THE COMBINED EFFECTS OF SOME ABIOTIC FACTORS AND NUTRIENT CONDITIONS ON THE GROWTH AND BIOCHEMICAL COMPOSITION OF FOUR SELECTED MARINE DIATOMS, ISOLATED FROM PENANG NATIONAL PARK COASTAL WATERS

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By

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KESAN BERSEPADU FAKTOR ABIOTIK DAN KEADAAN NUTRIENT KE ATAS PERTUMBUHAN DAN KOMPOSISI BIOKIMIA EMPAT DIATOM MARIN TERPILIH, YANG DIPENCILKAN DARI PERSISIRAN PANTAI TAMAN NEGARA PULAU PINANG

ABSTRAK

Dalam kajian ini, empat diatom marin *Nitzschia panduriformis* W. Gregory; *Nitzschia lorenziana* Grunow; *Cylindrotheca closterium* (Ehrenberg) dan *Navicula distans* (W. Smith), telah dipencilkan daripada sampel air yang dikutip daripada 8 lokasi di persisiran pantai Taman Negara Pulau Pinang. Identifikasi spesies yang dipencilkan dijalankan dengan berpandu pada morfologi dan struktur frustules yang halus di bawah penggunaan SEM; dan juga diperkukuhkan dengan bahagian DNA tertentu dengan menggunakan kaedah molekular PCR (Polymerase Chain Reaction).

Spesies terpencil itu dikaji di bawah keadaan kultur yang berbeza, iaitu suhu yang berbeza seperti 5, 15 dan 26±1˚C, saliniti seperti 6, 13, dan 27±1‰; 12:12 jam cerah:gelap dan 24 jam penggunaan lampu yang berterusan, penggunaan kandungan nitrogen dan phosphorus yang berbeza (media tanpa N dan P, 90% pengurangan, 50% pengurangan dan media yang mempunyai 100% kepekatan) dengan menggunakan media F/2 sebagai medium pertumbuhan. Kesan fluktuasi dalam keadaan kultur tersebut telah dikaji terhadap pertumbuhan kinetik, kadar pertumbuhan (d^{-1}), kandungan klorofil *a* (μgmL^{-1}), jisim kering (mgL^{-1}) dan komposisi biokimia; termasuk jumlah lipid, karbohidrat dan kandungan protein dalam peratus jisim kering (%). Di antara empat diatom yang dikaji, *Nitzschia panduriformis* dan *Navicula distans* mempunyai kadar pertumbuhan yang tertinggi (masing-masing 0.63 dan 0.61 d^{-1}); sementara kadar

pertumbuhan *Nitzschia lorenziana* tidak melebihi 0.44 d^{-1} walaupun dikultur di bawah keadaan pertumbuhan terbaik.

Secara umumnya, semua diatom yang dikaji tidak boleh hidup di bawah suhu rendah (5±1˚C) mahupun saliniti 6±1‰. Hanya spesies *Nitzschia panduriformis* yang menunjukkan kadar pertumbuhan yang tinggi (0.40 d^{-1}) di bawah suhu 15±1˚C, pada masa yang sama mempunyai susutan dalam kandungan klorofil *a*. Di bawah suhu 15±1˚C, fluktuasi dalam keadaan kultur yang lain menunjukkan impak yang kurang terhadap sifat algamikro yang dikaji. Saliniti yang pelbagai dan jangkamasa cahaya tidak menunjukkan kesan yang penting (signifikan) dalam pertumbuhan dan pengeluaran biojisim *Nitzschia lorenziana.* Walaupun kedua-dua diatom *Nitzschia panduriformis* dan *Navicula distans* menunjukkan kadar pertumbuhan yang agak tinggi di bawah sinaran lampu yang berterusan berbanding dengan 12:12 edaran cahaya: gelap, efisiensi pertumbuhan sebagai fungsi kadar cahaya adalah lebih tinggi di bawah rejim cahaya yang lebih rendah. *Cylindrotheca closterium* menunjukkan keutamaan terhadap rejim cahaya yang lebih rendah. Kandungan lipid dan karbohidrat selalunya diperhati dalam kultur yang dikultur di bawah rejim cahaya yang berterusan.

Nitrogen merupakan faktor utama yang mempengaruhi pertumbuhan dan komposisi biokimia. Pertumbuhan akan berhenti ketika sel hidup di bawah keadaan kurang (kelaparan) nitrogen; manakala kultur diatom di bawah keadaan nitrogen yang kurang boleh menyebabkan kadar pertumbuhan dan kandungan protin menurun secara drastik, di samping meningkatkan kandungan lipid dan karbohidrat. Kepekatan fosforus yang berlainan memberi impak yang kurang dalam kadar pertumbuhan dan komposisi biokimia berbanding dengan nitrogen, dan kesannya dapat diperhatikan ketika kultur diatom berada di bawah keadaan nitrogen yang mencukupi.

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ABSTRACT

In this study, four marine diatoms *Nitzschia panduriformis* W. Gregory; *Nitzschia lorenziana* Grunow; *Cylindrotheca closterium* (Ehrenberg) and *Navicula distans* (W. Smith), were isolated from water samples collected from eight stations located around the coastal waters of Penang National Park. Identification of the isolated species was carried out based on the morphological and fine characteristics of the frustules observed under SEM, as well as amplifying of specific region of the DNA using Polymerase Chain Reaction (PCR) molecular technique.

