EFFECTS OF SALINITY, LIGHT AND NUTRIENTS ON THE SPECIFIC GROWTH RATE, PIGMENT CONTENT AND TOTAL SOLUBLE PROTEIN OF *Gracilaria manilaensis* (Yamamoto & Trono, 1994)

By

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Thesis submitted in fulfilment of the requirements

for the degree of Master of Science

SEPTEMBER 2016

ACKNOWLEDGEMENT

First of all, I wish to express my acknowledgment and the foremost to God who has given me strength and good health throughout my study.

I would like to express my deepest gratitude to my supervisor, Assoc. Professor Dr. Misni Surif, for his invaluable guidance, supervision and patience, which make this thesis became a reality. I will always appreciate and remember the time that I spent under his supervision during my studies. Also, my sincere thanks to my co-supervisor Dr. Norsuhana Abdul Hamid which gave me good guidance and encouragement.

I would like to express our gratitude to School of Biological Science and Centre for Marine and Coastal Studies (CEMACS), Universiti Sains Malaysia which provide laboratory space, equipment and facilities at microalgae and microalgae culture laboratory and CEMACS Station. I would also to thank to all of the staff of CEMACS University Sains Malaysia (USM) which help my study.

My warmest thanks are dedicated to my wife, Raheleh for always being by my side, contribution, encouragement her never- ending love. Surely, this thesis would not be completed without her help, my son, Amirreaz and my daughter Niki and finally, without the support, patience and love of my family and friends, I could not have completed this program. The unwavering love and encouragement of my parents, Eskander and Nahid made it possible for me to persevere.

This work was funded by Fundamental Research Grant Schemes RU-PRGS (1001/PPANTAI/836011) and Department of Fisheries (304/PJJAUH /650569/ L119).

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LIST OF ACRONYMS AND ABBREVIATIONS

A_{645}	Light absorption at 645 nm
A_{665}	Light absorption at 665 nm
A_{564}	Light absorption at 564 nm
A_{592}	Light absorption at 592 nm
A_{455}	Light absorption at 455 nm
A_{592}	Light absorption at592 nm
A_{618}	Light absorption at 618 nm
A_{545}	Light absorption at 545 nm
A_{663}	Light absorption at 663 nm
ANOVA	Analysis of variance
ASW	Artificial sea water
BAP	6-Benzylaminopurine
BSA	Bovine serum albumin
Chl a	chlorophyll a
cm	Centimetres
d	Day
DW	Dry weight
FW	Fresh weight
g	Grams
g/L	Milligram per litre
h	Hour
kg	Kilograms
M	Molarity
min	Minute
m	Miligrams

mgl^{-1}	Milligrams per liter
ml	Millilitre
mM	Millimolar
mm	Millimeter
m^{-2}	Per square meter
nm	Nanomete
OD	Optical densities
PBPs	Phycobiliproteins
PC	Phycocynanin
PE	Phycoerythrin
ppt	parts per thousand
pH	Potential Hydrogen
PVP	Polyvinylpyrrolidone
s	Seconds
SD	Standard Deviation
SGR	Specific growth rate
TSP	Total soluble protein
SPSS	Statistical Package for Social Science
w/v	Weight over volume
UK	United Kingdom United
USA	United States
µg	Micrograms
μgL^{-1}	Micrograms per litter
µg/mL	Micrograms per millilitres
µM	Micro molar
µmol	Micromole

%	Percentage
°C	Degrees Celsius
rpm	Revolutions per min

KESAN KEMASINAN, CAHAYA DAN NUTRIEN TERHADAP KADAR PERTUMBUHAN SPESIFIK, KANDUNGAN PIGMEN DAN KANDUNGAN PROTEIN TERLARUT TOTAL PADA *Gracilaria manilensis* (Yamamoto & Trono, 1994)

ABSTRAK

Gracilaria merupakan genus alga merah (Rhodophyta) yang merupakan bahan mentah penting bagi industri agar dan beberapa industri lain. Permintaan pada *Gracilaria* adalah tinggi, tetapi stok semula jadi mengalami pengurangan kerana dituai secara berlebihan. Untuk menternak spesies ini pada skala komersial, parameter alam sekitar yang mempengaruhi pertumbuhannya perlu difahami dengan sebaiknya. Dalam kajian ini, kesan kemasinan, keamatan cahaya, kualiti cahaya, fotoperiod dan kandungan nutrien terhadap kadar pertumbuhan, kandungan pigmen (klorofil, phycoerythrin, phycocyanin) dan kandungan protein terlarut total dijalankan. Kesan kemasinan (15, 25, 30 dan 35ppt), kepekatan ammonium/kepekatan (0, 20/2, 50/5, 120/12 dan 300 / 30, mikron), keamatan cahaya (30, 50, 100.150 µmol foton $m^{-2}s^{-1}$), fotoperiod (8L / 16D, 12L / 12D, 16L / 8D, 24L) dan kualiti cahaya (hijau, biru, putih, merah dan gelap) terhadap pertumbuhan dan ciri-ciri biokimia *G.manilaensis* dikaji. Keputusan kajian menunjukkan bahawa (kecuali kesan pertumbuhan pada kemasinan 30 ppt) kemasinan mempengaruhi tumbesaran, kandungan pigmen dan kandungan protin terlarut total pada *G. Manilaensis*. Semakin tinggi kemasinan semakin tinggi kadar pertumbuhan, kandungan pigment dan kandungan protin terlarut total. Dalam kajian ini kadar pertumbuhan spesifik (KPS) tertinggi bagi *G. manilaensis* adalah 4.93% $hari^{-1}$ dan yang terendah adalah 2.63 % $hari^{-1}$ pada kemasinan 30 and 15 ppt setiap satunya dan

