CMFRI Training manual Ser. No.: 9/2016

Training Manual on Live Feed for Marine Finfish and Shellfish Culture

VISAKHAPATNAM REGIONAL CENTRE
ICAR-CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
OCEAN VIEW LAYOUT, PANDURANGAPURAM
VISAKHAPATNAM - 530003

Training Manual
On

Live feed for Marine finfish and shellfish culture

17th – 22nd March, 2016

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PREFACE

Fish food organisms are microscopic organisms naturally present in the aquatic environment as primary food for the larvae of finfish and shellfish. Their size ranges from a micron to few millimetres. They are tiny forms which suits the mouth size of all kinds of finfish and shellfish larvae. Fish food organisms are essential for the developmental stages of many aquatic organisms. They are very important in the critical phases of finfishes and shellfishes for their better survival and growth. They are rich in proteins, carbohydrates and essential fatty acids. The major expansion of finfish and shellfish aquaculture around the world is attributed to the development of standard mass production techniques of live feed. Though this field of fish food organisms forms a small part of the aquatic systems but due to increasing commercial interest in the hatchery development of aquatic finfish and shellfish for human needs lead to increasing urgent need for more development of live feed for providing feed for larval stages of fish. The availability of good quality and quantity of live foods contribute to successful operation of aqua-hatchery both for intensive and small scale operations. Visakhapatnam Regional Centre of Central Marine Fisheries Research Institute, under the administrative fold of Indian Council of Agricultural Research, possesses a rich tradition of expertise in live feed culture with pure stock cultures available of all commercially important species. The result of which, all hatcheries operating in the states of Andhra Pradesh, Odisha and West Bengal and the neighbouring country of Bangladesh depend totally on Visakhapatnam Regional Centre for meeting their live feed culture requirements. Keeping in view, the increasing demand of live feed culture, contributed by the increasing number of hatcheries; it was felt pertinent to have a hands-on training programme on the recent techniques in vogue on the various culture methodologies of all live feeds, so as to enlighten the researchers and hatchery operators on the nuances and intricacies of live feed culture. This training is the first of its kind in the region and the knowledge gained from this training programme will go a long way to boost the experience of hatchery operators, making them more confident of success in their hatchery operations.

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Shubhadeep Ghosh, Biji Xavier, Loveson L. Edward and Biswajit Dash

Introduction

Live food organisms include all plants (phytoplankton) and animals (zooplankton) which are used in finfish and shellfish larval rearing system. Live foods are able to swim in water column and are available to fish and shellfish larvae thereby stimulate the feeding response (David 2003). In nature, most of the finfish and shellfish larvae feed on small phytoplanktonic and zooplanktonic organisms. Phytoplankton comprises the base of the food chain in the marine environment. Micro algae have an important role in aquaculture as a means of enriching zooplankton for feeding fish and other larvae. In addition to providing protein (essential amino acids) and energy, they provide other key nutrients such as vitamins, essential poly unsaturated fatty acids (PUFA), pigments and sterols, which are transferred through food chain. Zooplankton forms the primary food for fish larvae. The dominant zooplankton groups are rotifers and copepods. These groups are the preferred prey for shrimp and fish larvae and are the most widely used live feeds by aquaculturists. The intensive larval culture of most marine fish depends on large supply of zooplankton.

Phytoplankton

Micro-algae are indispensable in the commercial rearing of various species of marine animals as a food source for all growth stages of bivalve molluscs, larval stages of some crustacean species, and very early growth stages of some fish species. Algae are furthermore used to produce mass quantities of zooplankton (rotifers, copepods, and brine shrimp) which serve in turn as food for larval and early-juvenile stages of crustaceans and fish. Besides, for rearing marine fish larvae algae are used directly in the larval tanks referred as "green water technique", where they are believed to play a role in stabilizing the water quality, nutrition of the larvae, and microbial control. All algal

species are not equally successful in supporting the growth and survival of a particular filter-feeding animal. Suitable algal species have been selected on the basis of their mass-culture potential, cell size, digestibility, and overall food value for the feeding animal. Various techniques have been developed to grow these food species on a large scale, ranging from less controlled extensive to mono specific intensive cultures.

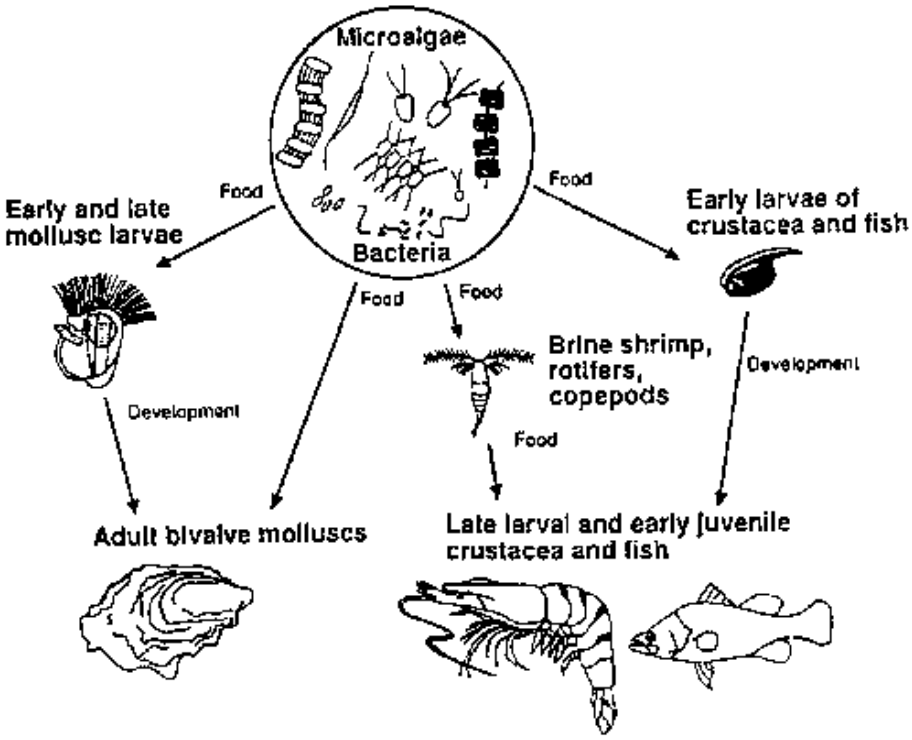

Fig 1. The central role of microalgae in mariculture (Brown *et al*., 1989). Source :FAO 1996

Cultured algal species - major classes and genera

Today, more than 40 different species of micro-algae, isolated in different parts of the world, are cultured as pure strains in intensive systems.

The list includes species of diatoms, flagellated and chlorococcalean green algae, and filamentous blue-green algae, ranging in size from a few micrometers to more than 100 μm.

Table 1. Major classes and genera of micro-algae cultured in aquaculture (modified from De Pauw and Persoone, 1988). Source: FAO 1996

Class	Genus	Examples of application
Bacillariophyceae	*Skeletonema*	PL,BL,BP
	Thalassiosira	PL,BL,BP
	Phaeodactylum	PL,BL,BP,ML,BS
	Chaetoceros	PL,BL,BP,BS
	Cylindrotheca	PL
	Bellerochea	BP
	Actinocyclus	BP
	Nitzchia	BS
	Cyclotella	BS
Haptophyceae	*Isochrysis*	PL,BL,BP,ML,BS
	Pseudoisochrysis	BL,BP,ML
	dicrateria	BP
Chrysophyceae	*Monochrysis (Pavlova)*	BL,BP,BS,MR
Prasinophyceae	*Tetraselmis(Platymonas)*	PL,BL,BP,AL,BS,MR
	Pyramimonas	BL,BP
	Micromonas	BP
Cryptophyceae	*Chroomonas*	BP
	Cryptomonas	BP
	Rhodomonas	BL,BP
Cryptophyceae	*Chlamydomonas*	BL,BP,FZ,MR,BS
	Chroomonas	BP
Xanthophyceae	*Olisthodiscus*	BP
Chlorophyceae	*Carteria*	BP
	Dunaliella	BP,BS,MR
Cyanophyceae	*Spirulina*	PL,BP,BS,MR
Eustigmatophyceae	*Nannochloropsis*	FL

PL, penaeid shrimp larvae; BL, bivalve mollusc larvae; ML, freshwater prawn larvae; BP, bivalve mollusc postlarvae; AL, abalone larvae; MR, marine rotifers (*Brachionus*); BS, brine shrimp (*Artemia*); SC, saltwater copepods; FZ, freshwater zooplankton ,FL, fish larvae

The most frequently used species in commercial mariculture operations are the diatoms *Skeletonema costatum*, *Thalassiosira pseudonana*, *Chaetoceros gracilis*, *C. calcitrans*, the flagellates *Isochrysis galbana*, *Tetraselmis suecica*, *Monochrysis lutheri* the chlorococcalean *Chlorella* spp. and eustigmatophycean, *Nannochloropsis* spp.

Nutritional value of micro-algae

The nutritional value of any algal species for a particular organism depends on its cell size, digestibility, production of toxic compounds, and biochemical composition. Although there are marked differences in the compositions of the micro-algal classes and species, protein is always the major organic constituent, followed usually by lipid and then by carbohydrate. Expressed as percentage of dry weight, the range for the level of protein, lipid, and carbohydrate are 12-35%, 7.2-23%, and 4.6-23%, respectively.

Table 2. Concentrations of chlorophyll a, protein, carbohydrate and lipid in 16 species of micro-algae commonly used in aquaculture (modified from Brown, 1991).Source: FAO 1996

Algal class Species	Dry weight (pg.cell-1)	Chl a	Protein	Carbohydrate	Lipid
	Weight of constituent (pg.cell-1)				
Bacillariophyceae					
Chaetoceros calcitrans	11.3	0.34	3.8	0.68	1.8
Chaetoceros gracilis	74.8	0.78	9.0	2.0	5.2
Nitzchia closterium	-	-	-	-	-
Phaeodactylum tricornutum	76.7	0.41	23.0	6.4	10.7
Skeletonema costatum	52.2	0.63	13.1	2.4	5.0
Thalassiosira pseudonana	28.4	0.27	9.7	2.5	5.5
Chlorophyceae					
Dunaliella tertiolecta	99.9	1.73	20.0	12.2	15.0
Nannochloris atomus	21.4	0.080	6.4	5.0	4.5
Cryptophyceae					

Chroomonas salina	122.5	0.98	35.5	11.0	14.5
Eustigmatophyceae					
Nannochloropsis oculata	6.1	0.054	2.1	0.48	1.1
Prasinophyceae					
Tetraselmis chui	269.0	3.83	83.4	32.5	45.7
Tetraselmis suecica	168.2	1.63	52.1	20.2	16.8
Prymnesiophyceae					
Isochrysis galbana	30.5	0.30	8.8	3.9	7.0
Isochrysis aff.	29.7	0.29	6.8	1.8	5.9
Galbana(T-so)	102.3	0.86	29.7	9.1	12.3
Pavlova lutheri	93.1	0.34	24.2	6.9	11.2
Pavlova salina					

The content of highly unsaturated fatty acids (HUFA), in particular eicosapentaenoic acid (20:5n-3, EPA), arachidonic acid (20:4n-6, ARA), and docosahexaenoic acid (22:6n-3, DHA), is of major importance in the evaluation of the nutritional composition of an algal species to be used as food for marine organisms. Significant concentrations of EPA are present in the diatom species (*Chaetoceros calcitrans, C. gracilis, S. costatum, T. pseudonana*) and the prymnesiophyte *Platymonas lutheri*, whereas high concentrations of DHA are found in the prymnesiophytes (*P. lutheri*, *Isochrysis* sp.) and *Chroomonas salina*. Micro-algae can also be considered as a rich source of ascorbic acid (0.11-1.62% of dry weight). The nutritional value of micro-algae can vary considerably according to the culture conditions like media in which the micro algae are cultured. The protein content per cell, which is considered as one of the most important factors determining the nutritional value of micro-algae as feed in aquaculture, was found to be more susceptible to medium-induced variation than the other cellular constituents. Moreover, the growth of animals fed a mixture of several algal species is often superior to that obtained when feeding only one algal species. A

particular algae may lack a nutrient, while another algae may contain that nutrient and lack a different one. In this way, a mixture of both algal species supplies the animals with an adequate amount of both nutrients.

Use of micro-algae in aquaculture

Micro-algae are an essential food source in the rearing of all stages of marine bivalve molluscs (clams, oysters, and scallops), the larval stages of some marine gastropods (abalone, conch), larvae of several marine fish species and penaeid shrimp, and zooplankton.

Bivalve mollusks

Intensive rearing of bivalves depend on the production of live algae, which comprises on average 30% of the operating costs in a bivalve hatchery. The relative algal requirements of the various stages of the bivalve culture process depend on whether the operation aims at the mass-production of larvae for remote setting or growing millions of seed till planting size.

Penaeid shrimp

Algae are added during the non-feeding nauplius stage so that algae are available immediately upon molting into the protozoea stage. Algal species most often used are *Tetraselmis chui*, *Chaetoceros gracilis*, and *Skeletonema costatum*. As feeding preference changes from primarily herbivorous to carnivorous during the mysis stages, the quantity of algae is reduced. Nevertheless, a background level of algae is maintained as this may stabilize water quality.

Marine fish

Apart from the requirement for micro-algae for culturing and/or enriching live prey organisms such as *Artemia* and rotifers, algae are often used directly in the tanks for

rearing marine fish larvae. This "green water technique" is part of the commonly applied techniques for rearing larvae of gilthead seabream *Sparus aurata* (50,000 cells $ml^{-1}$of *Isochrysis* sp.+ 400,000 cells.ml^{-1} of *Chlorella* sp. per day), milkfish *Chanos chanos* (between 500 and 3,500 *Chlorella* cells.ml^{-1} are added from hatching till day 21), Mahimahi *Coryphaena hippurus* (200,000 cells.ml^{-1} of either *Chaetoceros gracilis*, *Tetraselmis chui*, or *Chlorella* sp.), halibut *Hippoglossus hippoglossus* (*Tetraselmis* sp.), and turbot *Scophthalmus maximus* (60,000 cells.ml^{-1} of *Tetraselmis* sp. or 130,000 cells.ml^{-1} of *I. galbana*).

The effects of the presence of micro-algae in the larval rearing tank are still not fully understood and include:

- Stabilizing the water quality in static rearing systems (remove metabolic by-products, produce oxygen)
- A direct food source through active uptake by the larvae with the polysaccharides present in the algal cell walls possibly stimulating the non-specific immune system in the larvae;
- An indirect source of nutrients for fish larvae through the live feed (i.e. by maintaining the nutritional value of the live prey organisms in the tank);
- Increasing feeding incidence by enhancing visual contrast and light dispersion, and Microbial control by algal exudates in tank water and/or larval gut.

Replacement diets for live algae

The high costs associated with algal production, the risks for contamination, and temporal variations in the algal food value still pose problems for any aquaculture operation depending on the mass-cultures of unicellular algae. In order to overcome or reduce the problems and limitations associated with algal cultures, various investigators have attempted to replace algae by using artificial diets either as a supplement or as the main food source. Different approaches are being applied to reduce the need for on-site

algal production, including the use of preserved algae, micro-encapsulated diets, and yeast-based feeds.

