Nauplii Quality, Fry quality, Plankton quality, Fungus Checking, PL Checking, Artemia hatching rate. The present study was conducted in a leading commercial hatchery situated at Dandi village of Valsad District of Gujarat. From water quality monitoring to disease prevention, we explore best practices, regulatory compliance, and cutting-edge techniques. Quality of seeds from nauplii to Post Larvae 10 were checked by PCR throughout the culture at regular interval to ensure the disease free condition. The quality of seed meets the standard at larval and post larval stages.

Keywords: *Litopenaeus vannamei*, shrimp hatchery, post larvae, quality control standard, shrimp seeds

Introduction
The shrimps of the family penaeidae are known around the world as valuable resources for aquaculture. Majority of research and development efforts have been directed to few species (e.g., *Litopenaeus vannamei* and *Penaeus monodon*) that dominate world production. *L. vannamei* can be readily reproduced in captivity has wide tolerance to environmental parameters, better utilizes low-protein containing diets and grows fast compared to other penaeid shrimp species (Wyban 2007) [28]. Indian shrimp aquaculture has shifted from farming of giant tiger shrimp, *P. monodon*, to the exotic American white leg shrimp, *L. vannamei*, since 2008. Availability of quality shrimp seed was the main reason responsible for the shit from tiger shrimp to *L. vannamei* (Tandel 2017) [25]. However, disease outbreaks cause serious economic losses in India and other countries. The pathogens are often exotic viruses, fungus, bacteria and protozoans suspected to have spread through a variety of pathways (Soto-Rodríguez 2009) [22]. PCR-based methods are used by private and public service laboratories for the detection of white spot syndrome virus (WSSV) infection in penaeid shrimp post larvae (PL) before they are stocked in rearing ponds (Sritunyalucksana 2006) [23]. PCR is an important tool for detection of viral pathogens in shrimp aquaculture. It has been used for WSSV detection in brood stock and post larvae, for investigation of WSSV transmission routes among different hosts and for epidemiological studies (Lo 1996) [9]. PCR based methods have been successfully used to screen and eliminate WSSV from infected brood stock and PL before they enter the production system (Momoyama 1995) [14]. It stands for Polymerase chain reaction in which repeated replication of target sequence of desired sample takes place. It is used to amplify the particular target sequence for millions of times. PCR technology has commonly employed in the CPF India Pvt Ltd aquaculture industry for disease diagnosis and prevention of contamination and to sale the costumer quality Seed (Disease free seed).
Vibriosis is the only bacterial disease caused by Gram negative free-living bacteria described. Vibrio bacteria are one of the pathogenic factors, which cause high mortality among economically important species of farmed marine fish and shrimp.

During outbreaks in larval and post larval shrimp rearing, luminescent *Vibrio harveyi* (Lavilla-Pitogo 1990) [7] have been isolated and proven as true pathogens. Since no artificial feed formulation is yet available to completely substitute for Artemia, feeding live prey to young fish larvae still remains essential in commercial hatchery operations. Continuous disinfection of Artemia during hatching and enrichment is becoming a routine operation in hatcheries to keep larvae disease free. The use of the right size of Artemia for feeding ensures a better energy balance in food intake and assimilation, thereby improving the performance of the fish. Furthermore, its palatability induces a good and fast feeding response (Sorgeloose 2001) [21]. The efficient operation of a fish hatchery depends on water quality, adequate facility design, water supply structures and water sources. Among these, water quality determines to a great extent the success or failure of a fish culture operation. Physical and chemical characteristics such as suspended solids, temperature, dissolved gases, pH, mineral content and the potential danger of toxic metals must be considered in the selection of a suitable water source. Microalgae are the major food source for many aquatic organisms and the main live feed used in marine hatchery operations. The estuarine diatom *Thalassiosira weissflogii* (Fryxell & Hasle, 1979) [3] has been widely used as live feed in aquaculture. The growth rate and biochemical composition of microalgae are highly influenced by environmental factors such as, light, salinity and nutrient availability (Norma García 2012) [15].

