DEVELOPMENT OF CULTURE OF MICROALGAE AS LIVE FOOD SOURCE FOR LARVAE CULTURE IN BRACKISHWATER HATCHERIES

A Thesis

By

Md.Mahfuzur Rahman Shah

Roll No.: 990618
Session: 1998-99

The Thesis submitted to the Fisheries and Marine Resource Technology Discipline in partial fulfillment of the requirements for the degree of

Master of Science

in

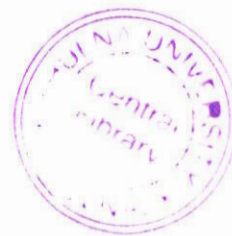
Fisheries and Marine Resource Technology

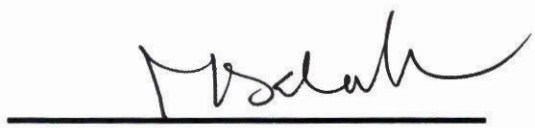
December 2003

DEVELOPMENT OF CULTURE OF MICROALGAE AS LIVE FOOD SOURCE FOR LARVAE CULTURE IN BRACKISHWATER HATCHERIES

Approved as to style and content

By





Professor Dr. Md. Saifuddin Shah
Head
Fisheries and Marine Resource Technology Discipline
Khulna University
Khulna, Bangladesh

DECLARATION

The results submitted in this thesis are entirely of the candidate's own investigations and no part of the results has been accepted for any degree, nor it is being concurrently submitted for any degree

(Supervisor)

Dr.Md.Saifuddin Shah
Professor
Fisheries and Marine Resource Technology Discipline
Khulna University
Khulna, Bangladesh

(Candidate)

Md.Mahfuzur Rahman Shah

Dedicated to

My Beloved Parents

Acknowledgement

It is my great pleasure for being able to complete this thesis. So first of all my appreciation and indebtedness go for the creator of this Universe, the almighty Allah.

I am pleased and felt proud to express my deep appreciation and profound respect to my supervisor Dr. Md. Saifuddin Shah, Professor and Head, Fisheries and Marine Resource Technology Discipline, Khulna University, Khulna for his scholastic guidance, constructive criticism and continued and effective help to execute this thesis work successfully.

I express my indebtedness to Dr. M. J. Alam, Chief Scientific Officer, Brackishwater Station, Bangladesh Fisheries Research Institute, Paikgacha, Khulna for providing me the necessary facilities and assistance to carryout the research work during my study period.

I convey my thanks to my wife Mrs. Rumana Sharmin Bindu, and to all of my friends especially Mr. Hindol Kumar Pal and whose inspirations were all along with me during the hard and tedious times.

Last of all, I express my deepest gratitude to my parents for standing all the way in my side.

December 2003 **Md.Mahfuzur Rahman Shah**

Abstract

Three standard microalgae species viz., *Nannochloropsis oculata*, *Tetraselmis chui* and *Chaetoceros muelleri*, commercially used in aquaculture in many countries, were cultured in small scale batch culture with initial inoculation density of 44 x 10^4 cells /ml for 28 days to determine the effect of salinities (05 ppt to 30 ppt) on the growth under laboratory condition. The BFRI (Bangladesh Fisheries Research Institute) microalgae culture nutrient medium was used as the culture medium. *N. oculata* showed maximum cell growth (345 x 10^4 cells /ml) at 20 ppt salinity. *T. chui* showed better growth (458 x 10^4 cells / ml) at 30 ppt but failed to grow at salinities from 5 to 10 ppt. *C. muelleri* did not tolerate a salinity of 5 ppt but grew well at the salinity range of 20-30 ppt and the highest cell density (395 x 10^4 cells / ml) of this alga was found at 25 ppt. *N. oculata* was the most euryhaline species of microalgae tested here and can tolerate a wide range (5-30 ppt) of salinities. This species is one of the most important species in aquaculture, so the findings from this study have positive implications for brackishwater hatcheries in Bangladesh.

The growth of three microalgae species mentioned above was also investigated using three different inorganic nutrient media: (i) Modified Guillard's f/2 medium; (ii) Rix Mix medium and (iii) BFRI medium. Each microalgae species was cultured at 25 ppt salinity for 24 days in small scale batch system with initial inoculation density of 17x 10^4 cells /ml in the three media with three replications in each. *N. oculata* cultured in modified Guillard's f/2 medium showed superior growth with a mean peak density of 221 x 10^4 cells/ml than in Rix Mix medium (141 x 10^4 cell/ml) and BFRI medium (47 x 10^4 cells/ml) on the 16^{th} day of culture at stationary phase. Considering the increase in cell density at the 20^{th} day of culture in Rix Mix medium, *C. muelleri* was significantly ($P<0.05$) highest than in other two media. *N. oculata* cultured in BFRI medium resulted in the poorest growth with a mean peak increase in density of 84 x 10^4 cell/ml at the 12^{th} day of culture. However, with an increase in cell density, growth of *T. chui* (182 x 10^4 cells /ml) was significantly ($P<0.05$) higher in BFRI medium than in modified Guillard's f/2 medium. *N. oculata* and *C. muelleri* can be grown very well in both the modified Guillard's f/2 medium and Rix Mix medium. Better growth of *T. chui* can be obtained while culturing either in BFRI and Rix Mix medium. These three nutrient media used in the present study may be useful for microalgae species culture for all the target zooplankton, fish and crustacean larvae in marine and brackishwater hatcheries.

Unicellular algae, *Chlorella* sp., were collected from the local brackishwater environment and then isolated by the combination of dilution, single cell collection by capillary glass pipette and agar media used techniques. Pure stock culture protocols of the isolated *Chlorella* sp. was developed to maintain stock culture in the microalgal culture laboratory by using modified Guillard's f/2 medium. The maximum cell density of *Chlorella* sp. reached at 16 X 10^6 cells /ml under pure culture condition within two weeks of culture period. Small scale culture of *Chlorella* sp. at 5-30 ppt salinities showed that it could grow equally well in all the salinities tested with significantly highest cell density (2530 X 10^4 cells /ml) at 20 ppt salinity, which might have the potentiality for development of medium scale culture and mass culture system of *Chlorella* sp. for different finfish / crustacean larvae rearing in hatchery.

CONTENT

List of Tables

List of Figures

List of Photographs

CHAPTER ONE

INTRODUCTION

1. Introduction

Brackishwater and Marine hatcheries rely upon live foods as the main source of feeds for larvae of the target species being cultured. Though micro encapsulated and other inert diets have been developed for some commercial species (e.g. *Penaeus monodon*), there is still a requirement for microalgae and a combination of inert diets along with *Artemia* in the early stages. One of the problems which has slowed down the development of fully artificial diets for many brackishwater and marine larvae is because larvae of the target culture species have very specific food requirements in their larval development and the only feeds which suit the requirements are the natural foods of the larvae (Hoff and Snell, 1989). The movement of the prey species, color, tastes and predator avoidance reactions are some of the factors, which must be considered. Studies have shown that a combination of artificial diets and live foods do equally as well as live foods alone, but artificial diets alone fail (Fulks and Main, 1991; Braley, 1992). Penaeid and freshwater shrimps are backbone of our export oriented shrimp culture industry. Although the species of cultured shrimp and its natural environments involved in its culture and larval rearing vary from one country to another, there are many technical problems shared by different countries. For mass production of penaeid shrimp larvae, mass production of different types of plankton or similar organisms such as a unicellular algae *chlorella,* unicellular yeast, diatom and zooplankton such as rotifer are essential to raise on mono-culture basis.

Microalgae is the base of the food chain for brackishwater and marine ecosystems. Carbon production starts here and many zooplanktons, which include the larvae of the target culture species, require the highly nutritious microalgae for their population growth. Larvae, which are predator species upon other prey zooplankton likewise, must receive their required nutrition from these prey species or else they will not develop successfully to metamorphosis. Prey zooplankton species such as rotifers (*Branchionus plicatilis*) hold the nutrition from their microalgae food for a short period only. Thus, they must be consumed by the predator larvae soon after the rotifer has eaten microalgae or the predator species will have nutrient-depleted food (Hoff and Snell, 1989; Braley, 1994).

Indoor / controlled cultures are now the necessary component of most commercial hatcheries. Monospecific cultures of microalgae can be kept uncontaminated and healthy in this situation. In the most modern hatcheries there are microbiologists who make bacterial plates daily from the working microalgal cultures so that disease species such as *Vibrio* do not

develop in the microalgae food cultures. A steady supply of clean, healthy microalgae can be generated from indoor / controlled cultures and used to inoculate the outdoor / open cultures. These outdoor / open cultures may be the main source of food for zooplankton prey species like rotifers, or may be food for spat of bivalve molluscs. In large hatchery / nursery operations there are plumbing systems set up to quickly transfer microalgae to mass culture tanks and from mass culture tanks to larval or juvenile culture tanks.

The tolerance of marine microalgae to changes in salinity is considered to be extremely broad. Most grow best at a salinity that is a bit lower than that of their native habitat. Different species have different nutrient, illumination, temperature, salinity and pH requirements for optimum growth (Chen and Long, 1991). Tolerate and optimum salinities have been investigated by a number of authors for a variety of commercially important species (Ukeles, 1976; Kaplan *et al.*; 1986; Fabreges *et al.*; 1984; Laing and Utting, 1980; Duerr and Mitsui, 1982).

Nutrients are very important environmental factors that influence the growth of any alga (Okaichi *et al.* 1989). Since the objective of growing microalgae in controlled condition is to obtain the highest density in the shortest possible time, utilization of natural seawater or freshwater without enrichment is not expected to yield significant results. Nutrient media used in the microalgae culture include Conway, Modified F or TMRL medium depending on the species cultured (Kongkeo, 1991). Microalgae grow best in media with quite different primary and trace nutrient composition than natural seawater (Hoff and Snell 1989). Marine or brackishwater microalgal species require a culture medium with a chemical composition similar to that of seawater. Besides carbon, the requirement of principal nutrients for phytoplankton are nitrogen and phosphorus, in an approximate ratio of 6:1 by weight, respectively. Trace minerals (iron, copper, zinc, cobalt, manganes and molybdenum) and vitamins (espccially B_{12} and thiamin and biotin) can also be added. These are necessary in most axenic cultures (Fulks and Main 1991) with addition of silicate for diatom.

Chlorella is primarily a freshwater genus, but some species adapt to a wide range of temperature and salinities, and can be culture in fertilizer enriched artificial saltwater (Wilkerson, 1998). The isolation of local species of microalgae (*Chlorella* sp.) has value for improving the diets, growth and survival of the larvae and post larvae of target predator species under culture. Techniques for isolation and culture of microalgae have been used for many years in different parts of the world. Many techniques for the culture of microalgae also have been developed. The type of culture can be divided into indoor/controlled culture and out door / open culture. Further, there are different approaches to culturing the microalgae,

which include 1) Batch culture; 2) Semi-continuous culture and 3) continuous culture. Batch cultures are the most consistent and reliable method. In batch culture, the microalgae started in one culture vessel and it is harvested completely, when the population reaches its near maximum density. Hoff and Snell (1989) presented a protocol for the small-scale culture of microalgae, which used equipment available for local sources. They suggested clear plastic cola bottle for small culture vessels. Small scale culture of *Chlorella* sp. in brackishwater is needed for upscaling the culture volume from small to medium scale carboy (20 liters) culture because in many research and small to medium size hatcheries all the algae to be used for larvae or post larvae of fish / crustaceans are produced in carboy in a temperature controlled algae culture room. Finally the *Chlorella* cells produced by the medium scale culture can be used for the mass culture in larger tanks. Numbers of research works have been carried out on the isolation and production of *Chlorella* sp. in different media (Hossain *et al,* 1998; Khatun, 1976; Khatun, 1993;Khatun *et al*., 1998).

Very little works so far been done on the culture of live food organisms in Bangladesh. Marine Fisheries and Technology Station of Bangladesh Fisheries Research Institute, Cox's Bazar had conducted experiments on isolation, propagation, mass seed production and analysis of nutritive value of some of the important planktonic feed organisms. Simple technique was developed for isolation of unicellular algae, *chlorella* sp. by dilution method (Hossain, *et al,* 1994). Many scientists worked on culture and other aspects of microalgae throughout the world (Chen, 1991, Chen, and Long, 1991, Dhart and Sorgellos, 1995, Hoff and Snell, 1989, Guillard and Ryther, 1962, Liao, Su. and Lin,1983, Lim, 1991, McLachlan,1961, Fox,1983, Okauchi,1991, Sato, 1991, Park,1991, Gladue,1991).Currently, there are several algae species popular in aquaculture including; several species of *Chlorella* sp, *Tetraselmis chui, Nannochloropsis oculata, Chaetoceros muelleri* etc.

