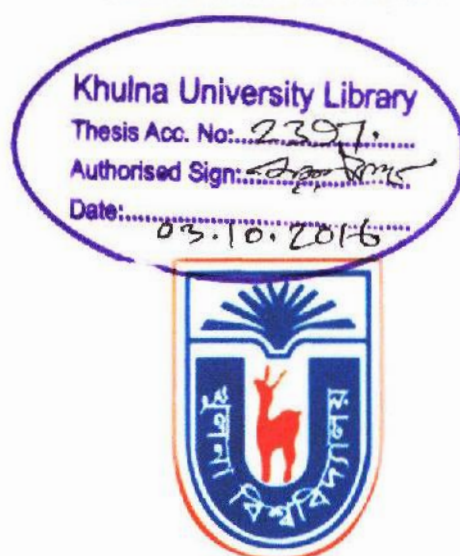


Growth performance of Marine microalgae *Chaetoceros gracilis* in different media for indoor culture system

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Growth performance of Marine microalgae *Chaetoceros gracilis* in different media for indoor culture system

A Thesis

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**Growth performance of Marine microalgae *Chaetoceros gracilis* in
different media for indoor culture system**

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Declaration

Growth performance of Marine microalgae *Chaetoceros gracilis* in different media for indoor culture system

The results presented in the project thesis with the above mentioned title are entirely of the candidate's own investigations and no part of the results has been accepted for any degree nor is it being concurrently submitted for any degree.

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Dedicated to

My Beloved Parents

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Abstract

The growth performance of algae, *Chaetoceros gracilis*, was studied using three nutrient media viz. (i) Walne's or Conway, (ii) Miquel's and (iii) TMRL, designated as Treatment-1 – 3 (T-1 –3) respectively, at laboratory condition for 27 days at 1600-2000 Lux light intensity. The culture was conducted in 1L. transparent plastic bottle with an initial cell density of 2×10^2 cells /ml. with three replicates of each treatment maintaining constant temperature of 25°C and 30 ppt salinity with continuous aeration and illumination. *C. gracilis* cultured in T-1 showed significantly ($P < 0.05$) higher growth with a mean peak density ($195 \pm 0.288 \times 10^2$ cells/ ml) than T-2 ($137 \pm 0.376 \times 10^2$ cells/ml) and T-3 ($117 \pm 0.527 \times 10^2$ cells/ml) on the 11th day of culture. Stationary growth phase among the Treatments varied from 11 to 16 days (T-1: 11-15days; T-2: 12-15 days and T-3: 12-16 days) after which declining phase started and no individual found alive at 27th day of culture. It resembles that Walne's or Conway media is suitable for the culture of *C. gracilis*.

Key Words: Microalgae, *Chaetoceros gracilis*, Nutrient media

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List of Abbreviations and non-English Words

Abbreviation	Elaboration
BFRI	Bangladesh Fisheries Research Institute
cm	Centimeter
DO	Dissolved Oxygen
DoF	Department of Fisheries
et al.	And Others
e. g.	Example Given/For Example
cell/ml	Cell per milliliter
Fig.	Figure
FMRT	Fisheries and Marine Resource Technology
mg	Milligram
g/gm	Gram
i. e.	Which to Say
KU	Khulna University
l	Liter
mm	Millimeter
MS	Microsoft
p.	Pages
PL	Post Larvae
SE	Standard Error
SPSS	Statistical Programme for Social Science
TDS	Total Dissolved Solids
viz.	Namely/That is to Say/As Follows

CHAPTER ONE



INTRODUCTION



Introduction

1.1. Background of the Study

In recent years, there has been renewed interest in producing single cell protein by mass culturing the unicellular micro-algae such as diatoms (species of *Chaetoceros* and *Skeletonema*) and nannoplankters (species of *Isochrysis*, *Chlorella* and *Tetraselmis*) for feeding the larvae of crustaceans, molluscs and fishes, as the success of any hatchery operation depends mainly on providing the required species of microalgae.

Microalgae (i.e. single-celled algae or phytoplankton) represent the largest, yet one of the most poorly understood groups of microorganisms on earth. As happens with plants relative to terrestrial animals, microalgae represent the natural nutritional base and primary source of bulk nutrients in the aquatic food chain. Microalgae play crucial nutritional role with regard to marine animals in the open sea, and consequently in aquaculture. Most marine invertebrates depend on microalgae for their whole life cycle, so commercial and experimental mollusc or fish hatcheries have included a microalgae production system in parallel to their animal production. Microalgae are utilized as live feed for all growth stages of bivalve molluscs (e.g. oysters, scallops, clams and mussels), for larval/early juvenile stages of abalone, crustaceans and some fish species, and for zooplankton used in aquaculture food webs at large. Productivity of any hatchery is directly related to the quantity and quality of the food source used therein.

Phytoplankton is photosynthesizing microscopic organism that inhabits the upper sunlit layer of almost all oceans and bodies of fresh water and from which the energy is transferred to higher organisms through food chain. Planktons are the basis of food chain in open water. They are agents for "primary production," the creation of organic compounds from carbon dioxide dissolved in the open water, a process that sustains the aquatic food web (Ghosal *et al.*, 2011). They have little or no resistance to currents and living as free-floating and suspended in open or pelagic water. It may be unicellular, colonial, or filamentous. Phytoplankton obtain energy through photosynthesis and must therefore live in the well-lit surface layer (termed the euphotic zone) of a water body.

Phytoplankton consists of one-celled marine and freshwater microalgae and other plant-like organisms. They are used in the production of pharmaceuticals, diet supplements, pigments,

and biofuels, and also used as feeds in aquaculture. Phytoplankton are cultured to feed bivalve molluscs (all life stages), the early larval stages of crustaceans, and the zooplankton (e.g., rotifers, copepods) that are used as live food in fish hatcheries. These zooplankton are the live food of crustaceans and fish. *Chaetoceros* sp. can be useful in establishing the rotifer culture protocol because of its high levels of vitamin B₁₂ and eicosapentaenoic acid (EPA) content (Okauchi *et al*, 1991).

The rotifer (*B. plicatilis*) is a rather sturdy species able to tolerate a wide range of salinity, temperature and ammonia levels. It can also use several food sources, provided that particle size remains within a 2-20 µm range. Obviously, the highest growth rate is achieved under more restricted environmental parameters, and is closely related to the selected rotifer strain and feeding provided. In particular, good yields are obtained with high dissolved oxygen levels, temperature at 25°C, pH at 7.5-8.5, salinity in a 20-30 ppt range, less than 1 mg/l free ammonia (NH₃), and moderate turbulence. Light is required only when rotifers are fed microalgae (FAO, 2006).

Rotifers are filter feeders and can be fed a variety of food types, including algae, yeast, bacteria or inert foods such as microcapsules and detritus. The type of feed used for culturing rotifers can have a significant effect on the cost of operations and on the nutritional value of rotifers. Micro-algae are the principal component of most cultured rotifer diet.

Therefore, micro-algae are indispensable in the commercial rearing of various species of marine animals as a food source for all growth stages of bivalve molluscs, larval stages of some crustacean species, and very early growth stages of some fish species. Algae are furthermore used to produce mass quantities of zooplankton (rotifers, copepods, brine shrimp) which serve in turn as food for larval and early-juvenile stages of crustaceans and fish. Besides, for rearing marine fish larvae according to the "green water technique" algae are used directly in the larval tanks, where they are believed to play a role in stabilizing the water quality, nutrition of the larvae, and microbial control.

All algal species are not equally successful in supporting the growth and survival of a particular filter-feeding animal. Suitable algal species have been selected on the basis of their mass-culture potential, cell size, digestibility, and overall food value for the feeding animal.

Marine microalgae play a key role in aquaculture development (Riquelme and Avendaño-Herrera, 2003). Unicellular marine algae are commonly grown as food for commercially valuable marine organisms (Utting, 1985). The diatom genus *Chaetoceros* is among the largest genera of marine planktonic diatoms and comprises several hundred species (Hasle and Syvertsen, 1997), some of which are important bloom formers in both oceanic and coastal habitats. The microalgal species *Chaetoceros* sp. for instance are frequently used in the culture of marine organisms. *Chaetoceros* sp. have good nutritional qualities, particularly highly unsaturated fatty acids and small cell size, so they are widely used in aquaculture as food in the early larval stages of mollusks, fish, and crustaceans (Godinez *et al.*, 2005). *Chaetoceros gracilis* is the most prominent marine microalgae. *C.gracilis*, in particular, has good nutritional value because of their small cell structures and unsaturated fatty acids.

Various techniques have been developed to grow these food species on a large scale, ranging from less controlled extensive to monospecific intensive cultures. However, the controlled production of micro-algae (*Chaetoceros gracilis*.) is a complex and expensive procedure. A possible alternative to on-site algal culture is the collection of algae from the natural environment where, under certain conditions, they may be extremely abundant. Furthermore, in order to overcome or reduce the problems and limitations associated with algal cultures, various investigators have attempted to replace algae using artificial diets either as a supplement or as the main food source.

The culture of microalgae began at the end of the 19th century, becoming one of the great discoveries of our time for Botany and for Science in general. In the beginning, research was focused on creating a method of culture that could propitiate the survival and maintenance of clowns in the laboratory, to develop knowledge about the photosynthetic mechanisms of the organisms. After some time, especially after the Second World War, new and varied proposals surged for the utilization of phytoplanktonic organisms.