Preserved algae:

A possible alternative to on-site algal culture could be the distribution of preserved algae that are produced at relatively low cost in a large facility under optimal climatological conditions and using the most cost-effective production systems. Centrifugation of algae into a paste form and subsequent refrigeration until required is widely applied in North America by oyster hatcheries using remote setting techniques. However, the limited shelf-life and/or the high prices of the presently available algal pastes (US$ 200 or more per kg dry weight) have discouraged many growers from using them.

Micro-encapsulated diets:

Through micro-encapsulation techniques dietary ingredients can be encapsulated within digestible capsules and delivered to suspension-feeders without losses of nutrients to the aqueous medium. Possible problems arising from the use of micro particulate feeds include settling, clumping and bacterial degradation of the particles, leaching of nutrients, and low digestibility of the cell wall material. In this regard, low susceptibility to bacterial attack and high digestibility for the filter-feeder may be conflicting requirements for a capsule wall.

Yeast-based diets:

Because of their suitable particle size and high stability in the water column yeasts can easily be removed from suspension and ingested by filter-feeding organisms. Furthermore, as opposed to most of the other alternatives to live algae, yeasts can be mass-produced at a relatively low cost. The potential of yeasts as a food in aquaculture has been proven by their successful application in the rearing of rotifers and some species of penaeid shrimp. However, a limited nutritional value of yeasts was reported for various species of filter feeders and attributed to their nutritionally deficient composition and/or undigestible cell wall. Despite this, the nutritional value and digestibility of yeast-

based diets can be improved through the addition of limiting essential nutrients and the chemical treatment of the yeast cell wall, respectively. In this way, about 50% of the algae can be substituted by yeast-based diets with minimal effects on the growth of juvenile hard clam, *Mercenaria mercenaria* (Coutteau *et al.*, 1994).

Zooplankton

Rotifers

The Rotifera (="wheel bearers") are a group of tiny animals first observed by early microscopists in the late 1600s. They are relatively small group of minute, unsegmented, pseudocoelomate, aquatic invertebrates with bilateral symmetry. Around 2,000 rotifer species have been described. Most species are in the range of 0.1 to 1 mm in length, although a few species may reach 2 to 3 mm. Different rotifer species display a striking variety of body forms and the morphology of individuals may be further altered (e.g, by growth of spines) in response to ecological cues indicating the presence in the environment of particular types of prey or predators (Wallace and Snell, 1991). Most rotifers are solitary, but there are a small number of colonial (mostly sessile, i.e. attached to the substrate) species. Although there are both solitary and colonial sessile rotifers, most rotifers are motile and very active (Brusca and Brusca, 2003).

Importance in mariculture

Rotifers are best suitable for early life stages of fish and methodologies have been devised for reliable supply of rotifers in large quantities. High numbers of rotifers, easily reaching several billions, may be required each day for raising fish larvae in commercial hatcheries. The amount needed ranges from 20000 to 100000 rotifers per fish larvae during the 20 – 30 days of culture.

Table 3. Characteristics of rotifers (Stottrup and McEvoy, 2003)

Size	90 – 350 µm
Body shape	Round and flat without spines
Distribution in water column and swimming	Usually planktonic and relatively slow growing
Density	Tolerance of high densities
Salinity	Tolerance to a wide range of salinities
Supply	Manipulated and regulated; reliable depending on culture facilities
Nutritional quality	Can be manipulated and regulated
Digestibility	Lorica and eggs not digested
Transmission of parasites and predators of fish larvae	Minimal
Transmission of therapeutic agents and probiotics	Feasible

Major fish species produced today using rotifers during the early developmental stages include yellowtail, red sea bream, Asian seabass, turbot, mullet, gilthead sea bream and European sea bass (FAO, 1998). Rotifers are also used as food for culturing penaeid shrimp (Samocha et. al., 1989) and crabs (Keenan and Blackshaw, 1999). Rotifers serve as "living capsule" providing the nutrients required by the cultured marine fish larvae for proper development. Research on *B. plicatilis* and *B. rotundiformis* has increased enormously during the past three decades and these two species are the best-studied rotifers so far. Rotifers will probably maintain their role as food organism for fish larvae, in spite of attempts to replace them with more accessible formulated food.

The rotifer *Brachionus rotundiformis* can be mass cultivated in large quantities and is an important live feed in aquaculture. This rotifer is commonly offered to larvae during the first 7–30 days of exogenous feeding. Variation in prey density affects larval fish feeding rates, rations, activity, evacuation time, growth rates and growth efficiencies. *B. rotundiformis* can be supplied at the food concentrations required for meeting larval metabolic demands and yielding high survival rates. Live food may enhance the digestive processes of larval predators. Larvae are first fed on a small strain of rotifers, and as larvae increase in size, a larger strain of rotifers is introduced. Rotifers are regarded as living food capsules for transferring nutrients to fish larvae. These nutrients include highly unsaturated fatty acids (mainly 20: 5 n−3 and 22: 6 n−3) essential for survival of marine fish larvae. In addition, rotifers treated with antibiotics may promote higher survival rates. The possibility of preserving live rotifers at low temperatures or through their resting eggs has been investigated.

An investigation of body size variability among 13 strains of the rotifer *Brachionus plicatilis* was conducted under controlled laboratory conditions. Lorica lengths ranged from 123 to 292 µm and lorica widths from 114 to 199 µm. An 85% increase in lorica length was recorded as females grew from birth to adulthood. Manipulation of rotifer size by culture conditions was investigated for various salinities, diets, and temperatures. In extreme cases, diet and salinity produced a 15% and 11% change in lorica length, respectively.

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Copepods are the most important group of zooplankton which forms the natural food for many fishes and invertebrates. Copepods, even the newly hatched nauplii are nutritious, rich in PUFA, DHA and EPA, in most desirable ratios (Watanabe et al., 1978, 1983; Sargent,1986; Watanabe and Kiron, 1994; Sargent et. al., 1997; Stottrup, 2000, 2006), easily digestible (Pederson, 1984; Stottrup, 2000) and rich in antioxidants, astaxanthine, vitamin C, D & E (Va der Meeren, 1991; McKinnon et. al., 2003). Copepods if fed during the larval phase, reduces malpigmentation and deformity rates, increases the pigmentation and survival (Bell et. al., 1997, Bell, 1998, Stottrup, 2000; Hamre et. al., 2005). Copepods are successfully cultured in fin fish hatcheries of many countries including India, especially for feeding atresial larvae of certain fishes like groupers. Mostly species belonging to the orders Calanoida, Cyclopoida and Harpacticoida are popular for hatchery production as live feed. Species belonging to the genera, *Acartia, Calanus, Temora, Paracalanus, Pseudodiaptomus, Pseudocalanus, Centropages, Eurytemora, Euterpina, Tigriopus, Tisbe, Oithona* and *Apocyclopus* are widely cultured for hatchery use (Stottrup and McEvoy, 2003; Stottrup, 2006). Many copepod nauplii are less than 100µ in size. Alone or as a supplement, in many cases copepods showed to improved primary growth than rotifers and brine shrimps. The smaller size of copepods enables their feeding by mouth gap-limited fish larvae like that of groupers and snappers (Fukuhara, 1989; Doi et. al., 1994).

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Microalgae culture media and glass ware

Biji Xavier, Sekar Megarajan and Vamsi B

Introduction

Microalgae culture with desired species of flagellates or diatoms is the basis of any finfish or shell fish larval rearing and culture system. Natural sea water is a complex culture medium containing more elements and organic compounds, and which supports mixture of all phytoplankton and zooplankton. But in the laboratory culture, monospecies culture of micro algae is being maintained to support different culture requirements. Natural sea water with added nutrients is recommended, because direct sea water may not provide the optimum nutritional requirement of specific algae. So enrichment of natural sea water is necessary with the addition of macro nutrients, micro nutrients, trace elements and vitamins. Each micro alga needs the specific culture media, with basic nutrients like nitrogen, phosphorous, vitamins and trace metals for better growth and multiplication.

Importance of Culture Medium

Algal nutrient solutions or culture or growth medium are made up of mixture of chemical salts and water. The culture medium provides the material needed for the growth of algae. These nutrients solutions are formulated specifically for its use in aquatic environments and their consistency is more precise is for laboratory culture.The culture medium constituted with the addition n of macronutrients, micro nutrients and vitamins. Micro nutrients include nitrate, phosphate and silicate. Micro nutrients contain various trace metals. Vitamins like thiamin (B1), cyanocobalamin (B12) and sometimes biotin are commonly required for the growth of most of the micro algae.

Macronutrients (Nitrogen, Phosphorus and Silicon)

Nitrogen and phosphorus are the important macronutrients, for the growth and metabolism of algal cells, which are added to the culture medium as Nitrate ($NaNO_3$ /KNO_3) and phosphate (NaHPO4.H2O).. Nitrogen is key element for the formation of protein and nucleic acids accounts to 7-20% of micro algal cell dry weight. Phosphorous play its major role in the formation of energy carrier molecule (ATP) forms 1% dry weight of the algae. Algae requires inorganic carbon source in the form of CO2, carbonate or bicarbonate for its photosynthesis.Silicate is necessary for the cell wall development of diatoms and is added in sodium silicate form (Na_2SiO_3 $9H_2O$) to the culture medium.

Micronutrients (Fe, Mn, Co, Zn, Cu and Ni)

Micro nutrients are trace metals which are present in algal cells in extremely small quantities (<4ppm), which are essential for the physiological growth of algae. Iron (Fe,) Manganese (Mn), Cobalt (Co), Zinc (Zn), Copper (Cu) and Nickel (Ni) are the most important trace metals required by algae for the various metabolic functions. Deficiencies in trace metals may lead to the slow algal growth and excess concentration may inhibit the growth, impair photosynthesis and finally damage the cell membrane of the algae. Typical trace metal stock solutions may consist of chloride or sulphate salts of zinc, cobalt, manganese, selenium, and nickel, and they are kept in a solution containing the chelator EDTA. Iron is an important trace metal required for the algae for its normal growth, photosynthesis and respiration. Iron is usually kept as a separate solution, may be added as ferric chloride or ferrous sulphate. EDTA is used as chelator and is available as disodium salt ($Na_2EDTA.2H_2O$) that is readily soluble in water.

Vitamins

Vitamins are organic micronutrients, which are essential for photosynthetic microalgae. Algal species require different combination of vitamins, mostly Vitamin B_{12} (Cyanocobalamin), Vitamin B_1(Thiamine) and Vitamin B_7 (Biotin). The general order of

vitamin requirements for algae is vitamin B_{12} > thiamine > biotin. Vitamins are normally added aseptically (through a 0.22-mm filter) after the medium has been autoclaved.

Some key aspects in medium preparation

In general, chemicals required for the microalgal culture media preparation are available from various chemical suppliers. Reagent grade salts from Merck showed good performance in the algal culture. The organic chemicals such as vitamins, buffer and chelators available from Sigma Chemical Company is better to use compared to others. Whenever the shortage of chemicals from the particular company occurs that also need to be taken care of seriously. Nutrients come with different salts and hydration, (copper and zinc) may be available as $CuSO_4$ or $CuCl_2$ and $ZnSO_4$ or $ZnCl_2$. Some nutrients also come with different hydrations (NH_2O). Substituting one form wth other due unavailability or shortage also may lead to poor growth or no growth of micro algae. Thus the change in chemical form and also different hydaration can lead to precipitation problems of the salts in the culture medium. Therefore the chemicals with correct form and correct recipes only will lead to a successful micro algal culture.

Stock solutions

Stock solutions are made with accurate weighing of the chemicals in the specific culture media, dissolved in the specific volume of distilled water. Some chemicals (EDTA) may need heat treatment to dissolve completely in the water, otherwise which may lead to unnecessary precipitation of the nutrients in the medium. But, vitamin stocks should be prepared with normal distilled water and should not be exposed to any heat treatments. The vitamin stokes are advised to keep in dark bottles. There are two terms used in stock preparation, as working stock and primary stocks. Working stocks are the small quantity (aliquot) of solution which are directly used for the preparation of final medium and Primary stocks are formed from several single substance solution and finally combined to form the working stock.

Water sources, treatment and storage

Successful micro algal culture needs good quality natural sea water free from pollution. The enrichment of the sea water can be done for specific algal species with the addition of nutrients trace metals and vitamins. The sea water from off shore area can be passed through slow sand filter in order to remove turbidity and pathogenic organisms through various biological and chemical processes. Furthur, the dissolved organic matter can be removed with high intensity ultra violet light. The UV sterilized water is stored and used for the regular micralgal culture. Furthur, chemical sterilization of the sea water using autoclave is practiced for the micro algal culture. Sometimes salinity of the sea water also needs to be adjusted with specific algal species. In general, sea water salinity varies from 32-35ppt and most of the algae grow with that salinity. But some algae species require low salinity; in that case salinity must be decreased by adding deionised water before the addition of any nutrients, trace metals to avoid dilution of these compounds.

Culture media recipes

The selection of culture media mainly depend on the type algae species cultured. Diatoms like *chaetoceros, skeletonema, Thalassiosera, tetraselmis* etc., need silicates for the formation of silicious cell wall in addition to nitrate, phosphate, trace metals and vitamins . Diatoms and nanoplankters performed better growth with Media like Erd-Schreiber's (Table 1)and Miquel's media (Miquel, 1892) (Table 2). Schreiber’s medium (modified serial dilution culture method) also available with the addition of some chaelators and vitamins along with basic Schreiber’s medium for the various micro algal culture.

Table: 1 Composition of Schreiber's medium

Potassium nitrate	0.1g
Sodium orthophosphate	0.02g
Soil Extract	50ml
Filtered and sterilized seawater	1L.

Soil extract is prepared by boiling garden soil (1kg in 1L freshwater) for one hour. Keep it overnight and decant the clear water and kept it in a bottle. 50ml of this extract is added to each litre of sterilized sea water. This media can be used as medium for isolating the micro algae.

Table 2. Composition of Miquel's medium

A	Pottassium Nitrate	20.2g
	Distilled water	100ml
B	Sodium orthophosphate	4g
	Calcium Chloride	2g
	Ferric Chloride	2g
	Hydrochloric acid	2ml
	Distilled Water	100ml

Table 3. Schreiber's medium (modified serial dilution culture method)

Potassium nitrate (5g in 100ml of DW)	0.25ml
Sodium orthophosphate (1g in 100ml)	0.25ml
EDTA (1.2g in 100ml	0.15ml
Vitamin Mixture (Thiamine,-200mg Biotin-1mg, Cyanocobalamin 1mg in 1L DW)	0.50ml
Soil extract	3ml
Sterilized seawater	250ml

The medium is autoclaved at 800C for 15 minutes, then cooled down to room temperature in running water. Vitamin mixture should be added after cooling the medium.

