Develop the suitable culture media for marine microalgae *Tetraselmis* species

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Sumaya Zamila

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Approved as to style and contents by

G.R.Banu

Dr. Ghausiatur Reza Banu
Professor and Head

Fisheries and Marine Resource Technology Discipline
Khulna University
Khulna

DECLARATION

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

Sumaya Zamila

(Candidate)

Sumaya Zamila

Examination Roll No: MS- 150630

30/01/2019

(Supervisor)

Dr. Mst. Muslima Khatun

Professor

Fisheries and Marine Resource Technology Discipline
Khulna University
Khulna

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Sumaya Zamila
ID: MS-150630

ABSTRACT

In the present study, the growth performance of *Tetraselmis* sp. was observed in different nutrient media (TMRL, Guillard, Conway, maxicrop, fertilizer and seawater) and in salt concentrations (15ppt, 20ppt, 25ppt and 30 ppt). This study also evaluated the growth performance of rotifer (*Brachionus plicatilis*) developed by microalgae cultured in various nutrient media. The microalgae, *Tetraselmis* sp. was collected from BFRI (Bangladesh Fisheries Research Institute), Marine Water Station, Cox's Bazar, Chittagong and the rotifer (*Brachionus plicatilis*) was collected from Bangladesh Fisheries Research Institute (BFRI), Brackish Water Station, Paikgacha, Khulna. The experiment was conducted in 500ml plastic bottles, with working volume of 200ml, temperature 25 ± 1°C, with constant light (1600-2000 lx) and aeration. Each experiment was conducted with the replication of three. The results evidenced that maximum cells number in stationary phase were noticed in Conway medium (241±5.178×10^4 cells/ml) followed by TMRL medium (225.148 ±4.62×10^4 cells/ml), Guillard f/2 medium (202.333±4.91×10^4 cells/ml), seawater (102.889±7.27×10^4 cells/ml), Maxicrop medium (99±3.46× 10^4 cells/ml) and commercial fertilizer (53.67±6.69× 10^4cells/ml). However, the production cost for the preparation of 100 ml TMRL medium was the lowest among three best medium, where *Tetraselmis* sp. growth was higher. The production cost of Guillard (298.45Tk/100ml) was 17.47 and 2.01 times higher than TMRL (17.08 Tk/100ml) and Conway (148.32 Tk/100ml) respectively. In the second experiment, the cell density of *Tetraselmis* sp. in 30ppt salinity was significantly ($p<0.05$) higher as 232.889±5.755×10^4 cells/ml, followed by 25ppt (207±6.11 × 10^4 cells/ml), 20ppt (92±2.309×10^4 cells/ml) and 15ppt salinity (84±4.73× 10^4 cells/ml). In the third experiment, the rotifer fed on *Tetraselmis* sp. cultured in Conway medium showed the highest ($p<0.05$) population growth (56.111±1.25ind./ml), compared to that fed on *Tetraselmis* sp. developed with TMRL medium (20.333±0.882ind./ml) and Guillard f/2 medium (18±1.15ind./ml). The results of the present study indicate that the TMRL medium could be a suitable and cheap culture medium for the enhancement of *Tetraselmis* sp. biomass in 30ppt salinity . The result also recommend that *Tetraselmis* sp. developed with Conway medium could be a suitable food for the stock culture of locally isolated rotifer *B. plicatilis.*

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Chapter- 1

Introduction

Microscopic organisms are found in both marine and fresh water environments (Richmon 2004; Gouveia 2011). Microalgae, also called phytoplankton are unicellular microscopic organisms. They are very small plant-like organisms between 1 to 50 micrometers in diameter without roots or leaves (Wolkers *et al.*, 2011). These organisms are found all over the world in both freshwater and seawater. They are crucial component in the marine food web. They drift near the water's surface, producing their own food and thus, providing meals for countless other aquatic dwellers. Most species contain chlorophyll, use sunlight as an energy source and carbon dioxide with photosynthetic efficiency higher than the plants for the production of biomass. Microalgae are mainly distributed in the waters, but also found on the surface of all type of soils. Normally they are not visible by the naked eye, but if the water is eutrophic, massive algal blooms occur, changing the water in green, brown, blue or orange liquid mass.

Marine microalgae play a vital role in the ecology of the planet, accounting for approximately 45% of global primary productivity (Andersen 2005). They are important for food source and feed additive in the commercial rearing of many aquatic animals, especially the larvae and spat of bivalve molluscs,crustaceans, penaeid prawn larvae and for live food organisms such as rotifers (Guedes *et al.*, 2010; Brown 2002; Lavens *et al.*, 1996; Lananan *et al.*, 2013). *Brachionus plicatilis* is a brackishwater rotifer, which has been used as food for marine fish larvae and planktonic crustaceans throughout the world (Watanable *et al.*, 1983). This microscopic organism has been widely used due to its ideal size, fast reproductive rate and its ability to feed on different unicellular algae (Witt et al., 1984), formulated diets (Lavens et al., 1994) and baker's yeast. They are also cultured to serve as water conditioner in fish larval rearing system (Palanichamy and Rani 2004). Although several alternatives for algae exist (such as yeasts and microencapsulated feeds), live algae are still recognized as being one of the best food sources and enrichment diets available (Arriada 2014). Many algae contain a considerable amount of high-quality oils, partly consisting out of omega-3 and omega-6 fatty acids, which are used as raw material for food supplements omega-3 fatty acids in fish originate from microalgae. The market of functional food, using microalgae in pasta, bread, yogurt

and beverage, has fast development in some countries like France, United States, China and Thailand. (Pulz and Gross 2004). Beside that microalgae are a potential source of biodiesel (Kalin *et al.*, 2005; Mallick 2002; Munoz and Guieysse 2006; Suresh and Ravishankar, 2004) and may become an alternative to fossil fuels in the future (Gavrilescu and Chist 2005).

Tetraselmis sp. is a genus (more than 50 species) of green flagellates. Most species of this microalga are known from inshore marine environments, tide pools in particular, but a few freshwater species are also known (Bold and Wynne 1985). It is a green four-flagellated motile prasinophyte. It is characterized by an ovoid body shape and a distinct curved body when viewed sideways. The micro alga measures 12-14μm in length, 9-10μm in width and belongs to the family Chlamydomonadaceae. It has very high lipid levels and also contains natural amino acids that stimulate feeding by other marine animals. *Tetraselmis chuii* is a species of this Microalga, which contains 10% lipid and is used to produce biodiesel. This specie is considered as an important source of polyunsaturated fatty acids (PUFA).

Bangladesh has both the world's largest delta system and the greatest flow of river to the sea. It is fortunate in having an extensive water resource in the form of ponds, natural depressions (haors and beels), lakes, canals, rivers and estuaries covering an area of 4.69 million ha (DoF, 2011). Total coastline of Bangladesh is 580 km (360 mi). For this wide range of aquatic environment, this country is enriched with large fish biodiversity. We have several important commercial fish and shell fish. These fishes need artificial breeding for commercial purposes and restocking in nature, but it could not possible due to lack of rearing facilities. In Bangladesh although Artemia is used for the propagation of shrimp larvae but this Artemia are imported from abroad by paying lot. On the other hand, at present, *B. plicatilis* has been recognized as a potential food for shrimp larvae in addition to or as a replacement for *Artemia* (Hirata *et al.*, 1985). Hence, the operation cost could be minimized by developing the rearing techniques of marine microalgae in the local conditions and replacing the rotifer. Presently, Bangladesh has an annual demand for fuel of around 37 lac tons, including 24 lac tons of diesel and imports the entire amount from Kuwait, Saudi Arabia, United Arab Emirates and India. In search of bio fuel energy resources, Bangladesh has potential in alternative fuel energy resources from algae (Muhit *et al.*, 2014). Records from the Ministry of Agricultural data show that Bangladesh has 0.73 million hectares of land that are not suitable for any crop production and can be used for algae production. The amount of sunlight we get is sufficient for microalgae production. The water required for microalgae can be used from the extensive

water source of this country. Moreover, microalgae culture system can be incorporated with the power plant flue gas in case of commercial production, which will reduce air pollution (Alam 2016).

Since aquaculture is one of the most rapidly growing industries in the field of food production, culture of micro algae has become an essential prerequisite for the rearing of economically important cultivable organisms. The success of any hatchery system (for crustaceans, oysters sea cucumbers or fishes) entirely depends on the availability of suitable microalgae for raising them in culture so as to be fed to larvae of cultivable shell or finfish species. Many techniques for the culture of microalgae have been used from many years in different parts of the world. The type of the culture is divided into indoor/controlled culture and outdoor/open culture. Further, there are different approaches to culturing of microalgae which include 1) Batch culture 2) Semi-continuous culture 3) Continuous culture (Fulks and Main 1991). All these culture require a suitable culture media to enrich with different nutrients, other cultural condition like illumination, temperature, salinity, pH and aeration that are the requirement for optimum growth of microalgae because environmental factors such as temperature, salinity, pH, and light have been reported to affect microalgae growth (Creswell 2010).

Natural seawater is a complex medium containing more than 50 known elements and a large number of organic compounds. For efficient cultivation of microalgae, direct use of natural seawater is seldom acceptable. Without the addition of nutrients and trace metals, the cell yield is usually very low. Thus, enrichment of sea water as cultivation medium for microalgae is required, especially macronutrients such as nitrogen, phosphorous, vitamins, buffers and chelators (Harrison *et al.*, 2005).

For indoor culture of *Tetraselmis* sp. different nutrient media can be used. Several media are used incorporating trace metals, vitamins and several organic and inorganic salts. Among these, f/2 (Guillard, 1975), PM (Pantastico, 1977), TMRL (Tung Kong Marine Research Lab., Tahiti) and Conway or Walne's (Walne, 1974) media are found to be effective for the maintenance of stock culture as well as for mass-culturing of various micro-algae. But for culture, selection of a suitable low-cost nutrient media with higher growth performance of the microalgae is very important, because of since the interaction of growing microalgae with the culture medium and with their own physical environment results in expressive changes in cell

density. Growth stage in microalgae cultures and the manipulation of the physical and chemical conditions of the cultures may result in differences in cell composition, i.e., variations in levels of lipid, protein, carbohydrate and other components of the cell. Therefore, it is highly feasible to study on growth performance of this phytoplankton in different nutrient media for its proper utilization. Thus, this study is aimed to explore the cost-effective and suitable nutrient media and suitable salinity for the growth of *Tetraselmis* sp. This study also identified the growth performance of zooplankton, rotifer (*Brachionus plicatilis*) fed *Tetraselmis* sp enriched with different nutrient media.

Objectives of the study:

The objectives of the study include the following:

- To explore the suitable and cost effective nutrient media for the growth of *Tetraselmis* sp.
- To determine the least suitable salinity for better growth of *Tetraselmis* sp.
- To compare the growth performance of Rotifer (*Brachionus plicatilis)* enriched with *Tetraselmis* sp. grown in different nutrient media.

Chapter-2

Review of Literature

2.1. Importance of microalgae

Planktonic micro algae, as primary producers, are the biological starting points of the aquatic food-chain and also help in the energy flow. They are utilized as live feed for all growth stages of bivalve and molluscs, for the larval/early juvenile stages of abalone, crustacean, some fish species and zooplankton in aquaculture food chains (Gopinathan 2009). As larval food microalgae (*Tetraselmis* sp.) is also important because it stimulates enzymatic synthesis and on-set of feeding in young larvae. Besides, it also acts as water conditioner by stripping off the nitrogenous substances. *Tetraselmis* sp. biomass is obtained from a renewable source, does not require a large area of land for cultivation, possesses a high growth rate and accumulates a satisfactory amount of nutrient for rotifer production (Danquah *et al.*, 2010).