The effect of salinity (5 ppt- 30 ppt) on the growth performance of the three microalgae viz. *Nannochloropsis oculata, Tetraselmis chui* and *Chaetoceros mulleri* by using modified Guillard's f/2 medium and the effect of the three selected inorganic nutrient media, viz., modified Guillard's f/2 medium, Rix Mix medium and BFRI medium on the growth of the above three species of microalgae at 25 ppt salinity in temperature controlled laboratory condition were studied. Beside these, the investigation were carried out to develop techniques and protocols for isolation, pure culture and small scale culture of *Chlorella* sp. at different salinities (5 ppt- 30 ppt).

N. oculata showed maximum cell growth at 20 ppt salinity. The maximum cell density of *T. chui* was observed at 30 ppt . *C. muelleri* did not tolerate a salinity of 5 ppt but grew well at

the salinity range of 20-30 ppt and the highest cell density of this alga was found at 25 ppt. *N. oculata* cultured in modified Guillard's f/2 medium showed superior growth than Rix Mix medium and BFRI medium . In Rix Mix medium, *C. muelleri* growth was higher than in other two media. Growth of *T. chui* was higher in BFRI medium than in modified Guillard's f/2 medium. Unicellular algae, *Chlorella* sp., cells were collected from the brackishwater pond and then isolated by the combination of dilution, single cell collection by capillary glass pipette and agar media used techniques. Pure stock culture protocols of the isolated *Chlorella* sp. was developed to maintain stock culture in the microalgal culture laboratory by using modified Guillard's f/2 medium. Small-scale culture of *Chlorella* sp. at 5 ppt-30 ppt salinities showed that it could grown equally well in all the salinities tested with highest cell density at 20 ppt salinity.

The collection of or isolation of local species of live food organisms and development of protocols to efficiently culture them will be of benefit for nutrition of the local target species in culture. The research program may assist in paving the way for the hatchery culture of species such as *Scylla serrata* and *Lates calcarifer* and other suitable finfish / crustaceans. Once the protocols have been developed for the culture of the appropriate live food species for each of the target culture species, the chances of success will increase.

The purpose of the research was to develop the suitable methods of culture of microalgae as live food source for brackishwater or marine hatcheries in Bangladesh so that the culture of a wider variety of target culture species may be possible in future. The specific objectives of the program were:

- To determine the effect of salinity and culture media on the growth of the three standard microalgae under small scale batch culture system.
- Isolation, maintenance of pure stock culture protocols of local *Chlorella* sp., collected from brackishwater environment and to develop its small scale culture method under variable salinity in laboratory condition.

CHAPTER TWO

CULTURE OF MICROALGAE AS LIVE FOOD

2.1. The role of microalgae:

Microalgae are the basic synthesizers of organic matter in aquatic environments. Microalgae utilize photosynthesis to construct complex carbon molecules like the more familiar land plants. Dissolved nutrients from various sources are absorbed by microalgae and in the presence of light, combined into more complex molecules necessary for survival. Microalgae are major contributors to the production of biomass in oceans, estuaries, lakes and reservoirs. Although small the combined effects of billions and billions of these cells provide most af the plant material consumed animals higher in the food web. The entire food web receives its energy from the biomolecules synthesized by these microscopic plants. In addition to the supplying food for animals, microalgae play a central role in nutrient cycling in aquatic habitats (Fig.1). Microalgae absorb primary nutrients such as ammonia, urea, nitrates, phosphates and potassium and metals such as iron, copper etc. Certain vitamins such as B_{12}, thiamin and biotin also have been found to be essential or beneficial to many algae species and are selectively absorbed. Removal and alteration of these nutrients by microalgae in the ocean is not permanent, since they are slowly but constantly being regenerated as microalgae cells die and decompose. In marine and freshwater aquaria or culture tanks, microalgae help to maintain water quality through their removal of excess nutrients and the regulation of pH. In effect, each algal cell is a living biofilter (Hoff and Snell, 1989).

2.2. Nutritional aspects of microalgae:

In general, the nutritional value of microalge is directly related to nutrient supply, light and other physical and chemical conditions during growth. Microalgae are direct food for all life stage of bivalves, for larvae of many crustaceans and very early growth stages of some fish. Microalgae are used to rear mass quantities of rotifers, copepods, and brine shrimp which in turn are used to feed late larval stages and juvenile of crustaceans and fish. The nutritional value of the algae is also passed on through the zooplankton to the fish or crustaceans being aquacultured. The size, digestibility and nutritional value of the microalgae determine how successful it is to rear various species in aquaculture (Brown *et al.*, 1989).

2.3. General principles:

2.3.1. Population dynamics:

In order to achieve sufficient production of culture on a consistent basis, an understanding of algal growth dynamics is required. The goal of any algal production facility concerned with

feeding shrimp is to supply as much algae as required as efficiently as possible. There are five typical phases during the growth of an algal culture (Fig.2.) (Holf and Snel, 1991):

1) Lag or induction phase where little increase in cell density occurs. This phase is easily observed when an algal culture is transferred from a plate to a liquid culture.
2) Once the lag phase is completed, the culture enters exponential phase where cell density increases rapidly according to a logarithmic function.
3) A significant slowing of cell division occurs when nutrients, light, carbon dioxide or other physical and chemical factors begin to limit growth.
4) Stationary phase is reached when cell density remains relatively constant for an extended period of time. Stationary phase is very short in batch cultures where nutrients are consumed without replacement, but it can be extended in continuous cultures.
5) As water quality deteriorates and cell starve, cell density begins to decrease rapidly, a situation called a " crush".

2.3.2. Types of culture:

Microalgae cultures may be coarsely divided into indoor and outdoor systems. Fulks and Main (1991) compare the two basic types of culture saying that indoor cuture has been fairly well worked out. Temperature, lighting and water quality can be controlled, while predators and diseases are kept out. In contrast, growing cultures outdoors for substantial periods have failed. Some species of microalgae dominate over others by natural selection. Outdoor cultures are more rapidly contaminated, but for large volumes this is still the only method available to us. The following description of three types of culture, whether indoor or outdoor, is paraphrased from Fulsk and Main (1991):

a) Continuous culture: There are many advantages to continuous cultures, including a steady supply of high quality, log phase cells; a grater rate of production; and automation (Table.1) and may be preferred for small volume and research purpose.
b) Semi-continuous culture: The microalgal population is allowed to grow to a desired cell density-partially harvested and fresh medium added. Indoor or outdoor is possible, but eventually predators, competitors, or other contaminants appear and metabolites build up.
c) Batch culture: Microalgae culture is started in one vessel and it is harvested completely when the population reaches its near maximum density. Batch cultures are consistent and the most reliable method. Contaminants are less than in old cultures of semi-continuous

systems. More tanks are required in batch cultures compared with semi-continuous because less algae is harvested from a tank than in a semi-continuous culture sys tem.

2.3.3. Species:

Chlorella, Chaetoceros, Isochrysis, Nannochloropsis,Tetraselmis and *Dunaliclla* are the genera of algae most commonly cultured for aquacultural purposes . Species are usually chosen on the basis of size, nutritional value and easy of culture (Persoone and Claus 1980, Ukeles 1980, Liao *et al* 1983). These are popular because they have specific nutritional qualities beneficial to many marine animals and can be grown to high densities in mass cultures.

***Chlorella* sp.:**

Chlorella sp. (3-9 μm) is often cultured as food for marine rotifers. It is primarily a freshwater genus, but some species adapt to a wide range of temperatures and salinities and can be cultured in fertilizer-enriched artificial saltwater. *Chlorella* is a non motile alga, which simply means that cells do not move by themselves, but depend upon water currents for mobility. This alga is lower in the ω–3 fatty acid EPA (eicosapentaenoic acid) content than most other marine algae (Wilkerson, 1998).

***Chaetoceros muelleri*:**

There are many species of *Chaetoceros* available as food for larval peaenids. *Chaetoceros muelleri* (4-5μm) is high in omega –3 HUFA's content and its overall nutritional value is also high (Fulks and Main, 1991)

***Tetraselmis chui*:**

Tetraselmis is an ancient genus, even by algal standards, which measured in billions of years. *T. chui* (7-10 μm) is a green flagellate and motile alga can be cultured in a nutrient mix similar to *Nannocholoropsis*, but it is nutritionally inferior to *Nannocholoropsis* as far as fatty-acid content is concerned. This large- celled alga may also prove useful as a direct food in culturing organisms that are too small to accept rotifers (Wilkerson, 1998).

Nannocholoropsis oculata :

N. oculata (2-4 μm) is widely used as a marine rotifer food. It is easily cultured and is high in both vitamin B_{12} and the highly unsaturated fatty acid EPA needed by larval and juvenile marine fishes. This is a dark green alga with a thick, tough cell wall that protects it from possible damage by overstirring during the micro culturing period (Wilkerson, 1998).

2.4. General Requirements and consideration:

2.4.1. Culture media / nutrients:

Most of the microalgae grown as feed for commercially important aquatic animals are marine or brackishwater species. Hence, they require a culture medium with a chemical composition similar to that of seawater (Fulks and Main, 1991). Nitrogen and phosphorus are the primary nutrients of microalgae, needed in a ratio of about 10:1. Algae also need trace minerals, (including iron, copper, zinc, cobalt, manganese and molybdenum) and vitamins (B_{12}, thiamin and biotin). In addition, brown or tan –colored micrialgae needs silicates. There are several papers that describe the classical microalgal media (Ukeles 1971, Walne 1974, Guillard 1975; Borowitzaka, 1998).

2.4.2. Light:

Light is the source of energy, which drives photosynthesis. In order to maximize yield, one must maximize the efficiency with which available light is converted into algal biomass. Maximum culture depth and cell density are the key variables regulating light utilization efficiency (Roels *et al.*, 1977). Hoff and Snell (1989) state that an intensity in the range of 2500-5000 lux is optimal. A photoperiod of 16 hours a day is adequate, but 24 hours a day is both convenient and suitable (Wilkerson, 1998).

2.4.3. pH:

A hydrogen ion concentration (p^H) that is too high or too low will slow algal growth by disrupting cellular processes. The optimum p^H range for most of the species cultured falls between 7 and 9. The "most optimum" range, furthermore, is reported to be 8.2- 8.7 (Ukeles, 1971) (Table.2). Complete culture collapse can result from a failure to properly monitor and maintain an acceptable p^H. Fortunately, this is easily accomplished in moderately dense cultures through aeration, a process which serves other purposes as well (Hoff and Snell, 1989).

2.4.4. Temperature:

Each species is regarded as having minimum, maximum and optimum temperature range for growth. In general, the most commonly cultured species of microalgae tolerate temperatures between 16 and 27 ° C. 20 to 24 ° C may be considered "optimum" (Guillard, 1975). Temperatures lower than 16 ° C will slow growth, but those higher than 35 ° C are lethal for a number of species (Hoff and Snell ,1989) .

2.4.5. Salinity:

The tolerance of marine algae to changes in salinity is considered to be extremely broad. Most species grow best at a salinity that is a bit lower than that of their native habitat. Tolerated and optimum salinities have been investigated by a number of authors for a variety of commercially important species (Ukeles, 1976; Kaplan, *et al.*, 1986; Duerr and Mitsui, 1982)

2.4.6. Aeration:

Good aeration enables carbon dioxide, found in relatively minute amounts in air but essential to the photosynthesis process, to be absorbed by culture water. If the culture water is not aerated, the carbon dioxide is quickly consumed and algal growth ceases. Aeration also maintains the p^H of the culture and keeps it uniform in terms of temperature and cell distribution (Wilkerson, 1998).

2.4.7. Monitoring:

Culture crushes can happen overnight, so it is important to closely monitor cultures for early warnings of a crush like declining cell densities or changes in culture color. Algal cells from declining populations also have little nutritional value for zooplankton (Hoff and Snell, 1989). Cell densities can be monitored in a variety of ways, but Secchi disks and haemocytometers are probably the most commonly used devices. Secchi disks are quick and easy to use, but haemocytometers provide more accurate measurements (Valero *et al.*, 1981)

2.4.8. Equipment and supply:

The equipment needed to culture microalgae is highly dependent on the type and scale of culture. Most of the equipment needed can be easily obtained from local sources. Many different shapes and sizes of vessels can be used to culture microalgae, from rectangular tanks to plastic buckets or suspended plastic bags. Generally, a cylinder shaped container with round or concave bottom, clear transparent sides and a restricted or covered opening is best. For small scale, home aquarium level culture we fell that glass, wide-mouth gallon jars or the clear plastic cola bottles are best (Hoff and Snell, 1989).

