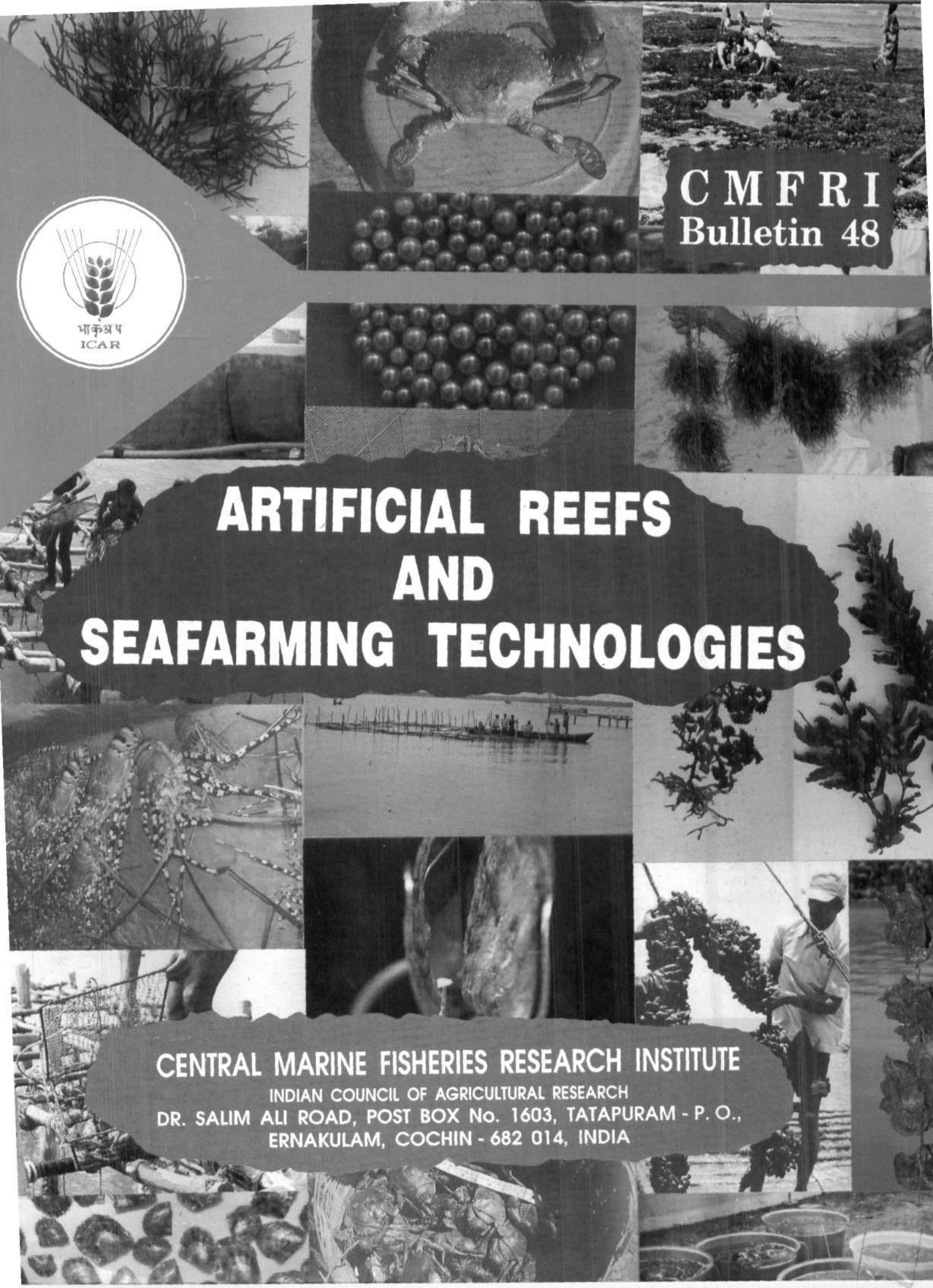

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CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
INDIAN COUNCIL OF AGRICULTURAL RESEARCH
DR. SALIM ALI ROAD, POST BOX No. 1603, TATAPURAM - P. O.,
ERNAKULAM, COCHIN - 682 014, INDIA

