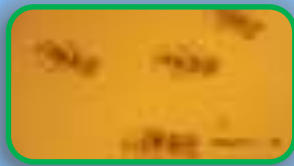
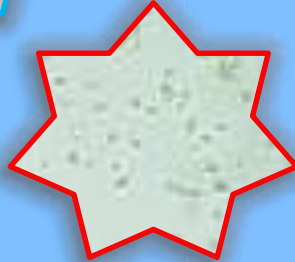


Training Manual on Live Feed for Marine Finfish and Shellfish Culture



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Microalgae culture media and glass ware

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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 ($\text{NaHPO}_4 \cdot \text{H}_2\text{O}$). 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 CO_2 , carbonate or bicarbonate for its photosynthesis. Silicate is necessary for the cell wall development of diatoms and is added in sodium silicate form ($\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$) 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 ($\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$) 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 B₁₂ > 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 CuSO₄ or CuCl₂ and ZnSO₄ or ZnCl₂. Some nutrients also come with different hydrations (NH₂O). Substituting one form with 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 hydration 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 stocks 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. Further, the dissolved organic matter can be removed with high intensity ultra violet light. The UV sterilized water is stored and used for the regular microalgal culture. Further, 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 chelators 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 *Nannochloropsis*, *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 ₂ PO ₄ .2H ₂ O)	20g
3.	EDTA di-sodium salt (Na ₂ EDTA)	45g
4.	Boric Acid (H ₃ BO ₃)	33.4g
5.	Ferric Chloride (FeCl ₃)	1.3g
6.	Manganous Chloride (MnCl ₂ .2H ₂ O)	0.36g
7.	Distilled Water	1L
Solution (B)		
1.	Zinc Chloride (ZnCl ₂)	4.2g
2.	Cobalt Chloride (CoCl ₂ .6H ₂ O)	4.0g
3.	Copper Sulphate (CuSO ₄ .5H ₂ O)	4.0g
4.	Ammonium molybdate ((NH ₄) ₆ Mo ₇ O ₂₄ .4H ₂ O)	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 ₂ SiO ₃ .5H ₂ O)	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 *Nannocloropsis*, *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 ₂ PO ₄ .H ₂ O)	5g	
P.S.Solution*		
1 Copper Sulphate (CuSO ₄ .5H ₂ O)	10g	1 L distilled water
2 Zinc Sulphate (ZnSO ₄ .7H ₂ O)	22g	1 L distilled water
3 Cobalt Chloride(CoCl ₂ .6H ₂ O)	10g	1 L distilled water
4 Manganous Chloride (MnCl ₂ .4H ₂ O)	180g	1 L distilled water
5 Sodium Molybdate (Na ₂ MoO ₄ .2H ₂ O)	6g	1 L distilled water
Prepare each solution separately in 1L bottles		
Solution (B)**		
1 EDTA di-sodium salt (Na ₂ EDTA)	4.36g	1 L distilled water
2 Ferric Chloride (FeCl ₃ .6H ₂ O)	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 ₂ SiO ₃ .9H ₂ O)	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 (<i>Chaetoceros</i>)		
Working Solution for mass culture: Add 1ml of each Solution A, B, C and D into 1 L sea water. D: Only for <i>Chaetoceros</i>		

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 requirement 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 minimum 1 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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