kandungan klorofil a tertinggi (7.37±0.25 mg g^{-1} DW) adalah pada kemasinan 30ppt. Keamatan cahaya, kualiti cahaya dan fotoperiod juga didapati memberi kesan terhadap kadar pertumbuhan, kandungan klorofil a, fikoeritrin, fikosianin dan kandungan protin terlarut total pada *G. manilaensis*. Kadar pertumbuhan *G. manilaensis* yang tertinggi yang dikultur di bawah pancaran cahaya yang berbeza (30, 50, 100and 150 µmol photons m^{-2} s^{-1}) adalah pada keamatan 100 µmol photons $m^{-2}s^{-1}$ (4.93±0.35 % $hari^{1}$) dan yang terendah adalah 3.60±0.2 % $hari^{-1}$ pada keamatan 30 µmol photons $m^{-2}s^{-1}$. Tumbesaran *G. manilaensis* yang dikultur pada empat fotoperiod yang berbeza (16L:8D, 12L:12D, 8L:16D, 24 L:0D) pada keamatan cahaya 50 µmol photons $m^{-2}s^{-1}$ menunjukkan fotoperiod 12L:12D memberikan kadar pertumbuhan tertinggi (4.48±0.14% $hari^{-1}$) dan kadar tumbesaran terendah (1.6±0.17% day^{-1}) adalah pada keadaan 24 L:0D. Kualiti cahaya (biru, putih, merah dan hijau) juga didapati mempengaruhi kadar tumbesaran, kandungan pigmen (klorofil a, fikoeritrin, fikosianin) dan kandungan protin terlarut total *G. manilaensis*. Kadar pertumbuhan di bawah kualiti cahaya yang berbeza daripada yang tertinggi kepada yang terendah adalah hijau > putih > merah > biru. kajian ini, ammonium dan fosfat juga didapati mempengaruhi kadar tumbesaran, kandungan pigmen (klorofil a, fikoeritrin, fikosianin) dan kandungan protin terlarut total *G. manilaensis*. Data kajian menunjukkan semakin tinggi kepekatan N/P (0/0, 20/2, 50/5, 120/12 dan 300/30 µM) semakin tinggi kadar pertumbuhan, kandungan pigmen (klorofil a, fikoeritrin, fikosianin) dan kandungan protin terlarut total *G. manilaensis*.

EFFECTS OF SALINITY, LIGHT AND NUTRIENTS ON THE SPECIFIC GROWTH RATE, PIGMENT CONTENT AND TOTAL SOLUBLE PROTEIN OF *Gracilaria manilaensis* (Yamamoto & Trono, 1994)

ABSTRACT

Gracilaria is a genus of red algae (Rhodophyta) which is an important raw material for agar industry and some other industries. Demand on the *Gracilaria* is high, but the wild stock became lesser due to over harvest. To culture this species on a commercial scale, environmental parameters that effect its growth must be well understood. In this study, the effect of salinity, light intensity and quality, photoperiod, and nutrient content on the growth, pigment content (chlorophyll a, phycoerythrin, phycocyanin) of *G.manilaensis* were carried out. Effects of salinity (15, 20, 25, 30 and 35ppt), ammonium/phosphate concentrations (0, 20/2, 50/5,120/12 and 300/30, μM), light intensity (30, 50, 100,150 μmol photons m^{-2} s^{-1}), photoperiod (8L/16D, 12L/12D, 16L/8D, 24L) and light quality (green, blue, white, red and dark) on the growth and biochemical characteristics of *G. manilaensis* were investigated. Results of the study revealed (except for effect of growth at 30ppt) that salinity influence the growth, pigments and total soluble protein of *G. manilaensis*. The higher the salinity the higher the growth rate, pigments and total soluble protein content. In this study the highest specific growth rate (SGR) of *G. manilaensis* was 4.93%d^{-1} and the lowest was 2.63 %d^{-1} under the salinity concentrations of 30 and 15 ppt respectively and the chlorophyll a content was highest (7.37±0.25 mg g^{-1} DW) at salinity 30ppt. Light intensity, light quality and photoperiod were also affected the growth rate, chlorophyll a, phycoerythrin, phycocyanin and total soluble protein content of *G.manilaensis*. The highest growth rate of *G. manilaensis* grew under four different irradiances (30, 50, 100and 150 μmol photons m^{-2} s^{-1}) was at

100 µmol photons $m^{-2}s^{-1}$ (4.93±0.35 % day^{-1}) and the lowest rate was 3.60±0.2 % day^{-1} at 30 µmol photons $m^{-2}s^{-1}$. Growth of *G. manilaensis* under four different photoperiods (16L:8D, 12L:12D, 8L:16D, 24 L:0D) at the light intensity of 50 µmol photons $m^{-2}s^{-1}$ revealed that 12L:12D photoperiod showed highest growth (4.48±0.14% day^{1}) and the lowest growth (1.6±0.17% day^{-1}) was under 24 L:0D condition. The quality of light (blue, white, red and green) was also affect the growth, pigments (chlorophyll a, phycoerithrin, phycocyanin) and total soluble protein content of *Gracilaria manilaensis*. The growth rate under different light quality from the highest to the lower were green > white > red > blue. In this study, ammonium and phosphate were found to affect the growth, chlorophyll a, phycoerithrin, phycocyanin and total soluble protein content of *Gracilaria manilaensis*. The data of the study showed that the higher the concentration of N/P (0/0, 20/2, 50/5, 120/12 and 300/30 µM) the higher the growth rate, chlorophyll a, phycoerithrin, phycocyanin and the total soluble protein content.

CHAPTER ONE

1.1 Introduction

Rhodophyta (red algae) is one of the oldest groups of eukaryotic algae. The order *Gracilariales* (*Gracilaria*) is the largest world-wide agar source for agar extraction. There are a lot of reports which indicated that the amount of agar produced from *Gracilaria* was the largest in the world with 53%. For several centuries seaweeds have been traditionally used as food. Nowadays, global utilisation of macroalgae has become a multibillion dollar industry. Among seaweeds, *Gracilaria* spp. has the highest commercial value because it is the most important raw material for producing agar and they are relatively easy and cheap to grow.

Seaweed farming has expanded rapidly as demand has outstripped the supply available from natural resources. Commercial harvesting occurs in about 35 countries, spread between the Northern and Southern Hemispheres, in waters ranging from cold, through temperate, to tropical. In 1999, about 63% of the total agar production was produced by *Gracilaria* and in 2009, *Gracilaria* contributed to 80 percent of the raw materials for agar production.