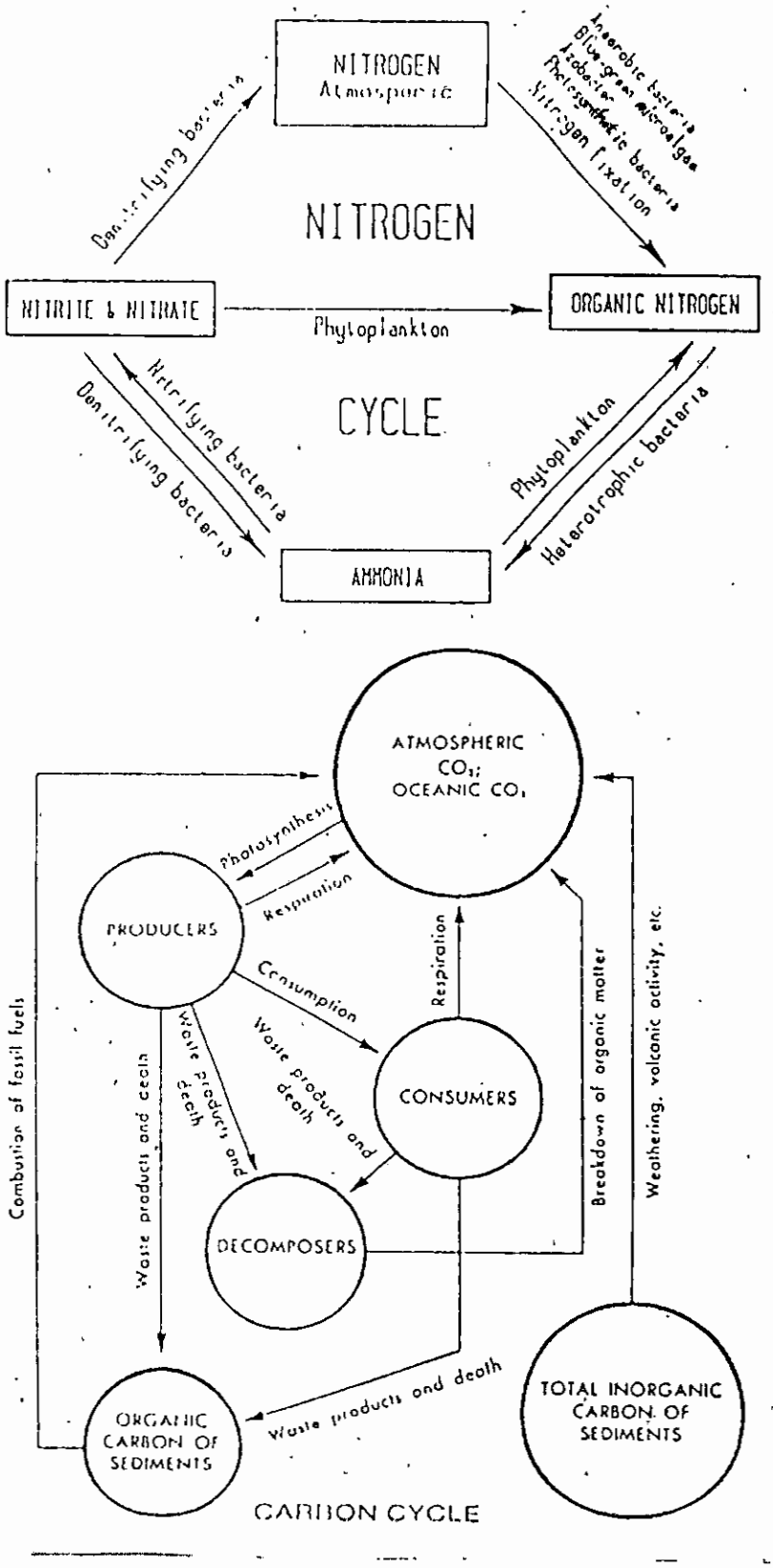


Figure 1. Nitrogen and carbon cycle in the aquatic food web.

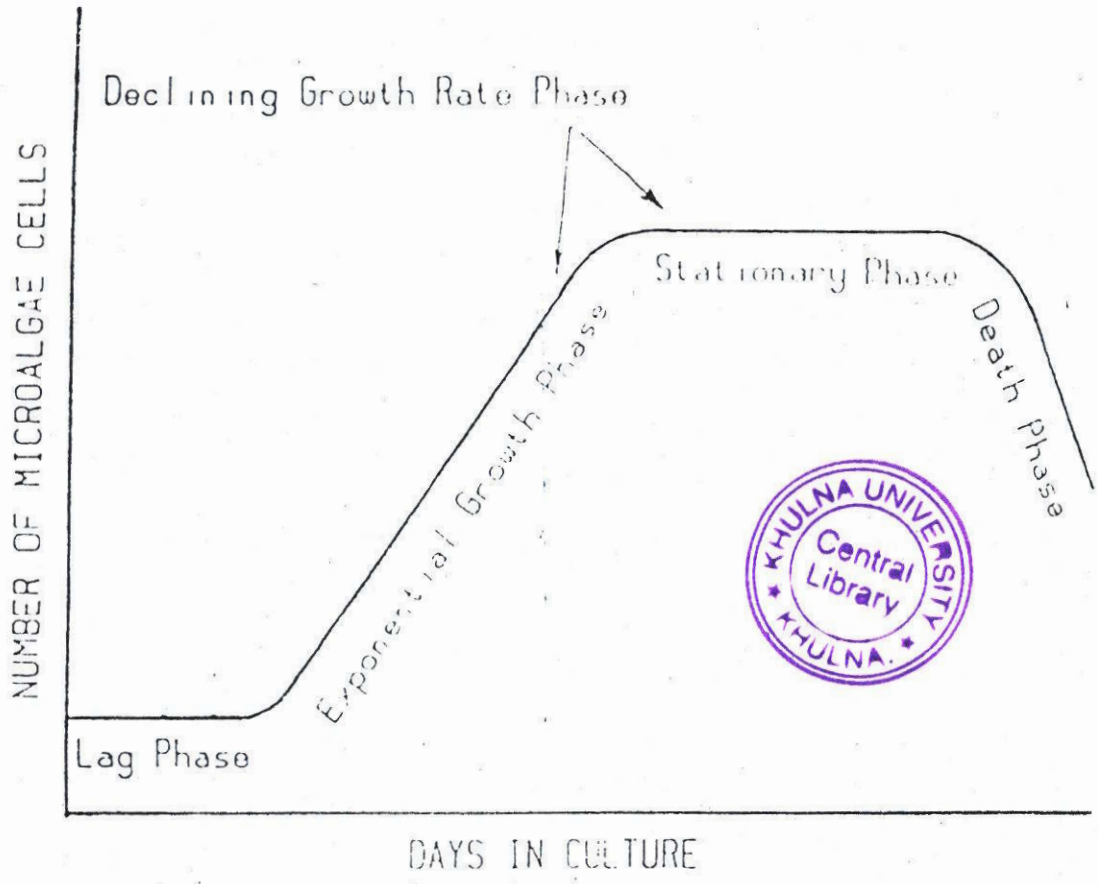


Figure 2. Five typical growth phases of microalgae cultures.

Table-1.The primary types of microalgae culture.

Culture type	Advantages	Disadvantages
Indoor Outdoors	A high degree of control (predictable) Cheaper	-Expensive -Little control (less predictable)
Closed Open	Contamination less likely Cheaper	-Expensive - Contamination more likely
Axenic Non axenic	Predictable, Less prone to crashes Cheaper, less difficult	-Expensive, difficult -More prone to crashes
Continuous	Efficient, provides a constant supply of high quality cells, automation, highest rate of production over extended periods	Difficult, usually only possible to culture small quantities, complex, equipment expenses may be high
Semi-continuous Batch	Easier, somewhat efficient Easiest, most reliable	-Sporadic quality, less reliable -Least efficient, quality may be inconsistent

Table-2. A generalized set of conditions for culturing microalgae

Parameters	Range	Optima
Temperature (° C)	16-27	20-24,18-22
Salanity (ppt)	12-40 (for neritic flagellates)	20-24
Light intensity (lux)	1,000-10,000 (depend on vol., density)	2,500-5,000
Photoperiod (light hours :dark)		16:8 (minimum) Continuous (maximum)
pH	7-9	8.2-8.7 4

CHAPTER THREE

REVIEW OF LITERATURE

3. Review of Literature:

Stein (1973) reported the use of many nutrient media for microalgae culture based on natural seawater enriched with soil extract, nitrates and phosphates.

Hoff and Snell (1989) reported that the Guillard's enrichment medium, which consists of inorganic nitrates, phosphates, trace metals and vitamins, has become popular in aquaculture.

Chen and Long (1991) stated that the tolerance of marine microalgae to changes in salinity is extremely broad and most grow best at a salinity that is a bit lower than that of their native habitat. They also stated that the suitable salinity ranges for growth of *Chaetoceros* sp. and *Chaetoceros simplex* were 18-22 ppt and 13-18 ppt in China.

Sato (1991) reported 32 ppt salinity for mass culture of *Nannochloropsis oculata* using F/2 medium with the maximum cell growth of 38×10^6 cells/ml in Hawaii.

Hur (1991) observed 30-35 ppt optimum salinity for the growth of blue green microalgae *Nannochloris oculata* in Korea.

Chen (1991) reported the optimum salinity to be 20-35 ppt for the growth of *Chaetoceros muelleri* and 30-40 ppt for *Tetraselmis subcordiformis* in China. He also observed a maximum of 200-250 x 10^4 cells/ml of *C. muelleri* in cemented ponds and tanks in large scale culture system using $NaNO_3$: 60g/ton, KH_2PO_4: 4 g/ton, Vit B_1: 100 mg/ton and Vit B_{12}: 0.5 mg/ton.

Chu et al. (1964) studied the effects of different nutrients on algal growth and reproduction and observed that N: P: Fe: Si = 120:12:1:10 showed better performances.

Chin et al. (1965) and Chen et al. (1982) investigated the effect of various illuminations, temperature and salinity regimes on the growth of cultured algae and found 4- 36 ppt salinity for *Nannochloropsis oculata* and salinity range of 8-80 ppt with optimum range of 30-40 ppt for *Tetraselmis* sp.

Konkeo (1991) observed 25-30 ppt optimum salinity for mass culture of *Chaetoceros calcitrans*. He also reported that nutrient media used in the microalgae culture include Conway, Modified F or TMRL media in Thailand

Liao *et al.* (1983) reported that the suitable salinity for culture of *Tetraselmis chui* in natural seawater to be 15 to 36 ppt and the minimal salinity for *Chaetoceros gracilis* is 6 ppt,but can grow well at salinities up to 50 ppt with optimum growth occurring between 17 and 25ppt .He also isolated *Chlorella* sp from the brackishwater fish ponds of the Tungkang Marine Laboratory, Taiwan and cultured at salinities between 0-35 ppt, with an optimum range of 10-20 ppt.

Anderson and Smith (1986) cultured *Chlorella* sp in 35 L cylinder at 20 ppt salinity using modified Guillard's F/2 nutrient media and obtained a peak density of 1.5 to 2.5 x 10^6 cells / ml in 5 days.

Hossain *et al.* (1998) worked on the isolation of *Chlorella* sp. and recorded the maximum cell density of 12 X 10^6 cells /ml through propagation and isolation by dilution method

Khatun *et al.*(1998) isolated *Chlorella* sp. by applying single cell isolation technique and cultured in mass scale using cheaper media (liquid extract of maskalai bran and ammonium phosphate) and obtained maximum yield of 4g/l/day (dry weight).

Laing and Utting (1980) found optimal salinity range of 25-30 ppt for growth in medium prepared from artificial seawater for *Tetraselmis suecia.*

Lim (1991) found 26-31 ppt optimal salinity for culture of *Tetraselmis tatrathele,* 22-25 ppt for *Nannochloropsis oculata* and observed a peak density of 20-25 x 10^6 cells/ml of *N. oculata* using Walne's medium in 3-L polyethylene bag.

Valero et al. (1981) reported that besides carbon, the principal nutrients for microalgae culture are nitrogen and phosphorus, in a ratio 6:1 by weight respectively.

Ukeles (1976) reported that the trace minerals (iron, copper, zinc, cobalt, manganese and molybdenum) and vitamins (B_{12} and thiamin and biotin) are necessary for microalgae culture. He also found the salinity range to be 12-40 ppt with optimum being 20-24 ppt for neritic flagellates microalgae culture.

Aquacop (1986) stated two isolation procedures (Gelose medium method and Dilution method) of *Chlorella* sp and observed 20 million cells /ml within 5 days while culturing in 2 liter flasks using KNO_3 -5.26%, $(NH_4)_2$ SO_4 - 3.69%, Superphosphate – 4.21%, Ca SO_4 - 2.63%, Mg SO_4 - 2.95%, SO_4 - 6.03% and Oligoelements-0.13%.

Fox (1983) reported several methods of isolation viz., simple capillary pipette isolation, streak plating followed by capillary pipette isolation, spray plating followed by capillary pipette isolation, dilution followed by capillary pipette isolation, treatment with antibiotics, ultrasonic vibration.