The self-rejuvenation and rapid growth (4 new cells every 17 to 20 hours) characteristics of microalgae help to produce lipids, proteins and carbohydrates in large amounts over a short period of time by using CO_2, sun light, sugar, N_2, P, K as nutrient. They also reported that, the microalgae have some remarkable advantages to be used as a biodiesel feedstock such as these are cultured to act as a low-cost raw material (feedstock), contain high energy per unit mass, renewable, environmentally friendly and can contribute to reduce CO_2 levels in the atmosphere by consuming for its own reproduction it and converting it to diesel oil (Hossain *et al.*, 2008). Algae biodiesel can be used as a successful alternative fuel for single cylinder Ricardo diesel engine. The methyl ester properties of the algae biodiesel are similar to the petroleum diesel. Haik *et al.*, (2011) reported that when they conducted an experimental study to use raw algae biodiesel and its methyl esters in an indirect injection diesel engine. They studied the effect on engine speed, load, injection timing of the algae biodiesel and engine compression ratio on the engine output torque, combustion characteristics.

2.2. Effect of culture media

Different culture media have been used from many years in different parts of the world for microalgae.because different culture media supply different nutrient that are important for the growth of microalgae .Fogg (1975) studied on algal cultures and phytoplankton ecology and found that growth rate decreases when the nutrient in shortest supply relative to its metabolic needs of the alga population.

A specific culture medium cannot be effective in all the cases. Goldman and Ryther (1976) formulated nutrient media used to culture marine microalgae in laboratories and hatcheries. They reported that Guillard and Ryther's f/2 media is the most widely used for microalgae culture because these are effective. On the contrary, Gopinathan (1986) reported that no available culture medium is uniformly effective in all the cases, unless supplemented with either trace metals or vitamins according to the requirements of the algae. Valenzuela-Espinoza *et al.*, (1999) reported that the production of *Isochrysis aff. galbana* biomass and nitrate, phosphate and ammonium uptake were determined by using batch culture techniques for 7 days, comparing commercially used fertilizer and f/2 media. It was found that the average cellular density of the algae when grown in the agricultural fertilizer showed no significant difference from that produced by the f/2 media. On the other hand, higher levels of nitrogen and ammonium can be toxic to some marine microalgae in some cases and speculated that this can be a reason that *Pavlova salina* does not grow well when it is cultured in Miracle-gro, a commercial agricultural fertilizer (Berges *et al.*, 2001).

Many experiments were conducted in many times by researchers to evaluate the growth performance of microalgae in different media. Shah *et al.*, (2003) conducted an experiment on the growth of three microalgae species, *viz.*, *Nannochloropsis oculata, Tetraselmis chui* and *Chaetoceros muelleri* which are commonly used in aquaculture using three different inorganic nutrient media: (i) Modified Guillard's f/2 medium (ii) Rix Mix medium and (iii) BFRI medium. *N. oculata* cultured in modified Guillard's f/2 medium showed superior growth with a mean peak density of 221 ±4.24 $\times 10^4$cell/ ml. *C. muelleri* attained the highest density of 321 ± 5.41$\times 10^4$cells/ml in Rix Mix medium and about same density (319±12.02 $\times$ 10^4cells/ml) at the 24th day of culture in modified Guillard's f/2 medium. However, with an increase in cell density, growth of *T. chui* (182 ± 6.26 $\times 10^4$cell/ml) was significantly ($P<0.05$) higher in BFRI medium.

In 2012, a researcher Kamal tried to develop a cheap and simple medium for *Nannochloropsis sp.* based on agricultural grade fertilizers available in the local market of Gaza Strip. Five different culture media, which consist of a combination of different agricultural fertilizers were tested and compared to F/2 medium, which is commonly used for microalgae cultivation in commercial aquaculture. One of the five medium which consists of ammonium sulfate (150 mg^{-1}), urea(7.5mg^{-1}), calcium super phospate (25 mg^{-1}), micronutrient solution (0.5 mg^{-1}) and vitamin solution (0.5 ml^{-1}) resulted in maximum average cell density

of 69 $\times 106ml^{-1}$. That medium was also found to be highly economical, since it is about thirteen times cheaper than F/2 medium. The result of this experiment indicated that culture media which consists of a combination of agricultural fertilizers such as urea, calcium superphospate, ammonium sulfate, micronutrient and vitamin solutions strongly support the growth of *Nannochloropsis sp.* and confirmed that using agricultural grade fertilizers can substitute the F/2 media which commonly used for culture in commercial aquaculture.

In an experiment Guillard f/2 and Conway media were used to evaluate the effect of nutrient depletion during the cultivation of the microalgae *Tetraselmis tetrathele*, and to verify the algal growth and productivity in different culture media. The experiment was conducted in, salinity 35 ± 2‰, temperature 28 ± 1°C, with constant light and aeration. The cultivation in Conway medium presented better results concerning microalgae growth, with a more pronounced exponential phase. This was due to the higher nitrate availability, which was reduced more rapidly in the culture medium Guillard f/2 (Coelho *et al.*, 2013). Lananan *et al.*, (2013) investigated the effect of the same two media, Conway and f/2 media on the growth of various microalgae genera (including *Chlorella sp., Dunaliella sp., Isochrysis sp., Chaetoceros sp., Pavlova sp. and Tetraselmis sp.*). It was found that the reproduction of *Tetraselmis sp., Dunaliella sp. and Pavlova sp.* could be sustained longer in the f/2 medium. Higher cell densities were achieved by the microalgal genera *Dunaliella*, *Chlorella* and *Isochrysis* in Conway medium. Different genera of microalgae had a preference for different types of cultivation media.

The cell number of *Skeletonema costatum* was maximum in exponential phase when they were cultured in Conway medium (600 cells/mL), followed by Miquel's medium (500 cells/mL), TMRL Medium (470 cells/mL) and TMRL medium with urea (450cells/mL) which clearly evidenced that the Convey medium was the most suitable medium for getting more algal biomass in low volume of area (Mayavan and Thangavel 2014).

Majumdar (2015) observed the growth performance of *Tetraselmis chuii* in three different nutrient media Conway or Walne medium, TMRL medium and Miquel's medium in laboratory condition. The experiment was conducted in salinity 30‰, temperature 25 ± 1°C, with constant light and aeration. Total culture period was 41days. TMRL medium showed the highest growth performance for these microalgae. In TMRL medium maximum cell density of *Tetraselmis chuii* was 169.00± 1.36423 $x10^2$ cells mL^{-1}. In the same year Barakoni with others carried out a study on growth performance of the marine microalgae *Pavlova salina*

and *Dunaliella tertiolecta* using different commercially available fertilizers in natural seawater. *P. salina* demonstrated significantly higher growth rates (as measured by determination of average cell densities) when provided with f/2 media, but no growth when cultured using commercial fertilizers, namely Miracle-gro and Maxicrop. In Maxicrop nitrogen and ammonium levels were much lower than f/2, which could have limited the growth of P. salina. In contrast, the average cell density of *D. tertiolecta* was found to be significantly higher ($P < 0.05$) when cultured using commercial fertilizers, as opposed to f/2media.

Same concentration of a specific nutrient is not needed for all species. In some cases extra concentrations of a specific nutrient enhance the growth rate. But, in other cases growth rate is same in high and low concentration. Rocha *et al.*, (2003) found that the addition of considerably extra concentrations of nitrogen sources to F/2 medium led to increase the growth rate of *Nannochloropsis gaditana* microalgae. Gopinathan (1986) also observed that *Chaetoceros calcitrans* showed some deviation from control growth in the absence of vitamin and trace elements. While, Sultana (2014) carried out a study on the effect of vitamin B_1 and B_{12} on growth performance of *Tetraselmis* sp. in stock culture with TMRL media. The results of that study also concluded that edition vitamin (B_1 and B_{12}) with TMRL media enhance the growth performance (cells/ml) of *Tetraselmis* in stock culture.

Bertrand *et al.*, (2007) demonstrated the importance of vitamins and iron to growth of phytoplankton species. He also said that the vitamins required by microalgae vary between species and it is possible to omit any of the vitamins from the culture media if the target species do not require that specific vitamin. However, in the same year Guzman-Murillo with others analyzed different culture media to the microalgae *Phaeodactylum tricornutum* and evidenced that cell density was not affected by different nitrogen concentrations, with high cell density both in test with high and low concentration of nitrogen.

2.3. Effect of salinity

To observe the effect of salinity different experiments were conducted and the result was not same in all case. In 1968, Johnson with others observed the effects of salts on algae. They observed that the optimum growth of *Tetraselmis chuii* occurred at salinity 25ppt and reduced growth rates at 40ppt. They concluded that faster nutrient depletion in the culture medium reduced the growth rate at higher salinity. Carvalho *et al.*, (2003) also quoted that in 6500 lux

light intensity and 25 ppt salinity, most cell density of the marine microalga *Tetraselmis chuii* induceded. On the other hand, the green microalga *Tetraselmis* sp. as a relatively very adaptable algal species and have been shown to be capable of growth at salinity levels varying from brackish (20ppt) up to hypersaline (50ppt), with maximum biomass reported at 40ppt (Vazquez and Arredondo 1991).

Many marine forms can survive at lower salinity, but for their optimum growth they express specific requirements for additional salts(Rippka *et al.*, 1979). The growth of marine *Chlorella saccharophila* linearly decreased with increasing salinities from 40 to 60 ppt. This suggests that Chlorella was affected by the overloading of the osmoregularoty mechanism therefore, affecting the growth, photosynthesis and respiration (Hirata *et al.*, 1981).

Costa *et al.*, (2004) analyzed the growth of the microalgae *Dunaliella viridis* and *T. chuii* in treatments with culture medium Guillard f/2 containing 10, 20, 30 and 40‰ of seawater. The authors observed higher growth rates of 2.11 divisions day-1with 30‰ of seawater added for *D. viridis*, and 2.28 divisions day-1with 40‰ of seawater added for *T. chuii*. Rao *et al.*, (2007) found that Salinity is a major factor in the retardation of central metabolic activities such as photosynthesis in plants. Microalgae species differ in their adaptability to Salinity and can be categorized as being halophilic and halotolerant based on their Salinity tolerance level.

In a researches work the growth and proximate composition of the *Chaetoceros calcitrans* f. *pumilus* was investigated under different salinity. Salinity of 25 and 35 ‰ had no significant effect on growth, maximum cell density, biomass and chlorophyll content of *Chaetoceros calcitrans f. Pumilus* although a tendency of higher growth, biomass and lower cell density was observed at lower salinity (Raghavan *et al.*, 2008).

Rai and Rajashekhar (2014) studied on the influence of pH, salinity and temperature on the growth of three species of cyanobacteria, two species of diatoms and one species of planktonic green algae. All isolates were able to grow at all salinities except diatom *Skeletonema costatum*, which did not show growth at 40 ppt. The best growth was obtained at 16ppt and 25 ppt .The cyanobacterium *Chroococcus turgidus*, diatom *Chaetoceros calcitrans* and planktonic green alga *Nannochloropsis oceanica* showed good growth at 16 ppt, where as cyanobactia *Lyngbya confervoides* and *Nostoc commune* and the diatom *Skeletonema costatum* showed maximum growth at 25 ppt. The growth was decreased at higher salinities. But, Shishir (2015) showed that 30ppt is the most optimum sailinlty for the culture of

Chaetoceros gracilis. She observed the growth performance of *Chaetoceros gracilis in* different salinity viz. 0ppt, 5ppt, 10ppt, 15ppt, 20ppt, 25ppt and 30ppt in laboratory condition. Culture period was 36 days. The maximum density at 30ppt was 7514.33 ±7.09 cell/ml.

