CMFRI

Course Manual

Winter School on

Recent Advances in Breeding and Larviculture of Marine Finfish and Shellfish

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MASS CULTURE OF MICROALGAE AS AN INHERENT PART OF HATCHERY OPERATION

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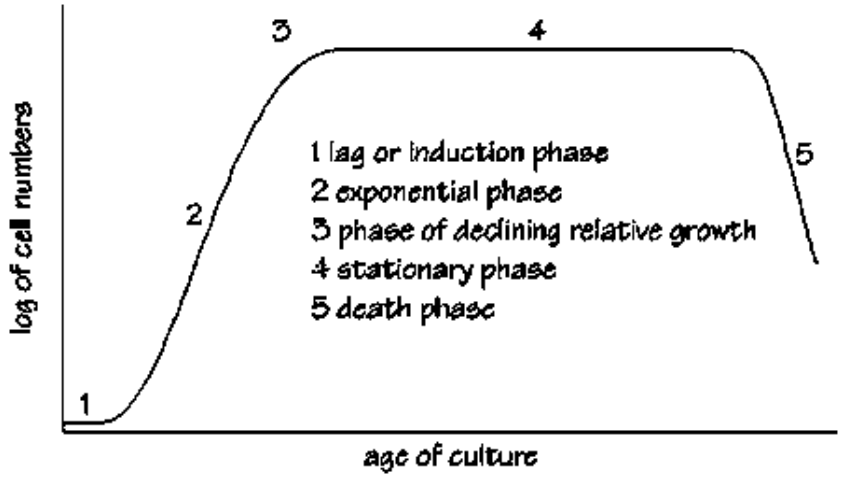
Introduction

Microalgae are microscopic unicellular phytoplankton which have less than 10 µ size. These floating planktons being the predominant component of the first tropic level in the aquatic food chain has got immense value as an aquaculture live feed and as a result the production of unicellular algae has gained importance in several countries due to their wide use as food in the hatchery. Most micro algae are rich sources of essential fatty acids; vitamins such as B12, B6, B1, biotin, riboflavin, nicotinic acid, pantothenate, C, E and A; chlorophyll 'a' and 'b' and carotenoids and these plankton plays a vital role in aquaculture to meet the nutritional requirements of the larvae as well as for bio-encapsulation. It is, therefore, its culture is an inherent part of aquaculture operation. The successful rearing, growth and survival of larvae in the hatchery depends upon the provision of suitable strain of these microalgae during their critical stages of life cycle. The algal species may vary in size, shape and nutritional quality, and are also used for generating "green water" system in many hatcheries. Inspite of all efforts to replace micro algae by artificial feeds, aquaculturists are still depending on the production and use of micro algae as live food for the fishes during their different stages of life cycles. In addition to these, micro algae together with bacteria have an important role in oxygen balance in fish culture system.

Over dependence for these inevitable plankton from the wild is an unreliable source for commercial seed production due to uncontrollable fluctuations in quality, quantity and to the drawbacks of collecting methods which do not excludes harmful organisms. More over, of the very many type of algae which live in the sea, only few can be cultured and certain type only will give good growth when fed to the organism andsuitable species for feeding are being selected based on their nutritive value, size, cell wall composition and growth characteristics. It is also revealed that larvae fed with natural concentration grow more slowly than larvae fed with cultured algae. Thus cultured algae are paramount important live feed in hatchery system for mass production of larvae of crustaceans, molluscs and fin fishes, and quality zooplankton which are essential part for successful hatchery operations. The growth of algal culture can be expresses in terms of cell division or doublings per day. Under suitable nutrient enrichment and favourable physical conditions, axenic cultures of algae will exhibit different growth stages: lag-phase or induction phase; exponential phase or log phase; phase of declining relative growth or transitional phase; stationery phase and death phase.



Type of culture

Maintenance of isolated algae in good growth condition as inoculum is the back bone of any algal production system. The major methods are indoor culture in which the isolated species are maintained as stock in small container under controlled condition in an aseptic algal culture laboratory whereas mass scale can be carried out indoor as well as outdoor in which production relies on natural conditions. In all the methods, the culture must be inoculated and allowed to grow and divide. The rate of growth varies depend upon the type of algae and its culture condition. The general culture methods are stock, sub and mass culture.