Diatoms are known to be a good source of food for marine invertebrates, supplying protein there is an increased trend of culturing them in mass scale. Research on mass culture of diatom has been carried out in many parts of the world for the past fifty years. The larvae of prawns and fishes prefer diatoms as basic food. The success of any hatchery operations depends mainly on providing the required food species of micro algae suitable for the larvae. Even after the two decades of research on the formulation of the micro diets to replace live

food in larviculture, there is limited success. Even though alternate feeds are available in market, being costly; the micro algal culture is most economical live feed in hatcheries.

Growth of phytoplankton is controlled by some environmental factors such as temperature, salinity, water turbulence and aeration etc. (Tomas, 1978, Uye and Takamatsu, 1990). However, nutrients are also very important environmental factors that influence the growth of any algae (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 includes Conway, Modified for 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 brackish water microalgal species require a culture medium with a chemical composition similar to that of seawater. Besides carbon, the requirement of principal nutrients for phytoplankton is nitrogen and phosphorus, in an approximate ratio of 6:1 by weight, respectively. Trace minerals and vitamins (especially B₁₁ 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. The need for microalgae to employ either as direct food for larvae and rotifers or in green water culture is important to consider in light of the results of trials to test growth of some well known micro algae species against varying microalgal culture media.

The effect of the three culture nutrient media, viz., Walne's or Conway medium, Miquel's medium and TMRL medium on the growth of microalgae species *Chaetoceros gracilis* which is commonly used in aquaculture, have not yet been studied in prevailing local condition. Considering the need for a thorough investigation with the suitable culture nutrient media for microalgae culture, the present study has been designed with the objective to determine the effect of three selected inorganic nutrient media on the growth performance of *Chaetoceros gracilis*.



Objectives

The long-term objective of the study is to gain the optimum growth performance of microalgae (*Chaetoceros gracilis*). However, the objective of this study is to:

- Study the growth performance of *Chaetoceros gracilis* in different media
- Find out a suitable media for optimum growth of *Chaetoceros gracilis*

CHAPTER TWO



LITERATURE REVIEW

Literature Review

Some studies on microalgae have been carried out by many researchers in various countries of the world, but no significant works have been yet done in Bangladesh. However, in recent time there has been rising consciousness among the researchers in our country to study about microalgae, their culture method, suitable culture media, different influential factors and ultimately growth performance. Some important studies those were conducted on the growth performance of microalgae in different media for indoor and outdoor culture are mentioned below-

Lincymol *et al.* (2012) reported that the microalgae (*Chaetoceros calcitrans*) cultured in Walne's medium gave high values of protein and lipid content. Miquel's medium gave high value of carbohydrate. Although cell count was the highest in f/2 medium, Walne's medium provided the best nutritional quality of algae.

Banerjee *et al.* (2011) examined the growth and proximate composition of two marine microalgae, *Chaetoceros calcitrans* and *Nannochloropsis oculata*, cultured outdoors under shade (24 to 36°C, 140 $\mu\text{mol}/\text{m}^2/\text{s}$) and laboratory conditions (environmental chamber, 23°C for *C. calcitrans* and 20°C for *N. oculata*, 150 $\mu\text{mol}/\text{m}^2/\text{s}$). Lipid content of *C. calcitrans* was significantly higher in outdoor culture. The experiment showed that both *C. calcitrans* and *N. oculata* grew faster in outdoors, producing more biomass within a shorter period of time.

Nezhad *et al.* (2012) showed that maximum growth rate of *C. calcitrans* was observed in 10 ppt salinity in Conway medium and highest cell density was achieved at 27 ppt salinity. The highest level of total carotenoid content was 5.8 mg/g fresh weight and the maximum protein content was at the 10 ppt in 60% of dry weight.

Homthong and Kongkangwal (2013) estimated the effect of various culture media, F/2 medium, Ketchum and Redfield medium, Miquel's medium, Sato and Serikawa medium, TMRL medium and Walne's medium on the growth of *Chaetoceros calcitrans* in the laboratory. From the experiment the best culture medium for *C. calcitrans* ($4.59 \pm 0.38 \times 10^6$ cells/ml) in the laboratory scale was Miquel's medium.

Raya *et al.* (2015) estimated on chlorophyll productivity and potency of hydrogen produced by phytoplankton *Chaetoceros calcitrans*, *Chlorella vulgaris*, *Dunaliella salina*, and

Porphyridium cruentum. Conway medium and vitamin was added with sea water as culture media on the fourth species of phytoplankton. The result indicated that phytoplankton *C. vulgaris* produced the most amount of the chlorophyll and the potency of hydrogen are 72.541 µg/ml and 6364 L/kg DW successively from the dry biomass 14187 g and the higher cell density 3.060×10^4 cells/ml.

Shah *et al.* (2003) discussed on the growth of three microalgae species, viz., *Nannochloropsis oculata*, *Tetraselmis chuii* 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 $221 \pm 4.24 \times 10^4$ cells/ml. *C. muelleri* attained the highest density of $321 \pm 5.41 \times 10^4$ cells/ml in Rix Mix medium and about same density ($319 \pm 12.02 \times 10^4$ cells/ml) at the 24th day of culture in modified Guillard's f/2 medium. However, with an increase in cell density, growth of *T. chuii* ($182 \pm 6.26 \times 10^4$ cells/ml) was significantly ($P < 0.05$) higher in BFRI medium.

A study was conducted by Sousa *et al.* (2014) on the growth potential of the green microalgae *Nannochloropsis* sp. using brackish ground water from a well in the semi-arid northeast region of Brazil as culture medium. The results showed that the culture tested was capable of growing in the brackish groundwater even at salinity levels as low as 2 ppt. Furthermore it was shown that municipal wastewater could be used as a sole nutrient source for *Nannochloropsis* sp.

Naghibi *et al.* (2015) stated that the growth of *Nannochloropsis oculata* has been evaluated in four different media (Conway, Guillard, N8, TMRL) and different salinity (20, 25, 30 ppt) with triplicate for treatments in laboratory (temperature was 25°C, pH=11, light intensity=2500LUX, light duration=18 hours and 6 hours darkness and also aeration using aquarium pump for 24 hours) During 14 days culturing. Significant differences between treatments with medium salinity differences exist ($p < 0.05$) and medium Conway salinity 25 ppt in terms of increased density and salinity 20 ppt the amount of a chlorophyll a in the first grade, medium Guillard with salinity 20ppt in terms of increased density and a chlorophyll a second level, medium TMRL salinity 25 ppt in terms of increased density and a chlorophyll a

third and medium N 8 with salinity 25 ppt in terms of increased density and salinity 20 ppt the amount of a chlorophyll a in fourth place fall.

Pacheco *et al.* (2010) showed that fatty acid content in microalgae can be modified by culture conditions. Three different concentrations of culture medium (f, f/2 and f/4) were used. The highest HUFA percentage was obtained with f/2 medium (8.65%) and the lowest was obtained with AF/2 medium (5.78%). *C. muelleri* cultured with f/2 medium or its equivalent AF/2 in an effort to obtain the highest HUFAs content. On the contrary, the use of AF/2 medium results in a more cost effective option.

Bindhu (2013) examined that the optimum concentration of culture media was required for the culture of *Chaetoceros calcitrans* under laboratory conditions to achieve a viable and economical culture method. The cell count, percentage of transmittance and the chlorophyll a values are significantly higher for the group in which the medium was provided on the initial day. The experiment indicating that the culture can grow upon the nutrients present in the seawater up to the 5th day and enrichment is needed only afterwards.

Raghavan *et al.* (2008) reported on the effects of temperature (20°, 25° and 30°C), salinity (25 and 35 ppt) and carbon dioxide addition (air1CO₂) on the growth and proximate composition of *C. calcitrans* under laboratory conditions. The addition of carbon dioxide affected the growth and biomass of *C. calcitrans*. At low temperatures (20° and 25°C) lipid and carbohydrate contents were higher but protein content was unaffected. Carbon dioxide addition increased protein, while lowering carbohydrates, but had no effect on lipid content. Carbohydrates were increased while lipids and protein decreased at the highest salinity 35 ppt.

Barros *et al.* (2014) discussed the growth and concentration of total lipids of the microalgae *C. muelleri* Lemmermann cultivated at three different salinities (15, 25 and 35 ppt). It was observed that the lipid yield (10.41±1.89, 10.87±2.10 and 12.33±1.81% at salinities of 15, 25 and 35, respectively) was not influenced by the salt concentration ($p>0.05$), but the maximum cell concentration was significantly higher ($p<0.05$) at salinities of 15 and 25, which was 16.3±1.50 and 17.6±2.90 x 10⁵ cell.ml⁻¹, respectively.

Costa *et al.* (1998) estimated the efficiency of additions of secondary sewage as a culture medium for *Chaetoceros gracilis* and *Thalassiosira* sp (Chrysophyceae) under laboratory conditions.). The best results in terms of cellular densities were observed at 40% addition of sewage. Maximum cellular densities were 4125.00×10^3 cells/ml for *Chaetoceros gracilis* on the ninth day and 834.00×10^3 cells/ml for *Thalassiosira* sp on the fifth day. Finally secondary sewage could be used as a culture medium for the species.

Cordova *et al.* (2006) described the effects of antibiotics on microalgae used as food for scallop larvae. Six different dose levels of chloramphenicol, erythromycin, and furazolidone were added to cultures of *Isochrysis galbana* and *Chaetoceros gracilis*. Chloramphenicol, erythromycin and furazolidone had adverse effect on *I. galbana* and *C. gracilis* microalgae but the positive effect on survival of the scallop larvae, decreasing bacterial population.