. The isolated species were studied under varied culture conditions; temperature 5, 15 and 26 ±1˚C; salinity 6, 13 and 27±1‰; light duration 12:12 h light: dark cycle and 24 h continuous light; various nitrogen and phosphorus concentrations (free N and P media, 90% reduction, 50 reduction and full strength media 100%) using F/2 media as a growth medium. The effects of fluctuation in these culturing conditions were investigated on the growth kinetic, growth rate (d^{-1}), chlorophyll *a* content (μgmL^{-1}), dry weight (mgL^{-1}) and the biochemical composition including total lipid, carbohydrate and protein content as percentage of the dry weight (%)

Among the four diatoms tested, *Nitzschia panduriformis* and *Navicula distans* recorded the highest growth rate (0.63 and 0.61 d^{-1} respectively); while the growth rate of *Nitzschia lorenziana* never exceeded 0.44 d^{-1} under optimum growth conditions.

Generally, all the investigated diatoms could not grow neither at low temperature conditions (5±1˚C) nor salinity 6±1‰. *Nitzschia panduriformis* was the only species that showed a relatively high growth rate (0.40 d^{-1}) under 15±1˚C, coincided with dramatically decrease in chlorophyll *a* content. Under 15±1˚C, fluctuation in other cultural conditions showed less impact on the investigated microalgal attributes. Varied salinities and light durations had no significant effects on the growth and biomass production of *Nitzschia lorenziana.* Although both diatoms *Nitzschia panduriformis* and *Navicula distans* showed higher growth rate under continuous light regime compared to 12:12 light:dark cycle, growth efficiency as a function of light duration was always higher under shortened light regime. *Cylindrotheca closterium* showed a preference for the shortened light regime. Higher lipid and carbohydrate contents were always observed under continuous light regime.

Nitrogen was the major factor controlling the growth and biochemical composition. Growth was ceased when the cells were grown under nitrogen starvation conditions; whereas maintaining diatoms under nitrogen limitation conditions caused drastically drop in growth rate and protein content, coupled with increase in lipid and carbohydrate content. Various phosphorus concentrations had lesser impact on the growth and biochemical composition than the nitrogen ones, and its effects were always observed under nitrogen replete conditions.

CHAPTER ONE

Introduction

Algae are a very diverse group of organisms, which inhabit a huge range of ecosystems ranging from marine and freshwater environments to desert sands, and from hot springs to snow and ice. They are an important constituents of aquatic ecosystems; as an autotrophic organisms, they are the main and primary producers in oceans, seas and water bodies, comprising more than half the total primary production at the base of the food chain worldwide (Guschina and Harwood, 2006; Matsunaga et al., 2005). The primary producers of oxygen in aquatic environments are algae, especially microalgae, as they play an important role in CO_2 recycling through photosynthesis (Matsunaga et al., 2005). In terms of photosynthetic efficiency, microalgae yields are greater than those of macroalgae and similar to those of higher plants, it has been estimated that up to 18% of the solar energy can be stored in algal cells in contrast to the 6% of higher plants in conventional agriculture (Aaronson and Dubinsky, 1982).

As they comprise the base of the food chain in the marine environment, therefore, microalgae are indispensable in the commercial rearing of various species of marine animals, and because of the high nutritional value and easy to culture, marine microalgae are increasingly used as a food supply in the aquaculture industry, especially mariculture, being the food source for larvae of many species of mollusks, crustaceans and fish. In addition, microalgae serve as a food source for zooplankton production (rotifers, copepods), which in turn are used as feed for rearing fish larvae, constituting both a source of energy as well as providing the essential vitamins and polyunsaturated fatty acids (PUFAs) (Harrison et al., 1990; Pulz and Gross, 2004). It has been reported that, more than 40 species of microalgae are used in aquaculture worldwide (Pulz and Gross, 2004). In addition to what has been mentioned, microalgae are an important source of valuable substances such as polyunsaturated fatty acids, antioxidants, colors and food-coloring products, toxins and other substances with biological activity (Pulz and Gross, 2004). Recently, microalgal biofuel applications have become increasingly important, and as an alternative biomass for biofuel production, microalgae have many benefits over land biomass. For example, they are not food; they are fast-growing, they have high oil content, and they can reduce CO_2 (Dragone et al., 2010).

Microalgae have served as human food in times of famine and also in times of plenty, the blue green microalga *Spirulina* is still eaten in the Lake Chad area of Africa when other foods are scarce, and the fresh red alga *Lemanea mamillosa* was eaten, after frying in India (Aaronson and Dubinsky, 1982). Other microalgae such as *Spirulina*, *Chlorella*, *Dunaliella*, *Haematococcus* and *Schizochytrium* are classified as food sources; among the most used microalgae are *Chlorella* and *Spirulina. Chlorella vulgaris* is a rich source of proteins, essential amino acids, vitamins (B-complex and ascorbic acid), minerals (potassium, sodium, magnesium, iron, and calcium), β-carotene and chlorophylls. *Chlorella* cells also contain β-1,3-glucan, an active immunostimulator, which acts as a free-radical scavenger and as a reducer of blood lipids. A polysaccharide also found in *Chlorella* has been linked to antitumor effects, as well as other health-promoting substances (Chac´on-Lee and Gonz´alez-Mari˜no 2010)