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Dr. K. Rengarajan

Editor

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LIFE-FEED CULTURE - MICRO ALGAE

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Introduction

Marine micro algae are the floating microscopic plant components of the seawater which forms the basic food of almost all the larval organisms, either crustaceans, molluscs or fishes. They are the primary producers of the sea belonging to various Classes of algae. The important components of micro algae are the diatoms, dinoflagellates, silicoflagellates (phytoflagellates), coccolithophores, blue-green algae and the 'hidden flora' the nanoplankters. Among these, the diatoms and phytoflagellates are significant organisms since they form the primary link in the food chain of the sea. It is known that the success of any hatchery operations depends mainly on the availability of the basic food, the micro algae.

Mass culture of micro-algae has been in prevalence in many research institutions, universities and hatcheries the world over, since the past 50 years. For many years, this has been experimented by Plant Physiologists, Algologists and Bioengineers as an alternate means of producing protein. But in the late 1960s the enthusiasm slowed down when it was found that the process was uneconomical due to technical problems especially in the recovery of the algal product and its subsequent conversion to human food supplement. Recently, there has been renewed interest in producing Single Cell Protein (SCP) by mass culturing the unicellular algae particularly the diatoms and phytoflagellates for feeding the larvae of crustaceans, molluscs, sea-cucumbers and fishes. In recent years, many other potential applications for large scale algal culture have been developed including waste-water treatment, production of extractable commercial chemicals, aquaculture and the bio-conversion of solar energy.

As is well known, the success of any hatchery system either crustaceans, oysters, sea-cucumbers or fishes, entirely depends on the availability of the suitable micro algae. In the natural environment, the larvae feed on any minute plant components which are readily available to them. But in a hatchery, the food which are acceptable to the larvae for their growth and further development have to be identified and isolated. In the early critical stages of the rearing larvae of finfishes and shellfishes, the phytoflagellates (species of *Isochrysis, Pavlova, Dicrateria, Chromulina* and *Tetraselmis*) and other nanoplankters (species of *Chlorella* and *Synechocystis*) form the basic food. But in the postlarval stages of crustaceans and spat juvenile stages of bivalves, the diatoms (species of *Chaetoceros, Skeletonema* and *Thalassiosira*) form the primary food. Hence the culture of micro algae is an essential prerequisite for the rearing operations of economically important cultivable organisms in a hatchery system.

Methodology

Isolation of the required species of micro algae can be done by one of the following methods.

1. *Pipette method* : Large organisms can be pipetted out using a micro-pipette under microscope and transfer to culture tubes having suitable culture media.

2. *Centrifuge* or *washing method* : By repeated centrifuging of the samples in different revolutions and by inoculating the deposits, it is possible to get different organisms.

3. *By exploiting the phototactic movements* : By this method, most of the phytoflagellates can be isolated. Make a dark chamber with a small hole on one side and keep the sample in a beaker nearer to the hole. Place a candle near

to the hole outside. Since the flagellates have a tendency to move towards the light, it is visible after sometimes that these organisms crowded near to the candle light. By pipetting, these organisms can be separated and by tube culture methods, it can be raised to a pure culture.

4. *By agar plating method* : For preparing the agar medium, 1.5 gm of agar is added to 1 litre of suitable culture medium *e.g.* Schreiber's medium, Miquel's medium, TMRL medium and Conway medium or even natural seawater. This agar solution is sterilized in an autoclave for 15 minutes under 60 kg pressure and 100°C temperature. Now this medium is poured in sterilized 15 cm diameter petri-dishes and kept for 24 hrs. For the isolation, the required species can be picked up by platinum needle or loop under microscope and streaked on the surface of agar plate. After inoculation, these petri-dishes are placed in an incubator for 7-8 days providing light (1000 lux) and constant temperature (25°C). Within this time, the required species, if it has grown into a colony, removed by platinum loop under microscope and transferred to culture tubes. Further, from culture tubes to small conical flasks and larger flasks, the algae can be grown on a mass scale.