Chen *et al.* (2012) studied on four species of microalgae evaluating their ability to remove ammonia from intensive marine fish/shrimp culture systems at different temperatures. Total ammonium nitrogen (TAN) uptake efficiencies of these organisms at different temperatures were 10.3 pg dry weights (DWs) per cell for *Nannochloropsis oculata*, 40.4 pg for *Isochrysisaff. galbana*, 39.7 pg for *Chaetoceros muelleri*, and 369 pg for *Tetraselmis chuii* at 25°C. Overall, *Tetraselmis chuii* is a good choice for removing ammonia from indoor intensive marine culture systems.

Pal *et al.* (2013) estimated that microalgae have the most significant contribution towards the global environment, oxygen production and carbon cycle. The study estimated the optimum light intensity (Lux) required for achieving maximum yield of *Chaetoceros muelleri* in a laboratory culture. Maximum cells were counted during the culture period (168 hours) at Light intensity 1000 Lux. However, the experiment showed that the cell growth with 1500 lux is very close and similar to 1000 lux.

Lombardi and Wangersky (1991) estimated on production of intracellular and extracellular lipid classes by *Chaetoceros gracilis* over a wide range of supply rates of inorganic phosphorus and silicon. The production of triglycerides was triggered by nutrient stress with either phosphorus or silicon demonstrated with nitrogen. The synthesis of phospholipids was reduced under phosphorus stress, while the synthesis of chlorophyll a was increased by

silicon stress. Particulate lipid yield was highest in intermediate concentrations of both nutrients.

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. The best growth was obtained at 16 ppt and 25 ppt indicated that isolated species prefer alkaline condition for their optimum growth. At higher salinity of 40 ppt, the growth was drastically decreased. All isolates showed best growth at 20°C and 30°C. The growth has ceased at 40°C and 50°C.

Kutlu (2010) reported that diatoms contribute to the global carbon cycle regulating climates by using 40 percent of carbon in oceans. *Chaetoceros gracilis* were exposed to various concentrations of $\text{NH}_4\text{-N}$ based on f/2 medium in order to determine which nutrients have a limited effect on the growth of *C. gracilis*. As a result, while μ values of the *C. gracilis* isolated from two different medium are approximate, their K_s values are different. This may be related to the water's nutrient value.

Adams and Bugbee (2013) examined that silicon deficiency is a lipid-promoting stress for many diatoms. It was hypothesized that lowering sodium chloride below ocean levels may thus be effective in creating silicon stress and enhancing lipid productivity. Sodium chloride/silicon had strong interaction on total and ash-free dry mass densities that arose because low sodium chloride concentrations were inhibitory to growth, but the inhibition was overcome with excessive silicon supply. Low level of sodium chloride may have affected metabolism of silicon.

William and Anne (1972) described that cells of the tropical Pacific diatom, *Chaetoceros gracilis*, were grown in a nitrogen limited chemostat at varying percentages of the maximum growth rate. Growth rates seemed to be controlled by internal supplies of nitrogen and the apparent half saturation constant "K" decreased with increasing growth rate.

Chen and Wangersky (1996) studied on the production of dissolved organic carbon (DOC) in cultures of the diatoms *Chaetoceros gracilis* and *Phaeodactylum tricornutum*, the flagellate *Isochrysis galbana*, the dinoflagellate *Alexandrium tamarense*. In both *C. gracilis* and *I.*

galbana a major portion (>65%) of the organic matter released to the medium during log-phase growth had mol. wts of <10 000 Da.

Brown *et al.* (1997) described that microalgae are used in mariculture as live feeds for growth stages of molluscs, larval stages of crustaceans and some fish species, and for zooplankton used in mariculture food chains. Microalgae varied in their proportions of protein (66.52%), carbohydrate (55.23%) and lipid (7-23%). All species had similar amino acid composition, and were rich in the essential amino acids. Microalgal polysaccharides were variable in sugar composition, but most had high proportions of glucose (21~87%). All species had relatively high concentrations of ascorbic acid (1-16 mg g⁻¹ dry weight) and riboflavin (20-40 µg g⁻¹).

Ritcharoen *et al.* (2014) studied on configurations and modes of airlift photobioreactors used in the cultivation of *Chaetoceros gracilis*. Among the four systems investigated, the continuous culture in airlift photobioreactors-in-series provided the best performance with the highest cell density of 12.12×10^6 cells mL⁻¹ incorporated with large productivity.

Sunlu and Buyukisik (2006) estimated on growth kinetics of *Chaetoceros gracilis* Schütt from the results of nutrient enrichment experiments under batch culture conditions. Monod curve (Monod, 1942) parameters were calculated by using transformations and the least square method. K_s (Half Saturation Constant) values calculated for each of nutrients were found as follows; $0.496 \mu\text{mol NO}_3^- \text{N L}^{-1}$; $0.862 \mu\text{mol NH}_4^+ \text{-N L}^{-1}$; $1.045 \mu\text{mol PO}_4^{3-} \text{-P L}^{-1}$ and $0.558 \mu\text{mol Si L}^{-1}$. Concerning the same nutrients, values of maximum growth rate were found as 1.611 day^{-1} , 1.332 day^{-1} , 1.662 day^{-1} and 1.757 day^{-1} respectively.

Oliveira and Crispim (2013) examined the influence of culture mediums on the growth and development of the microalgae *Dunaliella marina* and *Nitzschia closterium* on laboratory. Higher densities of *D. marina* were observed on the Conway medium, but densities above 1.5×10^6 cells.mL⁻¹ were frequently observed on the compost extract medium.

Rajeswari *et al.* (2014) studied on microalgae, *Skeletonema costatum* isolated from Vellar estuary, southeast coast of India were cultured under $28 \pm 1^\circ\text{C}$ and salinity 28 PSU with 50 µmol m⁻² s⁻¹ of light using different culture media such as TMRL medium, TMRL medium with urea, Miquel's medium and Conway of Walne's medium. The results evidenced that the

Conway medium was found to be the most suitable culture medium for the enhancement of algal biomass.

A study was conducted by Campana-Torres *et al.* (2012) on the effect of different culture media on the productive response and proximate composition of microalgae, *Nannochloropsis oculata*, and the subsequent effect of such microalgae on the productive response and proximate composition of the rotifer *Brachionus rotundiformis*. Microalgae were cultured in different media: Guillard F/2 (control), an agricultural fertilizer, and an aquacultural fertilizer. A better productive response was observed when microalgae were cultured in the agricultural fertilizer. When used as natural food for rotifers, higher productive response was observed in those rotifers fed with microalgae previously cultured in the aquacultural fertilizer. They were also included that the productive response and quality of microalgae depended on the medium in which they grew; also, the productive response and proximate composition of rotifers depended upon the nutritional quality of *N. oculata*.

A few information are available on the effect of four popular culture media in a biodiesel production study on the qualitative and quantitative lipid content, dry biomass, and lipid productivity of *Nannochloropsis oculata*. According to Malakootian *et al.* (2015) culture of microalgae was done separately in Walne, F/2, Sato, and TMRL media. *N. oculata* exhibited the highest rate of cell growth and biomass productivity of 0.2616 day⁻¹ and 2.652 g l⁻¹ in the Walne's medium. The highest level of biomass conversion into lipids in TMRL medium revealed a cell dry weight of 37.22%. Walne's medium proved to have the most efficient lipid productivity which was 0.1057 g l⁻¹ day⁻¹. The highest amount of triacylglycerol (TAG) was obtained in Sato medium in the stationary growth phase and was 75.25% of the fatty acids. The present study provides a practical benchmark, which allows the introduction of Walne's as a suitable culture medium for *N. oculata* in biodiesel studies.

Palanichamy and Rani (2004) revealed that the results of experiments on the viability and culture of the microalgae, *Nannochloropsis oculata* under preserved conditions. Uncentrifuged samples were kept in refrigerator (without cryoprotector) to observe the duration of viability and the algae could be maintained for 18 months by cooling. The cell quality under cooled culture condition was observed to be better and indicated the population growth similar to regular continuous culture after reinoculation. Variation in growth rate and cell concentration was observed between Walne's and Locally Developed Nutrient Medium (LDM). The cooling method preservation was most simple and effective for *Nannochloropsis*

oculata. Twelve months old preserved samples of cooled culture were raised and inoculated to outdoor mass culture using LDM.

A study by Coelho *et al.* (2013) aimed 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 (Guillard f/2 and Conway). The experiment was conducted in nine-liter containers, with working volume of eight liters, salinity $35 \pm 2\%$, temperature $28 \pm 1^\circ\text{C}$, with constant light and aeration, around $200\mu\text{E cm}^{-2} \text{ s}^{-1}$ and 3 L air min^{-1} . Regarding the culture media, a higher algal productivity was observed in the cultivation developed in Conway medium ($p < 0.05$), obtaining at the end of the cultivation, biomass of $3.38 \pm 0.02 \text{ g L}^{-1}$. In the Guillard medium, only $2.47 \pm 0.05 \text{ g L}^{-1}$ was obtained. 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.