Materials and Methods

The present study was carried out in CPF (Charion Pockphand Foods) India Pvt. Ltd., hatchery located at Dandi village in Valsad (Dist.), Gujarat. This hatchery is well designed, equipped and maintained for commercial production of *L. vannamei* seeds for the past Five years. There are Two production units operating simultaneously and the annual production is around 250-400 million seeds, and had 5 laboratories for water quality, microbial, Quality control, plankton and PCR. 1 batch stocked in 4 tanks having 6 million seeds was examined for sample collection.

Water treatment system

The seawater for the hatchery was pumped from the sea directly using 2 motors of 10 HP. The suction point is located about 100m from the shoreline. Before pumping water, the seawater pipeline and Aeration pipeline was disinfected with 0.2% NaOH solution and kept for 1 hour and then filled with acidified chlorine of 300 ppm for 24 hours and then rinsed with clean freshwater. Tanks were sprayed with solution of 0.2% NaOH and then sprayed with 1500 ppm Acidified chlorine and kept for 24 hours and then rinsed with freshwater. The water was initially pumped into Pressure sand filter. From the sand filter, water was shifted into chlorination tank using a 5 HP motor. Chlorination was done with 20ppm chlorine. After 24hrs, the chlorinated water was stored in Storage tank after passing through UF filter (Toray- HFU-2020AN) which can filter up to 0.04 Micron. The filtered water was passed through UV filter before filling into tanks. The residual chlorine available in the treated seawater was determined with chlorine test kits. After knowing the availability of excess chlorine in treated sea water sodium thiosulphate was used to neutralize the residual chlorine. The chelating agent, EDTA (10 ppm) was added in treated seawater to ensure clear seawater. A 15HP air blower and a 7.5HP standby provided continuous supply of air. The air generated by the blower was supplied to individual tanks through PVC pipes.

Production Unit Preparation, Seed Stocking and Larval rearing

Rearing tank, drained canal and Floor was sprayed with 0.2% NaOH solution then rinsed with Clean fresh water after an hour. It was again sprayed with 1500 ppm Acidified chlorine and rinsed with freshwater after 24 Hour then it was sprayed with and rinsed with Freshwater after an hour. Tanks were washed with 1:1:20 solution of Iodine:Soap oil:Water and then kept for drying for 24 hours. Fumigation was done with KMnO4 and Formalin and next day swab was taken from tank, floor, canal, aeration line, seawater line and vibrio test was conducted. If no vibrio colony is found on plates, then the section is ready to fill water and stocking seeds. Water sample from tanks was taken for testing of Salinity, pH, Temperature, Alkalinity, Ammonia, Nitrite, Chlorine.

Seeds were purchased from another hatchery of CPF India PVT LTD located at Chettikuppam Village, Taluk, Marakkanam, Tamil Nadu. Nauplius stage 3 was packed transported by flight which takes 20-22 hours to reach Valsad Hatchery. The plastic bags were rinsed with 20 ppm Iodine solution and then seeds were kept in Cement tanks of 20-ton capacity for temperature acclimatization. Water sample was collected from a bag and chemical parameters such as Salinity, pH, alkalinity, Ammonia and Nitrate were checked. Number of seeds were counted from randomly collected 3 bags. After acclimatization of temperature the seeds were stocked in prepared cement tanks with a density of 75 pcs/liter.

After stocking of nauplii, *Thalassiosira weissflogii* was filled twice a day for feeding. For zoea stages, Artemia with algae was fed. After conversion to Mysis stage the algae feeding was reduced and artemia quantity and frequency was increased, simultaneously liquid artificial was fed. Once larvae changes from mysis-3 to PL-1 artificial solid feed was fed 8 times/day along with Artemia 4 times/day. Probiotics, Minerals, Vitamins, and water color were used to maintain required water quality. Water exchange was carried out after PL-4 on a regular basis.