Amongst the microalgae, diatoms represent probably the largest biomass producers on earth, and considered as one of the most important groups, with enormous biological significance and great potential for biotechnology (Chepurnov et al., 2008; Trobajo et al., 2006). Diatoms (Bacillariophyceae) are a group of aquatic photosynthetic unicells organisms, within the eukaryotic division Heterokontophyta. As they present the most species-rich group of algae, including around 100,000 species in 250 genera, diatoms are immensely diverse and ecologically important; they are cosmopolitan, as they colonize nearly all habitats including marine, fresh and brackish waters, as well as damp places; they might be free-floating (plankton) or benthos, that live on surfaces of rocks, mud as they might live on plants, animals and other algae, where there is at least some sunlight to support their photosynthesis. Diatoms also inhabit sea ice, as there are ―air diatoms” (Hasle and Syvertsen, 1997). Their generally dominant brown coloring originates from the high content of fucoxanthin (an accessory photosynthesis pigment), in addition to chlorophylls *a* and *c*. Their main reserve products are chrysolaminarin and oil. Although some species form pesudofilaments or colonial aggregates, all species are unicellular and un-flagellated in their vegetative stage. One of the most unique and distinct features, that makes diatoms easily recognized is the cell's cover, which consists of two silica valves (like a Petri dish), usually with elaborate fine structure, that play an important role for identification of the diatoms. Diatoms are major players in oceans geochemistry and carry out more than 20% of the net primary production of the planet. Contributing around 40% of ocean primary productivity, they form the foundation of the trophic pyramid in many parts of the world ocean (Matsunaga et al., 2005; Scala and Bowler, 2001; Trobajo et al., 2006).

Diatoms can be plankton (free-floating) or benthic; benthic diatoms colonize a variety of living and nonliving surfaces submerged in aquatic environments, such as rocks, mud, plants, animals and other algae, where there is sunlight to support their photosynthesis. Benthic diatoms are the major group of photoautotrophic organisms inhabiting marine intertidal zone. The most ubiquitous component of the benthic microalgae, are the motile pennate diatoms, particularly those species belonging to the genera *Navicula*, *Nitzschia*, and *Amphora*. In coastal and estuarine areas, these diatoms are relevant primary producers, contributing an important fraction of biomass to the trophic chain; they also participate in the nutrient exchange between bottom sediments and the overlying water column, and contribute to sediment stabilization. Benthic diatoms represent an important part of the microphytobenthos in intertidal mudflats. Microphytobenthic biofilms exhibit high rates of primary production and can contribute up to 50% of estuarine primary production (Scholz and Liebezeit, 2012; Smith and Underwood, 2000; Underwood and Kromkamp, 1999). A substantial part of the carbon fixed by these organisms is excreted as extracellular products, which are mainly composed of polysaccharide, but small quantities of protein are present as well (Staats et al., 1999). The excretion of extracellular polymeric substances by these organisms often results in the formation of diatom biofilm on the surface of sediment of intertidal mudflats. Benthic diatoms are much less understood ecologically as compared to planktonic diatoms, but the benthos is more diverse than the plankton; it is not surprising that there are more species of benthic diatoms than planktonic ones (Harper and Harper, 2010).

Taxonomically, past and current classification systems for diatoms are mainly based on phenetic data confined to the structure of the siliceous parts especially the valves, however, other kinds of characteristics have been added for consideration, such as, the type of sexual reproduction, structure of the auxospore envelope and the type of habitat. Bacillariophyta are separated into two main groups: the centric diatoms (pill- shaped) cells, and the pennate diatoms (boat-shaped) cells, which might be with or without a raphe (Hasle and Syvertsen, 1997; Pulz and Gross, 2004). Both centric and pennate diatoms are widely used in aquaculture. Diatoms such as *Chaetoceros calcitrans*, *Chaetoceros gracilis*, *Skeletonema costatum* and others are commonly used as live feeds for all growth stages of bivalve mollusks (oyster, scallops, clams and mussels), while other genera such as *Navicula*, *Nitzschia*, *Cocconeis* and *Amphora* are used to feed juvenile abalone, where diatoms serve as the main source of nourishment during the early juvenile stages (Matsunaga et al., 2005; Pulz and Gross, 2004). On the other hand, several species belong to the genus *Pseudo-nitzschia* produce the neurotoxin domoic acid. Consumption of fish and shellfish, which have accumulated this potent excitotoxin has resulted in severe illness and even death in humans, marine mammals, and seabirds (Adams et al., 2009; Pulz and Gross, 2004).

Like any other organisms, microalgae cells are mainly comprised of carbohydrates, lipids and proteins, in varying proportions. Different microalgae belong to different divisions and classes tend to store different storage food products in their cells. The chemical nature of the storage products plays an important part in the definition of the various algal groups (Clayton and King, 1990; Hoek et al., 1995). Microalgae biomass usually have little ash content compared with macroalgae and higher plants, which might rich up to 50% of the dry weight (Aaronson and Dubinsky, 1982). Expressed as percentage of the dry weight, the ranges for the level of proteins, lipids, and carbohydrates are 6-52%, 7-23%, and 5-23%, respectively (Brown et al., 1997).