CHAPTER THREE



METHODOLOGY

Methodology

3.1 Study Area:

The culture of the microalgae was done in a temperature controlled (about 25°C) microalgae laboratory at Fisheries and Marine Resources Technology (FMRT) discipline of Khulna University, Khulna. The culture was maintained in 1 liter transparent mineral water bottles with sufficient aeration. The brackish water of 30 ppt was treated, for disinfection, with 30 ppm active chlorine by dissolving bleaching powder at the rate of 100mg/L and kept for 24 hours. Then vigorous aeration for 6 hours was done to eliminate residual chlorine from the water. The treated water was filtered through double rings 102 filter paper 12.5 cm qualitative (New, 2008). This disinfected water was used with different nutrient media (Table. 2) to prepare different culture media viz. Walne's or Conway medium, Miquel's medium and TMRL medium. The algal cells were inoculated into the bottles with equal initial concentration (2×10^2 cells/ml) for the species of microalgae. Aeration was moderate and the source was aquarium aerators. The light intensity of about 1600-2000 lux/m²/S. was maintained for culture.

3.2 Duration of Study

The duration of the study was 27 days.

3.3 Sampling Frequency

Samples were collected every day for three times.



3.4 Sample collection: The Sample (*Chaetoceros gracilis*) was collected from the Marine Fisheries and Technology Station, Cox's Bazar of Bangladesh Fisheries Research Institute (BFRI). Brine (100 ppt) was collected from salt pan or commercial prawn hatchery.

3.5 Preservation of sample:

Collected samples were preserved with Lugol Solution (20g potassium iodide (KI) and 10g iodine crystals dissolved in 200mL distilled water containing 20mL glacial acetic acid). Lugol solution was added in an amount of 0.7mL per 100mL of sample. The sample with solution was preserved at 4°C in refrigerator.

3.6 Necessary equipments: Culture, light, pH meter, aeration/mixing, saline water, autoclave, flask, microscope, foil paper etc. (FAO, 2006).

Table 1: Name of treatment in different culture bottles

Treatment	Bottle no	Replication
Conway medium(T-1)	1	T1R1
		T1R1
		T1R1
	2	T1R2
		T1R2
		T1R2
	3	T1R3
		T1R3
		T1R3
Miquel's medium(T-2)	1	T2R1
		T2R1
		T2R1
	2	T2R2
		T2R2
		T2R2
	3	T2R3
		T2R3
		T2R3
TMRL medium(T-3)	1	T3R1
		T3R1
		T3R1
	2	T3R2
		T3R2
		T3R2
	3	T3R3
		T3R3
		T3R3

3.7 Counting Cell Preparation and Counting Procedure:

The quantitative enumeration of the plankton was carried out with the help of a Sedgewick-Rafter (S-R) counting cell which is 50 mm long, 20 mm wide and 1 mm deep. Before filling the S-R cell with sample, the cover glasses were diagonally placed across the cell and then samples were transferred with a large bore pipette so that no air bubbles in the cell covers were formed. The S-R cell was let stunned for at least 15 minutes to settle plankton. Then plankton on the bottom of the S-R cell was enumerated by electronic microscope. By moving the mechanical stage, the entire bottom of the slide area was examined carefully. To achieve a random sampling, each time 3 fields were examined for each sample and an average of the

counts had been recorded. The organisms thus counted, were expressed as cells per liter of the sample. From each sample 30 cells counts in 3 slides have been made to achieve random counts and an average of the counts has been recorded. Number of plankton in the S-R cell was derived from the following formula.

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

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. The number of cells per mm was multiplied by a correction factor to adjust the number of organisms per liter (APHA, 1976).

3.8 Microalgae Identification

Chaetoceros gracilis was identified by using checklists of different scientists.

3.9 Statistical analysis:

Statistical analysis was performed by using SPSS Software Package Version 16.0 (SPSS Inc., Chicago, IL, USA). The validity of all the data was tested through Square, Square root, Cubic root, Reciprocal root, Reciprocal Square root, Box-Cox-transformation, GLMADMB model associated with R software. After validity testing statistical analysis was done. All the data were analyzed statistically using Analysis of Variance (ANOVA) and the significance test among mean values of treatments was done using Tukey and Duncun's New Multiple Range Test (Zaman *et al.* 1982). A value of ($P < 0.05$) was considered statistically significant.

Table 2: For the stock culture of microalgae, various culture media are prevalent

Ingredients	Amount of Ingredients		
	Miquel's medium	TMRL medium	Walne's or Conway medium
A. Potassium nitrate	20.2 gm	10.gm	100 gm
Sodium orthophosphate	-	1.0 gm	20 gm
EDTA (sodium salt)	-	-	45 gm
Boric acid	-	-	33.4 gm
Ferric chloride	-	.3 gm	1.3 gm
Manganese chloride	-	-	0.36 gm
Distilled water	100ml	100ml	1 lit
B. Calcium chloride	2.0 gm	-	-
Ferric chloride	2.0 gm	-	-
Hydrochloric acid	2.0 ml	-	-
Sodium silicate	-	.1 gm	-
Distilled water	100 ml	-	1 lit
Zinc chloride	-	-	4.2 gm
Cobalt chloride	-	-	4.0 gm
Ammonium molybdate	-	-	1.8 gm
Sodium orthophosphate	4.0 gm	-	-
C.1.Vitamin B ₁ (Thiamine HCL)	-	-	200mg dissolved in 100ml dist. water
2.VilamineB ₁₂ (Cyanacobalamine)	-	-	10 mg dissolved in 100ml dist water

(Reference: Kaladharan and Tara, 1997)

CHAPTER FOUR



RESULTS

Results

4.1 Growth Performance of *Chaetoceros gracilis* in Conway medium

The total culture period of *Chaetoceros gracilis* was 27 days in three culture media viz. Conway, Miquel's and TMRL. The initial cell density was 2×10^2 cells/ml at the time of stocking (inoculation density at zero days). The peak density of *C. gracilis* in Conway medium was observed $195 \pm 0.288 \times 10^2$ cells/ml at the 11th day of culture period, beginning of the stationary phase and the stationary phase found to continue up to 15th day of culture period (Fig.1) that showed no significant difference ($P > 0.05$) among these days (days 11 to 15), (Annex-2). Finally, declining phase started at 16th day and at 27th day of culture period all the cell became dead although it showed gradually declining trend in growth with significant difference ($P < 0.05$) among the days (day 16 to 26).

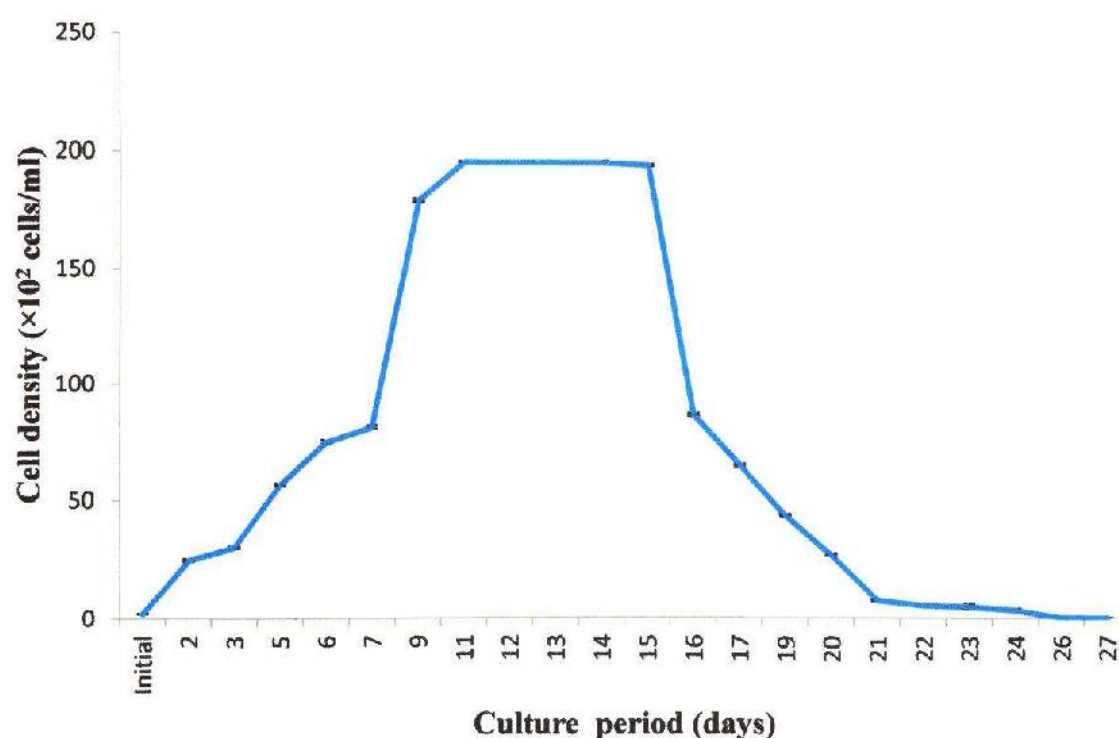


Fig 1: Growth Performance of *Chaetoceros gracilis* in Conway medium at different time interval.

4.2 Growth Performance of *Chaetoceros gracilis* in Miquel's medium

In Miquel's medium the initial cell density was same as the Conway medium at the time of stocking (inoculation density at zero day). The peak density of *C. gracilis* in Miquel's

medium was observed $137 \pm 0.376 \times 10^2$ cells/ml at the 12th day of culture period at the stationary phase. The stationary phase (lowest cell density $135 \pm 0.503 \times 10^2$ cells/ml) and highest cell density ($138 \pm 0.647 \times 10^2$ cells/ml) was started at the 12th day of culture period which continued up to 15th day (Fig: 2) that showed no significant difference ($P > 0.05$) among these days (day 12 to 15), (Annex-2). Finally the declining phase started at 16th day and at 26th day of culture period (declining phase) none found alive although it showed gradually declining trend in growth with significant difference ($P < 0.05$) among the days (day 16 to 26) in this media.

