Sanitary Measures in marine microalgae culture

Sekar Megarajan, Narsimhulu Sadhu, R D Suresh and P Damodara Rao
ICAR-CMFRI, Visakhapatnam Regional Centre, Pandurangapuram,
Andhra University Post, Visakhapatnam - 530003

The production of marine finfish and shellfish larvae in hatchery requires different types of phytoplankton and zooplankton. The quality of these live feeds directly affects the production cycle of finfish and shellfish larvae since these life feeds are used either directly or indirectly in larval rearing system. Thus proper sanitary measures for better management need to be taken at each level of culture operation of live feeds to maintain the quality and quantity of these live feeds, right from stock culture to mass culture.

Sanitary measures are routine biosecurity programmes that should be taken before, during and after production runs to maximize the health of the stock by minimizing the impact of contamination. These measures are taken to control the movement of different contaminants into and through the live feed stocks. This may begin with facility design, water intake and passes through the entire range of possible sources of contamination throughout the production cycle. Optimum level of sanitization in the system will result in good quality stock, which will reduce the failure risk of the larval production cycle. Hence, it reduces the cost of the production and helps in maximizing the profit from the production cycle.

Contaminants in phytoplankton

The major contaminants in phytoplankton stock and mass culture are as follows:

i. Microbes such as bacteria, virus etc

ii. Ciliates

iii. Other microalgal species

iv. Zooplankton in outdoor culture

Source of contamination

The following are the source of contamination in phytoplankton stock culture:

i. Glass ware

ii. Culture media

iii. Water

iv. Air

v. Personnel

Precautions/ sanitary measures

i. Phytoplankton stock culture

- It is necessary to practice aseptic techniques to protect the sterile culture media, glassware and finally the cultures from different contaminants.
- Personnel hygiene is the first important step to maintain sterile conditions in stock room.
- The person involved in maintaining stock room should first finish the work related to stock culture and then only carry out any other assigned work.
- He should always use sanitizer whenever he is handling the stock.
- The microalgae stock culture room needs to be kept clean and the door should be kept closed at all times to maintain desired temperature.
- The stock of the algae should be sub cultured under the laminar flow so that contamination in the stock can be avoided.
- The laminar flow UV light should be kept on at least half an hour before doing inoculation work.
- The equipments such as weighing balance, microscope and laminar flow need to be cleaned regularly.
- All glass wares such as culture containers, pipettes etc used in indoor culture are to be cleaned, washed with chromic acid and sterilized in hot air oven to avoid contamination.
- The culture media except heat labile media need to be autoclaved after preparation to avoid contamination. The heat labile chemicals need to be sterilized by 0.22 μm filtration techniques.
- Water is a major source of contamination. It needs to be treated with different filtration processes before sterilization.
- The culture container with water needs to be autoclaved for maintaining stock culture.
- The air supplied to the culture container must be passed through filters so that contamination can be avoided.

- Each and every step must be monitored carefully so the contamination can be stopped early on.
- The working area needs to be cleaned and wiped with alcohol to limit contamination.
- The air stone, pipes and air control need to be sterilized after each cycle by boiling in water for 15 minutes.
- Finally only authorized personnel should be allowed entry into algal stock culture room.

The ciliates are the major source of contamination in algal culture.

It can enter either though culture solution or air. The autoclaving of culture solution and use of filter before passing of air to the culture container can help in controlling ciliates in stock culture.

The contamination of one species of microalgae with another species of microalgae:

- It is common in a stock culture room if it is housing more than one species in the same room.
- This contamination can be controlled by the personnel involved in stock culture room.
- They should sub culture one species at a time and at least give half an hour time interval before starting sub culture of another species.
- In addition, they need to follow personal hygiene before starting sub culture.
- The pipette or the inoculum loop used for the sub cultures need to be sterilized each time before use.

ii. Phytoplankton mass culture

- Design of the water pumping system, water storage facility and culture tanks have effect on the contamination of phytoplankton culture.
- Proper design of the tanks is required to facilitate easy cleaning and drying so the contamination can be avoided.
- The tank inner surface needs to be smooth and the corners should be curved in such a way that cleaning should be easy.
- The bottom of the tank should have some slope so when the water is getting drain it should dry easily.