5. *Serial dilution culture technique* : This method is used mainly for the isolation of phytoflagellates (Sournia, 1971). In this method, mainly 5 dilution steps (the inocula corresponding to 1, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} or 4 steps - 0.001, 0.01, 0.1 and 1 ml) are involved for the isolation of the required species. For the serial dilution technique, nearly 25 culture tubes (15 ml) are required. After filtering the seawater through 10-20 micron seive, the filtrate has to be inoculated to five series of culture tubes in various concentrations. This has to be kept under sufficient light (1000 lux) with uniform temperature (25°C) conditions. After 15 days, some discolouration can be seen in the culture tubes, due to the growth of micro-algae. Further purification of this culture can be done by sub-culturing it in 500 ml or one litre conical culture flasks. Once the culture is fully purified, it can be transferred into a 3 or 4 litre Hafkin culture flasks and maintained as stock culture.

After the isolation of the required organisms in culture tubes, it may be sub-cultured again in few 50 ml test tubes. These test tubes are the base from which the algal food started producing and from where the continuous supply of non-contaminated algal feed is obtained for the operation of the large-scale culture systems. Once the system is started, the test tube culture can be transferred to small culture flasks and to bigger flasks by adding 3-5 ml of the stock culture. Therefore every two weeks a new set of 10 test tubes for each species should be inoculated from the previous set. The filtration of water and medium enrichment should be done not earlier than 3 days, prior to inoculation.

Culture media

For the successful culturing of the micro algae, either diatoms or nanoplankters, various chemical culture media have been used depending on the type of organisms cultured and their growth phases. Although most algae are photo-autotrophic and can grow in purely inorganic media, many other required organic compounds, the requirements of which may be either absolute or stimulatory. While most of the micro algae can be successfully cultured on synthetic inorganic media, a few genera require organic compounds for their rapid growth and therefore the culture are supplemented with soil extracts, yeast extracts or organic salts. Since the micro algae in any water body require the nutrients such as nitrates and phosphates roughly in a ratio of 10:1 (N:P) for its normal growth and reproduction, the culture media used in the laboratory should have sufficient quantities of these elements besides other growth promoting substances including trace metals, amino acids and vitamins. The absence of one or two growth promoting agents would defenitely reflect on the growth of micro algae especially in a culture system.

For culturing the micro algae, various culture media are in use depending on the organisms involved. Though Erd-Schreiber's and Miquel's media (Miquel, 1892) were found to be very effective for culturing the diatoms and nanoplankters, several other media are also available with the addition of trace metals, vitamins and other organic and inorganic salts.

Since the diatoms require silica for building up the cell walls, the culture media should have a compound of silicate besides the nitrates, phosphates, chlorides and trace metals. Usually for culturing the flagellates, 'Conway' or 'Walne's medium (Walne, 1974) is used in the laboratory for the maintenance of the stock culture as well as mass culture. Since this culture medium has got various chemicals, trace metals and vitamins (B1 and B12), the phytoflagellates such as species of *Isochrysis, Povlova, Dicrateria, Chromulina* and *Tetraselmis* are being cultured by using media alone. However, media like TMRL and PM (Gopinathan, 1982) are also found to be effective for the mass culture of nanoplankton flagellates. Still, the technique of culturing different micro algae requires a clear understanding of their nutritional requirements, especially during the various phases of growth. The important culture media used for the micro algae are given below.

Schreiber's medium

Potassium nitrate	0.1 gm
Sodium orthophosphate	0.02 gm
Soil extract	50 ml
Filtered and sterilized seawater	1 litre

Soil extract is prepared by boiling 1 kg of garden soil in 1 litre of freshwater for one hour. After 24 hrs, clear water is decanted and kept in a bottle. 50 ml of this soil extract can be added to each litre of sterilized seawater. This can be used as a medium while isolating the nanoplankton.

Miquel's medium

A.	Potassium nitrate	20.2 gm
	Distilled water	100 ml
B.	Sodium orthophosphate	4 gm
	Calcium chloride	2 gm
	Ferric chloride	2 gm
	Hydrochloric acid	2 ml
	Distilled water	100 ml

0.55 ml of 'A' and 0.50 ml of 'B' are added to one litre of filtered and sterilized seawater. This medium can be used for culturing various types of micro algae.