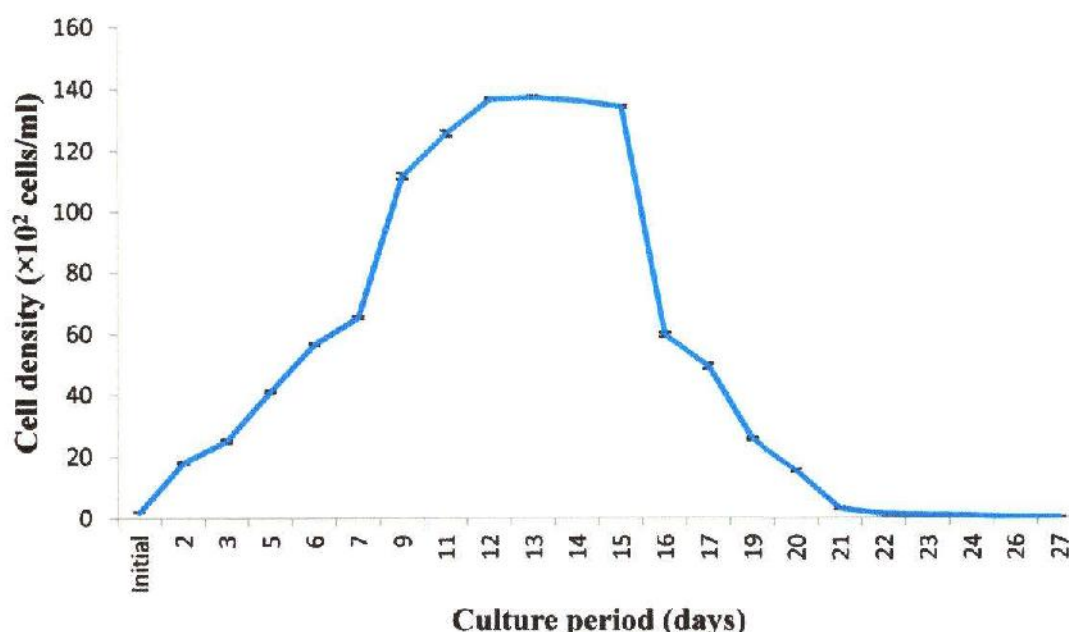


Fig 2: Growth Performance of *Chaetoceros gracilis* in Miquel's medium at different time interval.

4.3 Growth Performance of *Chaetoceros gracilis* in TMRL medium

In TMRL medium the initial cell density was same as the above two media at the time of stocking (inoculation density at zero day). The peak density of *Chaetoceros gracilis* in TMRL medium was observed $117 \pm 0.527 \times 10^2$ cells/ml at the 12th day of culture period at the stationary phase and the stationary phase was beginning at the 12th day that continue up to 16th day of culture period (Fig.3) that showed no significant difference ($P > 0.05$) among these days (day 12 to 16), (Annex-2). Finally the declining phase started at 17th day and at 26th day

of culture period (declining phase) none found alive although it showed gradually declining trend in growth with significant difference ($P < 0.05$) among the days (day 17 to 25).

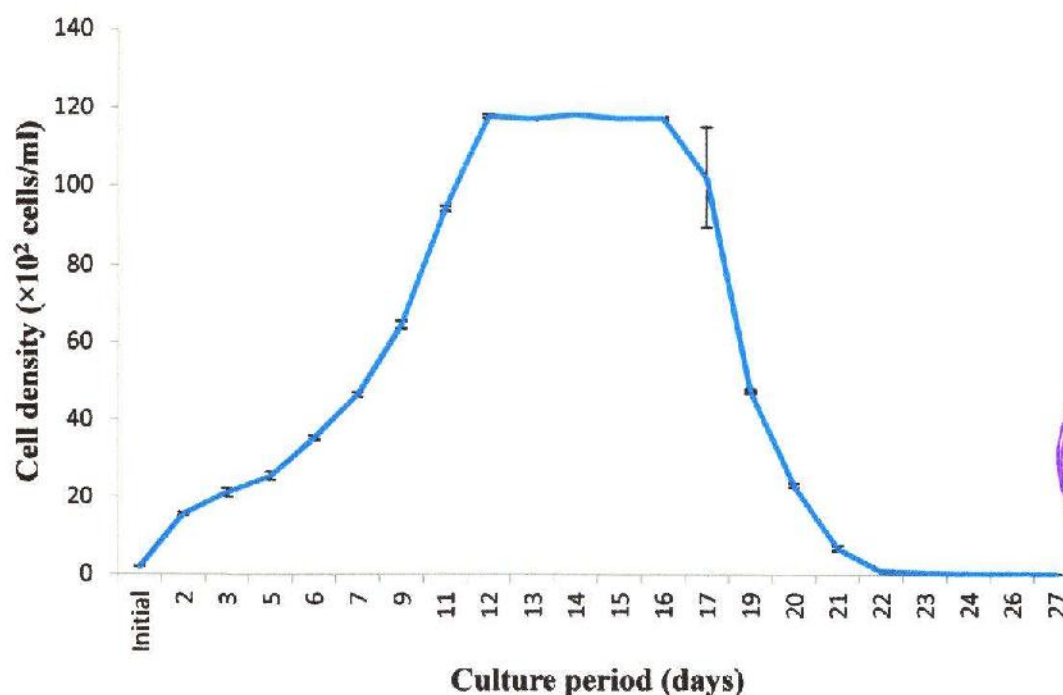


Fig 3: Growth Performance of *Chaetoceros gracilis* in TMRL medium at different time interval.

4.4 Comparison among the treatments:

Though the initial stocking density of *Chaetoceros gracilis* was same in all treatments (2×10^2 cells/ml; $P > 0.05$) yet it was observed different in stationary phase of cell growth among the treatments. The highest cell growth ($195 \pm 0.288 \times 10^2$ cells/ml) of the species was observed in treatment-1 (Conway) at the start of stationary phase that showed significant difference ($P < 0.05$) with the other two. The highest cell density ($117 \pm 0.527 \times 10^2$ cells/ml) of Treatment-3 (TMRL) in stationary growth phase showed lower growth ($137 \pm 0.376 \times 10^2$ cell/ml) than that of Treatment-2 (Miquel's) with no statistical difference ($P > 0.05$) but had difference ($P < 0.05$) with Treatment-1 (Fig. 4 and Table.-4).(Annex-2)

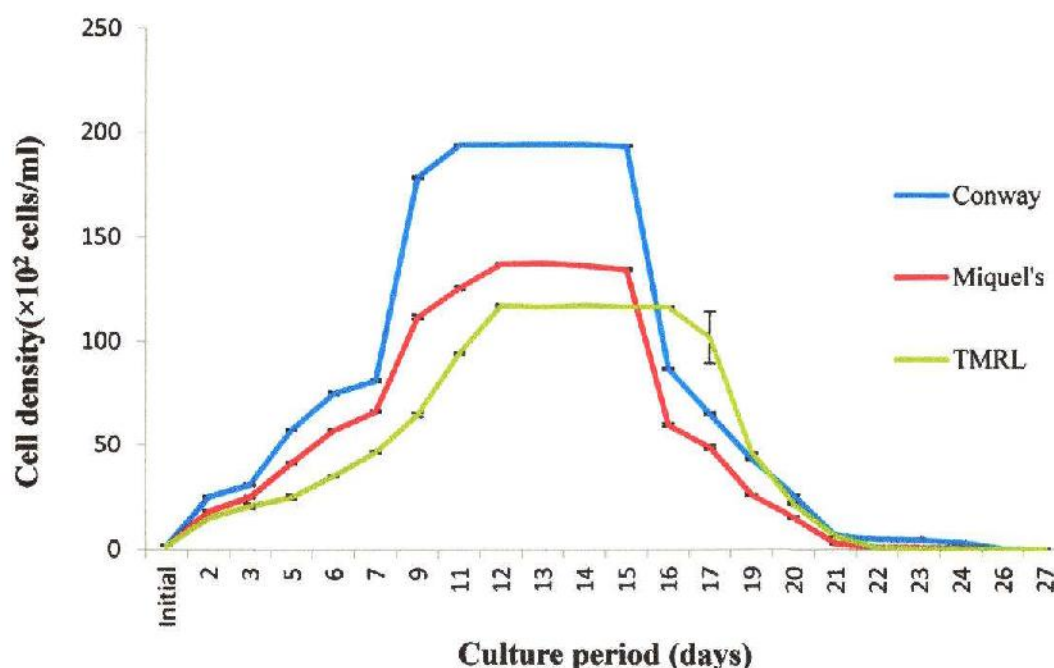


Fig 4: Growth Performance of *Chaetoceros gracilis* in three media at different time interval

The stationary growth phase of the species among the treatments was observed to start at different days of stocking and the duration of the phase among the treatments also varied. The earliest stationary phase was observed to achieve in Treatment-1 that started at the 11th day of culture and sustained for 5 days (15th day). The longest duration of the stationary phase of the species was observed in Treatment-1 and Treatment-3 that started at 11th and 12th day and sustained for 5 days (15th and 16th day) of culture period. The shortest stationary phase was observed in Treatment-2 that started at 12th day of culture and sustained for 4 days (15th day). At 27th day of culture period, at the end of the experiment/declining phase, none found alive irrespective of any media.