The productivity, survival and biochemical composition of microalgae varies with species, growth stage and are strongly affected by physiological and biochemical processes, as well as biotic and abiotic factors in the environment (Brown et al., 1997; Gatenby et al., 2003; Teoh et al., 2010). Variation in biochemical composition due to growth stages is frequently related to culture age and nutrient depletion, particularly if the organism is grown in batch culture (Harrison et al., 1977; Morris et al., 1983). However, it does not appear to be a strong correlation between the proximate composition of microalgae and nutritional value, since, algal diets with high levels of carbohydrates are reported to produce the best growth for juvenile oyster and larval scallops; in contrast, high dietary protein provided best growth for juvenile mussels (Whyte et al., 1989; Kreeger and Langdon, 1993). However, in early researches, the nutritional value of phytoplankton cultures in terms of biochemical composition was not considered, and instead, palatability and digestibility were suggested as being more significant. Later studies, benefitting from advances in analytical methods, emphasized the indisputable role of the microalgae biochemical composition in determining its nutritional value (Leonardos and Lucas, 2000).

Many microalgae accumulate large quantities of polysaccharides as reserve materials (Aaronson and Dubinsky, 1982). Green microalgae are plant-like, featuring rigid cellulose-based cell walls, and accumulating true starch (amylose, and amylopectin) inside the chloroplast as their main carbohydrate storage product. Several other algae commonly use starch for energy storage, including some red algae that store floridean starch as storage product. Other algae, for example many brown algae and diatoms (heterokont), accumulate carbohydrates such as laminaran, mannitol, chrysolaminarin or fucoidin as food reserves, and paramylon is stored by euglenoids (McFadden et al., 1997). Cyanobacteria (blue green algae) often store large quantities of glycogen, which was found to possess certain unique properties (Chao and Bowen, 1970). While other more complex carbohydrates, like cellulose and chitin are important structural molecules, which provide support and protection. Many algal cell walls from different groupings are cellulose-based, though their physical structure and the presence or absence of other structural polysaccharides varies greatly. There are also many algae that completely lack cellulose and have other polymers that provide structure to the cell (Raven et al., 1992), while some algae lack cell walls entirely.

Diatoms are also unique among algae for the presence of silica instead of cellulose in their cell walls. Some red algae also have a thick extracellular matrix composed of important products such as agar or carrageenan. Many diatoms and cyanobacteria produce diverse external carbohydrate structures, which sometimes contain pigments such as scytonemin (Hoiczyk and Hansel, 2000).

Lipids in living organisms can be divided into two main groups: the nonpolar lipids (acylglycerols, sterols, free fatty acids and wax) and polar lipids (phosphoglycerides, glycosylglycerides). Though some microalgae species contain unusual lipids, in general, algae have a lipid composition similar to that of higher plants (Guschina and Harwood, 2009). Lipids are important components of cell membranes, which act as a selective permeable barrier for cells and organelles, as some lipids play a role in responding to changes in the environment.

Algae average lipid content varies between 1 and 40 %, and under certain conditions it may be as high as 85% as percentage of the dry weight (Becker, 2004). The interest in phytoplankton lipid content and composition has increased dramatically in the past decades; much of this renewed interest is prompted by studies in applied phycology in areas such as aquaculture, the nutritional value and quality of foods fed to cultured animals and alternative energy source (Sicko-Goad and Anderson, 1991). Algal lipids are major dietary components for primary consumers, where they are a source of energy and essential nutrients. Lipids provide a substantial contribution to the food quality for invertebrates and are vital for maintaining somatic and population growth, survival, and reproductive success. Polyunsaturated fatty acids (PUFAs) which are one of the most important components of microalgal lipids, are essential nutritional components for most animals, because they cannot be synthesized *de novo*, PUFAs also involved in the regulation of physiological processes by serving as precursors in the biosynthesis of bioactive molecules (Guschina and Harwood, 2009). Lipids droplets are the storage products which distinguish some algal classes (Phaeophyta, Xanthophyta, Chrysophyta and Bacillariophyta) (Pulz and Gross, 2004).

Proteins are other complex organic compounds of high molecular. As with carbohydrates and fats; proteins contain carbon, hydrogen and oxygen, but in addition they all contain nitrogen and generally sulphur. Proteins are very important components of the enzymes, which control and regulate the chemical reactions inside the organisms.

Some hormones are also proteins. It has been reported that protein consists 6-52% of the dry weight of a microalgae biomass, which makes protein is the highest nutritional component quantity. Early and extensive nutritional studies of microalgae as foods and feeds, demonstrated that algal proteins are of high quality, comparable to conventional vegetables' proteins in terms of their content of essential amino acids, which mainly determine the nutritional quality of a protein source (Becker, 2007).

In addition to the previous substantial constitutes, algae are an important source for vitamins and trace metals. According to Becker (2007), microalgae contain high levels of essential vitamins and trace metals, similar to the best food sources such as baker's yeast and liver, as some microalgae species like *Chlorella* sp. and *Spirulina* sp. showed high vitamins and trace metals contents.

Effect of temperature, light (irradiance and duration), salinity, nutrients (nitrogen and phosphorus) limitation and starvation, on the growth and biochemical composition of microalgae have been investigated in previous numerous studies as independent factors. Either in nature or under controlled conditions for biomass production or experimental studies, microalgae are being exposed to the fluctuation of many factors. Although the microalgae under the controlled growth conditions are less affected, overlapping in the growth conditions definitely will affect their growth and biochemical composition.