A study was conducted on Fatty acid composition of the marine microalga *Tetraselmis chuii* in response to culture conditions. According to this study, a salinity of 25 ppt and light intensity 6500 lux seems more adequate for enhanced growth and fatty acid composition of this microalga (Mohammadi *et al.*, 2015).

2.4. Culture of Rotifer using microalgae

Many research works have been done to evaluate the growth of rotifer under different types of food. Villegas (1990) evaluated the effects of three selected algal species, *T. tetratheley Lgalbana and Chlorella* sp. on the population growth of *B. plicatilis* after 3, 5, and 7 days of culture. The rotifers fed on *T. tetrathele* showed superior growth with mean peak density of 92.5 ind. /ml to those fed on *L galbana* (48.2 ind./ml) and *Chlorella* sp. (47.2 ind./ml) in 5 days of culture. In 2004, Alam and Shah selected three types of algal species *Chlorella* sp. (T1), *Tetraselmis chui* (T2), *Nannochloropsis oculata* (T3) as feed to evaluate population growth and reproductive capacity of brackish water rotifer, *Brachionus plicatilis.* The duration of that experiment was 8 days. The feeding density of each algal species was maintained similar as of 4.5×10^6 cell/ml. The rotifer fed on *T. chui* showed the highest ($p<0.05$) population growth (131.5 ind./ml), compared to that fed on *Chlorella* sp. (45.67 ind./ml) *and N. oculata* (43.44 ind./ml). The abundance of egg bearing rotifers was also higher (35.77%) with *T. chui* than with *Chlorella* sp. (27.76%) and *N. oculata* (24.60%). The results of the study indicate that *T. chui* could be the most suitable algal food for the stock culture of locally isolated rotifer *B. plicatilis.*

In an experiment, the rotifer *Brachionus plicatilis* were cultured with powdered dried *Chlorella* in treatment 1, live or fresh cultured *Chlorella* in treatment 2, and baker's yeast in treatment 3. All the jars under three treatments were stocked with *B. plicatilis* at the initial density of 10 individuals per ml. The water temperature, air temperature, pH and dissolved oxygen were within the suitable range for *B. plicatilis* culture. The highest population densities of *B. plicatilis* in treatments 1, 2 and 3 were 60000, 50000 and 30000 (individual/L)

respectively. The powered dried *Chlorella* was comparable with live *Chlorella* and may be used successfully as a feed for *B. plicatilis* (Mostary *et al.*, 2010).

Malekzadeh (2012) studied two Iranian strains of *Brachionus* rotifers under different food and salinity regimes. The rotifers were fed with five algal types (freshwater and marine *Chlorella vulgaris, Nannochloropsis occulata, Isochrysisgalban* and *Scenedesmusobliquus*) at three different salinities (5, 15 and 25 g/L) and their reproductive and growth parameters were assessed. The maximum number of ovigerous females (73 ± 7 ind/mL), population density (354 ± 3 ind/mL) and specific growth rate (0.75 ± 0.01 / day) were obtained for the rotifers fed with freshwater *Chlorella*. After four years of that experiment a researcher evaluated the growth performance of rotifer (*Brachionus plicatilis*) developed by microalgae, *Nannochloropsis occulata* cultured in TMRL, Conway, and Gillard media. In that experiment, temperature (25 ± 1°C) and optimum salinity (25‰) were maintained for rotifer. At the final day (13th day) the number of rotifers in TMRL, Guillard and Walne were 79.78 ± 1.947ind./ml, 72.78 ± 1.567ind./ml and 62.561 ± 0.544 ind./ml, respectively (Sahiduzzaman 2016).

Chapter-3

Materials and Method

3.1. Microalgae and rotifer collection and maintenance

The microalgae, *Tetraselmis* sp. was collected from BFRI (Bangladesh Fisheries Research Institute), Marine Water Station, Cox's Bazar, Chittagong and the rotifer (*Brachionus plicatilis*) was collected from Bangladesh Fisheries Research Institute (BFRI), Brackish Water Station, Paikgacha, Khulna. After collection, microalgae were immediately brought to the Microalgae culture laboratory of Fisheries and Marine Resource Technology Discipline, Khulna University. The microalgae were maintained in refrigerator at 4°C until final experiment. Rotifers were used in experiment immediately after collection.

3.2. General experimental conditions

For the experiment, the microalgae culture room was kept under continuous lighting (24 hrs) facilities. Room temperature and light intensity were 25 ± 1°C and 1600-2000 lx, respectively. Continuous aeration was provided by an indoor compressor for faster algal growth, mixing and elimination of dead spot. pH in all the culture system was maintained as 8 to 8.5. The culture stock was kept at 30 ppt salinity with f/2 medium. To control salinity fluctuation, the opening of each bottle was covered firmly. Required glass materials were sterilized by autoclaving at a temperature of 121°C and a pressure of 15 pound/inch^2.

3.3. Preparation of seawater

Natural sea water was collected from Bay of Bengal with the assistance of "Bangladesh Navy", during their regular patrol. The sea water of 30 ppt was filtered with filter paper after being treated with 15 ppm active chlorine by dissolving required bleaching powder (Annex-1).

3.4. Preparation of nutrient media

In this study, Guillard f/2 (Hassan *et al.*, 2015), TMRL (Rajeswari and Balasubramanian 2014), Conway or Walne's (Walne, 1974), Maxicrop (Awal *et al.*, 2015) and Agricultural Fertilizer (Nabris, 2012) media were used for the enrichment of microalgae *Tetraselmis* sp. The preparation and compositions of each nutrient media is presented in Table 1.

Table 1: Nutrient composition of various culture media for culture of microalgae

Nutrient's name	Nutrient's concentration (gm. in 100 ml distilled water)				
	Guillard f/2	TMRL	Conway	Fertilizer	Maxicrop
$NaNO_3$	7.50	10.00	10.00		1.84
$NaH_2PO_4.H_2O$	0.50	1.00	2.00		0.48
KH_2PO_4					1.24
Na_2SiO_3		0.10			
K_2S					0.024
$CuSO_4$	1.00		0.20		0.02
$ZnSO_4$	2.20				0.012
$MgSO_4$					2.13
ZnCl			0.21		
$CoCl_2$	1.00		0.20		
$MnCl_2.4\ H_2O$	18.00		0.10		0.012
Na_2MoO_4	1.00				0.0001
$(NH_4)_2MoO_4$			0.20		
EDTA	0.44		4.50		
$FeCl_3.6\ H_2O$	0.32	0.30	0.13		0.02
Thiamine hydrochloride	2.00 Then 500µl in		0.20		
Biotin	0.01 100ml				
Cyanocobalamin	0.01 DW		0.01		
H_3BO_3			3.34		0.004
Ammonium Sulfate				1.50	
Urea				0.075	
TSP				0.25	
LiquidNutrient solution				0.5ml/L sea water.	

3.5. Preparation of salt water

Seawater was diluted with distilled water to prepare different concentration of saline water (15, 20, 25, and 30ppt). The sea water was filtered with filter paper after being treated with 15 ppm active chlorine by dissolving required bleaching powder. Then, vigorous aeration was done for 6 hours to eliminate residual chlorine from the water.

3.6. Microalgae enrichment with various nutrient media

190 ml of seawater and 10 ml of stock solution of microalgae were poured in 250 ml plastic bottle and enriched with five different nutrient media (Guillard, TMRL, Conway, Maxicrop and commercial fertilizer). Stock solution of nutrient media was kept separately in 250ml conical flask. Various media were added to the sample from the stock solution by using micropipette. Nutrient media were provided to the sample once at the starting of the experiment.The initial cell density of *Tetraselmis* sp. was 5.33 ± 0.882 $\times 10^4$ cells/ml at the time of stocking (inoculation density at initial day). Microalgae were also enriched (without nutrient media) with natural seawater as a control. Each treatment consists of three replications. The bottles were screwed carefully to avoid evaporation. The bottles were shaked vigorously once or twice everyday. The experiment was conducted for forty five days.

3.7. Microalgae culture with different salt concentrations

In the second experiment, *Tetraselmis* sp. was cultured for thirty one days in four (4) salinity conditions viz. 30, 25, 20 and 15ppt in TMRL nutrient media. To determine the least suitable salinity each treatment had three replications. The total volume of salt water was 190 ml and stock solution was 10 ml in each bottle. The initial cell density of *Tetraselmis* sp. was 3.33 ± 0.333 $\times 10^4$ cells/ml.

3.8. Rotifer culture and enrichment

Finally, a simple and randomized experimental design was performed to evaluate the effect of microalgae (*Tetraselmis* sp.) cultured in medium TMRL, Conway and Guillard medium on the growth performance of *B. plicatilis*. Three repetitions per treatment were performed. The experiment was conducted for 11 days in half liter plastic bottles with working volume of 200ml inoculated with rotifer at a density of 2ind./ml,with constant light and aeration. Temperature (25 ± 1°C) and optimum salinity (25‰) for rotifer were maintained according to the findings of Hassan *et al.* (2015). Microalgae were added daily as feed.

3.9. Growth evaluation

The growth evaluation of the microalgae was done by performing the cell count. During the culture period, number of *Tetraselmis* sp. cells was counted every 48 hours. The quantitative enumeration of plankton cell/ml for each flask was carried out with the help of a Sedgwick – Rafter (S-R) counting cell slide under compound microscope. As *Tetraselmis* sp. and rotifer (*Brachionus plicatilis*) are motile, first all of the sample was treated with Lugol Solution (20g

potassium iodide and 10g iodine crystals dissolved in 200ml distilled water containing 20ml glacial acetic acid) to kill the cells and stirred well. Before filling the S-R counting cell with sample, the cover glass was diagonally placed across the cell and then samples were transferred with a pipette so that no air bubbles in the cell can be formed. The S-R counting cell was stunned for at least 15 minutes to settle plankton. The entire bottom of the slide area was examined carefully. Thus the microalgae were counted and were expressed as cells per liter of the sample. Number of microalgae in the S-R cell was derived from the following formula (APHA 1999).

$$\text{No/ml} = \frac{C \times 1000\ mm^3}{L \times D \times W \times S}$$

Where,

C = Number of Organisms Counted;

L = length of each strip (S-R cell length) in mm;

D = depth of a strip (whipple grid image width) in mm;

W = Width in mm,

S = number of strips counted

3.10. Data analysis

Statistical analysis was performed using Microsoft Excel and IBM SPSS Software Package Version 21. The data didn't comply with normality (Shapiro-Wilk) test. The production parameters of microalgae and rotifers were analyzed by Friedman Test and Wilcoxon Signed Ranks Test (non-parametric tests). Stationary phase in different media of the microalgae was compared by using Mann- whitney test (non-parametric test). A significance level of $\alpha = 0.05$ was considered in all cases. Cell densities were expressed as the mean number of cells/ml ± standard error.

Chapter- 4

Result

4.1. Growth performance of *Tetraselmis* sp. in different culture media

The total culture period of *Tetraselmis* sp. was forty five days in different culture media e.g. seawater, Guillard f/2, TMRL, Conway or Walne's, Maxicrop and locally available commercial fertilizers. The cell density was $5.33\pm0.881\times10^4$cells/ml at initial day for all media.