CHAPTER FIVE



DISCUSSION

Discussion

In this experiment, *Chaetoceros gracilis* showed the best growth performance in Walne's or Conway medium ($195 \pm 0.288 \times 10^2$ cells/ml at the 11th day of the culture period. Lincymol *et al.* (2012) observed the best nutritional quality of algae in Conway medium. Nezhad *et al.* (2012) showed that maximum growth rate of *C. calcitrans* was in 10 ppt salinity in Conway medium and highest cell density was achieved at 27 ppt salinity. This experiment was conducted at 30 ppt salinity although the optimum salinity range for *C. gracilis* was 15-35 gTDSL⁻¹ (Anon, 1986). At higher salinity (40 ppt), the growth is drastically decreases (Rai and Rajashekhar, 2014).

Table 3: Growth performance of *Chaetoceros gracilis* in different media at different time interval ($\times 10^2$ cells/ml).

Day	Treatment(Media)		
	Conway(Treatment-1)	Miguel's(Treatment-2)	TMRL(Treatment-3)
	Mean $\pm$ Std. Error	Mean $\pm$ Std. Error	Mean $\pm$ Std. Error
Initial	2 ^a $\pm$.000	2 ^a $\pm$.000	2 ^a $\pm$.000
Day 2	25.00 ^a $\pm$ 0.408	18.222 ^b $\pm$ 0.323	15.22 ^c $\pm$ 0.464
Day3	30.9 ^a $\pm$ 0.423	25.222 ^b $\pm$ 0.464	21.00 ^c $\pm$ 1.118
Day5	57.11 ^a $\pm$ 0.423	41.333 ^b $\pm$ 0.408	25.333 ^c $\pm$ 1.130
Day6	74.9 ^a $\pm$ 0.771	56.9 ^b $\pm$ 0.423	35.333 ^c $\pm$ 0.577
Day7	81.11 ^a $\pm$ 0.715	65.8 ^b $\pm$ 0.464	46.333 ^c $\pm$ 0.577
Day9	179.00 ^a $\pm$ 0.587	112.00 ^b $\pm$ 1.027	64.33 ^c $\pm$ 0.971
Day11	195.00 ^a $\pm$ 0.288	126.00 ^b $\pm$ 0.949	94.44 ^c $\pm$ 0.668
Day12	195.00 ^a $\pm$ 0.408	137.44 ^b $\pm$ 0.376	117.666 ^c $\pm$ 0.527
Day13	195.00 ^a $\pm$ 0.408	138.00 ^b $\pm$ 0.647	117.00 ^c $\pm$ 0.440
Day14	195.00 ^a $\pm$ 0.806	137.00 ^b $\pm$ 0.222	118.00 ^c $\pm$ 0.288
Day15	194.00 ^a $\pm$ 0.323	135.00 ^b $\pm$ 0.503	117.00 ^c $\pm$.408
Day16	86.77 ^b $\pm$ 0.571	60.11 ^c $\pm$ 0.771	117.00 ^a $\pm$ 0.388
Day17	65.77 ^b $\pm$ 0.464	49.88 ^b $\pm$ 0.934	102.126 ^a $\pm$ 12.634
Day19	44.00 ^b $\pm$ 0.440	26.77 ^c $\pm$ 0.464	47.11 ^a $\pm$ 0.423
Day20	26.88 ^a $\pm$ 0.423	16.11 ^c $\pm$ 0.611	22.666 ^b $\pm$ 0.527
Day21	7.33 ^a $\pm$ 0.333	3.22 ^b $\pm$ 0.521	6.88 ^a $\pm$ 0.654
Day22	5.11 ^a $\pm$ 0.654	1.33 ^b $\pm$ 0.166	1.00 ^b $\pm$ 0.000
Day23	4.66 ^a $\pm$ 0.927	1.00 ^b $\pm$ 0.000	.5 ^c $\pm$ 0.094
Day24	3.33 ^a $\pm$ 0.577	.75 ^b $\pm$ 0.047	.3 ^b $\pm$ 0.091
Day26	.33 ^a $\pm$ 0.166	.15 ^a $\pm$ 0.017	.2 ^a $\pm$ 0.062
Day27	0 ^a $\pm$ 0	0 ^a $\pm$ 0	0 ^a $\pm$ 0

(Annex-3).

Values are Mean $\pm$ SE (n=9); different letter superscripts in the same row show significant difference. ($P < 0.05$)

Maximum cells were counted during the culture period (168 hours) at Light intensity 1000 Lux for *Chaetoceros muelleri* was reported by Pal *et al.* (2013) whereas the prevailing study was conducted at a light intensity of 1600-2000 Lux.

In this study three different media viz. Conway, Miquel's and TMRL were used for the culture of *Chaetoceros gracilis* where Treatment-1 (Conway) showed significantly higher ($P < 0.05$) growth performance ($195 \pm 0.288 \times 10^2$ cells/ml) than the rest two at 11th day of culture (Table. 2). Conway medium was found to be the most suitable culture medium for the enhancement of algal biomass, *Skeletonema costatum* (Rajeswari *et al.*, 2014). *Tetraselmis tetrathele* cultivated in Conway medium presented better results concerning microalgae growth, with a more pronounced exponential phase (Coelho *et al.*, 2013). Nutrient media used in the microalgae culture included Conway, Modified for TMRL medium depending on the species cultured (Kongkeo, 1991). Trace minerals and vitamins (especially thiamin and biotin) are necessary in most cultures (Fulks and Main, 1991) with addition of silicate for diatom. In this respect Conway or Walne's medium resembles the best medium for *Chaetoceros gracilis* culture as it contains different types of nutrients specially vitamin B₁ and B₁₂ (Table.2).

Costa *et al.* (1998) estimated maximum cellular density was 4125.00×10^3 cells/ml for *Chaetoceros gracilis* on the 9th day by additions of secondary sewage as a culture medium. *C. muelleri* attained the highest density of $321 \pm 5.41 \times 10^4$ cells/ml in Rix Mix medium and about same density ($319 \pm 12.02 \times 10^4$ cells/ml) at the 24th day of culture in modified Guillard's f/2 medium. The maximum cell density of *C. muelleri* ($135 \pm 6.95 \times 10^4$ cells/ml) in BFRI medium was observed at the 12th day of culture (Shah *et al.*, 2003). However, the peak cell density of this experiment was also achieved at the similar interval of the culture period.

Table 4 : Stationary Phase in different media for *Chaetoceros gracilis* ($\times 10^2$ cells/ml).

Days	Treatment(Media)		
	Conway Medium	Miquel's Medium	TMRL Medium
Day11	$195.00^a \pm 0.288$		
Day12	$195.00^a \pm 0.408$	$137.44^b \pm 0.376$	$117.666^c \pm 0.527$
Day13	$195.00^a \pm 0.408$	$138.00^b \pm 0.647$	$117.00^c \pm 0.440$
Day14	$195.00^a \pm 0.806$	$137.00^b \pm 0.222$	$118.00^c \pm 0.288$
Day15	$194.00^a \pm 0.323$	$135.00^b \pm 0.503$	$117.00^c \pm 0.408$
Day16			$117.00^a \pm 0.388$

(Annex-2).

The stationary phase in Conway medium started at 11th day and continued upto 15th day of culture (Fig. 1 and Table. 4). The duration of this phase existed for 5 days continuously for *C. gracilis*. The stationary phase in BFRI medium started at 12th day and continued upto 16th day of culture for *C. muelleri* (Shah *et al.*, 2003). For *Chaetoceros sp.* the stationary phase exists from 11th or 12th to 15th or 16th days of the culture period. No significant difference ($P > 0.05$; Appendix.2. Annex-2) in cell density within the same treatment at different days in the stationary phase was observed. However, it showed difference ($P < 0.05$) in growth among the treatments.

Chaetoceros gracilis showed less growth performance ($137.44 \pm 0.376 \times 10^2$ cells/ml) in Miquel's medium than Conway or Walne's medium. In contrary, Miquel's medium was suggested as the best culture medium for *C. calcitrans* ($4.59 \pm 0.38 \times 10^6$ cells/ml) in the laboratory scale (Homthong and Kongkangwal, 2013). It might be happened due to the difference between two species. The declining phase started after 15th days which were as similar as Conway or Walne's medium. At 27th day of the culture period all cells became dead.

Temperature has significant effect on the growth performance and proximate composition of algal species (Raghavan *et al.*, 2008). At low temperatures (20°C and 25°C) lipid and carbohydrate contents become higher but protein content remains unaffected. The optimum temperature range for *Chaetoceros gracilis* is 28-32°C (Anon, 1986). An average of temperature 25° C was maintained, in this experiment, for the culture under the laboratory condition. Isolated species prefer alkaline condition for their optimum growth (Rai and Rajashekhar, 2014). The pH range for *C. gracilis* is (7-9) optimum. 7 and 8 which was strictly maintained in the experiment (Anon, 1986).

From the experiment it was observed that TMRL medium showed the lowest growth performance of *Chaetoceros gracilis*. At 16th days of culture period cell density was highest ($117 \pm 0.388 \times 10^2$ cells/ml) among three media where Conway or Walne's ($195 \pm 0.288 \times 10^2$ cells/ml) and Miquel's ($137.44 \pm 0.376 \times 10^2$ cells/ml). Although the three media shows different growth performance during the culture period, at the 27th day of culture all the cells were crushed. So the shelf life of *C. gracilis* was similar for Conway, Miquel's and TMRL medium (27 days).