Most of the culture media for micro algal culture are composed of chemicals, trace metals and vitamins. Most commonly used culture media used for stock culture and mass culture of micro algae in the laboratory is **'Conway' or 'Walne's medium**

(Walne,1974) (Table 4). Mainly used for indoor culture of *Nannocloro*psis, *Chlorella, Diatoms like Chaetoceros, Skeletonema, halassiosera,Tetraselmis.*

Table 4. Composition of Conway / Walne's medium:

Solution (A)		
1.	Potassium Nitrate (KNO_3)	100g
2.	Sodium di-hydrogen orthophosphate ($NaH_2PO_4.2H_2O$)	20g
3.	EDTA di-sodium salt (Na_2EDTA)	45g
4.	Boric Acid (H_3BO_3)	33.4g
5.	Ferric Chloride ($FeCl_3$)	1.3g
6.	Manganous Chloride ($MnCl_2.2H_2O$)	0.36g
7.	Distilled Water	1L
Solution (B)		
1.	Zinc Chloride ($ZnCl_2$)	4.2g
2.	Cobalt Chloride ($CoCl_2.6H_2O$)	4.0g
3.	Copper Sulphate ($CuSO_4.5H_2O$)	4.0g
4.	Ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$)	1.8g
5.	Distilled Water	1L
6.	Concentrated HCl	
Solution (C)		
1.	Vitamin B_1 (Thiamine)	2g
2.	Vitamin B_{12} (Cyanocobalamin)	100mg
3.	Distilled Water	1L
Solution (D)		
1.	Sodium silicate ($Na_2SiO_3.5H_2O$)	40ml
2.	Distilled water	1L

Stock culture: only autoclaved seawater should be used.

Working Solution for mass culture: Add 1ml of each Solution A, 0.5ml of B, 0.1 ml of C and 1ml of D into 1 L sea water. D: Only for Diatoms (*Chaetoceros*)

Fig. 1. Conway medium

F/2 medium (Guillard, R. R. and Ryther, J. H. 1962) is widely used enriched seawater medium designed for growing marine algae culture. Commonly used for the indoor culture of *Isochrysis* and outdoor culture for the *Nannocloro*psis, *Chlorella*, *Diatoms* like *Chaetoceros, Skeletonema,Thalassiosera,Tetraselmis etc.*

Fig. 2. F/2 medium

Table 5. Composition of Guillard's F/2 media used for micro algal culture

Solution (A)			
1	Sodium nitrate ($NaNO_3$)	75g	1 L distilled water
2.	Sodium phosphate ($NaH_2PO_4.H_2O$)	5g	
P.S.Solution*			
1	Copper Sulphate ($CuSO_4.5H_2O$)	10g	1 L distilled water
2	Zinc Sulphate ($ZnSO_4.7H_2O$)	22g	1 L distilled water
3	Cobalt Chloride($CoCl_2.6H_2O$)	10g	1 L distilled water
4	Manganous Chloride ($MnCl_2.4H_2O$)	180g	1 L distilled water
5	Sodium Molybdate ($Na_2MoO_4.2H_2O$)	6g	1 L distilled water
Prepare each solution separately in 1L bottles			
Solution (B)**			
1	EDTA di-sodium salt (Na_2EDTA)	4.36g	1 L distilled water
2	Ferric Chloride ($FeCl_3.6H_2O$)	3.15g	
Add 1ml of each solution (P.S solution1-5) each to 1 L of EDTA & $FeCl_3$ mixed solution			
Solution (C)***			
1	Thiamin HCl	20g	1 L distilled water
2	Biotin	100mg	1 L distilled water
3	Cyanocobalamine (B_{12})	100mg	1 L distilled water
Add 5ml of each solution into 1L of sea water			
Solution (D)****			
1	Sodium Silicate ($Na_2SiO_3.9H_2O$)	35g	1 L distilled water
Stock culture: only autoclaved seawater should be used. *P.S. Solution: Each solution 1L should be prepared separately in different bottles **Solution B: 1L of EDTA & $FeCl_3$ mixed solution with P.S. solution (1ml of each) *** Solution C: 1L of sea water with Thiamin, Biotin and Cyanacobalamin solution (5ml each) **** Solution D: only for Diatoms (*Chaetoceros*)			
Working Solution for mass culture: Add 1ml of each Solution A, I C and D into 1 L sea water. D: Only for *Chaetoceros*			

For the mass culture of micro algae media named TMRL and PM (Gopinathan, 1982), is reported to be effective. So many media are available to culture the algae, but the exact requiremtnt during each growth stages need to be studied in detail.

Table 6. Composition of TMRL medium 100ml (Tung Kang Marine Res. Lab)

Potassium nitrate	10g
Sodium orthophosphate	1g
Ferric Chloride	0.3g
Sodium Silicate	0.1g

Glass wares used in media preparation

Reagent bottles (250 ml, 500 ml, 1000 ml & 2000 ml), culture tubes/test tubes (20 ml), conical flasks (100 ml, 250 ml, 2000 ml & 3000 ml), Haufkin culture flasks (3000 ml & 4000 ml). etc. In general Borosilicate glass ware should be used exclusively for all glassware, including stock bottles, beakers, test tubes and flasks. Teflon or plastics wares are also recommended, because they will reduce the breakage. Manufacturer's specifications for the particular glass ware usage such as storage for concentrated solutions and autoclaving also need to be considered. The glass wares and plastic wares used for culture medium preparation should keep separately from general purpose laboratory use. New glass wares and plastic wares need to be degreased with dilute NaOH, soaked in dilute HCL and then soaked in deionised water for several days before use. Glass wares should be autoclaved and cleaned glass wares and plastic wares should be stored in closed cupboards, and open vessels should be covered. Tubing used to siphon water from one bottle to another also should be cleaned properly. All containers used for culture and media stocks should be carefully selected to avoid toxic compounds. For general culture purpose, borosilicate glass wares and tissue culture grade polycarbonate or poly propylene plastic wares are recommended. Other accessories include,

micropipette, dropper with teats, tissue paper, copper wire /inoculation loop, spirit lamp, aluminum foil etc.

Cleaning of glass wares

The glass wares for the isolation, laboratory culture, maintenance and mass culture should be cleaned thoroughly prior to sterilization. The glass wares used for the indoor culture need to be cleaned with chromic acid. For outdoor culture of micro algae, the carboys used are cleaned by common salt and rinsing with tap water 4-5 times and keep for sun drying.

Preparation of Chromic acid:

5g of Potassium chromate partly dissolved in 5ml distilled water. Add 100 ml of Conc. H_2SO_4 with stirring and cooling and maintain as stock solution.

Chromic acid working solution:

Add 500 ml of the stock solution to 100 L of tap water.

Fig. 3. Cleaning of glass wares

The cleaning procedure is:

1. Rinse the glass wares with tap water.
2. Clean with brush and rinse again with tap water
3. Fill the glass wares with chromic acid (working solution) and keep it for minimum1 hr

4. Empty the Glass wares and rinse it with tap water 3-4 times
5. Drain out the water and keep the glass ware in the oven for drying.

Equipments in Micro algal culture

Analytical and top-loading balances	To weigh the chemicals accurately
p^H meter	To check the p^H of the culture media
Hot plate magnetic stirrer	To dissolve the chemicals in media completely
UV Filtration Unit	UV Filtered sea water is pre requisite for all micro algal culture.
Autoclave	Sterilization of UV filtered sea water using autoclave is necessary to prevent further contamination in the growing algal culture.
Hot Air Oven	Cleaned glassware and accessories for the inoculation of micro algae need to be sterilized with dry heat generated from Hot air oven.
Microscope	Identification of the micro-algae as well as for the determination of cell concentration of the culture, a good microscope is required. Since the flagellates are identified by noting the number of flagellae and other cell characteristics, a powerful microscope is advisable.
Haemocytometer	Counting chamber used to determine the cell counts in the micro algal culture.
Camera	Photographs of the cells can be documented

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Physico-chemical parameters for Micro algal culture

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Introduction

Marine microalgae are unicellular in nature, which are the primary producers of the sea. Among microalgae, green algae, flagellate and diatom species, are the primary producers at the base of the marine food chain. It's an important source of nutrition and is used widely in the aquaculture of other aquatic organisms like finfish and shellfish, etc., either directly or as an added source of basic nutrients. They are cultured in hatcheries for larval rearing of molluscs, crustaceans and fish as a source of nutrition. In hatchery systems, micro algae is also added to the larval rearing tanks to improve 'quality' of water. The production of micro algae is very critical in successful hatchery management. Suitably treated seawater enriched with nutrients, like nitrates, phosphates, essential trace elements, vitamins and carbon dioxide is a prerequisite for any successful algal culture. High micro algal biomass with low bacteria content is important to support the growth of finfish or shellfish larvae. In this context Physio-chemical parameters and its management in algal culture systems plays a vital role in enhancing the survival, growth and production of cultivable animals.

Parameters regulating algal growth

The most important parameters regulating algal growth are

1. Light
2. Temperature
3. Salinity
4. p^H and
5. Aeration and mixing

All these factors are interdependent and a parameter that is suitable for one type of algae is not necessarily suitable for another type of algae.

Table 1: Optimal conditions for culture of micro algae (modified Anonymous, 1991).

Parameters	Range	Optimum
Temperature (°C)	16-27	18-24
Salinity (g L^{-1})	12-40	20-24
Light intensity (Lux)	000-10000	2500-5000
Photoperiod Hrs (light: dark)	16:8 (min.)	24:0 (max.)
p^H	7-9	8.0-8.5

Light

It is one of the important factors for successful microalgae culture, but the requirements vary with the species, culture depth and the density of the algal culture. For maintaining the stock cultures of all micro-algae 500 Lux (one tube light) is essential while for the mass culture containers 2000 - 10000 Lux is necessary.

Fig. 1. Illumination in indoor algal culture

The light intensity must be increased and should be sufficient enough to penetrate through the culture, if cultured at higher depths, large volumes and higher cell concentrations (Example: Erlenmeyer flasks - 2000, Larger volumes - 5000-10000 Lux). Fluorescent white lights are mostly used in Indoor microalgal culture facilities, which can provide 2500 Lux, while outdoor systems and greenhouses gets ambient sunlight, if

needed fluorescent lights are used. Most of the flagellates require less light during the stationary and declining phase. Too much of light intensity will cause early declining of the culture. Fluorescent tubes should be preferred as these are the most active portions of the light spectrum for photosynthesis. The duration of artificial illumination should be 16 h (min.) of light per day, although cultivated phytoplankton develops normally under constant illumination.

Temperature

The optimal temperature for micro-algae cultures will vary with species and is generally between 20 and 24°C. Most commonly cultured species of micro-algae from tropical/subtropical regions tolerate temperatures between 16 and 27°C. This may vary with the composition of the culture medium, the species and strain cultured. Temperatures lesser than 16°C will slow down growth, whereas those more than 35°C are lethal for a number of species. If necessary, algal cultures can be cooled by a flow of cold water over the surface of the culture vessel or by controlling the air temperature with air - conditioning units.

Salinity

Marine phytoplanktons are extremely tolerant to changes in salinity. Most species grow best at a salinity that is slightly lower than that of their native habitat, which is obtained by diluting seawater with freshwater. Salinities of 20-24 g L^{-1} have been found to be optimal. But the salinity suitable for one algae may not be suitable for the other.

p^H

The p^H range for most cultured algal species is between 7 and 9, with the optimum range being 8.0-8.5. Changes in the p^H and culture condition due to precipitation of certain nutrients may lead to complete culture collapse. Reviving of the cultures affected by p^H changes can be accomplished by aerating the culture. Carbon dioxide plays a dual role in microalgal culture.

Table 2: Temperature, light, and salinity range for culturing selected microalgae species.

Species	Temperature (°C)	Light (Lux)	Salinity (ppt – ‰)
Chaetoceros calcitrans	25 - 30	2000-10000	20 - 35
Isochrysis galbana	25 -30	2000-10000	10 – 30
Skeletonema costatum	10 - 27	2500-5000	15 – 30
Nannochloropsis oculata	20 - 30	2500-8000	12 - 30
Pavlova sp	15 -30	4000-8000	10 – 40
Tetraselmis sp	20 -28	5000-10000	20 - 40
Chlorella sp	10 -28	2500-5000	26 - 30
Thalassiosira sp	25 - 30	2000-10000	20 - 35

It provides a source of carbon to support photosynthesis, and it helps maintain p^H at optimum levels. In the case of high-density algal culture, the addition of carbon dioxide allows to correct the increased p^H, which may reach up to p^H 9 during algal growth.

Aeration and mixing

Similar to light and temperature, aeration is also important for developing and maintaining healthy cultures, as well as to enhance the exponential phase of growth of micro-algae for a few days more. Air circulation is important to avert sedimentation of the algae and thermal stratification in the culture medium. It is also necessary to ensure that all cells of the algae get sufficient light and nutrients. Moreover it helps in enhancing the gas exchange activity between the culture medium and the air. The significance of aeration is that it contains carbon source in the form of carbon dioxide from atmospheric air, which is very much essential for photosynthesis of micro algae. For high density cultures, the CO_2 originating from the air may not be sufficient for the algal growth and pure carbon dioxide may be supplemented to the air supply. CO_2 addition, moreover supports the water by buffering action against p^H changes by maintaining the CO_2/HCO_3^-

balance. Based on the scale of the culture system and type of algal species, mixing of culture media can be done by daily hand stirring (test tubes, Erlenmeyers), aeration through air blower (bags, tanks), or by utilizing motor driven paddle wheels and jet pumps (ponds). However, it should be cautioned that few of the algal species can't tolerate vigorous mixing.

Turbidity in intake water can be reduced by passing through different filtration systems. The units other than algal culture don't require CO_2 in seawater. Hydrogen sulfide content should be nil in water used for hatcheries. NH_3 and NO_2 level in hatchery water should be below 0.1 mg L^{-1} and 0.01 mg L^{-1} respectively. Dissolved oxygen content in algal culture tanks should be above 5 mg L^{-1}. Alkalinity and p^H are interrelated while maintaining p^H, alkalinity will also remain under safe limits in most cases. Generally, the total alkalinity level of 80 – 120 mg L^{-1} is maintained in hatcheries. Water quality parameters like salinity, temperature, p^H and light intensity should be checked regularly. Generally the water intake system for any hatcheries should be devoid of pesticides and other organic and inorganic pollutants. Pathogens get entry into hatcheries through improper water quality maintenance and improper water treatment systems. So an effective water treatment system is very much essential for every hatchery.

Water Treatment

The micro algal culture water should be free of suspended solids, plankton (e.g., protozoans, ciliates and other algae species), bacteria, dissolved metals, pesticides and any other unacceptably high concentrations of dissolved organic compounds (DOC). Therefore, one of the most important prerequisites in successful micro algal culture is the pretreatment of water. Various standard pretreatment methods typically available are mechanical and chemical methods, sterilization or disinfection. Moreover, the choice of treatment method should be mainly based upon the type of species cultured, volume requirements, and cost.

Mechanical Filtration

Mechanical filtration removes suspended solids, plankton and bacteria, protozoa or another species of algae, which is a serious problem for monospecific/axenic cultures of micro-algae. The type of mechanical filtration used depends on the condition of the incoming water and the volume of water to be treated.

1. Sand filters or polyester filter bags (20 to 35µm) for large quantity.
2. Cartridge filters (10, 5, 1 µm) or diatomaceous earth (DE) filters for medium quantity.
3. To remove bacteria using 0.22 or 0.45 µm membrane cartridge filters for small quantity.

Heat Sterilization

Heat sterilization of filtered seawater can be either done by autoclaving (for small volumes) at 121°C (250°F) at 15 psi for 15 minutes or by a glass-lined water heater of 500 to 1000 W submersion heater (for large volumes).