4.1.1. Growth performance of *Tetraselmis* sp. in seawater

The highest density of *Tetraselmis* sp. in sea water was observed as $102.889\pm7.27\times10^4$ cells/ml at 25th day. The stationary phase started at 21th day ($101\pm4.50\times10^4$ cells/ml) and continued until day 27 of culture period ($102\pm6.80\times10^4$cells/ml) (Figure 1).

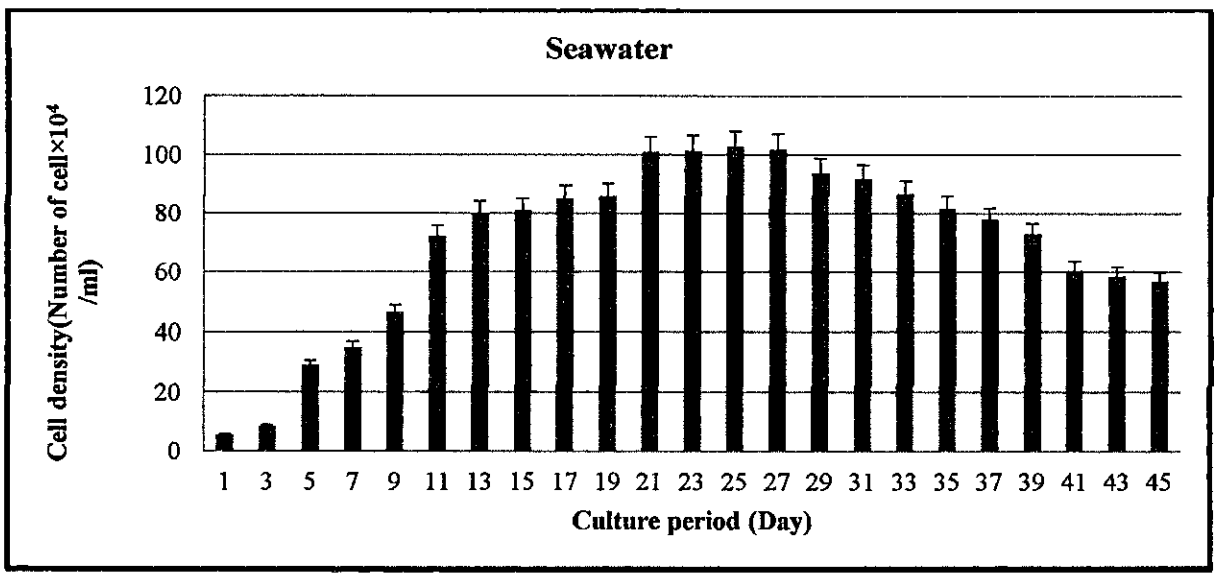

Figure 1: Growth performance of *Tetraselmis* sp. in seawater during culture time

4.1.2. Growth performance of *Tetraselmis* sp. in Guillard

The cell density was ranged from 5.333±.88×10^4 cells/ml to 65.00±4.04 ×10^4 cells/ml after stocking in Guillard medium. In this medium, the stationary phase continued from day 25 to 31 of culture period.The highest cell density of *Tetraselmis* sp. enriched with Guillard was found 202.333±4.91×10^4 cells/ml at the beginning of stationary phase. The declining phase started from day 33 (Figure 2).

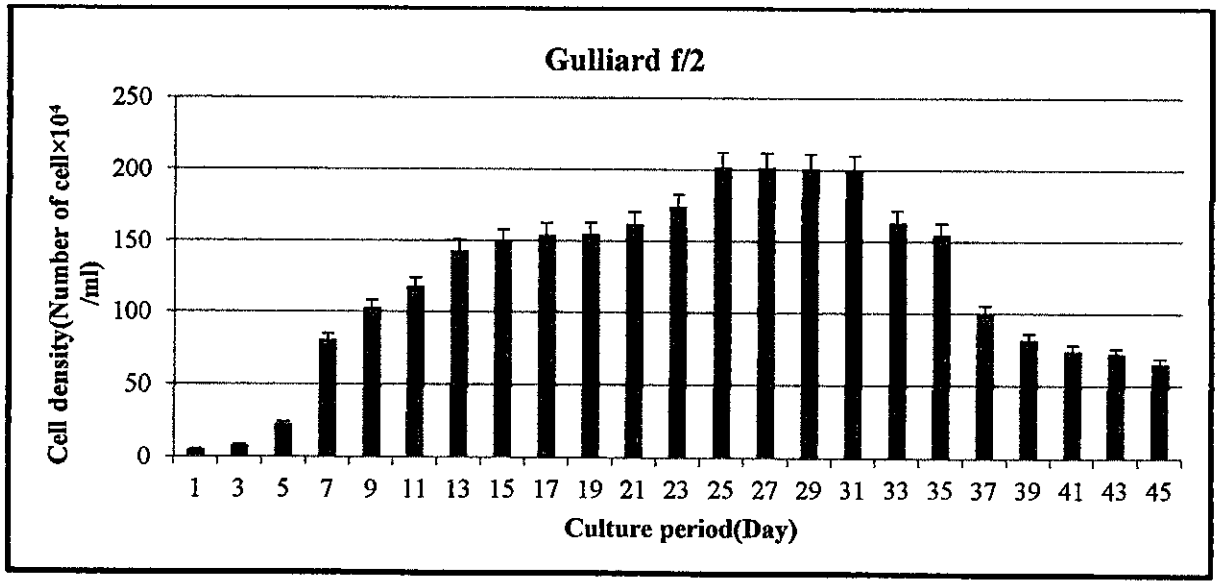

Figure 2: Growth performance of *Tetraselmis* sp. in Guillard during culture time

4.1.3. Growth performance of *Tetraselmis* sp. in Conway

Day 21th was the first day of stationary phase in Conway medium of *Tetraselmis* sp. which continued until day 29 of culture period. The highest cell density of *Tetraselmis* sp. in this medium was 241±5.178×10^4 cells/ml at the 25th day. At the last day of the observation, the cell density was 96.898±4.55×10^4 cell/ml (Figure 3).

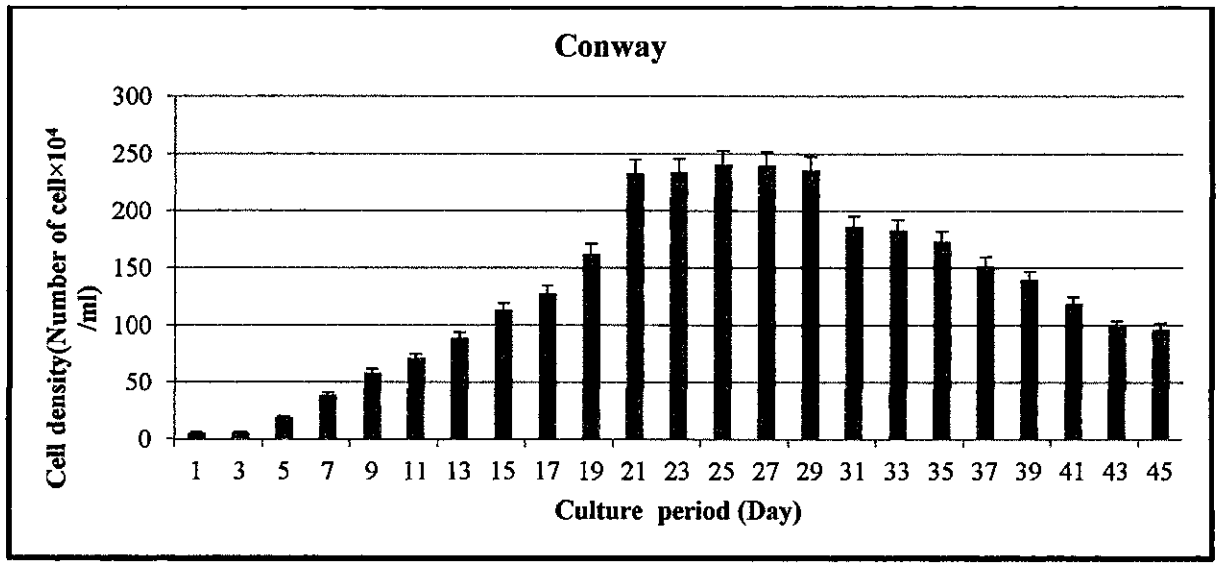

Figure 3: Growth performance of *Tetraselmis* sp. in Conway during culture time

4.1.4. Growth performance of *Tetraselmis* sp. in TMRL

The highest cell density of *Tetraselmis* sp. in TMRL was almost nearer to the highest cell density in Conway medium and it was 225.148 ±4.62×10^4 cell/ml at the 19th day. Day 17th (220±9.81× 10^4cells/ml) was the first day of the stationary phase that continued up to 27th day (207±4.93× 10^4 cells/ml) of culture period (Figure 4). Cell growth declined gradually from 29th day.

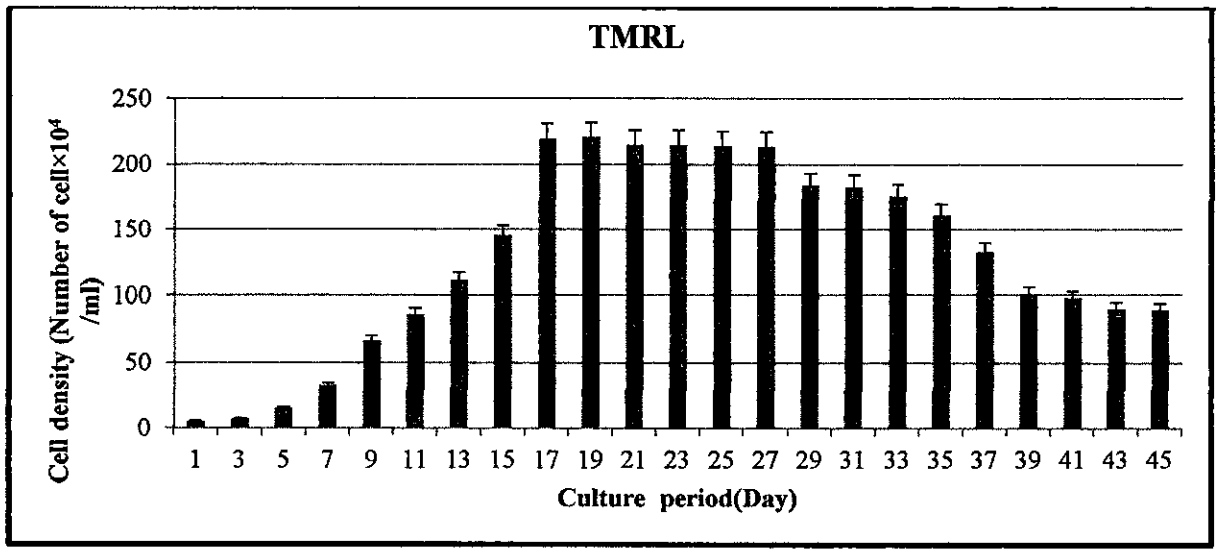

Figure 4: Growth performance of *Tetraselmis* sp. in TMRL during culture time

4.1.5. Growth performance of *Tetraselmis* sp. in Maxicrop

Figure 5 shows the growth pattern of *Tetraselmis* sp. enriched with Maxicrop. At the beginning of the stationary phase (19th day), the cell density was observed 97.232±3.128× 10^4 cells/ml and this phase continued up to 25th day (98±6.03× 10^4 cells/ml) of culture period. The maximum cell density of *Tetraselmis* sp. was observed 99±3.46× 10^4 cells/ml at day 21. The declining phase started at 27th day finally. Some cells were found still alive (10±.0577× 10^4 cells/ml) at the last of this phase.