Water Quality Parameters

Water sample were collected from culture tank, Storage tanks, Chlorination tanks, collection tanks, Direct Seawater and Fresh water. Physico-chemical parameters such as salinity, temperature, pH, dissolved oxygen, ammonia and alkalinity were monitored daily in the morning hours. Microbial test for yellow and green colony forming vibrio and Luminescent bacteria was carried out for same water samples. The optimum range of water quality parameters like salinity, alkalinity, pH, ammonia, and temperature for Nauplius to post larval 7 is mentioned is as mentioned in table 1.

Total Vibrio Count (TVC)

Vibrio species have become a major source of concern for shrimp culture because of their close association with low survival rates in hatcheries (Saulnier 2000) [19]. Larval mortalities associated with the presence of *Vibrio harveyi* have been reported in *Penaeus monodon* and *Litopenaeus*

vannamei in Indonesia (Sunaryanto and Mariam, 1986) [24], Thailand (Jiravanichpaisal *et al*., 1994) [4], India (Karunasagar *et al*., 1994) [6]. Main species to make problem in hatchery is luminescent *Vibrio harveyi.* Thiosulphate citrate bile salt sucrose (TCBS) agar was a media used to cultivate. TVC was conducted for determination of water quality and shrimp health monitoring. Water sample was collected from artemia culture, plankton culture and larvae rearing tanks was collected. Shrimp sample was collected from rearing tank no. 1, 2, 3, and 4. Range of the total Vibrio count should be as per table 1.

Table 1: Optimum water quality parameters

Stages of Seed	Salinity (ppt)	pH	Temp (°C)	Alkalinity	Ammonia	Total Vibrio Count
Nauplius 5	30	8-8.5	30	200-250	<1	$1X10^4$ - $1X10^5$ CFU/g
Zoea 1	30	8-8.5	30	200-250	<1	$1X10^4$ - $1X10^5$ CFU/g
Mysis 1	30	8-8.5	30	200-250	<1	$1X10^4$ - $1X10^5$ CFU/g
Post Larva1	30	8-8.5	28-32	200-250	<1	$1X10^4$ - $1X10^5$ CFU/g
Post Larva 4	25-30	8-8.5	28-32	200-250	<1	$1X10^4$ - $1X10^5$ CFU/g
Post Larva 7	20-30	8-8.5	28-32	200-250	<1	$1X10^4$ - $1X10^5$ CFU/g

PCR analysis

Sample was collected from water storage tanks, Nauplius 5, PL4, Artemia, feed, soil and swab from tanks. Samples were analyzed for WSSV, EHP, and IHHNV diseases. Due to the limitation of the silica resin and GT Buffer, the sample size for different sample source and different size of the shrimp should be adjusted to avoid inhibition of downstream application reaction. Silica, GT Buffer, DEPC ddH_2O was used for extraction of the template. Negative charge of DNA binds strongly with positive charged silica gel. Silica pellet was washed with GT buffer and then washed with 75% ethanol to removes Proteins, Fats, Carbohydrates and other impurities. DEPC Water is used to breakdown the silica-DNA bonds with help of heat. For amplification Real-time Premix, IQzyme DNA polymerase, RTzyme Mix, Nucleic acid was used. IQ RT-Pre Mix contains reaction buffer, dNTPs, specific primers, and fluorescent probes. The Optical Plate was placed in The RT-PCR Machine and programme run in EDS Format. The result came in graphical form and it was converted in Excel form for analysis. In the RT PCR we can analyse the sample in real time as the process take place and we can examine the magnitude of infection of different viruses.