Although the effects of the previous mentioned factors on the growth and biochemical composition were investigated; in many cases, contradictory results have been reported; for example, Harrison et al. (1990) reported increasing in total carbohydrate content and decreasing in total protein content, while the total lipid content remained constant, for some phytoplankton species grown under nitrogen starvation conditions. Contradictory results were obtained by Hu and Gao (2006) where, *Nannochloropsis* sp. grown under nitrogen limitation conditions showed increasing in total lipid content accompanied with decreasing in total protein content. Other conflicting results were obtained by Teoh et al. (2004) and Hu and Gao (2006).

Although improving optimum conditions for microalgae biomass production was not the main objective of the present study, the demand on high microalgae biomass production is an interesting area of study.

Renaud et al. (1999) reported the expanding in aquaculture worldwide, especially in tropical areas, and as a consequence, the demand on tropical microalgae is also on the increase and for environmental reasons, it is desirable to use microalgae species that are isolated from the same environment for use in aquaculture and biomass production.

In Malaysia, microalgae used as live feed are usually imported and the biochemical composition has not been characterized in detail (Chu et al., 1996).

Because of these reasons, species that have been investigated in the present study were isolated from Penang National Park coastal waters.

In this study, research focused upon elucidating the combined and overlapping effects in the fluctuation of some abiotic (temperature, light duration, salinity) factors and nutrients conditions on the growth, chlorophyll *a* content, dry weight and biochemical composition of some marine diatoms, that were isolated from Penang National Park coastal waters.

Therefore, the objectives of the present study were:

1. To determine the effects of combined fluctuations in temperature, light exposure duration, salinity and nitrogen, phosphorus limitation and starvation on the growth and biochemical composition of selected diatom species.
2. To quantify the species-specific response of different diatom species towards changing cultural conditions.
3. To determine which diatom species can stand the fluctuation with a minimum effect on the measured microalgal parameters
4. To determine whether the best growth conditions for a species as biomass production, will result in a high nutritional yield.

It has been reported that, studies on marine and brackish phytoplankton in the Malaysian coastal waters were very limited, particularly on the diatom. Although more than 100 diatom species in the Malaysian waters have been documented (Shamsudin (1990), but most of the diatom observed in that study were identified to only generic level due to lack of electron microscopic observation (Hilaluddin et al., 2011). Therefore, the present study will provide more knowledge about the diatom species inhabit a certain area of the coastal waters of Malaysia, and might be a cornerstone study, for further investigations that aim to set up a checklist for Malaysian diatom species. Furthermore, this study will provide useful information on the possibilities of enhancing the biomass and nutritional values of diatoms for aquaculture and nutraceutical applications.

Null hypotheses assume that, different culturing combination conditions have no effects on the growth and biochemical composition of the investigated diatoms, and different diatom species respond similarly to the fluctuation in the culturing conditions

Alternative hypotheses presume that, different culturing combination conditions affect the growth and biochemical composition of the investigated diatoms, and different diatom species respond differently to the fluctuation in the culturing conditions.

CHAPTER TWO

Literature review

Cultivation of microalgae indoors or outdoors, for biomass production or experimental purposes and regardless of the culture type (batch culture; semi-continuous culture); required suitable growth conditions. In general, temperature, light (intensity and exposure duration), salinity, pH, aeration (CO_2) and nutrients are the most important factors affecting growth, survival and the biochemical composition of a microalga. However, the range of each of these factors, in which active growth takes place, differs greatly among species.

The growth, dry weight or yield biomass, chlorophyll *a* content and biochemical composition of the microalgae are affected and subjected to the change in the fluctuation in the surrounding environmental and nutritional conditions, such as temperature, light (intensity and duration), salinity, pH, CO_2 and nutrients (Gatenby et al., 2003; Teoh et al., 2010; Brown et al., 1997). It has been reported that, in freshwater, estuarine and coastal environments, and under the normal ambient conditions, the most recognized growth-limiting nutrients are nitrogen and phosphate, as well as silica for diatoms (Roelke et al., 1999).

2.1 Temperature

Temperature controls the basic rates of all chemical reactions in microalgae cells and determines the general geographical distribution of certain algae (Sandnes et al., 2005; Okuyama et al., 1992). The optimal temperature for temperate phytoplankton cultured under laboratory conditions is generally between 20˚C and 25˚C, although this may vary with the species and strain. Most commonly cultured species of mesophilic microalgae tolerate temperatures between 16˚C to 27˚C (Lavens and Sorgeloos, 1996). Temperatures lower than 16˚C will slow down the growth, whereas those higher than 35˚C are lethal for a number of species. Some algae species are thermophilic (hot adapted microalgae), having high growth rates at high temperature, while other species are psychrophilic (cold adapted microalgae), which have growth temperature optima near 0˚C and cease growth at around 20˚C or lower for some snow algae (Smith et al., 1994; McLachlan et al., 1999).