(a) Stock culture

To start a new stock or starter culture, about 20 ml of algal inoculum of isolated species are required which are need to be cultured every 6 to 7 days in 250 ml flask plugged with cotton-wool containing sterilized cool seawater with suitable medium : Walnes or Guillard f or Gf/2 or Gf/4 or Miquels or TMRL or PM or Suto or SEAFDEC in appropriate proportion and need to be maintained in algal culture laboratory at temperature 21-25°C. These are need to be frequently subcultured to maintain the culture in the exponential growth phase which is the key factor for the successful and efficient algal production system for mass culture to feed the larvae of fishes.

(b) Sub culture

To maintain sufficient algal inoculum for upscaling algal production, the stock cultures must be sub-cultured once in 7 days or depending upon the growth phases of algae. Subculture can be carried out through inoculation of cells from an old stock culture into 3 or 4 l of haufkins flask with fresh culture medium to enable the cells to continue multiplication, growth and need to be maintained in algal culture laboratory**.**

(c) Mass culture

Since stock cultures are not sufficient to meet the requirements of zooplanktons, larvae of fishes in hatcheries, the algae are need to be multiplied in large quantity in minimum period of time either indoor or outdoor with suitable culture media. Indoor with transparent roofing is the ideal situation for getting non contaminated algae especially for the feeding the larvae of the fishes. Fully-grown culture from the stock culture can be used as inoculum to avoid contamination in mass culture. Mass culture can be carried out 10 l. plastic buckets or 20 l glass carbuoys or 100 and 250 l perspex and glass tanks or 500 l capacity FRP tanks kept in wooden or cement racks or elevated platform and in polyethylene bags with light, aeration, and suitable environmental parameters. To avoid settlement of algae, submersible pumps are need to be provided in each tank.

Methods for mass culture

There are many different ways of culturing algae. These range from closely-controlled methods on the laboratory bench top, with a few litres of algae, to less predictable methods in outdoor tanks, containing thousands of litres, in which production relies on natural conditions. Several methods have been developed for the production of algae for use as food for various marine animals. Under culture conditions, the rate of growth and division varies with different types of algae and also depends on how well the various culture conditions necessary for growth have been met. The major mass culture methods are detailed below.

Batch culture

Batch culture is a system where the total culture is harvested and used as food and a fresh culture of the same species is set up to replace it. When a small volumes of algae culture is required (2 to 10 L) per day, production is most conveniently achieved in flasks. A set of three, 250 ml stock cultures is started by inoculating from one existing 250 ml stock culture on each of 3 successive days. The new stock cultures are grown at a temperature of about 21°C, and at a distance of 15 to 20 cm from a 65 watt fluorescent tube. After 3 days, and then daily, each of these stock cultures is

used in turn to inoculate a new 250 ml stock culture and the remainder is added to the 3 l sea-water culture medium in the flasks, which have been prepared as follows: Three-litre borosilicate glass flasks with cotton wool plugs are filled with sea water. The contents are either autoclaved at 1.06 kg per square centimetre (15 psi) for 20 minutes, boiled for 30-45 minutes, or pasteurized and the sea water in the flasks should be allowed to cool before adding nutrient salts. To the 3 liters of sea water in the flask, 6 ml of solution A, 3ml of B 0.6ml of solution C and 0.6 ml of solution D of Walnes media are added. A fresh 3 L culture is started daily from a 3-day-old stock culture and aerated with a mixture of air and CO_2 at about 2 to 3 L/minute. The gas mixture is filtered through an in-line cartridge unit containing a 0.3-0.45 micron filter to reduce the risk of airborne contamination. The culture reaches a density of 45 000 to 60 000 cells/ml in 3 to 4 days at 21°C and 24 hrs illumination.

The batch cul-tures, which are started from 6 or 7 days old stock cultures, will take 7 to 8 days to grow to a density suitable for feeding. Larger containers of up to 10 L may be used for this method of culture. The batch cultures may be used directly as food or as inoculum to start larger volume batch, semi-continuous or continuous algal cultures.

Semi-continuous culture

Semi-continuous culture is a system where a part of the culture is harvested and used as food, and the amount taken is replaced with fresh culture medium. After allowing 2-3 days for the remaining cells to grow and divide, the process is repeated. Semi-continuous cultures may be operated for up to 7 to 8 weeks in a rum.