- An epoxy or fiber glass or plastic coating inside the tank will help in these things. Among these, epoxy coating is the best solution for making inner wall of tank smooth.
- The pumping facilities need to be treated every alternate month with chlorinated water (30-40 ppm) to avoid any contamination.
- The aeration system needs to be sanitized every alternate month by using formalin.
- After the harvest of live food organisms proper management measures for wastewater treatment is required for proper hatchery production.
- Drainage pipes carrying wastewater need to be of suitable diameter for water draining and for avoiding backflow.
- In outdoor culture system, carboys must be cleaned using common salt, rinsed with tap water and keep upside down for sun drying.
- All tanks should be cleaned, disinfected and dried before use.
- The water used for the mass culture of phytoplankton needs to be filtered before stocking in the tank.
- The chemicals required for the mass culture need to be prepared in filtered fresh water.

Conclusion

In order to bridge the gap between demand and supply of marine aquatic resources it is necessary to bring significant changes in live feed production technology. Appropriate sanitary measures are required to combat contamination and maintain hygiene. Technological developments for improving the sanitary conditions of marine hatcheries are critical. Further, awareness for sanitary cleanliness and personal hygiene among the workers is highly essential for improving the quality and quantity in the supply of live feed.

References

Cai Yimin, Pang Huili, Tan Zhongfang, Wang Yanping, Zhang Jianguo and Chuncheng Xu. 2014. Application of Lactic Acid Bacteria for Animal Production.Lactic Acid Bacteria, 443-491.

Loka Jayasree, Sonali, S. M., Saha Purbali and Philipose, K. K. 2016. Bacterial flora of water and rotifers in outdoor mass culture tanks fed with different microalgal diets. J. Life Sci., 10: 123-127.

Probiotics in live feed culture

Jayasree Loka, Ritesh Ranjan and Narsimhulu Sadhu
ICAR-CMFRI, Visakhapatnam Regional Centre, Pandurangapuram,
Andhra University Post, Visakhapatnam - 530003

Probiotics are considered as an excellent tool for microbial management in mariculture. They play a major role in mariculture either to establish optimal microbial communities in live feed cultures or as direct feed supplementation for fish larvae. In the long run, adding probiotics to the raising water of live meals may benefit the larvae in addition to enhancing their quality andquantity. Bioencapsulation of various live feeds, including copepods, rotifers, and *Artemia*, improves the nutritional status and general health of fish. Enrichment of live feed with probiotics has been applied for many years as microbial adjuncts with promising effects on the growth, health and culture environment of aquatic organisms (FAO & WHO, 2002), that resulted in a growing interest in learning about the advantages of probiotics in live feed cultures. Much attention has been devoted to manipulate the composition of the microbial community in order to improve the stability of cultures and mitigate the proliferation of harmful bacteria. Application of probiotics in live feed cultures enhance the density, swimming behaviour and feeding performance of phyto and zooplankton which will be used as feed for fish and shrimp larvae. Aquaculture faces a significant challenge with regard to organic enrichment and nitrogenous wastes which encompass ammonium (NH_3^+) and ammonia (NH_3). The present chapter deals with the role of probiotics in mass culture of marine microalgae, rotifers and copepods.