Fig. 2 Heat sterilization by autoclaving

Microwave sterilization is useful for small volumes of seawater. Nutrients can be added before microwaving since the temperature will not exceed 84 °C.

Chemical Methods

Dissolved inorganic and organic compounds (DOC), metals, pesticides, and other contaminants can prevent or retard microalgal growth, although detecting them can be complicated and costly. Activated carbon (charcoal) filtration is helpful in reducing DOC, while deionization resins are effective in removing metals and hydrocarbons. Activated carbon can be housed in a filter or a filter bag and all the water can be passed through it.

The most common and simplest method of chemical sterilization is chlorination. Mostly this type of sterilization is preferred for large volumes of atleast 4 L. An active chlorine level of 10-20 ppm in the water for 12 to 24 hours is sufficient to kill most pathogens. Filtered seawater can be sterilized (20 ppm active chlorine) with sodium hypochlorite solution in 200 ml of liquid chlorine (10% sodium hypochlorite) per 1000 liter of seawater. Sterilization occurs in a short period of time, usually 10 to 30 minutes, but a longer time without aeration (12 hours or overnight) is given for a margin of safety. Before use, neutralize the residual chlorine by adding a sodium thiosulphate solution at the rate of 1 ppm ($1g/m^3$) for every 1 ppm of chlorine left in the solution along with vigorous aeration for 2-3 hrs.

Disinfection

After the removal of suspended particulates through the mechanical filtration, disinfection of culture water can be done either through UV or ozone or by using both is found to be more effective. Ultraviolet radiation (germicidal energy) is an efficient, simple and reliable way to kill microorganisms in culture water. However, the killing power of UV is affected by turbidity/coloration of the incoming water, distance from the source, exposure time (flow rate) and species. Dosage of UV is measured as $mW\text{-}sec/m^2$. The dosage range varies from 2-230 $mW\text{-}sec/m^2$ at 254 nm. Minimum dosages vary widely for different microorganisms: 15 $mW\text{-}sec/m^2$ for most bacteria, 22 $mW\text{-}sec/m^2$ for

water-borne algae, 35 m W-sec/m^2 for bacteria/viruses, 100-330 m W-sec/m^2 for protozoans, fungi and moulds.

Fig. 3. Disinfection by Ultraviolet radiation and Ozone treatment

The ozone as a strong oxidizing agent is more effective in removing dissolved organics, pesticides, colour and nitrates. Due to unstable and highly corrosive nature of ozone, it should be handled with special materials because it gets quickly reverts back to O_2. Ozone oxidation can kill microorganisms, but for a given period of contact time; disinfection of water requires a certain dissolved ozone concentration. A residual ozone concentration of 0.1-2.0 mg L^{-1} for a period of 1-30 minutes is required to be maintained for complete disinfection. Moreover, disinfection also depends upon the target microorganism. Care should be taken since a residual level of even 0.01 mg L^{-1} can kill fish and shrimp larvae.

Mostly different hatcheries follow different types of mechanical filtration followed by Heat sterilization for indoor algal culture. For outdoor algal culture, filtration followed by Chemical methods or by disinfection with either UV or Ozonization is followed. For a complete treatment of seawater, filtration should be accompanied by either physical treatments like autoclaving, UV and ozonization or by employing chemical methods for greater degree of sterilization.

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Stock culture maintenance and mass culture of micro algae

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Introduction

Marine micro algae are found in marine systems living both in water as well as in sediment have an important role in ecological pyramid of the marine ecosystem. These are the base of the trophic web and provide energy for all successive trophic level in the marine eco system. These are single celled, chlorophyll bearing organisms uses solar energy and nutrients from water to convert it into organic matter. Out of 8 Lakhs species of different genera of algae available from marine ecosystem very few have been tapped for their use as availability of biomedical compounds and also as feed in different finfish, shellfish and molluscan hatchery. Their role is also very much vital and critical in a successful mariculture hatchery management. An alga serves as a feed for other zooplanktons and also added to the larval rearing tanks to improve quality of water as green water technology. Among microalgae, flagellate and diatom species are cultured in hatcheries in suitably treated seawater enriched with nutrients such as nitrates, phosphates, essential trace elements and vitamins. To support growth of high densities of larvae and juveniles reared in the hatchery high density of micro algal culture is essential. Considering the importance of marine algal culture, different indoor laboratories and outdoor mass culture systems being carried out in various hatcheries in world like Japan, Taiwan, China, Philippines, Indonesia as well as India.

Stock culture development and maintenance

Stock cultures of the preferred species are the basic foundation of culture. They are normally supplied as monospecific cultures without contaminations. Stock cultures are used as inoculum when required. For development of stock culture it is very much important to take up the required culture for a particular hatchery operation. There are thousands of microalgae that differ in size, color, shape, and habitat. Researchers have isolated and identified several species from natural waters that have proven to be nutritious food for growing and from them some are very much important for use in

larval rearing of fish, shrimp or molluscs. Different species of the genus mainly used are in fin fish hatchery are *Pavlova, Dicrateria, Thalassiosira, Isochrysis, Chaetoceros, Dunaliela, Nanochloropsis, Tetraselmis, Chlorella and Nannochloropsis.* Stock culture of a desired species of algae begins with a pure starter culture. It is important to collect starter culture from an established and running hatchery. Usually starter cultures transported in test tubes and inoculated in several new test tubes with a suitable media. Some of these cultures must be kept as stocks for unforeseen situations like old culture contaminated or lost etc. then again revival of the culture will restart and multiplied. The rest are used to inoculate progressively larger glasswares until there is enough culture to start mass production

To have a sustained production of algae main source is supply of good sea water free of any contaminant either chemical or biological. Sterilization of the seawater can be done by many ways like filtration, autoclaving, pasteurization, UV irradiation, chlorination, acidification or ozonization. The critical factors for a successful algal culture stock room which are to be maintained are mainly controlled light, available illumination with required lux, controlled aeration at different stages, stable temperature control, sterile sea water without any contamination with desired salinity, nutrient media for inoculation and skilled workers.

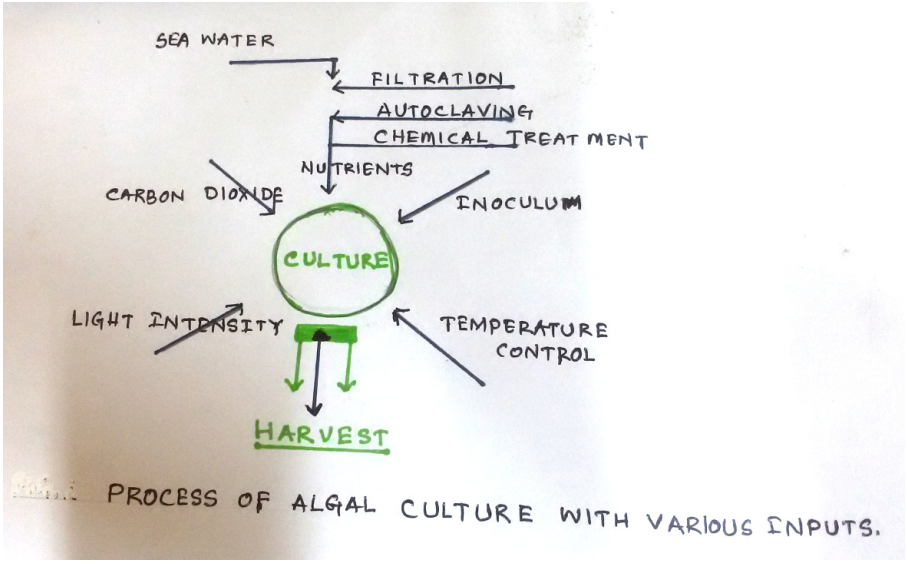


Fig. 1 . Process of Algal culture with various inputs

Always stock culture are maintained in small quantity and in gradual manner these are transferred to different containers observing the quality and density of the algae. In indoor culture it is maintained in a 10 ml test tube, then in 100 ml conical flask, then 250 ml conical flask, then 2 lt haufkin flask and finally to the 20 lt or pet jar or 50lit carboys. Care must be taken to minimize the risk of contaminating the stock and starter cultures with competing microorganisms. Each container should have half level sterilized media for inoculation of the algae and covered with cotton and gauge cloth to avoid contamination. Always harvest and transfer of algae is to be done during the peak of exponential phase so that maximal growth can be obtained.

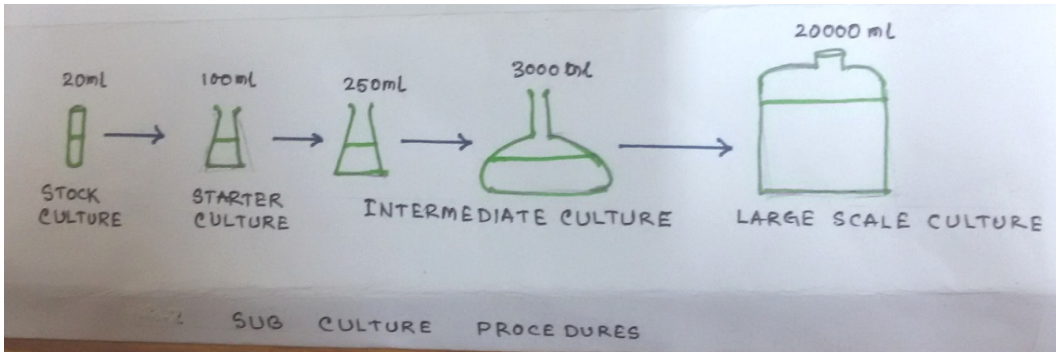


Fig. 2 . Sub culture procedures

Algal multiplication is normally dependant of various steps like sterilize the culture water, add nutrient enrichment, inoculate new culture from the pure algal strain, microscopic observations for growth of the algal counts, and multiply algae in larger containers. Finally the alga has to be supplied with a requisite quantity for outdoor mass culture. For all the above process to continue in a cyclical manner and it is very much essential to maintain different stock culture for a back up and for further use.

Control of light intensity is very much essential as less light is required during the stationary and declining phases. Too much of light will cause the culture for the early declining. For maintaining the stock cultures of all micro-algae during the declining and stationary phases of growth, light intensity of 500 lux is essential while for the

mass culture 2000-3000 lux is required. Maintenance of light and dark hours with timer control is ideal for maintaining the stock.

Since normal room temperature (28-30^{0}C) is not ideal for the maintenance and culture of micro-algae, air conditioned rooms are used for keeping the stock culture. It should have 23-25^{0}C during day time. Some of the A.Cs. can be switched off for few hours during night time to save current and maintaining temperature. All these should be done with automatic controls or timers.

Similarly, aeration is also important for developing and maintaining stock room cultures or mass outdoor culture. Aeration is very much essential to enhance the exponential phase of growth of micro-algae for required temperature. Aeration helps in many ways like the maintenance of oxygen sufficiency in the culture medium and a good agitation in the culture suspension for making the culture moving. Aeration also helps in mixing the nutrient in the culture media uniformly in the medium and preventing the algae from settling at the bottom of the culture tanks. Frequent checking of aeration pipes and decontamination with formalin also enhances the quality of stock. Also it is essential that separate person for maintaining separate culture of algae as it will reduce the chances of mixing by contamination.

Equipments required for the stock culture maintenance of microalgae

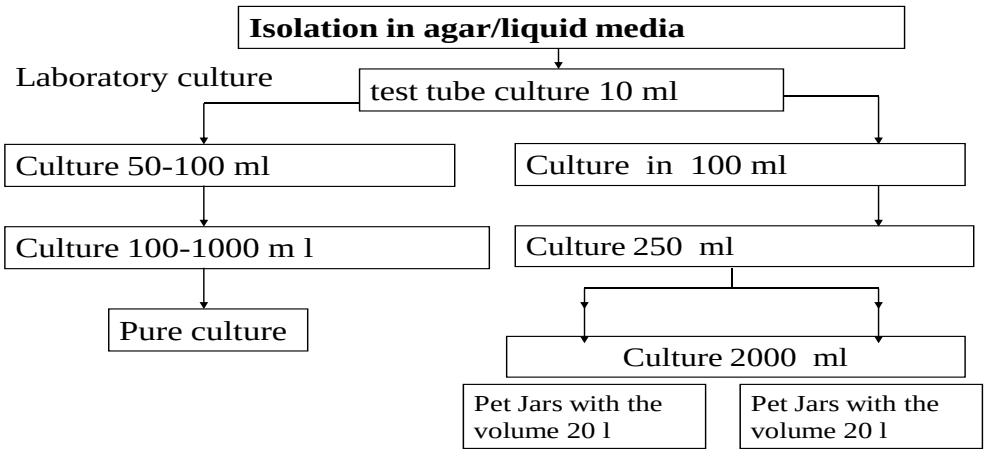
The minimal equipments required for a successful microalgal hatchery operation are numerous culture glasswares of different sizes, light, pure algal stock, seawater (or brackish water), seawater pump, blower or other aeration equipment, temperature controls or thermostats, chemicals, balance, autoclave, oven, salinometer, hydrometer, or refractometer, pH meter, sedgwick rafter or hemacytometer, microscope with digital counter with suitable magnification and most important is involvement of skilled or knowledgeable staff.

The glass wares or plastic wares for stock culture and mass culture in outdoor should be cleaned thoroughly prior to sterilization either by sun drying or by autoclaving. The glass wares used for the indoor culture need to be cleaned with chromic acid. For outdoor culture of micro algae, the carbuoys used are cleaned by

common salt and rinsing with tap water 4-5 times and keep for sun drying. The glass wares have to be kept in an oven at temperature 100 ^{0}C for an hour. The culture tubes, conical flasks and Haufkin culture flasks have to be plugged with cotton before keeping in the oven.

Procedure of inoculation

Add 10ml of the culture medium to the test tube (20ml) and inoculate the medium with single drop of the pure stock culture. Cover the mouth of the test tube with aluminium foil tightly. Keep the culture in the temperature controlled room with sufficient light intensity. Shake the culture gently everyday and observe for the culture. The culture will be observed from 5th to7 th day. The test tube culture will be served as the starter culture and precede the culture into 100 ml, 250 ml and 3000 ml conical flasks. A single test tube inoculum can be used for the inoculation of three or four culture (100 ml conical flask). The culture can be continued to 250 ml conical flask using the culture inoculum from 100 ml conical flask. Once the test tube inoculum is developed the culture will be observed on every third or fourth day depending on the quantity of inoculam was added to the culture medium. In every step the culture should be transferred aseptically without any contamination. The unicellular algae grow exponentially by subsequent multiplication of cells in suitable conditions of light, temperature and aeration.



Mass outdoor culture in 100 l transparent tanks and further in 1 ton taks

Table 1. Amount (ml/L) of medium added for the indoor culture of some specific algae

Algae	Convey medium (ml/L)				F/2 medium (ml/L)			
	A	B	C	D	A	B	C	D
Chaetoceros	-	-	-	-	1	1	1	1.5
Isochrysis	1	0.5	0.1	-	-	-	-	-
Nanochloropsis	1	0.5	0.1	-	-	-	-	
Thalasiosera	1	0.5	0.1	-	1	1	1	1.5
Skeletonema	1	0.5	0.1	-	1	1	1	1.5
Chlorella	1	0.5	0.1	-	-	-	-	-

Growth cycle of algal culture

After the inoculation of the cells with required culture medium the cells passes through various growth phases in the culture with suitable conditions of light, temperature and aeration. Cell count increases in a characteristic sigmoid fashion.