The growth mechanism of all of the seaweed are almost similar to each other and several parameters have been recognized as affecting the growth of seaweed, such as light, salinity, and nutrient. However, the influence of these parameters on the growth of different species is not fully understood. Salinity is an important factor which can affect photosynthesis and algae growth. Salinity of water in the pond where seaweed grow is subject to change due to water evaporation and raining. For marine life (including algae), the osmotic pressure affects moisture distribution

inside and outside of the semipermeable membrane and absorption of nutrients. The tolerant to salinity is also different for different species with different process of growth and development.

The light intensity in the water body where *G. manilaensis* grow is changing frequently because of climate and the amount of sunshine available is varies throughout the day. Light quantity and quality vary with water depth, light penetration and reflection, fluorescence, the density of particles suspension in the water (turbidity), day length, season, water surface conditions, human activities and pollutant input into the water. The study on the effect of light quality on the growth of *G. manilaensis* is equally important as the study on the effect of light intensity. The seaweed grow at the bottom of the pond may not receive all spectrum of light wave length (colour). The light that passed through the water body in the pond will lose some of the light wave length due to the absorption by particles and microorganisms (including microalgae) in the water body.

Nutrient availability is another key factor in regulating the main physiological responses of seaweeds. In water, nitrogen is available to seaweeds in three major forms: nitrate (NO_3^-), ammonium (NH_4^+) and urea (CH_4N_2O). The uptake rates of different nitrogen sources can be affected by environmental parameters as well as by the seaweed species and their respective biology. Seaweeds are efficient in taking up of nitrate, ammonium, and phosphate from seawater, and the assimilation of these nutrients into nitrogenous compounds (e.g., amino acids, proteins, pigments) stimulates seaweed growth. Run off water from land area especially from agriculture land during raining brings in high nutrients content into the sea and from the sea into the pond. Based on the field observation, *G.manilaensis* grow very well and faster during wet season. The more nutrients present in the pond,

the more algae species expected to be present. The macronutrient phosphorus most clearly supports algal productivity.

Malaysia is one of the potential areas for growing seaweed. However, little research has been done on the *G. manilaensis* which grows very well in Malaysian water. Thus, more research are needed to be done to evaluate the factor that affect the growth of seaweed especially *G. manilaensis* which has high demand.

1.2 Objectives of the Study

1 To investigate the effects of salinity on growth, pigments and total soluble protein content of red seaweed *G. manilaensis.*

2 To evaluate the effects of light intensity, light quality and light photoperiod on growth, pigment and total soluble protein content in red seaweed *G. manilaensis.*

3 To assess the effects of ammonium and phosphate concentrations on growth, pigment concentration and total soluble protein in *G.manilaensis.*

CHAPTER TWO

LITERATURE REVIEW

2.1 Rhodophyta

The Rhodophyta (red algae) are eukaryotes, and the great majority of the species are marine, photosynthetic, and macroscopic. Red algae are an ancient lineage (Xiao et al., 1998; Yoon et al., 2004), including what is generally believed to be one of the oldest taxonomically resolved eukaryotic fossils, the 1.2 billion year old angiomorpha pubescens Butterfield (Butterfield, 2000). Red algae are mostly benthic and possess the greatest diversity of seaweeds as 98% of the 6,000 species are from the marine environment (Karleskint, 2009). Along West European coasts, red algae generally do not grow below a few metres beneath the low-water mark, whereas, red species have been observed at depths of 268m off the coast of the Bahamas (van den Hoek et al., 1995).

2.1.1 Gracilariaceae

The family *Gracilariaceae* belongs to the red algal phylum Rhodophyta. The Rhodophyta is an ancient lineage and contains rich species ranging from unicellular to multicellular species and ecologically as primary producers in the marine environment throughout the world (Robba et al., 2006). Red algae are mostly benthic and possess the greatest diversity of seaweeds as 98% of the 6,000 species are from the marine environment (Karleskint, 2009). The cultivation of the agarophytic red alga *Gracilaria* has become economically important in some regions of the world, such as south Asia, South America and southern Africa (Santelices and Doty, 1989). *Gracilaria* is one of the seaweed genera most exploited worldwide (Yarish and Pereira, 2008). The genus *Gracilaria* (Rhodophyta) has been demonstrated to be the

most attractive candidate for intensive culture because of its ability to achieve high yields and produce commercially valuable products. *Gracilaria* species, being an efficient nutrient pump, offer both high bioremediation efficiency and commercial value in established markets, such as agar-agar, human consumption, and fodder for other high-valued aquaculture organisms, such as abalone (Fei, 2004). Other studies have also shown that *Gracilaria* has a good capability for removing nutrients from animal effluents (Nagler et al., 2003), and seaweed production is higher in the areas surrounding fish cages than in the areas far away from aquaculture operations (Fei et al., 2002).

2.1.2 *Gracilaria manilaensis*

Gracilaria manilaensis from the Philippines coast was first identified and introduced byYamamoto and Trono Jr (1994). Taxonomy of Economic Seaweeds is similar to Pacific species. (*Abbott, I.A. Eds*). The type species (*lectotype*) of genues *Gracilaria is Gracilaria compressa (C.Agardh) Greville.* This name *(G.manilaensis*) is originated form the taxonomy it belongs to and is a type of marine species. Moreover, *G. manilaensis* does not take any synonymy in Algae Base ((Tseng & Xia, 1999; Yamamoto & Trono Jr, 1994). There was no literature evidence found about the *G. manilaensis.*

Table 1-1: Classification of Red Seaweed *Gracilaria manilaensis* (http://www.marinespecies.org)

Rank	Name
Empire	Eukaryota
Kingdom	Plantae
Subkingdom	Biliphyta
Phylum	Rhodophyta
Subphylum	Eurhodophytina
Class	Florideophyceae
Subclass	Rhodymeniophycidae
Order	Gracilariales
Family	Gracilariaceae
Genus	*Gracilaria*
Species	*G. manilaensis*

2.2 Seaweed Pigment

Three different pigments are involved in algal photosynthesis. They are chlorophylls, phycobiliproteins, and carotenoids. Chlorophyll a is necessary in the reaction centre and is associated with all types of algae.