'Schreiber's medium (modified for serial dilution culture method)

Potassium nitrate (5 gm in 100 ml of DW)	0.25 ml
Sodium orthphosphate (1 gm in 100 ml)	0.25 ml
EDTA (1.2 gm in 100 ml)	0.15 ml
Vitamin mixture (Thiamine - 200 mg, Biotin - 1 mg, Cyanocobalamine - 1 mg in 1 litre of DW)	0.50 ml
Soil extract	3 ml
Sterilized seawater	250 ml

The medium is autoclaved at 80°C for 15 minutes, then cooled down to room temperature in running water. If possible add vitamin mixture to the medium after it is cooled.

***TMRL medium** (Tung Kang Marine Res. Lab.)*

Potassium nitrate	10 gm	/100 ml of DW
Sodium orthophosphate	1 gm	" "
Ferric chloride	0.3 gm	" "
Sodium silicate	0.1 gm	" "

The chemicals are kept separately in 100 ml reagent bottles. 1 ml each to 1 litre of sterilized seawater is added. This medium can be used for the mass culture of diatoms.

'Conway' or Walne's medium

A.	Potassium nitrate	100 gm
	Sodium orthophosphate	20 gm
	EDTA (Na)	45 gm
	Boric acid	33.4 gm
	Ferric chloride	1.3 gm
	Manganese chloride	0.36 gm
	Dist. water	1 litre
B.	Zinc chloride	4.2 gm
	Cobalt chloride	4.0 gm
	Copper sulphate	4.0 gm
	Ammonium molybdata	1.8 gm
	Dist water	1 litre
C.	Vitamin B1 (Thiamin)	200 mg in 100 ml dist. water
	Vitamin B12 (Cyanocobalamine)	10 mg in 100 ml dist. water

A, B and C (each) in different reagent bottles are prepared. 1 ml of A, 0.5 ml of B and 0.1 ml of C to 1 litre of filtered and sterilized seawater are added.

During the course of the larval rearing of oysters, the flagellates form the basic food upto its spat stage. However, for the better growth of the spat, the food is to be changed from flagellates to a mixture of diatoms and other nanoplankters. For the preparation of mixture of various phytoplankton organisms in the open tanks, using direct sunlight, the following media could be used.

Mixture culture medium

Potassium nitrate	1.2 gm
Sodium orthophosphate	0.66 gm
EDTA (Na)	0.66 gm
Sodium silicate	0.66 gm

The first 3 chemicals are dissolved in 25 ml of DW and sodium silicate alone is dissolved in 25 ml of DW. This is added to 100 litre of fresh unfiltered seawater (fresh seawater is filtered through organdy cloth, 0.33 mm mesh size to remove zooplankton). This water is poured in 3-4 white lined basins or fibre-glass tanks and kept in open sunlight. Within 24 hrs, a slight yellow colouration can be noted. On examination, it can be noticed the growth of mixture of planktonic diatoms and other nanoplankters. 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 serve as nutrients, commercial fertilizers can be used for the mass culture of diatoms and nanoplankters, in open tanks for economy purpose. The media used for the open culture are :

Urea 46	10 mg/l
16-20-0	10 mg/l
20-0-0	100 mg/l

Growth phases of the algal culture

The usual way of the laboratory culture of micro algae is one in which a limited volume of medium containing the necessary inorganic and organic nutrients, is inoculated with a relatively small number of cells and these exposed to suitable conditions of light, temperature and aeration. Increase in cell numbers in such a culture follows a characteristic pattern in which the following phases of growth may usually be recognised.

Lag or induction phase

The cells taken from the stock culture room and inoculated to a new flask have to acclimatise the surroundings or in the new medium. Hence, there will be no cell division for a few hours and this stage is known as lag or induction phase.

Exponential phase

Once the cells are acclimatised to the surroundings, they start multiplication and grow rapidly. It is assumed that within 8-16 hrs, the cell will divide into 2 and further these cells carry on the growth till the culture reached its maximum concentration. This growing phase is known as exponential phase.