Fungus analysis

Large scale hatchery losses of eggs and larvae to *Lagenidium sp.* and *Sirolpidium sp.* were reported (Meng and Yu, 1980) [13]. *Lagenidium* and *Sirolpidium* are two types of waterborne fungi that can significantly impact the zoea1 stage of shrimp. Excessive growth of *Lagenidium* can lead to larval mycosis, affecting the health and survival in shrimp hatcheries. To check fungus in nauplii 200-300 piece is packed with oxygen in polythene bag. The sample in the bag is checked for fungus (*Lagenidium* and *Sirolpidium*) by light microscope the next day i.e, in Zoea 1 stage at 40x lens.

Quality assessment in larval stages

Nauplius5, Zoea1, Mysis1, Post Larvae1 stages are the main checkpoint for health and growth monitoring. Larval stages were examined for its timely conversion using light microscope. Quality Standards for Nauplius-5 includes its quantity at the time of stocking, Stage of Nauplius, Total length of Nauplius (µm.), Deformity percentage, Photo taxis, and Total vibrio count. The scoring system detailed in Table 2 was then used to score the quality of each batch of post larvae produced. As per table 3 if Shrimp seeds which gets grade D should be discarded as per the standard procedure followed by disinfection of the tank.

Table 2: Point table for assessment of seed quality Monitoring

S. No.	Parameters for quality Monitoring	Points
1	Photo taxis	30
2	Total length of Nauplius	20
3	Stage of Nauplius	15
4	Percentage of Deformity	20
5	Total vibrio count	15
		100

Table 3: Grading of seed based on quality parameters

S. No.	Total points (%)	Class	Grade
1	95-100	Excellent	A
2	58-94	Good	B
3	75-84	Special handle	C
4	<75	Poor	D

Photo taxis

Phototaxis is the movement of larvae in response to light. The cylindrical pipe with a valve and transparent region was used to study phototaxis in shrimp and activeness of larvae in light. Take sample of 200 Nauplius in phototaxis pipe and keep it in front of halogen lamp for 15 minutes. Then the nauplii of lighten part was separated by the valve and its number was counted.

Calculation

$$\text{Nauplius Active (\%)} = \frac{\text{Count of strong Nauplius}}{\text{Total Nauplius}} \text{ X } 100$$