Semi-continuous culture : In Two hundred litre vessels

A method for large-scale production (mass culture) in 200 litre, internally illuminated, glass reinforced plastic (grp) vessels, using semi-continuous culture is described below.

The vessels are 150 cm high, 40-45 cm in diameter and each has a central lighting unit into which fluorescent lamps are fitted. A glass fibre cooling pipe is moulded onto the outer jacket. These vessels are most useful for growing diatoms, but they may also be used for flagellates. The vessels are sterilized by filling with a solution of sodium hypochlorite (50 parts per million (ppm) free chlorine concentration). They are allowed to stand for 2-4 hours and then drained, and flushed with filtered air for 24 hours to drive off residual chlorine.

The vessels are filled with 200 l of filtered sea water at 25 to 30 ppt salinity for diatom cultures or 30 to 35ppt for flagellate cultures. For diatom cultures, filtration to 2 microns is usually sufficient, while for culture of flagellates, filtration to half a micron is preferable. Two-hundred millilitres of solution A, 100 ml B, 20 ml of solution C and, for diatom cultures, 200 ml of solution D of Walnes medium are added to the ves-sels. The culture is inoculated with 2 to 5 l of a 4 to 8 day old batch culture, grown as described in the previous section and aerated with a filtered air/CO_2 supply at about 15 L / minute.

Cultures reach densities suitable for harvesting after 4 to 7 days depending upon the species cultured. After reaching suitable cell density, the amount of algae to be harvested can be calculated from the following equation:

$$\text{Vol. to harvest in lit.} = \frac{\text{200x density to which culture needs to be diluted}}{\text{actual culture density of algae harvested}}$$

After harvesting, the vessels are topped up to 200 l with filtered sea water of the correct salinity with required nutrient meida. It is usually more convenient to harvest the culture every 2 to 3 days. That part of the harvest which is required for feeding on the intermediate days can be aerated in a plastic container away from bright light and in a cool place. The length of culture period will vary with the type of algae cultured. Production of algae from 200 litre vessels should average the equivalent of 60-80 l/day.

Sixty litre polyethylene bags (PE Bags)

A further large scale production method utilizes 25 cm wide polyethylene tubing made into bags and hung from a framework. This simple method for producing either diatoms or flagellates is suitable for use in indoors with fluorescent lamps, and outdoors with natural daylight.

When setting up a system, a small hole is made in the top of each half of the bag and filtered sea water, at required salinity (for diatom and and flagellates) is pumped in until the bag is almost filled. To each half 30 ml of solution A, 15 ml B, 3 ml of solution C and (for diatoms) 150 ml of solution D of Walnes media are added, and then about 2 l of a batch culture that has grown for 4-8 days. An air line of 7 mm diameter Perspex tubing is fitted into each half of the bag and aerated vigorously. The culture is allowed to grow.

Inoculation of a PE bag from a flask or from another bag

1. Prepare the bag, either suspended to a G.I. rack or placing it inside a cylindrical frame made of wire.
2. Fill the bag with sterilized water; leaving enough free space for the volume to be inoculated; wait a couple of hours to check for possible leaks; if found, seal them or replace the bag.
3. Introduce nutrients and inoculum.
4. Fit two PVC tubes for aeration to the bottom of the bag, connect them to an air line and adjust the air flow to a gentle bubbling
5. Add the nutrients using a graduated cylinder at the usual rate of the required media.
6. Select a mature culture that will be used as inoculum, either from a large flask or from another bag, and check a sample under the microscope for contaminants.
7. In case a flask is used as inoculum, remove its cap and flame its neck; then close with a flamed aluminium foil stopper and let it cool.
8. Remove the stopper and pour the algal culture from the flask into the bag. The inoculum should be about 10% of the receiving volume.
9. If the inoculum comes from another bag, with a self-priming plastic pump transfer the inoculum from the old bag into the new one. The volume to be transferred should be about 15-20% of the receiving volume (a bigger inoculum is necessary to compensate for a less dense culture);
10. On each bag mark date, algal species, origin of the inoculum (bag or flask) and the bag serial number.