Declining phase

Once the cells reaches its maximum concentration, the growth and multiplication of the cells will be arrested and slowly the cells show the symptom of decline. This arrested growth of the cells in the culture is known as declining phase.

Stationary phase

After the arrested growth, the culture will be stationary without any further cell division for a few days. Actually, stationary phase is a prolonged one in the case of flagellates. For this they may develop some cover or cyst or matrix around its body for thriving in the unfavourable conditions. In the stationary phase, if the cells get a new environment, they start further growth and reproduction.

Death phase

After a long period in the stationary phase, the cells lose its viability and start to die and thus the culture becomes useless, either for reculturing or for feeding.

Determination of algal cell densities

Regular counts of the algal cells must be made in order to schedule inoculation of the various culture flasks as well as mass culture containers, to monitor growth of the algal cultures and to determine the quantity of algae to be fed to the rearing larval organisms.

In sampling the mass or tank cultures, a small length of rubber tubing is helpful to work with and to connect it to the mouth piece end of a sterile serological pipette. To get a representative sample, move the pipette around the tank while withdrawing algae upto the mark on the pipette and then place it in a flask. This is how the sample is to be counted.

Since most of the nanoplankters measure less than 10 μ a haemocytometer is used for counting the cells. First of all the sample is treated with a drop of eosin or formalin to kill the cells and stirred well, one drop is taken with sterilized pipette. After placing a cover-slip on the haemocytometer, the pipette is brought to the edge of the haemocytometer to touch it. The sample runs inside the cover slip and thus a thin film of the culture is maintained, and the cells are equally distributed. Since the haemocytometer has got 9 chambers, and 4 sides having 16 divisions and 5 chambers of multiple divisions, it is restricted the counting for at least 4 chambers. The average number of cells in 1 ml is calculated as shown below.

Average counts per chamber x 104 = Total number of cells/ml.

Stock culture maintenance

Stock culture of the micro algae are maintained in a special air conditioned room adjacent to the mass culture room. Stock cultures are kept in 3 or 4 litre Hafkin culture flasks. The autoclaved or boiled seawater after cooling is poured to the Hafkin flasks and required nutrients are added. Walne's medium enriched with vitamins is the quite ideal one to maintain the stock of all the phytoflagellates. About 10 ml of the inoculum in the growing phase is transferred to the culture flasks and the same is placed in front of 2 tube lights (1000 lux). After 8-10 days, when the maximum exponential phase has reached, light is reduced to 1 tube light for further growth. The time required for the maximum cell densities varies depending on the species. However, it was noticed that all the Haptophycean flagellates require 2 weeks for the completion of growth phase before entering into the declining phase. In the stationary phase, the flagellate can be kept for 2 months in the stock culture room, under controlled conditions of light and temperature, with or without aeration. At the time of maximum exponential phase of growth, the colour of the culture turns into dark brown and the cells are found as suspension without movement. The cells of flagellates produce a thick matrix or cyst around it in the stationary phase and when inoculate the same, these cysts break and the flagellates emerge out for its further growth and multiplication. A minimum of 5 Hafkin culture flasks were kept for each species as stock culture.

Mass culture

Large scale culture of micro algae, especially nanoplankton flagellates and selected species of diatoms, are necessary for feeding the larval forms in a hatchery. Since the molluscan larvae feed organisms measuring < 10 μ only, these forms are isolated from the seawater, maintained as stock culture and the inoculum, mass culture is done in the laboratory conditions as well as in the outdoor tanks.

The containers for the mass culture of micro algae are of 10 lt capacity polythene bags, 20 lt glass carbuoys, 100 lt persepex tanks and 250 lt cylindrical transparent FRP tanks for the indoor culture. These containers are kept in wooden racks with light and aeration. Fully grown culture from the stock culture room is used as inoculum for the mass culture in these containers. These tanks have the maximum concentration of the cells in the growing phase on the 5-7th day and harvested. After estimating the cell concentration using a haemocytometer, the culture is supplied to the hatchery for the rearing operations of the larval organisms. Leaving 2 lt of the culture, fresh enriched medium is added for further culture in the same container.