Growth and lipid content of the diatom *Nitzschia frustulum* were estimated under different temperatures (ranging from 12 to 37˚C) and silicate deficiency. As *N. frustulum* was pre-adapted to grow under relatively low temperature (22˚C), decreasing in growth rate was observed under low (12˚C) and high (32, 37˚C) temperatures; total lipid content increased to 21% of total dry weight for cells grown at a low temperature of 12˚C, and decreased drastically to 7% dry weight at high temperature of 37˚C (Ryu and Gregory, 2010).

Guan-Qun et al. (2008) studied the variation of lipid composition in *Nitzschia laevis* as a response to growth temperature change (15-27˚C); their results showed that, the highest biomass concentration was achieved at 23˚C and the maximum specific growth rate doubled from 0.28 d^{-1} to 0.58 d^{-1} when temperature increased from 15 to 23˚C. Further increase of culture temperature caused a serious inhibition of cell growth. Under all temperatures tested, triacylglycerol (TAG) was the predominant lipid constituent (64.5–69.1% of total lipid) and was highly saturated. However, no drastic effects of temperature change on the contents of the three major lipid components were detected (neutral lipids, glycolipids and phospholipids).

Effects of temperature on growth, chemical composition and fatty acid composition of tropical Australian microalgae grown in batch cultures were investigated by Renaud et al. (2002). Among the four species tested, there was only one diatom named *Chaetoceros* sp.. Within the range of investigated temperatures (25-35˚C), *Chaetoceros* sp., achieved the highest growth rate 0.87 d^{-1} at 27-30˚C. All tropical Australian species had significantly lower percentages of protein when cells were grown at temperatures above 27˚C, but there was no consistent trend in the percentages of carbohydrate. *Chaetoceros* sp. had highest percentage of lipid (16.8% dry weight), when cells were cultured at 25˚C, while *Rhodomonas* sp., *Cryptomonas* sp. and *Isochrysis* sp., had significantly higher amounts of lipid at temperatures within the range of 27–30˚C.

Hitchcock (1980) studied the influence of temperature on the growth rate of *Skeletonema costatum* in response to daylight intensity. Growth rate of *Skeletonema costatum* at 2˚C was controlled by the low temperature conditions, where the growth rates were essentially equal at all 3 intensities tested during the first 24 hour, with slightly increasing in the second day for cultures maintained at high intensity. While for the cells maintained at 20˚C under high light intensity, a significant increase in the growth rate was recorded within the first 24 hours, as only one day was required to attain maximum growth rate.

Thompson (1999) carried out an experiment to investigate the combined effects of daylength, irradiance and temperature on the growth and biochemical composition of the diatom *Thalassiosira pseudonana*. The results showed that, growth versus total daily irradiance received were not affected by temperature or daylength. Growth versus irradiance was best modeled as a hyperbolic function at short daylengths and better modeled as an exponential function at longer daylengths, while there were no interactions between the three factors.

Teoh et al. (2004) investigated the influence of temperature ranged from 4 to 30 ˚C, on the growth and biochemical composition of some Antarctic microalgae. They reported that all the species could grow at temperature up to 20˚C, as three of the studied species grew even at 30˚C. Increasing in total protein content was reported under low temperature (6-9 ˚C). Some of the species showed increasing in the total lipid contents under lower temperature conditions, others showed decreasing, while some other Antarctic microalgae did not show consistent and clear trend with respect to the variation in culture temperature.

Araújo and Garcia (2005) investigated *Chaetoceros cf. wighamii* for its potential use as food in mariculture, where the impact of temperature (20, 25, 30 ˚C), salinity (25, 35ppt) and carbon dioxide (with and without addition) on the growth and biochemical composition were studied. Temperature had a significant effect on the growth rate of *Chaetoceros cf. wighamii*, under salinity of 25˚C, but not at 35 ˚C. High temperature caused the growth to be lower at salinity of 25 ppt, chlorophyll *a* (per cell) was not affected by temperature as well as by any of the factors tested. Their results showed that salinity (25–35 ppt) had no significant effect on *Chaetoceros cf. wighamii*, growth, maximum cell density, biomass and chlorophyll per cell. At temperatures of 20˚C and 25˚C, lipids and carbohydrates were higher than at 30˚C, while protein was not significantly affected by temperature.

The effect of temperature and growth phase on biochemical composition and lipid profile of *Isochrysis galbana* were investigated by Zhu et al. (1997), where *Isochrysis galbana* was grown at 15˚C and 30˚C, and harvested in the exponential and early stationary phases. The highest protein content was found at the exponential growth phase at 15˚C, while the highest carbohydrate content was found at the stationary phase at 15˚C, while lipid was accumulated in the stationary growth phase, and its content was higher at 30˚C than at 15˚C, regardless of the growth phase.

Carvalho et al. (2009) studied effects of irradiance and temperature on the biochemical composition of the microalga *Pavlova lutheri.* Five levels were tested for each parameter (temperature,10, 14, 18, 22 and 26°C; irradiance, 60, 105, 150, 195 and 240 µmol photons m^{-2} s^{-1}), where carbohydrate, protein, lipid, pigments and elementary compound contents were measured as response variables. Results showed that, irradiance and temperature play a major role in the determination of the biochemical composition of microalgae. Increasing in carbohydrate content under low temperature at irradiance of 150 µmol photons m^{-2} s^{-1} was observed, whereas it decreased for an irradiance of 195 µmol photons m^{-2} s^{-1}. Cells maximized their protein content at environmental conditions of 14°C and 195 µmol photons m^{-2} s^{-1}, while the lipid content in general decreased with increasing temperature.