CHAPTER SIX



CONCLUSION

Conclusion

The growth performance of *Chaetoceros gracilis* was studied in three different nutrient media viz. Conway, Miquel's and TMRL in laboratory condition. Depending on the findings of this study it might be concluded as follows:

1. *Chaetoceros gracilis* needs a temperature range of 28°C - 32° C. But it can also grow well at 25° C.
2. The shelf life of *C. gracilis* is 27 days where the maximum cell density could be achieved at 11th day and onward up to the end of the stationary phase at 15th day of culture.
3. Conway media is suitable for the culture of *Chaetoceros gracilis*.
4. Harvesting time of *C. gracilis* for further uses would be 11 – 13 days of culture period.

Recommendation

- Further work on optimization of light intensity, photoperiod, air flow, pH and salinity could be done.
- Mass culture study should be done both in indoor and outdoor system.
- Further media development study could be taken where Conway would be used as reference media.

CHAPTER SEVEN



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Sample collection



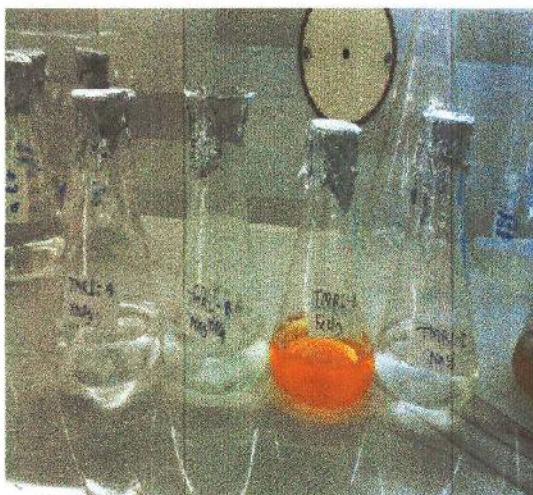
Media preparation



Conway medium



Miquel's medium



TMRL medium



Cell counting of *Chaetoceros gracilis* with electric microscope



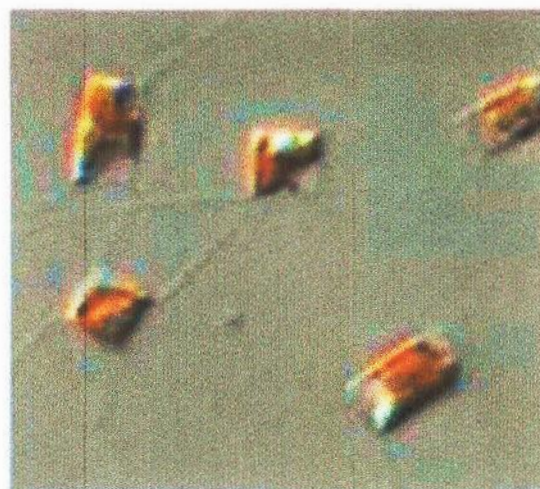
Growth performance of *C. gracilis* in Conway medium at Exponential phase



Growth performance of *C. gracilis* in Miquel's medium at Exponential phase



Growth performance of *C. gracilis* in TMRL medium at Exponential phase



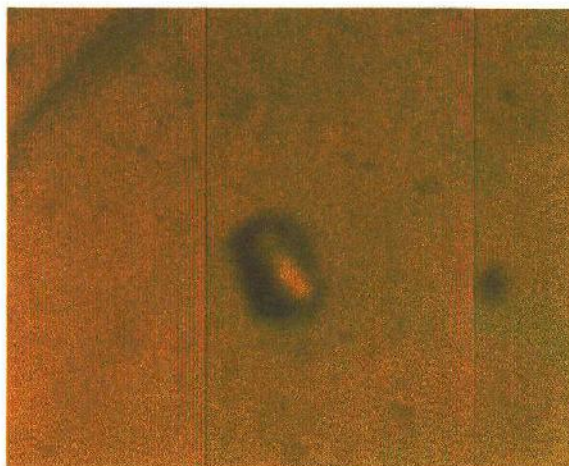
Microscopic figure of *C. gracilis*



Microscopic figure of *C. gracilis*



Microscopic figure of *C. gracilis*



Microscopic figure of *C. gracilis* with color



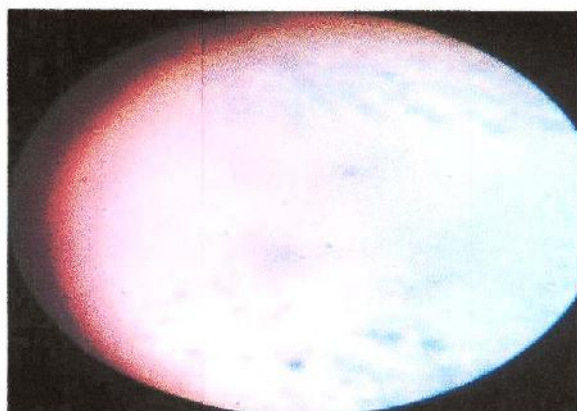
Growth performance of *C. gracilis* in Miquel's medium at decaling phase



Growth performance of *C. gracilis* in Conway medium at decaling phase



Growth performance of *C. gracilis* in TMRL medium at decaling phase



Microscopic figure of *C. gracilis*



Microscopic figure of *C. gracilis*

Appendix-2

Annex-1

Day2

Tukey HSD

Treat ment	N	Subset for alpha = 0.05		
		1	2	3
3	9	1522.22		
2	9		1822.22	
1	9			2500.00
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Day3

Tukey HSD

Treat ment	N	Subset for alpha = 0.05		
		1	2	3
3	9	2100.00		
2	9		2522.22	
1	9			3088.89
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Day5

Tukey HSD

Treat ment	N	Subset for alpha = 0.05		
		1	2	3
3	9	2533.33		
2	9		4133.33	
1	9			5711.11
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Day6

Tukey HSD

Treat ment	N	Subset for alpha = 0.05		
		1	2	3
3	9	3533.33		
2	9		5688.89	
1	9			7488.89
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Day7

Tukey HSD

Treat ment	N	Subset for alpha = 0.05		
		1	2	3
3	9	4633.33		
2	9		6577.78	
1	9			8111.11
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Day9

Tukey HSD

Treat ment	N	Subset for alpha = 0.05		
		1	2	3
3	9	6433.33		
2	9		1.12E4	
1	9			1.79E4
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.



Day11

Tukey HSD

Treat ment	N	Subset for alpha = 0.05		
		1	2	3
3	9	9444.44		
2	9		1.26E4	
1	9			1.95E4
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Day12

Tukey HSD

Treat ment	N	Subset for alpha = 0.05		
		1	2	3
3	9	1.18E4		
2	9		1.37E4	
1	9			1.95E4
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Day13

Tukey HSD

Treat ment	N	Subset for alpha = 0.05		
		1	2	3
3	9	1.17E4		
2	9		1.38E4	
1	9			1.95E4
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Day14

Tukey HSD

Treat ment	N	Subset for alpha = 0.05		
		1	2	3
3	9	1.18E4		
2	9		1.37E4	
1	9			1.95E4
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Day15

Tukey HSD

Treat ment	N	Subset for alpha = 0.05		
		1	2	3
3	9	1.17E4		
2	9		1.35E4	
1	9			1.94E4
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Day16

Tukey HSD

Treat ment	N	Subset for alpha = 0.05		
		1	2	3
2	9	6011.11		
1	9		8677.78	
3	9			1.17E4
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Day19

Tukey HSD

Treat ment	N	Subset for alpha = 0.05		
		1	2	3
2	9	2677.78		
1	9		4400.00	
3	9			4711.11
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Day17

Tukey HSD

Treat ment	N	Subset for alpha = 0.05	
		1	2
2	9	4988.89	
1	9	6577.78	
3	9		10212.67
Sig.		.293	1.000

Means for groups in homogeneous subsets are displayed.

Day20

Tukey HSD

Treat ment	N	Subset for alpha = 0.05		
		1	2	3
2	9	1611.11		
3	9		2266.67	
1	9			2688.89
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Day21

Tukey HSD

Treat ment	N	Subset for alpha = 0.05	
		1	2
2	9	322.22	
3	9		688.89
1	9		733.33
Sig.		1.000	.819

Means for groups in homogeneous subsets are displayed.

Day23

Tukey HSD

Treat ment	N	Subset for alpha = 0.05	
		1	2
3	9	53.33	
2	9	100.00	
1	9		466.67
Sig.		.815	1.000

Means for groups in homogeneous subsets are displayed.

Day22

Tukey HSD

Treat ment	N	Subset for alpha = 0.05	
		1	2
3	9	100.00	
2	9	133.33	
1	9		511.11
Sig.		.819	1.000

Means for groups in homogeneous subsets are displayed.

Day24

Tukey HSD

Treat ment	N	Subset for alpha = 0.05	
		1	2
3	9	33.33	
2	9	75.56	
1	9		333.33
Sig.		.657	1.000

Means for groups in homogeneous subsets are displayed.

Day26

Tukey HSD

Treat ment	N	Subset for alpha = 0.05
		1
2	9	15.56
3	9	20.00
1	9	33.33
Sig.		.455

Means for groups in homogeneous subsets are displayed.