Equipments and glasswares

For the identification of the micro algae as well as for the determination of cell concentration

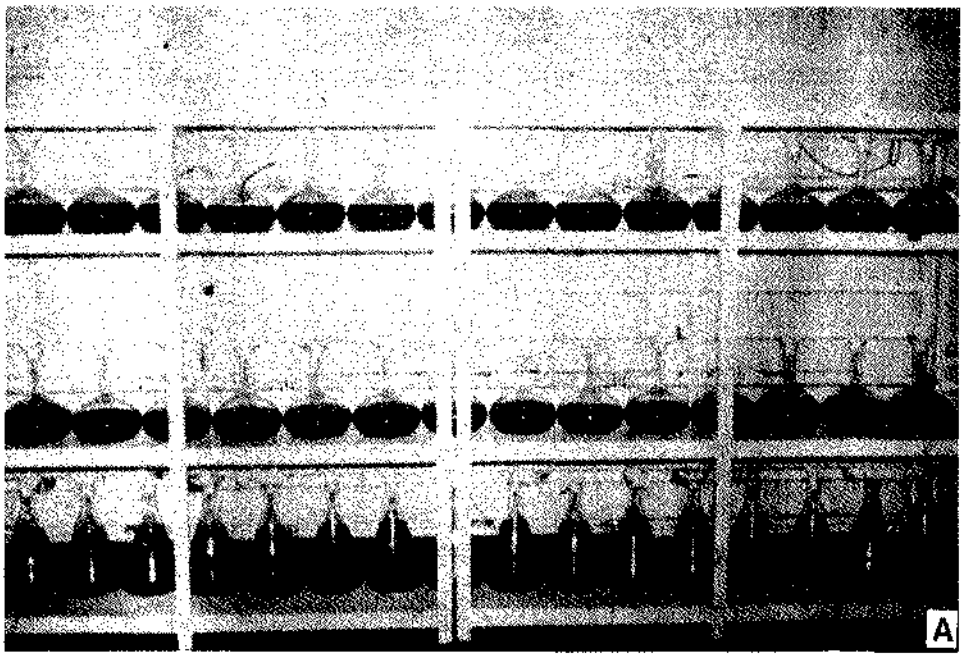
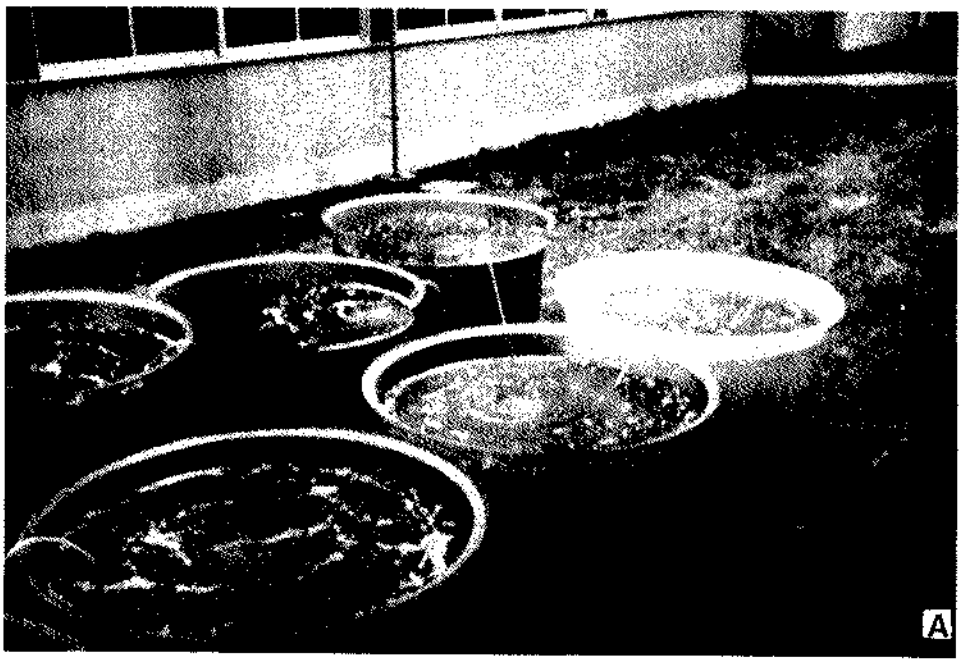


Plate I A. Stock culture maintenance of micro algae and B. Indoor mass culture of micro algae in 20 lt glass carbuoys and 100 lt perspex tanks.