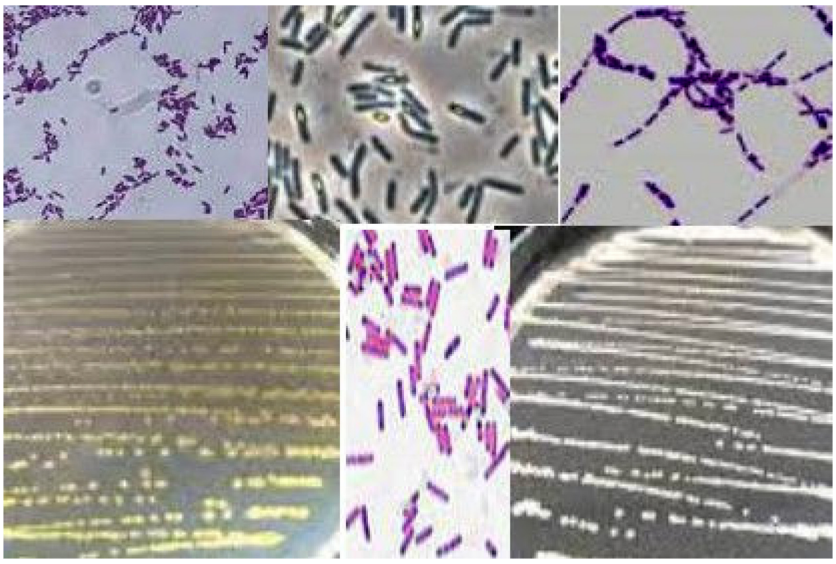
Potential probiotic bacteria used in live feed culture

Several probiotics are reported as beneficial for the enhancement of population density of rotifers and copepods. Supplementation of probiotics in *Artemia* increased HUFA and total lipids levels of live feed. Potential probiotic bacterial species that can be used as co-culture with microalgae and as enrichment feed for rotifer, copepod and *Artemia* are *Lactococcus* sp., *Lactobacillus* sp. *Pediococcus* sp., *Carnobacterium* sp., *Bacillus* sp., *Enterococcus* sp., *Pseudoalteromonas* sp., *Microbacterium* sp., *Ruegeria* sp., *Streptococcus* sp., *Thermophilus* sp., and *Shewanella* sp.

Probiotic selection

Potential probiotics can be selected by isolation of native species from live feed or can be procured from commercial companies. Before application of these

probiotics as enrichment in live feed, the probiotics can be tested for their antagonistic activity against pathogens and their efficiency in performance of other probiotic properties. The most efficient and potential probiotics can be selected as an enrichment medium for the culture of marine microalgae as water supplementation and as cofeed for rotifer, copepod and *Artemia* cultures.



Isolation and culture of beneficial bacteria

Probiotic enrichment methods in different live feed cultures

Probiotic enrichment in Rotifer culture

1. The rotifers should be washed with fresh seawater and stocked in a plastic bucket containing 50% volume of sterile seawater.
2. Probiotics (5 mg L^{-1}) are mixed with marine microalgae (single or in combination of two species with an approx. con of$1x10^8$cfu/ml) and are blended with 100 mL of deionized water.
3. This mixture is then poured into the bucket containing rotifers. Based on the stocking densities of rotifer, the feeding rate of this mixture can be adjusted.
4. Rotifers can be fed to fish larvae after 6 hours of enrichment

Enrichment of probiotics in Copepod and *Artemia* culture

Newly hatched copepod and *Artemia* are soaked in the probiotic suspension (10 mg L^{-1}) with strong aeration.

This enrichment process should be carried out for 1 h in order to avoid nutrient losses in copepod and *Artemia*.

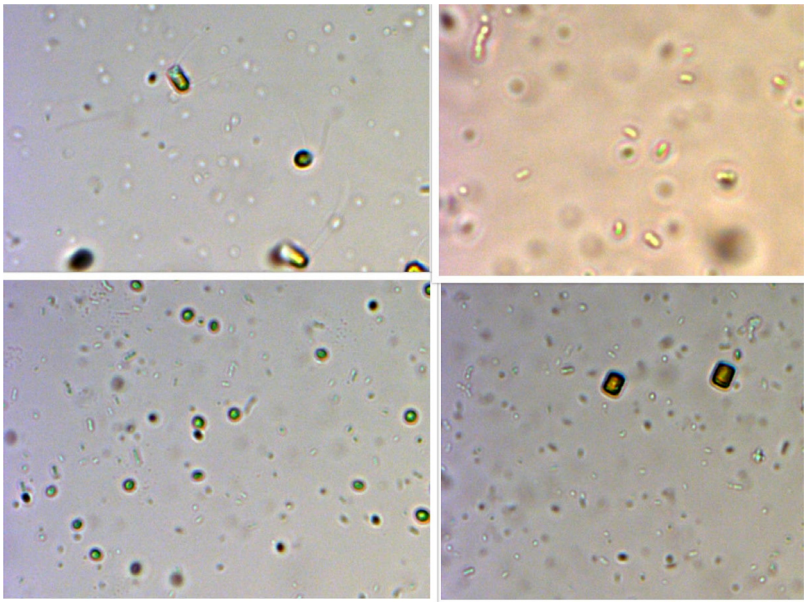
The enrichment protocol is as follows:

1. The Artemia that has just hatched is not completely developed and therefore cannot consume food from water. However, they are capable of absorbing water that contains probiotics.
2. Consequently, the *Bacillus* has the ability to inhabit both the gastrointestinal tract and the external surface of Copepod/*Artemia*.
3. In Long-term probiotic enrichment, the retention of probiotics in Copepod/ *Artemia*naupliiis compared over a long-term period of 24 hours which coincides with the commercial EFA enrichment period.
4. After the 22-hour hatch phase, copepod and *Artemia* nauplii are enriched with S. presso (INVE Aquaculture®, Belgium) and probiotics (1×10^{10} cfu/ ml).
5. Analyze *Artemia* and copepod samples at 10 min, 15 min and 24 hours probiotic enrichment following the methods described above.
6. Disinfection of samples with sodium hypochlorite (5 ppm) for two-time intervals, 10 and 15 minutes gives best results during enrichment process.
7. In short-term probiotic enrichment, the Copepod and *Artemia* are rinsed and concentrated.
8. The stock is inoculated with probiotics at concentration of 1mg/L.
9. The density of the Copepod and *Artemia* can be determined by diluting the culture 1:20 in aerated seawater and counting eight 100 μL samples of *Artemia* under a microscope.
10. *Artemia* and copepod nauplii can be disinfected with sodium hypochlorite (5 ppm) for 15 minutes prior to enrichment. Sodium thiosulphate (10 ppm) is used to neutralise the sodium hypochlorite.

11. Then the cultures are inoculated with the bacterial concentrate to attain 10^6 CFU/mL and remained at room temperature for 30 minutes after inoculation.
12. The enriched culture is stored in a refrigerator (4 p C) for 6 hours
13. Samples (water, copepods, *Artemia*) are taken at 0.5 and 6 hours for observation of enrichment and for determination of density, survival and also for elimination of pathogens in water.

Application of enriched probiotic consortium in larval rearing of finfish

1. Probiotic consortium (1.6 × 10^{10} CFU.g"1) which is already tested for its potential antagonistic and other probiotic characters towards the cultured larvae is supplemented with live feed diet to the fish larvae as a multi-strain probiotic.
2. The strains are kept in Modified Marine Broth, grown at 35æ%C for 24 h, and then centrifuged at 1800g for 15 min and resuspend in a sterile saline solution.
3. Then, the respective suspensions are sprinkled on the artificial feed.
4. The quantity of feed should be replenished every seven days on a weekly basis and stored at 4 C.



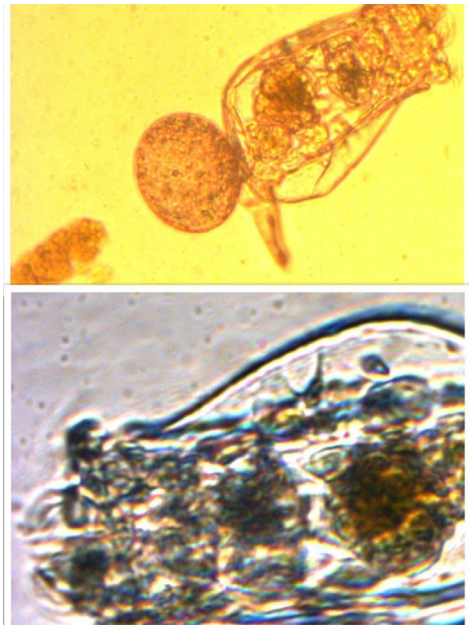
Co culture of probiotic consortia with marine microalgae