1. Lag phase or induction phase*:* After the addition of stoke culture inoculum for the subculture of micro algae, there will be a no cell division phase for few hours which is known as lag or induction phase.

2. Exponential phase: after the lag phase, the cells are acclimatized and start dividing, grow fast by utilizing nutrients, aeration and light. This growth phase is called exponential phase and reaches maximum cell concentration during this period.

3. Declining phase: After reaching the growth phase, the cells will be showing less growth or slow growth. This stunted growth stage is known as declining phase.

4. Stationary phase: The declining phase will be continuing for few days without any cell division and this period is known as stationary phase. Sometimes, the culture may start dividing the cells with suitable conditions.

5. Death phase*:* Prolonged stationary phase will lead to the death phase, where algal cells will lose their viability and the cells dies. This phase is called death phase.

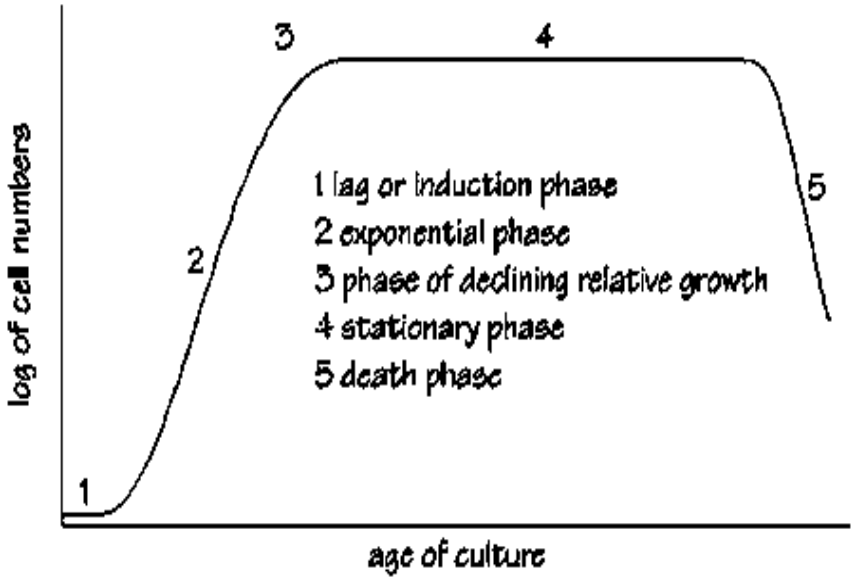


Fig. 3 . Growth cycle of algal culture

Growth slows as the algal population becomes more crowded. Nutrients are depleted, metabolites build, and light penetration decreases because of self-shading. The cultures have reached their stationary phase for the current conditions and will not increase in density. Algae harvested near this maximum density are a high quality food and provide the most efficient use of hatchery labor and space.

Fig. 4 . Stock culture and multiplication laboratory

Algal cell counting

Counting is normally done in Sedgwick Rafter Counting Chamber or Haemocytometers. Haemocytometers are thick glass slides with two chambers on the upper surface, each measuring 1.0 x 1.0 mm. A special cover slip is placed over these two chambers giving a depth of 0.1 mm making the total volume of each chamber 0.1mm^3. With the cover slip in position, one or two drops of the algal sample are introduced by means of a Pasteur pipette to fill both chambers. Cell density is estimated as follows. Haemocytometer has got 9 chambers (4 sides having 16 divisions and 5 chambers of multiple divisions) we have to restrict the counting for at least 4 chambers. Take the average number of cells in one ml.

Average counts per chamber x 10^{-4} = Total number of cells/ ml

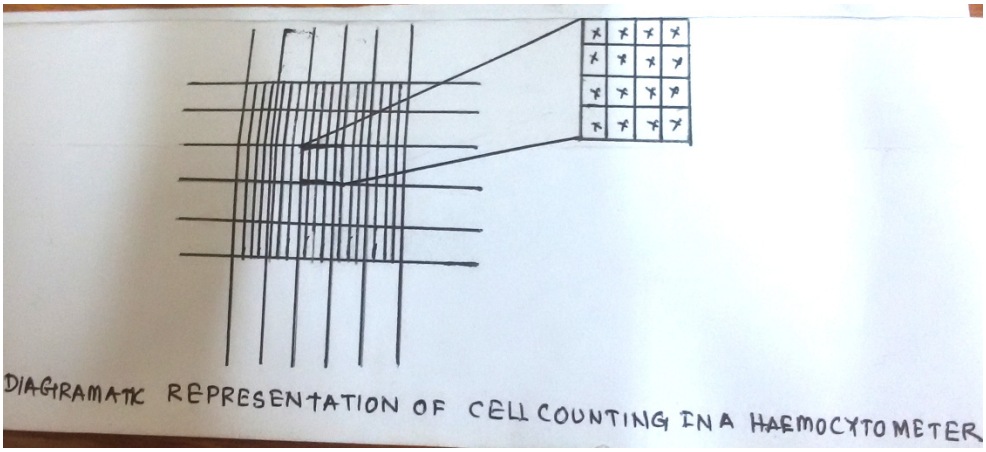


Fig. 5 . Diagramatic representation of cell counting in a haemocytometer

Mass culture of microalgae

Large scale culture of microalgae is normally taken up to obtain maximum possible yield per day, especially nannoplankton flagellates and selected species of diatoms are necessary for feeding the rearing larval forms in a hatchery. Mass culture can be done in the outdoor 100 L tanks taking the inoculum from the 20 L jar from indoor and further transferd to 1 tonne tanks. These containers will have the maximum concentration of the cells in the growing phase on the 5-6th day and can be harvested. After estimating the cell concentration, using a haemocyotometer, the culture is supplied to the outdoor algal hatchery for mass culture should have maximum concentration and purity to have good yield.

Mixed culture medium	
Potassium nitrate	1.2 g
Sodium Ortho-phosphate	0.66 g
EDTA (Na)	0.66 g
Sodium silicate	0.66 g

Dissolve the first 3 chemicals in 25 ml of dist. water and sodium silicate can be dissolved separately in 25 ml of dist. water. Add this to 100 L of fresh unfiltered seawater (freswater can filter through organdy net. 0.33 mm mesh size to remove zooplankton). Pour the water in 3-4 white lined basins or fibre-glass tanks and keep the same in open sunlight. Within 24 hrs, a slight yellow discoloration can be noted. On examination, we can notice the growth of planktonic diatoms and other nannoplankters. If the temperature is very high and sunlight is very bright. only the blooming of *Chaetoceros spp*. could be observed.

Besides the above mentioned laboratory prepared chemicals, which act as nutrients, commercial fertilizers can be used for the mass culture of micro-algae. In open tanks for economy purposes, the following fertilizing medium can be used:

Commercial fertilizer medium/ 1t	g
Urea	46
20-0-0	10
16-20-0	1

Commercial fertilizer medium/ 1t	g
Ammonium sulphate	100
Urea	10
Tripple super phosphate	10

The maximum density of algal cells in outdoor culture depends mainly on factors like atmospheric light and temperature, salinity of sea water without any contaminants suitable to the cultured species, and a good density of indoor inoculum. In outdoor culture with 100 L tanks around 10-15 million cells per ml can be achieved within 2 days of

inoculation and around 3-5 million cells per ml can be achieved in 1 ton outdoor tanks with suitable environmental conditions.

Fig. 6. Outdoor mass culture of micro algae in 100 L carboys

Fig. 7. Outdoor mass culture of micro algae in 1t FRP tanks

Harvest of the culture

The culture should be harvested during the exponential phase of the micro-algae after determining the cell concentration. If the culture has entered the declining or stationary phase, the metabolites will be very high and the cells may not be in healthy condition. The rearing larval organisms may not show the expected growth if fed with this feed. The trouble which comes frequently like air pressure, salinity, pH, culture contamination, nutrient media for culturing the algae needs immediate attention. Frequent

observation to avoid contamination is highly required as the algal feed is very much important in terms of nutrients for the other organisms like rotifers and copepods to feed and also pure culture will not contaminate while adopting the green water technology for the larval rearing system for marine fishes or shell fishes.

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Isolation and Purification of microalgae

Ritesh Ranjan, Chinnibabu B and Narsimhulu Sadhu

Introduction

Microalgae are diverse group of organisms, which found in various natural habitats. They exhibit variation in their nutritional requirements as well as metabolite production. Isolation is a necessary process to obtain pure culture of a micro algal species and it presents the first step towards the selection of desired microalgal species for culture. The sterile culture of desired microalgal species can be obtained from specialized culture collection centers. However, the isolation of endemic strain could be considered better due to their ability to grow under the local environmental conditions.

Sampling and isolation of microalgae from natural habitats is a well established procedure. Depending on their different habitats, algal strains vary in their ease of cultivation under laboratory conditions. It is important to provide culture conditions similar to their natural habitats while isolating microalgae. Microalgae are sensitive towards physiological conditions such as temperature, pH and salinity. Some microalgal species require specific nutrients, such as, diatoms need silica-supplemented media. Purification of cultures to obtain algal monocultures is another challenging step. Isolation of pure culture from its natural environment and maintenance of isolated pure cultures involve various techniques.

Sampling

The collection of microalgal samples is the first crucial step for isolation of microalgae from their natural environment. Microalgae are found in different environmental conditions and habitat such as ice, hot water springs, freshwater, rocks, saline water bodies, coastal areas, soil etc. Proper sampling technique, sampling season, habitat assessment and preservation of samples are essential factors for collection of microalgae. Different sampling techniques followed are syringe sampling, scraping,

brushing, inverted petri dish method etc. While collecting samples, it is necessary to record abiotic factors such as light, water temperature, dissolved oxygen, dissolved carbon dioxide, nutrient concentration, pH and salinity of the sample collection site. It is also important to record biotic factors such as pathogens and any competitors at the sampling site in order to mimic these conditions at the laboratory (Mutanda *et al*., 2011). Global positioning system (GPS) coordinates must be recorded for reference and re-sampling. Microalgae are often present in consortium with complex population dynamics in natural habitats, thus it becomes inevitable to isolate the strain of interest from the collected samples.

Isolation of microalgae species from collected samples is intricate because of their small cell size and the association with other epiphytic species. The isolation of the desired microalgae species can be carried out by any one or combination of the following methods:

1. Isolation of single cells
2. Serial dilution
3. Streak plating
4. Density centrifugation
5. Enrichment media
6. Micromanipulation
7. Automated Techniques

1. Isolation of single cells

Isolation of a single cell of microalgae is the technique whereby a cell is picked from the sample using a micropipette or glass capillary under microscopic observation. These single cells are transferred to sterile droplets of water or suitable media. The technique requires expertise and precision. The cells can get damaged due to shear stress caused by micropipette or capillary tips. Caution is necessary for successful

implementation of the present technique. Ultrapure droplets are required, especially for marine samples, to distinguish between microalgal cells and other particulate matters.

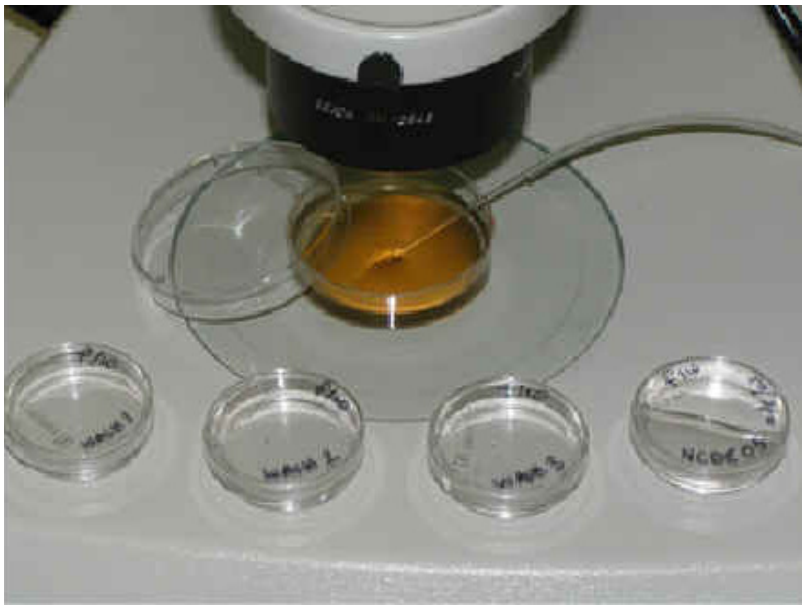
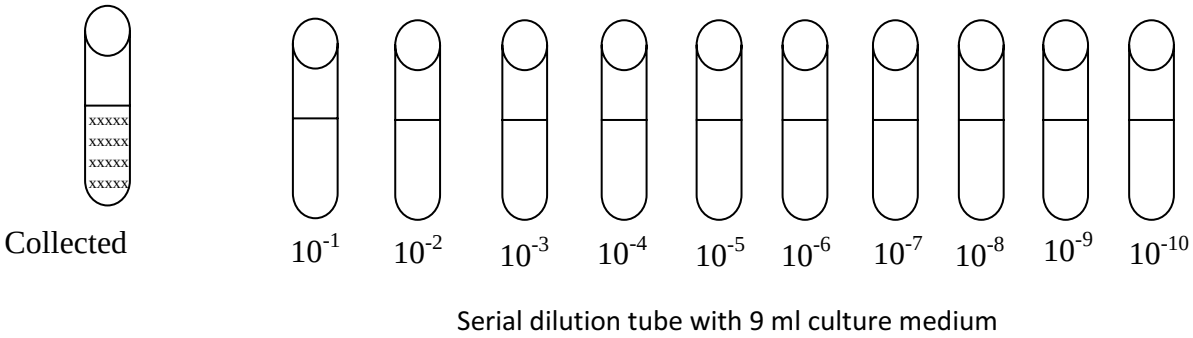


Fig. 1. Isolation of single cell with the help of microscope

2. Serial dilution

Serial dilution is the most common conventional and established method of microalgal isolation from collected samples. Serial dilution is stepwise dilution of a substance in sterile solution. The dilution factor is usually constant at each step, resulting in a geometric progression of the concentration in a logarithmic manner. A set of sterilized test tubes or flasks with nutrient media can be used for dilution. Dilution sets are determined on the basis of known number of cells in the enriched culture. Depending upon the habitat and specific requirements of desired microalgae species, the medium can be supplemented 'or' the physical environment can be set in such a way, to provide selective pressure towards the desired microalgae species. The success of the present technique is highly dependent on the accuracy of a measured amount of cell culture during transfer from one medium to another. The present method is helpful for production of algal monoculture; however these are generally not axenic cultures.