CHAPTER FOUR

MATERIALS AND METHODS

4. Materials and Methods

The experiments were carried out in a temperature controlled (about 25° C) Microalgal Laboratory at Brackishwater station (BS) of Bangladesh Fisheries Research Institute (BFRI), Paikgacha, Khulna during 2001-02, details of which are as follows:

4.1.Study-1: Effect of salinity on the growth performances of three microalgal species (*Nannochloropsis oculata, Tetraselmis chui* and *Chaetoceros muelleri*) by using BFRI medium under small-scale batch culture system.

4.1.1. Culture vessels:

The culture vessels, 1.5-L cola bottles available from local market were used to culture the microalgae followed by Hoff and Snell (1989) as the shape of the cola bottle is cylindrical and the bottom is round to provide good circulation. Cleaning of the culture vessels was performed according to the cleaning procedure for small-scale culture recommended by Hoff and Snell (1989) as it is essential for successful culture.

4.1.2. Treatment of water:

The water of different salinities (5-30 ppt) was filtered through 1-micron cartridge filter bag to remove particulate materials and treated with 30ppm chlorine at a rate of 0.1gm/l and dechlorinated by using 0.175 g/l of sodium thiosulphate. To avoid the excess sodium thiosulphate in prepared brackishwater media for inoculation of algal cells, the rate of application and period (30 minutes) of dechlorination was maintained properly.

4.1.3. Light:

The bottles were set up approximately 15 cm away from the two 'Daylight' fluorescent tube light sources (a new fluorescent bulb is about 2500 lux). Photoperiod was maintained as 16 hours light and 08 hours dark.

4.1.4. Aeration:

The cola bottles were provided with an aeration tube entering and an exit tube coming through the plastic screw cap of the bottle. Aeration by aquarium aerators was moderate

4.1.5. Nutrient medium:

BFRI medium was used as culture nutrient medium to study salinity effect. The BFRI microalgae culture nutrient medium was prepared at the Microalgal Laboratory, Brackishwater station of BFRI. The application rate of BFRI medium was 1ml/L seawater or

brackishwater. The trace metals and vitamins ingredients were similar to the standard microalgal culture media, Guillard's modified f/2 medium (Gillard & Ryther, 1962) but the amount was not the same.

4.1.6. Inoculation density and culture period:

The algal cells were inoculated with initial concentration of 44 x 10^4 cells /ml for all species of microalgae and the total culture period was twenty-eight days.

4.1.7. Estimation of growth:

Counts of microalgae cells were made at every four days. The cell concentration was determined by using an improved Neubauer Haemocytometer by applying the methodology used by Hoff and Snell (1989). The following is the procedure for using a Haemocytometer: A sample is taken with a pasteur pipette and placed into the V-well at one end of the two-sided chamber. The cover slip is over the slide and the liquid can be seen to flow under the cover slip and over the counting chamber area. If it has not filled the chamber then not enough liquid was added. A second sample can be placed in the chamber opposite this one to get more accurate estimate. There are 25 central squares in the etched lines in the counting chambers. These 25 square are further divided into smaller squares within the large 25, however, only the 25 squares need to be used. Using a compound electronics microscope on the 10 X objective count all cells within the 25 squares (or if the number is over 150 cells in these 25 squares, then 5 random squares can be counted and the sum is multiplied by 5). The total number of microalgae cells are ten multiplied by 10,000. This figure is the number of cells in one milliliter of microalgae culture.

4.1.8. Experimental Design:

Salinity range tested was 05- 30 ppt in 5 ppt intervals by using BFRI medium. There were three replications for each treatment i.e. each salinity.

4.1.9. Data analysis:

All the data were analyzed statistically for Analysis of Variance (ANOVA) was done and the correlations were seen by using Duncun's New Multiple Range Test (Zaman *et al.*, 1982).

4.2. Study-2: Effect of three inorganic media (modified Guillard's f/2 medium, Rix Mix medium and BFRI medium) on the growth performances of the three microalgal species (*N.oculata, T. chui* and *C. muelleri*) at 25 ppt salinity under small-scale batch culture system.

4.2.1. **Culture vessels:** Same as study-1.

4.2.2. Treatment of water:

The water of 25 ppt salinity was used for the culture of the algae species in this study and similar water treatment procedure as study –1 was followed.

4.2.3. Light: Same as study-1.

4.2.4. Aeration: Same as study-1.

4.2.5. Nutrient medium:

Media types used to study the effect of nutrient media were modified Guillard's f/2 media, Rix Mix, and BFRI (Bangladesh Fisheries Research Institute) medium. The standard nutrient medium used for algal culture was the Medium-F presented by Guillard and Ryther (1962). The Guillard's F medium suitable for the growth of most algae and is being used extensively (Fox 1983). The modified Guillard's f/2 medium used in this study was modification from Guillard's F medium for microalgal culture. The Rix Mix medium is used by the private laboratory in north Queensland, Australia for stocks and medium size working culture (Braley 2001). The composition of Rix Mix, BFRI medium and modified Guillard's f/2 medium is shown in Table-3. The application rate of BFRI medium and modified Guillard's f/2 medium was 1ml/L seawater or brackishwater and for Rix Mix it was 2 ml/L seawater or brackishwater. The application rate of sodium metasilicate was 1 ml to 1-L seawater or brackishwater for *Chaetoceros* only.

4.2.6. Inoculation density and culture period:

In case of nutrient media effect experiment, the algal cells were inoculated with initial concentration of 17 x 10^4 cell /ml for all species of microalgae and the total culture period was twenty-four days

4.2.7. Estimation of growth: Same as study-1.

4.2.8. Experimental Design:

Media types tested were modified Guillard's f/2 media, Rix Mix, and BFRI (Bangladesh Fisheries Research Institute) medium and 25 ppt water was used for culturing the algae species. There were three replications for each treatment i.e. each nutrient medium.

4.2.9. Data analysis: Same as study-1.

Table-3. Composition of Rix Mix, BFRI medium and Modified Guillard's f/2 medium.

Ingredients	Amount of ingredient (g or ml)			Comments	
	Rix Mix	BFRI medium	Modified Guillard's f/2 medium		
Thrive *	99g/1-L dw	-		***Thrive is common garden fertilizer in Australia having following chemical constituents:**	
Ammonium sulphate	27g/1-L dw	10 g/100 ml distilled water (dw)		Nitrogen (N) as Nitrate Nitrogen (N) as ammonia form Nitrogen as urea **Total Nitrogen (N)** **Phosphorus (P) as water soluble** **Potassium (K) as potassium nitrate** Magnesium (Mg) as magnesium sulphate Sulphur (S) as sulphates Copper (Cu) as copper sulphate Zinc (Zn) as zinc sulphate Boron (B) as sodium borate Manganese (Mn) as manganese sulphate Iron (Fe) as chelated iron Molybdenum (Mo) as sodium molybdate Maximum Biuret	3.0% 2.6% 21.4% **27.0%** **5.5%** **9.0%** 0.25% **0.22%** **0.005%** **0.02%** 0.005% 0.04% 0.18% 0.002% 0.4%
Urea	-	23 g/100 ml dw	-		
TSP	-	2g/100 ml dw	-		
Borax	-	1g/100 ml dw	-		
Sodium nitrate	-	-	75g/1000 ml dw		
Sodium dihydrogen orthophosphate	-	-	5g/1000 ml dw		
Citric acid	-	-	16.8 g/1000 ml dw		
Iron (ferric) citrate	-	-	3g/1000 ml dw		
Sodium metasilicate	20 g/L dw	20 g/L dw	20 g/L dw	For Diatom only	
Copper sulphate	-	1g/100 ml dw	1g/100 ml dw		
Zinc sulphate	-	2.2 g/100 ml dw	2.2 g/100 ml dw		
Sodium molibdate	-	0.6g/100 ml dw	0.6g/100 ml dw		
Manganese chloride	-	18g/100 ml dw	18g/100 ml dw		
Cobalt chloride	-	1g/100 ml dw	1g/100 ml dw		
Multi-Vitamin B	1- tab crushed/ 1-dw	-	-		
Vitamin B_1	-	10g/100 ml dw	10g/100 ml dw		
Vitamin B_{12}	-	0.05g/100 ml dw	0.05g/100 ml dw		
Vitamin Biotin	-	0.05g/100 ml dw	0.05g/100 ml dw		

4.3. Study-3: Isolation, maintenance of pure stock culture protocols of local *Chlorella* sp, collected from brackishwater environment and to develop its small scale culture method under variable salinity in laboratory condition.

4.3.1. Isolation of *Chlorella* sp.:

The water samples with microalgae were collected from the six ponds of Brackishwater pond complex and adjacent Shibsha River to isolate *Chlorella* sp. The water sample was passed through 30-micron mesh plankton net. Then the water, enriched with modified Guillard's f/2 media (@ 1ml/l) was cultured in 1.5 L cola bottle with aeration. After ten days microalgae were ready for isolation. During collection of sample battery operated acrator was used to carry the sample from field to the laboratory.

Step-1

The water sample (with confirmed microalgae inside) was diluted to several concentrations (1:25,1:50,1:100,1:200) with sterile water of the same salinity. One drop of plankton water was placed on a microscopic slide and checked under the compound microscope and individual cells removed by a specially designed capillary glass pipette (visible under the microscopic field of view) with a silicon tubing from the pipette end to the mouth of the observer. When a desired cell was seen and the pipette end was positioned in front of it, the operator sucked slightly on the tubing to pull the cell into the pipette. Selected cells were placed on a second slide with a few drops of sterile seawater for cleaning purpose. Approximately 50 cells of isolated algae were used to inoculate a 15-ml test tube in step-2.

Step-2

Selected microlagae (*Chlorella* sp.) from step-1 were inoculated in a 15 ml test tube filled with water medium (20ppt) and microalgae culture nutrient media, modified Guillard's f/2 media (@ ½ ml/l seawater) and incubated in incubator at 25°C for seven days. Then a loopful of culture was streaked on to the surface of an agar plate. The plate was then incubated in incubator at 25°C (upside down) for seven days. The whole process was repeated several times until a pure strain was obtained. The test tubes and water with culture media were sterilized (121°C temperature, 15lb pressure and 30 minutes) before inoculation of isolated individual cell.

4.3.2. Pure culture of *Chlorella* sp. in laboratory:

One loopful of *Chlorella* sp. from the best growth obtained above was inoculated in a sterile 15 ml test tube with enriched medium (water + modified Guillard's f/2 media @ ½ ml/l). Total 20 numbers of 15-ml test tubes were inoculated with isolated algae. The tubes were stoppered but not tightly with screw cap and kept on a lighted self at temperature controlled (25±1^{0}C) microalgal laboratory of Brackishwater station. The test tubes were agitated twice a day. After seven days, inoculated *Chlorella* sp. from the test tube was inoculated into 15 numbers of 250 ml and 15 numbers of 500-ml Erlenmeyer flasks containing sterilized water (20ppt) medium of 100 ml and 150 ml respectively (photograph 1.). The microalgae culture nutrient media, modified Guillard's f/2 media was used @ 1ml / l of water to propagate the algal cells. The mouth of the flasks was closed with cotton bung and the flasks were placed near a fluorescent lamp (light intensity 2500 LUX).

At every step, the cultures were examined under a compound microscope for contamination. No direct aeration was provided but routinely agitated 2 times a day throughout the culture period. After 2 weeks when the seawater media inside the flask turned green, these were examined under the microscope and counted the *Chlorella* cells with the help of a Neubaur haemocytometer according to the method described by Hoff and Snell. At every 12 days new water medium was prepared and the *Chlorella* cells were inoculated from test tubes to test tubes and flasks to flasks to continue the algal cell generation to maintain the pure culture of this strain. By repeating this process, pure stock culture of *Chlorella* were maintained in the microalgal laboratory of Brackishwater Station. A 15-ml test tube with seawater media was inoculated with 2-ml inoculent (algae) and a 15-ml test tube stock was used to inoculate a 100-ml flask. Before inoculation of algal cells, the seawater medium was treated with 30ppm chlorine (available in the local market) @ 0.1gm/l of water and after 30 minutes sodium thiosulphate (0.175gm/l) was mixed for proper dechlorination to disinfect and make the seawater free from contaminant.