To reduce the risk of contamination, smaller bags are usually inoculated from flasks, whereas larger bags are inoculated from smaller bags. To ensure a continuous supply of algae, inoculation may be done in such a way that, at a time in rotation (i.e. for every three bags of a particular species of algae, start one in the first week, another in the second week and the third the following week so that every day a certain quantity of algae can be harvested.

Production in outdoor bag cultures is variable, depending upon the amount of light avail-able and also on the temperature. Direct sunlight should be avoided, as this causes the cultures to become too hot and to collapse. When harvestable cell density reaches, the algae are harvested and top-up the bags with fresh medium. After about ten semi-continuous harvests, the culture and bag should be replaced.

The addition of CO_2 is important, as it increases yields five-fold in indoor cultures and by two and a half times in outdoor cultures. Outdoor cultures are less affected, as the CO_2 produced in cell respiration is not used during the night, allowing the natural CO_2 levels to build-up slightly.

Continuous culture

This falls into two categories:

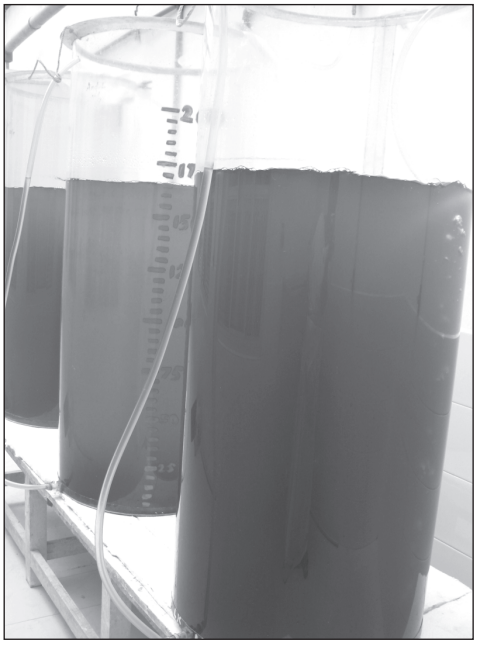
(i) **Turbidostat culture:** In this system, the number of algal cells in the culture is monitored and, as the cells divide and grow, an automatic system keeps the culture density at a pre-set level by diluting the culture with fresh medium.

(ii) **Chemostat culture**: In this method, a flow of fresh medium is introduced into the culture at a steady, pre-determined rate.

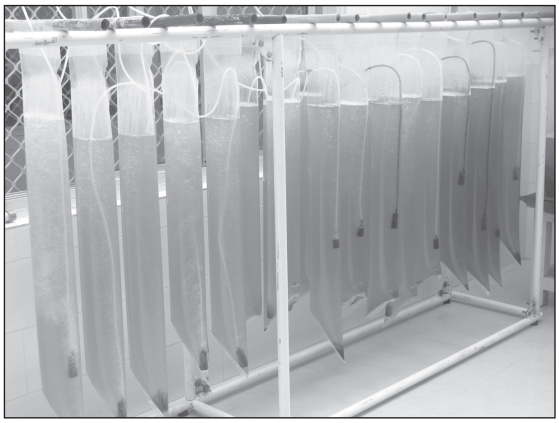
In both the above methods, the surplus culture overflows into a collecting container, from which it can be taken and used as food.

Mass culture in 10 L plastic buckets

Mass culture in 200 L Perspex columns



Mass culture of algae in polythene bags of different size



Continuous culture (40 l vessels)

This method is suitable for the culture of flagellates. The internally-illuminated, continuous culture vessels are made from polyethylene tubing supported by a metal framework. They consist of 160 cm lengths cut from 71 cm wide polyethylene, 'layflat' tubing. The tubing is free of potential contaminants due to the heat used in the manufacturing process and no further sterilization is necessary. The cut length is heat -sealed across the width of one end and positioned around the acrylic cylinder containing the lamps. The six nuts and bolts securing the outer supporting mesh jacket are fastened and the outer reflective sheet of white, corrugated plastic is held in place by 12.7 mm nylon power belting (nylon strapping), which also supports the sensor housing unit against the outer surface of the culture.

The polyethylene tubing is filled (38 l) with required salinity sea water filtered through a sterile, 0.45 micron, particle retention cartridge filter. If the water has a high silt load, it should first be passed through a 2 micron filter and required quantity of media are added to ensure that nutrient levels do not become limiting at the high cell densities at which the cultures are maintained.