The function of chlorophyll a is to harvest light (light-harvesting) in Photosystem I and II. Phycobilisome in red algae functions as a light-harvesting antenna. The structure of phycobilisome is made up of a core and a rod structure, with each made up of pigmented phycobiliproteins (PBPs) and connected linker proteins (Hirose et al., 2010). Phycobilisome are categorized into three main organizations, namely allophycocyanin (APC, λmax ~ 652 nm, blue pigment), phycocynanin (PC λmax ~ 615 nm, blue pigment), and phycocerythrin (PE, λmax ~ 560 nm, red pigment). PBPs are water-soluble and might make up 20% of cell dry weight. PBPs are utilized as colorants in food, beauty care, and pharmaceutic industry; and they have healing attributes. Because of their restricted supply and problems in their refinement, these pigments are rather high-priced and getting them in pure form is probably an appealing attempt (Ranjitha & Kaushik, 2005).

2.2.1 Effect of Salinity on Pigments

Influences of salinity on the alga pigment are specifically essential in open lake, tank farming, and coastal areas. In the open pond and tank, vaporization can lead to variations in salinity and in coastal areas rain and fresh water from the rivers can change the salinity of the water. Salinity variations can affect the functions of particular enzymes and proteins (Chen & Jiang, 2009). Influences of salinity on biochemical structure, in mixture with other environment variables are additionally noted for other green seaweed (Rao et al., 2007) and diatoms (de Castro Araújo & Garcia, 2005).

The observations on growth behaviour of the red algae in comparison to other seaweed groups such as *Phaeophyceae*, showed that they are sensitive to hypo-osmotic treatment (Bird & McLachlan, 1986).

Kumar et al. (2010a) investigated the effect of different salinities of 15 ppt, 25 ppt, 35 ppt, 45 ppt, and 55 ppt on the chlorophyll a content of red alga *Gracilaria corticata* and found that chlorophyll a content was different in all the salinities examined (15 ppt, 45 ppt, and 55 ppt) except for 25 ppt and 35 ppt. Phycoerythrin (PE) and Phycocyanin (PC) were different in their reactions at various salinity especially the amount PE at 45 ppt were considerably greater than the optimum content. This is equivalent to 71.42% and 52.12% higher than their concentration before the treatment (initial concentration). Phycocyanin has demonstrated a particular pattern on the reaction towards salinity changes and increases in salinity phycocyanin content increases. At salinities 45 and 55 ppt (hyper-salinity) phycocyanin content increases to 53.70% and 24.70% compared with their initial content, while phycocyanin content reduces by 30% from the initial content when treated with low salinity (15 ppt = hypo-salinity). Study done by Israel et al. (1999)

on *G. tenuistipitata* and study by Macler (1988) on *Gelidium coulteril* also showed that phycobiliprotein content was higher at higher salinity.

The changes in salinity usually affect oxidative stress and osmotic stress on the seaweeds (Kumar et al., 2010a). According to Kirst (1990), growth may be sacrificed around the salinity limits of tolerance in order to sustain osmotic realignment, which may assure survival for short periods. The growth decrease may additionally be a result of the cumulative effects of enzymes and decreased turgor stress that inhibits cell division (Lobban & Harrison, 1994).

2.2.2 Effect of Light on Pigments

Irradiance is considered as the most important ecological factor for algal survival in the marine environment (Shin et al., 2014). The quantity and composition of light is a function of water clarity, depth, season, time of day, cloud cover, and surface conditions (Wilson, 2010). Growth rates in algae generally increase with increasing irradiance until photosynthesis is saturated. As irradiance increases beyond saturating levels, decrease in photosynthesis (photoinhibition) may occur.

(a) Irradiance

Seaweeds as marine primary producers possess mechanisms that efficiently capture light at low irradiance levels to minimize damage of excessive radiation (Carvalho et al., 2011). Carvalho et al. (2011) also stated that under low irradiance some algal species develop adaptations for efficient harvesting of photons such as high accessory pigment to Chlorophyll a ratios. Duarte and Ferreira (1995) and Talarico and Maranzana (2000) stated that generally, during acclimation to light intensity, seaweeds change concentrations and ratios of their major photosynthetic pigments.

In red algae, photoacclimation to low light intensity results in increasing amount of chlorophyll a, phycoerythrin, and phycocyanin within the light-harvesting complex (Seckbach & Chapman, 2010). Photoacclimation in red algae occurs fast and may happen within hours; concentrations of pigments increase significantly during morning hours and decrease toward the afternoon (Sorek and Levy, 2012).

A longer-term adaptation to ambient light can be seen in the higher concentrations of PE from fronds of *Porphyra* growing on the surface of algal mats when compared with levels of PE from algae growing underneath, in which irradiance levels are very low (Merrill et al., 1983). Typically, during acclimation to high irradiance PE levels in red algae, decrease to a much larger extent than those of chlorophyll a, and there is a sharp reduction in the density of phycobilisomes on the thylakoid. In addition, acclimation to low light intensity results in decreased cell size to favour light capture. This is because for cells having similar quantities of photosynthetic pigments, the smaller cells absorb more light than the bigger ones on a surface basis (Zubia et al., 2014).

All three types of seaweed (Phaeophyceae, Chlorophyta, and Rhodophyta) showed increased antenna pigment content with decreasing light intensity, which results in an increase in the ratio of accessory pigment to chlorophyll a (Ramus et al., 1977). Rosenberg and Ramus (1982) found that R-phycoerythrin: Chl a ratio increases because of enhanced photosynthetic performance at subsaturating light intensities in *G. foliifera*. In *G. tenuistipitata*, carotenoid pigments were found to play an important role in protecting the photosynthetic apparatus by photoxidating reactions (Carnicas et al., 1999).

(b) Photoperiod

Kavishe (2015) stated that photoperiod refers to the daily ratio of hours of light to dark that seaweeds are exposed to during 24-hour period. Light: dark cycle is responsible for starting the different phases of life cycle in many types of seaweed. Photoperiod is one of the most important agents for reproduction control in seaweeds (Hwang & Dring, 2002). Dring was the first demonstrated in 1967 that photoperiodic response of *Porphyra* conchocelis. This response was found to be mainly due to the "short-day" photoperiods. Photoperiodic response was a response regulated by the phytochrome pigment in land plants. Dring stated that the main triggers for *Porphyra* life history are interactions of temperature, photoperiod and irradiance (Waaland et al., 1987).