Stage of Nauplius

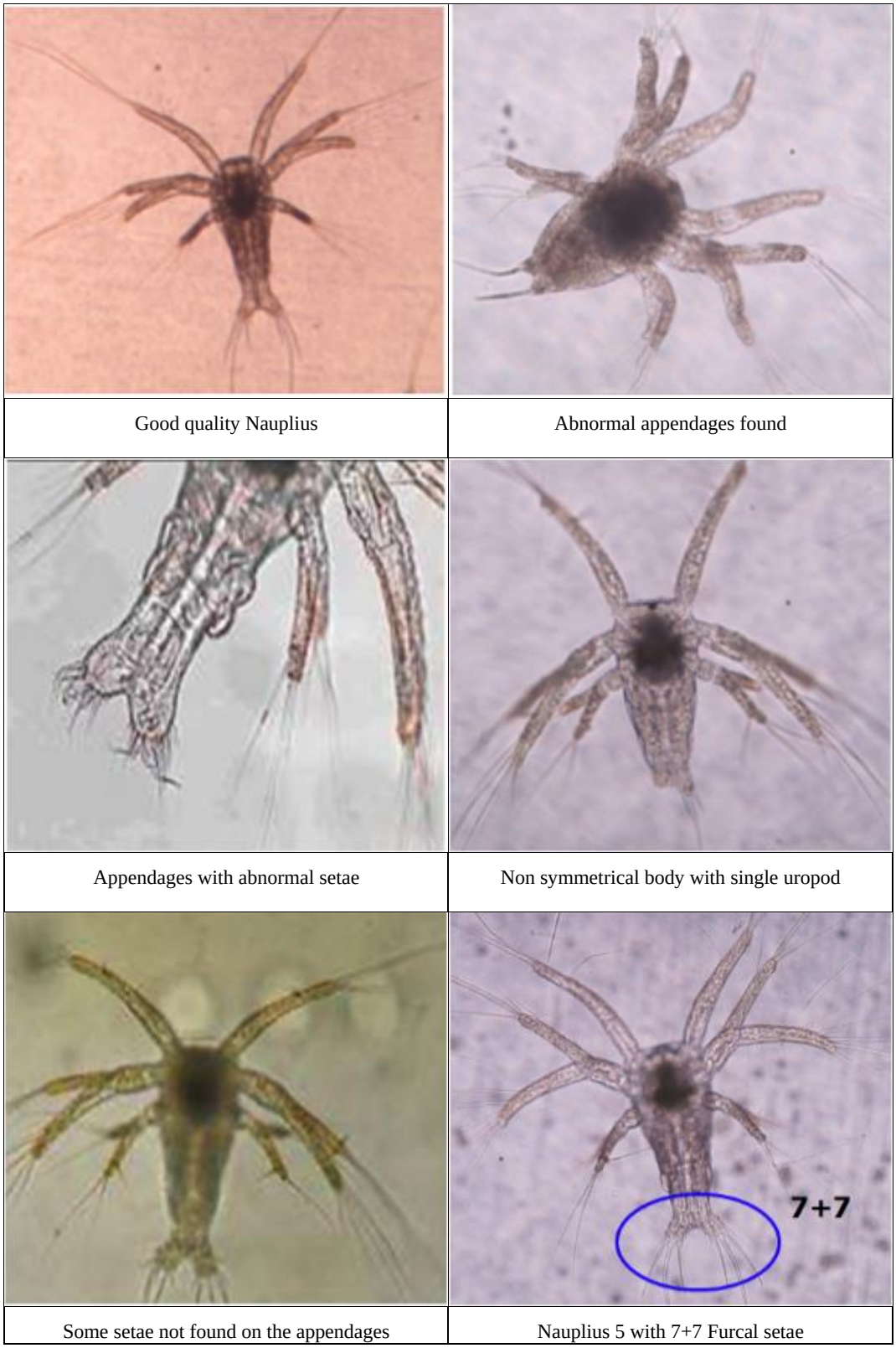
At the time of receiving 100 pcs of Nauplius was observed under microscope at 100x magnification by using ocular scale. The sample of NP5 should have 7+7 or 6+6 furcal setae to confirm its stage as showed in Figure 1. Sample was collected from tank in a 250 ml bottle by using net. Size of Nauplius was measured under light microscope at 100x magnification by using ocular scale. As per the company standards for Nauplius5, Zoea1, Mysis1 and PL1 stages total body length should be 400-450 µm, 0.8-1.3 mm, 2.7-3.4 mm, 5.0-5.5 mm respectively.

Deformity

Deformity is abnormalities in the body other than the original shape i.e. abnormal appendages, appendages with abnormal setae, non-symmetrical body, some setae not found on the appendage, Abdominal segment deformity Disease, Syn uropod disease as mentioned in Figure 1. From the rearing tank 100 larvae was collected on slide by using dropper then observed under microscope using 4x and 10x. 1 or 2 drops of lugols iodine was used to stop activity of the sample. 100 shrimp seed of PL7 stage was observed for necrosis, Fouling (Filamentous bacteria), lipid content and Muscle-gut ratio.

$$\text{Deformity (\%)} = \frac{\text{No. of Np. with deformity}}{\text{Total Nauplius}} \text{ X } 100$$

Standard: Np Deformity ≤ 5%
PL Deformity ≤ 5%



Good quality Nauplius	Abnormal appendages found
Appendages with abnormal setae	Non symmetrical body with single uropod
Some setae not found on the appendages	Nauplius 5 with 7+7 Furcal setae

Fig 1: Morphological structure and deformities in Nauplius

Post Larval Conversion analysis

PL1 is the important checkpoint to monitor larval health of shrimp. Sample was examined by Using light microscope at 4x and 10x for conversion from Mysis3 to PL1 by checking setae in pleopods and body length. If setae are present in the pleopods, the specimen is at the PL1 stage.