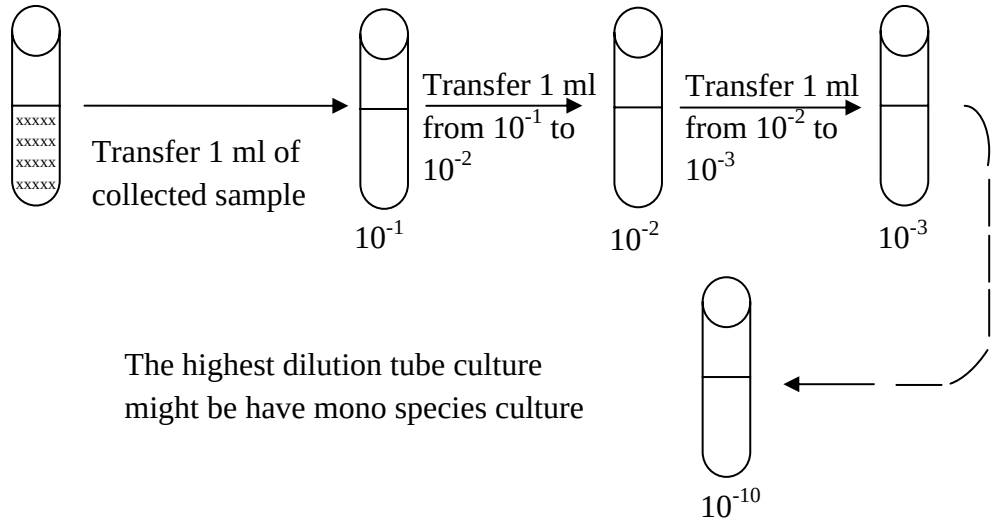


Fig. 2. and V. U. Mahesh

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3. Streak plating

Streaking is a common conventional established technique for isolation of a pure strain from a mixture of microalgae species for investigation and identification. This method requires the preparation of aseptic agar plates followed by plating of sample (0.1 ml) onto the agar surface by spread plate technique. Then the agar plates are usually incubated, providing optimum physiological conditions for the species or strain of interest, for one week followed by selecting a morphologically distinct colony from the

agar plate membrane surface with a sterile pin tool and streaking across on the agar plate. This technique is successful for most algal strains, especially coccoids, diatoms, microalgae and small cyanobacteria. The present technique is the best method to isolate axenic cultures without requiring any manipulation or modification of the technique. Agar concentration from 0.8 to 2 % is sufficient for growth of microalgae on its surface. Isolation is accomplished by streaking the independent microalgal colony on the agar surface. A loop containing a single microalgal colony is spread across the agar. After streaking agar plates are incubated, providing optimum physiological conditions for the species or strain of interest. Generally, the incubation period is 2 to 3 weeks for marine microalgae. The single cell algal strain can be further subcultured onto other agar plates.

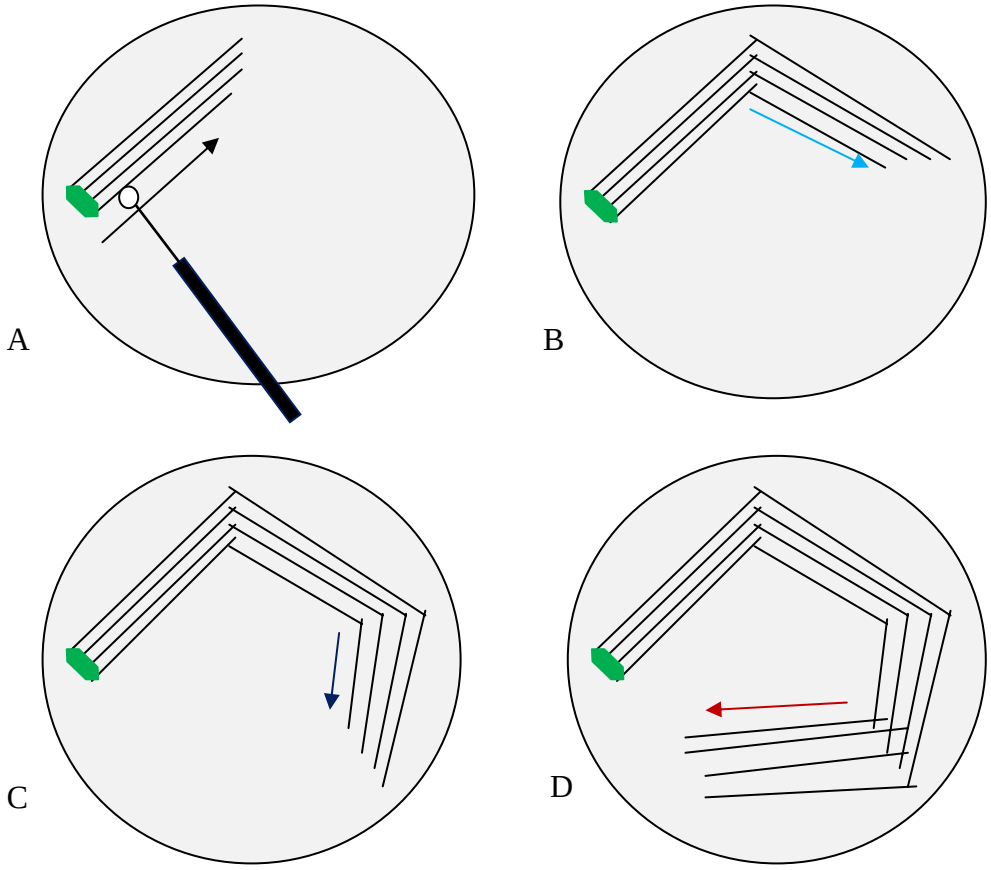

Fig. 3. Method of plate streaking technique

For preparing the agar medium, 0.8 to 2 g of agar is added to a litre of natural filtered seawater in a conical flask. The flask containing agar is usually heated on a flame and boiled twice till the agar is dissolved in water. The culture media 'or' nutrients (solution A and B of Conway media or F/2 media) are added to the agar solution. The flask mouth is usually covered with aluminium foil and then the flask is autoclaved for 15 minutes under 15 lbs pressure and 121°C temperature. The petri dishes are usually sterilized by keeping for 30 minutes at 150 ^{o}C in hot air oven. The solution C of culture media is added aseptically to the autoclaved agar flask once the temperature has brought down to 40 ^{o}C. Then, agar plates are prepared aseptically by pouring the warm autoclaved agar into the sterile petri dishes near a Bunsen flame or in a laminar flow, followed by covering up the petri dishes and leaving them to cool for about 2 h.

4. Density centrifugation

Density centrifugation applies gravity settling to separate organisms based on the cell size. This technique is usually applied to separate larger organisms from the microalgal cells. Density gradient (Example: Silica sol, Percoll) centrifugal technique separates the different species of microalgae into different bands. The present technique is primarily used to concentrate the number of cells of a desired microalgal strain. The isolation of cells of a desired microalgal strain can be achieved from the concentrated cells by use of other isolation techniques such as streaking plate or serial dilution. The caution has to be taken regarding centrifugation speed and time, which vary depending upon the target microalgal species. The delicate cells might get damaged due to shear stress generated during centrifugation.

5. Enrichment media

Nutritional requirements of microalgae species vary depending on their natural habitat and cellular physiology. The selection pressure can be used by enriching the culture medium to obtain single species. Commonly used enrichment substances include specific nutrient mediums, soil extracts, nutrients like nitrate and phosphate and trace

metals (Mutanda *et al*., 2011). Adjustment of pH is a commonly adopted strategy to obtain bacteria free cultures. Organic substances such as yeast extract, casein from various fruits and vegetable juices may also be added to the medium (Anderson, 2005). Natural habitats may be deficient in one or more nutrients required for microalgae growth. In nature, nutrients are recycled or provided by the physiological action of other organisms. Sampling reduces the recycling of nutrients and thus can cause death of microalgae. Enrichment substances are usually added in minimal quantities at sequential stages.

6. Micromanipulation

Micromanipulation is a powerful advanced microalgal isolation technique, which allows the movement and culturing of a single cell. Conventional techniques for microalgae isolation do not account for clumping and thus colony formation from a number of cells rather than a single cell (Frohlich and Konig, 2000) can be obtained. Traditionally, micromanipulation utilized capillary tube to target microscopically identified cell or cells and transfer these to sterile water or media. This is manual task that is laborious and requires skilled operator (Jacob, 2013). Modern micromanipulation utilizes a micromanipulator and stereomicroscope employing microcapillary tube or optical tweezers for cell separation with a high level of accuracy, making it an ideal tool for screening and isolation (Frohlich and Konig, 2000; Mutanda *et al*., 2011). Microcapillaries are usually made of glass with an outer diameter of 1 mm. These are melted under controlled conditions and stretched to give inner diameter of 2-10 µm (Ishoy *et al*., 2006). Optical tweezers use a focused laser to capture and move a cell of interest to a compartment from where it can be transferred to sterile media (Wright *et al*., 2007). The present technique is still in its infancy in most laboratories due to the level of skill required and time taken during the isolation process.

7. Automated Techniques

Flow cytometry coupled with fluorescence activated cell sorting (FACS) is a rapid method of microalgal isolation and purification from environmental samples (Jacob, 2013). FACS is based on light scatter and fluorescence, resulting from the passing of a thin fluid stream through one or more laser beams. Cell scatter and/or absorb the laser beam and emit fluorescence, which provides information on cell size, integrity and photosynthetic characteristics, which are closely related to morphological and photosynthetic characteristics that are utilized in conventional identification (Figueroa *et al*., 2010; La *et al*., 2012). One of the main advantages of FACS is the ability to obtain axenic cultures due to removal of bacteria (Pereira *et al*., 2011). The present method has gained popularity due to the efficiency and high throughput whereby cells in 5000 – 10000 cells per second can be characterized and sorted (Doan and Obbard, 2012). Efficiency of cell sorting is based on the original sample, especially with reference to the abundance, cell size, shape and hardiness of the algae being sorted. Greater efficiency is possible by enrichment of the culture/s prior to sorting. The technique is limited in terms of sorting of algae occurring as aggregates. This limitation may be overcome by sonication of the samples in order to disrupt cell to cell, cell to particle and particle to particle connections within microalgae communities (La *et al*., 2011). However, the sonication process is also having limitation, which might damage the algal cell.

Stock culture maintenance

The collected algal strains should be carefully protected against contamination during handling and sub-culture. To reduce risks of contamination, two series of stocks are often maintained, one which is usually used for the starter cultures for the production system and the other which is only subjected to the handling necessary for stock maintenance. Stock cultures are maintained in test tubes at a light intensity of about 1000 lux and a temperature of 16 to 19 °C. Constant illumination is usually provided for the maintenance of flagellates, but may result in decreased cell size in diatom stock cultures.

Stock cultures are maintained for about a month and then sub cultured to create a new culture stock.

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Zooplanktons for marine finfish and shellfish

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Introduction

Marine finfish seed production is gaining importance all over word in the recent years. However, seed production and culture practice is established for only limited number of marine fish species with variable success. Success of marine fin fish seed production is depends on several factors, and they directly or indirectly affect the mass rearing of marine finfish larvae, which include type of larvae feed, feed density, water quality and environmental factors. Among which, the major factor affecting the successful production of marine finfish larvae is the utilization of an appropriate larval feed during the initial feeding phase of the larval cycle. Feeding appropriate feed during initial period of larval cycle is very crucial for good survival and optimal development of marine fish larvae. Feed with the proper nutritional composition, having a suitable size range for larval mouth, and stimulating a feeding response is necessary to be an optimum feed for larvae. The commonly available zooplankton species such as rotifer, copepods and *Artemia* nauplii are considered to be most suitable feed for marine fin fish larvae (Delbos, 2009).

Selection of live feed for larval fry

The cultivation of larvae is generally carried out under controlled hatchery conditions and it requires specific culture techniques especially with respect to husbandry techniques, feeding strategies, and microbial/disease control, as the developing larvae are generally very small, extremely fragile, not physiologically fully developed and susceptible to different disease. Uncompleted development of their perception organs (i.e. eyes, chemoreceptors) and digestive system are limiting factors in selecting proper feed during the early first-feeding of the larval cycle. Therefore, while selecting live feed for initial larval feed, the size of live feed organisms and their ability to elicit a feeding response from fish larvae are important considerations for marine fish larviculture (Ohs *et al*., 2009). Moreover, mouth gape of the larvae and feeding response of the larvae to

various live feeds are species specific; and therefore these parameters should be very well identified for the candidate species to be cultured, since they will determine which live feed to be selected for their successful and continuous rearing.

Size at first feeding

The mouth size of first-feeding larvae usually mechanically restricts the size of the food particles which can be ingested by the larvae. In general, mouth size is correlated with body size, which in turn is influenced by egg diameter and the period of endogenous feeding (Ohs *et al*., 2009). In hatchery, while selecting first exogenous feed, it is important to look for the live feed size is lesser than mouth size of fish larvae. For example, the size of the mouth at first opening of humpback grouper is 180-200 µm, orange spotted grouper is 150-180 µm and silver pompano is 239 µm.

Feeding response

Ability of live feed to elicit a feeding response from fish larvae is an important consideration in marine fish larval culture. As the fish larvae has developed to feed on natural group of zooplankton, the stimuli produced by the movement of live feed organisms is needed for many marine fish larvae to elicit a feeding response (Ohs *et al*., 2009). The swimming activity of live food organisms generally assures a good distribution of food items in the water column, which in turn facilitating more frequent encounters to the developing larvae with low mobility in many cases.

Rotifers

Rotifers are one of the small sized zooplanktons and possess important characters like, swimming in slow speed, small size, tolerance to many different environmental conditions, ability to stay suspended in the water column and high reproduction rate compared to many other zooplanktons (Lubzens & Zmora, 2003). The positive and unique characters of different species of the rotifers help them to be a good prey for fish larvae during first exogenous feeding stage. They are also relatively easy to culture at high densities and can be enriched with fatty acids and antibiotics. Euryhaline rotifers, *Brachionus* spp. are currently essential for intensive culture of marine larval finfish in many hatcheries throughout the world. Only a few rotifer species in the genus

Brachionus are used in aquaculture, that are *Brachionus plicatilis* (L-strain) with a size range of 200 to 360 µm and *B. rotundiformis* (S-strain) with a size range of 150 to 220 µm.

Morphology

Rotifer's body is divided into three different parts such as head, trunk and foot. In the head the corona is found. It has an annular ciliation and is retractable, which allows them to move and makes easier the intake of small food particles by whirling water movement. The digestive tract, the excretory system and the genital organs are in the trunk region. The foot is a ring-type retractable structure without segmentation and ends in one or four toes. The body parts of a female and male from the *Brachionus plicatilis* strain is depicted in the fig 1.