Day length is a determinant factor for seaweed development. Day length influences the circadian rhythm of photosynthesis and seaweed growth rates (Bouterfas et al., 2006). Seyfabadi et al. (2011) analysed the effect of three different light intensities (37.5, 62.5, 100 µmol photon $m^{-2}s^{-1}$) and various photoperiods (8D:16L, 12D:12L, 16D:8L) on *G. persica*'s chlorophyll a content. They concluded that the maximum chlorophyll a content was observed at 37.5 µmol photons m^{-2} s^{-1} and 16D:8L photoperiod, while minimum chlorophyll a content was at 100 µmol photon $m^{-2}s^{-1}$ and 16L:8D photoperiod.

Zucchi and Necchi (2001) reported that effects of photoperiod and irradiance vary among the red algae species. The most significant difference in pigment content were related to temperature, irradiances, and photoperiods in treated alga. Phycocyanin was generally more concentrated than phycoerythrin and phycobiliproteins were more concentrated than chlorophyll a. They pointed out that total pigment concentrations in four red algae "*Chantransia*" stages of *A. pygmaea*,

C. coeruleus, *C. coeruleus* decreased sharply at 8:16 LD and 300 μmol photons m^{-2} s^{-1}. The highest total pigment contents were found in two species typical of shaded habitats: *A. hermannii* and *C. coeruleus*. The inverse relationship of pigment and irradiance was observed only in *C. coeruleus*. They concluded that the most favourable condition for growth did not coincide with those with highest pigment contents.

(c) Quality of Light

Light and chlorophyll are two most important factors that affect photosynthesis. Chlorophyll a acts as a primary light capture in the process of photosynthesis. Light sources with different wave lengths affect seaweed metabolism and growth, while different light wave lengths affect pigment composition. Light quality and irradiance greatly influence pigment composition, metabolism, and growth (Talarico & Maranzana, 2000). Franklin et al. (2001) stated that the pigment concentrations increase in *Chondrus crispus* grown under blue, red, and green light, respectively, when compared with white-light controls.

The manipulation of pigment composition by the use of different light qualities besides explaining physiological acclimation or adaptation processes in algae can be applied during cultivation of edible species either to improve appearance and attractiveness or to improve nutritional composition, particularly for potentially economically important species (Robledo & Freile, 1997). Mercado et al. (2002) pointed out that *Gracilaria tenuistipitata* showed lower maximal photosynthetic rates when grown under blue light compared with white light (controls).

Ramus et al. (1976a) showed that various macroalgae change their total pigment concentrations of their photosynthetic antennae and the relative proportion

of various pigments as a function of both the colour and the intensity of light. In addition to the red and green light regulation of phycobiliprotein synthesis, a red light induction and a partial reversion by far-red light are mostly detected. Since phytochrome is the only red-light-absorbing sensor pigment described that mediates red and far-red photoreversible responses, phytochrome or a phytochrome-like photoreceptor should be involved in the induction of photosynthetic pigment synthesis in this red alga (López-Figueroa, 1987).

Antoine and Benson-Evans, (1983) pointed out that changes in light quality alter pigment content in *Lemanea* sp.. The highest chlorophyll content and phycoerythrin were obtained at low irradiance (25 μmol photon m^{-2} s^{-1}) and in red light, while, the lowest values were found at high irradiance (94 μmol photon m^{-2} s^{-1}) and in yellow light.

2.2.3 Effect of Ammonium on Pigments

Chlorophyll a and PE are the key pigments that transfer light energy into chemical energy during photosynthesis in red algae. Pigment cellular level is an important physiological index for photosynthesis of seaweed (Korbee et al., 2005a; Yan et al., 2007). Nutrients such as nitrogen (N) and phosphorus (P) limit primary productivity, and changes in their availability can impact the growth and functioning of primary producers with implications to their growth and survival under extreme environments as well as to the communities they support (Perini & Bracken, 2014). On the other hand, photosynthetic metabolism appears to be an important determinant of the ability of intertidal seaweeds to survive abiotic stress (Davison & Pearson, 1996). Yu and Yang (2008) found that when N/P concentrations were lower than 400/25 μM, the chlorophyll a content showed an increasing trend with the increase of N/P concentrations. This suggests that raising the synthesis of

photosynthetic pigments and speeding up photosynthesis improved the growth of *G. lemaneiformis*. When N/P concentration reached 600/37.5 μM, chlorophyll a content dropped definitely. It maybe because high concentrations of N and P disturb the synthesis and normal metabolism of photosynthetic pigments and protein (Peng et al., 2007), which then restrict the growth of *G. lemaneiformis*. Similarly, Zubian (2014) stated that the amount of pigment of *G. tenuifrons* strongly depends on nitrogen availability.

Ribeiro et al. (2013) calculated the effect of nitrate, ammonium, and phosphate variations on soluble proteins and photosynthetic pigments of *Hypnea cervicornis* J. Agardh in laboratory conditions. They stated that excess nutrients accumulate as proteins and phycobiliproteins (mainly as allophycocyanin and phycoerythrin) with higher phosphate availability (N/P ratio of 10:1), and *H.cervicornis* tolerate with high ammonium and nitrate concentrations (50 and 500 μM, respectively). They also pointed out that under nitrogen and phosphate limitation, phycoerythrin concentrations are low because this pigment is preferentially metabolized to provide nutrients to sustain the algal growth. Contents of chlorophyll a are low and its accumulation is stimulated by high phosphate availability.

Bird et al. (1982) stated that chlorophyll a did not contribute greatly to the overall nitrogen accumulation in red alga *G. tikvahiae*, and this species showed higher nitrogen assimilation as phycoerythrin. Pereira et al. (2008) pointed out that nitrate is assimilated as phycocyanin and phycoerythrin by *Porphyra dioica* with the increase of nitrate availability, and phycoerythrin is the main form of nitrogen pool.