Standards:

Conversion of PL: >80%

Length: 5.0-5.5 mm at Doc 7

PL Quality Analysis Method

Length Analysis Method

Length of the post larvae was measured in the length machine which is comprises of 3 main components i.e. camera, water recirculating motor and software for data analysis. Camera clicks the photos of shrimp passing through the strip where water is continuously flowing by recirculating motor which is saved and analyzed by software and data is produced. Total length and percentage coefficient of Variation percentage are main parameters to check to check the uniformity of the larvae.

Stress Test Method

Acute stress tests have been described to distinguish between healthy and weak PL and used by commercial hatcheries to evaluate PL's hardiness. (Maugle, 1988) [12]. In most procedures, PL are subjected to sudden changes in salinity or to selected chemical solutions. However, because the response to osmotic or chemical stress (Samocha 1998) [17]. A Stress test was conducted once they reached PL4. Randomly collected sample of seeds were taken in a container out which 50 piece of sample separated from rearing water and release in to the 0 ppt water and kept for 30 minutes & then same seeds were released in 30ppt and kept for 30 minutes. Check for the dead pieces and remove them if there are any and record the survival rate.

Stress test standard: $\geq$ 90 = pass and < 90 = fail

Zooplankton Contamination analysis

One of the diseases that can cause the death of shrimp both in pond and hatchery is Zoothamniosis. This disease is one of the parasitic diseases found in vannamei shrimp caused by *Zoothamnium penaei*. This disease causes the shrimp to breathe hard, difficult to move and cannot find food (Sindermann 1997) [20], difficult to moult, inhibit growth, reduced economic value and cause death to 91% (Tonguthai 1991) [26]. *Zoothamnium* is a ciliate protozoan which attach themselves to surfaces, including plankton. an overgrowth of *Zoothamnium* can lead to contamination of the plankton culture. Their presence can negatively impact the health of larval shrimp by competing for resources and space. High ectoparasite infestation can cause an increase in mortality and have an impact on the organisms (Mahasri 2019) [11]. Ectoparasite infestation can also cause death, caused by secondary attacks by bacteria (Toriyanto 2004). Several genera from the Ciliate class that often infect pacific white shrimp are *Epistylis*, *Zoothamnium*, and *Vorticella* (Novita 2016) [16].

Phytoplankton Contamination analysis

Some *dinoflagellate* species can cause contamination in plankton cultures as they produce toxins that can harm other plankton species. Excessive growth of toxic *dinoflagellates* can negatively impact larval shrimp health and survival. *Navicula* (Diatom) is not suitable for the shrimp culture as it blooms fast and cause eutrophication which is not healthy for a shrimp culture.

Results & Discussion

Water quality of hatchery

Salinity of the direct seawater was 37 ppt and freshwater salinity was 5 ppt. Stocking water prepared for Nauplii, Plankton culture and artemia culture had 30 ppt salinity, 200-250 alkalinity, 8.1-8.5 pH and 30° C temperature. Rearing water for larval and post larval stages was maintained as mentioned in table 4. Once Larvae developed into PL1 the salinity of rearing water was reduced to 15 ppt till PL4 and raised up to 25 ppt till PL7. Sodium bicarbonate was used to increase Alkalinity. Sodium Hypochlorite was used to eliminate chlorine residue. Calcium Hydroxide was used to increase the pH of the water. Water exchange was done in case if ammonia level increase in rearing water. Exhaust fan was used during summer season and heater was used during winter to maintain temperature. 1 drop of treflan per ton of water was added to keep water fungus free.