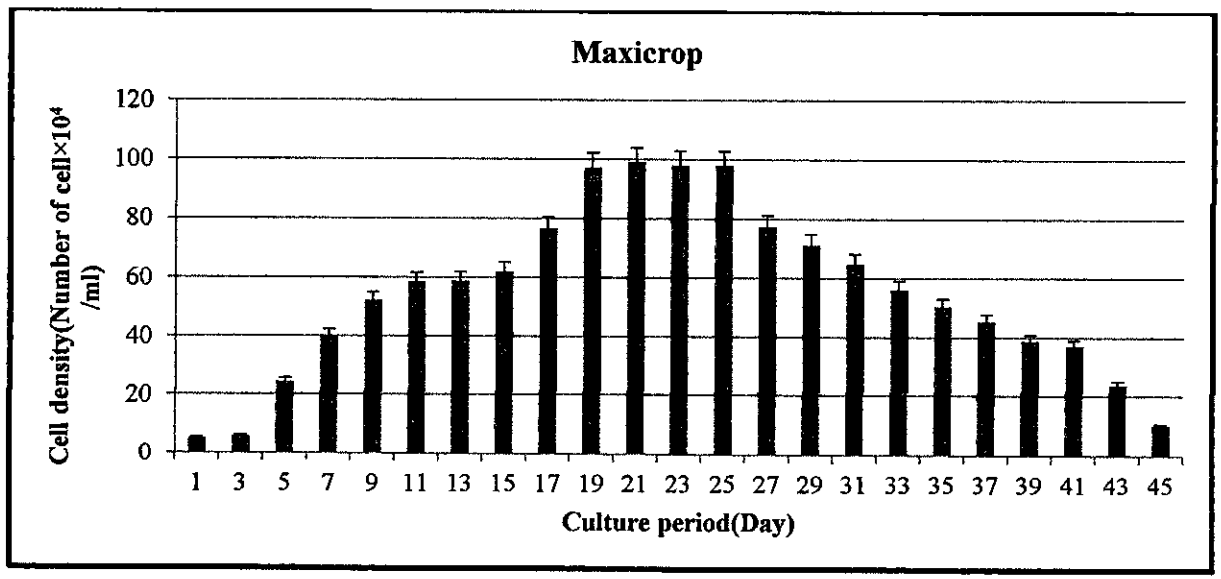

Figure 5: Growth performance of *Tetraselmis* sp. in Maxicrop during culture time

4.1.6. Growth performance of *Tetraselmis* sp. in fertilizer

The maximum cell density of *Tetraselmis* sp. in fertilizer was observed 53.67±6.69× 10^4 cells/ml at day19, which was the lowest maximum cell density among all the media. The stationary phase started at 17th day (53±6.557×10^4 cells/ml) and continued until day 21 of culture period (50.667±4.63×10^4cells/ml). Cell growth declined gradually from 23th day. At the last day of the observation, almost all the cell was dead and the cell density was 1±.0577×10^4 cell/ml (Figure 6).

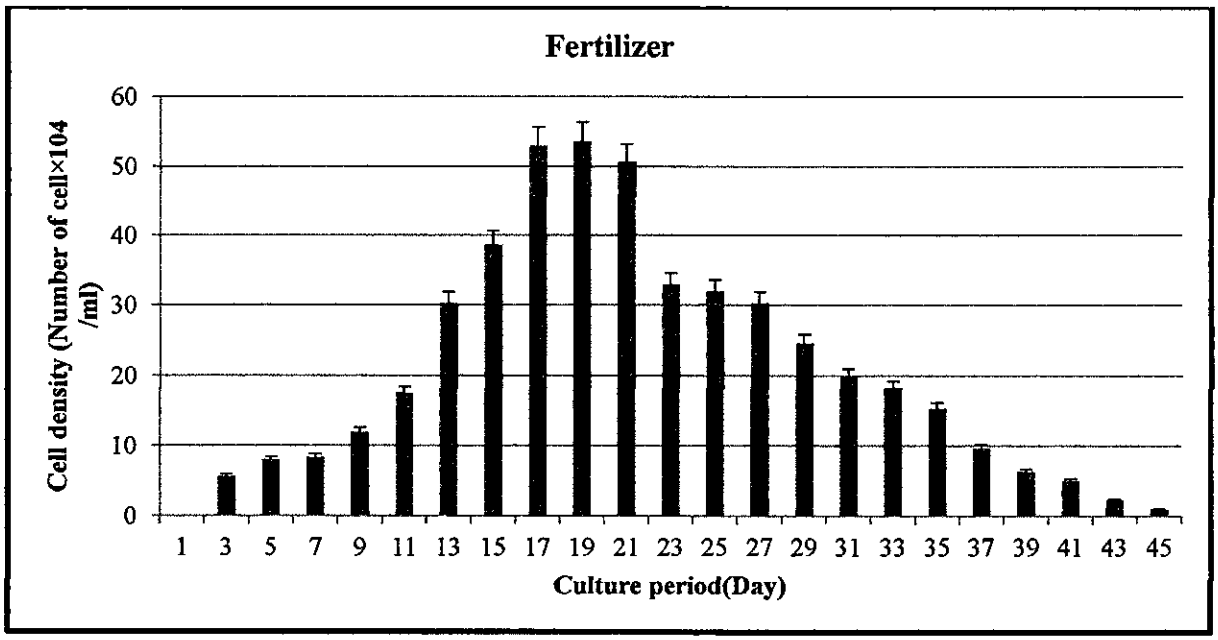

Figure 6: Growth performance of *Tetraselmis* sp. in fertilizer during culture time

4.1.7. Comparison of the growth of *Tetraselmis* sp. among different media

Figure 7 describes a comparison of the growth pattern of *Tetraselmis* sp. in seawater and different culture media. The duration of the stationary growth phase among the treatments varied from each other. Average *Tetraselmis* sp. concentration during stationary phase were also significantly different among different medium ($P < 0.05$).The average microalgae concentration during stationary phase in conway medium was 1.11 times higher than TMRL medium. The cell density of *Tetraselmis* sp. in this medium was 2.32, 1.18, 2.41, 4.51 times higher than respectively sea water, Guillard , maxicrop and fertilizer during stationary phase.The longest duration of the stationary phase of the species was observed in TMRL medium that sustained for 11 days. And the shortest stationary phase was observed in fertilizer medium that sustained only for 5 days. The duration of the stationary growth phase in conway and Guillard media was respectively 9 and 7 days.

Among all culture media *Tetraselmis* sp. in Conway medium showed the significantly highest growth performance ($241\pm5.178\times10^4$ cells/ml) followed by TMRL medium ($225.148 \pm4.62\times10^4$ cells/ml), Guillard f/2 medium ($202.333\pm4.91\times10^4$ cells/ml), seawater ($102.889\pm7.27\times10^4$ cells/ml), Maxicrop medium ($99\pm3.46\times 10^4$ cells/ml) and commercial fertilizer ($53.67\pm6.69\times 10^4$cells/ml). Both of the Gulliard and TMRL media showed significantly higher ($P < 0.05$) growth performance than seawater. Culture of *Tetraselmis* sp.

in Maxicrop ($99\pm3.46\times 10^4$ cells/ml) showed better performance than fertilizer ($53.67\pm6.69\times10^4$ cells/ml), but its performance was lower than the Guillard f/2 or TMRL culture medium. Growth performance in fertilizer was lowest than the others. In seawater, the highest density of this species was almost half of the highest density in Guillard medium.

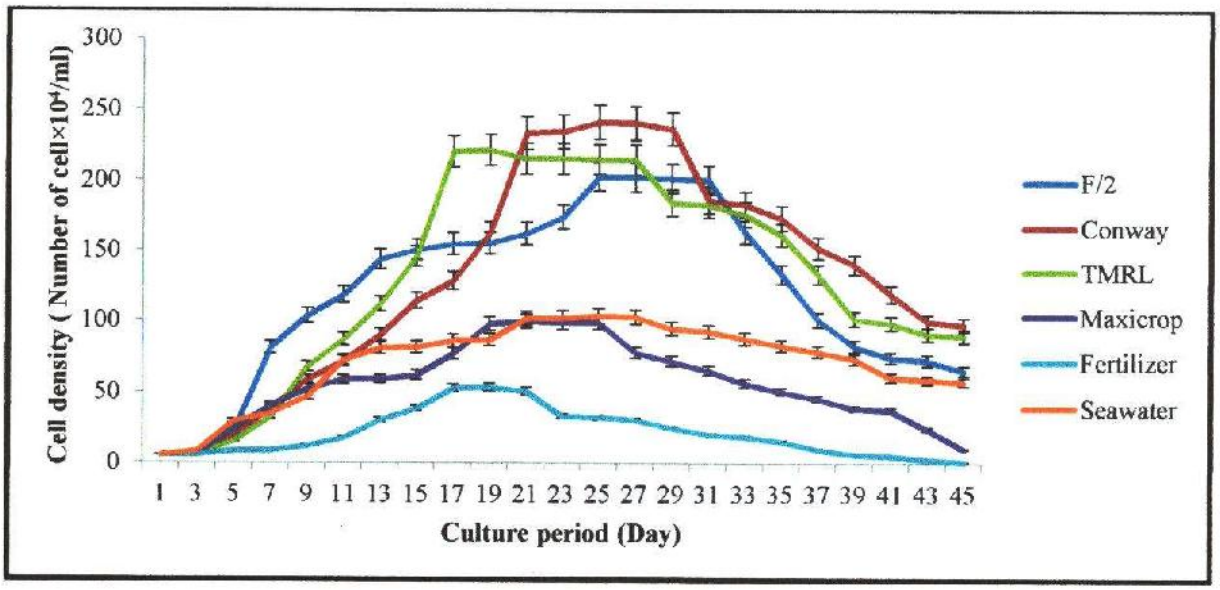

Figure 7: Growth performance of *Tetraselmis* sp. in different media during culture time

4.2 Growth performance of *Tetraselmis* sp. in different salinity

The total culture period of *Tetraselmis* sp. in four salinity viz. 15ppt, 20ppt, 25ppt, and 30ppt enriched with Conway medium was thirty one days. The initial cell density of *Tetraselmis* sp. was 3.33±.33× 10^4 cells/ml at the initial day of culture period.

4.2.1. Growth performance of *Tetraselmis* sp. in 15ppt salinity

In 15ppt salinity, the peak density (84±4.73× 10^4 cells/ml) was found at the 13th day of culture period, beginning of stationary phase. The stationary phase was continued from 13th to 17th days of culture period (Figure 8). Finally, the decline phase started after 17th day and it gradually declined trend in growth. At the last day (day 31) of the observation, some cells were still alive (13.111±2.31× 10^4 cells/ml).