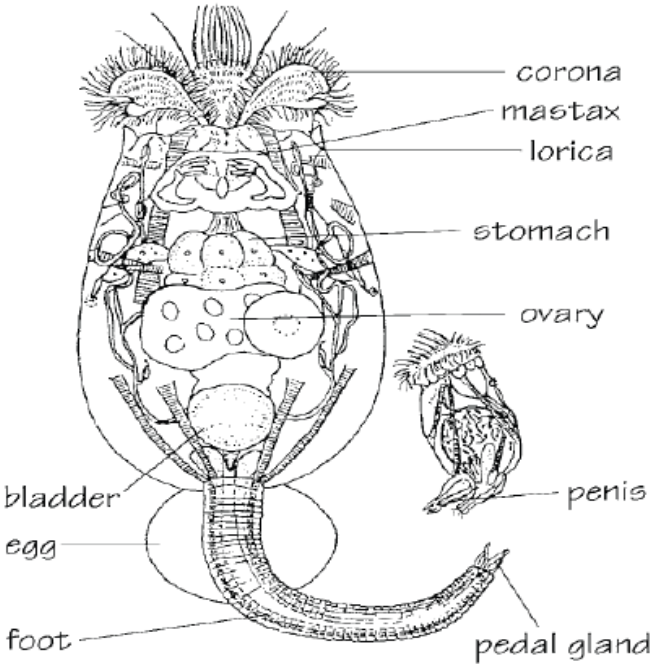

Fig. 1. Female and male of *Brachionus plicatilis* strain (from Dhert, 1996)

Life history

The life span of rotifers is depending on their reproduction cycle. Reproduction frequency of rotifer is varying according to the temperature of the culture environment for example at 25 ºC it is of about 4 - 5 days, while at a temperature of around 20–22 ºC the average life span is about 10.5 days. Generally, the larvae become adult after 0.5 to 1.5 days at 25^0C and then the females start to lay eggs approximately every four hours (Pastor, 2007). It is believed that females can produce ten generations of offspring and then eventually die. Reproduction in *Brachionus plicatilis* strain is being performed by two different modes i.e., both sexually (mictic) and asexually (amictic), depending on the conditions, environmental and also on the rotifer density of the population. During female parthenogenesis, the amictic females produce amictic (diploid, 2n chromosomes) eggs which develop and hatch into amictic females. Under specific environmental conditions the females switch to a more complicated sexual reproduction resulting in mictic and amictic females. The mictic females produce haploid (n-chromosomes) eggs. Larvae hatching out of these unfertilized mictic eggs develop into haploid males. These males are about one quarter of the size of the female, they have no digestive tract and no bladder but have single testis filled with sperm. During the mictic mode, resting eggs are produced that will only develop and hatch into amictic females, after they are exposed to specific conditions. This is probably a mechanism adopted by the rotifer to preserve the survival of the population even under unfavourable conditions.

Culture of rotifer

In a hatchery unit, the process of rotifer culture could be divided in to 4 different phases (Pastor, 2007).

1. Maintenance of stock cultures.
2. Inoculation phase: This phase is the start of new cultures based on inocula from stock cultures, or more commonly from production cultures.
3. Early growth phase: It is the critical phase when the food rations and rotifer density are increased gradually.

4. Late growth or production phase: It is the final phase when cultures are harvested.

Maintenance of stock culture

Maintenance of stock culture is important in rotifer culture, and it should be kept physically isolated from the production facility of microalgae, rotifers and other zooplanktons culture area in order to avoid contamination. Algal cultures used to feed the stock cultures of rotifers must also be free from harmful contaminants. Stock cultures of *Brachionus plicatilis* can be maintained in small units (0.1-1L). The water used must be free from contamination and sterilized. A stock culture is prepared by transferring 5-10 ml of mature stock culture to a beaker 0.1 – 1.0 L of sterilised water. The cultures can be maintained at room temperature with proper feeding. While maintenance, the stock should be renewed at particular interval to maintain the rotifer in good health. The stock cultures have to be renewed approximately once every month, or even less frequently at low temperatures. Feeding and renewal frequencies are low if the rotifers are kept in the light at low temperature (7-10 ºC) when compare to room temperature. If all the stock cultures become contaminated by other zooplankton, single rotifers should be selected carefully under the microscope, and repeatedly washed in sterilised water and then transferred to small units containing sterilised water and microalgae (Rombaut *et al*., 2003).

Rotifer culture methods

There are two general methods are followed for culturing rotifers:

1. Batch method: In this method, given volume of water is added or exchanged each day and the culture is restarted at regular intervals.
2. Continuous method: Recirculation-based technology is employed in this method to increase the density of rotifers cultured while minimizing the need to restart cultures.

Batch culture

Batch culture system normally follows a 4-5 day culture period. In batch culture method, a tank is inoculated with rotifers on day 1. The rotifers are then fed each day;

accordingly the volume of the culture is also increased to keep up with rotifer growth. Rotifer densities reached with this method is normally goes up to 500 numbers /ml. At the end of the cycle, most of the rotifers are harvested and fed to fish larvae. However, some of the rotifers are saved from the harvest for the next tank inoculation. The duration of the cycle can be extended slightly by performing regular water exchanges once a high terminal density is reached. When culture duration is increased, then 10 to 30 % of the water volume can be removed on a daily basis to keep water quality within desired parameters. However, the culture is to be restarted after particular time due to the accumulation of uneaten feed. Rotifer should be harvested after attaining maximum concentrations and while harvest 40-50 µ mesh to be used to filter the rotifer, so that the dust particle with less than 50 µ size will be removed and dust free rotifer will be collected.

Continuous culture

In continuous culture method, a supply of fertilized seawater is continuously pumped into a growth chamber and the excess culture is simultaneously washed out or harvested. This method permits the maintenance of cultures to the maximum growth rate using water recirculation. A typical rotifer recirculation system is having units like culture tank, bio filter unit, protein skimmer, algal storage tank and pumps. This system employs a standpipe with 50-55µ mesh screen located within the culture tank. The standpipe allows uneaten feed and ciliated protozoans to pass out of the culture tank when the rotifers are cultured in the tank. The waste coming out of the stand pipe travels through a biological filter and a foam fractionators (Protein skimmer) before returning back into the culture tank. Flow rates in this type of system are typically two to five tank turnovers per day through the recirculation system. Daily maintenance for this type of system involves cleaning the screened standpipe and floc traps and removal the settled materials from the bottom of the tank. In general, a daily water exchange of 20-30 % of the culture volume should be incorporated for long-term maintenance. This culture method gives production maximum of up to 1000 numbers /ml (Delbos, 2009).

Feeding of rotifers

Rotifers actively graze the water column and feeding on particles approximately 1 to 10 µm in size. Many species of microalgae such as *Nannochloropsis sp*, *Pavlova sp* and *Isochrysis sp*, etc, are good food for rotifers. In addition, there are a number of commercially available artificial feeds like yeast, algae based rations are suitable for culturing rotifers. Activated baker's yeast has been used successfully as an inexpensive grow-out diet when fed at approximately 0.5g/million rotifers. However, use of yeast along with microalgae gives better growth. It is important to note that the diet that is devoid of beneficial amino acid, fatty acids and lacks certain vitamins is need to be supplemented in order to achieve maximal culture performance of the rotifer and also to provide good survival to the fish larvae.

Fig. 2. (a): Feeding to rotifer **(b): Harvesting of rotifers**

Water quality parameters in rotifer culture

Rotifer culture environment is also play major role in maintaining proper multiplication of rotifers in culture tank. The water quality parameters such as salinity, temperature, dissolved oxygen, pH, ammonia need to be maintained and the optimum range of these parameters are follows.

Water quality parameters	Ranges
Salinity	10-35 ppt
Temperature	22-28°C
Dissolved oxygen	>4ppm
pH	7-8.5
Total Ammonia Nitrogen	≤5ppm

Important points to be considered in rotifer culture

- When starting a new culture, initial stocking densities should be ≥200 rotifers/ml of culture water. Lower stocking densities will result in delayed start-up time and may help promote the growth of unwanted contaminants.
- Rotifers need a consistent supply of free algae in the water at all times, allowing them to graze continuously. Therefore, frequent feeding need to be given in less concentration, so that feeds could be efficiently utilised and wastage also avoided.
- Batch culture method may be extended up to 6-7 days and during this period bottom should be siphoned at least once to avoid the building up of ammonia in the culture environment.
- Rotifers harvested between 4-5th day after inoculation gives maximum numbers in batch culture method.
- During the culture, counting the rotifer daily is essential for observation and enumeration. Rotifer is counted using counting cell (Sedgewick rafter cell) under the microscope. Counting is usually done with 1ml of sample collected from the particular culture tank and then the total number of rotifer for the particular tank is calculated accordingly.

Copepods

Copepods are small crustaceans, represent about 85% of the zooplankton exist in the sea, which form major component of the marine zooplankton community. They are one of most ubiquitous marine organisms with over 21000 described species. It is well documented that in the wild, copepods constitute a major link in the nutrient pathway from primary producers to marine fish larvae. Copepods are the natural food source for

many marine fish larvae, and in nature copepod nauplii consumed by marine fish larvae and adult copepods consumed by juvenile fish (Lee, 2005). The role of copepods in the marine trophic system is essential for the survival of many marine fish species. Unlike rotifers and brine shrimp, copepods are able to synthesize essential HUFAs, without enrichment, and maintain appropriate DHA: EPA and EPA: ARA ratios required by marine fish larvae. In addition, about 90% of the total fatty acids present in copepods are in the more easily used form of phospholipids. Therefore, copepods do not need to be enriched and will not lose their nutritional value quickly because of leaching or excretion. Fish larvae consume marine copepods from three main orders: Calanoida, Harpacticoida, and Cyclopoida. In which, several candidate species belonging to both the calanoid and the harpacticoid groups have been studied for mass production across the world. Calanoids groups can be easily recognized by their very long first antennae (16-26 segments), whereas the Harpacticoids have only a short first antennae (less than 10 segments). Some species of copepods from these groups have been under mass culture for hatchery operations including Calanoids: *Acartia tonsa, Eurytemora affinis, Calanus finmarchicus & C. helgolandicus,* and *Pseudocalanus elongates* and Harpacticoid: *Tisbe holothuriae, Tigriopus japonicas, Tisbenta elongate* and *Schizopera elatensis* (Lavens & Sorgeloos, 1999). Among these groups, the Harpactacoida are primarily benthic in nature, evidenced by their vermiform (worm-shaped) bodies and the Calanoida and Cyclopoida are primarily planktonic in nature and both are extremely important in food webs.

Morphology, life cycle and reproduction

The body of the most of the copepods is cylindriconical in shape, with a wider anterior part. The trunk region consists of two distinct parts: Cephalothorax (prosome); head is fused with the first six thoracic segments and the abdomen (urosome) is narrower than the cephalothorax. The head has a central naupliar eye and unirameous first antennae. The prosome & urosome regions are separated by the major articulation or flexing point in the body (Lavens & Sorgeloos, 1999). In general, most of the body segments come under prosome region but different copepod groups have different numbers of segments in the prosome, so no generalization can be made. Calanoid

copepods have enlarged first antennae which they often use for swimming. Calanoids can also be distinguished by a broad prosomes and narrow urosome whereas harpactacoids prosome and urosome are similar widths. In both groups all appendages are found on the prosomal segments except for the spiny appendages on the last body segment called the caudal ramus.

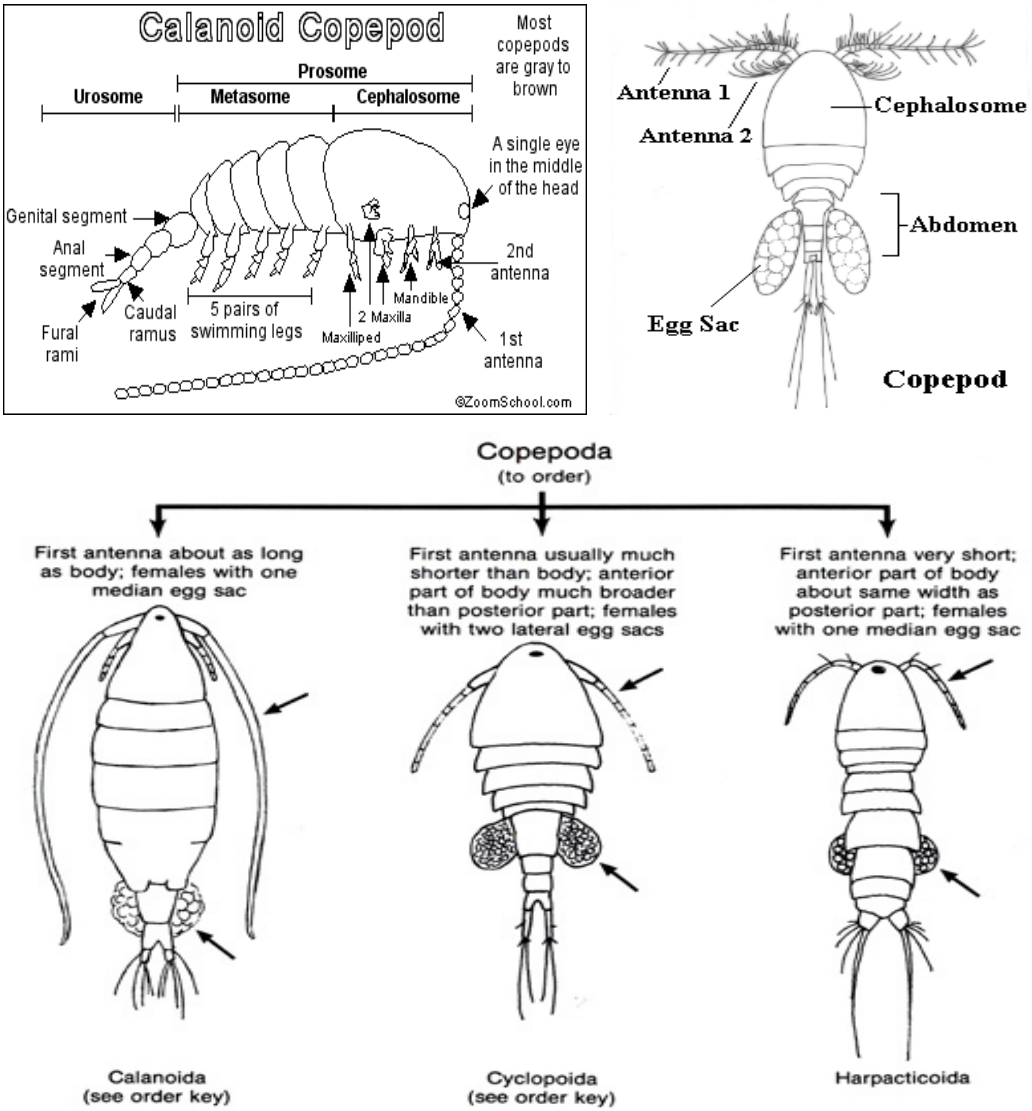

Fig.3. Copepod morphology (3a & 3b); Major differences among there different groups of copepods (3c) (Source; FAO, 1999)

Reproduction and life cycle

In copepods, sexes are separate and follows sexual mode of reproduction. In general, male copepods are commonly smaller than the females and during copulation the male grasps the female with his first antennae, and then deposits the spermatophores into seminal receptacle openings, where they are attached by means of special cement. The eggs are usually enclosed by an ovisac, which serves as a brood chamber and remains attached to the female's first abdominal segment and then slightly shed their eggs singly into the water. After fertilization the hatched out egg usually undergo different life cycle stages like nauplii, copepodites, and adults after five to six molting in every stages of the life cycle. The development may take from less than one week to one year in few species, accordingly life span of a copepod is ranging from six months to one year depending on the species. The size range of nauplii varies among species and generally ranges from 38-220 µm in body width. Commonly, the sizes of the copepods at the first nauplius stage are 125 µm long and 65µm wide and they grow to ~310 µm long by N6 stage. Jerk forward motion movement of copepods allow fish larvae to identify copepods as prey and elicits a feeding response. While measuring copepodids and adults, the length of the prosome is the most convenient descriptor of size.