Xu et al. (2014) investigated the effects of light irradiance and nitrogen contents on the photosynthesis and growth of the green-tide macroalga, *Ulva*

prolifera. They stated that thalli of *U. prolifera* grown in natural or NH_4^+ enrichment improves growth and photosynthetic under high light intensity.

Proteins or other N-containing compounds include proteinaceous phycobilins increase in high nitrogen levels in red algae. Thus, simple nutrient adjustments and balances may be used to increase phycocyanin and phycoerythrin levels (Prasanna et al., 2010). Alternatively, some responses to nutrient enhancement and interactions with other environmental parameters can be utilized to optimize seaweed protein levels (Fatma, 2009). Total protein levels can be directly and significantly increased in microalgae under various nutrient concentrations (Fabregas et al., 1986). Composition and level of polysaccharides of Arthrospira/Spirulina increase under nitrogen limitation (Nie et al., 2002).

Pigments are sensitive to the N position of the algae and decline due to lack of sufficient N for synthesising the new pigments. Increasing chlorophyll a content with increasing cellular N are well known for algae (Fogg, 1987). Studies of the red alga *Gracilaria tikvahiae* indicated that chlorophyll and carotenoid pigments did not contribute greatly to the overall N content (Bird et al., 1982). The ratio of PE to total protein decreases when tissue N content decreases below 1.8% (Bird et al., 1982). Perhaps, at incipient N limitation, these pigments are preferentially utilized to support continued growth.

2.3 Total Soluble Protein (TSP)

With the increase in the world's population and predictions that protein source will be inadequate, have led to a search for new options and unconventional protein sources. Some studies on seaweed indicated that seaweed has high amount of protein. For this reason, seaweed is a good candidate for this purpose. In general, red seaweeds contain high levels of protein; green seaweeds contain moderate

amounts, while brown algae contain much lower protein (Fleurence, 2004; Harnedy & Fitz, 2011). The red seaweeds possess significant levels of protein and in some cases contain higher quantities than some conventional protein-rich foods such as soybean, cereals, eggs, and fish (Fleurence, 2004; Kaliaperumal, 2003). Environmental conditions including light, nitrogen and salinity affect the level of the protein. To compare the total soluble protein (TSP) contents among seaweeds is difficult due to the different extraction and measuring methods (Berges et al., 1993). One of the main problems with protein analysis in seaweed is that the protein extraction has been done with different degrees of success by various researchers (Fleurence et al., 1995). Algal cell wall composition and protein extraction procedures affect the final results significantly (Fleurence, 1999). Sample preparation is one of the most critical steps in achieving high-quality resolution of proteins analysis (Jiang, He, & Fountoulakis, 2004).

However, various studies have previously been performed on red, brown, and green seaweeds to determine their soluble protein contents (Table 2.2). Ribeiro et al. (2013) investigated the effect of nitrate, ammonium, and phosphate variations on the level of soluble proteins in *Hypnea cervicornis* J. Agardh species. They stated that total soluble protein content in *H. cervicornis* is higher if cultured with nitrate and ammonium additions. They also pointed out that treatments including high phosphate concentration with ammonium and low phosphate concentration with nitrate resulted to high protein contents. Ribeiro et al. (2013) stated that total soluble protein is the largest pool of nitrogen storage in seaweeds, while phosphate availability increases the assimilation of nitrogen including proteins in *G. crinale*. Naldi & Wheeler (1999) and Bird et al. (1982) stated that additions of 1 mM ammonium resulted in increase of protein contents in *Gelidium pacifica* and

Gracilaria tikvahiae, respectively. Meanwhile, Buschmann et al. (2009) pointed out that low concentrations of ammonium and phosphate resulted in higher protein content in *G. chilensis*. Andria et al. (1999) also stated that *Gracilaria* sp. showed higher protein contents with addition of 75 μM nitrate in the N/P ratio of 37:1. Martins et al. (2009; 2011) stated that the main form of nitrate assimilation occurred as total soluble proteins and phycobiliproteins, mainly at the form of phycoerythrin in *H. cervicornis*. The higher phosphate availability did not influence this response in *H. cervicornis*. Martins et al. (2009, 2011) also pointed out that their results showed high protein contents in *H. musciformis*, when nitrate availability increased, but phosphate addition did no alter this response. Ribeiro et al. (2013) stated that the amount of the protein depends highly on the spices of the seaweed.

Light is another important parameter that affects total soluble protein (Korbee, 2005b). Korbee (2005b) showed that different light qualities (white, blue, green, yellow, and red) affect soluble protein contents in red alga *Porphyra leucosticta*. They stated that total soluble proteins also increased in all light treatments, reaching highest values under white and blue lights, while the lowest values occurred under red light. They concluded that blue light stimulated the accumulation of structural protein. Similarly, Zhang et al. (2010) examined the effect of different light intensities on *Potamogeton crispus*. They found that Total Soluble Protein content increases with increasing light intensity. Israel et al. (1999) investigated the effect of different salinity (20%, 30%, and 39%) on soluble proteins level of the *Gracilaria tenuistipitata* var. *liui.* They pointed out that salinity affects total soluble proteins contents significantly. They also concluded that that amount of total soluble proteins increases with increasing salinity concentrations.

Table 2-2 : Global Seaweed Soluble Protein Content Determined by Bradford and Lowry Aassy

	Species	Source of seaweed	Extraction solvent	Protein concentration mg/g(dw)
Red	*P.lanosa*	Fethard-on-Sea	Siobhan Ryan., 2010	10.80
	P.lanosa	Turkey	Dere et al., 2003	310.25±168.4
	P.arctica	Norwegian Arctic	Gordillo et al., 2006	7.51
	Gracilaria lemaneifomis	North Carolina	Vergara et al., 1995	5.54
	G. tenuistipitata	Tainan, Taiwan	Lee et al., 1999	≤25.00
	Gracilaria tenuistipitata var. liui	Luzon, Philippines	Israel et al,1999	≤27.5
	G.verrucosa	Turkey	Dere et al., 2003	9.40
	Ceramium strictum	Norwegian Arctic	Gordillo et al., 2006	24.17
	Devaleraea ramentacea	Norwegian Arctic	Gordillo et al 2006	56.85
	Odonthalia dentata	Norwegian Arctic	Gordillo et al., 2006	8.16
	Palmariapalmata	Norwegian Arctic	Gordillo et al., 2006	53.93
	Phycodrys rubens	Norwegian Arctic	Gordillo et al., 2006	24.41
	Ptilota plumosa	Norwegian Arctic	Gordillo et al., 2006	38.90
	Palmaria palmata	Brittany, France	Joubert & Fleurence 2008	8.26-13.2
	Palmaria palmata	Brittany, France	Joubert & Fleurence 2008	4.43

Table 2.2 continued.