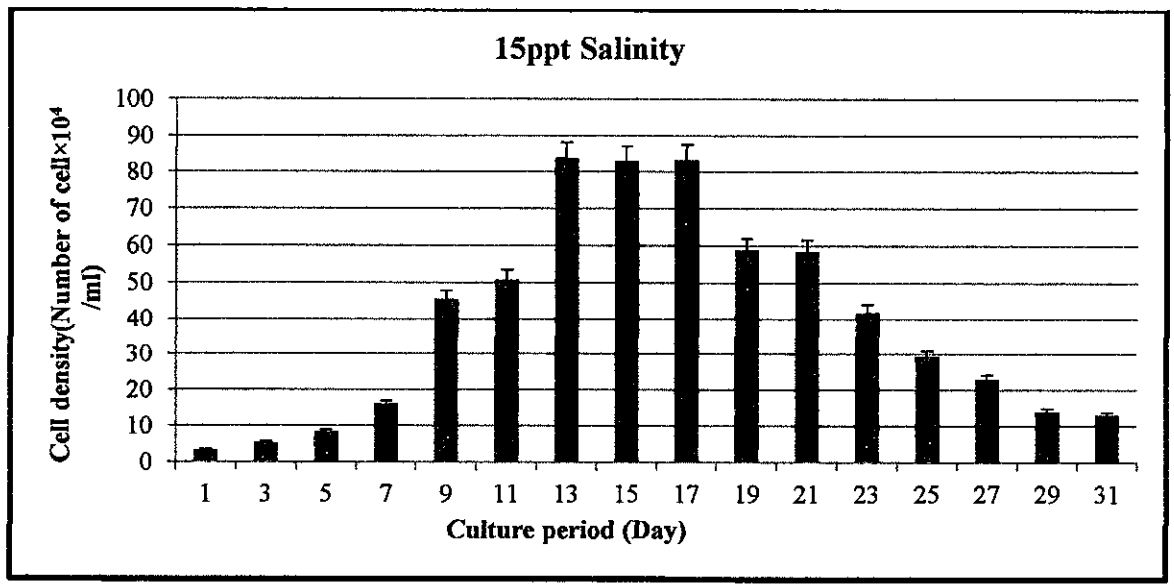

Figure 8: Growth performance of *Tetraselmis* sp. in 15ppt salinity during culture time

4.2.2. Growth performance *Tetraselmis* sp. of in 20ppt salinity

Figure 9 reveals that, the stationary phase of *Tetraselmis* sp. enriched with Conway medium in 20ppt salinity began at day 13 (89.5±3.75× 10^4 cells/ml) and remained almost similar up to 19th day (89±2.08× 10^4 cells/ml). The maximum cell density of *Tetraselmis* sp. was observed 92±2.309×10^4 cells/ml at day17. At the beginning of declining phase, the cell density was 61±4.041×10^4 cells/ml.

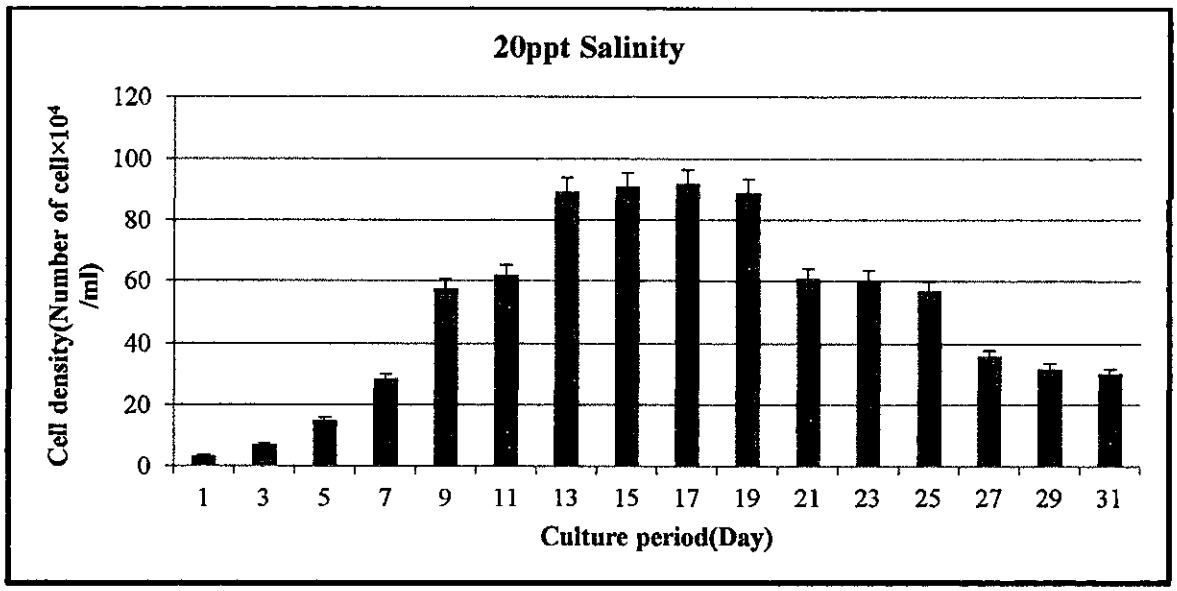

Figure 9: Growth performance of *Tetraselmis* sp. in 20ppt salinity during culture time

4.2.3. Growth performance of *Tetraselmis* sp. in 25ppt salinity

The peak density of *Tetraselmis* sp. was 207±6.11 × 10^4 cells/ml in 25ppt salinity that was counted at day 25 of culture period. Day 19th (198±4.73× 10^4cells/ml) was the first day of the stationary phase that continued up to 27th day (199.6667±6.17× 10^4 cells/ml). At the last day (day 31) of the observation many cell were still alive (164±8.96× 10^4 cells/ml) (Figure 10).

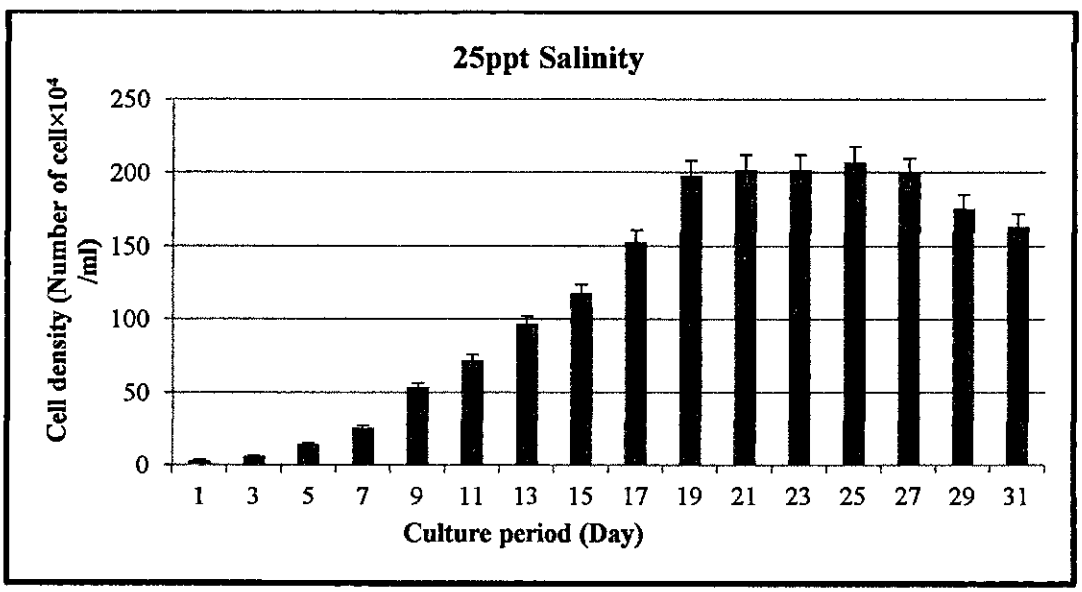

Figure 10: Growth performance of *Tetraselmis* sp. in 25ppt salinity during culture time

4.2.4. Growth performance of *Tetraselmis* sp. in 30ppt salinity

Among all the salinity, the highest cell density of *Tetraselmis* sp. was found in 30ppt salinity, which was significantly different from others ($P < 0.05$). Day 21th was the first day of stationary phase continued until day 27 of culture period. The highest cell density of *Tetraselmis* sp. in this medium was $232.889 \pm 5.755 \times 10^4$ cells/ml at the 23th day. At the last day of the observation, the cell density was $178.443 \pm 14.475 \times 10^4$ cell/ml (Figure 11).

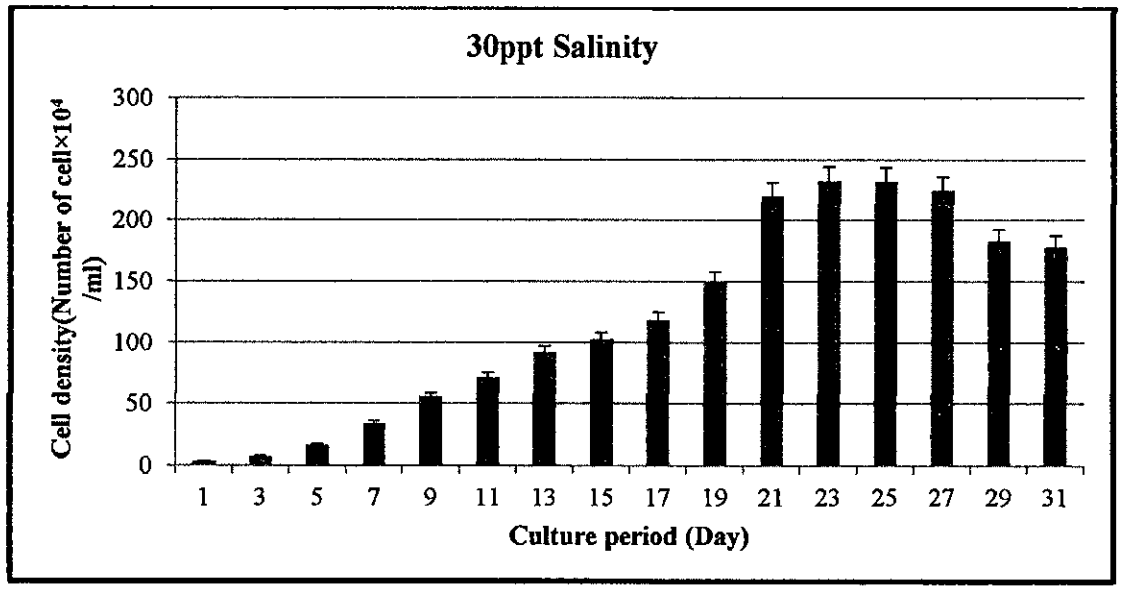

Figure 11: Growth performance of *Tetraselmis* sp. in 30ppt salinity during culture time

4.2.5. Comparison of the growth performance of *Tetraselmis* sp. among different salinity

The growth performance of *Tetraselmis* sp. in four treatments were compared in figure 12.The figure shows that density of *Tetraselmis* sp. is decreasing with reducing salt-concentrations. The average microalgae concentration in 30ppt was 1.13, 2.53 and 2.77 times higher respectively than 25ppt, 20ppt and 15ppt. The stationary growth phase of the species among the treatments started at different days of stocking and the duration of the phase among the treatments varied. The longest duration of the stationary phase of the species was observed in 25ppt salinity that sustained for 9 days. In addition, the shortest stationary phase was observed in 15ppt that sustained only for 5 days. But the duration of stationary phase of the species was same in 30ppt and 20ppt salinity. The growth of *Tetraselmis* sp. was significantly ($P < 0.05$) higher in 30ppt than 15ppt and 20ppt,while it was not significantly different($P > 0.05$) from 25ppt salinity. The highest cell density of *Tetraselmis* sp. in 30ppt

salinity was 232.889±5.755×10^4 cells/ml, followed by 25ppt (207±6.11 × 10^4 cells/ml), 20ppt (92±2.309×10^4 cells/ml) and 15ppt salinity (84±4.73× 10^4 cells/ml).