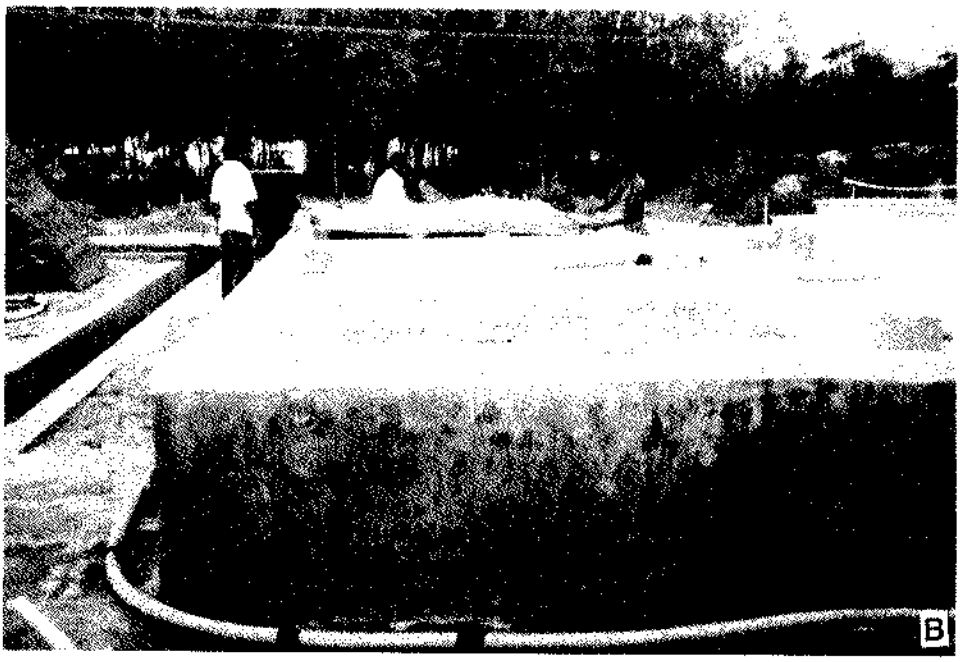


Plate II A. Outdoor mass culture of micro algae in FRP basins and B. Large-scale culture of *Chaetoceros* in 5 tonne concrete-white tile tanks.

of the culture, a powerful microscope is necessary. Since the flagellates are identified by noting the number of flagellae and other cell characteristics, a phase contrast microscope is advisable.

For the stock culture maintenance, micro-pipettes, droppers with teat, reagent bottles, culture tubes, conical flasks, Hafkin culture flasks, haemocytometer, etc. are required, in addition to the containers mentioned above.

Sterilization of glass containers

The glasswares used for the isolation, maintenance, laboratory culture and mass culture should be cleaned thoroughly prior to sterilization either by steam or by autoclaving. The cleaning procedures : (1) Treat with conc. hydrochloric acid and keep the same for 30 minutes, (2) Rinse 3 times with hot tap water, (3) Rinse 2 times with cold water, (4) Pour few drops of suitable soapwater (T-poll or Labolene) and rinse 6 times till the froth is completely removed and (5) Rinse 2 time with distilled water. After drying in the sunlight, the glasswares are kept in an oven at 100°C for an hour. The culture tubes, conical flasks and Hafkin culture flasks are to be plugged with cotton before keeping in the oven.

Illumination of algal cultures

One of the most important factors in determining successful culture of micro algae, is the type and quantum of illumination. Most of the flagellates require less light during the stationary and declining phases. Too much of light causes the culture for the early declining. Comparing the low light levels found in natural water, successful cultures of these algae in the hatchery also requires relatively low levels of light to achieve maximum growth and optimum densities of cells.