	Species	Source of seaweed	Extraction solvent	Protein concentration mg/g(dw)
Brown	*Sargassum filipendula*	North Carolina	Lee et al., 1999	1.50-2.70
	Cystoseira barbta	Turkey	Dere et al., 2003	10.49±6.15 -43.11±21.36
	Alaria esculenta	Norwegian Arctic	Gordillo et al., 2006	8.21
	Choraia flagelliformis	Norwegian Arctic	Gordillo et al., 2006	28.66
	Focus disticchus	Norwegian Arctic	Gordillo et al., 2006	43.56
	Laminaria saccharina	Norwegian Arctic	Gordillo et al., 2006	2.48
	Scytosiphon lomentaria	Norwegian Arctic	Gordillo et al., 2006	4.80
	Sphacearia plumosa	Norwegian Arctic	Gordillo et al., 2006	6.80
Green	*Ulva spp*	Turkey	Dere et al., 2003	78.58±75.39
	Enteromorphan linza	Turkey	Dere et al., 2003	277.58±135
	Enteromorphan compressa	Turkey	Dere et al., 2003	75.70±24.64
	Ulva rigida		Dere et al., 2003	82.00±6.98
	Acrosiphonia sp	Arctic	Gordillo et al., 2006	280.67±33.19
	Chaetomorpha melagonium	Arctic	Gordillo et al., 2006	49.49
	Monostroma arcicum	Arctic	Gordillo et al., 2006	4.56

2.4 Growth Rate

2.4.1 Effects of Salinity on Growth Rate

The chemical composition of the dissolved salts is relatively constant throughout the open oceans due to intensive mixing, and it varies only between 33 and 37 ppt, gradually decreasing from the subtropics toward the tropics and polar seas. However, salinity in ponds and lakes depends on many factors such as rain, snow, melting water, and evaporation, which lead to a wide range of changes in the salinity in these places.

One of the important factors for *Gracilaria* spp. to grow is salinity (Israel et al., 1999; Li-Hong et al., 2002). Low salinities usually prevent algal growth, affect branching, and promote variations in their chemical structure (Choi et al., 2006). Until now, numerous eco-physiological studies have been undertaken on the effects of salinity on seaweed growth (Krist, 1990; Karsten et al., 1991; Thomas & Kirst, 1991; Jacob et al., 1991; Karsten & West, 1993).

Kumar et al. (2010a) studied the impact of salinity on growth of *Gracilaria corticata*. They found that *G. corticata* grew very well in salinity between 25 ppt and 35 ppt, but the optimum salinity for the development was at 35 ppt. A similar study was carried out on the *G. chorda* and *G.salicornia.* The result showed that these spices also could grow well in salinities 25 ppt and 35 ppt (Choi et al., 2006; Phooprong et al., 2007). *Gracilaria verrucosa* and *G. tenuistipitata* can grow in a wide range of salinity ranging from 5 to 47 ppt (Israel et al., 1999). However, other researchers reported that these species grow in low salinity (Xu et al., 2009). The study by Yokoya et al. (1999) revealed that *G. vermiculophylla* can grow at salinity 5 - 60 ppt; however, it grows better in salinity 15-30 ppt.

Yokoya & Oliveira (1992) and Dawes et al. (1999) observed that *Gracilaria cornea* became white and died after a few days under hyposaline conditions (i.e., below 15 ppt). Meanwhile, Kumar et al. (2010a) observed that *Gracilaria corticata* grown at 15 ppt showed the loss of thallus rigidity and pigmentation after 9 days of salinity exposure. On the other hand, Macler (1988) stated that *Gelidium coulteri* cultured in 15 ppt of salinity or above showed gradual increase in growth rates, but salinities of 10 ppt or below were lethal to them. Macler (1988) also observed that chlorophyll and phycobiliprotein levels decreased with decreasing salinity at below 30 ppt. Furthermore, pigment content approaches zero at salinities of 10 ppt and below.

Salinity differentiation is stressful to most marine algae. Salinity fluctuations will change the cell osmolarity and impose stress on the organism. Salinity variation is followed by a flux of water along an osmotic gradient, which can affect turgor pressure and the cell volume. Algae may counteract these effects by different stress responses including cell ionic composition changes that would restore turgor pressure or volume of cell (Lobban & Harrison, 1994). Kirst (1990) stated that substantial water flux and ionic composition changes may result in irreversible damage including collapsed cell walls and damaged cell membrane permeability. Nevertheless, reversible consequences including affected enzyme kinetics and an enhanced metabolic cost due to enhanced ion transport and synthesis or degradation of organic osmolytes will happen at less stressful levels. Seaweeds differ in their osmoregulate capacity. However, generally estuarine species seem to be better able to regulate ions internal concentrations and osmolytes in response to salinity stress. Thus, they are more tolerant to low and different salinity than true marine species (Kirst, 1990). Kirst (1990) also indicated that tolerance to different salinity is

managed by the capacity to tolerate large changes in turgor pressure and cell volume without affecting cell integrity and metabolism.

2.4.2 Effect of Light on Growth Rate

Generally, "light" refers to radiation in that segment of the electromagnetic spectrum – about 400 to 700 mm, to which the human eye is sensitive. Furthermore, the waveband within which plants can photosynthesize corresponds approximately to that of human vision (Bonnett, 2000).

Irradiance is the most important physical factor affecting oxygenic photosynthesizers. Most forms of seaweeds would cease without the sun's energy. Generally, the irradiance wavelengths reaching the earth's surface ranged from 280 nm (UV-B) to 800 nm (infrared) (Bonnett, 2000). Shin et al. (2014) stated that solar angle and atmospheric conditions affect the total intensity and spectral energy quality. The irradiance quantity and quality is further changed when it reaches the air-water interface by reflection at the interface and absorption in the water column.