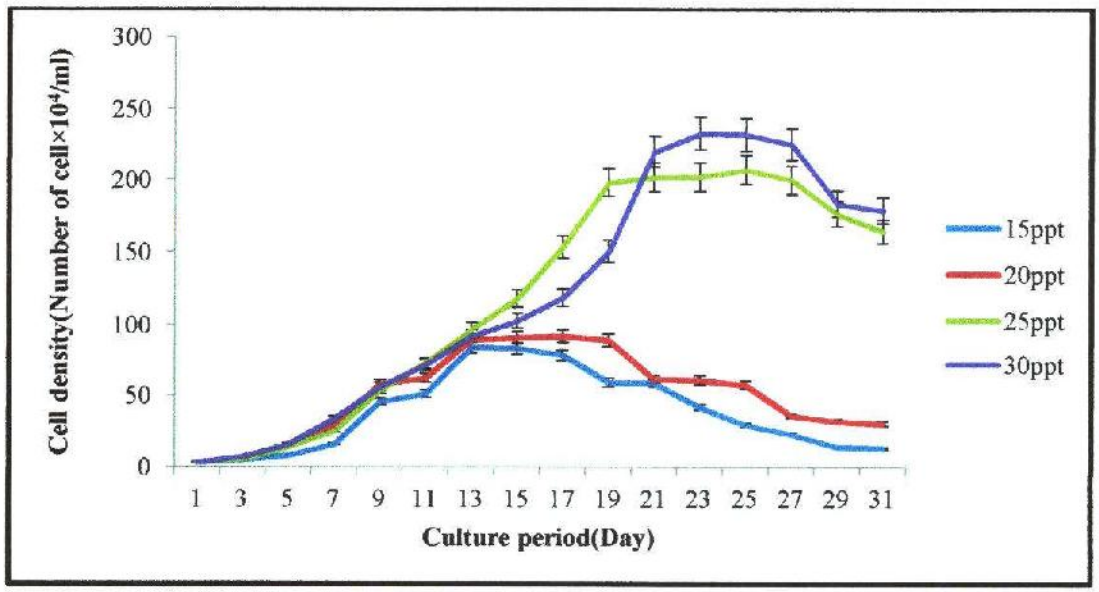

Figure 12: Growth performance of *Tetraselmis* sp. in different salinity during culture time

4.3. Rotifer culture and enrichment

4.3.1. Growth performance of rotifer (*Brachionus plicatilis*) fed with *Tetraselmis* sp. cultured in TMRL, Conway and Guillard media

The rotifers (*Brachionus plicatilis*) were fed with *Tetraselmis* sp. that was enriched with TMRL, Conway and Guillard media. Figure 13 describes a comparison of the growth pattern of rotifers fed with these three types of media. Up to day seven growth performance of rotifers in all media were almost equal, but distinct variations were noticed from day nine.

After day 7, number of rotifers in Conway medium increased very rapidly than others. Conway showed significantly highest ($P < 0.05$) growth rate followed by TMRL and Guillard. The population growth of rotifers fed on *Tetraselmis* sp. enriched with Guillard and TMRL media was not only similar ($p = 0.268$) with the growth values, but also nearly three times lower than that fed on *Tetraselmis* sp. enriched with Conway media at the end of the experiment(annex-3). At the last day (day 11) of the observation, the number of rotifers in Conway, TMRL and Guillard were 56.111±1.25 ind./ml, 20.333±.882 ind./ml and 18.0±1.15 ind./ml respectively.

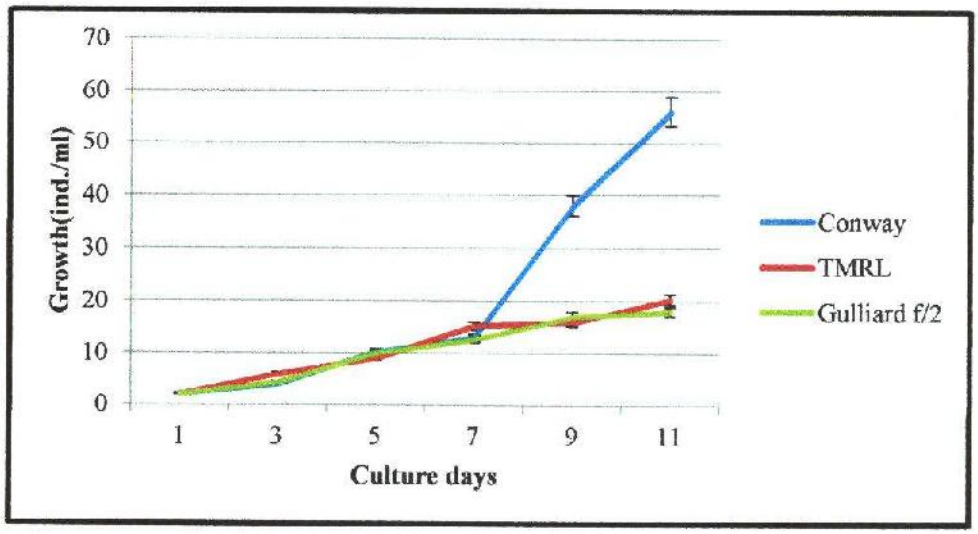

Figure 13: Growth performance of rotifer *B. plicatilis* under in different treatment

Chapter-5

Discussion

The culture of natural planktonic algae is essential because, in the early critical stages of rearing the larvae in hatcheries, some phytoflagellates (species of *Isochrysis, Pavlova, Dicrateria,Chromulina* and *Tetraselmis*) and some nannoplankters (species of *Chlorella* and *Synechocystis*) are used as basic food.

5.1. Growth performance of *Tetraselmis* sp. in different culture media

The present experimental study was conducted in order to find out most suitable medium for *Tetraselmis* sp. With least suitable salinity. The results of this investigation demonstrated a superiority of Conway medium in term of most suitable medium over other media. But the results also demonstrated that the production cost of Conway medium is almost 9 times higher than the TMRL medium and 0.497 times cheaper than Guillard medium.

Conway medium showed the highest population cell count in comparison to other culture media. The cell density of *Tetraselmis* sp. in this medium was ($241\pm5.178\times10^4$ cells/ml) followed by TMRL medium ($225.148 \pm4.62\times10^4$ cells/ml), Guillard f/2 medium ($202.333\pm4.91\times10^4$ cells/ml), seawater ($102.889\pm7.27\times10^4$ cells/ml), Maxicrop medium ($99\pm3.46\times 10^4$ cells/ml) and commercial fertilizer ($53.67\pm6.69\times 10^4$cells/ml). So the cell density in Conway medium was 2.34, 1.19, 2.43, 4.49 times higher than respectively sea water, Guillard , maxicrop and fertilizer. The result consistent to that of Rajeswari *et al.*,(2014), where he found the Conway medium to be the most suitable culture medium for the enhancement of algal (*Skeletonema costatum*) biomass among the TMRL medium (Tang Kang Marine Research Lab. Medium), TMRL medium with urea, Miquel's medium and Conway or Walne's medium. Although growth performance in Conway medium was 1.07 times higher than TMRL, its production cost was 8.68 times higher than TMRL. Growth performance of *Tetraselmis* sp. in Conway medium was significantly ($P< 0.05$) different from the other media. Similarly, Coelho *et al.*, (2013) used Guillard and Conway media to verify the algal growth and productivity in different culture media. Where the average cell density of *Tetraselmis tetrathele* in Guillard f/2 (2.28 ± 0.08 g L-1) and in Conway (3.37 ± 0.03 g L-1) were statistically different ($p < 0.05$) from each other. Cell density of *Tetraselmis* sp. in Guillard and TMRL is statistically different and both of them are 1.96 and 2.19 times higher than seawater respectively. The mean growth performance of this species with Guillard

medium was significant ($P = 0.000$), being considerably higher than Maxicrop and fertilizer. Similar result was also obtained with *P. salina* where the mean growth performance of this species with Guillard medium was significant ($P = 0.0001$), being considerably higher than Maxicrop (Awal *et al.*, 2015). Growth performance of this species in maxicrop is 1.84 times higher than fertilizer. In this experiment, the performance of fertilizer as a culture media was lowest among all the media. This result is consistent with previous study of Kanlis *et al.*, (2004) who indicated that, the components of the NPK fertilizers are mostly suitable for crop (land) agriculture and provide only the barest essentials for the growth of algae. Leal et al., (2004) also reported that the excessive use of fertilizer (Nutrilake) resulted in lower production of the microalgae *Tetraselmis suecica*, compared to other nutrient sources. On the other hand, it was predicted that Conway medium is the most suitable medium for growing more algal biomass which contains vitamin B1and B12 and several micro and macro nutrients (Mayavan and Thangavel, 2014). Besides that, it has been stated that the absence of meta-silicate in Guillard could have a negative effect on the growth of microalgae. Because silicate is, an essential nutrient and its deficiency may negatively affect the growth process of diatoms (Wen and Chen, 2000). This may be the reason why cell dencity is higher in TMRL, between TMRL and Guillard.

The stationary phase of the *Tetraselmis* sp. in TMRL was significantly different ($P< 0.05$) from Conway and Guillard media. The stationary phase of this species in Guillard was also different from seawater. The duration of the stationary phase in TMRL medium was 1.22 and 2.20 times higher than Conway and fertilizer respectively. The duration of stationary phase in this medium was also 1.57 times higher than both Guillard medium and seawater. The duration of this phase in Conway medium was 1.28 times higher than the Maxicrop.

This result showed the superiority of Conway to TMRL in term of growth performance and it was 1.07 times higher than TMRL, but its production cost was almost nine times higher than TMRL. Growth performance in TMRL was also higher than the other media. However, this study revealed that TMRL could be the most suitable and cost effective nutrient media for the growth of *Tetraselmis* sp.

5.2. Growth performance of *Tetraselmis* sp. in different salinity in TMRL medium

In the second experiment, four different salinity viz. 30 ppt, 25 ppt, 20 ppt and 15 ppt, were used. *Tetraselmis* sp. showed that the growth rate decrease with decreasing salinity. The

results of this investigation demonstrated that 30ppt showed the highest cell density in comparison to other salinity, but statistically it was comparable with 25ppt. The maximum cell density of *Tetraselmis* sp. in 30ppt was 1.13, 2.53 and 2.77 times higher respectively than 25ppt, 20ppt and 15ppt. The mean growth performance of this species in 30ppt salinity was significant ($p < 0.05$), being considerably higher than 15ppt and 20ppt. Khatoon *et al.*, (2014) also showed that *Nannochloropsis* sp. and *Tetraselmis* sp. had significantly higher ($p < 0.05$) cell density, lipid and carbohydrate content under control condition at 30 ppt. Although, The peak density of *Tetraselmis* sp. was $207 \pm 6.11 \times 10^4$ cells/ml in 25ppt salinity and in 30ppt it was $232.889 \pm 5.755 \times 10^4$ cells/ml , but it was statistically similar ($P = 0.084$) to each other. The growth performance in 15ppt salinity was significant being considerably lowest among all the salinity.

During the culture period, the stationary phase of *Tetraselmis* sp. in these four media was significantly different ($P < 0.05$) from each other. The duration of this phase in 25ppt was almost two times higher than 15ppt. The duration of this phase in the same salinity was 1.20 times higher than both the 20ppt and 30ppt. the stationary phase in both 20ppt and 30ppt was 1.40 times higher than 15ppt.

5.3. Growth performance of rotifer (*Brachionus plicatilis*)

Next, this study also addressed the influence of differently grown *Tetraselmis* sp. (grown in different media) on growth of the rotifer *Brachionus plicatilis*. Quantitative changes of zooplankton fed with different diets is one of the common approaches to evaluate the diet effect on the zooplankton growth because, food type can affect growth rate of rotifers (Malekzadeh, 2012).