2.2 Salinity

Microalgae living in marine environments such as estuaries, tide pools, and brackish water ponds can tolerate a wide range of external salinities (Ahmad and Hellebust, 1984). A considerable number of microalgae species are considered euryhaline, since they can adapt to varying external salinity (Hellebust, 1985). However, the salinity range over which active growth takes place differs greatly among species. It has been observed that microalgae differ in their adaptability to salinity and other stress conditions, as producing of some osmoregulators metabolites to protect from salt stress has been recorded (Ghezelbash et al., 2008). Laing (1991) has reported that, salinities of 25 to 30 psu are generally best for culturing marine flagellates, while 20 to 25 psu for the culture of marine diatoms. It has been reported that typical estuarine algae tolerate low salinities better than the oceanic species; and coastal phytoplankters take an intermediate place.

The best microalgae growing conditions for most species for culture purpose is at salinity level that is slightly lower than that of their native habitat, which is obtained by diluting sea water with tap water or distilled water. Salinities of 20-24 g l^{-1} have been found to be optimal for most of the marine species (Lavens and Sorgeloos, 1996).

Pugh (1975) investigated the variations in the biochemical composition of diatom *Coscinodiscus eccentricus*, with culture age and salinity. Carbohydrates content as % dry weight, significantly increased at salinity 25‰ compared with 20‰, 30‰ and 35‰, as the carbohydrates content was influenced by the culture age. Proteins content increased at salinity 20‰, during early growth stage, which later on, became influenced by culture age.

Nielsen and Carl (1991) studied temperature (12.5-22.5˚C) and salinity (17.8- 34‰) effects on growth of *Gyrodinium aureolum*. The results showed that the highest growth rate measured was 0.61 (dd^{-1}) at 20˚C and salinity 22.3‰, *Gyrodinium aureolum* does not grow at temperature ≤ 10˚C or ≥ 25˚C and at salinities ≤ 12‰.

Renaud and Parry (1994) studied the effect of salinity (10-35 ppt) on growth and gross biochemical composition of some marine microalgae. Their results showed that, the growth of *Isochrysis* sp. and *Nitzschia (frustulum)* were not affected by the fluctuation in the salinity, while *Nannochloropsis* sp. had a significantly slower growth rate at salinity 35 ppt. *Isochrysis* sp. and *Nannochloropsis* sp. showed linear increase in total lipid content with increasing salinity over the tested range. Variations in the salinity had only a slight effect on the total protein, soluble carbohydrate and chlorophyll *a* content.

Thessen *et al*. (2005) studied the effect of salinity on different species belonging to the diatom genus *Psedo-nitzschia*, which contains a number of species that produce the neurotoxin and domoic acid. Although most of the tested species showed varied abilities to grow over a wide range of salinity (6.2- 45 psu), they showed higher growth rate at high salinity rather than low salinity.

Hu and Gao (2006) investigated the responses of growth and biochemical composition of *Nannochloropsis* sp. to the fluctuation in salinity within the range from 22 to 88 g l^{-1}. The maximum biomass recorded (308 mgl^{-1} wet weight) achieved at salinity 30 g l^{-1}, while remarkably drop in the biomass yield (36 mgl^{-1}) at higher salinity (64 gl^{-1}) coupled with increasing in total lipid content (% w/w) was observed.

Abu-Rezq *et al*. (1999) reported the optimum production conditions for a number of high-quality marine microalgae, *Tetraselmis* sp., *Nannochloropsis* sp. and *Isochrysis* sp.; for *Nannochloropsis* sp. and *Tetraselmis* sp. the maximum cell density and growth rate were obtained at the salinity range from 20‰ to 40‰ and 20‰ to 35‰, respectively, and temperature range 19-21˚C for both species. However, both of the species could grow within a salinity range between 5‰ to 40‰, while salinity range from 25‰ to 35‰ and temperature from 24 to 26˚C was the optimum conditions for *Isochrysis* sp., which could not grow at salinity less than 20‰.

Ghezelbash et al. (2008) studied the combined effects of salinity (20, 30, 40 and 50 ppt) at different luminance on the biochemical composition of *Tetraselmes chuii*. Their results showed no significant change in protein contents under the tested conditions, whereas carbohydrates and proline (as osmoregulators) increased at low and high salinity, as well as under high irradiance conditions.