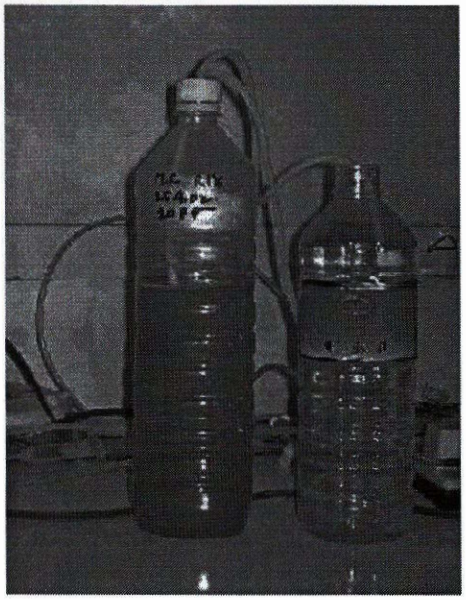
4.3.3. Small scale culture of *Chlorella* sp. under variable salinity:

Small-scale batch culture of isolated *Chlorella* sp. was done in a temperature controlled (about 25 ° C) Microalgal laboratory of BS, BFRI. The culture vessels, 1.5 -L cola bottles available from local sources were used to culture the microalgae. The cola bottles were provided with an aeration tube entering and an exit tube coming through the plastic screw cap of the bottle (photograph 2.). The water of different salinities (05-30 ppt) were filtered through 1 micron cartidge filter bag to remove particulate and treated with 30ppm chlorine (@0.1gm/l of brackishwater) for 30 minutes and then dechlorinate with sodium thiosulphate (0.175gm/l). The salinity of 5 ppt, 10 ppt, 15 ppt, 20 ppt, 25 ppt and 30 ppt were tested for this small-scale culture trial for 30 days. Culture media type used was modified Guillard;s f/2 media (Guillard and Ryther 1962)(the compositions of modified Guillard;s f/2 media is shown in Table-3 and photograph 3.). The modified Guillard;s f/2 media was applied at the rate of 1ml/ L of seawater or brackishwater. There were three replications for each treatment i.e. each salinity. The cola bottles with 1.5 liter prepared water medium were set up approximately 15 cm away from the two "Day light" fluorescent tube light sources (a new fluorescent tube light is about 2500 LUX). The photoperiod was maintained as 16 hours light in a 24 hours period. The *Chlorella* cells were inoculated from the pure stock culture into the cola bottles with an equal initial concentration of 55 X 10^4 cell / ml. Moderate aeration was provided from the aquarium aerators into the cola bottles through aeration tubing. Counts of algal cells were made at every 03 days throughout the culture period using an improved Neubaure Haemocytometer according to the methodology described by Hoff and Snell (1989). The average number of cell division per day (K) for the 15 days growth period was calculated from:

$$K \text{ (division/day)} = 3.322 \; \frac{\text{Log} \; \frac{N_t}{N_o}}{t_o - t_l}$$

Where. N_t and N_o are cell concentration at time t and 0 respectively(Guillard,1973).

Photograph 1. Pure stock culture of *Chlorella* sp .



Photograph 2. Small scale culture of *Chlorella* sp .

Photograph 3. Culture media for *Chlorella* sp .

CHAPTER FIVE

RESULTS AND DISCUSSION

5. Results and Discussion

5.1. Study-1: Effect of salinity on the growth performances of three microalgal species (*Nannochloropsis oculata, Tetraselmis chui* and *Chaetoceros muelleri*) by using BFRI medium under small-scale batch culture system.

A summary of salinity effects on cell growth (cells x 10^4/ml) of the three microalgae species is presented in Table-4. At the 16th day of culture the peak cell growth of *N. oculata* and *C. muelleri* were attained in all salinity treatments during the study period. When a one-way ANOVA was run comparing the maximum cell densities attained at various salinities for *N. oculata,*, there was significant ($P<0.05$) difference between the means and this species showed maximum cell growth (345 X 10^4 cells /ml) at 20 ppt salinity. One-way ANOVA showed that the difference in maximum cell density of and *C. muelleri* at various salinities was highly significant ($P<0.05$). Significantly highest (395 X 10^4 cell/ ml) cell density of this alga was found at 25 ppt which was not significantly higher than 30 ppt. Peak growth of *T. chui* was observed at 20 days in 15 ppt & 20 ppt but in 25 ppt and 30 ppt it was found at 24 days. *T. chui* showed significantly ($P<0.05$) highest cell density (458 X 10^4 cells /ml) at 30 ppt salinity at 24 days during the culture period. The 5 ppt salinity treatment for both *C. muelleri* and *T. chui* had a reduced density by the fourth days of culture after inoculation and crashed by 12 and 16 days of culture respectively indicated a lethal reaction to the low salinity.

Marine unicellular algae are generally considered to be tolerant of and adaptive to a wide range of salinities. The salinity tolerance and the optimum salinity for growth of marine microlagae varies with species and strains (McLachlan, 1961). Maximum cell growth of *N. oculata* was found at 20 ppt on the 16th day of culture (Fig.3.). The plankton could tolerate a wide range (5-30 ppt) of salinities of brackishwater. It grew well at the salinity range of 15-25 ppt. Lim (1991) observed the optimum salinity 22-25 ppt for *N. oculata*, which is very close to the present study (15-25). Sato (1991) reported 32 ppt salinity for mass culture of ***N. oculata*** in Hawaii with the maximum cell density 38 X 10^6 and it does not agree with present study. Hur (1991) observed 30-35 ppt optimum salinity for the growth of another blue green microalgae *Nannochloris oculata* in Korea . No lag phase was exhibited at salinities from 10-30 ppt with the exponential growth from the 4th to 16th day. At 5-10 ppt salinity the maximum cell growth of *N. oculata* was more or less similar.

The maximum cell density of *T. chui* was observed at 30 ppt on the 24th day (Fig.4.).This plankton species grew well at the salinity range of 25-30 ppt. The suitable culture salinity 15 to 36 ppt for *T. chui* in natural seawater is reported by Liao *et al.* (1986), was close to the present study (25-30 ppt). Optimal salinity range for growth in medium prepared from artificial seawater was found to be 25-30 ppt for *T. suecia* (Laing and Utting, 1980). Lim (1991) found 26-31 ppt optimal salinity for the culture of *T. tatrathele* in Singapore. Chen (1991) reported suitable salinity 30-40 ppt for *T. subcordiformis* culture in China. No lag phase was exhibited at salinities from 25-30 ppt with the exponential growth from the 4th to 24th day. The exponential growth was found from the 8th to 20th day with maximum growth on the 20th day at the salinity range 15-20 ppt. The alga failed to grow below 15 ppt but cells were able to survive up to 16 days at 5-10 ppt. At 5 and 10 ppt no living cells were found after 16 days.

The maximum cell density of *C. muelleri* was observed at 25 ppt on the 16th day (Fig.5.). This species also grew well at the salinity range of 20-30 ppt . Chen (1991) reported that the optimum salinity for the growth of *C. muelleri* was 20-35 ppt which was within the optimum salinity range (20-30 ppt) in cultures of the present study. Chen and long (1991) stated that the suitable salinity ranges for growth *Chaetoceros sp* and *C. simplex* were 18-22 ppt and 13-18 ppt in China. Konkeo (1991) observed 25-30 ppt optimum salinity for mass culture of *C. calcitrans* in Thailand but in case of *C. radicuns* it was only 15 ppt in Japan (Takano, 1963). The minimal salinity for *C. gracilis* is 6 ppt,but can grow well at salinities up to 50 ppt with optimum growth occurring between 17 and 25ppt (Liao *et al.*, 1983). The exponential growth was found from the 4th to 16th day with maximum growth on the 16th day at the salinity range 20-30 ppt. *C. muelleri* failed to grow at 5 ppt but cells were able to survive up to 16 days and thereafter no living cells were found.

In this experiment it was found that in the culture of these three microalgae species at different range of salinities the exponential growth began without passing any lag phase and that might be due to the inoculation of the culture at its exponential phase of growth. According to Spencer (1954) the length of the lag phase is least when the inoculum is in its exponential phase of growth. Ammini (1984) and Gopinathan (1984) have observed similar results in microalgae culture.

Nannochloropsis oculata was the most euryhaline species of microalgae tested here and can tolerate a wide range (5-30 ppt) of salinities. This species is one of the most important species in aquaculture so the findings from this study have positive implications for brackishwater hatcheries in Bangladesh.

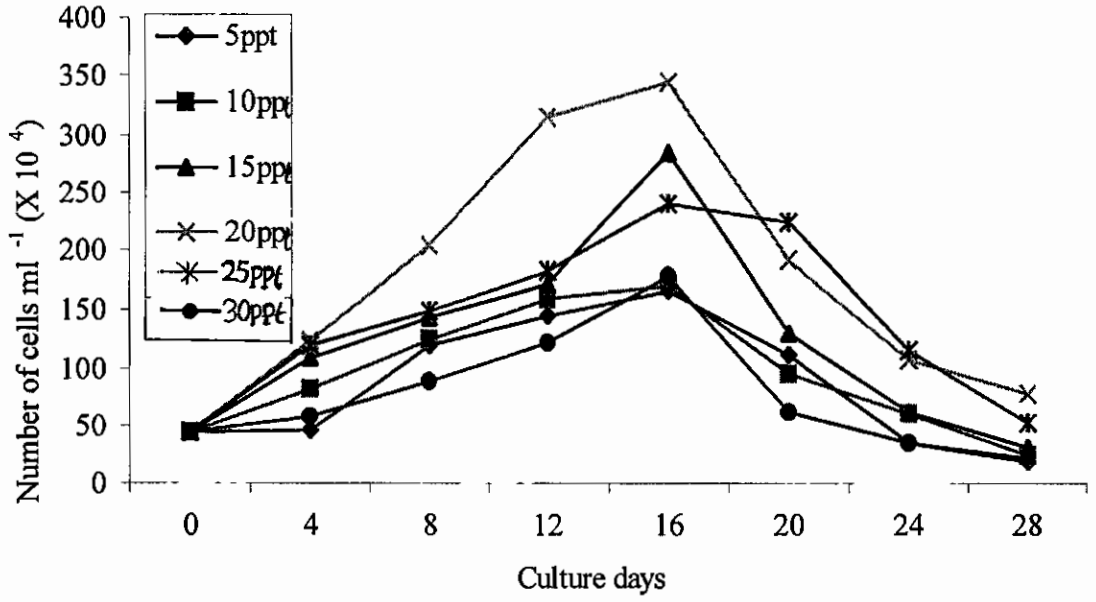


Fig.3. Growth curves of *Nannochloropsis oculata* at different salinities by using BFRI medium.

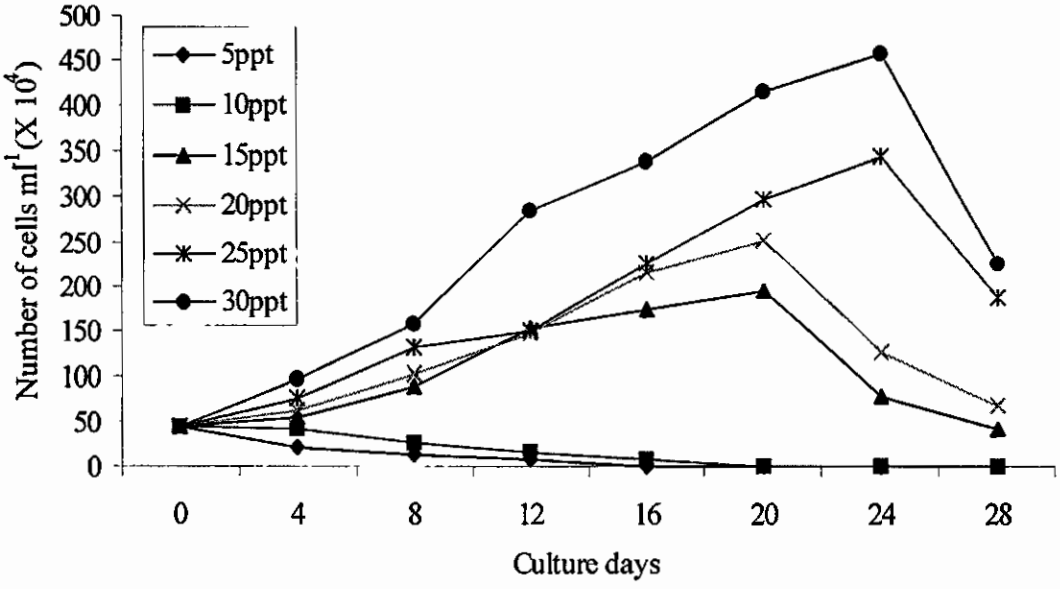


Fig. 4. Growth curves of *Tetraselmis chui* at different salinities by using BFRI medium.