Advantages of Probiotic supplementation in live feed

Probiotics consist of live microbial supplements designed to optimize the host's microbial equilibrium for its potential benefit. Most high-intensity live food culture systems see a decrease in yield due to the accumulation of organic carbon, nitrates and phosphorous, which can cause high levels of these nutrients to be released, which can eventually cause the culture to break down. The removal of particulate matter and excess nutrients continues throughout the harvesting period in many culture systems. Hence, to improve the live feed culture condition, probiotically enriched system will be the best solution for constant collapse of the live feed cultures. Therefore, the constant collapse of live food cultures may be remedied by a bacterially mediated mechanism for enhancing the condition of the culture and maximizing the utilization of nutrients. Adding carbon in this system can restore the correct C: N ratio, enabling bacteria to transform solid waste into biomass.

Bacterial communities have the potential to shape the environmental conditions and population expansion of live food cultures. When bacteria and microalgae are combined, the resulting population growth of rotifers is significantly higher compared to using bacteria alone as the primary diet. In the case of larger live food sources, single bacteria cells may prove to be an inadequate diet, and the process of flocculation can be employed to boost the growth of these organisms. Enhanced utilization of microalgae due to its increased digestibility of probiotic bacteria which can enhance amictic reproduction with the availability of increased food.

Enrichment of microalgae with probiotic bacteria enhances number of eggs, population density, locomotion and swimming behaviour of rotifers. Enrichment of probionts helps in dominating their population in culture systems and decreases or inhibits growth of other microbes. Probiotics play a significant role in balancing microbial community of rotifer/copeod culture systems and also on the population density. Probiotic application enhances rotifer/copeod density significantly and the concentration of probiotics and strains vary significantly with initial density of zooplankton and also mode of culture.



Rotifer enrichment with probiotic consortium

The density of copepod nauplii increase significantly (2.4 times) in tanks supplemented with probiotics. Water quality of live feed culture systems also improve with probiotic inoculation in culture tanks. A decrease in *Vibrio* count andincrease in the density with increase of probiotic loads and in the mass culture tanks of copepod and rotifers supplemented with probiotic enriched diets. The total bacterial loads and ammonia levels of water in copepod tanks decrease in probiotic treated tanks and eliminates *Vibrios* from tanks fed with marine microalgae supplemented with probiotics.

Conclusion

The usage of probiotics as supplementary diet along with microalgae is suggested to improve the quality of water and density of copepod/rotifer in mass scale production which in turn helps to improve the survival rates of finfish larvae in early stages of development.

References

Douillet, P. A. 2000. Bacterial additives that consistently enhance rotifer growth under synxenic culture conditions - Evaluation of commercial products and pure isolates. *Aquaculture*, 182: 249-260.

Jayasree Loka, S. M. Sonali, purbali saha, K. Devaraj and K. K. Philipose. 2016.Use of commercial probiotics for the improvement of water quality and rotifer density in outdoor mass culture tanks. Indian J. Fish., 63(4): 145-149, 2016. DOI: 10.21077/ijf.2016.63.4.60352-24

Lara-Lores. M. 2011. The use of probiotic in aquaculture: an overview International Research Journal of Microbiology, 2 (2011), pp. 471-478

Verdonck, L., Swings, J., Kersters, K., Dehasque, M., Sorgeloos, P. and Leger, P. H. 1994. Variability of the microbial environment of rotifer *(Brachionus plicatilis) and Artemia* production systems. *J. World Aquac. Soc., 25: 55-59*.

Vine, N. G., Leukes, W. D. and Kaiser, H. 2006. Probiotics in marine larviculture. *FEMS Microbiol.* Rev., 30: 404-427.

Yun-Zhang Sun, Hong-Ling Yang, Kun-Peng Huang, Ji-Dan Ye, Chun, Xiao Zhang. 2013. Application of autochthonous *Bacillus* bioencapsulated in copepod to grouper *Epinephelus coioides* larvae 2013. Aquaculture392–395: 44-50

Zink, I. C., Douillet, P. A. and Benetti, D. D. 2013. Improvement of rotifer, *Brachionus plicatilis* population growth dynamics with inclusion of Bacillus spp. probiotics. *Aquac*. Res., 44: 200-211.