For maintaining the stock cultures of all micro algae during the declining and stationary phases of growth, one tube light (500 lux) is essential while the mass culture containers require 1000-2000 lux. Twelve hours of light and 12 hrs of darkness is ideal for maintaining the stock as well as mass culture, which can be controlled by auto-timer control switch mechanism.

Temperature control

Normal room temperature (28-30°C) is not ideal for the maintenance and culture of micro algae. Hence air conditioned rooms are used for keeping the stock as well as indoor mass culture of this live feed. Both the rooms should have 23-25°C during day time. Since there is no light during night hours, air conditioners can be switched off for few hours.

Aeration

Similar to light and temperature, aeration is also important for developing and maintaining healthy cultures, as well as enhance the exponential phase of growth of micro algae for few days more. It was noticed that if aeration is given to the mass culture tanks, cultures remain in the growing phase 3-4 days more than the tanks with no aeration. Moreover, aeration is more required during day time when there is light when compared to night. Aeration not only helps the culture, but also keeps the culture always in suspension. Aeration also helps the nutrient salts to distribute uniformly in the medium and supplying of CO_2 required for photosynthesis. Lastly, aeration prevents the settling of the cells at the bottom of the culture tanks, causing eventual death due to the lack of supply of CO_2.

Anti-contamination procedure

In culturing of various species of micro algae, the most important aspect is the cleanliness of all surfaces. Personnel should not work in areas where there is raw ambient seawater prior to working in the algal section. All works with algae should be done with one species at a time. Neither samples from, nor inoculation of culture containers containing different species of algae should not be done at the same time. In addition, personnel should wash their hands after working with one species and before starting to work with another species of algae. Transfer of tube cultures should take place where there is a minimal movement of air to reduce chance of contamination. All glasswares should be sterilized before use.

Further, all stock cultures in Hafkin culture flasks should be checked in a week under microscope using sterilized pipette for contamination. The checking of these cultures is to be

combined with the counting procedure on a regular basis.

Harvest

The fully grown 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.

Preservation

The maintenance and constant supply of the culture whenever required is a problem in the hatchery especially during adverse weather conditions. In this case, the preservation of the algae either by freezing or by sun-drying is done in the sense that during scarcity of the feed, the rearing operations may be successfully controlled. For the method of freezing, the culture is flocculated either by adding lime or by adjustment of pH using sodium hydroxide. Usually, the addition of alum or lime will not give satisfactory results, but manefestation of pH with sodium hydroxide is the advanced technique conducted in the laboratory. After knowing the quantity of the culture to be flocculated, the volume of sodium hydroxide solution needed to flocculate to get one degree raise in pH is measured. Suppose the pH of the culture is 8.4, by adding sufficient quantity of sodium hydroxide solution raised to 9.4. After vigorous stirring, the culture is left for one hour to settle the algal mass at the bottom. Slowly decanted the clear water and the mass in a plastic bucket is collected. Then the pH of the culture to the original level is brought slowly by adding dilute hydrochloric acid. Now the algae is ready for freezing or sun-drying. The algae is dried by pouring the mass in white enamel trays and keeping the same in the bright sun-light. If the algae dried thoroughly, the powder from the enamel tray is scraped and kept in glass bottles.

Before freezing the algal mass, some protective reagents like Dimethyl sulphoxide or Glycerol (a few drops) are added. The concentrate is then poured into polythene bags after measuring. The polythene bags are labelled and kept in deep-freezers. The frozen algae do not have the same protein content as in the live condition. Whenever adverse condition arise, the frozen food are used for rearing the larval organisms.

Future scope of micro algal culture

The development of mass culture of micro algae offers immense scope and can run concurrently with mariculture programmes. At the same time it offers a challenge for the phycologists. Considerable research is needed for elucidation of the key problem such as the growth kinetics of different species, the period of economically viable harvest, etc. In the matter of waste-water recycling, unicellular algae holds out much promise. Maximisation of solar energy utilization under controlled conditions of temperature, light and aeration, development of viable methods for intensive culture, maintenance, formation of extracellular products and their significance are promising lines of investigations.

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