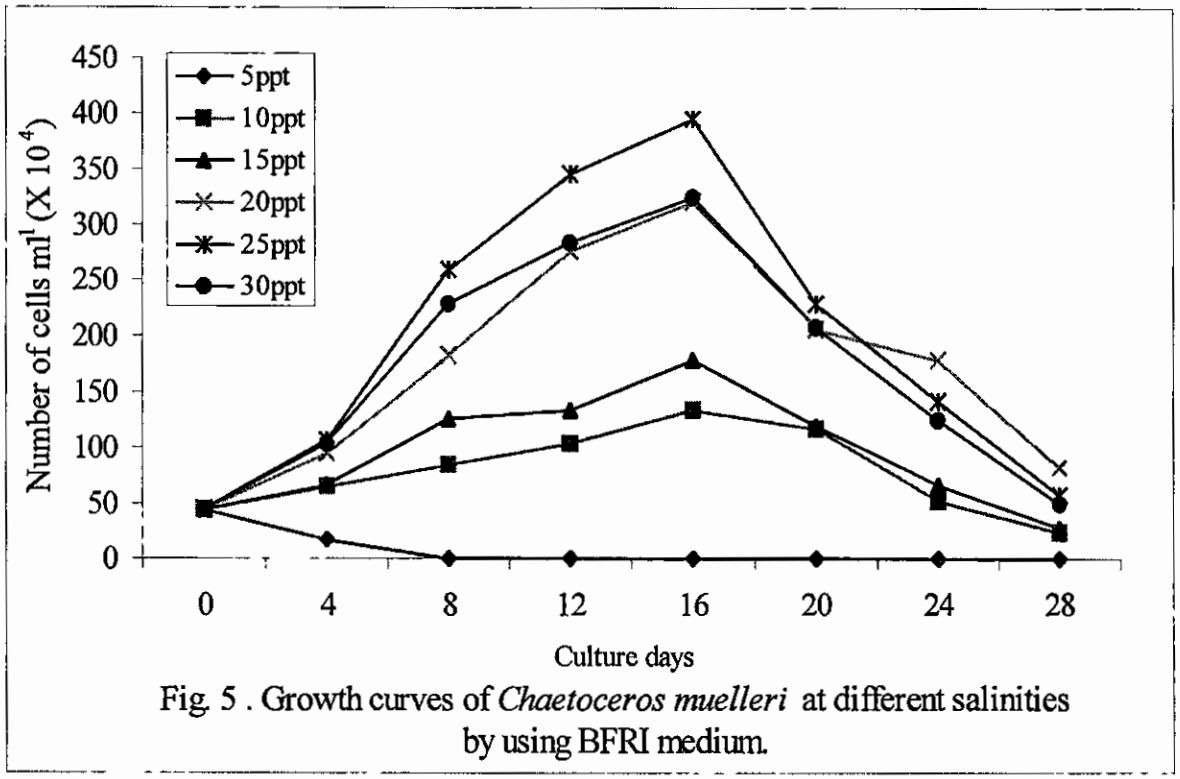


Fig. 5 . Growth curves of *Chaetoceros muelleri* at different salinities by using BFRI medium.

Table- 4. Effect of salinity on the growth (cells x 10^4/ml) of three standard microalgae species by using BFRI medium. Values are means ± SD from triplicate observations.

Culture days	05ppt			10ppt			15ppt			20ppt			25ppt			30ppt		
	* NO	** TC	*** CM	NO	TC	CM	NO	TC	CM	NO	TC	CM	NO	TC	CM	NO	TC	CM
0	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44
4	46 ±6.51	22 ±7.34	17 ±6.73	82 ±4.75	42 ±6.23	65 ±5.23	109 ±8.12	56 ±9.83	67 ±6.68	124 ±5.52	62 ±5.52	95 ±4.98	120 ±4.49	76 ±8.31	107 ±6.61	58 ±9.18	96 ±6.83	105 ±5.87
8	120 ±9.16	12 ±5.91	2 ±7.82	125 ±9.89	26 ±5.78	85 ±3.46	144 ±7.73	89 ±12.23	126 ±7.89	204 ±7.16	102 ±5.78	182 ±6.91	149 ±5.29	132 ±6.23	259 ±4.28	89 ±4.39	156 ±4.78	228 ±4.96
12	145 ±8.21	8 ±4.73	0 ±0	160 ±7.63	16 ±5.34	105 ±4.23	172 ±6.52	152 ±6.76	135 ±6.81	316 ±5.28	147 ±6.87	276 ±8.23	182 ±7.72	148 ±8.59	346 ±5.94	122 ±5.28	285 ±6.74	284 ±6.61
16	167^c ±4.35	1^c ±5.94	0 ±0	170^c ±5.52	8 ±4.45	134^c ±3.28	285^a ±6.79	$172b^c$ ±8.17	179^b ±6.12	345^a ±5.23	214^b ±7.91	321^a ±7.92	240^{ab} ±4.56	225^b ±7.24	395^a ±6.76	178^c ±6.16	341^a ±6.17	325^a ±5.65
20	112 ±11.18	0 ±0	0 ±0	95 ±3.23	0 ±0	117 ±2.39	130 ±7.12	195^c ±4.36	120 ±9.58	192 ±8.27	259^b ±12.16	206 ±5.67	224 ±3.68	298^b ±6.72	228 ±4.38	62 ±4.97	416^a ±4.36	208 ±4.96
24	35 ±8.29	0 ±0	0 ±0	60 ±5.28	0 ±0	52 ±2.98	62 ±9.81	78^d ±7.71	66 ±5.35	108 ±4.29	125^c ±9.91	179 ±9.78	115 ±4.35	345^b ±8.52	142 ±5.35	35 ±4.39	458^a ±7.41	125 ±3.28
28	19 ±3.29	0 ±0	0 ±0	24 ±4.87	0 ±0	24 ±4.67	31 ±7.23	42 ±5.87	28 ±4.89	78 ±6.34	67 ±5.81	82 ±6.34	53 ±6.37	185 ±6.38	58 ±5.23	21 ±5.45	224 ±5.87	49 ±5.37

NO=*Nannochloropsis oculata*, **TC=*Tetraselmis chui*, CM=*Chaetoceros muelleri***

Note: Different superscript in the same row for same species indicates significant variation and same superscript in the same row for same species means insignificant at 5 % level of significant.

5.2. Study-2: Effect of three inorganic media (modified Guillard's f/2 medium, Rix Mix medium and BFRI medium) on the growth performances of three microalgal species (*N. oculata, T. chui* and *C. muelleri*) at 25 ppt salinity under small-scale batch culture system.

N. oculata showed the best growth performance in modified Guillard's f/2 medium among the three media tested in this study (Fig. 6). The peak density of *N. oculata* in modified Guillard's f/2 medium was 221±4.42 x 10^4 cell/ml at 16 days of culture before stationary phase. Lim (1991) found a peak density of 20-25 x 10^6 cells/ml using Walne's medium in 3 L polyethylene bag. In Rix Mix medium the growth increased to the highest density of 185±9.26 x 10^4 cell/ml at 12 days of culture. BFRI medium showed the poorest growth performance for this species.

C. muelleri showed unconventional growth pattern in f/2 and Rix Mix media, but followed the typical growth pattern of microalgae in BFRI medium (Fig. 7). The reason of this is less understood. *C. muelleri* attained the highest density of 321± 5.41 x 10^4 cells/ml in Rix Mix medium and about same density (319 ±12.02 x 10^4 cells/ml) at the 24th day of culture in modified Guillard's f/2 medium. The maximum cell density of *C .muelleri* (135 ± 6.95 x 10^4 cells/ml) in BFRI medium was observed at the 12th day of culture. Chen (1991) observed a maximum 200-250 x 10^4 cells/ml of *C. muelleri* in cemented ponds and tanks in large scale using $NaNO_3$:60g/ton,KH_2PO4:4 g/ton, Vit B:100 mg/ton and Vit B_{12}:0.5 mg/ton.

T. chui was grown reasonably well in both BFRI and Rix Mix media compared to modified Guillard's f/2 medium (Fig. 8). As water quality deteriorates and algal cells starve with the increase of density, death and breaking of cells occur after exponential growth phase of microalgae (Hoff and Snell 1991, Fox 1983). *T.chui* is less sensitive to environmental stress (Liao *et al*, 1991), which could be responsible for the extended exponential growth phase of this species in all the media. The maximum cell density of 182± 6.26 x 10^4 cell/ml was in BFRI medium at the final day (24th day) of culture. The highest density of the species in Rix Mix medium was 169 ±4.48 x 10^4 cells/ml at the final day (24th day), which was close to the maximum value in BFRI medium at the same day. *T. chui* gave reduced growth in modified Guillard's f/2 medium during the entire culture period, reaching a maximum cell density of only 78 ± 4.24 x 10^4 cells/ml at the final day (24th day) of culture.

Different microalgal culture test media resulted in varying growth rates of cultured microalgae species (Table- 5). Considering the increase in density at the 16th day of culture in

modified Guillard's f/2 medium the cell growth of *C. muelleri* (222 ± 4.24 x 10^4 cell/ml) was significantly ($P<0.05$) higher than the other two species. After 16 days, *N. oculata* and *T. chui* were reduced in their growth, but *C. muelleri* increased at the highest density up to the last day of culture.

In BFRI medium, considering the increase in cell density at the 12th day, *C. muelleri* growth (135 ± 6.95 x 10^4 cell/ml) was significantly ($P<0.05$) higher than other species, but at the 24th day *T. chui* showed significantly ($P<0.05$) higher density (182 ± 6.26 x 10^4 cells/ml) than other two species. *N. oculata* and *C. muelleri* concentration decreased after 12 days and reached at minimum level at the last day of culture. At 12 days of culture in Rix Mix medium, *N. oculata* concentration was significantly ($P<0.05$) higher (185 ± 9.28 x 10^4 cells/ml) than other two species. After 12 days this species gradually decreased but *T. chui* increased up to the end and *C. muelleri* reached the highest density (321 ± 5.41 x 10^4 cell/ml) at the 20th day and then decreased.

The growth of *N. oculata* reached a higher density of 221 ± 4.42 x 10^4 cells/ml in modified Guillard's f/2 media at the 16th day than that of (141 ± 10.54 x 10^4 cell/ml) in Rix Mix medium. Modified Guillard's f/2 medium promoted significantly ($P<0.05$) higher growth than other media. Hur (1991) observed the highest cell density of 197.5 x 10^5 of *N. oculata* cultured in F/2 medium, which is much higher than the density obtained in present study and this might be due to the changes in chemical composition of nutrient medium. *N. oculata* grew reasonably well in modified Guillard's f/2 and Rix Mix media among the three media tested in this study.

The highest cell density of *N. oculata* was attained between 12 and 16 days of culture period in three culture media. On the other hand, the cell number of *T. chui* increased up to the 24th day (final) day of culture and this difference might be due to the difference in exponential growth phase of these two microalgal species. *C. muelleri* showed difference in maximum cell density at different days of culture.

In the above view, it may be concluded that, *N. oculata* and *C. muelleri* might be grown very well in both the modified Guillard's f/2 and Rix Mix media. Better growth of *T. chui* might be obtained while growing in BFRI and Rix Mix media. These three microalgal culture media used in the present study may be useful for culture of different species to supply as live food for all the target zooplankton and larvae of fish, crustaceans, etc in marine and brackishwater hatcheries.

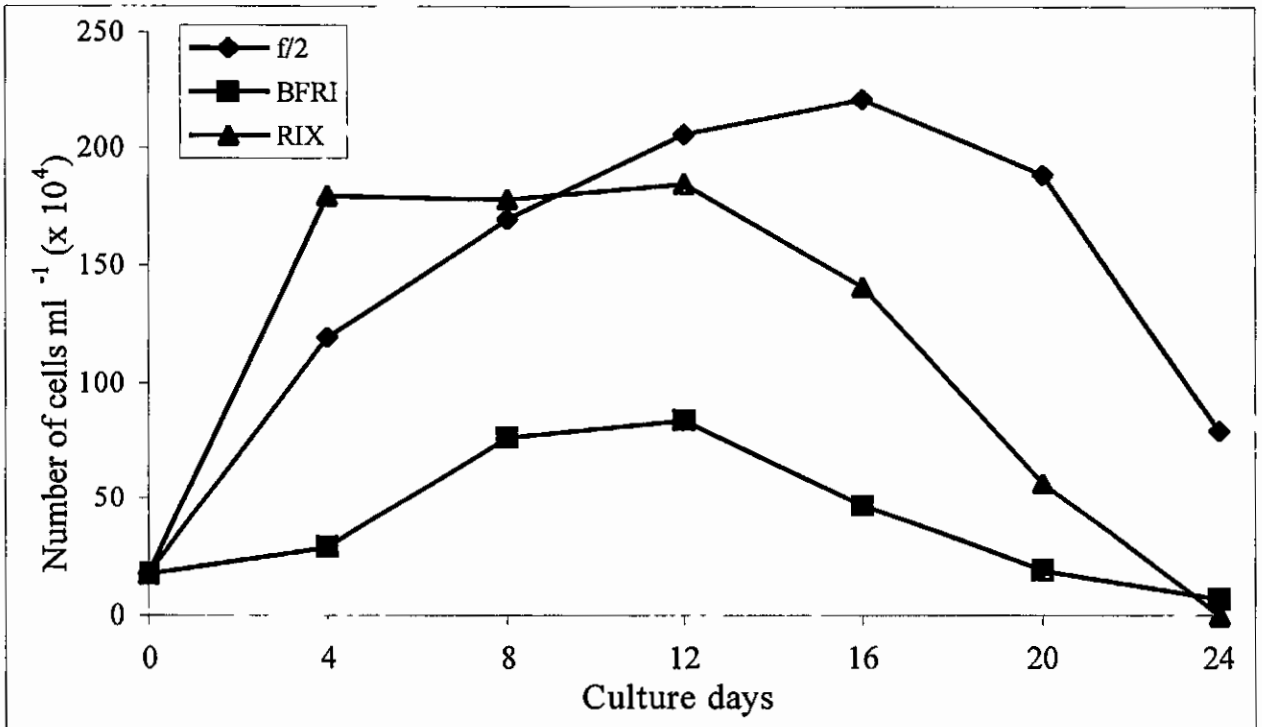


Fig.6. Growth curves of *Nannochloropsis oculata* in three culture media at 25 ppt salinity