Annex-2

Conway

Tukey HSD

Treat ment	N	Subset for alpha = 0.05													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
27	9	.00													
26	9	33.33													
0	9	200.00	200.00												
24	9		333.33	333.33											
23	9			468.67											
22	9			511.11	511.11										
21	9				733.33										
2	9					2500.00									
20	9					2688.89									
3	9						3088.89								
7	9							3533.33							
18	9								4400.00						
5	9									5711.11					
17	9										6577.78				
6	9											7488.89			
16	9												8877.78		
9	9													1.79E4	
15	9														1.94E4
14	9														1.95E4
11	9														1.95E4
12	9														1.95E4
13	9														1.95E4
Sig.		.413	.961	.647	.221	.529	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Miquel

Tukey HSD

Treatment	N	Subset for alpha = 0.05											
		1	2	3	4	5	6	7	8	9	10	11	12
27	9	.00											
26	9	15.56											
24	9	75.56	75.56										
23	9	100.00	100.00										
22	9	133.33	133.33										
0	9	200.00	200.00										
21	9		322.22										
20	9			1611.11									
2	9			1822.22									
3	9				2522.22								
19	9				2677.78								
5	9					4133.33							
17	9						4988.89						
6	9							5688.89					
16	9								6011.11				
7	9									8577.78			
9	9										1.12E4		
11	9											1.28E4	
15	9												1.35E4
14	9												1.37E4
12	9												1.37E4
13	9												1.38E4
Sig.		.501	.137	.391	.893	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.391

Means for groups in homogeneous subsets are displayed.

TMRL

Tukey HSD

Treat ment		Subset for alpha = 0.05										
	N	1	2	3	4	5	6	7	8	9	10	11
27	9	.00										
26	9	20.00										
24	9	33.33										
23	9	53.33										
22	9	100.00										
0	9	200.00										
21	9		688.89									
2	9			1522.22								
3	9				2100.00							
20	9				2266.67	2266.67						
5	9					2533.33						
6	9						3533.33					
7	9							4633.33				
19	9							4711.11				
9	9								6433.33			
11	9									9444.44		
17	9										1.15E4	
15	9										1.17E4	1.17E4
13	9										1.17E4	1.17E4
16	9										1.17E4	1.17E4
12	9											1.18E4
14	9											1.18E4
Sig		.608	1.000	1.000	.879	.111	1.000	1.000	1.000	1.000	.223	1.000

Means for groups in homogeneous subsets are displayed.

Annex-3

Descriptives									
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	
					Lower Bound	Upper Bound			
Day0	1	9	200.00	.000	.000	200.00	200.00	200	200
	2	9	200.00	.000	.000	200.00	200.00	200	200
	3	9	200.00	.000	.000	200.00	200.00	200	200
Total	27	200.00	.000	.000	200.00	200.00	200	200	200
Day2	1	9	2500.00	122.474	40.825	2405.68	2584.14	2300	2700
	2	9	1922.22	97.193	32.394	1747.52	1996.92	1700	2000
	3	9	1522.22	139.443	46.481	1415.04	1629.41	1300	1700
Total	27	1949.15	432.675	83.268	1778.99	2119.31	1300	2700	2700
Day3	1	9	3089.89	126.930	42.310	2991.32	3186.46	2900	3300
	2	9	2522.22	139.443	46.481	2415.04	2629.41	2300	2700
	3	9	2100.00	335.410	111.803	1642.18	2357.82	1700	2700
Total	27	2570.37	464.770	89.445	2386.61	2754.23	1700	3300	3300
Day5	1	9	5711.11	126.930	42.310	5581.54	5808.68	5500	5900
	2	9	4133.33	122.474	40.825	4039.19	4227.48	4000	4300
	3	9	2633.33	339.118	113.039	2272.67	2794.00	2000	2900
Total	27	4125.93	1338.941	267.679	3598.26	4655.59	2000	5900	5900
Day6	1	9	7488.89	231.541	77.180	7310.81	7666.87	7100	7800
	2	9	5688.89	126.930	42.310	5591.32	5786.46	5500	5900
	3	9	3533.33	173.205	57.735	3400.20	3666.47	3400	3900
Total	27	5570.37	1667.109	319.911	4914.84	6225.90	3400	7800	7800
Day7	1	9	8111.11	214.735	71.578	7946.05	8276.17	7800	8400
	2	9	6577.78	139.443	46.481	6470.59	6684.96	6400	6900
	3	9	4633.33	173.205	57.735	4500.20	4766.47	4300	4900
Total	27	6440.74	1480.311	281.027	5893.08	7018.42	4300	8400	8400
Day9	1	9	1.79E4	176.363	58.794	17775.53	18046.69	17600	18100
	2	9	1.12E4	309.221	102.740	10929.76	11403.59	10700	11500
	3	9	6433.33	291.548	97.183	6209.23	6657.44	6000	6900
Total	27	1.10E4	4908.178	924.949	9936.70	12738.30	6000	18100	18100
Day11	1	9	1.95E4	86.603	28.869	19433.43	19566.57	19400	19800
	2	9	1.26E4	294.600	94.933	12392.18	12830.03	12100	12900
	3	9	9444.44	200.893	66.899	9290.18	9598.71	9100	9700
Total	27	1.39E4	4292.486	824.161	12157.76	15545.94	9100	19800	19800
Day12	1	9	1.95E4	122.474	40.825	19405.86	19594.14	19300	19700
	2	9	1.37E4	113.039	37.860	13657.56	13831.33	13600	13900
	3	9	1.18E4	158.114	52.705	11645.13	11888.20	11500	12000
Total	27	1.50E4	2345.201	643.794	13690.39	16327.02	11500	19700	19700
Day13	1	9	1.95E4	122.474	40.825	19405.86	19594.14	19300	19700
	2	9	1.39E4	104.365	34.789	13806.15	13904.96	13500	14000
	3	9	1.17E4	132.288	44.096	11598.31	11801.69	11500	11900
Total	27	1.50E4	2367.001	647.980	13853.24	16317.13	11500	19700	19700
Day14	1	9	1.95E4	242.097	60.699	19302.80	19674.98	19100	19900
	2	9	1.37E4	66.667	22.222	13626.53	13729.02	13600	13800
	3	9	1.16E4	66.603	28.869	11700.10	11833.74	11700	11900
Total	27	1.50E4	2349.685	644.643	13652.69	16302.86	11700	19900	19900
Day15	1	9	1.94E4	97.193	32.394	19347.52	19486.92	19300	19600
	2	9	1.35E4	150.923	50.308	13428.43	13680.45	13400	13800
	3	9	1.17E4	122.474	40.825	11572.62	11760.81	11500	11800
Total	27	1.49E4	3366.672	649.301	13545.18	16210.38	11500	19600	19600
Day16	1	9	8677.78	171.594	57.198	8545.88	8809.68	8400	8900
	2	9	8011.11	231.541	77.180	5933.13	8109.09	5700	6400
	3	9	1.17E4	116.667	38.889	11621.43	11800.79	11500	11900
Total	27	8900.00	2379.237	457.884	7858.81	9741.19	5700	11900	11900
Day17	1	9	8577.78	139.443	46.481	8470.59	8684.96	8400	8900
	2	9	4888.89	280.377	93.459	4773.37	5204.41	4600	5400
	3	9	1.02E4	3790.217	1263.406	7299.25	13126.09	114	11700
Total	27	7259.78	3069.355	590.505	6045.98	8473.58	114	11700	11700
Day19	1	9	4400.00	132.288	44.096	4298.31	4501.69	4200	4600
	2	9	2877.78	139.443	46.481	2570.59	2784.96	2500	2900
	3	9	4711.11	126.930	42.310	4613.54	4808.68	4500	4900
Total	27	3929.63	920.297	177.094	3555.61	4293.65	2500	4900	4900
Day20	1	9	2669.89	126.930	42.310	2591.32	2766.46	2500	2900
	2	9	1611.11	183.333	61.111	1470.19	1752.03	1300	1800
	3	9	2266.67	158.114	52.705	2145.13	2398.20	2000	2500
Total	27	2189.89	476.833	91.728	2000.34	2377.44	1300	2900	2900
Day21	1	9	733.33	100.000	33.333	656.47	810.20	600	900
	2	9	322.22	166.347	52.116	202.04	442.40	100	800
	3	9	688.89	196.497	65.499	537.65	839.93	300	900
Total	27	581.48	240.252	49.228	486.44	676.52	100	900	900
Day22	1	9	511.11	196.497	65.499	380.07	652.15	300	900
	2	9	133.33	50.000	16.667	94.90	171.77	100	200
	3	9	100.00	.000	.000	100.00	100.00	100	100
Total	27	249.15	220.788	42.490	180.81	325.48	100	900	900
Day23	1	9	466.87	278.388	92.796	252.68	680.65	100	900
	2	9	100.00	.000	.000	100.00	100.00	100	100
	3	9	53.33	28.284	8.428	31.59	75.07	20	90
Total	27	206.67	244.069	49.971	110.12	303.22	20	900	900
Day24	1	9	333.33	173.205	57.735	200.20	466.47	100	800
	2	9	75.56	14.240	4.747	64.61	86.50	50	90
	3	9	33.33	27.386	9.129	12.28	54.38	0	70
Total	27	147.41	185.678	32.077	81.47	213.34	0	800	800
Day26	1	9	33.33	50.000	16.667	-5.10	71.77	0	100
	2	9	15.66	5.270	1.767	11.50	19.61	10	20
	3	9	20.00	16.708	5.236	5.62	34.38	0	50
Total	27	22.96	30.738	5.915	10.80	35.12	0	100	100
Day27	1	9	.00	.000	.000	.00	.00	0	0
	2	9	.00	.000	.000	.00	.00	0	0
	3	9	.00	.000	.000	.00	.00	0	0
Total	27	.00	.000	.000	.00	.00	0	0	0