A 2.5 cm diameter circle is cut from the tubing, with its centre about 7 cm above the water level. Into this is fitted a 1.9 cm rigid, PVC tank connector, from which a 150 cm length of 1.5 cm bore flexible PVC tubing is run into a 125 litre collecting vessel. The overflow allows for automatic harvesting of the culture into the reservoir.

A supply of filtered air, enriched with sufficient carbon dioxide to maintain culture pH at 7.6-8.2 (about 0.25% $C0_2$ by volume) is introduced through a 0.4 cm bore, 150 cm long, acrylic tube inserted into the top of the culture. A flow rate of about 15 litres per minute ensures efficient mixing of the culture. The 40 l culture should be inoculated with a 2 l batch culture that has gown for 7 to 8 days.

Automatic harvesting of the culture is controlled by the following method. A cadmium sulphide photo conductive cell is enclosed in a light-proof housing against the outer surface of the cul-ture. The housing is placed about 50 cm from the base of the vessel and positioned so that the stream of air bubbles rising through the culture does not interfere with its operation. The resist-ance of the photo-conductive cell will increase as the light intensity reaching it from the lamps falls, when density of the culture increases, due to growth and division of the algal cells. A circuit switching the peristaltic pump will be energized when the resistance of the cell becomes greater than a present value on a relay sensitive to input resistance in the range 50-5000 ohm. Sea water, at required salinity and enriched with 2.5 ml of solution A per l and 0.25 ml of solution C per l, is then pumped from the culture medium reservoir through the filter into the vessel and the volume is maintained by an overflow. The outflow of algae culture from the vessel is collected in an aerated container. As the culture is diluted, the decrease in resistance of the photo-conductive cell, caused by the higher light intensity now reaching it, sensed by the relay and the pump circuit is switched off. The relay should be set so that automatic harvesting of the culture occurs at the density that gives the most yield. Production of 30-40 litres per day, can be expected from these 40litre vessels. When the yield begins to fall appreciably, all of the culture should be harvested for feeding and the bag dis-carded. A new, clean bag should be fitted to the vessel and the above operating procedure repeated.

Though various culture methods are described in detail, the most suitable method for any application will depend on the resources and facilities available and the amount of algae required. There are other similar, methods for growing marine algae that may be more appropriate in certain circumstances. For example, externally-illuminated 20 litre glass jars can be operated as batch cultures or semi-continuous cultures for a variety of algae. Another, widely used, type of culture container includes a mesh framework to support a 200-400 litre externally-illuminated polyethylene bag. Both of these methods have the disadvantage of being less efficient, as the light energy cannot penetrate the culture as well as the internally-illuminated unit, but they do have the advantage of being easier and cheaper to construct. In small production units and backyard hatcheries , the mass culture can also be done in transparent (Perspex)tanks of assorted size depending upon the requirements.

Monitoring algal populations

A regular check of microalgae cultures is essential to prevent crashes and to keep high quality standards. The main parameters to be monitored are: colour, density, pH and contaminant levels. As an example, a change in colour to opaque grey and a pH level lower than 7.5 may indicate a high degree of bacterial contamination. A lighter colour than normal may reveal insufficient nutrients or poor lighting. Mass cultures are normally checked at naked eye by experienced staff and strict controls are usually restricted to pure strains and small vessels. The algal culture growth can be monitored daily by counting the number of cells per ml with a haemocytometer (see below), and their average growth curves can be plotted against values obtained with a colorimeter.

These test cultures are useful for a number of other reasons as it is possible:

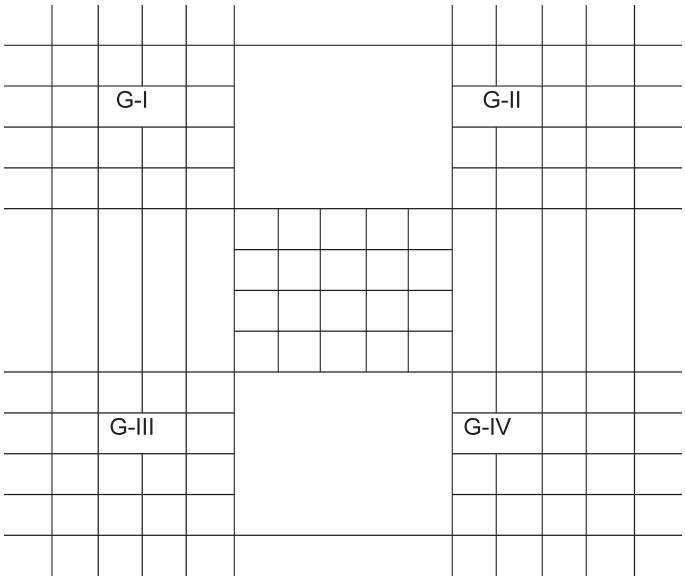
- To determine the growth curve for each species of algae under local conditions;
- To devise criteria for quick identification of possible troubles (eg.; presence of foam, sedimentation pattern, changes in colour, etc.);
- To determine optimal utilization time, i.e. the age at which the algal population reaches the peak of the log-phase;

- To adjust environmental conditions to maximize production;
- To control possible contaminants and try counter measures.

Determination of algal cell density

In order to monitor growth of the algal cultures in various culture flasks as well as mass culture tanks, regular counts of the algal cells need to be conducted. Sampling can be done with sterile serological pipette which can be used for dragging the sample. To get the uniform sample, mild agitation with help of sterilized rod to be done and move the pipette around the tank while withdrawing algae upto the mark on the pipette. Samples can be taken from each corner of the tank and then treated with a drop of eosin or 1% formalin to kill the cells and stirred well. Draw sample in pipette and place the tip of the pipette near the V shaped notch of heamocytometer. The sample runs inside the cover slip and thin film of the culture is formed and the cells are equally distributed. In the same way load the second chamber also and allow to remain for 10minutes. Since the haemocytometer has got 4 grid in 4 corners with 16 divisions in each grid, the counting is restricted to 4 grids at four corners of the haemocytometer chamber . The cell density in 1 ml is calculated as shown below.

$$\frac{\text{Total number of cells in 4 grid}}{4} \times 10^4 = \text{Total number of cells /ml}$$



Surface view of haemocytometer showing grid areas (G=Grid)

If the cell density is in the higher levels (1-25x10^6 ells/ml) count the 8 grids(Both sides) of the haemocytometer.

$$\text{Average No. of cells counted (A)} = \frac{\text{Total No. of cells counted}}{8}$$

$$\text{Cell density (cells/ml)} = \frac{Ax25}{100} \times 10^6 \text{ (cells/ml)}$$

If the cell density is greater than 25x10^6 ells/ml, observe the central square of haemocytometer which is divided in to 25 smaller square, and each of which is further sub divided into 16 sub –squares. Count the number of algal cells in 5 of the 25 squares. Five such squares are counted on each side of the haemocytometer (10 per sample).

$$\text{Average No. of cells counted (A)} = \frac{\text{Total No. of cells counted}}{10}$$

$$\text{Cell density (cells/ml)} = \frac{Ax25}{100} \times 10^6 \quad \text{(cells/ml)}$$

Extensive methods

Outdoor tanks are nutrient-en-riched with agricultural fertilizers for extensive method . Adding (per 1000 l of sea water) 1.5 g of urea ($NH_2CON\ H_2$:46% nitrogen), 1.6 g of triple superphosphate (P_20_5:19.9% phosphorous) and 10.6 g of sodium metasilicate ($Na_2Si0_3.5H_20$:13% silica) will provide the required amounts of nitrogen, phosphorous and silica to stimulate growth and division of algal cells and, depending on the temperature of the sea water and the amount of sunshine on the tanks, blooms of algae will develop. This system can be operated as a batch culture or managed as a semi-continuous or continuous culture with an in-flow of nutrient enriched sea water to provide fresh medium as an impetus to further algal growth. The method is especially suitable for providing algae for feeding to bivalve mollusc spat held in nursery systems, or for mass 'grow-out' of brine shrimps (Artemia) and mass culture of rotifers.

Conclusions

Though different method of large-scale algae culture is adopted, it is liable to be expensive and techni-cally difficult to operate and the success in mass production depend upon the provision of suitable condition. As a result a great deal of research is now being directed towards a suitable algae replacement diet for use in commercial aquaculture however none of them are found suitable than algae for the larviculture, and the demand for algae remains the same in the hatcheries world wide.