The quantity of irradiance in the water column reduces with depth enhancement. This may be the reason why irradiance in marine environment is more important ecological factor (Shin et al., 2014). In general, seaweed growth rates enhance with increasing irradiance until photosynthesis is saturated. A decrease in photosynthesis (photoinhibition) might occur when irradiance increases beyond saturating levels. Lapointe (1981) indicated that *Garcilaria foliifera* growth increased linearly with increasing irradiance to 0.43, doubling per day at high light levels in situ. Bunsom and Prathep (2012) reported *Gracilaria tenuistipitata* under different light intensities under 700 mmol photons m^{-2} s^{-1} increases its weight gain; and the highest pigment contents were phycoerythrin and phycocyanin produced in

low light conditions and at 150 mol photons $m^{-2}s^{-1}$. Jensen et al. (2011) examined *G.vermiculophylla* responses under various irradiances and found that *G.vermiculophylla* grows. It is able to utilise low amount of light, while maintaining maximum growth rate.

(a) Light Intensity

Yu et al. (2013) examined the effects of irradiance and salinity on the growth of *Gracilaria edulis and Gracilaria tenuistipitata var liui* and found both species increased their daily growth rates with increasing irradiance. Yu et al. (2013) also indicated that *G. edulis* grows best at 100 μmol photons $m^{-2}s^{-1}$, while *G.tenuistipitata* var liui achieves its optimal growth at 60–130 μmol photons $m^{-2}s^{-1}$. However, Xu et al. (2009) stated that irradiance of 200 μmol photons $m^{-2}s^{-1}$ is the ideal irradiance for the growth of *G. tenuistipitata var liui*. Adaptation to the different levels of turbidity in their respective habitats and the existence of ecotypes might be reasons for the differences in the optimal irradiance for different seaweeds. Xiaolei et al. (2008) found that the optimum irradiance for conchospore release in *P. yezoensis* is 200 μmol photons $m^{-2}s^{-1}$ and observed that conchospore division increased with increased irradiance and wavelength.

Roleda et al. (2006) stated that generally, the seaweed light requirement is related to its geographical position, with lower values in Polar Regions and higher values in warmer regions. Seaweed species growing in Polar Regions tend to adapt themselves to shady conditions (Wiencke et al., 2007). This shows that seaweeds need less light and reach photosynthetic saturation between 14 and 52 μmol photons $m^{-2}s^{-1}$ (Hanelt et al., 2003). However, subtropical seaweed species need more light. For instance, Stekoll et al. (1999) reported that growth inhibition of Alaskan

P.abbottae was at irradiance over 40 µmol photons $m^{-2}s^{-1}$ at 15 °C or higher. Meanwhile, Waaland et al. (1990) showed good growth rates for the same species collected in Washington, in the range 10 to 15 °C and 100 µmol photons $m^{-2}s^{-1}$. Furthermore, Zacher et al. (2007) stated that *P. endiviifolium* from Antarctica also reaches photosynthetic saturation at low irradiance (33 µmol photons $m^{-2}s^{-1}$). However, Zou and Gao (2002) indicated that species from warmer areas can only reach photosynthetic saturation at high irradiances; for instance, at 200 µmol photons $m^{-2}s^{-1}$. However, light requirements of seaweed may be species-specific and may differ with different life history stages (Katz et al., 2000). Katz et al. (2000) found that cultured blades of *P. linearis* from the Meditereranean Sea reach photosynthetic saturation at 20 µmol photons $m^{-2}s^{-1}$. They also associated themselves with the shade-adapted growth habit of this species in the wild rather than in their geographical location. Lin et al. (2008) also indicated that the photosynthetic saturation for *P. pseudolinearis*, *P.abbottiae* and *P. torta* are species specific and sometimes not significantly related to variations in temperature.

(b) Photoperiod

Photoperiod refers to hours' ratio of light: dark that the seaweeds are exposed to over a 24-hour period. Gaines and Lubchenco (1982) stated that the light: dark cycle is responsible for the induction of different phases of life cycle in many types of seaweed. Critchley and Russell (1994) reported that the roles of light in biological systems can be considered in terms of wavelength, intensity, and photoperiod. Matinfar et al. (2013) reported that the maximum growth in *Gracilaria persica* occurs at 16:8 hr light/dark photoperiod, while Fortes and Lfining (1980) reported that the growth rate increases linearly with day lengths up to 24 hr light per day in

Chondrus crispus and Laminaria *saccharina*. In *Ulva lactuca* and *Porphyra umbilicalis*, the growth versus day length begins to flatten out at day lengths greater than 16 hr (Markham & Hagmeier, 1982).

Khoeyi et al. (2012) also found that by increasing the photoperiod from 8:16 to 16:8 hr, plant biomass production can be increased. The necessity of a dark phase is explained by the photosynthesis reactions. Photosynthesis reactions include two phases, namely photochemical phase that is light dependent and biochemical dark phase which is light independent.

Krzeminska et al. (2014) investigated the effect of photoperiod on the four different green algae. They observed that continuous illumination increased the growth of *B. braunii* and *S. obliquus* more effectively than the growth of the microalgal species from the genus *Neochloris*. However, under shorter duration of light with similar light intensity, the growth of all the three species of *Neochloris* was stimulated. Under continuous illumination, the specific growth rate in the first phase of *B. braunii* and *S. obliquus* cultures was higher than the growth rate of *Neochloris*, whereas under the 12L:12D cycle, the specific growth rate of all the three *Neochloris* species was generally higher than that in *B. braunii* and *S. obliquus*.

(c) Quality of Light

Light quality is one of the important factors that affects seaweed growth rate. Ramus et al. (1976a) and Gómez et al. (2004) stated that seaweeds that grow at deeper depths normally synthesise more pigments as a compensatory effect for low light availability, since environmental parameters especially light quality change with depth. Mercado et al. (2002) stated that the growth of *Gracilaria tenuistipitata* exposed to blue light showed lower maximal photosynthetic levels compared with the ones that grew under white light. Furthermore, Leukart and Lüning (1994)