2.3 Light duration

Light and temperature are major processing factors that affect overall biomass productivity in photoautotrophic cultures, where light provides the energy source to the growing culture and is indispensable to photoautotrophic cell (Sandnes et al., 2005). It has been mentioned that, among the most important factors that affect growth and biochemical composition of microalgae, is light, which directly influences the photosynthesis mechanism (Bouterfas et al., 2006). For culturing purpose, light may be natural or artificial (supplied by fluorescent bulbs). Too high light intensity may result in photo-inhibition, while too low light intensity might be inadequate for growth. On the other hand, many microalgae species do not grow well under continuous light conditions, and hence a light/dark cycle is used to maintain such species. It has been reported that the photoperiod may also limit phytogeographic distribution, as light:dark cycle is responsible for the induction of different phases of life cycle (Andersen and Masanobu, 2005; Dhargalkar, 2004). At the polar latitude, where the summer days of nearly continuous, with low intensity daylight, and the little sunlight available during the rest of the year, result in a short period of rapid growth during the midsummer. At the middle latitude the sunlight intensity and duration vary with the seasons; the spring increase the solar radiation and lengthening of the day light result in increase in the phytoplankton biomass; and a second increase in phytoplankton population occurs during the summer. On the other hand, an extended lower growth of phytoplankton over the whole year in the tropics, where the sun-light provides abundant high intensity solar energy, and the population increase or decrease associated with the seasonal change in tropic-latitude sunlight is slight (Duxbury and Alyn, 1996). Both light intensity and duration are important factors for growth (cell division). Day length is a determinant factor for microalgae development, as the day length influences the circadian rhythm of photosynthesis, respiration as well as cell division and the growth rates (Seyfabadi et al., 2011; Vaulot and Chisholm, 1987). Beside controlling cell divisions and the pigment content, variations in light:dark regimes impose changes in the cellular contents of unsaturated fatty acids, proteins, carbohydrates and lipids (Renaud et al., 1991; Sukenik and Carmeli, 1990).

A study was carried out by Sicko-Goad and Anderson (1991) to investigate the effect of growth and different light:dark cycles on some diatom’s lipid contents. The results showed that light/dark cycles preferences are different among the diatoms species, although two of the diatoms could grow over different light/dark cycles, they showed strong day length preferences for growth, where they grew best under long-day conditions of 20:4 L:D cycle, while the other diatom grew best under short-day condition.

Meseck et al. (2005) investigated the effects of different photoperiods (24:0, 16:8, 12:12 and 8:16 light/dark cycles) and light intensities (range from 73 to 220 µmol photons m^{-}2 s^{-1}) on the growth and utilization of nutrients by *Tetraselmis chui*. Results showed that, longer day length and higher light intensity resulted in higher biomass production and complete utilization on nitrate and phosphate in less time, as compared with shortened day length and less intensity.

Bouterfas et al. (2006) studied different irradiances and photoperiod's effects on the growth rate of three freshwater green microalgae. The results indicated a linear increase in the growth rate with the day length, where the maximum growth rate achieved was under continuous light conditions.

Effect of two light regimes, 12:12 h light:dark cycle and 24 h continuous light regime, on the diatom *Skeletonema costatum* (Cleven), grown in batch cultures had been studied by Brunet et al. (1996). Particulate organic carbon (POC), particulate organic nitrogen (PON) and photosynthetic pigments were monitored for 7 to 9 days. Under continuous light regime, POC and PON production were always lower, but much higher proportion of chlorophyll degradation products such as chlorophyll *a*, phaeophytin and phaeophorid were observed under same light conditions.

According to Dickman et al. (2006), the stoichiometric composition of phytoplankton and other autotrophs can vary greatly in response to variation in light and nutrient availability. The light-nutrient hypothesis states that, phytoplankton C:nutrient ratios are driven by the ratio of available light and nutrients, as example, high light or low nutrients supply (nitrogen or phosphorus) can result in high C:N or C:P ratios respectively.

The combined influence of light intensity ranges from 34 to 80 µmol photons $m^{-2}s^{-1}$ and temperature within the range 14 to 35.7˚C, on the growth rate of *Nannochloropsis oceanica* had been studied. The results showed an increasing growth rate as a function of temperature, with a peak at 25-29˚C, and light irradiance 80 µmol photons $m^{-2}s^{-1}$, while at low intensity the growth became less affected by the fluctuation in the temperature (Sandnes et al., 2005).

Changes in the biochemical composition of marine microalgae *Nannochloropsis gaditana* during 12:12 light:dark cycle were investigated by Fabregase et al. (2002). Results showed increasing in protein content and decreasing in lipid content during the dark phase. Changes of cell density were not observed during the light phase. The maximal contents of all components (protein, carbohydrate and lipid) were observed at the end of the light phase.

2.4 Nutrients

2.4.1 Sources of nutrients in nature

In nature, nutrients come naturally from the weathering of rocks and from the conversion of atmospheric nitrogen gas (N_2) into biologically usable forms. Human activity has dramatically added to these inputs. In many coastal waters, river input of nutrients is the most important source, including sewage, animal wastes, ground water inflow as well as agriculture and other fertilizers runoff, which resulted in stimulating and may causing abnormal algal blooms. It has been reported that the export of phosphorus to the oceans has increased 3-folded compared to pre-industrial, pre-agricultural levels, and the nitrogen has increased even more dramatically, especially over the last 4 decades (Anderson et al., 2002).

Although nutrients are present in many forms in the aquatic system, only some inorganic dissolved forms are directly available for algal growth; for nitrogen these include ammonia, nitrate, nitrite and orthophosphate for phosphorus.

2.4.2 Nitrogen

Nitrogen is an essential element to all life. It is an important component of proteins, genetic material, chlorophyll and many other organic molecules. In fact, nitrogen is the fourth most common element in living tissues (after oxygen, carbon and hydrogen). In nature, nitrogen exists in several dissolved forms in the oceans, including nitrate NO^{-3}, nitrite NO^{-2} and ammonium NH^{+4}, typically present at 0-40, 0-1 and 0-1 μmol N kg^{-1} respectively, and are easily taken up by phytoplankton; while dissolved organic nitrogen