Copepod culture process

Collection and isolation

Copepod samples are normally collected from the coastal/estuary waters during early morning using plankton net of less than 200 µm in mesh size. This mesh size may vary depending on the species of interest. After collection, the collected samples should be provided with vigorous aeration using aerators and samples should be immediately transported to laboratory and then thoroughly filtered to reduce the contamination from other zooplanktons (Joseph *et al*., 2016). From the collected samples the copepods are isolated using size fraction method. At first, the collected samples need to be sieved through a 500 or 250 micron mesh to remove the eggs and larvae of fish & shrimp. Then

the sieved samples are to be collected and again sieved through 200 micron mesh to remove smaller zooplanktons like rotifers. This process should be repeated for three to four times and then the samples need to be observed under microscope using established key for identification. After confirmation, the copepods should be transferred to stock culture container for further maintenance.

Maintenance of stock culture

Stock cultures are maintained physically separated from other zooplanktons and algal culture area. In general stock culture is maintained in 5-20 L glass containers or in plastic buckets with continuous aeration and proper feed (Jayasingam *et al*., 2015). Periodic water exchange with filtered seawater helps to reduces contamination of the culture. The culture needs to be frequently filtered through 200 micron mesh for maintaining purity of the stock. Water quality parameters should to be maintained by regular monitoring of p^H, salinity, temperature, dissolved oxygen etc. The algal feeds including *Chlorella sp*, *Isochrysis galbana*, *Chaetoceros calcitrans* are commonly used. Scaling up of culture is done by inoculating into bigger containers (Joseph *et al*., 2016).

Culture of copepods

Batch culture is the relatively easy if proper environmental and nutritional conditions are maintained in the culture environment. In this method, the adult copepods (10-25 individuals/ml) are stocked in a culture container containing filtered sea water and feeding with microalgae in the ratio 1:25 (v/v) (Joseph *et al*., 2016). The capacity of culture container varies depending on the requirement; for commercial hatchery operation 100 L to 5000 L capacity tank is generally suitable. After stocking, the copepods are fed with algae on daily or alternate day basis based on the feed consumption level. During the culture, the culture is maintained by increasing the level of water by adding the feed. Expansion of culture is done by collecting the eggs, nauplii, and adults from one tank and inoculating into other tanks or otherwise each culture tank is stocked separately and maintained for particular period and then harvested whenever required. After inoculation, the adult would begin producing eggs/ sperms in 9-12 days; thereafter egg production

would initially rise, then reaches the peak and finally falls. Once the hatching success falls below 75%, it is time to terminate the culture batch. For continuous production of nauplii, sequential batch cultures have to be initiated at every 5-7 day intervals. When timed correctly, one tank of a series will be at the maximum productivity at any given time. In starting cultures, high copepod densities are obtained in the first few days and then the production rate is decrease and stabilizes at low rate. Maximum production capacity of copepods varies depending on the species, for eg., *Eurytemora affini*, can be produced in the range of 100-2000 no/L; maximum of 30 no/ml for *Parvocalanus*; maximum of 5 no/ml for *Acartia sp* (Stottrup, 2006) observed at high population densities by different studies.

In mass culture, chlorinated and dechlorinated sea water is used. The cultures can be maintained in tanks with continuous aeration. Copepods are daily fed with a mixture of micro algal diet (*T. gracilis, C. calcitrans, I. galbana*) and baker's yeast. For culture of calanoid species the initial concentration of *Isochrysis* is given at 1000 cells.ml^{-1} and after 10 days a mixture of algae given at slightly lower concentration (Joseph *et al*., 2016). The generation time (period needed to reach 50% fertilized females) is varies for different groups, about 20 days with a constant mortality rate of about 5%/day for species in calanoids. The generation time for the species in harpacticoids under optimal conditions is about 8-11 days at 24-26 °C.

Feeding

Feeding copepods with an optimal diet is an important factor for culturing copepods. Copepods in pelagic nature must be fed by planktonic algal species. The minimum algal concentration is required to achieve maximum growth rates depend on the size of algae provided as feed (Stottrup, 2006). Generally around 10^5 cells/ml is sufficient when using small size algae (<5µ). and 10^4 cells/ml is required when using algae slightly bigger in size (>5µ). By means of gut analysis and faecal examination the feeding of copepod can be determined. While selecting food for a species the particle size, digestibility and chemical composition of the feed has be taken into consideration for

their multiplication and nutritional value. In general the particle size of the feed should not be more than 10 µ. The ideal algal feed is *I. galbana* and *N. oculata* (~2-5 µ). The amount of food required is directly proportional to the copepod biomass present in the culture media. Feed of 2 x 10^{-4} cells/ml/day is sufficient for young nauplii. Then, it can be gradually increased up to 1 x 10^{-5} cells/ml/day till they mature (Santhosh & Anil, 2013). Daily assessment of population density is essential. If the water appears cloudy, the feed rate should be decreased. The supply of sufficient amount of feed will successfully facilitate the peak production.

Brine shrimp (Artemia)

The rotifer is the most commonly used live feed upon transition of the larvae from endogenous (internal energy reserves) to exogenous (external) feeding in larviculture. Upon completion of the rotifer stage, the most commonly used live feed prior to conversion of the larva to a dry diet is *Artemia (*Brine shrimp*)* and it represent the transitional feed for the larvae, after which artificial dry feeds can be used. Brine shrimp is typically a primitive crustacean belonging to the class Branchiopoda with a total length of about 07-1.2 mm. This is a unique marine organism, which can extremely withstand and survive in a wide range of salinity. It is widely distributed and more than 50 strains have so far been recorded across the world. Among the live diets used in the aquaculture, *Artemia* nauplii are the most and widely used food item mainly due to its convenience and availability (Treece & Davis, 2000). The unique property of the *Artemia* is the formation of dormant embryos, called ‘cysts’. Cysts are available year-round in large quantities along the shorelines of hypersaline lakes, coastal lagoons, which can be collected, processed and stored or commercially available. *Artemia* cysts have remarkable shelf life and can be stored in containers for years and utilized as a ready-made live food source. Upon some 24-h incubation in seawater, these cysts release free-swimming nauplii that can directly used as a nutritious live food source to the larvae of a several variety of marine organisms. This excellent property of *Artemia*, make them as the most convenient, least labour-intensive live food available for aquaculture. Brine shrimp cysts are purchased from commercial suppliers and hatched in the tanks. Like rotifers, brine

shrimp must be enriched to increase their nutritional value before they are fed to the fish larvae. *Artemia* are nonspecific feeders and will ingest a wide variety of foods. This feeding habit helps for nutritional enrichment of *Artemia* to enhance the levels of important marine-based highly unsaturated fatty acids (HUFA) to match the nutritional properties of many copepod species.

Life cycle

The *Artemia* life cycle begins from the hatching of dormant cysts. The cysts are metabolically inactive embryos that can remain dormant for many years, as long as they are kept dry and oxygen free. It resumes its development when it is re-hydrated. Upon hydration, the cyst bursts and the embryo leaves the shell after 15 to 20 hrs at 25^{o}C (Lavens & Sorgeloos, 1999). After which, the embryo hangs underneath the empty shell called as umbrella stage, development of the nauplius is completed during the period. Immediately within a short period of time the hatching membrane is ruptured (hatching) and the free-swimming nauplius is born. The first larval stage is called Instar-I (400 to 500 µm in length), where the nauplii are a brownish-orange color because of their yolk reserves. Newly hatched *Artemia* do not feed because their mouth and anus are not fully developed. Approximately 12 hours after hatching, the animals molt into the second larval stage called as Instar-II. In this stage, small food particles ranging in size from 1 to 50 µm are filtered into the digestive tract. During the next eight days, the nauplii grow and progress through 15 molts before reaching adulthood.

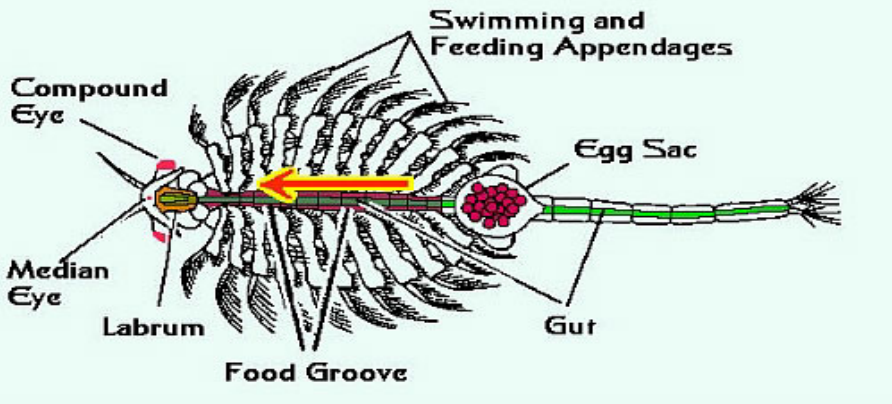

Fig: 4. Adult female Brine shrimp (Source – Dumitrascu, 2011)

Adult *Artemia* size is an average about 8 mm long, but can reach lengths up to 20 mm in optimal environments. An adult brine shrimp is approximately 20 times longer and 500 fold larger in biomass than a nauplius. Male brine shrimp possess a paired penis in the posterior part of their trunk, and adult female *Artemia* can easily be recognized by the brood pouch.

In nature, fertilized females usually produce free-swimming nauplii at a rate of up to 75 nauplii per day (ovoviviparous reproduction) under suitable environment. Normally, the average life span of the female is about 50 days. But under ideal conditions, an adult *Artemia* can live as long as three months and produce up to 300 nauplii or cysts in every 4 days (Lavens & Sorgeloos, 1999). Normally in the wild, the cyst production is induced under unfavorable environmental conditions, such as high salinity, chronic food shortages and/or cyclic oxygen stress, where embryos develop up to the gastrula stage, and then surrounded by a thick shell. Formation of this shell initiates a state of metabolic dormancy (diapause), and the cysts released by the female (oviparous reproduction), float to the shoreline and dehydrate.

Hatching/Production

Proper hatching and harvesting of *Artemia* nauplii are very much important to maximizing quality. Standardization of protocols for *Artemia* hatching and culture is important, as slight deviations in the process will extremely affect the hatching rate, nutritional makeup, and final size of the harvested nauplii. *Artemia* cysts are expensive, is one of the largest variable costs for a hatchery. Therefore, every attempt must be made to maximize hatch rate and quality. In the hatching process, cysts are hatched out after the decapsulation.

Decapsulation

The hard egg shell that covers cyst is to be e completely removed by short exposure to a bleach solution and this procedure is called decapsulation. Decapsulation of the cyst is always necessary for the following reasons.

1. When normal cysts (non-decaptulated) hatched, the separation of nauplii from their shells is not always possible. Un-hatched cysts and empty shells can cause deleterious effects in the larval tanks when they are ingested by the fry. The egg casing cannot be digested and may obstruct the gut.
2. Nauplii produced from decapsulated cysts have a higher energy content and individual weight (30-55% depending on strain) than the nauplii from the normal cyst.
3. Decapsulation results in a disinfection of the cysts. Therefore, infection due to contamination by dirty brine shrimp eggs is avoided.
4. The illumination requirements for hatching decapsulated cysts are lower.

Decapsulation process

In this, the cysts are initially hydrated because the complete removal of the envelope can only be performed when the cysts are spherical in shape. After hydration, decapsulation starts with removal of the brown shell in a bleach solution, followed by washing and neutralization of the remaining bleach. These decapsulated cysts can be hatched into nauplii immediately, or dehydrated in concentrated brine solution and then stored for later hatching. Decapsulated *Artemia* cysts could be stored for a few days in the refrigerator without any noted decrease in hatching rate.

1. Hydrate cysts by placing them for 1 h in water (< 100 gm/l), with aeration at 25°C.
2. Collect cysts on a 125 µm mesh sieve, rinse, and transfer to the hypochlorite solution. (The hypochlorite solution can be made up of either liquid bleach NaOCl

(fresh product; activity normally =11-13% w/w) or bleaching powder Ca(OCl)2 (activity normally ± 70%).

3. Add the hydrated cysts and then keep them in suspension using aeration tube for 5-15 min. Check the temperature regularly, since the reaction is exothermic; never exceed 40°C (if needed add ice to decapsulation solution).
4. During the process, the cysts turn grey (with powder bleach) or orange (with liquid bleach), after 3-15 min. Remove the cysts from decapsulation suspension and rinsed with water on a 125 µm screen until no chlorine smell is detected. It is very important that, embryos should not be for long time in the decapsulation solution, since this will affect their viability.
5. Deactivate all traces of hypochlorite by dipping the cysts (< 1 min.) either in 0.1 N HCl or in a 0.1% $Na_2S_2O_3$ solution, then rinse again with water. Hypochlorite residues can be detected by putting some decapsulated cysts in a small amount of starch-iodine indicator. When the reagent turns blue, washing and deactivation has to be continued.

Hatching

For hatching, the decapsulated cysts are to be transferred to the hatching container and suitable environment need to be provided. The best hatching results are achieved in conical bottom containers, aerated from the center bottom. Cylindrical or square-bottomed tanks will have dead spots in which *Artemia* cysts and nauplii accumulate, so using of the particular shaped container is need to be avoided. During the hatching the intensity of aeration should be sufficient to maintain high oxygen levels, because increased hatching has been reported with increasing oxygen level. As for temperature, optimal hatching occurs in the range of 25-28°C; below 25°C cysts hatch more slowly and above 33°C the cyst metabolism is stopped. Reports from commercial hatcheries suggest that strong illumination (approximately 2000 Lux at the water surface) is essential for maximal hatching, and that this lighting is essential during the first hours

after complete hydration, in order to initiate embryonic development. The optimal conditions for hatching *Artemia* are: Temperature: 26-30°C, salinity: 25-35 ppt, pH range 8-9, light level**:** ~2000 lux with heavy continuous aeration (Do : > 4 mg/L)

Harvesting of nauplii

After hatching, the nauplii are harvested by simply turning off the air, and allow the culture to settle for approximately 10 minutes. Hatched, empty shells float to the surface, and unhatched cysts will sink to the bottom. The newly hatched nauplii will concentrate just above the unhatched cysts on the bottom. Since the newly hatched nauplii are attracted to light, using flashlight at the center of the container will concentrate the nauplii where it is easy to siphon them off.

Feeding and enrichment

Artemia are non-selective filter feeders and therefore will ingest a wide range of foods. The main criteria for food selection are particle size, digestibility, and nutrient levels. The best feeds for *Artemia* are live microalgae such as *Nannochloropsis*, *Tetraselmis*, *Isochrysis,* and *Pavlova*. Moreover, the combinations of live phytoplanktons fed to *Artemia* cultures have demonstrated superior enrichment characteristics (i.e., increased HUFAs) over feeding single phytoplankton species. But some unicellular algae are not appropriate for sustaining *Artemia* growth. For example, *Chlorella,* and *Stichococcus* have a thick cell wall that cannot be digested by *Artemia*. In addition to live algae, *Artemia* cultures can be enriched by feeding a wide variety of processed foods, including yeasts, fish meal, soybean powder, egg yolk, and micronized rice bran. Like rotifers, the inherent nutritional value of *Artemia* is low, so enrichment is suggested to enhance the nutritional quality of the *Artemia* before feeding to larvae. *Artemia* are enriched with commercially available enrichment medium (DC DHA Selco, algamag). Once enriched, *Artemia* are rinsed, concentrated, enumerated, and then fed to larvae.

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- http://216.25.82.220/Rotifer.PDF, Online scientific article on mass culture of rotifers.

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