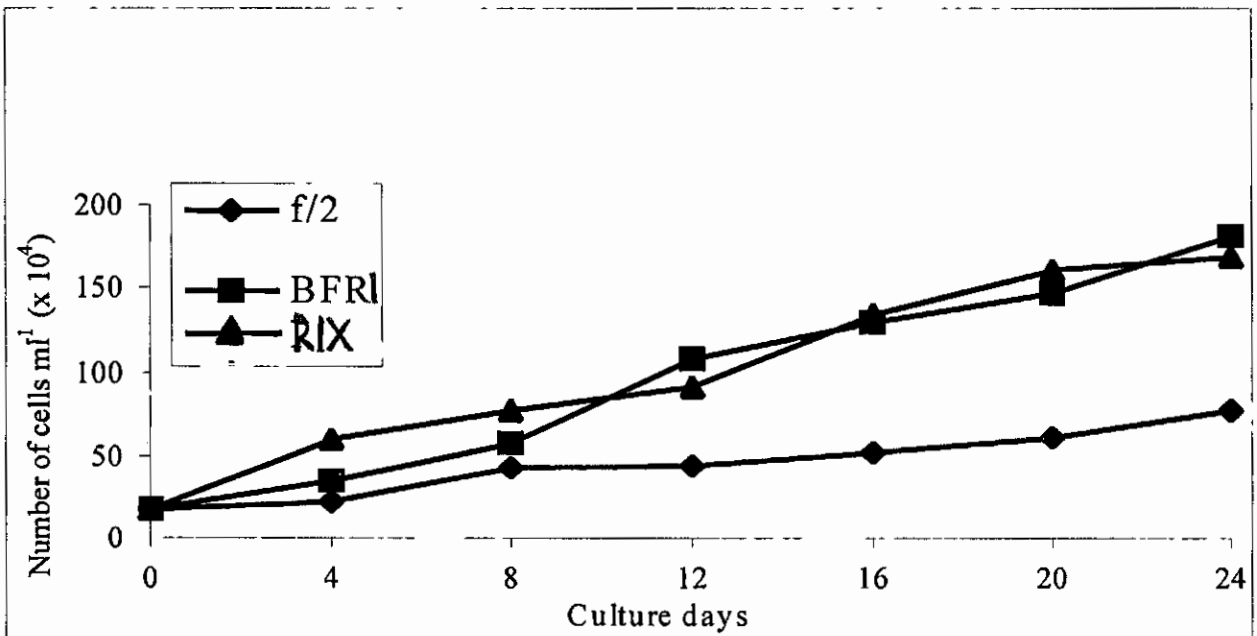


Fig.7. Growth curves of *Tetraselmis chui* in three different culture media at 25 ppt salinity

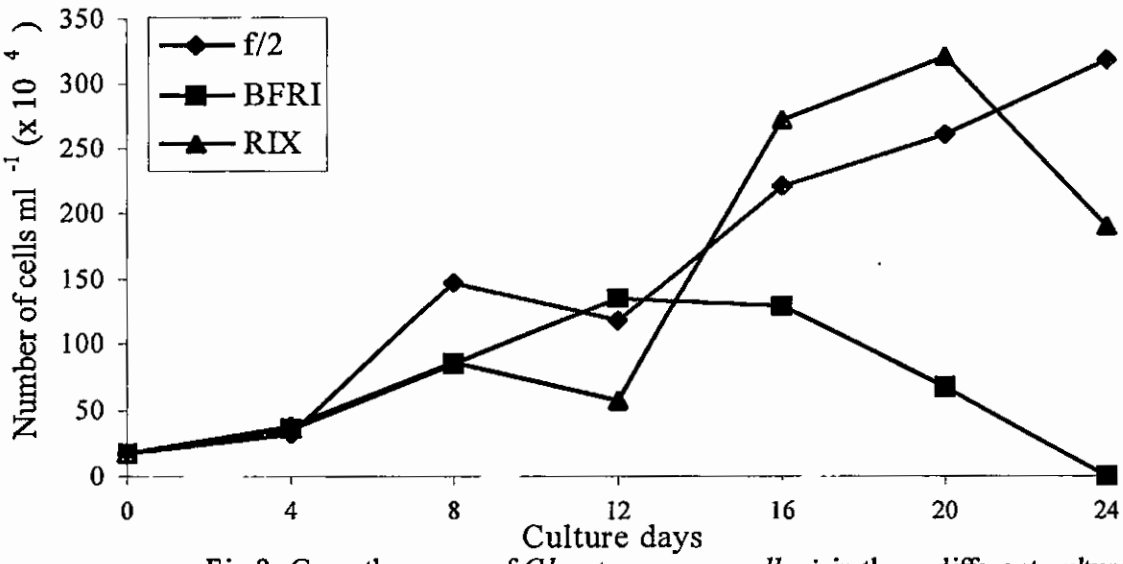


Fig.8. Growth curves of *Chaetoceros muelleri* in three different culture media at 25 ppt salinity

Table-5. Effect of different microalgae culture nutrient media on the growth (cells x 10^4/ml) of three microalgae species at 25 ppt salinity. Values are means ± SD from triplicate observations.

Culture days	Modified Guillard's f/2 media			BFRI medium			RIX MIX medium		
	*NO	**CM	***TC	NO	CM	TC	NO	CM	TC
0	17	17	17	17	17	17	17	17	17
4	119±7.07	33±9.19	22±2.82	29±5.65	36±10.06	35±9.89	180±7.28	39±8.20	60±4.59
8	170±1.31	147±12.84	43±6.36	76±4.94	85±4.24	58±7.27	178±4.56	86±5.19	77±6.71
12	206±9.19^a	117±10.60^a	44±8.48^b	84±9.19^b	135±6.95^a	108±10.12^a	185±9.28^a	57±8.31^b	91±4.81^a
16	221±4.42^a	222±4.24^b	52±7.77^b	47±4.94^c	128±11.11^c	129±5.62^a	141±10.54^b	273±9.61^a	134±9.27^a
20	189±11.55^a	262±6.36^b	61±2.12^b	19±4.98^c	67±9.01^c	147±8.18^a	57±6.81^b	321±5.41^a	161±7.61^a
24	79±7.08	319±12.02	78±4.24^b	7±1.41	0±0	182±6.26^a	0±0	191±7.79	169±4.48^a

NO=*Nannochloropsis oculata*, **TC=*Tetraselmis chui*, ***CM=*Chaetoceros muelleri

Note: Different superscript in the same row for same species indicates significant variation and same superscript in the same row for same species means insignificant at 5 % level of significant.

5.3. Study-3: Isolation, maintenance of pure stock culture protocols of local *Chlorella* sp., collected from brackishwater environment and to develop its small scale culture method under variable salinity in laboratory condition.

5.3.1. Isolation and Pure culture of *Chlorella* sp.:

Isolation of unicellular alga, *Chlorella* sp., from natural environment is complex and time consuming due to its smaller size but the isolation procedure described in the present study is appear to be significant to isolate the *Chlorella* for maintaining pure stock culture and for small to large culture. The growth of unicellular alga *Chlorella* sp. under pure culture conditions are presented in Table-6. The height density of 16 X 10^6 cells /ml was found in 6 flasks followed by 12-14 X 10^6 cells /ml in 4 flasks and 10-12 X 10^6 cells /ml in 6 flasks. While the 8-10 X 10^6 cells /ml were recorded in 6 flasks and the remaining flasks contained less than 8 X 10^6 cells /ml as shown in Table-6.

Hossain *et al.* (1998) recorded the maximum cell density of 12 X 10^6 cells /ml of *Chlorella* sp. through propagation and isolation by dilution method. Hur (1991) observed a maximum cell density 26.86 x 10^6 cells/ml of *Chlorella ellipsoidea* using modified Guillard's F/2 media under pure stock (100 ml flask) culture condition. The present study reveals that a *Chlorella* population of 16 X 10^6 cells /ml appear to be promising and significant in isolation and pure culture through the process described above. The results also showed that presence of protozoa and bacteria in pure stock culture seemed to be inversely related to the growth of *Chlorella* cells and vice-versa (Table-6).

5.3.2. Small scale culture under variable salinity:

Growth of *Chlorella* sp. at different salinities is shown in Fig.9. The plankton species could tolerate and grew more or less well at all the salinities tested. The maximum cell density of 2530 x 10^4 cells / ml was found at 20 ppt salinity following the maximum density (1775 x 10^4 cells / ml at 25 ppt. At 20 ppt , the exponential growth phase was found from the 3rd to 15th day with maximum growth on the 15th day and the stationary phase was extended up to the 24th day and then death phase up to end of the culture days. At 20 ppt, the cell density was always highest among the salinities in all phases of growth of this alga. No lag phase was exhibited at all salinities and that might be due to the inoculation of the culture at its exponential phase of growth.

Mean highest cell density attained at different days throughout the entire culture period and mean daily division rate (0- 15 days) of *Chlorella* sp. cultured at different salinities (5-30 ppt) are presented in Table-7. When a one-way ANOVA run, comparing the highest density attained during the whole culture period at different salinity treatments, there was significant ($P<0.05$) difference between means and 20 ppt salinity promoted the highest density at the 15th day of culture period. Liao *et al* (1986) isolated *Chlorella* sp from the brackishwater fish ponds oh the Tungkang Marine Laboratory cultured at salinities between 0-35 ppt, with an optimum range of 10-20 ppt. Anderson and Smith (1986) cultured *Chlorella* sp in 35 L cylinder at 20 ppt salinity using modified Guillard's f/2 nutrient media and obtained a peak density of 1.5 to 2.5 x 10^{6} cells / ml in five days. The highest mean daily division rate (0.36 divisions day $^{-1}$) was observed at 20 ppt which was not significantly ($P>0.05$) higher than the other salinity (Table-7). The division rate of the plankton in relation to different salinities showed that the growth tends increase at all the salinities.

The isolation technique/procedure described in the present study can be applied for the isolation of *Chlorella* sp. The pure culture protocol will help to maintain the pure stock culture of *Chlorella* in microalgae culture laboratory for up scaling the culture volume from small to mass culture in larger tank. As the *Chlorella* sp. was grown equally well under small scale in all the salinities tested with highest cell density at 20 ppt. This microalgal species might be useful in establishing greenwater culture at 10-14 ppt for *Macrobrachium rosenbergii* larval culture. Likewise, it may be used at these salinities for the culture of zooplankton (Rotifer and Copepods). Small-scale culture of *Chlorella* sp. at 20 ppt salinity might be helpful for future establishment of medium scale culture and mass culture of *Chlorella* sp.