Table 4: Water quality parameters of water samples

Stages of Seed	Salinity (ppt)	pH	Temperature (^{0}C)	Alkalinity	ammonia	Total Vibrio count
Nauplius 5	30	8.1	30	210	0	0
Zoea 1	30	8.2	31	204	0	$1.21x10^4$
Mysis 1	30	8.1	31	218	0.1	$1.3x10^4$
Post Larva 1	28	8.5	30	220	0.2	$1.36x10^4$
Post Larva 4	17	8.3	31	270	0.2	$1.75x10^4$
Post Larva 7	22	8.2	32	235	0.3	$2.05x10^4$

Seed quality parameters

From all tanks after 2 hours of acclimatization 4 samples were collected for Phototaxis test that shows the active number of larvae was 198, 196, 199 and 195 out of 200 from tank number 1, 2, 3 and 4 respectively. Nauplius of 5th stage had 6 furcal setae (98%) while 7 furcal setae (2%). Average body length of the larval and post larval stages was as mentioned in table 5. In analysis of Nauplius 5 stage, out of 100 seed sample 1 larva was found with appendages with abnormal setae, 1 with non-symmetrical body and some setae was not found on appendages of 1 out of 100 larvae. While analyzing Zoea1 stage, out of 100 seed sample 1 larva was found with abdominal segment deformity Disease and some setae not found on the appendage of 1 larva. No deformity was found on Mysis1, PL1 and PL4 stage of shrimp seeds.

No vibrio colony was observed for stocking water of artemia culture, plankton culture, and larvae rearing tanks. No green colonies were found from rearing tanks throughout the culture. Average yellow colony for larval sample from Zoea1, Mysis1, PL1, PL4 and PL7 stage was $2.57x10^4$, $7.9x10^4$, $9.29x10^4$, $1.21x10^5$, and $1.43x10^5$ respectively.

Water, Nauplius 5, PL 4, Artemia, shrimp feed, soil and swab from tanks was analyzed for the various diseases like WSSV, EHP, and IHHNV and none of these was detected during the culture. No fungus was observed during the examination of larva of Zoea1 stage.

All the examined Post larvae had setae on their pleopods which confirm that seeds are 100% converted from mysis3 to PL1.

Number of Active PL4 after stress test of tank no. 1, 2, 3, and 4 was 49, 47, 47, and 48 respectively. Number of active PL7 after stress test of tank no. 1, 2, 3, and 4 was 48, 47, 48 and 49 respectively. Remaining pieces was alive but it was barely moving hence it was not counted. Active number of seeds in PL4 and PL7 was >90% which makes it passed for stress test as per standards. Approximate survival of shrimp seed till the PL7 stage was 80%.

Table 5: Average body length of seeds at various stages

Tank No.	Nauplius 5	Zoea 1	Mysis 1	Post larva 1	Post larva 4	Post larva 7
1	0.45 mm	1.1 mm	3.2 mm	6.92 mm	7.8 mm	9.1 mm
2	0.44 mm	1.0 mm	3.0 mm	6.95 mm	8.1 mm	9.0 mm
3	0.44 mm	1.0 mm	3.0 mm	6.85 mm	8.1 mm	9.1 mm
4	0.45 mm	1.05 mm	3.3 mm	6.89 mm	8.0 mm	8.9 mm

Conclusion

Standard operating procedures were followed in the hatchery. Excellent water treatment system, disinfection procedures and strict biosecurity rules were followed. It ensures proper conversions, better growth and disease free culture of shrimp seeds. Salinity of rearing water was reduced by adding freshwater in seawater. Analytical tests conducted to monitor the quality of the seed shows that Larvae of the shrimp was of an excellent quality as it was of A grade. Water quality management by Regular water exchange, proper feeding management and daily assessment of water and seed was the key to achieve such milestones. These are crucial parameters which must be followed for culture of disease free and healthy seeds. Even after following strict rules for biosecurity, clean water and health assessment 20% of the seeds couldn't survive which leaves a further study for the better survival of seeds. Whether you're a seasoned hatchery manager or a newcomer to the industry, this article equips you with the knowledge needed to maintain excellence in shrimp production. For the researcher it gives an idea about the standards followed by well-equipped shrimp hatcheries.

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