The present study revealed that *Tetraselmis* sp. grown in Conway medium possesses the dietary superiority over *Tetraselmis* sp. grown in TMRL and Guillard when these are used as feed of rotifers. At the last day of the experiment, the number of rotifers in Conway medium was 2.76 times and 3.12 times higher respectively than TMRL and Guillard media. And the number of rotifers in TMRL medium was 1.13 times higher than Guillard medium. This result is consistent with previous study of Sahiduzzaman (2016) where he also showed that the number of rotifers (*Brachionus plicatilis*) fed on *N. oculata* enriched with TMRL medium was 1.09 times higher than fed on *N. oculata* enriched with Guillard medium.

Several nutrient media are being used to enrich microalgae and rotifer. The present experiment reveals that although the growth performance of microalgae in Conway medium possesses superiority over TMRL and other media. The result also reveals that, production cost of Conway is too much higher than TMRL medium.

However, further study is needed to observe the growth performance of *Tetraselmis* sp. in TMRL medium and Conway medium in large volume of water area. Study also needed to observe the growth performance of crustaceans and fish post larvae fed with rotifer enriched with *Tetraselmis* sp. grown in TMRL medium and Conway medium in the best laboratory conditions of this study.

Chapter – 6

Conclusion

The growth performance of *Tetraselmis* sp. was studied in six different nutrient media viz. Guillard, TMRL, Conway, Maxicrop, seawater and locally available commercial fertilizers in laboratory condition. The growth performance of *Tetraselmis* sp. was also studied in different salinity viz. 15ppt, 20ppt, 25ppt and 30ppt in TMRL medium to determine suitable salinity. Besides these, the growth performance of rotifer (*Brachionus plicatilis)* enriched with *Tetraselmis* sp. was evaluated in best three nutrient media viz. Guillard, TMRL and Conway.

Depending on the findings of this study, it might be concluded as:

- *Tetraselmis* sp and rotifer (*Brachionus plicatilis)* can grow well at 25 ± 1°C and 1600-2000lx.
- TMRL medium is a suitable and cheap medium for the culture of *Tetraselmis* sp. in laboratory.
- Medium prepared with locally available commercial fertilizers is totally unsuitable for the culture of this microalgae.
- 30ppt is a suitable salinity for the culture of *Tetraselmis* sp. However, growth performance in this salinity is not significantly different from the growth performance in 25ppt.
- Conway medium is suitable for the culture of rotifer (*Brachionus plicatilis)* fed with Conway-enriched-microalgae in laboratory.

Chapter-7

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ANNEX 01

Measuring the level of chlorine in commercial liquid bleach or bleaching powder:

- Dissolve 0.5-1.0 g potassium iodide crystals (KI) in 50 ml distilled water and add 5 ml of glacial acetic acid (CH_3COOH), or enough to reduce the pH to between pH 3.0-4.0.
- Add 1.0 ml of either the commercial liquid bleach (NaOCl) or 1.0 ml of a solution of the bleaching powder [consisting of 15.00 g of the bleaching powder ($Ca(OCl)_2$) in 100 ml of distilled water].
- Using standard 0.1N sodium thiosulphate ($Na_2S_2O_3$. $5H_2O$), titrate (away from direct sunlight) until the yellow colour of the liberated iodine has almost disappeared, add 1 ml starch indicator21, and titrate until the blue colour disappears.
- Calculate the level of active chlorine in the bleach solution. 1 ml of 0.1 N sodium thiosulphate = 3.54 mg active chlorine.

Reading:

Reading	Initial	Final	Difference	Average
1st	22.1	16.5	5.6	5.6
2nd	16.5	11.0	5.5	
3rd	11.0	5.4	5.6	

Calculation for commercial liquid bleach:5.6 ml of sodium thio-sulphate are required to reach the end point of the titration. So, there is 5.6 x 3.54 = 19.824 mg of active chlorine in 1 ml of the commercial bleach solution.

So, the level of active chlorine in the liquid commercial bleach is therefore -(19.824 x 100) ÷ (15 x 1000) x 100% = 13.216% Cl.

So, amount of required bleaching powder is –(100 x 15) ÷ 13.216 = 113.50 mg

ANNEX 02

Growth comparison of *Tetraselmis* sp. among different nutrient media

Related- Samples Friedman's Two way Analysis of Variance of Ranks

Ranks

	Mean Rank
Seawater	3.13
Gulliard	4.68
Conway	4.84
TMRL	4.72
Maxicrop	2.47
Fertilizer	1.16

Test Statistics[a]

Total N	**207**
Chi-Square	**707.751**
Degrees of Freedom	**5**
Asymp. Sig.	**.000**

a. Friedman Test

Wilcoxon Signed Ranks Test
Test Statistics

	Sea - TMRL	Sea - Con	Sea- Gulliard	Maxi - TMRL	Maxi - Con	Gulliard - TMRL	Gulliard - Con	Ferti- Maxi	Ferti - TMRL	Ferti - Con
Z	-11.582[a]	-11.360[a]	-11.732[a]	-11.667[a]	-11.569[a]	-3.810[a]	-3.740[a]	-12.153[a]	-12.148[a]	-12.172[a]
Asymp. Sig. (2-tailed)	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000

a. Based on negative ranks.

	Sea - Maxi	Sea - Ferti	Gulliard - Ferti	Gulliard - Maxi	Con - TMRL
Z	-9.648[b]	-12.171[b]	-12.195[b]	-11.992[b]	-2.508[b]
Asymp. Sig. (2-tailed)	.000	.000	.000	.000	.012

b. Based on positive ranks.

Mann-Whitney Test
Ranks

Test Statistics[a]

	Con-TMRL	Gulliard-Con	Gulliard-Sea	Gulliard-TMRL	Maxi-Fertilizer
Mann-Whitney U	390.500	50.500	.000	145.000	.000
Wilcoxon W	1875.500	716.500	666.000	811.000	378.000
Z	-5.798	-7.223	-7.303	-6.817	-6.755
Asymp. Sig. (2-tailed)	.000	.000	.000	.000	.000

a. Grouping Variable: Media

ANNEX 04

Growth comparison of Rotifer *(Brachionuspicatilis)*

Related- Samples Friedman's Two way Analysis of Variance of Ranks

Ranks

	Mean Rank
Conway	2.27
TMRL	1.91
Gulliard	1.82

Test Statistics[a]

Total N	**54**
Chi-Square	**7.704**
Degrees of Freedom	**2**
Asymp. Sig.	**.021**

a. Friedman Test

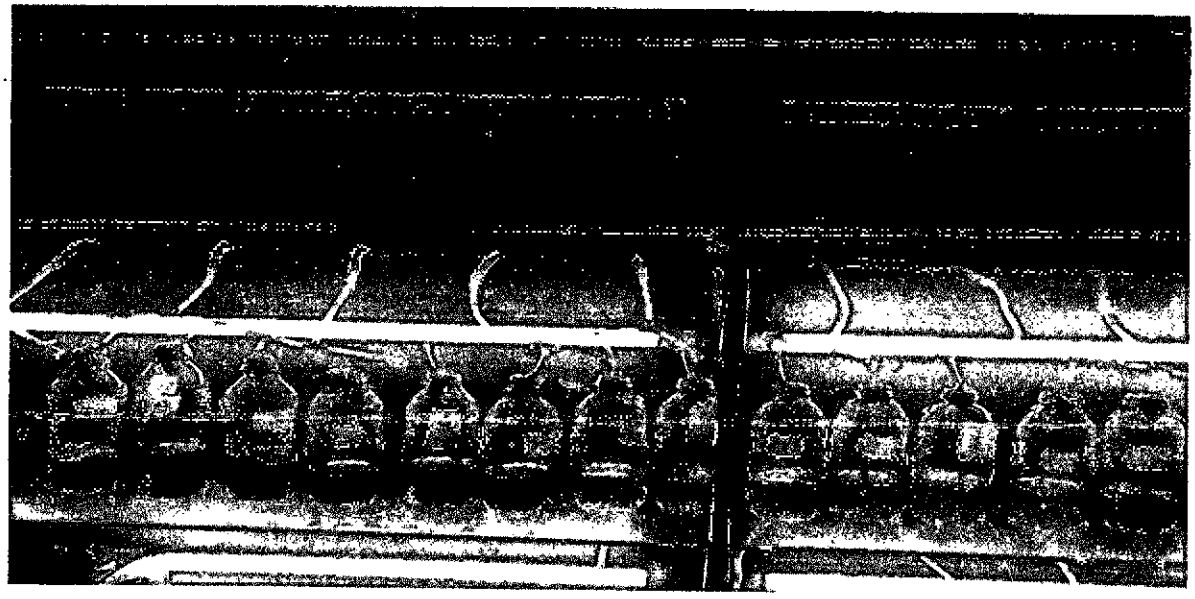
Wilcoxon Signed Ranks Test
Test Statistics

	TMTL - Conway	Guillard - Conway	Guillard - TMTL
Z	-3.231[a]	-4.027[a]	-1.107[a]
Asymp. Sig. (2-tailed)	.001	.000	.268

a. Based on negative ranks

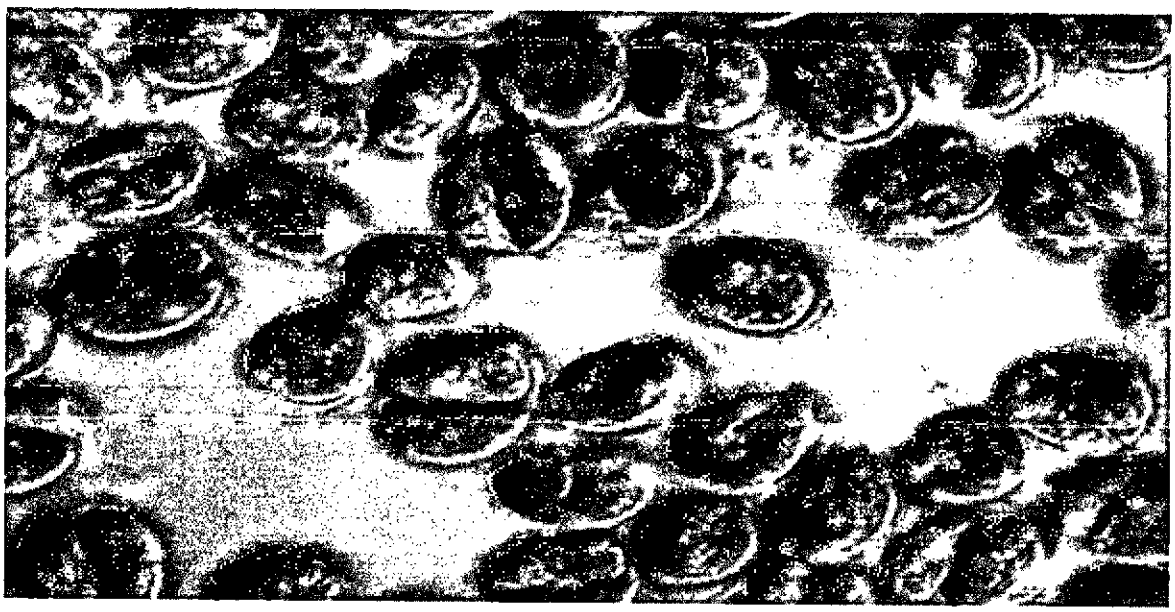
Photo Gallery

Photograph-1:Stock solution of *Tetraselmis* sp.



Photograph-2:Culture of *Tetraselmis* sp. in different media

Photograph-3:Counting of rotifer (*Brachionus plicatilis*) by using Microscope and Sedgwick–Rafter(S-R) counting cell



Photograph-4:Microscopic view of *Tetraselmis* sp.