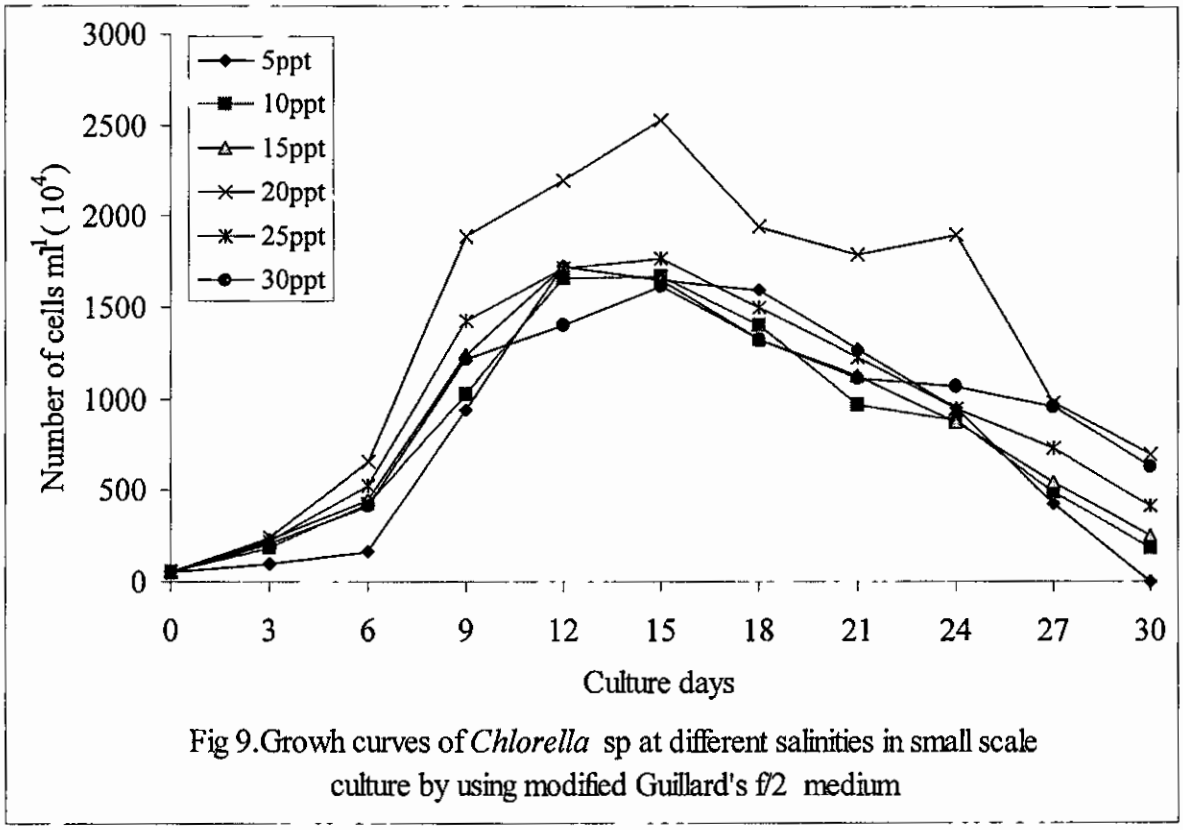


Fig 9.Growh curves of *Chlorella* sp at different salinities in small scale culture by using modified Guillard's f/2 medium

Table- 6. Growth of *Chlorella* sp. under pure stock culture condition.

Conditions	Density (Cells X 10^6 /ml)						
	2-4	4-6	6-8	8-10	10-12	12-14	14-16
No of Flasks	2	2	4	6	6	4	6
*Protozoan contamination	+++	++	+	-	-	-	-
*Bacterial contamination	+++	++	+		-	-	--
Color	Greenish yellow	Dull Green	Green	Green	Green	Green	Green

*+++: High, ++: Little, +: Poor, - : Very poor.

Table-7. Mean (± SD) highest cell density and mean (± SD) daily division rate of *Chlorella* sp. at different salinities cultured in modified Guillard's f/2 medium. (Cell numbers reported in units of 10^4 cells / ml).

Treatments (Salinity)	Highest cell density	Daily division rate (0 to 15 days)
5 ppt	1725 ± 3.03^b	0.32 ± 0.02
10 ppt	1675 ± 4.28^b	0.32 ± 0.02
15 ppt	1730 ± 3.96^b	0.32 ± 0.02
20 ppt	2530 ± 6.48^a	0.36 ± 0.01
25 ppt	1775 ± 8.89^b	0.33 ± 0.01
30 ppt	1625 ± 3.65^b	0.32 ± 0.02

Means with different superscripts are significantly different (Duncan's multiple range test, $P<0.05$)

CHAPTER SIX

CONCLUSION

6. Conclusion

Live foods especially microalgae production is one of the most important operations of shrimp and fish hatcheries in Bangaladesh. It is crucial that suitable foods be produced in sufficient amounts for the different larval stages. The food organisms must be of the appropriate size, digestible and of high nutritional quality. In addition, the food species must be hardy, able to reproduce rapidly and amenable to mass production under controlled conditions. In recent years the problem of producing microalgae as live larval food has become important due to the rapid expansion of hatchery operations for shrimp and prawn in Bangladesh. There are many problems to solve for the development of the culture of microalgae to met the requirement for larval rearing in brackishwater or marine hatcheries in our country. These are:

-Lack of appropriate scientific knowledge.

-Lack of skill manpower.

-Insufficient required facilities and equipment.

-Lack of private enterprnarship to culture microalgae commercially.

To overcome the above-mentioned problems, government organizations, and private companies should expand their activities to culture microalgae as well as other live food organisms. Research activities on various aspects of microalgae culture should be given priorities by research institutes, universities and Department of Fisheries. If the necessary step is taken to overcome the problem of culturing microalgae as live food, the shrimp and prawn larvae production in hatcheries will obviously increase , which will ultimately help to promote our national economy.

REFERENCES

References

Ammini, J., 1984. Culture and growth kinetics of selected Nannoplankters. Ph.D. Thesis, Cochin University of Science and Technology, Cochin, India ,185 pp.

Anderson, D.W and Smith, N., 1986. The culture of larval foods at the Oceanic Institute, Waimanalo, Hawai. In: J.P. McVey (ed.), *CRC Handbook of Mariculture. Crustacean Aquaculture. (V-1).* CRC Press Inc., Boca Raton, Florida, pp. 97-101.

Aquacop, 1983. Algal Food Cultures at the Centre Oceanologique Du Pacific. In: J.P. McVey (ed.), *CRC Handbook of Mariculture. Crustacean Aquaculture. (V-1).* CRC Press Inc., Boca Raton, Florida, pp. 2-14.

Braley, R.D., 2001. Manual for the operation of the hatchery at Brackishwater station, Paikgacha, Khulna. ARMP report. Winrock International Institute for Agricultural Development, Arkansas, U.S.A, pp. 108.

Brown, M.R., Jeffery, S.W. and Garland, C.D., 1989. Nutritional Aspects of Microalgae used in Mariculture; a Literature Review. CSIRO Laboratories Report 205, Australia, pp.44

Chen,J.F.,G.Y.Tan and Y.Y. Pan.1982.The effect of temperature ,light intensity and photoperiod on the growth *Tetraselmis* sp. *Mar.Sci.*2:38-40. (in Chinese with English abstract).

Chen, J.F., 1991. Commercial Production of Microalgae and Rotifer in China. In: W, Fulks. and K. L. Main (eds.),Rotifer and Microalgae culture systems. Proceedings of a U.S.-Asia workshop, 28-31 January 1991, The Oceanic Institute, Honolulu, pp. 105-111.

Chen,X.Q. and Long, L.J.,1991. Research and Production of live feeds in China. In: W, Fulks. and K. L. Main (eds.),Rotifer and Microalgae culture systems. Proceedings of a U.S.-Asia workshop, 28-31 January 1991, The Oceanic Institute, Honolulu, pp. 187-201.

Chin,T.G., Chen, C.F., Liu, S.C. and Wu, S.S.,1965.Influence of temperature and salinity on the growth of three species of planktonic diatoms.*Oceanol.Limnol.Sinica.*7 (4):373-384. (in Chinese with English abstract).

Chu, S.P., Liu, C., Hasiang, P.C and Wang, S.J., 1964.Effects of soil extracts, its microelements and different concentrations of vitamin B12 and cobalt on the growth of *Nitzschia closterium* W.Smith.*J.Fisher.China.*1 (1-2): 19-37.

Dhart, P and Sorgellos, P., 1995. Live feeds in Aquaculture. *INFOFISH International,* 2/95:31-39.

Durer, E.O and Mitsui, A., 1982. Salinity preference and tolerance of aquatic photosynthetic organisms. In: A. Mitsui and C.C. Black (eds.), *CRC Handbook of biosolar resources.Vol.1 Basic principles, Part 2*. CRC Press Inc., Boca Raton, Florida, pp. 223-244.

Fabergas, J., Abalde, J., Herrero, C., Cabezas, B. and Veiga, M., 1984. Growth of marine microalga *Tetraselmis suecica* in batch cultures with different salinities and nutrient concentrations. *Aquaculture,* 42:207-215.

Fox, J. M., 1983. Intensive algal culture Techniques. In: J.P. McVey (ed.), *CRC Handbook of Mariculture. Crustacean Aquaculture. (V-1).* CRC Press Inc., Boca Raton, Florida, pp. 15-41.

Fulks, W. and K.L. Main (Eds), 1991. Rotifer and Microalgae Culture Systems. Proceedings of a U.S. – Asia Workshop, Honolulu, Hawaii, 28-31 January 1991,3-12,The Oceanic Institute, Honolulu, pp. 364

Gopinathan,C. P.,1984. Growth characteristics of some nannoplankters. *J. Mar.Biol.Assoc. India*, 26: 89-94.

Guillard,R.R.L.and Ryther, J.H.,1962. Studies of marine planktonic diatoms.1.*Cyclotella nana* Hustedt and *Detonula confervaces* (Cleve) Gran. *Can. J.Microbiol.*, 8: 229-239.

Hoff, F.H. and Snell, T.W., 1989. *Plankton Culture Manual.* Florida Aqua Farms, Inc., 126 pp.

Horstmann, U.1985. The use of microalgae in aquaculture. *Arch. Hudrobiol. Beth.Ergebn. Limnol,* 20: 153 -156.

Hossain, M.A., Sultana, N., Hossain, A., Islam, S.Q. and Alamgir, M., 1998. Simple methods of isolation and propagation of *Chlorella,* Yeast and Rotifer. *Bangladesh J. Sci.Ind.Res.*,33(2) : 290-295.

Hur, S.B., 1991. The selection of optimum phytoplankton species for rotifer culture during cold and warm seasons and their nutritional value for marine finfish larvae. In: W, Fulks and K. L. Main (eds.),Rotifer and Microalgae culture systems. Proceedings of a U.S.-Asia workshop, 28-31 January 1991, The Oceanic Institute, Honolulu, pp. 163-173.

Kaplan, D., Cohen, Z. and Abeliovich, A., 1986. Optimal growth conditions for *Isochrysis galbana. Biomass,* 9: 37-48.

Khatun, M., 1976. Pure culture of *Chlorella. East Pakistan Scientific Research,* 4(1): 73.

Khatun. M., 1993. Pure culture of *Chlorella* in Bangladesh. Part viii: culture of *Chlorella vulgaris* in the extracts of different brans of pulses. *Bangladesh J.Sci. Ind.Res.*,28 (4) : 155-158.

Khatun .M and Kamal, A.S.M., 1995. Mass Culture of *Chlorella vulguris* in natural media, Cow-pea, Pea shell and Spinach stalk. *Bangladesh J.Sci.Ind.Res.*,30 (1): 1-11.

Khatun, M.,Kamal, A.S.M., Haque, K.S., Chowdhury, S.A. and Nahar,Q., 1998.*Chlorella* and *Scenedesmus:* Isolation, identification and Mass Cultivation for Feeding Cattle. *Bangladesh J. Sci.Ind.Res.* 33(4): 527-531.

Kongkeo, H., 1991. An overview of live feeds production systems design in Thailand. In: W, Fulks. and K. L. Main (eds.),Rotifer and Microalgae culture systems. Proceedings of a U.S.-Asia workshop, 28-31 January 1991, The Oceanic Institute, Honolulu, pp. 175-186.

Laing, I. and Millican, P.F., 1986. Relative growth and growth efficiency of *Ostrea edulis* L. spat fed various algal diets. *Aquaculture, 54* : 245-262.

Laing, I. And Utting, S.D., 1980. The influence of salinity on the production of two commercially important unicellular algae. *Aquaculture,* 21: 79-86.

Liao, I.C., Su, HM. and Lin, J,H.,1986. Larval foods for Penaeid prawn. In: J.P. McVey (ed.), *CRC Handbook of Mariculture. Crustacean Aquaculture. (V-I).* CRC Press Inc., Boca Raton, Florida, pp. 43-69.

Lim, L.C, 1991. An overview of live feeds Production systems in Singapoor. In:W, Fulks. and K. L. Main (eds.),Rotifer and Microalgae culture systems.Proceedings of a U.S.-Asia workshop, 28-31 January 1991, The Oceanic Institute, Honolulu, pp. 187-201.

Mazid, F.Z and Khatun M., 1992. Culture of algae in Aquatic weed extract media. *Bangladesh J. Sci. Ind.Res.*,27 (1-2)): 163-165.

McLachlan, J., 1961. The effect of salinity on growth and Chlorophyll content in representative classes of unicellular marine algae. *Can .J. Microbiol.*, 7:399-406.

Okaichi, T., Montani, S., Hiragi, J. and Husui, A., 1989. The role of iron in the outbreaks of *Chattonella* red tide. In: T. Okaichi, D.M. Andersons and T. Nemoto (eds.), *Red tides: biology, environmental science and toxicology.* Elsver, New York, pp.353-356.

Okauchi, M., 1991. The status of phytoplankton production in Japan. In:W, Fulks. and K. L. Main (eds.),Rotifer and Microalgae culture systems.Proceedings of a U.S.-Asia workshop, 28-31 January 1991, The Oceanic Institute, Honolulu, pp. 247-255.

Pauw, N.D and Pruder, G., 1986. Use and production of microalgae as food in aquaculture: practices, problems and research needs .In: M. Bilio, H. Rosenthal and C. J. Sinderman (eds.), Realism in Aquaculture: Achievements, Constraints, and Perspectives. European Aquaculture Society, Bredene, pp.77-106.